VNIVERSITAT DE VALÈNCIA

**FACULTAT DE FARMÀCIA**

Departament de Medicina Preventiva i Salut Pública, Ciències de l´Alimentació, Toxicologia i Medicina Legal

**PROGRAMA DE DOCTORAT AMB MENCIÓ CAP A L'EXCEL·LÈNCIA EN CIÈNCIES DE L'ALIMENTACIÓ**

# FRUIT BASED BEVERAGES TREATED BY NON-THERMAL TECHNOLOGIES: BIOACTIVE COMPOUNDS AND BIOACCESSIBILITY

## BEBIDAS A BASE DE FRUTAS PROCESADAS POR TECNOLOGÍAS NO TÉRMICAS: COMPUESTOS BIOACTIVOS Y BIOACCESIBILIDAD

**TESI DOCTORAL INTERNACIONAL**

València, 2016

Presentada per:

**Juana Mª Carbonell Capella**

Dirigida per:

**Dra. Ana Mª Frígola Cánoves**

**Dra. Mª José Esteve Mas**

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Las Dras. Ana Mª Frígola Cánoves y Mª José Esteve Mas, profesoras titulares del Área de Nutrición y Bromatología,

**CERTIFICAN QUE:**

**Dª Juana Mª Carbonell Capella**, licenciada en Farmacia y graduada en Nutrición Humana y Dietética, ha realizado bajo su dirección el trabajo que lleva por título **"Bebidas a base de frutas procesadas por tecnologías no térmicas: compuestos bioactivos y bioaccesibilidad"** y autorizan su presentación para optar al título de Doctora por la Universidad de Valencia.

Y para que conste a los efectos oportunos,

Burjassot (Valencia), Febrero 2016

**Dra. Ana Mª Frígola Cánoves** **Dra. Mª José Esteve Mas**

Este trabajo ha dado lugar a 9 artículos y 2 capítulos de libro, publicados o que se publicaran en las siguientes revistas y libros:

1. Emerging role of *Stevia rebaudiana* Bertoni as source of natural food additives. In Wenbiao Wu (Ed.), *Leaf Sweeteners: Resources, Processing and Health Effects* (pp. 59-76). New York: Nova Publishers (2014).
2. Novel beverages sweetened with *Stevia rebaudiana* as sources of bioactive compounds. *Journal of Food Science and Technology (under review). Impact factor: 2.203.*
3. Automating a 96-well microtiter plate assay for quick analysis of chemically available lysine in foods. *Food Analytical Methods, 6, 1258-1264 (2012). Impact factor: 1.969.*
4. High pressure processing of fruit juice mixture sweetened with *Stevia rebaudiana* Bertoni. Optimal retention of physical and nutritional quality. *Innovative Food Science and Emerging Technologies, 18, 48-56 (2013). Impact factor: 2.248.*
5. Ascorbic acid in orange juice based beverages processed by pulsed electric fields. In Katherlyn Elizabeth Elder (Ed.), *Fruit Juices: Types, Nutritional Composition and Health Benefits. Nutrition and diet research progress* (pp. 103-124). New York: Nova Publishers (2014).
6. *Stevia rebaudiana* Bertoni as a natural antioxidant/antimicrobial for pulsed electric field processed fruit juice: Processing parameter optimisation. *Food Control (under review). Impact factor: 2.806.*
7. Influence of pulsed electric field processing on the quality of fruit juices sweetened with *Stevia rebaudiana*. *Innovative Food Science and Emerging Technologies (under review). Impact factor: 3.273.*
8. Changes of antioxidant compounds in a fruit juice-*Stevia rebaudiana* blend processed by pulsed electric technologies and ultrasound. *Food and Bioprocess Technology, DOI: 10.1007/s11947-016-1706-1. Impact factor: 2.691.*
9. "Ice" juice from apples obtained by pressing at subzero temperatures of apple pretreated by pulsed electric fields. *Innovative Food Science and Emerging Technologies, DOI:10.1016/j.ifset.2015.12.016. Impact factor: 3.273.*
10. Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: A review. *Comprehensive Reviews in Food Science and Food Safety, 13, 155-171 (2014). Impact factor: 4.182.*
11. Effect of *Stevia rebaudiana* on bioaccessibility of bioactive compounds and antioxidant activity of beverages based on exotic fruits mixed with oat following simulated human digestion**.** *Food Chemistry, 184, 122-130 (2015). Impact factor: 3.391.*

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**INFORMAN QUE:**

**Dª Juana Mª Carbonell Capella**, licenciada en Farmacia y graduada en Nutrición Humana y Dietética, ha contribuido en el trabajo que lleva por título "*Automating a 96-well microtiter plate assay for quick analysis of chemically available lysine in foods"* a partes iguales con D. Francisco J. Barba Orellana.

Burjassot (Valencia), Febrero 2016

**Dra. Ana Mª Frígola Cánoves** **Dra. Mª José Esteve Mas**

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Burjassot (Valencia), Febrero 2016

**Dra. Ana Mª Frígola Cánoves** **Dra. Mª José Esteve Mas**

La presente tesis doctoral internacional se enmarca dentro del proyecto de investigación AGL2010-22206-C02-01: Combinación de ingredientes naturales y tecnologías no térmicas en la estabilización de bebidas de origen vegetal. Aspectos microbiológicos, nutricionales y fisicoquímicos.

La estudiante de doctorado, Juana Mª Carbonell Capella, agradece al Ministerio de Educación la beca predoctoral del Programa de Formación del Profesorado Universitario (F.P.U.) AP2010-2546.

La doctoranda agradece al Ministerio de Educación las ayudas de movilidad para estancias breves (2013 y 2014) para la mención internacional del título de Doctora, realizadas en el Département de Génie des Procédés Industriels, Laboratoire Transformations Intégrées de la Matière Renouvelable, Université de Technologie de Compiègne (Francia), bajo la dirección del Dr. Eugène Vorobiev.

*One never notices what has been done;*
*one can only see what remains to be done.*
Marie Curie (1867-1934)

**AGRADECIMIENTOS**

Me gustaría agradecer en primer lugar a mis directoras, las profesoras Ana Mª Frígola y Mª José Esteve, no sólo por brindarme la oportunidad de realizar la tesis, sino también por dirigir mi trabajo con sabios consejos e infinita paciencia. A la Dra. Ana Mª Frígola, por su profesionalidad y apoyo. Por su gran dedicación a la docencia y a la investigación, su excelente labor formativa y su concepto de trabajo en equipo. Pero sobre todo, por guiarme día a día tanto a nivel laboral como personal. A la Dra. Mª José, por su eficiencia y calidad humana. Por preocuparse por mí y poder acudir a ella siempre que lo he necesitado. Por atenderme y recibirme con una sonrisa aunque estuviera cargada de faena.

A todo el profesorado del Área de Nutrición y Bromatología, integrado por grandes profesionales que también han sido partícipes de mi paso por el laboratorio y de los que he podido aprender humana y científicamente.

A mis compañeros de laboratorio, Francisco, Magda, Kamila, Lorena, María, Andrea, Islam, Toni, Gabi, Virginia, Elisa, Abigail, Chelo, Sonia y Laura, por haber sido mis compañeros y amigos durante estos 4 años. Por vuestra sonrisa diaria, por ese buen humor que se respira trabajando a vuestro lado y que transmitís con entusiasmo, por vuestros ánimos y consejos y por las largas charlas en los momentos de cafetería y en las quedadas fuera del laboratorio, en las que os he podido conocer mejor y descubrir en cada uno de vosotros un gran número de virtudes. A Francisco, por enseñarme las técnicas de laboratorio y transmitirme tu espíritu crítico experimental. Y gracias también al resto de personas que han pasado por el departamento durante mi periodo formativo.

Y por supuesto a los laborales y técnicos, con especial mención a Carmen y Amparo, que tantas dudas me han resuelto y que desempeñan una gran labor en nuestra formación.

Asimismo, agradezco a la Dra. Dolores Rodrigo y al Dr. Antonio Martínez por permitirme el uso de sus instalaciones en el IATA-CSIC, y a María, Nieves, Clara, Chelo, Alejandro y Chechu. Gracias por los conocimientos transmitidos, el trato recibido y por hacerme partícipes de vuestro grupo.

I would also like to thank Dr. Vorobiev and Dr. Grimi for allowing me to carry out my research stay at the University of Compiègne, France, and to all my lab-partners for their hospitality and excellent predisposition, especially to Oleksii, for instructing me in the use of equipment and result analysis.

Gracias a todas mis amigas, que sin pertenecer a este mundo de la investigación os habéis interesado por mis progresos y habéis estado a mi lado cuando os he necesitado, con consejos, risas, buenos momentos, recuerdos que quedan en mí y me llenan.

Finalmente, quisiera agradecer esta etapa de mi vida, a mi familia, el pilar básico de mi vida, a los que quiero con toda el alma, por cuidar de mí y de vuestro nieto y sobrino con tanto amor cuando yo estaba inmersa en el desarrollo de esta tesis. Nunca podré pagaros todo lo que habéis hecho. A mis padres, por inculcarme valores, por vuestro amor incondicional y por haber sido los primeros en animarme a realizar esta tesis. A mis hermanos, JoseVi y Francisco, con los que puedo contar cuando lo necesito, por sacarme tantas sonrisas, por contagiarme tantas veces de vuestra alegría. Por hacer de la vida un lugar tan bonito.

A mis suegros, cuñados y sobrinos, que os habéis ganado todo mi cariño y realmente os siento como lo que sois, mi familia. Gracias Vicente y Aurora por haber hecho posible que hoy Jose esté aquí a mi lado.

A las tres personas que más quiero. A ti, Jose, por el mejor regalo que nunca he soñado, la suerte de mi vida, tenerte a mi lado. Soy consciente de todo lo que haces día a día, de cada pequeño detalle que tienes. Por eso te quiero dar las gracias, por ser mi confidente, mi amigo, mi marido. Por tu ayuda informática que tan útil me ha sido. Porque cada día aprendo de ti. Porque eres el ejemplo que quiero seguir.

A Nacho, que ha transformado radicalmente mi vida llenándola de alegría. Tu risa es el sonido más bonito que puede existir. Y al peque que viene en camino.

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## ABREVIATURAS / ABBREVIATIONS

| | |
|---|---|
| AA | Ascorbic acid |
| ABTS | 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) |
| AIJN | Association of the Industry of Juices and Nectars |
| $A_M$ | Measured activity |
| ANOVA | Analysis of variance |
| APH | Altas presiones hidrostáticas |
| $A_T$ | Theoretical calculated activity |
| AUC | Area under the curve |
| BHA | Butylated hydroxyanisole |
| BI | Browning index |
| $C_c$ | Carbohydrate concentration |
| CECU | Confederación Española de Consumidores y Usuarios |
| CIELAB | Commission International d'Eclairage L-a-b Representation |
| CSIC | Consejo Superior de Investigaciones Científicas |
| CTE | Catechin equivalent |
| D | Decimal reduction time |
| d | Diameter |
| DEAV | Descargas eléctricas de alto voltaje |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| E | Electric field strength |
| $E^{1\%}$ | Extinction coefficient |
| EC | European Comission |
| ER | Equivalente de retinol |
| Eq. | Equation |
| EU | European Union |
| EEUU | Estados Unidos |
| FDA | Food and Drug Administration |
| FSA | Food Standards Agency |
| FT | Freeze-thawed |
| GAE | Gallic acid equivalent |
| GE-EP | Glycostevia-EP® |
| GE-R60 | Glycostevia-R60® |
| GRAS | Generally recognised as safe |
| h | Height |
| HPP | High pressure processing |
| HVED | High voltage electrical discharges |

| | |
|---|---|
| HWE | Heating washing extract |
| %I | Percentage of inhibition |
| IAN | Industrias Alimentarias de Navarra |
| IATA | Instituto de Agroquímica y Tecnología de los Alimentos |
| IDA | Ingesta diaria admisible |
| IF | Interaction factor |
| JECFA | Joint FAO/WHO expert comittee on food additives |
| LOD | Limit of detection |
| Log S | Decimal logarithm of the survival fraction |
| LOQ | Limit of quantification |
| LSD | Least significant difference |
| Lys | Lysine |
| MeOH | Methanol |
| $m_m$ | Maximum juice mass |
| n | Number of pulses |
| N | Series of pulses |
| ND | Not detected |
| NEBI | Non-enzymatic browning index |
| OPA | *ortho*-phthaldialdehyde |
| ORAC | Oxygen radical absorbance capacity |
| P | Pressure |
| PCA | Principal component analysis |
| PEAI | Pulsos eléctricos de alta intensidad |
| PEF | Pulsed electric fields |
| PME | Pectinmethyl esterase |
| POD | Peroxidase |
| PPO | Polyphenoloxidase |
| QC | Quality control |
| $R^2$ | Correlation coefficient |
| RA | Relative activity |
| RE | Raw extract |
| Reb | Rebaudioside |
| rpm | Revolutions per minute |
| RSD | Relative standard deviations |
| RSM | Response surface methodology |
| $R_t$ | Retention time |
| S.A. | Sociedad anónima |
| SDS | Sodium dodecyl sulfate |
| SENC | Sociedad Española de Nutrición Comunitaria |
| *SR* | *Stevia rebaudiana* |

| | |
|---|---|
| SWE | Stevia water extract |
| TA | Total anthocyanins |
| TAC | Total antioxidant capacity |
| TC | Total carotenoids |
| TE | Trolox equivalent |
| $t_e$ | Transition time |
| TEAC | Trolox equivalent antioxidant capacity |
| $t_f$ | Effective freezing time |
| TI | Turbidity index |
| TPC | Total phenolic compounds |
| UHT | Ultra high temperature |
| UK | United Kingdom |
| USA | United States of America |
| USN | Ultrasounds |
| UTC | Université de Technologie de Compiègne |
| UV-vis | Ultraviolet-visible |
| W | Energy input |
| WE | Washing extract |
| $W_{PEF}$ | Pulse energy |
| Y | Yield |
| $Y_m$ | Maximum yield |
| $Z_E$ | Sensitivity to electric field strength |
| $\tau$ | Pulse length |
| $\sigma$ | Conductivity |

# Summary

## *Resumen*

**RESUMEN**

La política en alimentación de la Unión Europea debe garantizar los mayores niveles de seguridad y calidad alimentaria, lo que supone proteger y promover la salud de los consumidores. A éstos se les debe ofrecer un abanico amplio de alimentos seguros y de elevada calidad, por lo que el objetivo de la presente tesis es el análisis de compuestos bioactivos y parámetros de calidad en bebidas a base de zumos de frutas y sus modificaciones tras el procesado por tecnologías no térmicas, así como el estudio de la bioaccesibilidad de estos compuestos.

Se ha formulado y caracterizado un alimento nuevo, una bebida a base de zumo de papaya, mango, naranja y bebida de avena adicionado con açaí deshidratado y extracto acuoso de stevia como edulcorante acalórico natural con capacidad antimicrobiana y antioxidante.

Se ha evaluado la aplicación de distintas tecnologías no térmicas (altas presiones hidrostáticas, pulsos eléctricos, descargas eléctricas de alto voltaje y ultrasonidos) en la obtención de estas bebidas a base de zumo de frutas. Los resultados muestran que la aplicación conjunta de dos estrategias de conservación (aplicación de tecnologías no térmicas junto con el empleo de stevia) permite un mayor contenido de compuestos bioactivos y capacidad antioxidante total en la bebida final. De entre los pulsos eléctricos, descargas eléctricas de alto voltaje y ultrasonidos, los pulsos eléctricos son en general la tecnología que permite la obtención de una bebida a base de zumo de papaya, mango y stevia con el mayor contenido de compuestos bioactivos y capacidad antioxidante. Además, la aplicación de pulsos eléctricos como pretratamiento al prensado a temperaturas bajo cero evidencia una mejora en la cinética de obtención de zumo de manzana y en su contenido en compuestos bioactivos. Los resultados avalan la aplicación de tecnologías no térmicas para obtener bebidas a base de frutas de mayor calidad.

Se ha realizado una revisión de los métodos de determinación de bioaccesibilidad y biodisponibilidad de los compuestos bioactivos en alimentos, para posteriormente, estudiar la bioaccesibilidad de los compuestos bioactivos de la bebida a base de zumo de frutas edulcorada con stevia a través de una

simulación gastrointestinal. El aumento en la concentración de extracto acuoso de stevia da lugar a un incremento en la bioaccesibilidad de compuestos fenólicos, antocianinas, capacidad antioxidante total y glucósidos de esteviol, mientras que repercute negativamente sobre la bioaccesibilidad del ácido ascórbico, evidenciando de este modo la importancia de realizar estudios de bioaccesibilidad de los compuestos bioactivos y no únicamente la determinación de su contenido en cada matriz y técnica empleada en el procesado de los alimentos.

**SUMMARY**

European Union food policy must ensure the highest levels of food safety and food quality, protecting and promoting consumers health. Consumers should be offered a wide range of safe and high quality food products, which is why the aim of this thesis is the analysis of nutritional and quality parameters of beverages based on fruit juices and their modifications after non-thermal processing technologies, along with the bioaccessibility study of these compounds.

A novel beverage based on papaya, mango and orange juice, oat beverage mixed with açaí and stevia water extracts as a natural non-caloric sweetener with antimicrobial and antioxidant capacity was formulated and analysed.

The application of non-thermal technologies (high pressure processing, pulsed electric fields, high voltage electrical discharges and ultrasound) in the obteinment of these beverages was assessed. Results show that the joint application of two conservation strategies (application of non-thermal technologies along with the use of stevia) allowed a higher content of bioactive compounds and total antioxidant capacity in the final beverage. When comparing pulsed electric fields, high voltage electrical discharges and ultrasound, pulsed electric fields was generally the technology which led to the papaya and mango juice beverage sweetened with stevia with the highest bioactive compounds content and total antioxidant capacity. Furthermore, pulsed electric fields applied as pretreatment to pressing at subzero temperatures showed an improvement in the kinetics of production of apple juice and in the bioactive compounds content. Results support the application of non-thermal technologies for higher quality fruit beverages.

A review of the methods for determining bioaccessibility and bioavailability of bioactive compounds was performed, so as to later study bioaccessibility of bioactive compounds in the beverages based on fruit juices sweetened with stevia through a gastrointestinal simulation. Higher stevia concentration led to an increase in the phenolic compounds, anthocyanins, total antioxidant capacity and steviol glycosides bioaccessibility, while stevia addition had a negative impact on ascorbic acid bioaccessibility, demonstrating the importance of bioactive

compounds bioaccessibility studies and not only bioactive compounds content determination with each food matrix and technology applied in the food processing.

# 1. General introduction

*Introducción general*

# 1. INTRODUCCIÓN GENERAL

## 1.1. Bebidas funcionales

Dado que la prevención de enfermedades crónicas constituye una mejor estrategia que su tratamiento, reducir el riesgo de sufrir enfermedades cardiovasculares o cáncer es de gran interés para profesionales de la salud, científicos y para la industria alimentaria. Por esta razón, la industria alimentaria ha centrado sus esfuerzos en la producción de alimentos con "propiedades saludables", conocidos como alimentos funcionales (Zawistowski, 2014). El término alimento funcional se refiere a un alimento o ingrediente capaz de mejorar la salud y/o reducir el riesgo de padecer enfermedad (Corbo y col., 2014). Estos alimentos se consumen como parte de la dieta y producen efectos beneficiosos que van más allá de los requerimientos nutricionales tradicionales. Puede tratarse de alimentos naturales enteros, alimentos con algún componente añadido, o productos alimentarios a los que se les elimina algún componente. Asimismo, se incluyen alimentos con uno o más componentes modificados, alimentos cuya biodisponibilidad de uno o más componentes ha sido modificada, o cualquiera de las combinaciones anteriores. Éstos pueden estar dirigidos a toda la población en general o a grupos particulares, por ejemplo, por edad o constitución genética (Roberfroid, 2002). Junto con un estilo de vida saludable, los alimentos funcionales pueden contribuir de forma positiva a la salud y al bienestar de las personas. Ya no basta con evitar déficits nutricionales, sino asegurar una alimentación “óptima”, donde la identificación de los compuestos biológicamente activos en los alimentos, con potencial para optimizar el bienestar físico y mental y reducir el riesgo de enfermedad, son claves.

Según la “Guía de Alimentos Funcionales”, publicada por la Sociedad Española de Nutrición Comunitaria (SENC), la Confederación Española de Consumidores y Usuarios (CECU) y el Instituto Omega 3 de la Fundación Puleva, en España existen más de 200 alimentos funcionales a la venta y se calcula que este tipo de productos, que en 2003 apenas representaban un 5% del mercado español, hoy suponen alrededor de un tercio del mismo (Özen y col., 2014). La mayoría de ellos pertenecen al grupo de los lácteos, aunque también existen alimentos infantiles,

platos preparados, productos de panadería y cereales, productos cárnicos y bebidas con algún componente modificado, como los ácidos grasos o la fibra, o con alguna sustancia biológicamente activa adicionada, como compuestos fitoquímicos, esteroles, antioxidantes, prebióticos o probióticos.

Concretamente, las bebidas son con diferencia el grupo con mayor interés por su facilidad de manejo, distribución y almacenamiento y posibilidad de incorporar nutrientes deseables y compuestos bioactivos. Específicamente, el sector de las frutas y verduras presenta una gran oportunidad en el mercado actual, donde las frutas y sus derivados están ganando cuota de mercado (Corbo y col., 2014). Su consumo resulta beneficioso, no sólo por su aporte en nutrientes, sino por su capacidad para disminuir el riesgo de padecer enfermedades como cáncer, diabetes y enfermedades cardiovasculares, así como de retrasar procesos degenerativos, entre los cuales se encuentra el envejecimiento. Estos efectos se atribuyen principalmente a sus componentes biológicamente activos como la fibra, compuestos fenólicos, carotenoides, vitamina A, C y E, glucosinolatos y compuestos organosulfurados, entre otros. En España y en la mayoría de países de la Unión Europea se recomienda un consumo de 5 piezas de frutas y verduras diarias (five-a-day) (Braesco y col., 2013), excluyendo patatas y otras raíces amiláceas e incluyendo una ración de zumo de frutas. En términos de salud pública, se ha demostrado que el consumo de zumos de frutas y verduras puede ser tan eficaz como el consumo de frutas y verduras enteras en cuanto a la reducción del riesgo de enfermedades degenerativas (Bhardwaj y col., 2014).

En este contexto, la investigación científica está encaminada hacia la optimización de la producción y formulación de nuevas bebidas funcionales. Cuando se diseña una nueva bebida, es importante el estudio de las interacciones que pueden ocurrir al mezclar los distintos ingredientes, ya que la funcionalidad de estos productos se puede perder o reducir por formación de precipitado, oxidación o degradación de sus componentes. Asimismo, es importante el estudio de la bioaccesibilidad (fracción del componente alimentario que se libera en el intestino para su absorción en la mucosa intestinal) y biodisponibilidad (fracción del componente alimentario que alcanza su lugar de acción) de los compuestos bioactivos para asegurar la mejora efectiva de la funcionalidad de bebidas.

Además, se debe garantizar su presencia en la matriz de la bebida durante el procesado. Por ello, algunos autores se centran en la aplicación de tecnologías no convencionales, tales como el procesado por altas presiones hidrostáticas (APH), pulsos eléctricos de alta intensidad (PEAI) y ultrasonidos (USN) para mejorar la producción de alimentos funcionales sin comprometer sus propiedades sensoriales y funcionales (Zulueta y col., 2013). En esta línea, los ingredientes naturales con actividad antioxidante y antimicrobiana podrían utilizarse en el diseño de estas bebidas, representando una alternativa a los conservantes químicos y ampliando el mercado de las bebidas funcionales (Sun-Waterhouse, 2011). Por tanto, la investigación encaminada hacia la búsqueda de estrategias exitosas que mejoren el atractivo de las bebidas funcionales y demuestren su inequívoca eficacia en la promoción de la salud se presenta como necesaria.

Dentro del grupo de bebidas funcionales se encuentran los zumos funcionales, que suelen ser mezclas de productos que contienen sustancias activas que protegen la salud, sobre todo con capacidad antioxidante. En la elaboración de estos zumos se emplean frutas u otros ingredientes que destacan por su riqueza en nutrientes y compuestos antioxidantes beneficiosos para la salud, basándose en la evidencia científica. Entre ellos figuran los siguientes:

La papaya es apreciada por su sabor, calidad nutricional y propiedades digestivas. Tiene un alto contenido en carotenoides, flavonoides, potasio, fibra y ácido ascórbico. Contiene 108 mg de ácido ascórbico por 100 g de fruta fresca, valor superior al que presenta la naranja (67 mg/100 g) (Gayosso-García y col., 2011) y es una de las frutas con mayor capacidad antioxidante (Vij & Prashar, 2015). Por otra parte, contiene papaína, una enzima proteolítica similar a la pepsina que le confiere propiedades digestivas.

El mango es una fruta tropical que destaca por su color, sabor y por su alto contenido en fibra, vitamina C, β-caroteno, vitamina E y compuestos fenólicos (Li y col., 2014), que le aportan una alta capacidad antioxidante. Representa para los consumidores una nueva fuente natural de β-caroteno (provitamina A) y vitamina C (44 mg/100 g), que, como antioxidantes, contribuyen a reducir el riesgo de múltiples enfermedades crónicas. Entre sus compuestos fenólicos se encuentran el ácido tánico, con propiedades antioxidantes y antibacterianas, el ácido elágico y

la manguiferina, específica del mango. El ácido elágico y la manguiferina poseen propiedades antiinflamatorias y anticancerígenas que ayudan a fortalecer el sistema inmunológico del organismo. Su contenido en fibra le confiere propiedades laxantes. Existen numerosos estudios sobre el valor nutritivo y el efecto del procesado sobre los compuestos bioactivos y capacidad antioxidante del mango (García-Magaña y col., 2013; Kaushik y col., 2014).

La naranja, por su parte, es rica en vitamina C, ácido fólico y minerales como el potasio, magnesio y calcio. También aporta cantidades importantes de β-caroteno, lo que hace de ella una fruta con un gran poder antioxidante. Contiene fibra que se encuentra concentrada en el albedo, entre la pulpa y la piel. Su zumo es el de mayor elaboración y consumo a nivel mundial (Zvaigzne & Karklina, 2013). Se han realizado diversos estudios sobre el efecto que las tecnologías térmicas y no térmicas pueden tener sobre su contenido en compuestos bioactivos (Cortés y col., 2008; Esteve y col., 2009).

La manzana presenta un alto contenido en minerales, fibra y compuestos bioactivos como el ácido ascórbico y compuestos fenólicos con capacidad antioxidante (Wu y col., 2007). El zumo de manzana es el tercero en orden de consumo (después del de naranja y el multisabor) en el mercado europeo (AIJN, 2015). Sin embargo, presenta el inconveniente de pardearse con facilidad, por lo que se han llevado a cabo numerosos estudios para el desarrollo de estrategias capaces de prevenir su pardeamiento (Sun y col., 2015).

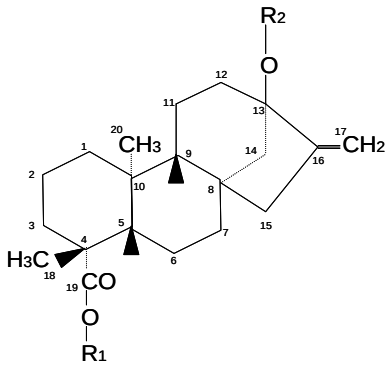
Por otra parte, la avena es un cereal con un índice glucémico bajo, un alto contenido de proteínas (16,9%) y alta digestibilidad. Contiene todos los aminoácidos esenciales, aunque no en la proporción óptima, ya que es relativamente pobre en lisina y treonina, mientras que tiene un exceso de metionina. Aunque la avena contiene alrededor de 7 g/100 g de grasa, predominan los ácidos grasos insaturados (80%), entre los que destaca el linoleico. Presenta también vitaminas del grupo B (niacina, vitamina B6 y ácido fólico), vitamina E, fósforo, y hierro (4,72 mg/100 g). Destaca por su contenido en fibra soluble, cuyo principal componente es el β-glucano, derivado soluble de la celulosa. Esto le confiere un efecto laxante suave, pero sobre todo, capacidad de disminuir el contenido de colesterol (Zheng y col., 2015).

Otro ingrediente es el extracto de açaí (*Euterpe oleracea*). El açaí es el fruto de la palmera originaria de la región brasileña del Amazonas. En los últimos años se ha asociado con beneficios para la salud por su importante contenido en compuestos bioactivos (vitamina C, E y A y compuestos fenólicos) que le confieren una alta capacidad antioxidante y una potencial capacidad antimicrobiana (Schauss, 2010), por lo que se ha incluido en lo que se denominan "superfrutas". En Brasil, forma parte de distintos tipos de bebidas y refrescos y en el mercado europeo y en EEUU, su presencia es creciente. Existe una gran demanda de nuevos sabores y que proporcionen un valor añadido, si es posible, a alimentos ya conocidos y hacerlos más atractivos, como yogures, batidos o helados, y en la industria y comercio alimentarios, el açaí ocupa ese espacio emergente (Yamaguchi y col., 2015).

*Stevia rebaudiana* Bertoni, una planta originaria de la cordillera de Amambay, entre Paraguay y Brasil, utilizada como edulcorante por los indios guaraní durante siglos, también presenta interés como ingrediente en la elaboración de bebidas. Sus hojas constituyen la parte más dulce de la planta (unas 10-30 veces más dulces que el azúcar). Contienen glucósidos de esteviol y un gran número de micronutrientes como flavonoides, ácidos fenólicos, β-sitosterol, vitamina C, hierro, fósforo, calcio, potasio, sodio, magnesio y zinc. En las hojas es donde se encuentran estos compuestos que tienen propiedades beneficiosas. Sin embargo, dado que normalmente se consume en bajas cantidades, su aporte apenas es significativo. Además, los extractos comercializados de stevia son más refinados y su contenido en estos compuestos es menor (Wölwer-Rieck, 2012).

Los glucósidos de esteviol son las moléculas responsables del sabor dulce de la stevia y son entre 100 y 300 veces más dulces que la sacarosa. Éstos pueden utilizarse como edulcorantes acalóricos en sustitución de la sacarosa, fructosa o glucosa. Son resistentes al calor y a condiciones ácidas, lo que les permite ser utilizados en alimentos y bebidas sin apenas degradación. Su esqueleto está compuesto por el esteviol, cuyo grado de glucosilación da lugar a los distintos glucósidos de esteviol (Figura 1). Los principales son el estevióside (5-10%), el rebaudiósido A (2-4%), el rebaudiósido C (1-2%) y el dulcósido A (0,5-1%). Ya en menor proporción se encuentran el esteviolbiósido, el rubusósido y los

rebaudiósidos B, D, E y F (Gasmalla y col., 2014). Se ha visto que el esteviósido y el rebaudiósido A no se absorben, sino que se convierten en esteviol en la flora intestinal. En los seres humanos y ratas, el esteviol se convierte rápidamente en el glucurónido, y éste no se metaboliza sino que es excretado (Renwick & Tarka, 2008). Por tanto, son acalóricos y no proporcionan valor energético.




**Esteviósido** — **R1: Glu**, **R2: Glu-Glu**

**Rebaudiósido A** — **R1: Glu**, **R2: Glu-Glu** (**Glu**)

**Rebaudiósido C** — **R1: Glu**, **R2: Glu-Rha** (**Glu**)

**Rebaudiósido F** — **R1: Glu**, **R2: Glu-Xyl** (**Glu**)

**Figura 1.** Estructura química de los glucósidos de esteviol.

El uso en alimentos de edulcorantes derivados de stevia se ha permitido durante años en América del Sur y en varios países de Asia, incluyendo China, Japón y Corea del Sur. Más recientemente, se ha aprobado su uso en México, Australia, Nueva Zelanda y Hong Kong. En Estados Unidos, desde 1995 se han utilizado glucósidos de esteviol como suplementos dietéticos, y desde 2008, la FDA (Food and Drug Administration) reconoce su estatus de GRAS (Generally Recognised As Safe) y se permite su uso como edulcorante en alimentos, concretamente, los glucósidos de esteviol o el rebaudiósido A con un nivel de pureza superior al 95% (FDA, 2008). Se permite específicamente para alimentos que se producen en gran volumen, como productos horneados y bebidas no alcohólicas y se excluye su uso en productos cárnicos y aves de corral, así como en preparados para lactantes. En 2008, la JECFA (Joint FAO/WHO Expert Committee on Food Additives) aprobó una ingesta diaria admisible (IDA) de 0-4 mg/kg peso corporal, expresada como esteviol (JECFA, 2008). En Europa, en Noviembre del

2011 se autorizaron los glucósidos de esteviol como aditivos alimentarios (E 960) (EC, 2011). Esto no incluye las hojas, que deben considerarse nuevos alimentos, todavía pendiente de autorización.

En el año 2014, se consumieron unas 4670 toneladas de glucósidos de esteviol, un 14% superior al año anterior (Zenith International, 2014). Los principales países productores son China y Japón (80%), mientras que la India y América del Sur comparten el resto de la producción. En Japón, donde el consumo de edulcorantes artificiales como la sacarina o ciclamato está limitado ante la sospecha de su efecto cancerígeno, su uso representa actualmente el 41% de mercado de edulcorantes (Zenith International, 2014). Además, los glucósidos de esteviol ya se incluyen como ingredientes en una amplia variedad de productos en la industria alimentaria (Figura 2): bebidas como zumos y refrescos, productos lácteos, salsas, confitería, galletas, etc. y productos bajos en calorías (Nachay, 2015).


**Figura 2.** Productos comercializados a nivel mundial que contienen *Stevia rebaudiana* en su composición.

## 1.2. Compuestos bioactivos

Hoy en día, el efecto beneficioso de las frutas y verduras en la prevención de enfermedades crónicas se atribuye principalmente a sus compuestos bioactivos, que se definen como constituyentes fitoquímicos presentes en los alimentos capaces de modular procesos metabólicos y de esta forma mejorar la salud de las personas. Son sintetizados en pequeñas cantidades en la naturaleza y entre sus funciones destacan su capacidad antioxidante, inhibición o inducción de enzimas, inhibición de actividades del receptor, e inducción e inhibición de la expresión génica (Correia y col., 2012). Dentro de este grupo se incluyen una gran variedad de compuestos con distinta estructura química (hidrófilos/lipófilos), distribución en la naturaleza, rango de concentración, lugar de acción, eficacia contra especies oxidativas, especificidad y acción biológica (Porrini & Riso, 2008).

Según su estructura química se pueden agrupar en compuestos fenólicos, vitaminas antioxidantes, derivados de terpeno, compuestos de azufre, fitoesteroles, péptidos y aminoácidos, minerales, ácidos grasos poliinsaturados, fibra dietética, ácido fítico y bacterias lácticas (Gil-Chávez y col., 2013).

### 1.2.1. Compuestos fenólicos

Los compuestos fenólicos son metabolitos secundarios de las plantas formados químicamente por un anillo aromático unido a uno o más grupos hidroxilo. Se pueden clasificar según su estructura química en ácidos fenólicos (ácidos hidroxi-benzoicos y ácidos hidroxi-trans-cinámicos), cumarinas, lignanos, flavonoides (flavonas, flavonoles, flavanonas, flavanololes, flavanoles y antocianidinas), estilbenos, isoflavonoides y polímeros fenólicos (proantocianidinas y taninos hidrolizables) (Craft y col., 2012) (Figura 3).

La presencia de compuestos fenólicos en las plantas es muy variada y depende de factores como la especie vegetal, variedad, parte de la planta, condiciones agroclimáticas y aspectos tecnológicos relacionados con el procesado y conservación. En frutas exóticas, se encuentran en el rango de 13,5 a 159,9 mg ácido gálico/100 g. Su concentración en zumos de frutas oscila entre 2 y 500 mg/L, dependiendo del tipo de fruta, aunque en zumos que contienen naranja su

concentración es mayor (hasta 700 mg/L) (Suna y col., 2013). Su distribución en tejidos vegetales y células varía considerablemente según su estructura química. Se sitúan en el interior de la célula o en la pared celular y son secretados como mecanismo de defensa ante situaciones de estrés de la célula vegetal.

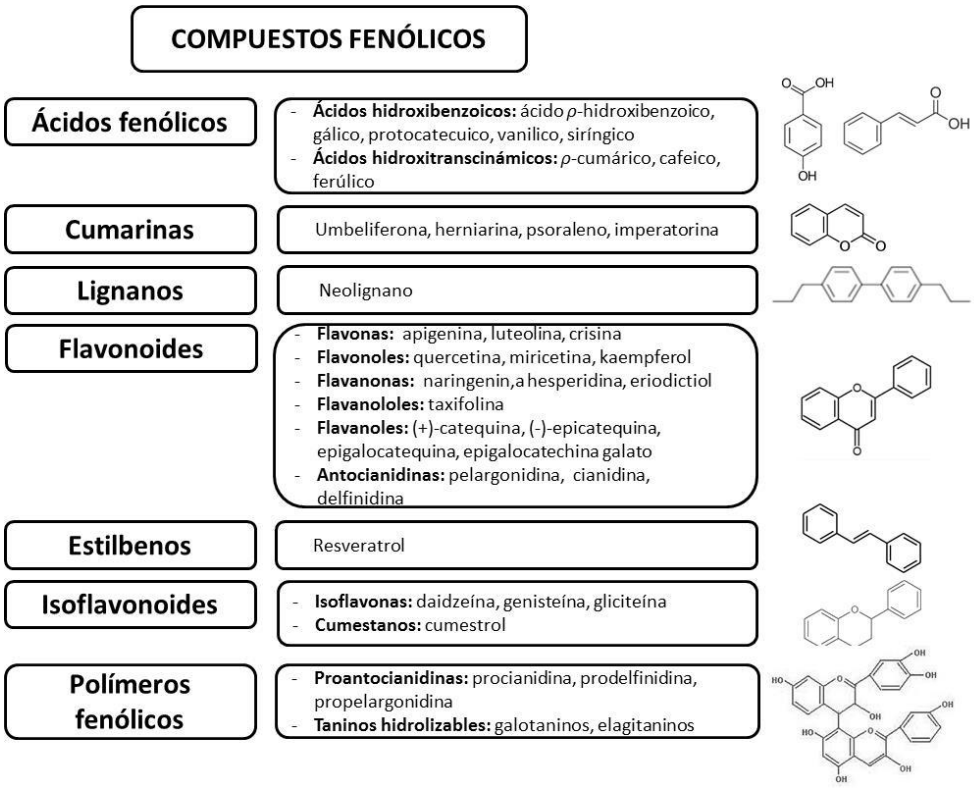



**Figura 3.** Clasificación y estructura de los compuestos fenólicos.

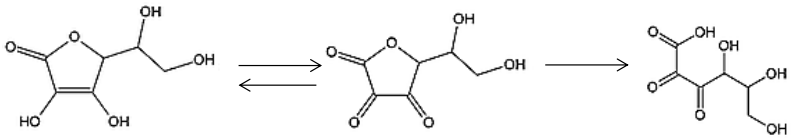
Estos compuestos desempeñan funciones protectoras en las plantas frente a patógenos y depredadores, radiaciones ultravioleta u otras situaciones de estrés físico. También contribuyen al color y características sensoriales de frutas y verduras. En los seres humanos, estos compuestos pueden tener implicaciones sobre su salud, concretamente en la reducción de enfermedades degenerativas como ciertos tipos de cánceres y enfermedades cardiovasculares, efectos atribuidos a sus múltiples propiedades fisiológicas (antialergénicos, antiinflamatorios, antimicrobianos, antioxidantes, antitrombóticos,

antiarterogénicos, vasodilatadores y cardioprotectores) (Heleno y col., 2014). La capacidad antioxidante de estos compuestos depende de su estructura, en particular del número y posición de los grupos hidroxilo y de las sustituciones en los anillos aromáticos. Pueden ejercer su acción a través de varios mecanismos: reaccionando contra radicales libres, protegiendo y regenerando otros antioxidantes de la dieta o por quelación de iones metálicos (Craft y col., 2012). La importancia de estos compuestos también radica en la formación de quinonas cuando son oxidados por la enzima polifenoloxidasa, produciendo el pardeamiento enzimático de los alimentos y afectando así a la calidad de muchas frutas y verduras durante su procesado (Criado y col., 2014).

Los flavanoides constituyen el grupo mayoritario de compuestos fenólicos englobando a flavonas, flavonoles, flavanonas, flavanololes, flavanoles y antocianidinas y desempeñan diferentes funciones. Dentro de este grupo, se encuentran las antocianinas (glucósidos de antocianidinas), que contribuyen al color rojo-azul de muchas frutas y verduras, dependiendo del pH, como uvas, arándanos, cerezas, coles rojas, judías y batatas púrpuras y se han relacionado con numerosas propiedades saludables, entre las que destacan la antiinflamatoria, antioxidante y anticancerígena (Pojer y col., 2013).

### 1.2.2. Ácido ascórbico

El ácido ascórbico posee una estructura de enodiol que se halla conjugada con el grupo carbonilo en el anillo lactona. Los dos átomos de hidrógeno enólicos son los que confieren a este compuesto su carácter ácido y proporcionan los electrones para su función antioxidante. Es un ácido inestable, fácilmente oxidable y se puede destruir en presencia de oxígeno y altas temperaturas. En presencia de oxígeno, el ácido ascórbico se oxida a ácido dehidroascórbico, que tiene su misma actividad vitamínica. Esta actividad se pierde después de la hidrólisis a pH fisiológico del ácido dehidroascórbico a ácido 2,3-dicetogulónico. El término vitamina C incluye ácido ascórbico, ácido dehidroascórbico y sales de ascorbato (Figura 4).



**Ácido ascórbico** **Ácido dehidroascórbico** **Ácido 2,3-dicetogulónico**

**Figura 4.** Estructura química del ácido ascórbico y sus derivados.

El ácido ascórbico es esencial para el ser humano ya que no podemos sintetizarlo por la falta de la enzima gulonolactona oxidasa. La ingesta diaria recomendada para la población española es de 60-90 mg al día (Cuervo y col., 2010), aunque en personas fumadoras o expuestas a tabaco estos requerimientos son superiores. Se encuentra principalmente en frutas y verduras frescas, aunque su contenido varía en función de la variedad, madurez, clima, condiciones del suelo y condiciones de procesado y almacenamiento. Su concentración es elevada en cítricos, pimientos, fresas, kiwis, tomates, brócoli y coles de Bruselas. La ingesta de zumos de frutas proporciona en torno a un 21% de la ingesta diaria recomendada (Almeida, 2014) y en frutas exóticas, se encuentra dentro del rango de 1,2 a 96,3 mg ácido ascórbico/100 g (Almeida y col., 2011).

El ácido ascórbico es termolábil y su concentración disminuye durante su almacenamiento, en función de las condiciones de temperatura, presencia de oxígeno y luz (Esteve y col., 1996). Por tanto, puede utilizarse como indicador de la pérdida de otras vitaminas o compuestos como pigmentos naturales y sustancias aromáticas. Su absorción intestinal se lleva a cabo mediante transporte activo, siendo muy variable la concentración de ácido ascórbico en los diferentes órganos y tejidos.

Se considera uno de los antioxidantes naturales más eficaces y es necesario para el funcionamiento normal del organismo ya que está implicado en muchas funciones fisiológicas y protege el cuerpo de los efectos nocivos de radicales libres y contaminantes. Participa en el crecimiento del tejido y curación de heridas, mantenimiento de la integridad del tejido conjuntivo, especialmente de las paredes capilares, prevención del escorbuto y actúa como cofactor de varias enzimas implicadas en la biosíntesis de carnitina y ciertos neurotransmisores y en

el metabolismo de la tirosina, ácido fólico y triptófano, además de favorecer la absorción del hierro iónico (Grosso y col., 2013).

### 1.2.3. Carotenoides

Los carotenoides son pigmentos liposolubles de color rojo-amarillo ampliamente distribuidos en muchas frutas y verduras que se dividen en carotenos (hidrocarbonos insaturados: α-caroteno, β-caroteno y licopeno…) y xantófilas (derivados oxigenados de los carotenos: luteína, zeaxantina…). Están formados por largas cadenas de dobles enlaces conjugados siendo su estructura de naturaleza isoprénica. Los principales carotenoides presentes en verduras son la luteína y el β-caroteno, mientras que en las frutas las xantófilas se encuentran generalmente en mayor proporción.

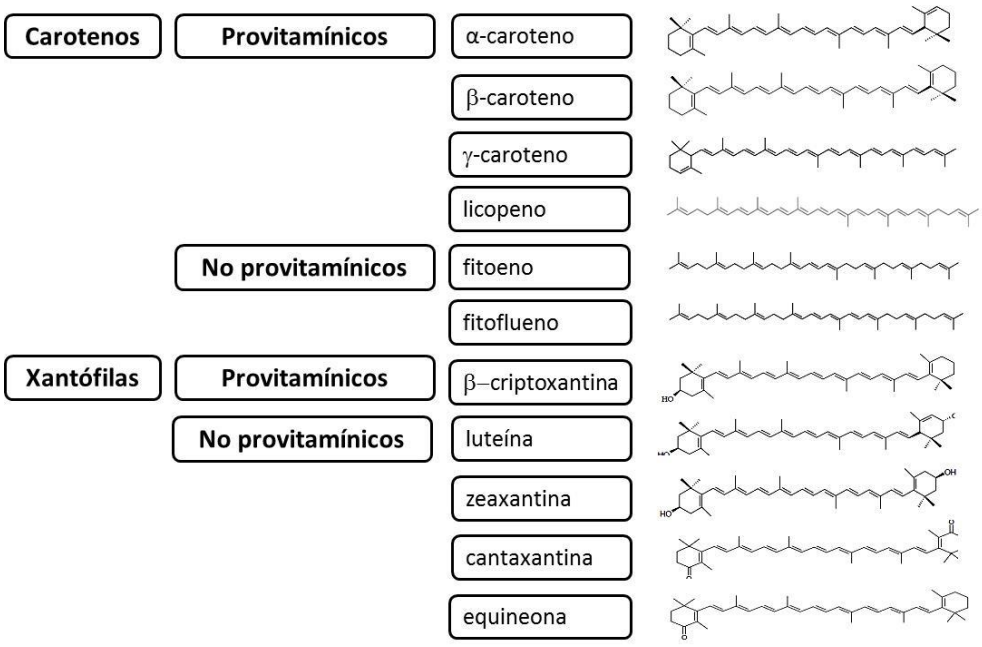



**Figura 5.** Estructura de algunos de los principales carotenoides.

En los seres humanos, algunos carotenoides contribuyen al aporte de vitamina A al ser metabolizados a retinal o retinol (Simons y col., 2015), lo que permite clasificarlos en dos grandes grupos: provitamínicos y no provitamínicos

(Figura 5). El número de carotenoides precursores de la vitamina A oscila entre 50-60, destacando los α, β y γ-carotenos y algunas xantofilas como la β-criptoxantina.

Los carotenoides se absorben por un proceso pasivo en el que influyen varios factores como la ingesta dietética de grasa, el consumo de fibra o el aporte de vitamina A. Tras la ingesta, el 10-50% de los carotenoides se absorben en la mucosa intestinal incorporados en micelas lipídicas junto con otros compuestos lipofílicos (Read y col., 2015). Los quilomicrones son los responsables del ulterior transporte de los carotenoides al sistema linfático. Tras su liberación al torrente sanguíneo, los carotenoides participan en la síntesis de retinoides o se unen a lipoproteínas de muy baja densidad, que posteriormente pasan a lipoproteínas de baja densidad, siendo ésta su principal forma de transporte en sangre.

Dependiendo de la posición de los sustituyentes y los dobles enlaces, los carotenoides presentan dos configuraciones químicas y se denominan E/Z o cis/trans estereoisómeros. Los carotenoides predominantemente se encuentran en la naturaleza en configuración trans, termodinámicamente más estable (Rodríguez-Amaya, 2003). Debido a su insaturación, los carotenoides son propensos a la isomerización y/o la oxidación.

El β-caroteno se absorbe con menos facilidad que el retinol y debe ser convertido a retinal y retinol por el organismo. Por ello se emplea el término equivalente de retinol (ER) que tiene en cuenta la bioeficacia de los carotenoides: 1 ER=1 μg de retinol=12 μg de β-caroteno=24 μg de α-caroteno=24 μg de β-criptoxantina. Se ha estimado una ingesta recomendada de vitamina A de 600-800 equivalentes de retinol por día (Cuervo y col., 2010). Aproximadamente, el 26-34% de la vitamina A proviene de los carotenoides.

El β-caroteno tiene gran importancia desde el punto de vista nutricional dado que es el precursor de la vitamina A. En las células vegetales, los carotenoides protegen de la oxidación y su descomposición. En el organismo humano, actúan como antioxidantes reaccionando contra radicales libres. Se ha demostrado que existe una asociación inversa entre la ingesta de carotenoides y el riesgo de padecer determinados tipos de cánceres (estómago y pulmón), calcificación ósea, degeneración ocular y daño neuronal (Bovier y col., 2014; Kaur y col., 2011). Es

por ello que se recomienda aumentar el consumo de frutas y verduras ricas en carotenoides.

### 1.2.4. Capacidad antioxidante

La mayoría de compuestos bioactivos presentan una marcada actividad antioxidante, que se pone de manifiesto en su capacidad para atrapar radicales de oxígeno, nitrógeno y radicales orgánicos. Un compuesto antioxidante se define como aquel que está presente en una concentración baja con respecto a un sustrato oxidable y es capaz de retrasar o inhibir su oxidación (Brewer, 2011). La actividad biológica de antioxidantes naturales presentes en los alimentos, como el ácido ascórbico, tocoferoles, carotenoides, compuestos fenólicos, aminoácidos, fosfolípidos y esteroles, ha sido ampliamente demostrada *in vitro* e *in vivo* (Heleno y col., 2014; Wan y col., 2015). Las frutas y verduras se caracterizan por tener una alta concentración de estos compuestos y por tanto, una alta capacidad antioxidante.

La capacidad antioxidante de los alimentos depende de muchos factores, entre los que destacan las propiedades coloidales de los sustratos, las condiciones de oxidación y el estado y ubicación de los antioxidantes en los alimentos (Choe & Min, 2009). Dada la complejidad de los sistemas antioxidantes del organismo humano, cada tipo e incluso cada compuesto presenta un mecanismo de acción y una actividad propia, por lo que a la hora de medir la capacidad antioxidante en general, no basta con el empleo de un único método de ensayo, sino que en general, se recurre a la combinación de dos o más métodos de evaluación (Zulueta y col., 2009).

Los métodos empleados para medir la capacidad antioxidante se pueden clasificar en dos grupos, atendiendo a su mecanismo de acción. En primer lugar se encuentran los métodos basados en la transferencia de átomos de hidrógeno. Entre ellos se incluye el método ORAC (Oxygen Radical Absorbance Capacity), que consiste en la determinación fluorimétrica de la disminución de la fluorescencia en presencia de antioxidantes. Además, existen métodos basados en la transferencia de electrones. Entre éstos, se incluyen los métodos de DPPH (2,2-diphenyl-1-picrylhydrazyl) y TEAC (Trolox Equivalent Antioxidant Capacity), que

consisten en la determinación espectrofotométrica para conocer la disminución de la absorbancia de los radicales DPPH y ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) en presencia de antioxidantes.

### 1.3. Tecnologías de conservación no térmicas

Desde hace varias décadas, el tratamiento por calor ha sido la tecnología más empleada en la conservación de alimentos. A través de este proceso se consiguen alimentos microbiológicamente seguros y con baja actividad enzimática. Sin embargo, en ocasiones, conlleva un detrimento de la calidad sensorial y nutricional del producto, causando modificaciones de aroma, cambios de color, desnaturalización de proteínas, cambios físicos y degradación de vitaminas termolábiles.

Este hecho ha llevado a la industria alimentaria a la búsqueda de tecnologías de conservación no térmicas (aquellas que no emplean la temperatura como principal forma de inactivación de microorganismos y enzimas) que aseguren que las cualidades nutricionales y organolépticas de los distintos alimentos no se vean prácticamente afectadas. Si bien es cierto que en la mayoría de estos tratamientos se produce un leve incremento de la temperatura, éste nunca llega a ser tan elevado como en un tratamiento térmico. El objetivo principal de estas tecnologías no térmicas es lograr alimentos que conserven al máximo su calidad sensorial y nutricional sin renunciar a la seguridad alimentaria, como alternativa a los tratamientos térmicos tradicionales y algunas de ellas además ofrecen mejoras en la eficiencia energética y reducción de residuos. Entre dichas tecnologías destacan las altas presiones hidrostáticas, campos eléctricos de alta intensidad, ultrasonidos, irradiación y campos magnéticos oscilantes. En ocasiones se recurre a la aplicación de procesos combinados, donde la asociación o aplicación simultánea de varios procedimientos permite potenciar el efecto de cada uno de ellos y reducir el impacto adverso en las características de los alimentos tratados (Barba y col., 2014).

### 1.3.1. Altas Presiones Hidrostáticas (APH)

La aplicación de altas presiones hidrostáticas (APH), también conocida por las siglas HHP (High Hydrostatic pressure), HPP (High Pressure Processing) y UHP (Ultra High Pressure), se basa en someter un producto a elevadas presiones hidrostáticas (entre 100 y 800 MPa) de forma continua durante un cierto tiempo (desde pocos segundos hasta 20 minutos) junto con un líquido presurizante, normalmente agua, pudiéndose combinar con la aplicación de calor, alcanzando temperaturas que pueden ir desde los 0 °C hasta los 100 °C. Esta técnica se asienta fundamentalmente en dos principios: a) Principio de Le Chatelier, que enuncia que cualquier fenómeno (reacciones químicas, cambios moleculares, etc.) que va acompañado de una disminución de volumen se ve favorecido por la presión y viceversa. Según este principio la aplicación de la alta presión desplaza el equilibrio de un proceso hacia el estado que ocupa menos volumen. b) Ley de Pascal, según la cual una presión externa aplicada a un fluido confinado se transmite de forma uniforme e instantánea en todas las direcciones. Ello evita la deformación del producto, a pesar de estar sometido a tan altas presiones, y hace que éste sea muy homogéneo y no presente zonas sobretratadas. De acuerdo con este último principio, esta tecnología puede aplicarse directamente a alimentos líquidos o a cualquier producto envasado, sumergidos en un fluido de presurización de baja compresibilidad. La presión aplicada al sistema permite un tratamiento isostático y uniforme independientemente del tamaño, forma y volumen del material procesado.

El tratamiento con APH puede afectar, en mayor o menor grado, la viabilidad de microorganismos y la actividad enzimática, así como modificar los componentes de los alimentos y cambiar sus características organolépticas (Terefe y col., 2014). Presiones inferiores a 1000 MPa no afectan a enlaces covalentes, dado que tienen baja compresibilidad, por lo que la estructura primaria de las moléculas no se ve modificada. Sin embargo, sí que contribuyen a la disociación de grupos ácidos de las cadenas laterales de aminoácidos y a la ruptura de puentes salinos intramoleculares (Chakraborty y col., 2014). Asimismo, son capaces de reducir o incluso inhibir reacciones de Maillard relacionadas con la

mala conservación de alimentos. Entre los mecanismos por el que las APH producen estos efectos destacan el aumento de la porosidad y consecuente permeabilidad de las membranas celulares, disminución de la síntesis de ácido desoxirribonucleico y desnaturalización de biopolímeros y proteínas, incluida la inactivación de enzimas por cambios en la estructura intramolecular (>300 MPa). En los microorganismos, la estructura afectada en primer lugar es la membrana celular, con formación de poros en ella o modificación de sus sistemas de transporte, lo que en definitiva implica un intercambio entre el citoplasma y el medio extracelular distinto del intrínseco, que afecta a la homeostasis celular y, en consecuencia, las posibilidades de supervivencia de la célula.

Los equipos de APH empleados en el procesado de alimentos constan de una cámara de presión (cilíndrica de acero de elevada resistencia), un generador de presión (generalmente un sistema de bombeo constituido por una bomba hidráulica y un sistema multiplicador de presión), un sistema de control de temperatura y un software (controlador), tal como aparece en la Figura 6.

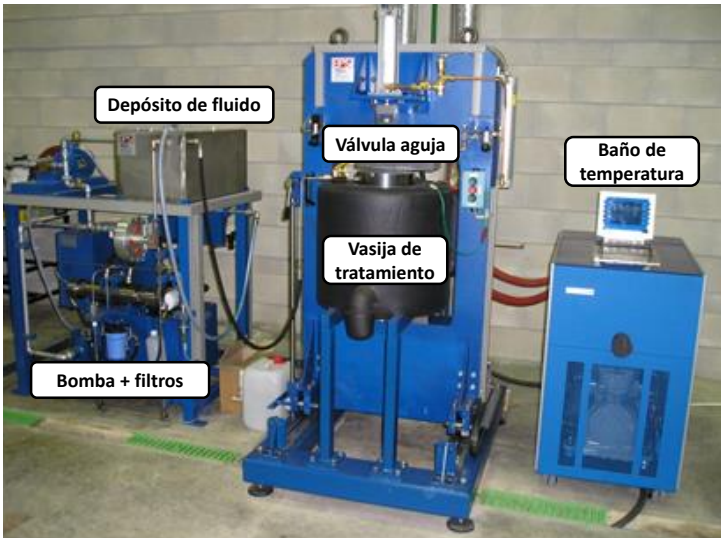



**Figura 6.** Equipo de tratamiento de APH del IATA (CSIC).

Para proceder al tratamiento por APH, se coloca la muestra en el interior de la cámara de presurización. En el caso de alimentos envasados, el envase debe ser

flexible y deformable, tolerando reducciones de volumen de hasta un 15%. Con el fin de asegurar la máxima eficacia de presurización, es especialmente importante la evacuación de gases. Una vez que la cámara se carga con el alimento envasado y se cierra, se llena con el medio de presión-transmisión, generalmente agua. El sistema de bombeo va sustituyendo el aire de la cámara por el fluido de presurización hasta su total llenado y posteriormente, incrementa la presión hasta los niveles establecidos. Una vez alcanzada la presión deseada, una válvula que cierra el circuito permite el mantenimiento de la presión, sin necesidad de aporte adicional de energía, durante el tiempo estipulado. El registro de la temperatura en el interior de la vasija se realiza mediante termopares inmersos en el fluido presurizante. La compresión aumenta uniformemente la temperatura de los alimentos, aproximadamente 3 °C por cada 100 MPa. Un aumento en la temperatura del alimento por encima de la temperatura ambiente aumenta la tasa de inactivación de microorganismos durante el procesado por APH. El tiempo de tratamiento depende del tipo de alimento y de la temperatura del proceso. Una vez finalizado el tiempo de procesado se descomprime la cámara para sacar el alimento tratado (Figura 7).

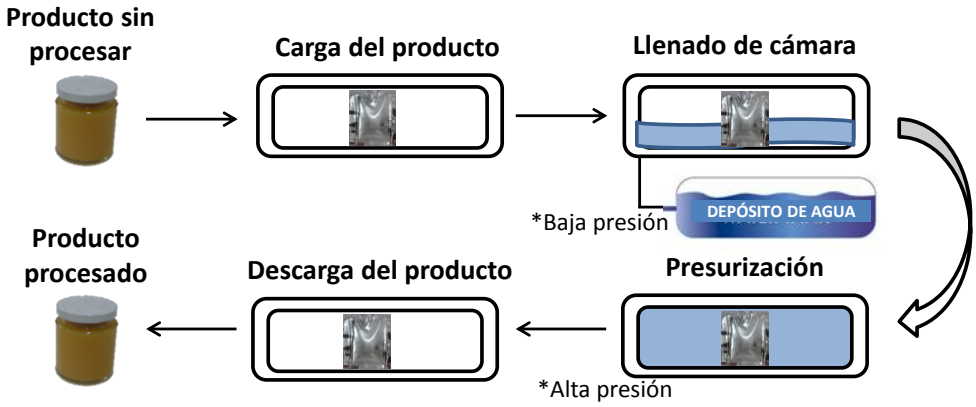



**Figura 7.** Esquema de funcionamiento de un equipo de APH.

En el procesado por APH influyen principalmente los parámetros de tiempo y presión, además de la temperatura del tratamiento, incluyendo la correspondiente al calentamiento adiabático. Además, intervienen otros factores

críticos como el "come-up-time" (tiempo empleado en aumento del nivel de presión hasta la presión de tratamiento), tiempo de descompresión (tiempo requerido para reducir la presión desde el nivel de presión de tratamiento hasta presión atmosférica), temperatura del producto inicial, cambios de temperatura producidos durante la compresión/descompresión (calentamiento/enfriamiento adiabático), pH, composición y actividad de agua del producto, integridad del material de empaquetado y contribuciones del proceso coexistentes. Todos estos factores pueden influir en las características microbiológicas, nutricionales y organolépticas del producto final.

En general, presiones de 400 y 600 MPa en frío (4-10 °C) o a temperatura ambiente producen importantes reducciones (4 unidades logarítmicas) de la mayoría de microorganismos en su forma vegetativa (bacterias, virus, levaduras, mohos y parásitos), mientras que las esporas pueden requerir presiones superiores a los 1000 MPa. La inactivación de esporas es mayor cerca de la neutralidad y más pequeña a niveles extremos de pH. En productos vegetales de pH 4, es necesaria la combinación de los tratamientos de APH con otras tecnologías de conservación como el calor suave o aditivos (Tola & Ramaswamy, 2014). Por ello, con las presiones y temperaturas empleadas en equipos industriales se suele hablar de pasteurización por alta presión, ya que no se destruyen las células vegetativas de los géneros más resistentes o las esporas. Es por esto que los alimentos presurizados comerciales deben mantenerse en refrigeración. Sin embargo, la combinación de presión y temperatura moderada (60-80 °C) o alta (100-110 °C) puede llegar a afectar, parcial o totalmente, a las esporas. En este caso se habla de esterilización por presión y temperatura (pressure assisted thermal sterilisation).

En cuanto a los efectos en los componentes y características de los alimentos, en condiciones habituales de procesado, el efecto de las APH es mínimo, ya que los enlaces covalentes no se ven afectados y en general, los compuestos de bajo peso molecular (vitaminas, compuestos responsables del aroma y pigmentos) no suelen verse modificados. Aunque se produce un ligero aumento de la temperatura durante la aplicación de APH, este aumento no es suficiente para la degradación de estos compuestos. De esta forma, se pueden obtener productos

con propiedades nutricionales y sensoriales prácticamente iguales a la de los productos frescos, satisfaciendo la demanda del consumidor de alimentos nutritivos, saludables, y naturales, además de asegurar su calidad, seguridad y una mayor vida útil. Sin embargo, en ocasiones sí que se pueden producir cambios de color, apariencia y modificaciones de textura, aunque depende en gran medida de la matriz alimentaria y las condiciones de tratamiento (Barba y col., 2012).

La aplicación de APH está aprobada como técnica de pasteurización de productos empaquetados para alimentos de baja acidez en Estados Unidos. En España, la comercialización de productos sometidos a altas presiones está regulada por el Reglamento Comunitario EC 258/97 y EC 424/2001 (EC, 1997, 2002), que contemplan los "nuevos alimentos y nuevos ingredientes alimentarios" y que consideran a los productos presurizados como productos sometidos a pasteurización por presión (baropasteurización). Existe ya una amplia gama de alimentos comercializados, aunque el coste asociado a la instalación de un equipo comercial de alta presión es una barrera importante para su implantación en la industria alimentaria.


**Figura 8.** Productos tratados por altas presiones comercializados en España.

Entre los alimentos comercializados tratados por APH se incluyen alimentos sólidos, preferentemente envasados al vacío (productos cárnicos cocidos o curados, quesos, pescado, marisco, platos preparados, salsas, frutas, mermeladas

y verduras) y alimentos líquidos, en envase suficientemente flexible (productos lácteos, zumos de frutas y preparados nutraceúticos). No son alimentos adecuados para este tipo de tratamiento alimentos sólidos con aire incluido (pan y mousse), alimentos envasados en envases totalmente rígidos (de cristal o lata) ni alimentos con muy bajo contenido en agua (especias y frutos secos). En la Figura 8 se recogen algunos de los productos comercializados actualmente en nuestro país.

En zumos de frutas es una de las técnicas de procesado no térmico más utilizadas en las últimas décadas, ya que puede contribuir a extender su vida útil y conservar su textura, sabor, vitaminas y otros compuestos (Rao y col., 2014). Al ser de naturaleza ácida, la pasteurización de zumos de frutas por APH no requiere temperaturas elevadas, sino que se puede lograr a temperatura ambiente y presión inferior a 300 MPa. A nivel industrial, se suelen aplicar presiones de 400-600 MPa. Mediante esta técnica, los compuestos bioactivos y capacidad antioxidante no se ven prácticamente alterados con respecto al producto fresco, aunque los resultados dependen de las condiciones de procesado y de la matriz alimentaria (Barba y col., 2014). Además, también se ha observado que estos compuestos se conservan mejor durante el almacenamiento refrigerado en comparación con aquellos sometidos a una pasteurización convencional (Liu y col., 2014).

### 1.3.2. Pulsos eléctricos de alta intensidad (PEAI)

La tecnología de PEAI consiste en la aplicación de pulsos eléctricos de corta duración (1- 2500 µs) e intensidades de campo altas (10-80 kV/cm) a un alimento con una conductividad eléctrica adecuada situado entre dos electrodos. Se basa en la propiedad que tienen los alimentos fluidos, compuestos principalmente por agua y nutrientes, de ser buenos conductores eléctricos debido a las altas concentraciones de iones y a su capacidad de transportar cargas eléctricas. La aplicación de PEAI genera la polarización de moléculas bipolares y el movimiento de electrones en el interior de los alimentos, induciendo una corriente eléctrica que puede dar lugar a la destrucción mecánica de la membrana celular de microorganismos, la electrólisis de sustancias y la producción de calor por el

efecto Joule, propiciando la destrucción de microorganismos (Barbosa-Cánovas y col., 2000). Sin embargo, esta técnica se considera un método de procesado no térmico, ya que los alimentos tratados se mantienen a temperatura ambiente o en todo caso a temperaturas inferiores a las de pasteurización (Terefe y col., 2015a).

La destrucción de microorganismos se basa en la deformación o destrucción de la pared celular al aplicar pulsos eléctricos de alta intensidad. Cuando la diferencia de potencial entre ambos lados de la membrana (potencial transmembrana) alcanza un valor crítico, que varía en función del tipo de microorganismo, se originan poros en la membrana (electroporación) y se facilita su permeabilización. Estos poros pueden ser reversibles o irreversibles, según si se excede o no un determinado umbral de intensidad de campo eléctrico.

Aunque el mecanismo de inactivación enzimático no está completamente dilucidado, esta tecnología puede desnaturalizar enzimas, romper sus enlaces covalentes u originar reacciones redox entre los grupos sulfuro y los enlaces disulfuro y con ello, dar lugar a un cambio de conformación con la consecuente pérdida de actividad enzimática (Buckow y col., 2013). Sin embargo, en algún caso, no se han producido modificaciones de la actividad enzimática o incluso ésta ha aumentado con tratamientos suaves (Van Loey y col., 2001). En general, se requieren condiciones más drásticas para la inactivación de enzimas que para la destrucción de microorganismos.

El nivel de inactivación microbiana y enzimática alcanzado depende de una serie de parámetros técnicos (intensidad del campo eléctrico, duración, número y forma del pulso, temperatura y tiempo de tratamiento), de la naturaleza del producto a tratar (pH, conductividad, resistencia y propiedades dieléctricas del alimento) y de la enzima o microorganismo de referencia. La inactivación microbiana aumenta al disminuir la conductividad y la presencia de nutrientes, así como al aumentar la acidez y la temperatura de tratamiento. Aunque esta técnica permite la inactivación de células vegetativas de hongos y bacterias, las esporas bacterianas presentan resistencia a la acción de los campos eléctricos.

Básicamente, un equipo de PEAI consta de un generador de pulsos, una cámara de tratamiento, un sistema de impulsión del alimento y sistemas de control del tratamiento (Figura 9).


**Figura 9.** Equipo de tratamiento de PEAI del IATA (CSIC).

El generador de pulsos está formado por un generador de energía y una serie de condensadores, resistencias e interruptores. El generador de energía es el encargado de suministrar energía eléctrica a un voltaje seleccionado. Para ello transforma la corriente alterna de la red en corriente continua con la que se carga el condensador. Los condensadores son los componentes encargados de almacenar la energía eléctrica que se va a descargar a través del interruptor. El interruptor libera la energía en forma de pulso con las características deseadas. Toda esta combinación de condensadores, resistencias e interruptores se denomina red formadora de pulsos (pulse-forming network). La distinta disposición y número de componentes de cada elemento de la red dan lugar a las distintas formas de los pulsos (Figura 10).

La cámara de tratamiento es el recinto donde se sitúa el alimento entre dos electrodos, uno conectado al condensador a través del interruptor y otro conectado a tierra, separados por un aislante (1-50 mm). Según el régimen de trabajo, las cámaras pueden aplicar tratamientos estáticos o continuos, siendo estos últimos los más indicados para equipos de conservación de alimentos.

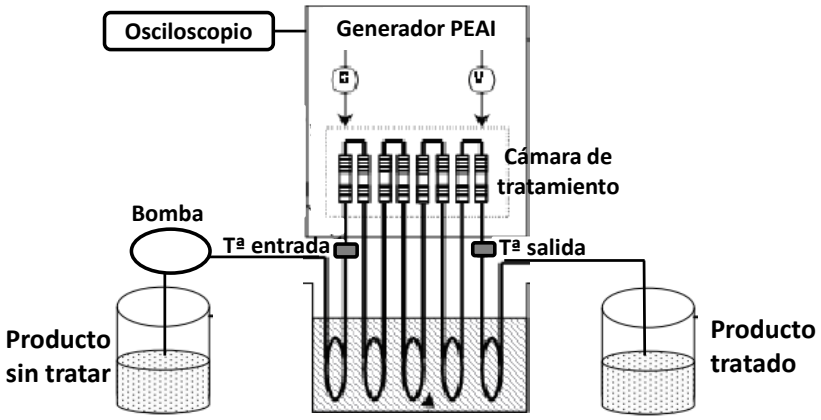



**Figura 10.** Esquema de funcionamiento de un equipo de PEAI.

El sistema de impulsión del alimento permite el suministro del alimento a las cámaras de tratamiento. Además, el equipo debe disponer de un sistema que registre los parámetros del proceso tales como la forma del pulso, número de pulsos, voltaje, intensidad de corriente que circula a través de la cámara de tratamiento, temperatura de tratamiento y caudal del producto en el caso de un proceso en continuo. Para el registro de los parámetros eléctricos, las sondas se conectan a un osciloscopio.

Después de los tratamientos por PEAI, los alimentos deben ser envasados asépticamente y almacenarse refrigerados, para prevenir deterioros enzimáticos o germinación de esporas bacterianas. Hay que destacar que la presencia o formación de burbujas en un alimento es un inconveniente si se quiere aplicar PEAI, ya que si el campo eléctrico supera la resistencia dieléctrica de las burbujas de gas, puede generar problemas durante el procesado.

La aplicación de PEAI se adecua mejor a alimentos líquidos, aunque también se han tratado algunos semisólidos y pulverulentos (Góngora-Nieto y col., 2002). El tamaño de las partículas de los alimentos es una limitación para la aplicación de los pulsos ya que el máximo admisible debe ser menor que el espacio que hay entre los electrodos de la cámara de tratamiento. Además, los alimentos a tratar deben tener baja conductividad eléctrica, fuerza iónica y viscosidad, gran

homogeneidad y bajo riesgo de ruptura dieléctrica. Los zumos de fruta son alimentos idóneos para ser tratados por esta tecnología (Elez-Martínez y col., 2012). Alimentos con una alta conductividad eléctrica reducen la resistencia de la cámara y requieren mayor energía para conseguir un campo eléctrico adecuado.

Frente al tratamiento térmico convencional, esta técnica permite una menor degradación de las características organolépticas y valor nutricional de los alimentos, una menor duración del tratamiento y una mayor eficiencia energética (Guo y col., 2014; Mena y col., 2014; Zulueta y col., 2013). Se ha empleado satisfactoriamente (a escala piloto o de laboratorio) en alimentos líquidos o semilíquidos como zumos de frutas y verduras, leche y derivados, huevo líquido y derivados, vino, cerveza, horchata, salsas y sopas (Espina y col., 2014; Milani y col., 2015; Sharma y col., 2014; Timmermans y col., 2014), aunque también existen varios productos tratados por PEAI comercializados a nivel mundial. En la actualidad, se está investigando la combinación de PEAI con otras tecnologías (antimicrobianos, pH, actividad de agua, temperatura suave…), para incrementar la efectividad de esta técnica (por ejemplo, para inactivar esporas) y permitir su aplicación en diferentes alimentos líquidos.

Esta tecnología también se emplea en la extracción de compuestos de interés en los alimentos (Parniokov y col., 2016). La formación de poros en la membrana celular permite la extracción de sustancias como pigmentos, azúcares y otros compuestos, pudiéndose aplicar PEAI como pretratamiento en la obtención de zumos de frutas ricos en compuestos bioactivos (Bobinaite y col., 2015). También se han empleado en la mejora de procesos de marinado y sazonado y en la obtención de mostos, al reducir el tiempo de maceración e incrementar el color de los vinos y la extracción de componentes intracelulares (Puértolas y col., 2010).

### 1.3.3. Descargas eléctricas de alto voltaje (DEAV)

Esta tecnología (DEAV) consiste en la aplicación de descargas eléctricas de alto voltaje localizadas en un alimento que se sitúa entre dos electrodos, uno de los cuales está conectado a tierra y el otro a un generador de alta tensión, dando lugar a la ruptura eléctrica del agua (descarga electrohidráulica), y con ello una serie de procesos físicos (ondas de choque) y químicos (formación de $O_3$) que

afectan a las células y a los microorganismos y enzimas presentes en alimentos. Aunque los mecanismos de formación de la descarga eléctrica en el agua no están totalmente elucidados, la primera hipótesis, conocida como teoría de la burbuja, supone el desarrollo de burbujas de aire, donde tiene lugar el movimiento de electrones. La segunda hipótesis, conocida como modelo de ionización por impacto directo, postula que no es necesaria la formación de la fase gaseosa, sino que la ruptura del agua se rige por la multiplicación de los transportadores de electrones causados por la ionización del líquido. La descarga eléctrica conduce a la generación de plasmas calientes localizados que emiten radiación UV de alta intensidad, producen ondas de choque que se propagan radialmente en el medio líquido y generan radicales hidroxilo durante la fotodisociación del agua, causando la fragmentación de partículas y dañando la estructura celular (Boussetta & Vorobiev, 2014).

A través de estos fenómenos físicos y químicos, se produce la fragmentación y desintegración celular, dando lugar a la inactivación microbiana e inhibición enzimática y por tanto, a un aumento del tiempo de vida útil del producto tratado. Sin embargo, se necesitan más estudios acerca del efecto de esta tecnología sobre las propiedades sensoriales y nutricionales del producto procesado. A diferencia de los PEAI que resultan en la pérdida de la integridad de las membranas celulares al someter a un campo eléctrico externo (electroporación), las DEAV afectan tanto a las paredes de las células como a las membranas, con lo que se puede causar un mayor daño al producto.

Un equipo de DEAV consta básicamente de un generador de descargas eléctricas, una cámara de tratamiento y equipos de control del tratamiento, tal como se aprecia en la Figura 11. El generador de descargas eléctricas de alto voltaje consta a su vez de un electrodo de aguja y un electrodo de placa conectado a tierra, un banco de condensadores y un interruptor. El producto se coloca en la cámara de tratamiento donde se le suministran las descargas eléctricas. En todo momento se controla la temperatura del alimento durante el tratamiento.

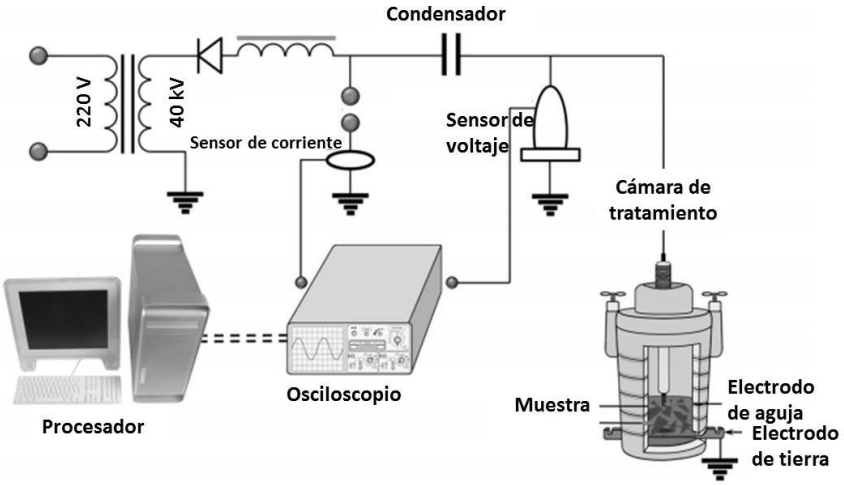



**Figura 11.** Esquema de funcionamiento de un equipo de DEAV (Koubaa y col., 2015).

El proceso de descomposición del agua consta de dos fases: una fase de pre-ruptura (corriente eléctrica) y una fase de descomposición (arco eléctrico) (Boussetta & Vorobiev, 2014). Durante la fase de pre-ruptura, se inicia una corriente eléctrica en un punto localizado al aplicar un campo eléctrico de alto voltaje. Esta corriente eléctrica se propaga hacia el electrodo opuesto a través de canales de plasma ionizados. Cuando la corriente eléctrica alcanza el electrodo opuesto se produce un arco eléctrico (fase de descomposición). Dado que la resistencia del arco disminuye en un tiempo muy corto (ns), la corriente aumenta y el voltaje cae muy rápidamente, entonces, la corriente se ve limitada principalmente por el circuito eléctrico externo. La energía eléctrica total por pulso es la suma de la energía liberada con la propagación de la corriente eléctrica más la energía disipada en el arco.

Además de la ruptura eléctrica del agua, se producen procesos físicos y químicos que incluyen ondas de choque y formación de especies reactivas. La formación de arcos eléctricos está asociada con la emisión de una onda de choque de gran alcance que se multiplica radialmente en el agua. La onda de choque de elevada presión es seguida por una onda de rarefacción que produce cavitaciones.

El colapso de estas cavitaciones crea fuertes choques secundarios de muy corta duración que pueden afectar a las células.

Esta técnica se ha empleado para la extracción de compuestos de alimentos vegetales (Parniakov y col., 2014; Sarkis y col., 2015), inactivación de microorganismos (Butscher y col., 2016), trituración electrohidráulica de sólidos y limpieza de agua con impurezas orgánicas (Lu y col., 2015).

### 1.3.4. Ultrasonidos (USN)

Los ultrasonidos son ondas sonoras con una frecuencia superior a la perceptible por el oído humano, mayor de 20 kHz. Estas ondas, a su paso por los alimentos, producen fenómenos de cavitación (formación, crecimiento e implosión de diminutas burbujas de gas en el líquido), colapso de burbujas (al producir extremos incrementos de temperatura y presión en puntos localizados) y sonólisis (formación de radicales libres oxidantes), provocando daños en las membranas de los microorganismos y su destrucción. Por tanto, esta técnica se puede emplear en la conservación de alimentos dado que es capaz de inactivar microorganismos en forma vegetativa y reducir la resistencia térmica de esporas a través de fenómenos de cavitación.

En general, la frecuencia de los USN se encuentra en el intervalo de 20 kHz-10 MHz, dando lugar a tres regiones: USN de baja frecuencia y alta potencia (20-100 kHz), USN de frecuencia intermedia y media potencia (100 kHz-1 MHz) y USN de alta frecuencia y baja potencia (1-10 MHz). En función de los requisitos del tratamiento, se seleccionará un rango de frecuencia u otro. Para la conservación de alimentos suelen emplearse ondas de baja frecuencia (18-100 kHz; $\lambda$=145 nm) y alta intensidad (10 a 1000 W/cm$^2$) (Chandrapala & Leong, 2015).

Ahora bien, el empleo de USN como única técnica de conservación en muchas ocasiones es insuficiente para la inactivación microbiana, ya que se requieren niveles de intensidad muy elevados, lo que puede repercutir negativamente en las propiedades nutricionales y sensoriales de los alimentos. Es por ello que esta técnica se suele asociar a otros métodos de conservación como calor, alta presión, antimicrobianos naturales o luz ultravioleta (Gabriel, 2015). La combinación de USN con temperaturas moderadas inferiores a 100 °C (termosonicación), presión

inferior a 500 MPa (manosonicación) y combinación de USN, calor y presión (manotermosonicación) han sido efectivas en la inactivación microbiana y enzimática (Lee y col., 2013).

Un equipo de USN consta básicamente (Figura 12) de un generador eléctrico, un transductor, una cámara de tratamiento y un sistema de control. El generador produce pulsos de energía de alto voltaje a la frecuencia establecida en el transductor. El transductor piezoeléctrico trasforma la energía eléctrica en oscilaciones mecánicas. Existe otro tipo de transductor, denominado magnetoestrictivo, que se basa en generar distorsiones mecánicas en los alimentos cuando se les somete a un campo magnético intenso, aunque éste es menos frecuente. La onda ultrasónica se transmite a través del líquido, generalmente agua, en el que se encuentra inmerso el producto a tratar. El sistema de control es el encargado de asegurar que la temperatura alcanzada no sobrepase un determinado valor.

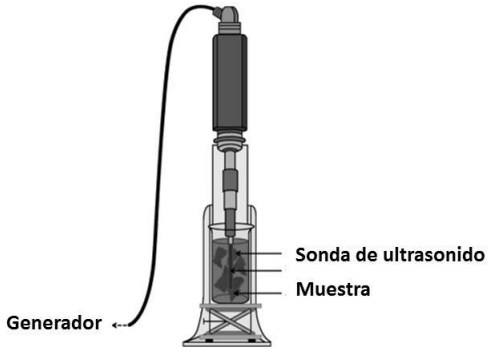



**Figura 12.** Esquema de un equipo de ultrasonidos.

En un medio líquido, los ultrasonidos generan ciclos alternativos de compresión y expansión. Cuando la presión negativa en el líquido, creada por el ciclo de expansión alternativo, es baja y supera las fuerzas intermoleculares (fuerza de tensión), aparecen pequeñas burbujas de gas inmersas en la matriz líquida tratada. Tras sucesivos ciclos, dichas burbujas crecen, alcanzan un tamaño crítico y, al superarlo, se colapsan. Al chocar entre sí las moléculas del líquido, como consecuencia del colapso, se producen ondas de presión que se transmiten por el medio, inactivando bacterias y disgregando la materia en suspensión,

fenómeno que se conoce como cavitación. Uno de los fenómenos resultantes de la cavitación acústica es el aumento de temperatura en puntos localizados alrededor de la burbuja, además de turbulencias, fuerzas de cizallamiento, choques de ondas, presiones altas en puntos localizados y generación de radicales libres altamente reactivos (Chandrapala & Leong, 2015).

Entre los parámetros que definen las ondas ultrasónicas destacan la amplitud, que es el valor máximo que toma una magnitud oscilante en un semiperiodo y la frecuencia, referida al número de oscilaciones o vibraciones de un movimiento por unidad de tiempo. La frecuencia es inversamente proporcional al tamaño de burbuja formado. Por tanto, el empleo de USN de baja frecuencia genera grandes burbujas de cavitación y altas temperaturas y presiones en la zona de cavitación. A medida que la frecuencia aumenta, el mecanismo principal es la transmisión acústica.

La eficacia del proceso depende de factores técnicos (frecuencia, potencia de irradiación y tiempo de tratamiento), del alimento a tratar (volumen, composición, pH, presencia de gases disueltos y tensión superficial) y del microorganismo o enzima en cuestión. La fuerza de tensión de líquidos puros es muy alta y difícil de superar. Sin embargo, la mayoría de alimentos líquidos contienen burbujas de gas que pueden actuar como núcleos de cavitación.

Puede considerarse como técnica de pasteurización de alimentos ya que cumple con el requisito de la FDA de alcanzar una reducción de 5 ciclos logarítmicos de *Escherichia coli* en zumos de frutas (Patil y col., 2009). Sin embargo, las formas esporuladas son muy resistentes a la acción de los ultrasonidos y se requieren horas para su inactivación (Ferrario y col., 2015). Respecto a la inactivación enzimática, los estudios son contradictorios, ya que pueden producirse activaciones e inactivaciones dependiendo de diversos factores, entre ellos la estructura molecular de la enzima (Terefe y col., 2015b).

Esta técnica se ha empleado con éxito en la pasteurización de la leche. La leche tratada muestra un mayor grado de homogenización, color blanco y mejor estabilidad después del procesado. Empleando esta técnica, la pasteurización y homogeneización se completan en un solo paso, lo que resulta provechoso para minimizar problemas de sinéresis de algunos productos lácteos como el yogur o el

queso fresco (Bermúdez-Aguirre & Barbosa-Cánovas, 2010). También se ha investigado su aplicación en zumos de frutas, intentando alcanzar la letalidad de una pasteurización y una esterilización sin que las características organolépticas se vean modificadas (Martínez-Flores y col., 2014). Se ha observado que el contenido de ácido ascórbico apenas se ve modificado en zumos de frutas tratados por USN e incluso su estabilidad es mayor durante el almacenamiento en comparación con los tratados térmicamente. Este efecto se atribuye a la eliminación del oxígeno ocluido en los zumos (Knorr y col., 2004), dado que éste es un parámetro crítico que influye en la degradación del ácido ascórbico.

Los ultrasonidos de alta intensidad se emplean para limpieza de equipos (Tuziuti, 2016), desgasificado de líquidos, homogeneización (Zisu & Chandrapala, 2015), inducción de reacciones de oxidación/reducción, extracción de compuestos alimentarios (Parniakov y col., 2015) e inducción de la nucleación durante la cristalización (Cogné y col., 2016). Hasta el momento, se han desarrollado equipos a escala semi-industrial e industrial encaminados a la eliminación de espumas y deshidratación de vegetales.

### 1.4. Ingredientes naturales con capacidad antimicrobiana

Los aditivos de origen natural incluyen una gran variedad de compuestos antimicrobianos naturales (lisozimas, aceites aromáticos, compuestos fenólicos…) que inhiben el crecimiento de ciertos microorganismos alteradores y patógenos. Constituyen una alternativa para ofrecer productos sanos y seguros, ante la creciente inquietud de los consumidores en cuanto al uso de conservantes químicos artificiales (Calo y col., 2015). Estas sustancias, además de ser ingredientes que actúan en la formulación del alimento, aportan un cierto carácter funcional y a su vez pueden actuar previniendo el desarrollo de microorganismos de riesgo (Pina-Pérez y col., 2014). La gran ventaja que tiene usar ingredientes naturales con capacidad antimicrobiana es su estatus legal. Su uso en alimentación está autorizado y aunque muchas veces su fin no es contribuir a la inocuidad del alimento, sí es interesante aprovechar su capacidad antimicrobiana como medida de control adicional (Sanz-Puig y col., 2015).

Muchos de estos ingredientes pueden reducir de 1 a 2 ciclos logarítmicos el crecimiento microbiano, pero tienen que actuar sinérgicamente con otros tratamientos térmicos o no térmicos. Dependiendo del tipo de material vegetal utilizado (especias, extracto o aceite esencial), origen (país, altitud a la que crece, temporada de cosecha), proceso de producción, grado de pureza y conservación, el efecto antimicrobiano puede verse intensificado o disminuido (Sánchez & Aznar, 2015). Además, la actividad antibacteriana de estos ingredientes varía de acuerdo con el tipo de microorganismo y se ve influenciada por la temperatura de almacenamiento del alimento. Las bacterias Gram positivas son generalmente más sensibles a los antimicrobianos naturales que las Gram negativas. Se ha demostrado que el uso de altas concentraciones de antimicrobianos naturales puede prolongar considerablemente la fase de latencia de microorganismos e incluso su inhibición (Belda-Galbis y col., 2015).

Los antimicrobianos naturales están compuestos por diferentes grupos químicos, por lo que su actividad antimicrobiana no se debe a un mecanismo específico, sino a la acción combinada de varios de ellos sobre distintas localizaciones de la célula bacteriana (Farzaneh & Carvalho, 2015). Se ha atribuido a los compuestos fenólicos gran parte del poder antimicrobiano de las sustancias naturales, aspecto relacionado a su vez con las características de sus grupos hidroxilo (He y col., 2014). Los compuestos fenólicos son capaces de causar la degradación de la pared celular, daño en la membrana citoplasmática y en las proteínas de membrana, pérdida del contenido intracelular al exterior, coagulación del citoplasma y pérdida de la fuerza motriz de protones, dando lugar a la inactivación bacteriana.

Entre los distintos ingredientes con actividad antimicrobiana de origen vegetal se encuentran los aceites esenciales, también conocidos como aceites volátiles, como los de canela, orégano, romero, albahaca, y subproductos cítricos, así como compuestos activos específicos como el timol, eugenol, carvacrol y geraniol, entre otros (Raybaudi-Massilia y col., 2009). También destacan las hierbas y especias como la menta y canela en polvo. Recientemente, se está investigando la actividad antimicrobiana del extracto de stevia y del extracto de açaí para su

potencial uso como ingrediente natural con capacidad antimicrobiana, además de antioxidante (Belda-Galbis y col., 2014; Belda-Galbis y col., 2015).

Cuando se aplican dos tecnologías de conservación de alimentos conjuntamente, se puede producir un efecto sinérgico y conseguir un mayor efecto que la simple suma de efectos atribuidos a cada acción por separado. Este hecho se ha observado también en el uso de ingredientes naturales combinados con otras técnicas de conservación de alimentos, demostrando la aparición de sinergismos sobre la inactivación microbiana, reduciendo la necesidad de tratamientos más severos y aumentando la efectividad del conjunto (Pina-Pérez y col., 2013). El efecto sinérgico entre tecnologías no térmicas de conservación y sustancias naturales con propiedades antimicrobianas además de conferir efectos saborizantes y aromatizantes, abre un campo potencial de trabajo hacia posibles tratamientos más suaves combinados con concentraciones moderadas de estas sustancias de gran aceptación entre los consumidores (Martín-Belloso & Sobrino-López, 2011).

### 1.5. Bioaccesibilidad y biodisponibilidad

La bioaccesibilidad se refiere a la cantidad o fracción de un compuesto que se libera de la matriz alimentaria en el tracto gastrointestinal, quedando disponible para su absorción intestinal e incorporación al torrente sanguíneo (Heaney, 2001). Esta definición incluye todos los procesos que tienen lugar durante la digestión del alimento, absorción/asimilación de las células epiteliales y el metabolismo pre-sistémico (tanto intestinal como hepático). El término biodisponibilidad incluye también en su definición la utilización del nutriente y por tanto, se puede definir como la fracción del compuesto o nutriente ingerido que alcanza la circulación sistémica y ejerce su acción. Es decir, la biodisponibilidad incluye la digestión gastrointestinal, absorción, metabolismo, distribución tisular y bioactividad. La bioactividad, por su parte, es el efecto específico que provoca la exposición al compuesto e incluye la captación tisular y la respuesta fisiológica consiguiente (Fernández-García y col., 2009).

En el estudio de la biodisponibilidad existen factores intrínsecos (fisiológicos) y extrínsecos (dietéticos) al organismo que pueden modificarla en función de interacciones (sinérgicas o antagónicas) que tengan lugar. Entre los factores intrínsecos se encuentran la variabilidad interindividual, edad, estado fisiológico y nutricional, flora intestinal y capacidad individual de adaptación a aportes variados de nutrientes, susceptibles de influir en su disponibilidad para su posterior absorción y metabolismo. Entre los factores extrínsecos se incluyen el aporte total de la dieta, forma química en la que se encuentra el compuesto, solubilidad e interacción con otros componentes del alimento o de la dieta. Además, debe tenerse en cuenta la estructura química de los compuestos alimentarios, ya que condiciona en gran medida su posterior biodisponibilidad (Velderrain-Rodríguez y col., 2014).

Los métodos empleados en los estudios de bioaccesibilidad y biodisponibilidad pueden clasificarse en dos grandes grupos*: in vivo* e *in vitro*. En general, la bioaccesibilidad suele evaluarse a través de procedimientos *in vitro*, mientras que la biodisponibilidad se evalúa *in vivo*.

### 1.5.1. Métodos *in vivo*

Los métodos *in vivo* estudian la biodisponibilidad de un compuesto mediante su administración a un organismo vivo. Dado que los factores fisiológicos del organismo humano son difíciles de reproducir en el laboratorio, se puede decir que los métodos *in vivo* proporcionan la mejor estimación de la biodisponibilidad de nutrientes a partir de alimentos. Entre dichos métodos se encuentran el balance químico, el empleo de isótopos y los ensayos con animales. En este último caso, el principal inconveniente radica en la dificultad de extrapolar los resultados al hombre, dadas las diferencias entre el metabolismo animal y humano.

En el caso de los compuestos bioactivos, la evaluación *in vivo* de su biodisponibilidad se lleva a cabo de forma indirecta, mediante el incremento de la capacidad antioxidante en plasma tras el consumo de alimentos ricos en estos compuestos o de forma directa midiendo su concentración en plasma u orina tras la ingesta tanto de compuestos puros como de alimentos con una cantidad conocida de los compuestos de interés (Heleno y col., 2014). Ahora bien, muchos

de estos compuestos bioactivos sufren modificaciones a lo largo del proceso de digestión, absorción y distribución, por lo que las formas que alcanzan la sangre y tejidos difieren de su forma original en la que se encuentran en los alimentos, dificultando su identificación.

### 1.5.2. Métodos *in vitro*

Los sistemas *in vitro* simulan las condiciones fisiológicas y son útiles para predecir situaciones *in vivo*. Estos estudios presentan como ventajas un menor coste, sencillez, rapidez, mejor control de las variables experimentales, además de permitir el barrido de múltiples muestras. Todo ello ha propiciado su creciente uso en la evaluación de la bioaccesibilidad de distintos componentes alimentarios.

Mediante los estudios *in vitro* se pueden simular las condiciones fisicoquímicas del tracto gastrointestinal (pH, temperatura y tiempo de incubación) y evaluar las posibles transformaciones de los compuestos presentes en el alimento, así como las interacciones entre los distintos componentes que puedan tener lugar durante su tránsito intestinal. Sin embargo, las estimaciones que proporcionan estos métodos son relativas y no absolutas, dado que no tienen en cuenta todos los factores fisiológicos del organismo (estado nutricional, secreción gastrointestinal, flora intestinal, transporte activo, interacciones con la mucosa, cinética del tránsito intestinal, etc.).

Se basan, generalmente, en la simulación del proceso de digestión gastrointestinal humano. Consiste en un tratamiento secuencial enzimático en etapas, comenzando con la salivar, con la adición de α-amilasa y mucina a un pH en torno a 6,5-7, seguida de la etapa gástrica, con la adición de pepsina a un pH entre 1,8 y 2,5 y finalmente la etapa intestinal, en la que se añaden pancreatina y sales biliares a pH 5-6,5. Tras este procedimiento, se estima la fracción del compuesto de interés soluble o dializable a través de una membrana de determinado tamaño de poro (fracción bioaccesible).

Los métodos basados en la solubilidad determinan la cantidad del compuesto que se encuentra en el sobrenadante obtenido por centrifugación o filtración (Brandon y col., 2014). De este modo se estima la fracción máxima del compuesto ingerido disponible para ser absorbido.

Los métodos basados en la diálisis incorporan una membrana de diálisis durante el proceso de digestión intestinal que simula la difusión pasiva a través del epitelio intestinal. El proceso de diálisis puede llevarse a cabo en equilibrio (Miller y col., 1981) o en continuo (Promchan & Shiowatana, 2005). Estos métodos proporcionan información relativa a la fracción del componente soluble que se encuentra potencialmente disponible para la absorción, con tamaño inferior al de poro de membrana empleada.

En ocasiones se realizan estudios con cultivos celulares que permiten evaluar la captación y transporte en el epitelio intestinal a partir de la fracción bioaccessible obtenida del alimento. Las más empleadas son las células Caco-2, provenientes de carcinoma de colon, que crecen en monocapas y presentan muchas de las características funcionales y morfológicas de los enterocitos intestinales (Alemany y col., 2013).

Los métodos *in vitro* son difíciles de extrapolar *in vivo* y no reproducen todas las condiciones dinámicas y mecanismos de homeostasis del organismo humano. A pesar de esta limitación, los estudios *in vitro* de compuestos bioactivos proporcionan datos útiles que permiten establecer comparaciones entre distintos alimentos, así como determinar el efecto causado por diversos factores en la bioaccesibilidad de los compuestos bioactivos (Rodríguez-Roque y col., 2013), por lo que son los procedimientos más empleados cuando se realiza un estudio de bioaccesibilidad de compuestos bioactivos.

Existen numerosos estudios para determinar los factores críticos de la bioaccesibilidad de compuestos bioactivos, de gran importancia cuando se formula una matriz alimentaria. Así, el contenido de grasa y el perfil lipídico parecen ser determinantes en la absorción de carotenoides (Anese y col., 2015). El procesado del alimento (por tecnologías térmicas y no térmicas) también influye en la bioaccesibilidad de carotenoides, compuestos fenólicos y ácido ascórbico (Fonteles y col., 2016; Wang y col., 2014). Estos estudios ponen de manifiesto la necesidad de obtener información sobre la concentración de compuestos bioactivos que está disponible para ejercer su función biológica en el organismo humano, y no sólo su concentración en el alimento correspondiente.

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# 2. Objectives

*Objetivos*

## 2. OBJETIVOS

El **objetivo general** del presente trabajo es el estudio de compuestos bioactivos y parámetros de calidad en bebidas a base de frutas adicionadas de stevia, sus modificaciones tras el procesado por tecnologías no térmicas así como su bioaccesibilidad.

Para alcanzar este objetivo general se plantean los siguientes **objetivos específicos**:

1. Formulación de bebidas a base de frutas (papaya, mango, naranja) con un alto contenido de compuestos bioactivos y capacidad antioxidante, adicionadas o no de stevia.
2. Evaluación del efecto de la aplicación de distintas tecnologías no térmicas (APH, PEAI, DEAV y USN) sobre compuestos bioactivos y capacidad antioxidante de las bebidas formuladas.
3. Estudio de la combinación de los PEAI y congelación para obtener un zumo de fruta con un alto contenido en compuestos bioactivos.
4. Evaluación de la bioaccesibilidad de compuestos bioactivos y capacidad antioxidante en las bebidas formuladas a base de frutas adicionadas o no de stevia.

## 2. OBJECTIVES

The **overall objective** of this work is the study of bioactive compounds and quality parameters from fruit based beverages with stevia, their changes after non-thermal technologies processing and their bioaccessibility.

To reach this goal, the following **specific objectives** are proposed:

1. Formulation of fruit based beverages (papaya, mango, orange) with a high content of bioactive compounds and antioxidant capacity, with or without stevia addition.
2. Evaluation of the effect that different non-thermal processing technologies (HPP, PEF, HVED and USN) exert on the bioactive compounds content and antioxidant capacity of the formulated beverages.
3. Assessment of the combination of PEF and freezing in order to obtain a fruit juice with a high content of bioactive compounds.
4. Evaluation of the bioaccessibility of bioactive compounds and antioxidant capacity in the formulated fruit based beverages with or without stevia addition.

# 3. Results

*Resultados*

## 3.1. Emerging role of *Stevia rebaudiana* Bertoni as source of natural food additives

*Leaf sweeteners: resources, processing and health effects, Wenbiao Wu*
*New York: Nova Publishers (2014) 59-76*

# Emerging role of *Stevia rebaudiana* Bertoni as source of natural food additives

Juana M. Carbonell-Capella, María J. Esteve, Ana. Frígola*

University of Valencia, Department of Nutrition and Food Science, Av. Vicent Andrés Estellés, s/n, 46100 Burjassot, Spain

*Corresponding author:
Ana Frígola
e-mail: ana.frigola@uv.es
Phone: +34 963544955, Fax: +34 963544954

**ABSTRACT**

*Stevia rebaudiana* leaf extract, used as a vegetable-based sweetening additive in drinks and other foods due to steviol glycosides content, has been demonstrated to exhibit extremely high antioxidant capacity due to its high content in potential antioxidant food compounds such as phenolic compounds. However, concentration of bioactive compounds and total antioxidant capacity in stevia products may depend on the origin of the product. For this reason, stevia leaves direct infusions, stevia crude extract (Glycostevia-EP®), purified steviol glycosides (Glycostevia-R60®), and commercialised stevia powdered samples in different countries (PureVia, TruVia and Stevia Raw) were evaluated for their content in ascorbic acid (AA), total carotenoids (TC), total phenolic content (TPC), phenolic profile, total anthocyanins (TA), steviol glycosides profile, and antioxidant capacity (trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC)). Eleven phenolic compounds, including hydroxybenzoic acids (2), hydroxycinnamic acids (5), flavones (1), flavonols (2) and flavanols (1) compounds, were identified in stevia-derived products. Of these, chlorogenic acid was the major phenolic acid. Rebaudioside A and stevioside were the most abundant sweet-tasting diterpenoid glycosides. Total antioxidant capacity (TEAC and ORAC) was obtained to be correlated with TPC. From all of the analysed samples, stevia leaves direct infusions and stevia crude extract (Glycostevia-EP®) were found to be a good source of sweeteners with potential antioxidant capacity.

**Keywords:** *Stevia rebaudiana*, food additives, steviol glycosides, phenolic compounds.

## 1. Introduction

In recent years, growing awareness in human health, nutrition and disease prevention has enlarged consumers' demand for functional foods with a high nutritional and sensory quality. Food industry has shown increased interest in plant food materials, as they can be a useful tool in order to provide new food products of proven nutritional quality, thus increasing added value [1-3].

New products with functional properties based on exotic and innovative ingredients are becoming common in Europe and the North American market, with a good consumer acceptance and a high nutritional value, largely due to its high content in bioactive compounds and antioxidant capacity. Demand for these products is growing and thus, a thorough study on the characteristics and benefits attributed to such ingredients is necessary [4].

Recently, there has been an increasing interest in the use of a natural sweetener obtained from the leaves of the plant called *Stevia rebaudiana*, which contain twelve known leaf sweetening diterpenic glycosides (200 times sweeter than sucrose), as it can be a nutritional strategy in order to replace or substitute sugar energy content with one or more ingredients of low-calorie content [5]. Stevia has attracted economic and scientific interests due to the sweetness and the supposed therapeutic benefits of its leaf. FDA approved stevia for commercialisation in 2008 and more recently, in November 2011, the European Commission has approved steviol glycosides as a new food additive (E 960) [6-7]. In recent years, food industry is developing an array of new products based on stevia plant extracts in order to satisfy the demand of consumers concerned with healthier eating. Many of these new low-sugar products are not just the old standbys like diet sodas and sugarless gum, but foods and drinks like cereals, fruit juices, cookies, bread, ice cream, flavoured milk, pasta sauce and even bottled water [8]. The products may range from crude stevia extracts to Reb A, which is a highly purified ingredient that contains the best-tasting component of the stevia leaf. In Europe, the recent green light will probably lead to wide-scale use [9]. So far, little data has been available regarding the practical applications in foods [10].

*Stevia rebaudiana* yields a sweet aqueous extract containing various glycosides. Coca-Cola Company and Cargill use stevia in Japan for its Diet Coke and are seeking exclusive rights to develop and market *Stevia rebaudiana* derived sweetener rebaudioside A, Truvia, for use in drinks [11]. Furthermore, no significant photodegradation in acidic beverages containing rebaudioside A or stevioside, when exposed to light, has been reported. Stevioside is stable during different processing and storage conditions, which is essential for its effective application in processed beverages [12].

Moreover, *Stevia rebaudiana* water extracts have been demonstrated as a good source of antioxidant additives such as vitamin C and phenolic compounds [13] which can serve as potential additives for preventing quality deterioration or to retain the quality of different food products [14] and are beneficial components which have been implicated in the reduction of degenerative human diseases, mainly because of their antioxidant potential [15-17]. Moreover, these bioactives can be used as natural food additives. Due to the growing popularity of phenolic antioxidants over the past 2 decades, an increasing interest in determining the antioxidant activities exhibited by phenolic acids and their derivatives should also be noted [18]. Their protective effect can be ascribed to their capacity to transfer electron free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals, and inhibit oxidases [19].

In the literature available at present, there is a lack of information about the natural potential food additives found in *Stevia rebaudiana* products. Thus, at this stage of development, it is necessary to evaluate their content for a promising use of *Stevia rebaudiana* in the formulation of new food products.

## 2. Materials and methods

### *2.1. Samples*

The research was conducted on seven different stevia-derived products. Samples were prepared in accordance with manufacturer's instructions. Stevia leaves, Glycostevia-EP® (GE-EP) and Glycostevia-R60® (GE-R60) were supplied by Anagalide, S.A. (Huesca, Spain). To prepare a stock solution of stevia water extract

at 1%, w/v ($SWE_1$), 100 mL of bottled water at 100 ºC were added on the dried leaves (1 g) and were kept for 30 min. The infusion was vacuum filtered using filter paper (Whatman No. 1). A sample of Glycostevia-EP® (GE-EP), which was a crude extract outcome of the industrial water extraction of stevia leaves, at 1% w/v; and a sample of Glycostevia-R60® (GE-R60), which was a purified extract with 95% of Rebaudioside A (1% w/v), were also studied.

Moreover, a stevia water extract 2 ($SWE_2$) was prepared from *Stevia rebaudiana* leaves purchased from a local supermarket (Navarro Herbolario, Valencia). Following the manufacturer's instructions, the sample (1 g) was mixed with 100 mL of boiling water for 30 minutes with constant shaking and the samples where then filtered through Whatman No. 1 filter paper.

In addition, different stevia-derived products from local and international supermarkets: TruVia (Azucarera, Madrid, Spain), PureVia (Whole Earth Sweetener Company, Paris, France) and stevia extract in the Raw (Cumberland Packing corp., Brooklyn, USA) were also studied and stored at room temperature. Each sample (1 g) was mixed with 100 mL of distilled water. Samples were prepared in triplicate just before use.

*2.2. Chemicals and reagents*

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), as a standard substance (2 mM) to measure TEAC, 2,2´-azobis(2-methylpropionamidina) dihydrochloride (ABTS), fluorescein sodium salt, 2,2´-azobis(2-amidinopropane) dihydrochloride (AAPH), disodium metabisulfite, Folin-Ciocalteau (ammonium molibdotugstat) reagent, chlorogenic acid, *ρ*-coumaric acid, (+)-catechin, ferulic acid, 3,4-dihydroxybenzoic, trans-cinnamic acid, caffeic acid, rebaudioside A, stevioside hydrate and steviol hydrate were purchased from Sigma (Steinheim, Germany). Gallic acid 1-hydrate in distilled water, as a standard (10 mg/mL) for phenolic compounds, was purchased from UCB (Brussels, Germany). Oxalic acid, acetic acid, chlorhidric acid, acetone, sodium acetate, potassium persulphate ($K_2S_2O_8$), sodium di-hydrogen phosphate (anhydrous) ($NaH_2PO_4$) and di-potassium hydrogen phosphate ($K_2HPO_4$) were purchased from Panreac (Barcelona, Spain), and ethanol, methanol, acetonitrile, hexane, sodium

carbonate anhydrous ($Na_2CO_3$), trichloroacetic acid and sodium sulphate from Baker (Deventer, The Netherlands). Ascorbic acid was obtained from Merck (Darmstadt, Germany), rutin trihydrate and quercetin dehydrate from Hwi analytic GMBH (Rülzheim, Germany) and rebaudioside C and rebaudioside F from Wako (Osaka, Japan).

*2.3. Liquid chromatographic analysis of steviol glycosides*

The method of JECFA [20], with various modifications, was used. Samples were filtered through a Sep-Pak® cartridge (a reverse-phase C-18 cartridge; Millipore, MA, USA) which retains steviol glycosides. Cartridges were previously activated with 10 ml of methanol (MeOH) and 10 ml of water. Every 10 ml of sample was eluted with 2 ml of MeOH, and all methanolic fractions were collected, filtered through a 0.45 µm membrane filter Millex-HV13 (Millipore) and then analysed by liquid chromatography. Kromasil 100 C18 precolumn (guard column) (5 µm, 150 x 4.6 mm) and Kromasil 100 C18 column (5 µm, 150 x 4.6 mm) (Scharlab, Barcelona, Spain) were used. The mobile phase consisted of two solvents: Solvent A, acetonitrile and Solvent B, 10 mmol/L sodium phosphate buffer (pH=2.6) (32:68, v/v). Steviol glycosides were eluted under 1 mL/min flow rate and temperature was set at 40 °C. Triplicate analyses were performed for each sample. Chromatograms were recorded at 210 nm. Identification of steviol glycosides was carried out by the addition of authentic standards, while quantification was performed by external calibration with standards.

*2.4. Polarographic determination of ascorbic acid*

The method used was in accordance to Barba et al. [21]. Plant food material (5 mg) was diluted to 25 ml with the extraction solution (oxalic acid 1%, w/v, trichloroacetic acid 2%, w/v and sodium sulphate 1%, w/v). After vigorous shaking, the solution was filtered through a folded filter (Whatman No. 1). Oxalic acid (9.5 ml) 1% (w/v) and 2 ml of acetic acid/ sodium acetate 2 M buffer (pH=4.8) were added to an aliquot of 0.5 ml of filtrate and the solution was transferred to the polarographic cell. A Metrohm 746 VA Trace Analyser (Herisau, Switzerland) equipped with a Metrohm 747 VA stand was used for the polarographic

determination. The working electrode was a Metrohm multi-mode electrode operated in the dropping mercury mode. A platinum wire counter electrode and a saturated calomel reference electrode were used. The following instrumental conditions were applied: $DP_{50}$, drop mercury mode, drop size 2, drop time 1 s, scan rate 10 mV/s, initial potential -0.10 V. Determinations were carried out using the peak heights and standard additions method.

*2.5. Total carotenoids*

Extraction of total carotenoid was carried out in accordance with the method of Lee and Castle [22]. An aliquot of sample (2.5 mL) was homogenised with 5 mL of extracting solvent (hexane/acetone/ethanol, 50:25:25, v/v) and centrifuged for 5 min at 6,500 rpm at 5 °C. The top layer of hexane containing the colour was recovered with a Pasteur pipet and transferred to glass tubes protected from light and homogenised. After that, 1 mL of this supernatant was transferred to a 25 mL volumetric flask, and volume was completed with hexane. Total carotenoid determination was carried out measuring the absorbance of an aliquot of the hexane extract at 450 nm. Total carotenoids were calculated according to Ritter and Purcell [23] using an extinction coefficient of β-carotene of $E^{1\%}$=2505.

*2.6. Phenolic compounds*

2.6.1. Liquid chromatographic analysis of phenolic profile

HPLC analysis was performed in accordance to Kelebek et al. [24], with some modifications. Samples were filtered through a Sep-Pak® cartridge (a reverse-phase C-18 cartridge; Millipore, MA, USA) which retains phenolic compounds. Cartridges were previously activated with 10 ml of methanol (MeOH) and 10 ml of water. Every 10 ml of sample was eluted with 2 ml of MeOH and all methanolic fractions were collected, filtered through a 0.45 µm membrane filter Millex-HV13 (Millipore) and then analysed by liquid chromatography. The liquid chromatography system consisted of two isocratic pumps (Prostar 210, Varian Inc, California, USA) with degasser (Degassit, MetaChem, USA), column thermostat (Prostar 510, Varian) and UV-vis detector (Varian Inc, California, USA). The whole liquid chromatography system was operated by a Varian STAR Chromatography

Workstation Ver. 6.0 (Varian Inc, California, USA). Luna PFP(2) precolumn (guard column) and Luna 100 PFP(2) column (5 µm, 150 x 4.6 mm) (Phenomenex, Spain) were used. The mobile phase consisted of two solvents: Solvent A, water/formic acid (95:5; v/v) and Solvent B, acetonitrile/solvent A (60:40; v/v). Phenolic compounds were eluted under the following conditions: 1 mL/min of flow rate, 40 °C of temperature and isocratic conditions from 0 to 10 min with 0% B, gradient conditions from 0% to 15% B in 20 min, from 15% to 22% B in 45 min, from 22% to 100% B in 15 min, from 100% to 0% B in 5 min, followed by washing and reconditioning of the column. Triplicate analyses were performed for each sample. Chromatograms were recorded at 280 nm. Identification of phenolic compounds was carried out by using authentic standards and by comparing retention times, while quantification was performed by external calibration with standards. A known quantity of each of the phenolic standards was added to each of the samples analysed in order to confirm the identification of this compounds and the method described was applied. Furthermore, in order to verify phenolic compounds, UV-vis spectra was determined with a diode-array detector.

2.6.2. Total phenolic compounds

Total phenolic compounds were determined according to the method reported by Georgé et al. [25], with some modifications. Briefly, 10 mL of sample were homogenised with 50 mL of a mixture of acetone/water (7/3, v/v) for 30 min. Mixture supernatants were then recovered by filtration (Whatman No. 2, England) and constituted the raw extracts (REs). REs (2 mL) were settled on an Oasis cartridge (Waters). Interfering water-soluble components (steviol glycosides, reducing sugars, ascorbic acid) were recovered with 2 x 2 mL of distillate water. The recovered volume of the washing extract (WE) was carefully measured. In order to eliminate vitamin C, heating was carried out on the washing extract (3 mL) for 2 h at 85 °C and led to the heated washing extract (HWE). All extracts (RE, WE, and HWE) were submitted to the Folin-Ciocalteu method, adapted and optimised [26]. Gallic acid calibration standards with concentrations of 0, 100, 300, 500, 700 and 1000 ppm were prepared and 0.1 mL were transferred to borosilicate tubes. 3 mL of sodium carbonate solution (2%, w/v)

and 0.1 mL of Folin–Ciocalteu reagent (1:1, v/v) were added to 0.1 mL of all gallic acid standard and sample tubes. The mixture was incubated for 1 h at room temperature and absorbance was measured at 765 nm.

*2.7. Total anthocyanins*

Total anthocyanins were determined using a modified method of Mazza et al. [27]. A 10-fold diluted sample of 100 µL was mixed with 1700 µL of distilled water and 200 µL of 5% (v/v) HCl. The sample was hold at room temperature for 20 min before measuring the absorbance at 520 nm in a 10 mm cuvette. Calculations of total anthocyanins were based on cyanidin-3-glucoside (molar absorptivity 26900 L/mol·cm). All spectrophotometric analyses were performed using a UV–visible spectrophotometer Lambda 20 (Perkin-Elmer, Überlingen, Germany).

*2.8. Total antioxidant capacity*

2.8.1. Trolox Equivalent Antioxidant Capacity (TEAC)

The method used was described by Re et al. [28], based on the capacity of a sample to inhibit the ABTS radical ($ABTS^{\bullet+}$). The radical was generated using 440 µL of potassium persulfate (140 mM). The solution was diluted with ethanol until an absorbance of 0.70 was reached at 734 nm. Once the radical was formed, 2 mL of $ABTS^{\bullet+}$ was mixed with 100 µL of appropriately diluted beverage (1:25, v/v), and absorbance was measured at 734 nm for 20 min in accordance with Zulueta et al. (2009).

2.8.2. Oxygen Radical Absorbance Capacity Assay (ORAC)

The oxygen radical absorbance capacity (ORAC) assay used, with fluorescein as the “fluorescent probe”, was that described by Ou et al. [29] The automated ORAC assay was carried out on a Wallac 1420 VICTOR$^2$ multilabel counter (Perkin-Elmer, USA) with fluorescence filters, for an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The measurements were made in plates with 96 white flat bottom wells (Sero-Wel, BibbySterilin Ltd., Stone, UK). The reaction was performed at 37 °C, as the reaction was started by thermal decomposition of AAPH in 75 mM phosphate buffer (pH 7.0). The final reaction tested and the

concentrations of the different reagents were determined following Zulueta et al. [30].

*2.9. Statistical analysis*

All determinations were performed in triplicate. An analysis of variance (ANOVA) was applied to the results obtained in order to verify whether there were significant differences in the parameters studied in relation to sample analysed and to ascertain possible interactions between factors (differences at $p<0.05$ were considered significant). Where there were differences, an LSD test was applied to indicate the samples in which differences were observed. A multiple regression analysis was performed in order to study the influence of the potential natural food additives in the antioxidant capacity (results are shown in the significant cases, $p<0.05$). Finally, a study was conducted with the aim of determining whether there were correlations between a pair of variables. All statistical analyses were performed using SPSS® (Statistical Package for the Social Sciences) v.20.0 for Windows (SPSS Inc., Chicago, USA).

**3. Results and discussion**

*Stevia rebaudiana* has many different functions in foods, such as sweetening, preserving and flavouring properties, along with antioxidant and antimicrobial activity. Some of the compounds responsible of these properties were studied in the present research.

More than 100 compounds have been identified in *Stevia rebaudiana*, the best known being the steviol glycosides, particularly stevioside and rebaudioside A which are the most abundants (Wölver-Rieck 2012). Four different steviol glycosides were detected (Table 1, Figure 1) with the high-performance liquid chromatography (HPLC), although the actual JECFA analytical method [20] lists nine different steviol glycosides. Their concentrations vary widely depending on the genotype, cultivation conditions and preparation of the sample. Stevia water extract 2 showed the highest yield of the four steviol glycosides analysed. Stevioside was found to be the major compound (411.9 mg/100 g) in stevia water extract 2, followed by rebaudioside F and rebaudioside A (26.6 and 26.1 mg/100 g

respectively). In stevia water extract 1, concentrations of rebaudioside A and stevioside were similar (22.5 and 22.0 mg/100 g respectively). In purified steviol glycosides, only rebaudioside A and stevioside in the case of TruVia were detected. Rebaudioside A ranged from 0.7 mg/100 g in Glycostevia-R60® up to 411.9 mg/100 g in stevia water extract 2. These results were in accordance with Gardana et al. [32], who studied steviol glycosides in stevia leaves from southern Italy and commercial preparations (Truvia).

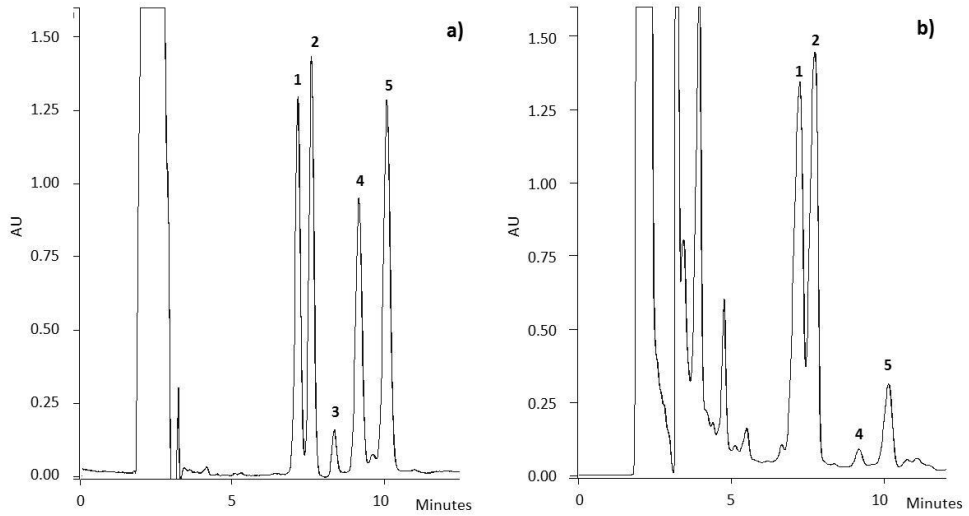


**Figure 1.** Chromatogram HPLC analysis of steviol glycosides 1: Rebaudioside A, 2: Stevioside hydrate, 3: Steviol hydrate, 4: Rebaudioside F, 5: Rebaudioside C in a) standard mixture and b) Stevia water extract 1.

Ascorbic acid was only detected in stevia water extracts (SWE) obtaining the higher values in $SWE_1$ (11.3±0.1 mg/100 g) in comparison to $SWE_2$ (9.8±0.1 mg/100 g). These findings were in accordance with those obtained by Kim et al. [33], when they studied stevia leaf and callus extracts. They found a vitamin C content of 14.97 mg/100 g in stevia leaf extract.

In addition, experimental results showed that carotenoids were not detected in the samples analysed in the present research. These results were similar to those found by Muanda et al. [34] in different stevia-derived products.

**Table 1.** Concentration of steviol glycosides (mg/100 g) in different samples.

| Sample | Reb A | Ste | Reb F | Reb C |
|---|---|---|---|---|
| $SWE_1$ | 58.9±0.9 | 255.0±1.8 | 5.79±0.08 | 23.74±0.45 |
| $SWE_2$ | 26.1±0.7 | 411.9±21.9 | 26.64±0.55 | 8.82±0.2 |
| GE-EP | 24.3±0.2 | 22.8±0.9 | 1.17±0.11 | 5.00±0.05 |
| GE-R60 | 0.7±0.1 | 0.5±0.1 | 0.03±0.01 | 0.10±0.01 |
| PureVia | 16.7±0.8 | - | - | - |
| TruVia | 11.2±0.6 | 12.0±0.9 | - | - |
| Stevia extract raw | 48.1±0.3 | - | - | - |

$SWE_1$: stevia water extract 1. $SWE_2$: stevia water extract 2. GE-EP: Glycostevia-EP®. GE-R60: Glycostevia-R60®. Reb A: rebaudioside A. Ste: stevioside. Reb F: rebaudioside F. Reb C: rebaudioside C.

Phenolic compounds are beneficial components mainly found in plant food products [35]. Among the different phenolic compounds, anthocyanins contribute significantly to the antioxidant capacity of plant products. Glycostevia-EP® exhibited the highest value of total phenolic compounds (20.85±27.80 g gallic acid equivalents (GAE)/100 g), followed by $SWE_1$ (12.64±10.81 g GAE/100 g) and $SWE_2$ (10.46±32.22 g GAE/100 g), whilst no phenolic compounds were detected in purified stevia extracts (Glycostevia-R60®, PureVia, TruVia and Stevia extract raw), just containing steviol glycosides (>95%). These values were in the range of those previously reported by other authors [34, 36-37] in different stevia-derived products (2-24 g gallic acid/100 g). A significant difference of total phenolic compounds between the different water extracts was observed due to the different variety of the stevia leaves.

In order to make a deeper study of the phenolic compounds, an HPLC analysis of the phenolic profile was performed. Figure 2 shows a chromatogram of stevia water extract 1. A total of 11 phenolic compounds were identified in stevia-derived products and quantified, including hydroxybenzoic acids (2), hydroxycinnamic acids (5), flavones (1), flavonols (2) and flavanols (1).

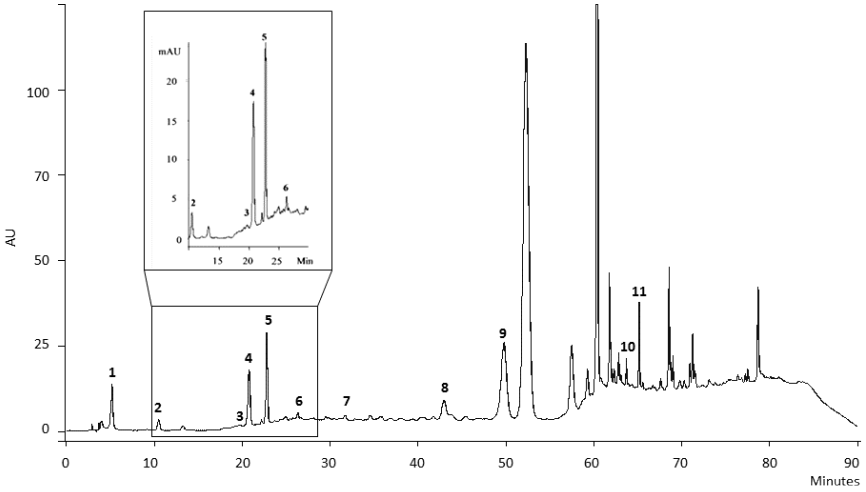


**Figure 2.** Chromatogram HPLC analysis of stevia water extract 1: Gallic acid, 2: Protocatechuic acid, 3: Catechin, 4: Caffeic acid, 5: Chlorogenic acid, 6: Coumaric acid, 7: Ferulic acid, 8: Transcinnamic acid, 9: Rutin, 10: Quercetin, 11: Apigenin.

**Table 2.** Phenolic content (mg/100 g) of *Stevia rebaudiana* water extracts (SWE) and Glycostevia-EP® (GE-EP).

| Compound | $R_t$ (min) | $SWE_1$ | $SWE_2$ | GE-EP |
|---|---|---|---|---|
| Gallic acid | 5.3 | 1.8±0.1 | 15.7±1.1 | 49.5±7.1 |
| Protocatechuic acid | 7.9 | 8.6±0.3 | 3.7±0.2 | - |
| Catechin | 19.3 | 494.2±23.3 | 905.0±45.2 | 13.4±0.3 |
| Caffeic acid | 21.2 | 76.9±1.0 | 118.5±3.3 | 250.2±6.2 |
| Chlorogenic acid | 23.2 | 343.4±32.0 | 293.1±6.3 | 668.4±65.3 |
| Coumaric acid | 26.3 | 50.8±4.4 | 37.0±0.6 | 212.1±9.6 |
| Ferulic acid | 34.1 | 141.6±1.3 | 10.4±0.2 | 270.4±34.2 |
| Transcinnamic acid | 44.5 | 14.4±0.1 | 403.6±5.4 | 101.1±17.3 |
| Rutin | 50.2 | 2797.1±28.9 | 401.0±4.4 | 10972.4±504.8 |
| Quercetin | 64.0 | 3619.4±80.0 | 3342.5±9.4 | 3077.7±25.2 |
| Apigenin | 67.2 | 1186.6±43.1 | 933.9±31.0 | 1383.3±28.1 |
| Total phenolics (Sum up) | | 8734.8±121.0 | 6464.3±32.1 | 16998.4±637.0 |

$R_t$: retention time $SWE_1$: stevia water extract 1. $SWE_2$: stevia water extract 2. GE-EP: Glycostevia-EP®.

Phenolic profile obtained in the present study for stevia samples was similar to the one found by different authors in stevia-derived products [32-33]. As can be shown in Table 2, quercetin and rutin were the predominant phenolic compounds in stevia-derived products, followed by apigenin, catechin and chlorogenic acid. A different phenolic profile was obtained for each sample. In addition, the major hydroxybenzoic acid was gallic acid (3, 4, 5-trihydroxybenzoic acid). This compound is present in foods of plant origin, and since it was found to exhibit antioxidative properties, it has attracted considerable interest. Except for protocatechuic acid and transcinnamic acid, a significant correlation was found between each phenolic compound and total phenolic contents measured using Folin-Ciocalteu method. A significant correlation was also found between the sum up of the eleven phenolic compounds identified and the total phenolic compounds measured both with Folin-Ciocalteu ($R^2$=0.998).

Within the phenolics, total anthocyanins were also measured, showing that these compounds were only detected in stevia water extracts, obtaining the higher concentrations in the $SWE_2$ (0.975±0.008 g/100 g) in comparison to $SWE_1$ (0.802±0.003 g/100 g). Results were in accordance with those found by Muanda et al. [34] who reported values of 0.35 mg total anthocyanins/g dry matter when they studied the chemical composition of water extracts from *Stevia rebaudiana* Bertoni.

Total antioxidant capacity values of stevia-derived products measured both by TEAC and ORAC assays are given in Figure 3. Remarkable antioxidant capacities were found in stevia extracts, with a high correlation to the total phenolic contents measured with the Folin-Ciocalteu method. These results were in accordance to those reported by Kim et al. [33] in stevia products. Both antioxidant assay systems showed comparable values ($R^2$=0.995, $p$<0.05). As can be expected, Glycostevia-EP® with the highest total phenolic contents had the highest antioxidant capacity using TEAC and ORAC assays. The nearly twice higher ORAC values (201.7 mmol TE/100 g) in Glycostevia-EP® compared to TEAC values (105.9 mmol TE/100 g) showed the excellent ability of phenolic compounds to scavenge peroxyl radicals. Meanwhile purified steviol glycosides, without total phenolic compounds detected, did not display any antioxidant capacity using

TEAC assay. However, remarkable antioxidant capacity was detected with ORAC assay, revealing 64.1, 1.16, 1.62 and 2.32 mmol TE/100 g in Glycostevia-R60®, Purevia, Truvia and Stevia extract raw, respectively. TEAC assay is suitable for compounds such as phenols, which have a redox potential lower than that of $ABTS^{\bullet+}$. Only then can a reduction of $ABTS^{\bullet+}$ occur [38]. Other compounds, such as butylated hydroxyanisole (BHA) may contribute to the total antioxidant capacity measured with ORAC in Glycostevia-R60®. These results were in accordance to those previously reported by other authors who found that phenolic compounds are strongly related to antioxidant activity [39]. In addition, a Pearson test was conducted in order to establish the possible correlation between the phenolic profile with the total antioxidant capacity (TEAC and ORAC method) (Table 3).

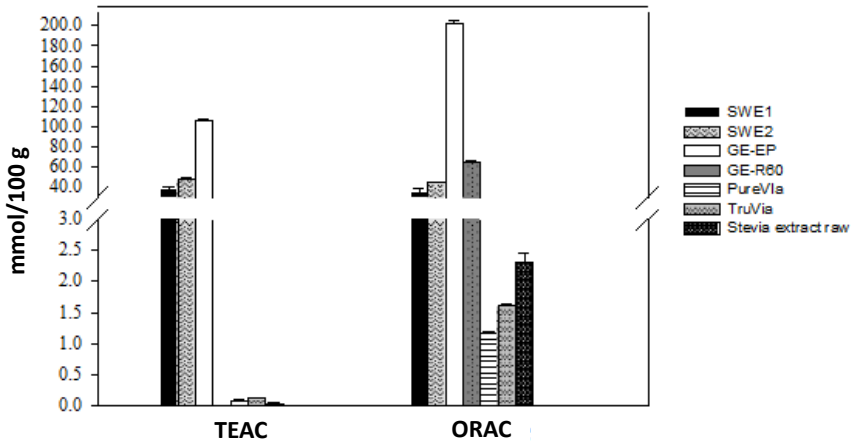


**Figure 3.** TEAC and ORAC values (mmol TE/100 g) in *Stevia rebaudiana* water extracts (SWE), Glycostevia-EP® (GE-EP), Glycostevia-R60®, PureVia, TruVia, and Stevia extract raw.

A strong correlation was found for TEAC and ORAC method with specific phenolic compounds (gallic acid, caffeic acid, chlorogenic acid, coumaric acid and rutin) in stevia herbal products, whereas protocatechuic acid, catechin, transcinnamic acid and quercetin turned out to be negatively correlated with TEAC and ORAC values. The results revealed significant differences between samples from different origin and were not comparable as the based chemical reactions and the parameters being determined varied considerably. As a result,

no single antioxidant method accurately reflects all antioxidants, which shows the necessity to standardise the methods in order to determine antioxidant capacity [38].

**Table 3.** Correlations of phenolic compounds with TEAC and ORAC in stevia-derived products.

| Compound | TEAC | ORAC |
|---|---|---|
| Gallic acid | 0.6617 | 0.6754 |
| Protocatechuic acid | 0.2053 | -0.0739 |
| Catechin | 0.2277 | -0.0537 |
| Caffeic acid | 0.9768 | 0.8461 |
| Chlorogenic acid | 0.8906 | 0.9039 |
| Coumaric acid | 0.8774 | 0.8327 |
| Ferulic acid | 0.8718 | 0.8900 |
| Transcinnamic acid | 0.7475 | 0.6395 |
| Rutin | 0.7622 | 0.7200 |
| Quercetin | 0.5654 | 0.3165 |
| Apigenin | 0.7999 | 0.8093 |

TEAC: trolox equivalent antioxidant capacity. ORAC: oxygen radical antioxidant capacity.

Furthermore, concentration curves for steviol glycosides standards (10-50 mg/100 mL) were also prepared in order to verify the response of the two antioxidant methods to different concentrations of these compounds (Figure 4). When the Reb A concentration increased, the antioxidant capacity was higher with the ORAC method ($p$<0.01), ($R^2$=0.949), but no antioxidant activity was detected applying the TEAC method. The same results were observed for stevioside ($R^2$=0.942), rebaudioside F ($R^2$=0.968), rebaudioside C ($R^2$=0.990) and steviol ($R^2$=0.990) applying the ORAC method. As the standard line slopes indicate, same concentration produces a higher increase in total antioxidant capacity with rebaudioside C and steviol.

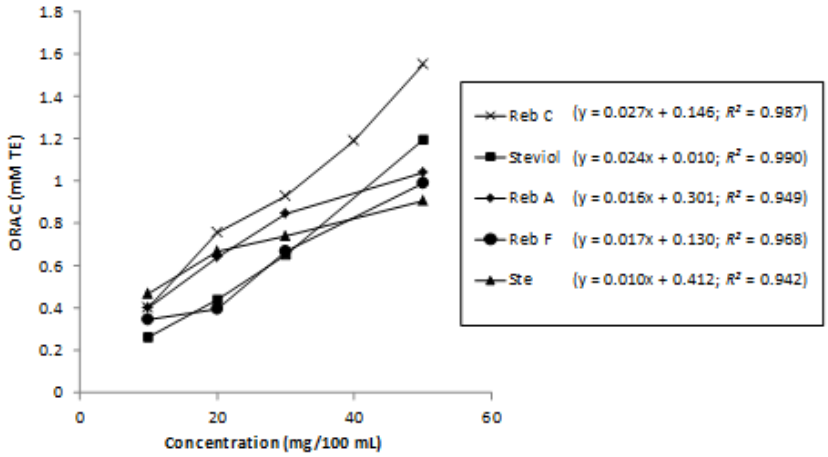


**Figure 4.** Antioxidant capacity of reference substances evaluated by ORAC (oxygen radical antioxidant capacity) method. Reb A: rebaudioside A. Ste: stevioside. Reb F: rebaudioside F. Reb C: rebaudioside C. TE: trolox equivalent.

This observation suggests that the antioxidant capacity found in steviol glycosides must be assayed with ORAC method and not with TEAC method, due to the nature of steviol glycosides compounds. Chaturvedula and Prakrash [39] described the presence of three anomeric glucose protons in diterpene glycosides from stevia. As the ORAC method is a reaction based on the transfer of H atoms [30], these compounds present in *Stevia rebaudiana* may be better represented by this assay.

## 4. Conclusion

Stevia water extracts can be considered a good source of natural sweeteners and antioxidants, especially phenolic compounds. Overall, components of stevia products are clearly attractive targets for the scientific community to develop novel food products with a given added value. Consequently, *Stevia rebaudiana*, a natural acaloric sweetener, considered an exogenous dietary antioxidant, can be used as a nutraceutical ingredient in food products in order to provide new functional foods of proven nutritional quality, thus increasing added value.

**Acknowledgements**

This research project was supported by the Spanish Ministry of Science and Technology and European Regional Development Funds (AGL2010-22206-C02-01). Carbonell-Capella, J.M. holds an award from the Spanish Ministry of Education (AP2010-2546).

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## 3.2. Novel beverages sweetened with *Stevia rebaudiana* as sources of bioactive compounds

*Journal of Food Science and Technology (Under review)*

# Novel beverages sweetened with *Stevia rebaudiana* as sources of bioactive compounds

Juana M. Carbonell-Capella, Magdalena Buniowska, María J. Esteve, Ana. Frígola*

University of Valencia, Department of Nutrition and Food Science, Av. Vicent Andrés Estellés, s/n. 46100 Burjassot, Spain

*Corresponding author:
Ana Frígola
e-mail: ana.frigola@uv.es.
Phone: +34 963544955 Fax: +34 963544954

**ABSTRACT**

New developed functional beverages, with a high content in bioactive compounds, based on exotic fruits (mango juice, papaya juice and açaí) mixed with orange juice, oat and sweetened with different concentrations (0, 1.25 and 2.5% w/v) of *Stevia rebaudiana* extracts, a natural source of non-caloric sweeteners (steviol glycosides), were formulated and studied. Ascorbic acid, total carotenoids, total phenolics, anthocyanins, total antioxidant capacity (TEAC and ORAC methods) and steviol glycosides were evaluated. Beverages sweetened with 1.25 and 2.5% (w/v) stevia showed a significant increase (≈3-fold and 4-fold higher, respectively) in phenolic compounds as well as in antioxidant properties in comparison with the beverage without stevia. The displayed results indicate the potential use of stevia as an alternative non-caloric sweetener in the preparation of beverages based on fruit juice mixtures and oat, as it may provide good nutritional and physicochemical properties and also enhance the already existing beneficial effects of fruit juices. Synergistic interactions observed between phytochemicals and steviol glycosides in the complex food beverages when TEAC method was used suggest an improved solubility, stability and/or different mechanisms of action of antioxidant compounds and hence, the combined antioxidant capacity measured with TEAC assay is potentiated in the complex food matrix.

**Keywords:** Exotic fruits, *Stevia rebaudiana*, bioactive compounds, antioxidant capacity, interaction factor.

**Introduction**

During the last decade, research dealing with health promoting features of so-called functional foods is increasing, with a promising future and a challenging development. A change in food consumption trend is observed, mainly due to lifestyle changes. Proportionally, juices obtained from exotic fruits is the group with the highest growing rate, both in Europe and in North America (Heckman et al. 2010). In terms of public health, drinking fruit and vegetable juices may well be as effective as consuming whole fruits and vegetables with regard to reducing the risk of chronic disorders (Ruxton et al. 2006). Many fruits including acerola, açaí, avocado, durian, kiwi, mango, papaya, oranges, etc., have attracted much attention because of their health benefits due to the wide range of bioactivities (Dembitsky et al. 2011), containing a large quantity of bioactive compounds such as ascorbic acid, phenolic compounds and carotenoids, which have been shown to be good contributors to total antioxidant capacity of foods (Barba et al. 2013; Zulueta et al. 2009).

Meanwhile, açaí (*Euterpe oleracea*) berry, native of Brazil, has been acclaimed to have a wide range of health-promoting and therapeutic benefits due to its reportedly high levels of antioxidants, with a relative high content of polyphenols, mainly anthocyanins (de Rosso et al. 2008). Findings demonstrate that açaí pulp improves biomarkers of physiological oxidative stress (de Souza et al. 2010).

Furthermore, the use of natural green plant extracts or their derived products in foods and beverages is also becoming an increasing trend in food industry (Bhardwaj and Pandey 2011). *Stevia rebaudiana*, an herb native to South America, is used as a natural source of non-caloric sweeteners (steviol glycosides) and is thought to possess antioxidant, antimicrobial and antifungal activity (Lemus-Mondaca et al. 2012). Although stevia-derived products have been used in different countries for several years, in Europe they have not been used extensively. FDA approved stevia for commercialisation in 2008 (FDA GRAS 275 and 323) and more recently, in November 2011, the European Commission (EU) has approved steviol glycosides as a new food additive (E 960). Recent green light will probably lead to wide-scale use of stevia-derived products (Stoyanova et al.

2011). *Stevia rebaudiana* may be used as an alternative of synthetic additives in marketed food products (Food Consulting, 2010; FSA, 2010). So far, little data has been available regarding practical applications in foods (Nehir El and Simsek 2012).

Differently from synthetic pharmaceuticals, based upon single chemicals, many phytomedicines exert their beneficial effects through additive or synergistic action of several bioactive compounds. Following research focused on increasing antioxidant consumption in a healthy diet, as well as providing alternatives for decreasing sugar consumption, the aim of this work was to study the potential use of *Stevia rebaudiana* water extracts as sources of non-caloric sweeteners to formulate novel beverages based on exotic fruits (papaya, mango and açaí), orange juice and oat beverage, as well as to evaluate antioxidant capacity and synergistic interactions between bioactive compounds in these new beverages.

**Materials and methods**

*Preparation of orange, mango and papaya juice, oat beverage, açaí and stevia stock solution*

Cultivars of papaya (*Carica papaya*), mango (*Mangifera indica*), oranges (*Citrus sinensis* L.), Navel variety and oat beverage (Santiveri, Lérida, Spain) were purchased from a local supermarket. Papaya, mango and orange juices were extracted after appropriate washing and hygienisation of the fruits and the pulp was removed. Açaí (containing 450 mg of açaí berries extract, with 10% of polyphenols) was provided by Nature's Way Products Inc. (Utah, USA).

*Stevia rebaudiana* leaves were supplied by Anagalide, S.A. (Barbastro, Huesca, Spain) and stored at room temperature. A stock solution (8.33%, w/v) of *Stevia rebaudiana* was prepared in order to formulate the beverage. For this purpose, 100 mL of bottled water at 100 ºC were added on the dried leaves (8.33 g) and were kept for 30 min. Infusion was vacuum filtered using filter paper (Whatman No. 1) and the filtrate obtained was stored at –40 ºC.

*Preparation of beverages*

Beverages were prepared by mixing 32.5%, 10%, and 7.5% (v/v) of papaya, mango and orange juices, respectively, with the pulp removed, 20% (v/v) of oat beverage and 30% (v/v) of water (0% stevia) or the different stevia leaves infusion (1.25 and 2.5%, w/v). Finally, açaí (1% w/v) was added to the beverage. Solid ingredients were placed in water in the weight proportions indicated. The beverage was prepared just before use. Each sample was prepared in triplicate. The maximum stevia concentration (2.5%) was selected taking into account the sucrose concentration of commercial fruit based beverages and the sweetness equivalence stevia/sucrose (Savita et al. 2004).

*Chemicals and reagents*

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), as a standard substance (2 mM) to measure TEAC, 2,2´-azobis(2-methylpropionamidina)dihydrochloride (ABTS), fluorescein sodium salt, 2,2´-azobis(2-amidinopropane)dihydrochloride (AAPH), disodium metabisulfite, Folin-Ciocalteu (ammonium molibdotugstat) reagent, rebaudioside A, stevioside and steviol hydrate were purchased from Sigma (Steinheim, Germany). Gallic acid 1-hydrate in distilled water, as a standard (10 mg/mL) for phenolic compounds, was purchased from UCB (Brussels, Germany). Oxalic acid, acetic acid, chlorhidric acid, acetone, sodium acetate and potassium persulphate ($K_2S_2O_8$) were purchased from Panreac (Barcelona, Spain). Di-sodium hydrogen phosphate (anhydrous) ($Na_2HPO_4$) and potassium di-hydrogen phosphate ($KH_2PO_4$) were obtained from Scharlau (Barcelona, Spain). Ethanol, methanol, acetonitrile, hexane, sodium carbonate anhydrous ($Na_2CO_3$), trichloroacetic acid and sodium sulphate proceeded from Baker (Deventer, The Netherlands), while rebaudioside C and rebaudioside F from Wako (Osaka, Japan). L(+)-ascorbic was obtained from Merck (Darmstadt, Germany).

*Polarographic determination of ascorbic acid*

Beverage (5 mL) was diluted to 25 ml with an extraction solution (oxalic acid 1%, w/v, trichloroacetic acid 2%, w/v, and sodium sulphate 1%, w/v). After

vigorous shaking, the solution was filtered through a folded filter (Whatman No. 1). Oxalic acid (9.5 ml) 1% (w/v) and 2 ml of acetic acid/ sodium acetate 2 M buffer (pH=4.8) were added to an aliquot of 0.5 ml of filtrate and the solution was transferred to the polarographic cell. A Metrohm 746 VA Trace Analyser (Herisau, Switzerland) equipped with a Metrohm 747 VA stand was used for polarographic determination. Working electrode was a Metrohm multi-mode electrode operated in the dropping mercury mode. A platinum wire counter electrode and a saturated calomel reference electrode were used. The following instrumental conditions were applied: $DP_{50}$, drop mercury mode, drop size 2, drop time 1 s, scan rate 10 mV/s, initial potential -0.10 V. Determinations were carried out by using the peak heights and standard additions method (Barba et al. 2013).

*Total carotenoids*

An aliquot of sample (2 mL) was homogenised with 5 mL of extracting solvent (hexane/acetone/ethanol, 50:25:25, v/v) and centrifuged for 5 min at 4000 rpm at 5 ºC. The top layer of hexane containing the colour was recovered and transferred to a 25 mL volumetric flask. The volume of recovered hexane was then adjusted to 25 mL with hexane. Total carotenoid determination was carried out on an aliquot of the hexane extract by measuring absorbance at 450 nm (Barba et al. 2013). Total carotenoids were calculated using an extinction coefficient of β-carotene, $E^{1\%}$=2505.

*Phenolic compounds*

Total phenols were determined according to the method reported by Georgé et al. (2005), with some modifications. Briefly, 10 mL of sample were homogenised with 50 mL of a mixture of acetone/water (7/3, v/v) for 30 min. Mixture supernatants were then recovered by filtration (Whatman No. 2, England) and constituted the raw extracts (REs). REs (2 mL) were settled on an Oasis cartridge (Waters). Interfering water-soluble components (reducing sugars, ascorbic acid) were recovered with 2 x 2 mL of distillate water. The recovered volume of the washing extract (WE) was carefully measured. In order to eliminate vitamin C, heating was carried out on the washing extract (3 mL) for 2 h at 85 °C

and led to the heated washing extract (HWE). All extracts (RE, WE, and HWE) were submitted to Folin-Ciocalteu method, adapted and optimised (Barba et al. 2013): Sodium carbonate solution (3 mL) 2% (w/v) and 100 µL of Folin–Ciocalteau reagent were added to an aliquot of 100 µL of sample. The mixture was incubated for 1 h at room temperature. Absorbance was measured at 765 nm.

*Total anthocyanins*

Total anthocyanins were determined using a modified method of Mazza et al. (1999). A 10-fold diluted sample of 100 µL was mixed with 1700 µL of distilled water and 200 µL of 5% (v/v) HCl. Sample was hold at room temperature for 20 min before measuring the absorbance at 520 nm in a 10 mm cuvette. Calculations of total anthocyanins were based on cyanidin-3-glucoside (molar absorptivity 26900 L/mol·cm). All spectrophotometric analyses were performed using a UV–visible spectrophotometer Lambda 20 (Perkin-Elmer, Überlingen, Germany).

*Total antioxidant capacity*

Trolox Equivalent Antioxidant Capacity (TEAC) assay: The TEAC test was determined according to the method reported by Barba et al. (2013), based on the capacity of antioxidants to inhibit the radical cation 2,2-azino-bis(3-ethylbenzothiazoline6-sulphonate) (ABTS), which has a characteristic long-wavelength absorption spectrum, showing a maximal peak at 734 nm. The ABTS radical cation is formed by the interaction of ABTS (7 mM) with $K_2S_2O_8$ (2.45 mM).

Oxygen Radical Absorbance Capacity (ORAC) Assay: The ORAC assay used, with fluorescein as the “fluorescent probe”, was that described by Barba et al. (2013). Automated ORAC assay was carried out on a Wallac 1420 VICTOR$^2$ multilabel counter (Perkin-Elmer, USA) with fluorescence filters, for an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Measurements were made in plates with 96 white flat bottom wells (Sero-Wel, BibbySterilin Ltd., Stone, UK). Reaction was performed at 37 °C, as the reaction is started by thermal decomposition of AAPH in 75 mM phosphate buffer (pH 7.0).

*Physicochemical properties*

pH was determined in a Crison GLP 21 pH-meter (Barcelona, Spain) equipped with a temperature compensation sensor at 20 °C. Brix was determined with an Atago RX-1000 digital refractometer (Atago Company Ltd., Tokyo, Japan). To measure turbidity index (TI), samples were centrifuged (618×g, 10 min, 20 °C), supernatant was taken and absorbance at 660 nm was measured (Krop and Pilnik 1974). To determine browning index (BI), samples were centrifuged (824×g, 20 min, 18 °C), supernatant was taken and diluted with ethanol (1:1, v/v). The mixture was filtered with Whatman No. 42 filters and absorbance of the filtrate was measured at 420 nm (Meydav et al. 1977). Hydroxymethylfurfural (HMF) content was measured using the method described by International Federation of Fruit Juice Producers (1984). The colour analysis was performed using a Hunter Labscan II spectrophotometric colorimeter (Hunter Associates Laboratory Inc., Reston, U.S.A.) controlled by a computer that calculates colour ordinates from the reflectance spectrum. Results were expressed in accordance with the Commission International d'Eclairage LAB (CIELAB) system with reference to illuminant D65 and with a visual angle of 10°. Three consecutive measurements of each sample were taken and $L^*$ (lightness [0=black, 100=white]), $a^*$ ($-a^*$=greenness, $+a^*$=redness) and $b^*$ ($-b^*$=blueness, $+b^*$=yellowness) values were measured (Calvo 2004).

*Liquid chromatographic analysis of steviol glycosides*

The method of Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA 2010) with various modifications was used. Samples were filtered through a Sep-Pak® cartridge (a reverse-phase C-18 cartridge; Millipore, USA) which retains steviol glycosides. Cartridges were previously activated with 10 ml of methanol (MeOH) and 10 ml of water. Every 10 ml of sample was eluted with 2 ml of MeOH and all methanolic fractions were collected, filtered through a 0.45 µm membrane filter Millex-HV13 (Millipore) and then analysed by liquid chromatography. Kromasil 100 C18 precolumn (guard column) (5 µm, 150 x 4.6 mm) and Kromasil 100 C18 column (5 µm, 150 x 4.6 mm) (Scharlab, Barcelona,

Spain) were used. The mobile phase consisted of two solvents: Solvent A, acetonitrile and Solvent B, 10 mmol/L sodium phosphate buffer (pH=2.6) (32:68, v/v). Steviol glycosides were eluted under 1 mL/min flow rate and temperature was set at 40 °C. Chromatograms were recorded at 210 nm. Identification of steviol glycosides were obtained using authentic standards and comparing retention times, while quantification was performed by external calibration with standards.

*Theoretical approach*

The interaction factor (IF), which provides an explanation for the mode of interaction, was determined, according to Eq. (1) (Gawlik-Dziki 2012):

$$IF=A_M/A_T \quad (1)$$

where $A_M$=measured activity of a mixture of samples, and $A_T$=theoretically calculated mixture activity (based on the dose response of single components at various concentrations). IF value>1 indicates synergistic interaction; IF<1 indicates antagonism; IF≈1 indicates additional interactions.

*Statistical analysis*

All determinations were performed in triplicate. Normality and homoscedasticity (variance homogeneity) were assayed as premises prior to parametric statistical tests using a Shapiro-Wilk test and a Levene test, respectively, as described by Granato et al. (2014). When variances were heterogeneous, dependent variables were transformed by the Box-Cox transformation. An analysis of variance (ANOVA) was applied in order to verify whether there were significant differences in the parameters studied in relation to the sample analysed and to ascertain possible interactions between factors (differences at $p<0.05$ were considered significant). Where there were differences, an LSD test was applied to indicate the samples in which differences were observed. A multiple regression analysis was performed to study the influence of bioactive compounds to antioxidant capacity (results are shown in the significant cases, $p<0.05$). Finally, a study was conducted with the aim of

determining whether there were correlations between a pair of variables (Pearson´s test). All statistical analyses were performed using Statgraphics® Centurion XVI (Statpoint Technologies Inc., USA).

**Results and discussion**

*Physicochemical and nutritional characterisation of single components*

Advantages of fruit mixtures are widely known, such as organoleptic improvements (aromas and flavours combination) and the synergy effects between their nutritional components. In the present study, nutritional and physicochemical characterisation of papaya, mango and orange juices as well as oat beverage, *Stevia rebaudiana* extracts and açaí capsules were conducted in order to determine potential interactions among the antioxidant compounds when the fruits were combined (Table 1). Mango and orange juices had pH values of 3.53 and 3.90, respectively, while papaya juice (5.40) stevia (6.97) and oat beverage (7.52) had higher pH values. pH is related to the stability of bioactive compounds in fruit-derived products. Thus, the combination of different ingredients with different pH can avoid oxidation reactions and microbial deterioration of food by decreasing pH values (Gao and Vasantha 2012). Total soluble solids content of fruits varied from 10.70 to 14.30 °Brix; the products are thus indicated for different consumers, submitted to diets with different calories. These contents were similar to those found by other authors in mango and papaya purées (Guerrero and Alzamora 1998; El-Mansy et al. 2005).

As can be seen in Table 1, the selected fruits constitute a good source of ascorbic acid (22.99-57.81 mg/100 mL), carotenoids (421.90-708.75 µg/100 mL), phenolic compounds (84.90-132.84 mg gallic acid equivalents (GAE)/L) and antioxidant capacity (TEAC (4.75-27.63 mM TE) and ORAC (2.78-8.22 mM TE)). Fruit juices were mixed with oat beverage, which is a good source of proteins from vegetal origin and showed also a high content in carotenoids (369.60±28.28 µg/100 mL).

**Table 1.** Bioactive compounds, total antioxidant capacity (TAC), physicochemical properties and steviol glycosides profile of the different ingredients used in the formulation of samples.

| | Orange juice | Mango Juice | Papaya juice | Oat beverage | Stevia infusion 8.33% (w/v) | Açaí Capsules* |
|---|---|---|---|---|---|---|
| **Bioactive compounds and antioxidant capacity** | | | | | | |
| Ascorbic acid (mg/100 mL) | 31.85±0.28 | 22.99±0.60 | 57.81±0.78 | - | - | - |
| Total carotenoids (µg/100 mL) | 421.90±31.82 | 639.00±36.77 | 708.75±46.57 | 369.60±28.28 | - | 97.33±3.50 |
| TPC (mg GAE/L) | 132.84±3.59 | 84.90±2.26 | 105.23±0.47 | 79.45±1.48 | 1216.30±12.30 | 100.55±0.07 |
| TA (mg/100 mL) | - | 251.86±1.62 | - | - | 0.22±0.03 | 280.45±13.46 |
| TEAC (mM TE) | 27.63±4.28 | 4.75±0.75 | 7.30±0.78 | 2.16±0.06 | 61.30±0.28 | 18.98±0.05 |
| ORAC (mM TE) | 8.22±0.35 | 2.78±0.85 | 6.11±0.47 | 0.93±0.11 | 122.05±0.78 | 18.94±0.33 |
| **Physicochemical properties** | | | | | | |
| pH | 3.90±0.04 | 3.53±0.04 | 5.40±0.03 | 7.52±0.01 | 6.97±0.06 | 6.35±0.07 |
| ºBrix | 11.90±0.14 | 14.30±0.14 | 10.70±0.14 | 11.75±0.07 | 1.15±0.07 | - |
| HMF (mg/L) | 0.07±0.01 | 0.16±0.02 | 0.14±0.02 | 0.14±0.01 | - | - |
| Turbidity index | 0.20±0.01 | 2.48±0.02 | 0.33±0.01 | 0.25±0.01 | 2.44±0.01 | 1.15±0.01 |
| Browning index | 0.09±0.01 | 0.10±0.01 | 0.08±0.01 | 0.05±0.01 | 2.77±0.03 | 3.01±0.02 |
| Lightness (*L**) | 48.36±0.05 | 36.47±0.03 | 52.08±0.02 | 68.55±0.05 | 22.53±0.03 | 31.66±0.50 |
| Redness (*a**) | 3.72±0.04 | 23.46±0.04 | 25.79±0.01 | -1.94±0.04 | 12.50±0.02 | 8.82±0.13 |
| Blueness (*b**) | 50.36±0.05 | 46.79±0.04 | 61.74±0.02 | 6.38±0.02 | 20.36±0.04 | 18.40±0.02 |
| **Steviol glycosides** | | | | | | |
| Reb A (mg/100 mL) | - | - | - | - | 1061.24±40.85 | - |
| Reb C (mg/100 mL) | - | - | - | - | 222.08±7.46 | - |
| Ste (mg/100 mL) | - | - | - | - | 1881.70±1.47 | - |
| Reb F (mg/100 mL) | - | - | - | - | 56.04±3.51 | - |

*per mg/g/kg product. -. Non-detectable. TPC: total phenolic compounds. GAE: gallic acid equivalent. TA: total anthocyanins.TEAC: trolox equivalent antioxidant capacity. ORAC: oxygen radical antioxidant capacity. Reb: rebaudioside. Ste: stevioside.

In addition, *Stevia rebaudiana* extract can be used as a natural sweetener, due to its high content in steviol glycosides (rebaudioside A (1061.24±40.85 mg/L), rebaudioside C (222.08±7.46 mg/L), stevioside (1881.70±1.47 mg/L), and rebaudioside F (56.04±3.51 mg/L)) with sweeting properties, and can also be a useful tool in order to increase phenolic consumption (1216.30±12.30 mg/100 mL) and total antioxidant capacity of food products (TEAC (61.30±0.28 mM TE) and ORAC (122.05±0.78 mM TE)). Moreover, açaí fruit is another exotic fruit which has attracted researcher's interest as to its high nutritional properties (Yamaguchi et al. 2015). For this reason, it was selected as an ingredient in the formulation of highly nutritional antioxidant beverages. As can be seen in Table 1, açaí capsules had a high content in anthocyanins and total antioxidant capacity.

*Phenolic compounds, total anthocyanins, ascorbic acid and total carotenoids in the formulated beverages*

Total phenolic content (TPC) in the beverage based on exotic fruits (mango juice, papaya juice and açaí) mixed with orange juice, oat and without stevia was of 66.4 mg gallic acid equivalents (GAE)/L. As can be seen in Table 2, TPC were significantly ($p$<0.05) higher when stevia at 1.25% (≈3-fold) and 2.5% (≈4-fold) was added to the formulation. These results were in concordance to those obtained by different authors who reported *Stevia rebaudiana* as an excellent source of phenolic compounds (Kim et al. 2011; Muanda et al. 2011).

Total anthocyanins (TA) in the beverage without stevia were found in a concentration of 22.0±1.4 mg cyanidin-3-glucoside/L. However, this value was 1.3- and 1.4-fold higher when stevia at 1.25% and 2.5% stevia (w/v) was used as a sweetener, respectively (Table 2). Muanda et al. (2011) reported values of 0.35 mg total anthocyanins/g dry matter when they studied the chemical composition of water extracts from *Stevia rebaudiana* Bertoni and de Rosso et al. (2008) obtained values of total anthocyanins ranging from 282 to 303 mg/100 g in açaí. The proposed beverages can be considered an excellent source of total anthocyanins, mainly due to the presence of açaí in its composition. A significant correlation between anthocyanins and total phenolic compounds ($p$=0.0073) was found when the Pearson test was studied for the different stevia concentrations.

**Table 2.** Bioactive compounds, total antioxidant capacity, physicochemical properties and steviol glycosides of three different beverages mixture of exotic fruit juices and oat beverage, sweetened with 0%, 1.25% and 2.5% stevia.

| | % *SR* | | |
|---|---|---|---|
| Parameter | 0 | 1.25 | 2.5 |
| **Bioactive compounds and antioxidant capacity** | | | |
| Ascorbic acid (mg/100 mL) | 24.8±0.2[a] | 24.9±0.2[a] | 24.6±0.2[a] |
| Total carotenoids (µg/100 mL) | 436.6±17.6[a] | 399.2±35.3[a] | 424.2±35.3[a] |
| TPC (mg GAE/L) | 230.8±10.9[a] | 2353.8±16.1[b] | 4715.4±15.4[c] |
| TA (mg/L) | 22.0±1.4[a] | 27.8±1.4[b] | 29.7±0.3[c] |
| TEAC (mM TE) | 6.4±0.3[a] | 20.3±2.2[b] | 30.4±0.7[c] |
| ORAC (mM TE) | 5.1±0.1[a] | 23.5±0.1[b] | 36.1±0.1[c] |
| **Physicochemical properties** | | | |
| pH | 4.38±0.20[a] | 4.50±0.10[a] | 4.49±0.10[a] |
| ºBrix | 7.70±0.14[a] | 8.70±0.14[b] | 9.70±0.20[c] |
| HMF (mg/L) | 0.057±0.003[a] | 0.099±0.004[b] | 0.224±0.031[c] |
| Turbidity index | 2.430±0.006[a] | 2.620±0.005[b] | 2.798±0.005[c] |
| Browning index | 0.080±0.003[a] | 1.140±0.004[b] | 2.416±0.005[c] |
| Lightness (*L**) | 59.0±0.1[a] | 40.7±0.2[b] | 33.5±0.1[c] |
| Redness (*a**) | 11.9±0.1[a] | 8.5±0.1[b] | 9.2±0.2[b] |
| Blueness (*b**) | 38.5±0.1[a] | 31.8±0.2[b] | 30.8±0.1[c] |
| **Steviol glycosides (mg/100 mL)** | | | |
| Rebaudioside A | - | 171.5±1.5 | 286.9±8.4 |
| Rebaudioside C | - | 30.1±0.6 | 63.6±0.1 |
| Stevioside | - | 363.8±2.6 | 637.5±3.0 |
| Rebaudioside F | - | 7.5±0.1 | 14.6±0.1 |

a–c Different letters in the same row indicate a significant difference in function of the samples analysed ($p$<0.05). TPC: total phenolic compounds. GAE: gallic acid equivalent. TA: total anthocyanins. TEAC: trolox equivalent antioxidant capacity. ORAC: oxygen radical antioxidant capacity. HMF: hydroxymethylfurfural.

Ascorbic acid concentration in the beverage without stevia was 24.8±0.2 mg/100 mL (Table 2). These results were in close agreement with the values obtained by other authors in papaya, mango and orange (Burdurlu et al. 2006;

Almeida et al. 2011). Non-significant modifications of ascorbic acid content resulting from the presence of stevia water extracts (1.25% and 2.5% (w/v)) were found.

Total carotenoids content was of 436.6±17.6 µg/100 mL in the sample without stevia and these values did not increase with the addition of stevia to the beverage. Experimental results did not show any presence of carotenoids in stevia-derived products. These results were in accord to those of Muanda et al. (2011) when they studied the chemical composition stevia water extracts.

*Antioxidant capacity*

The beverage without stevia added exhibited a more elevated antioxidant activity ($p$<0.05) when measured with TEAC method (6.4±0.3 mM TE) compared to values obtained with ORAC assay (5.1±0.1 mM TE) (Table 2). However, when stevia was added, antioxidant capacity was higher using ORAC method. The different increase in antioxidant values of stevia-sweetened samples measured with both TEAC and ORAC methods can be explained considering that the two tests, apart from differing in the reactive species, are performed at different reaction phases. This consideration suggests that the two tests furnished a diverse kind of information as they emphasised differently the antioxidant capacity of hydrophilic and hydrophobic antioxidants compounds. Results indicated that in stevia-sweetened beverage, hydrophobic constituents contributed to antioxidant capacity in higher amounts than water soluble antioxidants.

Independently of the method employed, antioxidant capacity of beverages sweetened with stevia at 1.25% and 2.5% (w/v) was higher than that obtained in beverages without stevia (Table 2). This was more evident when ORAC method was used. ORAC values were 5 and 7 times higher for beverages with 1.25% and 2.5% (w/v) compared to the beverage without stevia while TEAC values were about 3 and 5 times greater for samples with 1.25% and 2.5% (w/v) stevia, respectively. Therefore, the addition of stevia contributed to a considerable increase of the antioxidant activity in both of the beverage types.

A significant correlation between TEAC and ORAC values ($p$<0.05) was found. These results were in accordance with those found by other authors in different

liquid food matrices (Proteggente et al. 2003). Furthermore, Pearson´s test showed that antioxidant activity determined by TEAC and ORAC was significantly correlated ($p$<0.05) with total phenolic compounds and total anthocyanins. This was also observed by Granato et al. (2015) when they analysed various juices from different botanical origins.

*Physicochemical properties*

Results obtained for physicochemical properties of the beverages analysed in the present study are shown in Table 2. The values of pH and ºBrix in the beverage without stevia were 4.38±0.20, and 7.70±0.14, respectively. Non-significant changes were found in pH values when stevia was added as a sweetener, while a significant increase was found in ºBrix. The values of hydroxymethylfurfural (HMF), turbidity index and browning index of the samples without stevia added were 0.057 mg/L, 2.430, and 0.080, respectively. As can be observed in Table 2, a significant increase was obtained in HMF content, turbidity and browning index of samples when stevia percentage was higher in comparison to the beverage without stevia. However, parameters which define the colour of the samples ($L^*$, lightness; $a^*$, redness; and $b^*$, blueness) decreased significantly when increasing stevia percentage compared to sample without stevia added ($L^*$=59.0; $a^*$=11.9; $b^*$=38.5).

*Steviol glycosides*

Results obtained for steviol glycosides of the beverages analysed in the present study are shown in Table 2. Four different steviol glycosides (rebaudioside A (reb A), rebaudioside C (reb C), rebaudioside F (reb C), and stevioside (ste)) were detected and quantified (Table 2, Figure 1) in samples containing stevia. Stevioside was the predominant steviol glycoside identified in the beverages with stevia at 1.25% (363.8 mg/100 mL) and 2.5% (637.5 mg/100 mL), while the lower values were found for rebaudioside F. As can be expected, the ANOVA analysis showed a significant increase ($p$<0.05) in reb A, reb C, reb F and ste when stevia percentage used in the formulation of the beverages was increased, enhancing the sweetening properties of the beverages. In a previous study, Carbonell-

Capella et al. (2014) demonstrated that steviol glycosides standards showed antioxidant capacity using the ORAC method, so the presence of these compounds in the beverages could explain in part the increase of the antioxidant capacity measured with ORAC method.

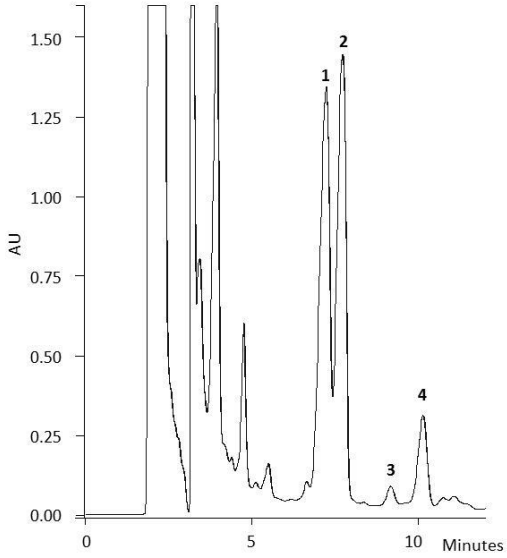


**Figure 1.** Chromatogram HPLC analysis of steviol glycosides 1: Rebaudioside A, 2: Stevioside, 3: Rebaudioside F, 4: Rebaudioside C in a beverage mixture of exotic fruit juice and sweetened with *Stevia rebaudiana* Bertoni at 2.5% (w/v).

*Interaction assay*

In order to investigate deeply the antioxidant capacity of bioactive compounds found in the orange, mango and papaya juice combined with oat beverage and açaí and sweetened with different concentrations of stevia water extracts, the interaction factor (IF) was determined (Table 3). This assay is a simple way to explain the mode of interaction and may be used to make a preliminary assessment of the types of interactions between the examined extracts or chemical compounds. Antioxidant activity of beverages and theoretically calculated mixture activity (based on the dose response of single components at various concentrations) was calculated.

**Table 3.** Comparison of interaction factors (IF) in the beverages sweetened with *Stevia rebaudiana* (0%, 1.25% and 2.5%).

| ***Stevia rebaudiana*** | Activity | $A_M$ | $A_T$ | IF |
|---|---|---|---|---|
| **0%** | TEAC | 6.44 | 5.54 | 1.16 |
| | ORAC | 5.05 | 3.25 | 1.55 |
| **1.25%** | TEAC | 20.30 | 14.73 | 1.38 |
| | ORAC | 23.46 | 21.56 | 1.09 |
| **2.5%** | TEAC | 30.41 | 23.93 | 1.27 |
| | ORAC | 36.09 | 39.87 | 0.91 |

$A_M$: Measured activity. $A_T$: Theoretical calculated activity. TEAC: Trolox equivalent antioxidant capacity. ORAC: Oxygen radical antioxidant capacity.

When examining the antioxidant capacity using the TEAC method, antiradical scavengers included in the fruit juices, oat beverage and açaí acted synergistically in the beverages without stevia added. In the case of 1.25% and 2.5% stevia beverages, the same kind of interaction was observed. Unlike in the case of synthetic pharmaceuticals based on the activity of single active compounds, numerous phytochemicals act in a beneficial manner via an addition of synergistic activity in target sites connected to physiological processes. Synergistic interactions observed when TEAC assay is employed in the beverage sweetened with 2.5% stevia suggest an improved solubility or stability of the antioxidant compounds and therefore, the combined antioxidant capacity of the mixture measured by TEAC method is potentiated. These results are in line with those reported by Gawlik-Dziki (2012), who observed synergistic interactions for constituents within the total extracts of a single plant-derived product, as well as between different plant products in a formulation, obtaining that the whole or partially purified extract of a fruit and vegetables offers advantages over a single isolated ingredient.

The antioxidant capacity measured using the ORAC method also revealed synergistic interactions between the single components of the beverages without stevia. However, the addition of 1.25% and 2.5% of stevia to the beverages indicated additional interactions (≈1) between the individual components of the

beverages. Unlike TEAC values, antioxidant activity measured with ORAC method when stevia is included in the formulation was similar to that predicted, revealing that the presence of stevia water extract may be engaged in shaping the potential antioxidant activity of the studied beverages. Several studies conducted on food products fortified with phenolic-rich ingredients such as onion skin (Gawlik-Dziki et al. 2013), quinoa leaves (Świeca et al. 2014) and parsley leaves (Sęczyk et al. 2016) show that part of the antioxidant activity may be masked as a result of interactions between selected phenolic compounds with other food components. Nevertheless, despite these possible interactions, the addition of phenolic rich ingredients results in the promotion of the total antioxidant capacity of foods.

**Conclusions**

The beverage based on exotic fruits (mango juice, papaya juice and açaí) mixed with orange juice, oat and sweetened with *Stevia rebaudiana* water extracts at 2.5% (w/v) was found to contain the highest amount of total phenolic compounds, almost 4-fold higher than the sample without stevia, and consequently presented the highest antioxidant capacity measured both with ORAC and TEAC method. From these results, it can be concluded that the use of *Stevia rebaudiana* as a natural non-caloric sweetener can also be a good source of bioactive compounds. Synergistic interactions observed for phytochemicals and steviol glycosides in the complex food beverages when TEAC method was used suggest an improved solubility, stability and/or different mechanisms of action of antioxidant compounds and hence, the combined antioxidant capacity of the mixture measured by TEAC assay is potentiated in the complex food matrix.

**Acknowledgments**

This research project was supported by the Spanish Ministry of Science and Technology and European Regional Development Funds (AGL2010-22206-C02-01). Carbonell-Capella, J.M. holds an award from the Spanish Ministry of Education (AP2010-2546). Buniowska, M. holds an award from the European Union (8.2.2/IV.26/217/11/U/155/12).

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## 3.3. Automating a 96-well microtiter plate assay for quick analysis of chemically available lysine in foods

*Food Analytical Methods 6(5) (2013) 1258-1264*

# Automating a 96-Well Microtiter Plate Assay for Quick Analysis of Chemically Available Lysine in Foods

Francisco J. Barba[a] · Juana M. Carbonell-Capella[b] · María J. Esteve[b] · Ana. Frígola[b]*

[a]Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Cientificas (CSIC), Av. Agustín Escardino, 7, 46980 Paterna, Spain.

[b] University of Valencia, Department of Nutrition and Food Science, Av. Vicent Andrés Estellés, s/n, 46100 Burjassot, Spain.

*Corresponding author:
Ana Frígola
e-mail: ana.frigola@uv.es.
Phone: +34 963544955 Fax: +34 963544954

**ABSTRACT**

A new method for quick analysis of available lysine content in different food products has been developed by automating a 96-well microtiter plate assay. Although manual fluorometric methods validated in order to determinate available lysine content already existed for this compound, the benefits of applying appropriate automation should provide continuous operation, increased precision, an affordable electronic audit trail and significantly reduced time and reagent consumption. The objective of this work was to adapt the *ortho*-phthaldialdehyde (OPA) fluorometric method to an automated workstation. Considerable effort went into developing and validating an automated method. The analytical parameters of linearity ($R^2$=0.999), the precision of the method (relative standard deviations (RSD)=2.8-3.0% for the different samples) and the results of the comparison with the corresponding OPA manual fluorometric method show that the studied method is useful for the measurement of available lysine in several food products from different natural origins such as liquid foods (soy, oat, quinoa beverages and ultra-high temperature (UHT)/sterilised milk) and powdered samples (powdered adapted, powdered follow-up and junior milk infant formulas) with reduced time and reagent consumption.

**Keywords:** Lysine, 96-well microtiter plate assay, automation, Maillard reaction, fluorometric determination.

## 1. Introduction

Maillard browning is one of the main chemical reaction causing deterioration of proteins during processing and storage of foods. This reaction between free amino groups and reducing sugars reduces protein digestibility and amino acid availability (Malec et al. 2002).

Available lysine content is an indicator of early and advanced Maillard reaction phases (Ferrer et al. 1999) and can be a useful tool in order to predict nutritional losses. The quantitative analysis of available lysine content together with its degradation products have been used as a chemical marker of protein quality (Meade et al. 2005).

Different methods like spectrophotometric (Carpenter 1960; Kakade and Liener 1969; Vigo et al. 1992), chromatographic (Albalá-Hurtado et al. 1997; Fernández-Artigas et al. 1999; McEwen et al. 2010), and fluorometric (Ferrer et al. 2003; Goodno et al. 1981; Morales et al. 1995) have been proposed for the determination of available lysine content in food products. Conventionally, the fluoro-2,4-dinitrobenzene (FDNB) assay has been the most extensively used method (Smith 2010). However, Vigo et al. (1992) and Morales, Romero, & Jiménez-Pérez (1995) demonstrated that this method was time consuming and special precautions were necessary. Dialysis of carbohydrate-rich samples is recommended since it avoids the uncertainty inherent in applying correction factors for reaction interference, but this adds two or three days to each assay (Tomarelli et al. 1985). In order to eliminate possible interferences by spectrophotometric methods, Goodno et al. (1981) established a fluorometric analysis, using *ortho*-phthaldialdehyde (OPA) for estimating reactive lysine in proteins, which has been used widely in the published literature (Morales et al. 1995; Swaisgood and Ctagnani 1985; Vigo et al. 1992). The use of OPA does not require hydrolysis or amino acid analysis of the sample and does not require heating or solvent extraction that can release lysine from modified forms (Ferrer et al. 2003). The OPA reaction is rapid and complete at room temperature, and the complex formed by lysine and OPA is fluorescent, while side-products are not fluorescent. Furthermore, the amount of sample needed is small, sugars do not

interfere, and the assay is reproducible and easy to perform. The main disadvantage is the instability of the fluorescent complex (Goodno et al. 1981). More recently, chromatography has been used for determination of available lysine. Chromatographic methods offer good linearity and reproducibility but they are time and solvent consuming. The aim of the current study was to develop and validate a new method (sensitive, economical and with minimal solvent consumption), for quick analysis of available lysine content in different food products by automating a 96-well microtiter plate assay.

## 2. Materials and methods

### *2.1. Samples*

Three units from each of two batches of different food products marketed in Spain were purchased from a local supermarket (Valencia, Spain) and were analysed: liquid foods (soy, oat, quinoa beverages and UHT/sterilised milk) and powdered samples (powdered adapted, powdered follow-up and junior milk infant formulas) were used. The powdered samples were rehydrated in accordance with the manufacturer's instructions (130 g/L). Table 1 gives details (as indicated on the label) of each of the samples analysed.

**Table 1.** Nutritional composition of the commercial samples analysed as indicated on the labels.

| | Sample | Proteins | Carbohydrates | Fat |
|---|---|---|---|---|
| Liquid samples (g/100 mL) | Soy beverage | 3.4 | 3.7 | 0.9 |
| | Oat beverage | 1.0 | 6.1 | 1.0 |
| | Quinoa beverage | 3.0 | 3.7 | 2.8 |
| | Liquid UHT milk | 3.0 | 4.9 | 1.6 |
| | Liquid sterilised milk | 3.0 | 4.6 | 1.5 |
| Powdered samples (g/100 g) | Powdered adapted infant formula | 9 | 58 | 26 |
| | Powdered follow-up infant formula | 10 | 62 | 19 |
| | Junior milk infant formula | 13 | 56 | 25 |

UHT: ultra-high temperature.

*2.2. Materials and reagents*

All reagents were of analytical reagent grade. Anhydrous ethanol, sodium tetraborate, and sodium hydroxide were from Panreac (Barcelona, Spain). Casein from bovine milk was from Sigma-Aldrich (Steinheim, Germany). Intermediate (10 mg/mL casein) and working standard solutions were prepared in sodium tetraborate buffer (pH=9). Trichloroacetic acid was from Fluka (Buchs, Switzerland). Hydrochloric acid, β-mercaptoethanol, sodium dodecyl sulfate (SDS) and ethanol were from Merck (Darmstadt, Germany). *Ortho*-phthaldialdehyde (OPA) reagent was prepared daily according to Goodno et al. (1981) as follows: 80 mg OPA 99% (Merck) in 2 mL ethanol, 50 mL 0.1 M sodium tetraborate buffer (pH 9.7–10.0), 5 mL SDS (200 g/L), and 0.2 mL β-mercaptoethanol. Ninety-six-well black bottom plates were from Sero-Wel, Bibby Sterilin Ltd. (Stone, UK).

*2.3. Instrumentation*

All analysis were performed on a Spectrofluorophotometer (RF-5000 Shimadzu Corporation, Kyoto, Japan) for the OPA manual method and on a microplate spectrofluorometer Wallac 1420 VICTOR$^2$ multilabel counter from Perkin–Elmer (California, USA) for the OPA automated method. Setting was: $\lambda_{excitation}$=340 nm and $\lambda_{emission}$=455 nm.

*2.4. Procedure for analytical optimisation of the automated method*

Preparation of samples, interferences determination, preparation of blanks, calibration and calculations of available lysine determination were already optimised during the previous development of the manual method of the OPA assay (Ferrer et al. 2003). The amount of sample and the volume of water needed to obtain a solution containing 0.6-3% of proteins were used. Then, 1 mL of SDS solution (120 g/L) was added to 950 µL of water and 50 µL of liquid food or powdered sample (0.3-1.5 mg of proteins). In order to eliminate possible interferences caused by small peptides, 2 mL of trichloroacetic acid were added to 2 mL of liquid samples or powdered samples, and then centrifuged at 3000 rpm for 15 min. Nine hundred µL of water and 1 mL SDS solution (120 g/L) were added

to 100 µL of supernatant. For the preparation of blanks, 1 mL of SDS solution (120 g/L) was added to 1 mL of water. The tubes from samples, interferences and blanks were allowed to cool at 4 ºC for 12 h and the sonicated for 15 min at 25 ºC. Figure 1 shows a diagram of the preparation process. A standard of casein bovine milk was used to prepare a calibration curve. A set of casein working standards (0.1–1 mg/mL casein in assay solution), with lysine contents ranging from 0.0085 to 0.0850 mg lysine/mL assay solution, were prepared using 0.1 M sodium tetraborate buffer (pH=9) as solvent. The conversion factor of casein to lysine was calculated considering that: $a_{s1}$-casein, $a_{s2}$-casein, β-casein and κ-casein have, respectively, 14, 24, 11 and 9 residues of lysine/protein molecule, that the $\alpha_{s1}$-casein:$\alpha_{s2}$-casein:β-casein:κ-casein ratio was 0.45:0.12:0.33:0.10, the molecular weights were 23615, 25230, 23983 and 19007 daltons (Da), respectively, and the molecular weight of lysine was 146.1 Da (Eigel et al. 1984; Modler 1985). Thus, mg lysine/mg casein (F)=$\Sigma$ ($R_L$ x $W_L/Wc$ x $Rp$)=0.08484, where $R_L$ are the residues of lysine in each casein; $W_L$ is the molecular weight of lysine; *Wc* is the molecular weight of each casein, and *Rp* is the protein ratio in each casein.

## 3. Results and discussion

### *3.1. Adaptation and optimisation of the OPA method by automating a 96-well microtiter plate assay*

There is no methodology available to analyse available lysine using 96-well microtiter plates, the use of which would allow a rapid determination of available lysine in different samples after processing or storage at the same time. The described method is an adaptation of the OPA fluorometric assay for the determination of available lysine proposed by Ferrer et al. (2003) with modifications. After applying the method described, it was seen that with the conditions applied it was not possible to determinate available lysine content due to the reduced volume of wells. Sample quantity was decreased (5-20 µL). When 8 µL of sample was used, the best reproducibility of the results was found. The plate allows the analysis of a high number of samples at the same time.

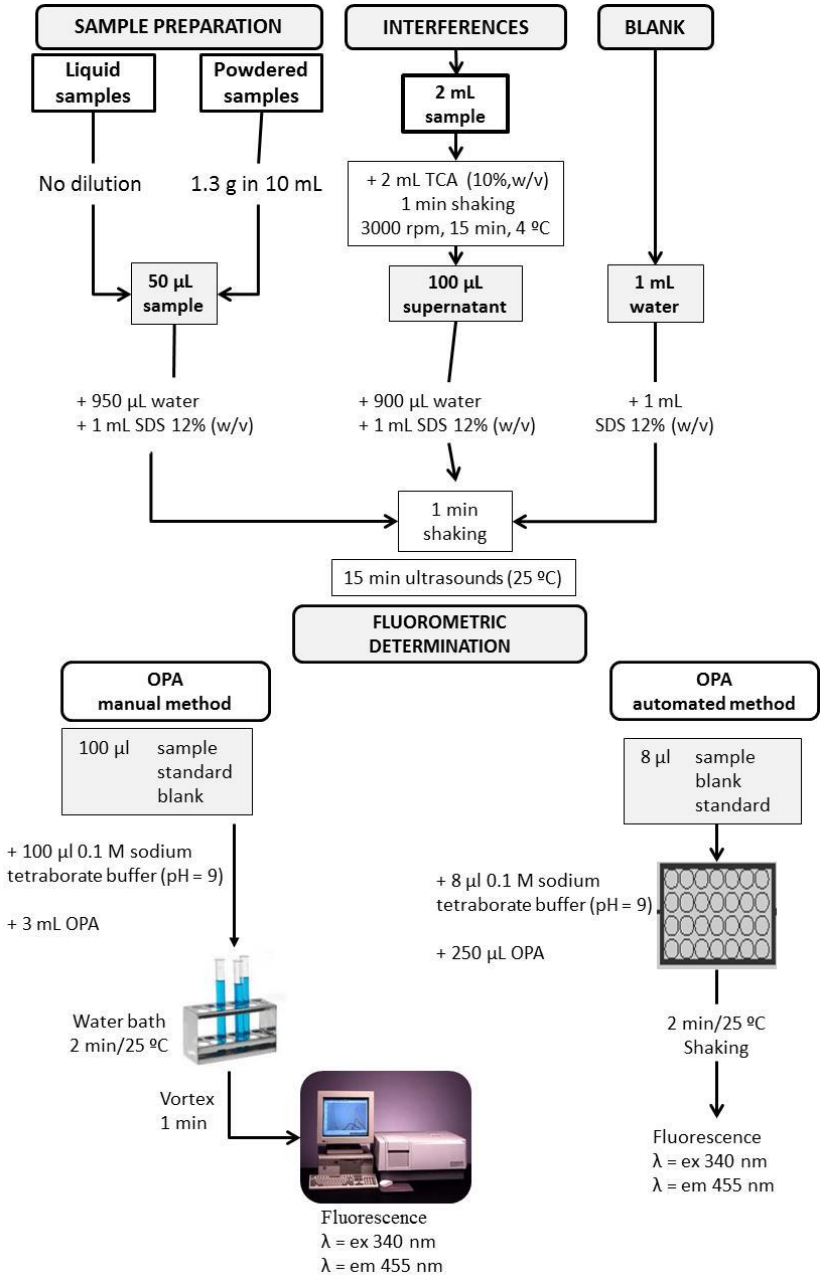


**Figure 1.** Experimental procedure scheme for available lysine determination.

The spectrofluorometer Wallac 1420 VICTOR$^2$ multilabel was programmed to use a two-reagent system. The reaction mode pipetted and transferred the sample (8 µL), 0.1 M sodium tetraborate buffer (pH=9) (8 µL), and main reagent (250 µL OPA) into the main reagent wells of their respective cuvette rotor positions. With the spinning of the rotor, the reagents were mixed and incubated for 2 minutes at 25 ºC and fluorescence was measured. Figure 1 shows a schematic of the experimental arrangement for automated determination of available lysine. The 0.1 M sodium tetraborate buffer (pH=9) was used as a blank, and 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL casein were used as standards. A sample of 0.3 mg/mL casein was used as quality control (QC). Samples and standard calibration solutions were always analysed in duplicate in a "forward-then-reverse" order as follows: blank, 0.1 mg/mL casein, 0.2 mg/mL casein, 0.4 mg/mL casein, 0.6 mg/mL casein, 0.8 mg/mL casein and 1 mg/mL casein, QC, sample 1 ... sample 1, QC, 1 mg/mL casein, 0.8 mg/mL casein, 0.6 mg/mL casein, 0.4 mg/mL casein, 0.2 mg/mL casein, 0.1 mg/mL casein, blank. This arrangement can correct possible errors due to the signal drifting associated with the different positions of the same sample. Determinations were carried out in quadruplicate. The absorbance of samples was corrected with the absorbance of the blank and of the interferences. Lysine content (mg) was obtained by interpolation in the calibration curve.

### *3.2. Validation of the OPA automated using a 96-well microtiter plate assay*

To verify the quality and usefulness of the method, the analytical parameters linearity, sensitivity, precision and percentage of recovery were determined for all the matrices mentioned in section 2.1. Linearity was checked in the range of 0.1-1 mg standard casein/mL, corresponding to 0.0085-0.085 mg available lysine/mL. Good linearity was obtained for the studied range of available lysine contents for the OPA fluorometric automated ($y$=7·10$^6$ $x$ + 6135.9; $R^2$=0.999) and manual ($y$=217.890 $x$ + 0.554; $R^2$=0.999) methods.

$S_{n-1}$ value was estimated based on the standard deviation of the blank obtained by analysing $n$=10 blanks. Detection limit values of the automated method were calculated in the cuvette and in the samples (liquid and powdered).

Detection limits in cuvette were 0.00008 mg and 0.00264 mg for automated and manual methods, respectively. In addition, detection limits of liquid samples were 0.0090 g/L and 0.0331 g/L for automated and manual methods, respectively. Meanwhile, detection limit values of powdered samples were 0.2544 g/kg and 0.6881 g/kg for automated and manual methods, respectively. These results showed that the OPA automated method was more sensitive than manual method for liquid and powdered samples, although in all cases, values obtained allowed the detection of available lysine in the studied samples. In addition, the OPA automated method was more sensitive for liquid foods than for powdered samples.

LOQ corresponds to the minimum quantity with which it is possible to quantify without uncertainty ($LOQ=10 \cdot S_{n-1}/m$). Detection limit values for the automated method were calculated in the cuvette and in the samples (liquid and powdered). Quantification limits in cuvette were 0.00026 mg and 0.00879 mg for automated and manual method, respectively. In addition, quantification limits for liquid samples were 0.1102 g/L and 0.2982 g/L for automated and manual method, respectively. Meanwhile, quantification limit values for powdered samples were higher in automated (0.8480 g/kg) and manual (2.2937 g/kg) method. These results showed that the OPA automated and manual method allowed the quantification of all the samples analysed, in the present study, without problems.

Instrumental precision was checked from six consecutive analysis of a sample extract and was expressed as relative standard deviations (RSD%). The instrumental precision values of liquid foods were 2.5% and 3.1% for automated and manual method, respectively. With regard to powdered samples, instrumental precision values given by the automated (2.1%) and manual method (2.9%) were in the range of those previously reported in liquid samples.

The precision of the method was determined by preparing six aliquots of the sample and was expressed as RSD (%). The RSDs for 6 replicates of liquid samples given by automated and manual methods were 3.0% and 3.8%, respectively. With regard to powdered samples, method precision values were 2.8% and 4.3%, for automated and manual methods, respectively. Due to the difficulty in finding

certified samples similar to those used in this study for evaluating method conditions, a recovery assay was carried out. A known quantity of casein standard (1 mg/mL casein assay; 0.085 mg/mL lysine of assay) was added to each of the samples analysed in this study and the method described was applied. The recovery percentages obtained in liquid samples were 100.3±1.9% and 101.0±1.2% for automated and manual method, respectively. Results obtained when the recovery assays were performed in powdered samples were in accord to those obtained for liquid samples. In this case, automated and manual methods quantified the 101.2±1.0% and 103.3±3.4% of available lysine, respectively.

In order to estimate if the studied methods can be used with the same confidence, precision and accuracy of the different samples were compared (Tables 2-3).

**Table 2.** *F* test for comparison of the precision of the OPA automated and manual fluorometric methods.

| Samples | $S'_1$ | $S'_2$ | *F* test[a] |
|---|---|---|---|
| Soy beverage | 0.0013 | 0.0027 | 0.4316 |
| Oat beverage | 0.0007 | 0.0009 | 0.7576 |
| Quinoa beverage | 0.0038 | 0.0065 | 0.5787 |
| Liquid UHT and sterilised milk | 0.0026 | 0.0031 | 0.8321 |
| Powdered adapted formula | 0.0103 | 0.0214 | 0.4800 |
| Powdered follow-up formula | 0.0293 | 0.1098 | 0.2665 |
| Junior milk formula | 0.0481 | 0.0720 | 0.6679 |

*n*=6. $S'^2$: corrected sample variance. UHT: ultra-high temperature. [a]$F=S_1'^2/S_2'^2$. tabulated *F* values: $F_{0.05(5,5)}$=5.05 (*p*=0.05). $F_{0.01(5,5)}$=10.97 (*p*=0.01).

In the comparison of precision, six batches of sample were analysed for the studied methods. A comparison of variance by an *F* test showed that the methods were similar in precision ($p>0.05$) (Table 2). In the comparison of accuracy (*t* test), six batches of the different samples were analysed by each method, twice and on different days (Table 3). From these results it should be noted that non-significant

differences ($p$<0.05) among the OPA manual and automated fluorometric assays were obtained for the samples analysed.

**Table 3.** *t* test for comparison of the accuracy of the OPA automated and manual fluorometric methods.

| Samples | Paired data *t*-test | | |
|---|---|---|---|
| | $\bar{d}$ | $S_{n-1}$ | $\frac{\lvert\bar{d}\rvert}{S_{n-1}} \cdot \sqrt{n}$ |
| Soy beverage | -0.04 | -0.06 | 1.60 |
| Oat beverage | 0.02 | 0.04 | 1.45 |
| Quinoa beverage | 0.10 | 0.09 | 2.30 |
| Liquid UHT and sterilised milk | 0.08 | 0.07 | 2.52 |
| Powdered adapted formula | -0.04 | 0.16 | 0.61 |
| Powdered follow-up formula | -0.19 | 0.34 | 1.23 |
| Junior milk formula | 0.03 | 0.32 | 0.19 |

$n$=6. UHT: ultra-high temperature. $\bar{d}$: mean value of differences (OPA automated method – OPA manual method). $S_{n-1}$: standard deviation of differences. $t_{n-1}^{0.05/2}$=2.571.

*3.3. Comparison of the available lysine contents after determination with OPA method using automated 96-well microtiter plate assay and traditional OPA method*

Table 4 shows the available lysine contents in the samples analysed (expressed as g/L for liquid foods and g/kg for powdered samples). In general, the average available lysine value in dairy proteins based formulas was in the range of the standard values of amino acid requirements established by the Institute of Medicine (2005) (children≤2 years 58, 10-12 years 44 and adults 16 mg lysine/g reference protein) and the mean value accepted for human milk (66 mg/g) established by WHO (2000). Available lysine contents of these formulas was lower than that for powdered milk prepared in a laboratory studied by Pereyra-Gonzáles et al. (2003) (80.4 mg/g), or those reported in the literature (76.6–85.4 mg/g protein) by different authors (Erbersdobler and Hupe 1991; Ferrer et al. 2003; van Mil and Jans 1991; Vigo et al. 1992). However, powdered adapted infant formula,

containing soy protein, had a lower content in available lysine ($p$<0.05) in comparison to the other formulas. Pereyra-Gonzáles et al. (2003) also observed significantly lower values in formulas containing soy protein with regard to dairy protein based formulas, obtaining similar values (45.0±8.3 mg/g protein) to those found in the present study. In addition, powdered infant formulas had lower contents of available lysine than those reported for the corresponding protein sources: soy protein (63.4–64.1 mg/g protein); casein (79.8–85.0 mg/g protein); milk (76.6–85.4 mg/g protein); whey protein (80–97.5 mg/g protein) (Erbersdobler and Hupe 1991; Friedman and Brandon 2001; Souci et al. 2000; Vigo et al. 1992). The losses were probably a consequence of the combined effects of the type of protein, the treatments during the manufacture and the different time and conditions of storage.

**Table 4.** Chemically available lysine content by OPA manual and automated fluorometric methods.

| Samples | Manual | | Automated | |
|---|---|---|---|---|
| Liquid samples | g/L | mg lys/g ref protein | g/L | mg lys/g ref protein |
| Soy beverage | 1.09±0.04 | 31.91±1.04 | 1.07±0.03 | 31.47±0.83 |
| Oat beverage | 0.67±0.02 | 67.50±1.41 | 0.66±0.02 | 66.00±2.83 |
| Quinoa beverage | 2.08±0.07 | 69.17±2.59 | 2.22±0.05 | 74.33±1.41 |
| Liquid UHT and sterilised milk | 2.02±0.04 | 68.00±1.89 | 2.12±0.04 | 71.00±2.36 |
| Powdered samples | g/kg | mg lys/g ref protein | g/kg | mg lys/g ref protein |
| Powdered adapted formula | 3.72±0.09 | 41.22±1.41 | 3.45±0.07 | 38.33±0.79 |
| Powdered follow-up formula | 5.97±0.22 | 59.45±2.47 | 5.42±0.13 | 54.25±1.06 |
| Junior milk formula | 8.56±0.20 | 65.81±1.03 | 8.54±0.17 | 65.58±0.82 |

$n$=6. Lys: lysine. Ref: reference. UHT: ultra-high temperature.

Amigo-Benavent et al. (2008) found available lysine values ranging from 1.54 to 9.76 mg/g of protein in soy products. When they studied a liquid soymilk beverage similar to the soymilk studied in the present study, they reported

available lysine content (29.2 mg/g) very closed to that found in this study. In addition, data on lysine content of the soymilk studied in this work compared well with previously published data (Kwok et al. 1998; Souci et al. 2000).

In the published literature, it was not possible to find any studies of chemically available lysine contents in oat or quinoa-based beverages in order to make a comparison with results obtained in this study. However, bearing in mind the nutritional purpose of these foods in some population groups, it should be noted that compliance in available lysine content is essential to ensure nutritional quality and to avoid potential harmful effects in target groups. Moreover, taking into account the lysine content of oat and quinoa beverages, it can be concluded that generally the protein quality of these beverages was comparable to that of cow's milk (74.3 mg/g of protein) and egg whites (64.9 mg/g of protein) (Souci et al. 2000).

**4. Conclusion**

The analytical parameters: linearity, detection limit, precision and accuracy of the assay showed that the automated method studied was useful for measuring the available lysine content in food products. The method was easy to perform and the fact that sample preparation was the same for all the samples makes it suitable as a method for routine determinations. This method was valid in order to quantify the changes in the available lysine content of liquid foods and powdered samples after processing/preservation treatments and subsequent storage.

**Acknowledgements**

This research project was supported by the Spanish Ministry of Science and Technology and European Regional Development Funds (AGL2010-22206-C02-01). Barba, F.J received a contract employment from this project to carry out the study. Carbonell-Capella, J.M. holds an award from the Spanish Ministry of Education (AP2010-2546).

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## 3.4. High pressure processing of fruit juice mixture sweetened with *Stevia rebaudiana*: Optimal retention of physical and nutritional quality

*Innovative Food Science and Emerging Technologies 18 (2013) 48-56*

# High pressure processing of fruit juice mixture sweetened with *Stevia rebaudiana*: Optimal retention of physical and nutritional quality

Juana M. Carbonell-Capella[a], Francisco J. Barba[b], María J. Esteve[a], Ana Frígola[a]*

[a]University of Valencia, Department of Nutrition and Food Science, Av. Vicent Andrés Estellés, s/n, 46100 Burjassot, Spain

[b]Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas (CSIC), Avda. Agustín Escardino, 7.46980 Paterna. Spain

*Corresponding author:
Ana Frígola
e-mail: ana.frigola@uv.es.
Phone: +34 963544955 Fax: +34 963544954

**ABSTRACT**

The impact of high pressure processing (HPP) technology on physicochemical properties (colour, browning index, turbidity index), bioactive compounds (ascorbic acid, total phenolic compounds, total anthocyanins, total carotenoids) and antioxidant capacity of a fruit juice mixture (papaya (32.5%, v/v), mango (10%, v/v) and orange (7.5%, v/v)) sweetened with *Stevia rebaudiana* Bertoni at different percentages was studied. The experimental design comprised a response surface methodology according to a central composite face-centred design. The variable ranges were 300–500 MPa (pressure), 5-15 min (time), 0-2.5% stevia percentage. This design was used to determine the optimal high pressure-stevia concentration in order to obtain the best retention of physicochemical and nutritional quality in the beverage following high pressure. HPP conducted at 300 MPa for 14 min led to a beverage with the greatest presence of antioxidant compounds and total colour differences lower than 3.

**Industrial relevance:** There has been an increased interest in the use of non-caloric sweeteners from plant sources, among them is *Stevia rebaudiana* Bertoni, due to the growing evidence of its health benefits. Combined mixtures of *S. rebaudiana* water extracts and fruit juice can be a useful tool in order to provide new food products with increased nutritional properties. Moreover, high pressure processing (HPP) allows the acquisition of drinks that keep their characteristics similar to the fresh product. A deeper knowledge of the effect of HPP on the nutritional and physicochemical characteristics of these new beverages processed by HPP with regard to unprocessed juices is necessary.

**Keywords**: *Stevia rebaudiana***,** exotic fruits**,** orange**,** bioactive compounds**,** total antioxidant capacity**,** high pressure processing.

## 1. Introduction

In the last years, new functional juices without sugar and/or obtained from exotic fruits are becoming common in Japanese, US and EU markets and have been receiving considerable attention as their market potential grows (Perumalla & Hettiarachchy, 2011; Puri, Sharma, & Tiwari, 2011). In addition, food industry has shown increased interest in plant extracts from *Stevia rebaudiana*, because it can be a nutritional strategy in order to replace or substitute sugar energy content due to its high content in non-nutritive sweeteners (Nehir El & Simsek, 2012). Currently, stevia in leaf or extracted forms was approved by FDA as a dietary supplement in the US, and under similar classifications in several other countries. In November 2011, the European Commission approved steviol glycosides as food additives (European Commission, 2011), which will probably lead to wide-scale use in Europe (Stoyanova, Geuns, Hideg, & Van den Ende, 2011). So far, little data has been available regarding the practical applications in foods and stability under different processing and storage conditions (Nehir El & Simsek, 2012). The leaves of stevia have functional and sensory properties superior to those of many other high-potency sweeteners, and is likely to become a major source of high-potency sweetener for the growing natural food market (Goyal, Samsher, & Goyal, 2010). Moreover, it has been reported that stevia is nutrient-rich, containing substantial amounts of minerals, vitamins, polyphenols and other antioxidant compounds. In some countries, stevia has been consumed as a food and medicine (ethnobotanical) for many years, including most notably Japan and Paraguay (Lemus-Mondaca, Vega-Gálvez, Zura-Bravo, & Ah-Hen, 2012). In addition, stevia sweetener extractives are suggested to exert beneficial effects on human health, as they have anti-hyperglycaemic, anti-hypertensive, anti-inflammatory, anti-tumour, anti-diarrhoeal, diuretic, and immunomodulatory effects (Chatsudthipong & Muanprasat, 2009).

On the other hand, orange and different exotic fruits such as mango and papaya are a good source of bioactive compounds like ascorbic acid, polyphenols, and carotenoids. These compounds have been shown to be good contributors to the total antioxidant capacity of foods (Zulueta, Esteve, & Frígola, 2009; Vijaya,

Sreeramulu, & Raghunath, 2010) and have been involved in the prevention of some degenerative diseases (Devalaraja, Jain, & Yadav, 2011).

The development of non-thermal processing technologies combined with natural additives with antioxidant activity to obtain healthier and safer food products is one of the major challenges facing the food industry in the new century (Barros-Velazquez, 2011; Norton & Sun, 2008). So, high pressure processing (HPP) can be a useful tool in order to achieve this goal. With this kind of treatment it is possible to inactivate and inhibit microorganisms, and it can activate or inactivate enzymes at low temperatures (Saucedo-Reyes, Marco-Celdrán, Pina-Pérez, Rodrigo, & Martínez-López, 2009; USFDA, 2000), while compounds of low molecular weight, such as vitamins and compounds related to pigmentation and aroma, remain unaltered (Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007). In fluid foods, pressure is transmitted uniformly and instantly, that is, there are no gradients (it follows the so-called isostatic rule) (Thakur & Nelson, 1998; Toepfl, Mathys, Heinz, & Knorr, 2006). Unlike what happens with heat processes, HPP is independent of the size and geometry of the product, which reduces the time required to process large quantities of food (Rastogi et al., 2007).

Response surface methodology (RSM) has been used in the optimisation of food processes to define the relationships between the responses and independent variables. RSM has important application in the design, development, and formulation of new products (Bas & Boyaci, 2007). Different authors have used RSM to evaluate the HPP conditions such as the pressure, temperature and time on antioxidant compounds and physicochemical parameters of food products (Roldán-Marín, Sánchez-Moreno, LLoría, De Ancos, & Cano, 2009; Terefe, Matthies, Simons, & Versteeg, 2009). These authors concluded that all the factors, pressure, temperature, and time, significantly influenced different nutritional and quality parameters.

The aims of the present study were (1) to investigate the effects of the HPP conditions, specifically pressure and time, as well as the influence of stevia concentrations on bioactive compounds (ascorbic acid, total phenolics, total anthocyanins, total carotenoids), antioxidant capacity, and physicochemical

properties (turbidity, browning, colour) of a fruit juice mixture sweetened with stevia and (2) to determine optimum conditions in order to obtain a fruit juice mixture beverage sweetened with stevia with the highest levels of health-related compounds and the best physicochemical properties.

## 2. Materials and methods

### *2.1. Samples*

#### 2.1.1. Fruit juice mixture

Oranges (*Citrus aurantium*, cultivar Salustiana), mango (*Mangifera indica*), and papaya (*Carica papaya*) were purchased from a local supermarket (Valencia, Spain). Orange, mango and papaya juices were extracted after appropriate washing and hygienisation of the fruits, then the pulp was removed. The fruit juice mixture was prepared by mixing 32.5% (v/v) of papaya juice, 10% (v/v) of mango juice, 7.5% (v/v) of orange juice, and water to 100%.

#### 2.1.2. Stevia infusion

A stock solution of 8.33% (w/v) was prepared from dried leaves. One hundred mL of boiling distilled water were added to the dried leaves (8.33 g), the mix was covered and let infuse for 30 min. The infusion was vacuum filtered using filter paper (Whatman® No. 1, Whatman International Ltd., UK) and the filtrate obtained was stored at –40 ºC.

#### 2.1.3. Fruit juice-stevia mixture (FJ-stevia)

Different volumes of stevia stock solution (3 and 6 mL) were added to 14 mL of fruit juice mixture to obtain stevia concentrations of 1.25 and 2.50%, respectively. Water was added when necessary to complete a final matrix volume of 20 mL. In parallel, a blank sample without stevia in its composition (0% stevia) was formulated with 14 mL of fruit mixture juice and 6 mL of water. The maximum stevia concentration (2.5%) was selected taking into account the sucrose concentration of commercial fruit based beverages and the sweetness

equivalence stevia/sucrose (Savita et al., 2004). Under these conditions, samples were rated as excellent and were characterised by higher acceptability.

*2.2. Chemicals*

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS (2,2´-azinobis(3-ethylbenzothiazoline 6-sulfonate)), Folin-Ciocalteu reagent, and fluorescein sodium salt were purchased from Sigma-Aldrich (Steinheim, Germany). Gallic acid was purchased from UCB (Brussels, Belgium). Hexane (LC grade), potassium hydroxide, and hydrogen peroxide were purchased from Scharlau (Barcelona, Spain). Sodium and disodium phosphate, L(+)-ascorbic acid, acetonitrile (special grade), magnesium hydroxide carbonate (40-45%), and 2,2´-azobis-(2-amidinopropane) dihydrochloride (AAPH) were purchased from Panreac (Barcelona, Spain). Ethanol, diethyl ether, methanol, hydrochloric acid, and sodium chloride (special grade) from Baker (Deventer, The Netherlands). Chloroform was obtained from Merck (Darmstadt, Germany).

*2.3. HPP Equipment*

Samples, inserted in low density polyethylene bottles, were placed in polyethylene bags filled with water and heat-sealed (MULTIVAC Thermosealer, Hünenberg, Switzerland) before being placed in the HPP unit (High-Pressure Food Processor; EPSI NV, Belgium). The equipment consists on a vessel with an internal diameter of 100 mm and 300 mm high, with an operation pressure vessel of 689 MPa and an operation temperature vessel of −20 to 100 °C and a volume of 2.35 L. The pressure medium was a water–ethylenglycol mixture (70:30). Samples were pressurised at 300, 400 and 500 MPa for specific times in a range of 5, 10, and 15 min. Pressure level, pressurisation time, and temperature were controlled automatically. Pressure increase rate was 300 MPa/min and depressurisation time was less than 1 min. Initial temperature was 15 °C, final temperature after pressurisation at highest pressure was 32 °C, final temperature after holding time at highest pressure was 26.6 °C and final temperature after decompression at highest pressure was 12.5 °C. Come-up time was 90 s and decompression time was 15 s. All treatments were applied in duplicate, with three bottles per

treatment. Immediately after pressurisation, samples were transferred to an ice/water bath (Armfield FT61, UK), packed, and then stored under refrigeration (4±1 °C) until needed for analysis. For HPP, literature reports 5-15 min at 300-500 MPa to achieve 5-log reduction of different foodborne pathogens in different liquid foods (Alpas, Kalchayanaud, Bozoglu, & Ray, 2000; Donsì, Ferrari, Di Matteo, & Bruno, 1998).

*2.4. Determination of ascorbic acid*

A Metrohm 746 VA Trace Analyser (Herisau, Switzerland) equipped with a Metrohm 747 VA stand was used for the polarographic determination. The working electrode was a Metrohm multi-mode electrode operated in the dropping mercury mode. A platinum wire counter electrode and a saturated calomel reference electrode were used. The following instrumental conditions were applied: $DP_{50}$, dropping mercury mode, drop size 2, drop time 1 s, scan rate 10 mV/s, initial potential −0.10 V. Beverage (5 mL) was diluted to 25 mL with the extraction solution (oxalic acid 1% w/v, trichloroacetic acid 2% w/v, sodium sulfate 1% w/v). After vigorous shaking, the solution was filtered through a folded filter (Whatman® No. 1, Whatman International Ltd., UK). Oxalic acid (9.5 mL) 1% (w/v) and 2 ml of acetic acid/sodium acetate 2 M buffer (pH=4.8) were added to an aliquot of 0.5 mL of filtrate and the solution was transferred to the polarographic cell. Determinations were carried out by using the peak height and standard addition method in accordance to Barba, Esteve, Tedeschi, Brandolini, and Frígola (2013).

*2.5. Total Phenolic Compounds*

Total phenols were determined according to the method reported by Georgé, Brat, Alter, and Amiot (2005). Briefly, 10 mL of sample was homogenised with 50 mL of a mixture of acetone/water (7/3, v/v) for 30 min. Mixture supernatants were then recovered by filtration (Whatman® No. 2, England) and constituted the raw extracts (REs). REs (2 mL) were settled on an Oasis cartridge (Waters). Interfering water-soluble components (Steviol glycosides, reducing sugars, ascorbic acid) were recovered with 2 x 2 mL of distillate water. The recovered

volume of the washing extract (WE) was carefully measured. In order to eliminate vitamin C, heating was carried out on the washing extract (HWE). All extracts (RE, WE, and HWE) were submitted to the Folin−Ciocalteu method, adapted and optimised (Barba et al., 2013). Gallic acid calibration standards with concentrations of 0, 100, 300, 500, 700 and 1000 ppm were prepared and 0.1 mL was transferred to borosilicate tubes. 3 mL of sodium carbonate solution (2% w/v) and 0.1 mL of Folin–Ciocalteau reagent (1:1, v/v) were added to 0.1 mL of all gallic acid standard and sample tubes. The mixture was incubated for 1 h at room temperature and absorbance was measured at 765 nm.

*2.6. Total anthocyanins*

Total anthocyanins were determined using a modified method of Mazza, Fukumoto, Delaquis, Girard, and Ewert (1999). A 10-fold diluted sample of 100 µl was mixed with 1700 µl of distilled water and 200 µl of 5% (v/v) HCl. The sample was hold at room temperature for 20 min before measuring the absorbance at 520 nm in a 10 mm cuvette. This reading corresponds to the total anthocyanins content after considering the relevant dilution. Calculations of total anthocyanins were based on cyanidin-3-glucoside (molar absorptivity 26900 L/mol·cm).

*2.7. Total Carotenoids*

Extraction of total carotenoid was carried out in accordance with Lee and Castle (2001). An aliquot of sample (2 mL) was homogenised with 5 mL of extracting solvent (hexane/acetone/ethanol, 50:25:25, v/v) and centrifuged for 5 min at 4000 rpm at 5 ºC. The top layer of hexane containing the colour was recovered and adjusted to 25 mL with hexane. Total carotenoid determination was carried out on an aliquot of the hexane extract by measuring the absorbance at 450 nm. Total carotenoids were calculated according to Ritter and Purcell (1981) using an extinction coefficient of β-carotene, $E^{1\%}$=2505.

### *2.8. Total Antioxidant Capacity*

#### 2.8.1. $ABTS^{\bullet+}$ test

The method used was described by Re et al. (1999), based on the capacity of a sample to inhibit the ABTS radical ($ABTS^{\bullet+}$) (Sigma-Aldrich, Steinheim, Germany) compared with a reference antioxidant standard (Trolox®) (Sigma-Aldrich, Steinheim, Germany). The radical was generated using 440 µL of potassium persulfate (140 mM). The solution was diluted with ethanol (Baker, Deventer, The Netherlands) until an absorbance of 0.70 was reached at 734 nm. Once the radical was formed, 2 mL of $ABTS^{\bullet+}$ was mixed with 100 µL of appropriately diluted sample and the absorbance was measured at 734 nm for 20 min in accordance with Zulueta, Esteve, and Frígola (2009). Results, obtained from duplicate analyses, were expressed as: mM TE (millimolar Trolox equivalents).

#### 2.8.2. ORAC (Oxygen Radical Absorbance Capacity) assay

The ORAC assay used, with fluorescein (Sigma-Aldrich, Steinheim, Germany) as the "fluorescent probe," was that described by Ou, Hampsch-Woodill, and Prior (2001). The automated ORAC assay was carried out on a Wallac 1420 VICTOR$^2$ multilabel counter (Perkin-Elmer, Valencia, Spain) with fluorescence filters, for an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The measurements were made in plates with 96 white flat-bottom wells (Sero-Wel, Bibby Sterilin Ltd., Stone, UK). The reaction was performed at 37 °C as the reaction was started by thermal decomposition of AAPH in 75 mM phosphate buffer (pH 7.0) because of the sensitivity of fluorescein to pH. The final reaction tested and the concentrations of the different reagents were determined following Zulueta, Esteve, and Frígola (2009).

### *2.9. Physicochemical Properties*

To measure turbidity index (TI), samples were centrifuged (618 g, 10 min, 20 °C), supernatant was taken, and absorbance at 660 nm was measured (Krop & Pilnik, 1974). To determine browning index (BI), samples were centrifuged (824×g, 20 min, 18 °C), and the supernatant was taken and diluted with ethanol (1:1, v/v).

The mixture was filtered with Whatman® No. 2 filters and absorbance of the filtrate was measured at 420 nm (Meydav, Saguy, & Kopelman, 1977). Colour analysis was performed using a Hunter Labscan II spectrophotometric colourimeter (Hunter Associates Laboratory Inc., Reston, VA., U.S.A.) controlled by a computer that calculates colour ordinates from the reflectance spectrum. Results were expressed in accordance with the Commission International d'Eclairage LAB (CIELAB) system with reference to illuminant D65 and with a visual angle of 10°. Three consecutive measurements of each sample were taken. CIE $L^*$ (lightness [0=black, 100=white]), $a^*$ ($-a^*$=greenness, $+a^*$=redness) and $b^*$ ($-b^*$=blueness, $+b^*$=yellowness) values were used to calculate the total colour differences ($\Delta E^*=[(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$), where $\Delta L^*$, $\Delta a^*$, and $\Delta b^*$ are differences between the untreated HP-treated beverage (Calvo, 2004).

*2.10. Experimental design and statistical analysis*

A face-centred central composite response surface analysis was used to determine the effect of pressure (MPa) ($P$), time (minutes) ($t$) and *S. rebaudiana* concentration (%, w/v) (*%* stevia) on the health-related compounds (ascorbic acid, total phenolic compounds, total anthocyanins, total carotenoids), antioxidant capacity (TEAC and ORAC methods), and physicochemical properties (colour, turbidity and browning index) of the beverage. Response surface methodology (RSM) consists of a set of mathematical and statistical methods developed for modelling phenomena and finding combinations of a number of experimental factors (variables) that will lead to optimum responses. With RSM, several variables are tested simultaneously with a minimum number of trials, according to special experimental designs, which elucidates interactions between variables. This is not an option with classical approaches. In addition, RSM has the advantage of being less expensive and less time-consuming than the classical methods. The independent variables of the RSM were: pressure (from 300 to 500 MPa), time (from 5 to 15 minutes) and stevia concentration (from 0 to 2.5%, w/v). The levels for each independent parameter were chosen considering sample and equipment limitations. Three (maximum, minimum and central) values of each factor were considered, leading to 26 experiments (Table 1).

**Table 1.** Experimental design matrix in terms of actual variables and the average values of the response for experiments on the effect of combined high pressure-*Stevia rebaudiana* concentration on the antioxidant activities of a fruit juice mixture.

| Run[a] | Pressure (MPa) | Time (min) | Stevia (%) | TC[b] (µg/100 mL) | TPC[b] (mg GAE/L) | TA[b] (mg/L) | TEAC[b] (mM TE) | ORAC[b] (mM TE) |
|---|---|---|---|---|---|---|---|---|
| | ($X_1$) | ($X_2$) | ($X_3$) | ($Y_1$) | ($Y_2$) | ($Y_3$) | ($Y_4$) | ($Y_5$) |
| 1 | 500 | 15 | 0 | 364.3±15.1 | 165.7±9.8 | 21.4±1.0 | 2.91±0.21 | 3.41±0.41 |
| 2 | 300 | 5 | 0 | 294.6±17.3 | 184.5±10.2 | 23.2±0.4 | 2.68±0.12 | 4.67±0.50 |
| 3 | 500 | 15 | 0 | 349.3±15.4 | 143.7±8.6 | 22.9±0.5 | 2.92±0.31 | 3.20±0.25 |
| 4 | 500 | 15 | 2.5 | 329.3±18.3 | 3683.1±151.5 | 46.4±1.1 | 23.54±1.01 | 34.95±0.71 |
| 5 | 300 | 15 | 0 | 279.4±15.3 | 164.2±9.6 | 22.3±0.3 | 2.34±0.32 | 4.87±0.43 |
| 6 | 300 | 5 | 2.5 | 304.6±20.4 | 4494.6±201.5 | 30.7±0.6 | 18.73±0.81 | 33.33±0.32 |
| 7 | 400 | 10 | 1.25 | 319.4±16.1 | 2756.5±156.4 | 26.3±0.4 | 18.47±0.72 | 19.22±0.63 |
| 8 | 300 | 15 | 2.5 | 304.4±15.6 | 4146.5±210.3 | 28.9±0.3 | 26.32±1.01 | 30.73±0.81 |
| 9 | 500 | 5 | 2.5 | 330.3±12.7 | 3815.8±198.4 | 39.1±1.0 | 34.03±1.03 | 37.68±0.85 |
| 10 | 400 | 10 | 1.25 | 300.6±18.3 | 2869.2±156.1 | 26.2±0.5 | 19.24±0.81 | 20.81±0.75 |
| 11 | 400 | 5 | 1.25 | 289.4±20.1 | 2837.1±149.3 | 27.1±0.6 | 17.35±0.92 | 24.13±0.67 |
| 12 | 400 | 10 | 2.5 | 305.3±21.1 | 3901.0±205.6 | 36.6±0.8 | 29.64±0.71 | 29.81±0.65 |
| 13 | 300 | 15 | 2.5 | 284.4±13.6 | 4220.6±210.9 | 33.2±1.1 | 26.32±0.81 | 30.73±0.81 |
| 14 | 500 | 5 | 2.5 | 325.6±12.5 | 4000.2±208.7 | 39.6±1.0 | 34.05±0.83 | 36.09±1.00 |
| 15 | 300 | 10 | 1.25 | 289.5±14.6 | 3057.9±132.6 | 27.5±0.9 | 16.21±0.61 | 24.09±0.71 |
| 16 | 500 | 5 | 0 | 295.4±17.0 | 170.7±9.6 | 23.0±1.0 | 2.80±0.24 | 4.33±0.21 |
| 17 | 500 | 15 | 2.5 | 314.4±17.2 | 4060.4±263.4 | 45.6±2.0 | 23.54±0.71 | 34.95±0.86 |
| 18 | 500 | 5 | 0 | 300.7±13.6 | 173.8±10.0 | 23.3±1.8 | 2.93±0.10 | 4.07±0.15 |
| 19 | 300 | 15 | 0 | 289.4±15.3 | 184.8±7.6 | 23.6±1.0 | 2.72±0.31 | 4.64±0.16 |
| 20 | 400 | 15 | 1.25 | 315.4±14.4 | 2919.3±123.6 | 28.1±0.9 | 20.07±0.72 | 21.63±0.51 |
| 21 | 400 | 10 | 0 | 279.9±16.6 | 169.5±8.3 | 24.0±0.7 | 3.67±0.21 | 3.95±0.17 |
| 22 | 300 | 5 | 2.5 | 248.6±15.3 | 4355.5±213.1 | 29.3±0.8 | 18.92±0.80 | 29.43±0.61 |
| 23 | 400 | 10 | 1.25 | 339.3±16.1 | 2638.8±160.5 | 26.7±0.9 | 19.22±1.01 | 20.47±0.55 |
| 24 | 400 | 10 | 1.25 | 349.3±15.2 | 2728.9±149.7 | 27.9±0.7 | 18.89±0.92 | 19.26±0.43 |
| 25 | 300 | 5 | 0 | 304.6±15.2 | 177.9±7.3 | 22.3±0.8 | 2.37±0.34 | 3.78±0.12 |
| 26 | 500 | 10 | 1.25 | 315.8±12.3 | 2818.5±162.5 | 25.5±0.6 | 20.43±0.81 | 22.05±0.46 |

TC: total carotenoids. TPC: total phenolic compounds. TA: total anthocyanins. TEAC: trolox equivalent antioxidant capacity. ORAC: oxygen radical antioxidant capacity.[a] Order of the assays was randomised.[b] Data shown are the mean±SD of two treatment repetitions, each assay was performed in triplicate.

The experimental design was performed twice, resulting in two blocks of experiments. The combinations included HPP-stevia conditions with an intermediate level (central point) of the three variables replicated 4 times, which was used to determine inherent variance in the technique. Experiments were randomised to minimise the systematic bias in the observed responses due to extraneous factors and to increase precision. Experimental data were fitted to a polynomial response surface. The second-order response function was predicted using the following equation (Eq. (1)):

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{i=i+1}^{3} b_{ij} X_i X_j \qquad (1)$$

where $Y$ represents a response variable, $b_0$ is the centre point of the system, $b_i$, $b_{ii}$, and $b_{ij}$, are coefficients of the linear, quadratic and interactive effect, respectively; $X_i$, $X_i^2$, and $X_i X_j$ represent linear, quadratic and interactive effect of the independent variables, respectively.

The non-significant terms were deleted from the second-order polynomial model after an ANOVA test, and a new ANOVA was performed to obtain the coefficients of the final equation for better accuracy. The experimental design and the data analysis were performed were performed using SPSS® (Statistical Package for the Social Sciences) v.19.0 for Windows (SPSS Inc., Chicago, USA). The optimisation was done following the method proposed by Derringer and Suich (1980). All the individual desirability functions obtained for each response were combined into an overall expression, which is defined as the geometrical mean of the individual functions. The nearer the desirability value to the unit, the more adequate the system (Ross, 1996). In the present study, desirability functions were developed in order to obtain the beverage with the highest levels of antioxidant compounds and the best physicochemical properties. Subsequently, an ANOVA of three factors (pressure, time, and *Stevia rebaudiana* concentration) was applied, and in the parameters for which significant differences were obtained with more than two levels, Tukey's test was applied to ascertain the range of values in which the differences were located. Finally, a study was conducted with the aim of determining whether there were correlations between a pair of variables (Pearson's test).

## 3. Results and discussion

### *3.1. Effect of HPP and* stevia *concentration on nutritional qualities and antioxidant capacity of the beverages*

The ascorbic acid concentration in the fruit juice blend (papaya, mango and orange) without stevia was 25.5±0.3 mg/100 mL (Table 2). These results were in close agreement with the values obtained by other authors in papaya, mango and orange (Beserra-Almeida et al., 2011; Burdulu, Koca, & Karadeniz, 2006; U.S. Department of Agriculture (USDA) & Agricultural Research Service, 2012). In addition, similar results were found for the fruit juice mixtures sweetened with stevia at 1.25% (w/v) (25.3±0.2 mg/100 mL), and 2.5% (w/v) (25.3±0.1 mg/100 mL), respectively. Immediately after HPP, ascorbic acid retention was higher than 92% in all cases (data not shown). Several authors have reported that ascorbic acid of fruit and vegetable juices was minimally affected by HPP at mild temperatures (Barba, Esteve, & Frígola, 2010; Barba, Esteve, & Frígola, 2011a; Bull et al., 2004).

**Table 2.** Physicochemical and nutritional characteristics of untreated fruit juice mixture sweetened with *Stevia rebaudiana* Bertoni.

| Parameters | Beverage with stevia (%) | | |
|---|---|---|---|
| | 0 | 1.25 | 2.50 |
| Ascorbic acid (mg/100 mL) | 25.5±0.3$^{a}$ | 25.3±0.2$^{a}$ | 25.3±0.1$^{a}$ |
| Total phenolics (mg GAE/L) | 166.9±11.7$^{a}$ | 2509.5±142.5$^{b}$ | 3824.4±100.0$^{c}$ |
| Total anthocyanins (mg/L) | 21.8±0.1$^{a}$ | 24.6±0.5$^{b}$ | 28.8±0.3$^{c}$ |
| Total carotenoids (μg/100 mL) | 329.3±14.1$^{a}$ | 337.6±10.1$^{a}$ | 324.4±7.1$^{a}$ |
| ORAC (mM TE) | 4.5±0.5$^{a}$ | 22.2±0.7$^{b}$ | 32.8±1.7$^{c}$ |
| TEAC (mM TE) | 2.3±0.1$^{a}$ | 17.9±1.4$^{b}$ | 20.5±0.8$^{c}$ |
| Browning index | 0.097±0.003$^{a}$ | 2.313±0.033$^{b}$ | 1.581±0.004$^{c}$ |
| Turbidity index | 0.075±0.001$^{a}$ | 0.613±0.004$^{b}$ | 0.316±0.003$^{c}$ |
| Lightness (*L**) | 72.4±0.2$^{a}$ | 39.3±0.1$^{b}$ | 36.7±0.1$^{c}$ |
| Redness (*a**) | -1.9±0.1$^{a}$ | 10.8±0.2$^{b}$ | 10.1±0.1$^{c}$ |
| Blueness (*b**) | 8.5±0.2$^{a}$ | 50.4±0.2$^{b}$ | 39.3±0.1$^{c}$ |
| ºBrix | 6.4±0.1$^{a}$ | 7.4±0.1$^{b}$ | 7.8±0.1$^{c}$ |

$^{a-c}$ Different letters in the same file indicate significant statistical differences in function of the stevia percentage.

Results obtained for total phenolic compounds, total anthocyanins, total carotenoids and antioxidant capacity in the untreated and HPP samples are shown in Tables 1-2. Total phenolics content (TPC) of untreated fruit juice mixture without stevia sweetened was 166.9±11.7 mg GAE/L. However, TPC values were 15 and 23-fold higher when stevia at 1.25% and 2.5% stevia (w/v) used as a sweetener, respectively. These results were in close agreement with some previous studies that have reported high levels of phenolic compounds in *S. rebaudiana* products. Tadhani, Patel, & Subhash (2007) and Abou-Arab & Abu-Salem (2010) obtained that total phenolic compounds in stevia water extracts were 25.18 and 24.01 mg gallic acid equivalents (GAE)/g dry weight basis, respectively. In addition, Shukla, Mehta, Mehta, & Bajpai (2011) found 56.74 mg GAE in 1 g of aqueous leaf extract while ethanolic leaf extract of *S. rebaudiana* has been reported to show 61.50 mg GAE of phenols (Shukla, Mehta, Bajpai, & Shukla, 2009).

Three-way ANOVA showed that the preservation treatment applied (pressure, time) and the percentage of stevia had a significant influence ($p$<0.05) on the values of total phenolics. As can be seen in Figure 1, the behaviour of TPC was different after applying HPP depending on stevia concentration used in the formulation of the beverages. In addition, a multiple linear regression equation of a second order polynomial model was generated in order to elucidate the effects of % stevia and HPP (pressure and time) on the beverages. The reduced regression model presented in the Eq. (2) allowed for prediction of the effects of independent variables on total phenolic compounds.

$$\text{TPC (mg GAE/L)} = -14240.5 + 91.9 \cdot P - 557.7 \cdot t + 2432.2 \cdot \%stevia - 0.1\, P^2 + 1.3 \cdot P^* t - 1.1 \cdot P^* \%stevia + 24.4 \cdot t^* \%stevia - 286.0 \cdot \%stevia^2 \quad (2)$$

The statistical analysis indicates that the quadratic model proposed for TPC was adequate ($p$<0.05) in order to evaluate the changes after applying HPP, with satisfactory determination coefficients ($R^2$=0.961, $p$<0.05, standard error=1.761). No significant lack of fit of the model was found, showing that it fits properly within the range of HPP-stevia assayed conditions.

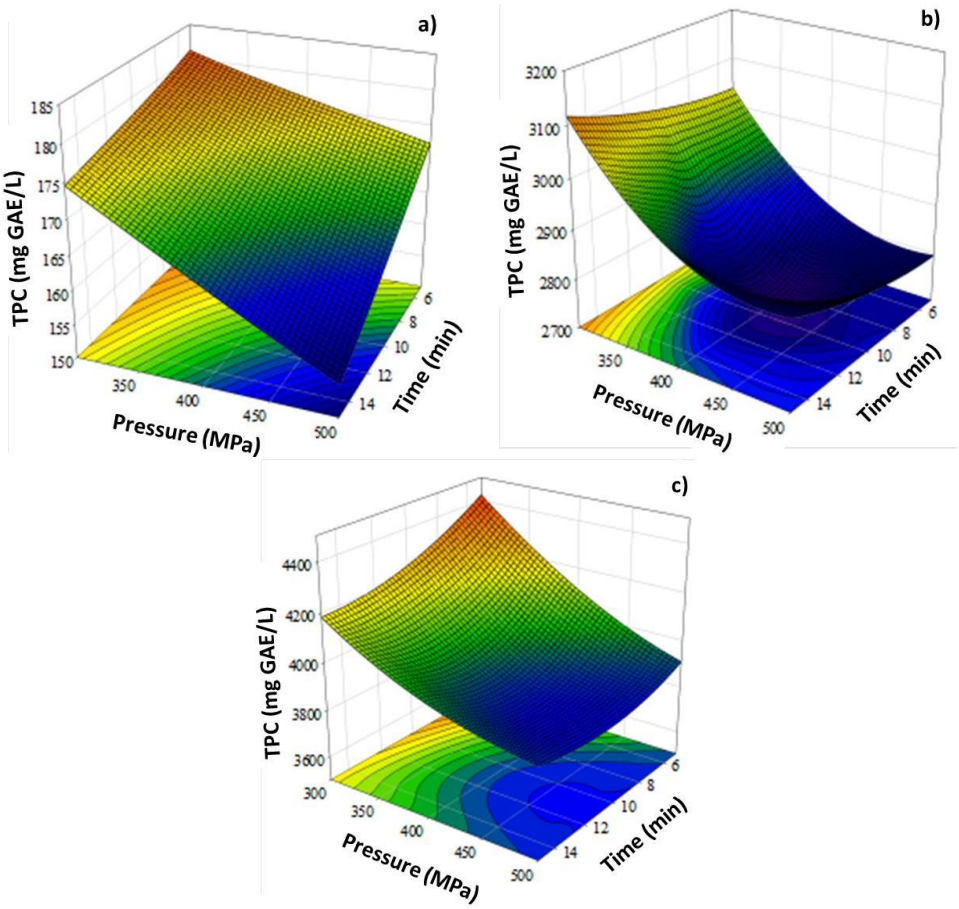


**Figure 1.** Effects of pressure and time on total phenolic compounds (mg GAE/100 mL) of a fruit juice sweetened with *Stevia rebaudiana* at: a) 0%, b) 1.25%, and c) 2.50%.

Phenols appeared to be relatively resistant to HPP and were even significantly increased (22%) after HPP (300 MPa/10 min) when the fruit juice mixture was sweetened with stevia (1.25%, w/v) and after 300 MPa for 5 min (18%) when the highest concentration of stevia (2.5%, w/v) was used. This increase in total phenolic content may be related to an increased extractability of some of the antioxidant components following high pressure processing. These results were in accord with those found by Plaza et al. (2011) in orange juice processed at 400 MPa/40 ºC/1 min, Barba, Esteve, & Frígola. (2011a) in orange juice mixed with

milk processed at 100-400 MPa/20-42 °C/2-9 min, and Barba, Esteve, and Frígola (2011b) in blueberry juice after HPP at 200-600 MPa/20-42 ºC/5-15 min. They reported an increase in phenolic content following HPP.

Total anthocyanins concentration in the fruit juice blend without stevia was 21.8±0.1 mg/L. However, total anthocyanin concentration was higher when the fruit mixture was sweetened with 1.25% (w/v) stevia (24.6±0.5 mg/L) and 2.5% (w/v) stevia (28.8±0.3 mg/L), respectively. Muanda, Soulimani, Diop, & Dicko (2011) reported values of total anthocyanins (measured as cyanidin-3-glucoside) of 0.35±0.01 and 0.67±0.09 when they studied stevia water extracts and methanol-water extracts, respectively. In addition, the anthocyanin content, expressed as relative retention, ranged between 98% and 161% in HPP samples under the studied experimental conditions. The reduced regression model for total anthocyanins is presented in Eq. (3):

$$TA\ (mg/L) = 197.736 - 1.337 \cdot P + 15.983 \cdot t - 1.854 \cdot \%stevia + 0.002 \cdot P^2 - 0.030 \cdot P*t + 0.010 \cdot P*\%stevia - 0.348 \cdot t^2 \quad (3)$$

Figure 2 indicates a positive influence of the pressure on the total anthocyanin content. The highest anthocyanin content was observed at the highest levels of both pressure and time (500 MPa/15 min) as well as stevia concentration (2.5%, w/v). This result indicates that in this particular range of processing conditions the HPP mainly modifies the mechanism of anthocyanin degradation by affecting the molecules involved in the kinetics of reaction, such as enzymes. Barba, Esteve, and Frígola (2011b) and Ferrari, Maresca, and Ciccarone (2010) observed similar results in HPP (200-600 MPa/15 min) blueberry juice and HPP (400-600 MPa/5-10 min) pomegranate juice, respectively.

Total carotenoid (TC) content in the untreated beverage sweetened with 0, 1.25 and 2.5% (w/v) were 329.3±14.1, 337.6±10.1, and 324.4±7.1 µg/100 mL, respectively. The analysis of variance showed that the regression model was accurate enough ($R^2$=0.921, $p$<0.05, standard error=28.744). The relationship between the independent variables and total carotenoids can be described by the Eq. (4):

$$TC\ (\mu g/100\ mL) = -270.886 - 0.297 \cdot P - 0.141 \cdot P*t - 0.050 \cdot P*\%stevia - 2.276 \cdot t^2 \quad (4)$$

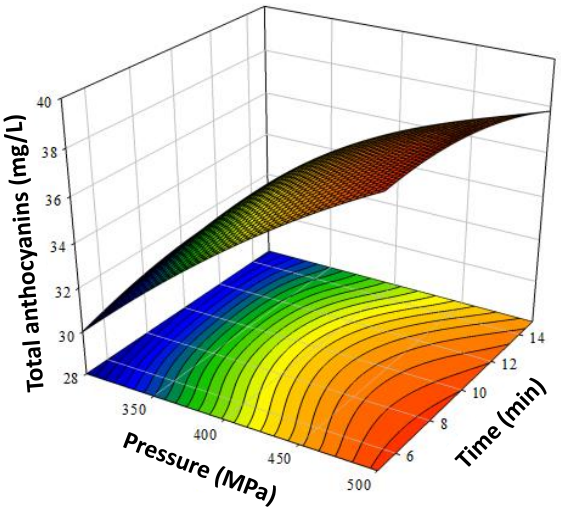


**Figure 2.** Response surface plots for total anthocyanins content of the beverage with 2.5% *Stevia rebaudiana* as affected by HPP at different pressures (300-500 MPa) and times (5-15 minutes).

Pressure had a significant positive effect ($p$<0.05) on the total carotenoids of the beverage independently of the stevia concentration used. Overall, at higher pressures, the values of total carotenoids were higher, reaching a maximum (4% increase) when pressures of 450-500 MPa were used (Figure 3), indicating that the beverages treated at higher pressure had an increased nutritional value.

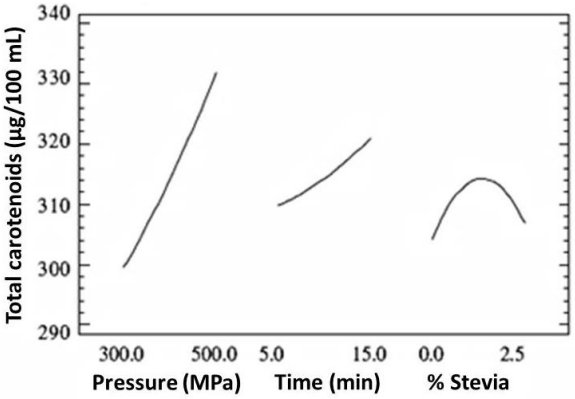


**Figure 3.** Effect of % *Stevia rebaudiana* and HPP (pressure and time) on total carotenoids (µg/100 mL): interactions.

Trolox equivalent antioxidant capacity (TEAC) and oxygen radical antioxidant capacity (ORAC) methods have been used widely for the determination of total antioxidant capacity (TAC) after applying HPP in fruit juice complex mixtures (Barba, Esteve, & Frígola, 2012b). In the present study, TAC values of untreated samples without stevia were 4.5±0.5 and 2.3±0.1 mM TE for ORAC and TEAC assays, respectively. ANOVA results indicated an increase in ORAC and TEAC values when stevia percentage was increased, independently of the preservation treatment applied. These results were in accordance to those found by different authors who have demonstrated the high antioxidant capacity of *S. rebaudiana* products (Muanda et al., 2011; Shukla et al., 2009, 2011; Tadhani et al., 2007). In addition antioxidant capacity values measured with ORAC assay were significantly higher ($p$<0.05) for the samples with stevia at 1.25% (22.2±0.7 mM TE) and 2.5% (32.8±1.7 mM TE) than those obtained with TEAC method (17.9±1.4 and 20.5±0.8 mM TE for the beverages sweetened with 1.25 and 2.5% stevia, respectively). The antioxidant activity of some stevia-derived products has been attributed to the scavenging of free radical electrons and superoxides (Thomas & Glade, 2010). As the ORAC method is a reaction based on the transfer of H atoms, these compounds present in *S. rebaudiana* may be better represented by this assay.

The results obtained for the three-way ANOVA showed that pressure, time and stevia percentage had a significant influence ($p$<0.05) on the total antioxidant capacity of the beverages measured as TEAC values. However, when ORAC assay was used, only stevia percentage had a significant effect. Moreover, the regression analysis test showed that a second-order model fits well the antioxidant capacity (ORAC and TEAC) after applying HPP. The determination coefficients were ($R^2$=0.960, $p$<0.05, standard error=1.823) and ($R^2$=0.970, $p$<0.05, standard error=2.220) for TEAC and ORAC methods, respectively. Experimental data were fitted by a second-order polynomial model (Eqs. (5)-(6)):

$$\text{TEAC (mM TE)} = -24.048 - 0.013 \cdot P + 6.504 \cdot t + 5.270 \cdot \%stevia - 0.007 \cdot P*t + 0.005 \cdot P*\%stevia - 0.218 \cdot t^2 + 0.075 \cdot t*stevia^2 \quad (5)$$

$$\text{ORAC (mM TE)} = 33.38820 + 15.61100 \cdot \%stevia - 1.99497 \cdot \%stevia^2 \quad (6)$$

When the possible correlation (Pearson test) between the various parameters that contribute to antioxidant capacity (ascorbic acid, total carotenoids, total phenolics and total anthocyanins) was studied for the different stevia concentrations, it was found that there was a positive correlation between total phenolics and TEAC ($R^2$=0.920, $p$<0.05) and total phenolics with ORAC ($R^2$=0.961). In this line, Cai, Luo, Sun, and Corke (2004) and Silva, Souza, Rogez, Rees, and Larondelle (2007) obtained a high correlation between TEAC and TPC when they studied 112 traditional Chinese medicinal plants and 15 Brazilian plants from Amazonian region, respectively. In addition, Ehlenfeldt and Prior (2001) and Bisby, Brooke, and Navaratnam (2008) also found a positive correlation between TPC and ORAC assays when they studied different plant food materials. Moreover, in the present study a positive correlation was found between ORAC and TEAC methods ($R^2$=0.905, $p$<0.05). These results were in accord to those found by Barba, Esteve, and Frígola (2010) when they treated a vegetables beverage with HPP.

### *3.2. Effect of HPP and stevia concentration on physicochemical properties of the beverages*

Results obtained for turbidity index (TI), browning index (BI) and colour parameters ($a^*$, $b^*$ and $L^*$) in the untreated and HPP samples are shown in Tables 2-3 and Figure 4. As can be expected, among the studied parameters, stevia percentage had the greatest effect on the quality parameters of the beverages. Compared to untreated samples, lower browning index values were obtained for the HPP samples when 0 and 1.25% (w/v) stevia percentages were used, however a significant increase in BI was observed after applying HPP in the samples with 2.5% (w/v) stevia.

The regression model was accurate enough ($R^2$=0.962, $p$<0.05, standard error=0.181). The regression equation describes the following model (Eq. (7)):

$$BI = 8.066 - 0.0551 \cdot P + 0.4856 \cdot t + 0.6965 \cdot \%stevia + 0.0001 \cdot P^2 - 0.0009 \cdot P^*t + 0.0009 \cdot P^*\%stevia - 0.0108 \cdot t^2 - 0.0092 \cdot t^*\%stevia \quad (7)$$

**Table 3.** Experimental design matrix in terms of actual variables and average values of the response for experiments on the effect of combined high pressure-*Stevia rebaudiana* concentration on the physicochemical parameters of a fruit juice mixture.

| Run[a] | Pressure (MPa) | Time (min) | Stevia (%) | BI[b] | TI[b] | $L^*$[b] | $a^*$[b] | $b^*$[b] | $\Delta E$ |
|---|---|---|---|---|---|---|---|---|---|
| | ($X_1$) | ($X_2$) | ($X_3$) | ($Y_1$) | ($Y_2$) | ($Y_3$) | ($Y_4$) | ($Y_5$) | ($Y_6$) |
| 1 | 500 | 15 | 0 | 0.083±0.003 | 0.078±0.004 | 70.4±0.1 | -1.5±0.1 | 11.3±0.2 | 3.5±0.2 |
| 2 | 300 | 5 | 0 | 0.054±0.006 | 0.076±0.008 | 71.9±0.2 | -1.8±0.1 | 8.3±0.2 | 0.5±0.1 |
| 3 | 500 | 15 | 0 | 0.086±0.005 | 0.082±0.004 | 70.4±0.3 | -1.5±0.1 | 11.3±0.3 | 3.5±0.3 |
| 4 | 500 | 15 | 2.5 | 2.256±0.010 | 0.700±0.005 | 31.7±0.2 | 11.1±0.2 | 37.7±0.3 | 5.3±0.3 |
| 5 | 300 | 15 | 0 | 0.056±0.004 | 0.070±0.004 | 72.1±0.1 | -1.6±0.1 | 5.5±0.2 | 3.0±0.2 |
| 6 | 300 | 5 | 2.5 | 1.890±0.009 | 0.429±0.008 | 36.0±0.2 | 9.1±0.2 | 38.8±0.3 | 1.3±0.1 |
| 7 | 400 | 10 | 1.25 | 1.460±0.011 | 0.327±0.005 | 33.9±0.1 | 9.4±0.1 | 37.1±0.4 | 14.4±0.4 |
| 8 | 300 | 15 | 2.5 | 1.641±0.012 | 0.414±0.006 | 37.2±0.2 | 10.4±0.1 | 44.5±0.4 | 5.2±0.2 |
| 9 | 500 | 5 | 2.5 | 1.458±0.010 | 0.452±0.004 | 35.7±0.1 | 10.3±0.2 | 40.7±0.5 | 1.7±0.1 |
| 10 | 400 | 10 | 1.25 | 1.468±0.009 | 0.333±0.006 | 35.0±0.2 | 9.0±0.1 | 33.0±0.2 | 18.0±0.5 |
| 11 | 400 | 5 | 1.25 | 1.467±0.007 | 0.319±0.007 | 35.5±0.2 | 8.9±0.1 | 34.1±0.3 | 16.8±0.3 |
| 12 | 400 | 10 | 2.5 | 1.990±0.009 | 0.445±0.006 | 33.8±0.2 | 9.5±0.1 | 37.0±0.2 | 3.7±0.3 |
| 13 | 300 | 15 | 2.5 | 1.648±0.010 | 0.416±0.008 | 37.6±0.4 | 10.4±0.2 | 44.3±0.1 | 5.1±0.3 |
| 14 | 500 | 5 | 2.5 | 1.472±0.011 | 0.466±0.009 | 35.8±0.3 | 10.3±0.1 | 40.3±0.2 | 1.4±0.1 |
| 15 | 300 | 10 | 1.25 | 1.388±0.015 | 0.345±0.010 | 35.1±0.2 | 9.1±0.1 | 35.5±0.3 | 15.6±0.4 |
| 16 | 500 | 5 | 0 | 0.083±0.003 | 0.076±0.003 | 70.6±0.3 | -1.5±0.1 | 9.1±0.4 | 1.9±0.1 |
| 17 | 500 | 15 | 2.5 | 2.235±0.010 | 0.697±0.011 | 31.7±0.2 | 11.1±0.2 | 37.4±0.2 | 5.4±0.2 |
| 18 | 500 | 5 | 0 | 0.086±0.004 | 0.078±0.003 | 70.6±0.4 | -1.5±0.1 | 9.1±0.2 | 1.9±0.1 |
| 19 | 300 | 15 | 0 | 0.059±0.005 | 0.076±0.004 | 72.1±0.4 | -1.6±0.1 | 5.5±0.1 | 3.0±0.1 |
| 20 | 400 | 15 | 1.25 | 1.465±0.008 | 0.337±0.009 | 34.8±0.3 | 8.8±0.1 | 32.8±0.3 | 18.3±0.4 |
| 21 | 400 | 10 | 0 | 0.201±0.006 | 0.115±0.009 | 71.0±0.5 | -1.1±0.1 | 11.4±0.2 | 3.3±0.2 |
| 22 | 300 | 5 | 2.5 | 1.910±0.010 | 0.426±0.011 | 36.0±0.3 | 9.0±0.2 | 38.5±0.2 | 1.5±0.2 |
| 23 | 400 | 10 | 1.25 | 1.456±0.013 | 0.330±0.010 | 35.0±0.2 | 9.0±0.1 | 33.1±0.3 | 17.9±0.5 |
| 24 | 400 | 10 | 1.25 | 1.463±0.012 | 0.335±0.010 | 35.0±0.2 | 9.0±0.1 | 33.0±0.4 | 18.0±0.6 |
| 25 | 300 | 5 | 0 | 0.057±0.006 | 0.078±0.003 | 71.9±0.2 | -1.9±0.1 | 8.3±0.1 | 0.5±0.1 |
| 26 | 500 | 10 | 1.25 | 1.470±0.010 | 0.342±0.011 | 34.8±0.4 | 8.6±0.2 | 32.8±0.3 | 18.3±0.6 |

BI: Browning index. TI: Turbidity index. ΔE: Total colour differences.[a] Order of the assays was randomised. [b] Data shown are the mean±SD of two treatment repetitions, each assay was performed in triplicate.

Overall, no statistically significant changes were observed in TI values of the HPP samples in comparison with the untreated beverage when stevia was not added. However, there was a statistically significant ($p$<0.05) decrease in the HPP samples with 1.25% (w/v) stevia in comparison with the unprocessed beverage.

In addition, the opposite trend was obtained after applying HPP when stevia percentage was 2.5% (w/v) (Eq. (8)).

$$TI = -0.4166 + 0.0141 \cdot \%stevia + 0.0004 \cdot P*\%stevia - 0.0152 \cdot t^2 \quad (8)$$

With regard to lightness ($L^*$), the three-way ANOVA showed that pressure, time and stevia concentration had a significant influence ($p$<0.05) on this parameter. Compared to the untreated beverages, lower $L^*$ values were found for samples treated by HPP independently of the stevia used in the formulation of the beverages. In addition, the response surface equation obtained in the present study described the experimental data adequately ($R^2$=0.921, $p$<0.05, standard error=5.324), which also was confirmed by the insignificant lack of fit ($p$=0.810). The Eq. (9) was as follows:

$$L^* = -82.361 + 0.963 \cdot P - 6.430 \cdot t - 34.891 \cdot \%stevia - 0.001 \cdot P^2 + 0.013 \cdot P*t - 0.020 \cdot P*\%stevia + 0.238 \cdot t*\%stevia + 9.834 \cdot \%stevia^2 \quad (9)$$

As can be observed, $L^*$ value decreased for all the HPP treatments applied, obtaining a higher decrease at higher pressure and longer time range. These results were in accord to those previously reported by Barba, Esteve, & Frígola (2011a) in HPP orange juice mixed with milk. These authors attributed it to partial precipitation of unstable particles in the juices after processing.

With regard to $a^*$ values, the behaviour was different depending on stevia concentration of the untreated samples. The reduced regression model presented in the Eq. (10) allowed the prediction of the effects of independent variables on the $a^*$ values:

$$a^* = 41.967 - 0.304 \cdot P + 3.046 \cdot t + 11.683 \cdot \%stevia + 0.001 \cdot P^2 - 0.006 \cdot P*t + 0.002 \cdot P*\%stevia - 0.067 \cdot P^2 - 3.313 \cdot \%stevia^2 \quad (10)$$

There were significant differences ($p$<0.05) in the $a^*$ values at different HPP conditions. Overall, the $a^*$ value changed toward a more positive direction for the HPP beverage with no stevia added for all high pressure treatments and the beverage with 2.5% (w/v) of stevia when HP treatment at 500 MPa/15 min was applied. The increase in CIE $a^*$ values were similar to the results found by Barba et al. (2010) when they studied the effects of HPP in a vegetables beverage and Patras, Brunton, Da Pieve, Butler, and Downey (2009) for high pressure processed tomato and carrot purées. However, the opposite trend was obtained when stevia at 1.25% (w/v) was added for all HPP conditions. The highest decrease in CIE $a^*$ values was observed at 500 MPa/10 min. The decrease in $a^*$ values was similar to the results found by Patras, Brunton, Da Pieve, and Butler (2009) in HPP strawberry and blackberry purées and by Barba, Cortés, et al. (2012) in HPP orange juice mixed with milk samples.

The yellowness ($b^*$ values) of untreated samples were 8.5±0.2, 50.4±0.2 and 39.3±0.1 when 0, 1.25 and 2.5% (w/v) of stevia was added. Overall, longer treatment times and processing at the highest pressure resulted in the highest decrease in $b^*$ values (see Table 3). These results were in accord with those found by Saldo, Suárez-Jacobo, Gervilla, Guamis, and Roig-Sagués (2009) in apple juice processed at 300 MPa/4 °C, Daoudi et al. (2002) in white grape juice processed at 500 MPa/600 s/2 °C, and Barba, Cortés, et al. (2012) in orange juice-milk samples after HPP. Furthermore, statistical analyses showed a positive correlation between browning index and $b^*$ value ($p$=0.809). In addition, relatively low correlation coefficient was observed ($R^2$=0.873) with a non-significant lack of fit ($p$=0.85). The regression Eq. (11) describes by the following model:

$$b^* = -48.276 + 29.040 \cdot \%stevia - 0.026 \cdot P^*\%stevia + 0.435 \cdot t^*\%stevia - 6.242 \cdot \%stevia^2 \quad (11)$$

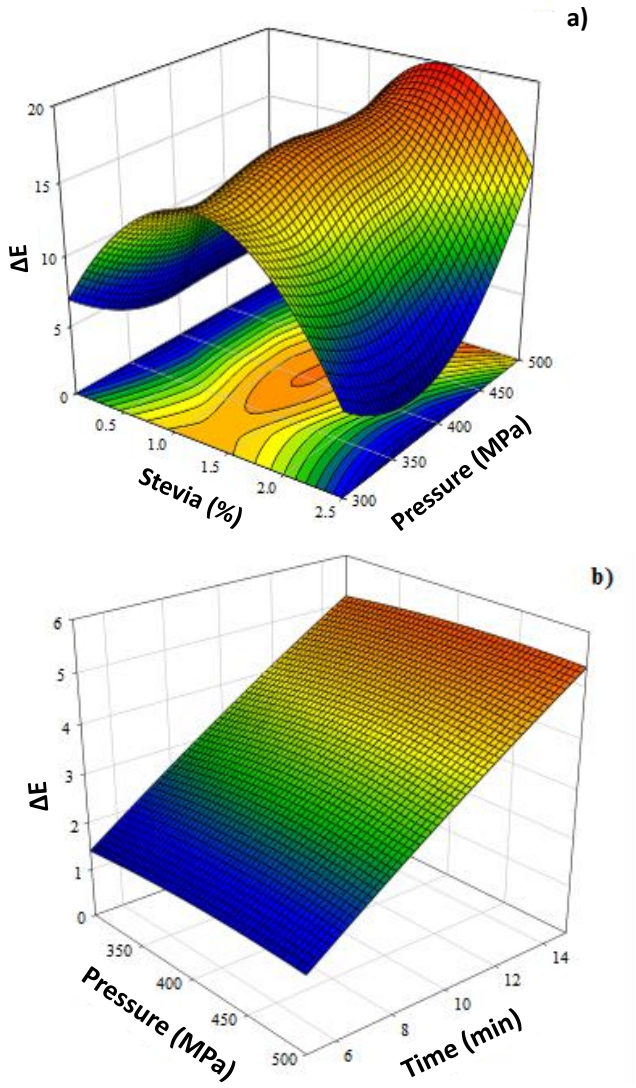


**Figure 4.** The effect of combined high pressure-time and % of *Stevia rebaudiana* on the colour of a beverage mixture of fruit juice sweetened with stevia. a) Effects of pressure and % stevia on total colour differences (ΔE) of the beverage for 10 min treatment time. b) Effects of pressure and time on total colour differences (ΔE) of the beverage for 2.5% stevia.

As can be expected, the linear and quadratic effects of stevia percentage were very significant for this model. In addition, the combined effect of time and stevia also had a significant effect.

The total colour difference (*ΔE**) indicates the magnitude of the colour difference. Depending on the value of ΔE, the colour difference between the treated and untreated samples can be estimated such as not noticeable (0–0.5), slightly noticeable (0.5–1.5), noticeable (1.5–3.0), well visible (3.0–6.0) and great (6.0–12.0) (Cserhalmi, Sass-Kiss, Tóth-Markus, & Lechner, 2006). As can be seen in Table 2, stevia addition had a strong effect of the beverage colour, increasing significantly ($p$<0.05) the browning of the juices. Moreover, in the present study, ΔE values were found to be different in behaviour, depending on treatment time or HPP intensity level (Table 3, Figure 4). Colour changes increased when pressure and treatment times were higher independently of the stevia percentage used, with the highest differences appearing at 500 MPa (3.3, 18.3 and 5.3 for 0, 1.25 and 2.5% (w/v) stevia). The regression Eq. (12) describes the following model:

$$\Delta E = 141.297 - 0.858 \cdot P + 5.299 \cdot t + 13.257 \cdot \%stevia + 0.001 \cdot P^2 + 0.019 \cdot P^*\%stevia - 0.218 \cdot t^*\%stevia\ 6.603 \cdot \%stevia^2 \quad (12)$$

*3.3. Optimisation and validation of the HPP and stevia concentration conditions*

The combination of HPP critical parameters that lead to a beverage (fruit juice mixture sweetened with stevia) with the highest nutritional and physicochemical quality was determined. The same priority was assigned to each dependent variable in order to obtain a beverage with maximal retention of bioactive compounds (ascorbic acid, total phenolic compounds, total anthocyanins, and total carotenoids) and antioxidant capacity. Likewise, the conditions that lead to a beverage with total colour differences lower than 3 were selected. Therefore, the optimal conditions of HPP in the present study were as follows: 1.7% (w/v) of stevia concentration, and 300 MPa of high hydrostatic pressure for 14 min. Under such conditions the greatest retention of bioactive compounds, antioxidant capacity as well as physicochemical properties were achieved, with an overall score of 0.626.

## 4. Conclusion

The results obtained in the present research suggested that optimising pressure–time conditions after HPP can be considered as a factor of great interest in order to obtain a better retention of bioactive compounds and physicochemical characteristics. HPP combined with *S. rebaudiana* water extracts can be a useful tool in order to provide new functional foods of proven physical and nutritional quality, thus increasing added value. In any case, more studies on the combined effect of pressure and time are required to elucidate the effects of HPP parameters on bioactive compounds and colour in foods, and further studies dealing with the effects of HPP in liquid foods during storage are needed.

## Acknowledgements

This research project was supported by the Spanish Ministry of Science and Technology and European Regional Development Funds (AGL2010-22206-C02-01). Carbonell-Capella, J.M. holds an award from the Spanish Ministry of Education (AP2010-2546). Barba, F.J. received a contract employment from this project to carry out the study.

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## 3.5. Ascorbic acid in orange juice based beverages processed by pulsed electric fields

*Fruit juices. Types, nutritional composition and health benefits. Nutrition and diet research progress, Katherlyn Elizabeth Elder New York: Nova Publishers (2014) 103-124*

# Ascorbic Acid in Orange Juice Based Beverages Processed by Pulsed Electric Fields

Juana M. Carbonell-Capella, Francisco J. Barba, Ana Zulueta, María J. Esteve, Ana. Frígola*

University of Valencia, Department of Nutrition and Food Science, Av. Vicent Andrés Estellés, s/n, 46100 Burjassot, Spain

*Corresponding author:
Ana Frígola
e-mail: ana.frigola@uv.es
Phone: +34 963544955, Fax: +34 963544954

**ABSTRACT**

Citrus fruits have been widely used as ingredients in fruit juices and beverages. However, vitamin C content may be reduced by thermal processing, thus reducing beneficial health effects. For this reason, the degradation kinetics of ascorbic acid were determined in orange juice based beverages after treatment by pulsed electric field, a novel emerging technology with increasing interest in the conservation of juices. The orange juice and orange-carrot juice mixture fitted a linear model, while the orange juice–milk beverage followed an exponential model. The degradation constants obtained in the orange juice were $0.00046\pm1\cdot10^{-4}$, $-0.00051\pm5\cdot10^{-5}$, $-0.00074\pm8\cdot10^{-5}$, and $-0.00095\pm1\cdot10^{-4}$ $\mu s^{-1}$ for fields of 25, 30, 35, and 40 kV/cm, respectively. In the case of the orange–carrot juice they were $-0.00037\pm3\cdot10^{-5}$, $-0.00042\pm5\cdot10^{-5}$, $-0.00119\pm7\cdot10^{-5}$, and $-0.00091\pm2\cdot10^{-4}$ $\mu s^{-1}$ for fields of 25, 30, 35, and 40 kV/cm, respectively, and for the orange juice–milk beverage they were $-0.00012\pm3\cdot10^{-5}$, $-0.00022\pm5\cdot10^{-5}$, $-0.00042\pm7\cdot10^{-5}$, and $-0.00061\pm6\cdot10^{-5}$ $\mu s^{-1}$ for fields of 15, 25, 35, and 40 kV/cm, respectively, demonstrating the use of PEF as an alternative to pasteurisation treatments. The *D* value increased with electric field strength, and the $Z_E$ values were 42.9, 30.6, and 35.1 kV/cm for the orange juice, orange–carrot mixture, and orange juice–milk beverage, respectively. Therefore, the orange juice was more resistant to changes in electric field strength in PEF treatment, while the treatment time required to produce the same degradation of ascorbic acid was greater in the orange juice–milk beverage than for the orange juice or the orange–carrot juice mixture, showing the need to optimise treatment conditions whenever there is a change in the food matrix.

**Keywords:** Ascorbic acid, pulsed electric fields, orange juice, carrot, milk, degradation kinetics.

**Introduction**

Fruit juices constitute one of the most widespread food-products in the manufacturing industry. They contain compounds that have a protective effect against degenerative diseases, known as phytochemicals or bioactive compounds (phenolic compounds, carotenoids, vitamins A, C, glucosinates, etc.), whose biological activity has been studied in numerous *ex vivo*, *in vitro* assays and by tests on humans. Epidemiological studies also show that the consumption of fruit juices has a considerable protective effect against the risk of certain diseases such as cancer, cataract, macular degeneration, and cardiovascular diseases [1-10]. Not only fruit juices are rich in bioactive compounds; vegetable juices, dairy products and milk fractions (milk, whey, casein, and lactoferrin) have a powerful biological and functional activity and contain high concentrations of antioxidant compounds. The antioxidants in milk play an important part in preventing lipid peroxidation and maintaining milk quality [11, 12].

Growing consumer demand for safe processed foods requiring minimum preparation time and presenting maximum similarity to the fresh product has led the food industry to increase production of fruit juices and seek ways of ensuring that bioactive compounds and nutrients are retained or modified only minimally during processing and storage, until they reach the consumer [13-14]. Considerable importance is currently being gained by fruit juices not derived from concentrates, enriched or mixed with vegetable juices or milk.

Moreover, technological innovation in the food industry is one of the pillars that form a basis for increased competitiveness and the provision of microbiologically safe food products that offer a nutritional quality and availability acceptable to the modern consumer. Apart from the improvements that have gradually been made in preservation process involving heat, such as continuous high temperature short time treatments, UHT, and aseptic packaging, new non-thermal technologies are emerging to respond to the need for greater nutritional and sensory quality in certain manufactured foods whose freshness characteristics are particularly affected by heat treatment [15].

During heat treatment, in addition to the inactivation of microorganisms, varying percentages of desirable constituents such as nutrients, color, aroma, and texture are destroyed [16-18]. Very high temperatures (135-150 ºC) are applied during sterilisation, with very short heating times (4-15 seconds). Most commercialised sterile foods have a shelf life of two years or more; any deterioration occurring after that time is due to changes in texture or aroma, not to microbial growth [19, 20]. Pasteurisation is a mild heat treatment causing minimal losses of organoleptic characteristics and nutritional quality. There are two major groups of pasteurisation technologies, those that use low temperatures (60-65 ºC) for fairly long times, and those that use higher temperatures (75-99 ºC) for short times. Currently, fruit and vegetable juices are subjected to temperatures ranging between 90 and 99 ºC for 15-60 seconds, and hot-filled aseptically. They are then cooled and stored in refrigeration for subsequent marketing [21].

Non-thermal preservation technologies such as high-intensity pulsed electric fields (PEF) are emerging in this context, with the aim of obtaining microbiologically safe foods with physicochemical, nutritional, and quality characteristics that are more like those of the fresh product. Treatment by high-intensity pulsed electric fields is an emerging technology with promising results for the inactivation of microorganisms and enzymes, preserving the organoleptic and nutritional characteristics of the treated product [22-25], and therefore interest in this technology is increasing [26]. The electric field affects cell membranes, causing irreversible damage, alteration of ion transport, and changes in enzyme structure [27-29].

A study by the Institute of Food Technology [30] highlights the research needs for emerging preservation technologies, especially the identification of how they may affect bacterial inactivation, quality, nutritional value, and shelf life of foods, changes in critical processing factors, and the introduction of new factors. Several studies have been conducted on the effect of PEF on nutrients in various food matrices in recent years [22, 31-35]. The type and physicochemical characteristics of the product (conductivity, pH, ionic strength, water activity, presence of particles, etc.) influence the effectiveness of the process [36, 37].

Studies on the loss and/or modification of vitamin contents are necessary to learn how treatment affects the product's nutritional characteristics that are of particular importance for the consumer. Vitamin C is a thermolabile vitamin that is especially affected by heat treatment [38], and it has been used as a quality indicator after application of a preservation process [39-42]. Vitamin C degradation indicates not only loss of quality but also loss of other nutrients and organoleptic components. However, with appropriate stabilisation procedures and suitable storage conditions (e.g. temperature and light), ascorbic acid can be stable for long periods of time.

The aim of the present study was to determine the degradation kinetics of ascorbic acid in various orange juice based beverages treated by high-intensity pulsed electric fields, and to analyse the possible influence of the product's characteristics on the variation in ascorbic acid after the preservation treatment.

**Materials and Methods**

*Samples*

Orange juice: The orange juice (*Citrus sinensis L*., Navel variety) was obtained by squeezing (FMC juice extractor with 2 mm perforated plates) and passed through a filter with pores having a diameter of 0.23 mm. The juice was packaged aseptically and stored at –40 ºC until the time for the analysis.

Orange–carrot juice mixture: The orange juice was prepared as described above. To obtain the carrot juice, the carrots were washed first with a solution of sodium hydroxide and then with drinking water. The juice obtained was sieved and mixed with the orange juice in the following proportion: orange–carrot, 80:20 (v/v). It was packaged aseptically and frozen at –40 ºC until the time for the analysis.

Orange juice and milk beverage: The beverage was prepared by mixing 50% (v/v) of orange juice prepared as described above, 20% (v/v) of UHT skim milk (Grupo Leche Pascual S.A., Burgos, Spain), and 30% (v/v) of distilled water. Then 7.5% of saccharose (w/v) (Panreac, Barcelona, Spain), 0.1% of citric acid (w/v) (Panreac, Barcelona, Spain), and 0.3% of high methoxyl pectin (w/v) (Unipectine

AYD 250 Degussa Food Ingredients, Boulogne, France) were added as sweetener, preservative, and stabiliser of the samples, respectively. The beverage was prepared just before treatment.

A sufficient quantity of each product was prepared and divided into three parts: one was assigned as a control, one was processed by PEF, and one was pasteurised thermally.

*Heat Treatment*

The heat treatment was applied in an Armfield FT74P plate exchanger. The beverage was placed in a feed tank and driven by a pump towards the heat exchanger, where it attained the heat conditions selected (90 ºC, 20 s for the orange juice and the juice–milk beverage; 98 ºC, 21 s for the orange–carrot juice mixture, heat treatments of 90–99 ºC for 15–30 s being customary in the industry [21]. The beverage was cooled after treatment by means of a cooler (Armfield FT61), packaged aseptically, and stored in refrigeration (4±1 ºC) until it was analysed. The treatment was carried out in duplicate.

*PEF Treatment*

The treatment was applied in continuous mode in an OSU-4D system designed by the University of Ohio (USA) and installed at the Instituto de Agroquímica y Tecnología de los Alimentos (CSIC) in Valencia. The pulse system consisted of six "co-field" treatment chambers connected in series, having a diameter of 0.23 cm and a distance of 0.293 cm between electrodes, and two cooling coils submerged in a refrigerated bath (Polystat, Cole Parmer, IL, USA) connected before and after each pair of treatment chambers in order to keep the temperature within the designated range. The initial and final treatment temperatures were recorded by thermocouples placed at the entrance to the first treatment chamber and at the exit of the last chamber, respectively. The temperature, waveform, voltage, and treatment intensity were recorded with a digital oscilloscope (Tektronix TDS 210, Tektronix, OR, USA). The flow rate was set at 60 mL/min and controlled by a peristaltic pump (Cole-Parmer 75210-25, Cole-Parmer Instruments, IL, USA). The pulse duration selected was 2.5 μs. The treatment time ranged from 30 to 700 μs,

and the electric fields assayed were in the range 15– 40 kV/cm. The samples were collected after each treatment and stored in refrigeration (4±1 ºC) until they were analysed. The treatment was performed in duplicate.

**Table 1.** Treatment conditions applied to the orange juice.

| *E* (kV/cm) | $\tau$ (μs) | $\nu$ (pulses/ chamber) | *T* (ºC) | Conductivity (mS/cm) | *Q* (J/mL) | *W* (J/mL/ pulse) |
|---|---|---|---|---|---|---|
| 25 | 40 | 16 | 37 | 4.56 | 41.04 | 2.57 |
| 25 | 100 | 40 | 44 | 5.13 | 115.52 | 2.89 |
| 25 | 170 | 68 | 52 | 5.79 | 221.47 | 3.26 |
| 25 | 240 | 96 | 64 | 6.77 | 365.80 | 3.81 |
| 25 | 300 | 120 | 67 | 7.02 | 473.85 | 3.95 |
| 25 | 340 | 136 | 72 | 7.43 | 568.39 | 4.18 |
| 30 | 40 | 16 | 38 | 4.64 | 116.05 | 4.18 |
| 30 | 80 | 32 | 46 | 5.30 | 264.90 | 7.25 |
| 30 | 100 | 40 | 49 | 5.54 | 346.51 | 8.28 |
| 30 | 150 | 60 | 58 | 6.28 | 588.94 | 8.66 |
| 30 | 190 | 76 | 63 | 6.69 | 794.67 | 9.82 |
| 30 | 240 | 96 | 69 | 7.18 | 1077.60 | 11.23 |
| 35 | 40 | 16 | 41 | 4.89 | 239.51 | 10.46 |
| 35 | 60 | 24 | 46 | 5.30 | 389.40 | 11.23 |
| 35 | 80 | 32 | 51 | 5.71 | 559.38 | 14.22 |
| 35 | 100 | 40 | 55 | 6.04 | 739.41 | 16.23 |
| 35 | 140 | 56 | 61 | 6.30 | 1119.55 | 16.98 |
| 35 | 170 | 68 | 67 | 7.02 | 1461.92 | 21.50 |
| 40 | 30 | 12 | 41 | 4.89 | 234.62 | 19.24 |
| 40 | 40 | 16 | 44 | 5.13 | 328.58 | 20.49 |
| 40 | 60 | 24 | 49 | 5.54 | 532.22 | 22.00 |
| 40 | 80 | 32 | 55 | 6.04 | 772.61 | 19.55 |
| 40 | 100 | 40 | 61 | 6.53 | 1044.48 | 20.54 |
| 40 | 130 | 52 | 69 | 7.18 | 1494.27 | 28.74 |

The energy of the electric pulse was calculated by means of the following equation: $W$ (J·L$^{-1}$)=$E^2 \cdot \sigma \cdot \tau$, where E is the electric field strength (V·m$^{-1}$), $\sigma$ is the electric conductivity of the product (S·m$^{-1}$) calculated for each temperature attained during processing, and $\tau$ is the length of the pulse (s).

When $\tau$ and $E$ are constant, $W$ depends only on $\sigma$, so that application of the same number of pulses generates greater energy ($Q$) when $\sigma$ is greater: $Q$ ($J \cdot L^{-1}$)=$n \cdot W$, where $n$ is the number of pulses. Tables 1 to 3 show the treatment conditions for each of the samples studied.

**Table 2.** Treatment conditions applied to the orange–carrot juice.

| $E$ (kV/cm) | $t$ (μs) | $\nu$ (pulses/ chamber | $T$ (ºC) | Conductivity (mS/cm) | $Q$ (J/mL) | $W$ (J/mL/ pulse) |
|---|---|---|---|---|---|---|
| 25 | 60 | 24 | 40 | 6.12 | 229.58 | 9.57 |
| 25 | 110 | 44 | 44 | 6.54 | 449.49 | 10.22 |
| 25 | 200 | 80 | 51 | 7.27 | 908.25 | 11.35 |
| 25 | 280 | 112 | 58 | 7.99 | 1398.95 | 12.49 |
| 25 | 340 | 136 | 65 | 8.72 | 1853.43 | 13.63 |
| 30 | 60 | 24 | 42 | 6.33 | 341.82 | 14.24 |
| 30 | 110 | 44 | 47 | 6.85 | 678.15 | 15.41 |
| 30 | 170 | 68 | 56 | 7.79 | 1191.26 | 17.52 |
| 30 | 200 | 80 | 61 | 8.31 | 1495.08 | 18.69 |
| 30 | 220 | 88 | 64 | 8.62 | 1706.36 | 19.39 |
| 35 | 60 | 24 | 44 | 6.54 | 480.54 | 20.02 |
| 35 | 90 | 36 | 50 | 7.16 | 789.61 | 21.93 |
| 35 | 110 | 44 | 55 | 7.58 | 1021.14 | 23.21 |
| 35 | 130 | 52 | 59 | 8.10 | 1289.61 | 24.80 |
| 35 | 150 | 60 | 64 | 8.62 | 1583.56 | 26.39 |
| 40 | 30 | 12 | 43 | 6.43 | 308.83 | 25.74 |
| 40 | 60 | 24 | 53 | 7.47 | 717.50 | 29.90 |
| 40 | 80 | 32 | 61 | 8.20 | 1049.86 | 32.81 |
| 40 | 90 | 36 | 60 | 8.31 | 1196.06 | 33.22 |
| 40 | 110 | 44 | 65 | 8.72 | 1535.07 | 34.89 |

**Table 3.** Treatment conditions applied to the orange juice–milk beverage.

| *E* (kV/cm) | *t* (μs) | *ν* (pulses/ chamber | *T* (ºC) | Conductivity (mS/cm) | *Q* (J/mL) | *W* (J/mL/ pulse) |
|---|---|---|---|---|---|---|
| 15 | 40 | 16 | 35 | 3.89 | 35.04 | 2.19 |
| 15 | 80 | 32 | 37 | 4.04 | 72.69 | 2.27 |
| 15 | 130 | 52 | 38 | 4.15 | 121.31 | 2.33 |
| 15 | 300 | 120 | 45 | 4.62 | 311.81 | 2.60 |
| 15 | 500 | 200 | 55 | 5.35 | 601.36 | 3.01 |
| 15 | 700 | 280 | 59 | 5.67 | 893.36 | 3.19 |
| 25 | 40 | 16 | 35 | 4.11 | 893.36 | 6.42 |
| 25 | 80 | 32 | 41 | 4.33 | 102.78 | 6.76 |
| 25 | 130 | 52 | 45 | 4.62 | 216.45 | 7.22 |
| 25 | 200 | 80 | 51 | 5.13 | 375.33 | 8.01 |
| 25 | 280 | 112 | 57 | 5.53 | 640.95 | 8.64 |
| 25 | 310 | 124 | 59 | 5.67 | 1098.97 | 8.86 |
| 35 | 40 | 16 | 40 | 4.29 | 210.34 | 13.15 |
| 35 | 60 | 24 | 44 | 4.58 | 336.86 | 14.04 |
| 35 | 80 | 32 | 47 | 4.80 | 470.49 | 14.70 |
| 35 | 100 | 40 | 51 | 5.09 | 623.68 | 15.59 |
| 35 | 130 | 52 | 59 | 5.67 | 903.28 | 17.37 |
| 35 | 180 | 72 | 63 | 6.29 | 1387.91 | 19.28 |
| 40 | 40 | 16 | 43 | 4.47 | 286.35 | 17.90 |
| 40 | 60 | 24 | 48 | 4.84 | 464.37 | 19.35 |
| 40 | 80 | 32 | 53 | 5.20 | 665.63 | 20.80 |
| 40 | 90 | 36 | 55 | 5.35 | 769.74 | 21.38 |
| 40 | 110 | 44 | 58 | 5.41 | 952.66 | 21.65 |
| 40 | 130 | 52 | 62 | 5.90 | 1227.26 | 23.60 |

*Determination of Physicochemical Parameters*

The physicochemical parameters were determined as follows: Electric conductivity by a Crison 525 conductivity meter (Crison Instruments S.A., Alella, Barcelona, Spain); pH by a Crison 2001 pH meter (Crison Instruments S.A., Alella, Barcelona, Spain), and soluble solids (ºBrix) by an Atago RX-1000 refractometer (Atago Company Ltd, Tokyo, Japan).

*Polarographic determination of ascorbic acid*

The polarograph used comprised the following units: Ω Metrohm 746 VA Trace Analyser, Ω Metrohm 747 VA Stand, Ω Metrohm PC Software 693 VA Back Up. The working electrode was mercury operated in the continuous drop mode. An auxiliary platinum wire electrode and a saturated calomel reference electrode were used (Ag/AgCl, KCl 3 M). Five mL of the product was diluted and made up to 25 mL with the extraction solution: 1% oxalic acid (w/v), 2% trichloroacetic acid (w/v), and 1% sodium sulfate (w/v). After vigorous shaking the solution was filtered through Whatman No. 1 paper. Then 9.5 mL of a solution of 1% oxalic acid (w/v) and 2 mL of acetic acid/sodium acetate 2 M buffer solution (pH=4.8) was added to 0.5 mL of the filtrate in the polarographic cell [43,44].

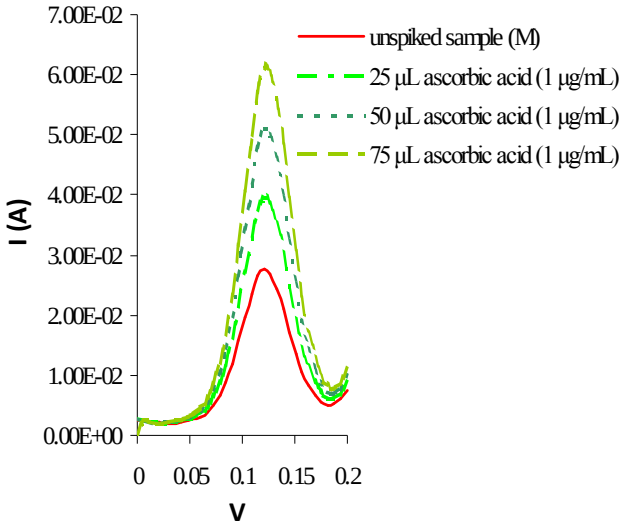


**Figure 1.** Polarogram of a sample of the orange juice–milk beverage.

To avoid possible interference caused by the presence of oxygen, the sample was purged with extra pure nitrogen for 5 minutes before the analysis. The polarogram was recorded using a pulse width of 50 mV, a drop time of 1 second, and a scan rate of 10 mV/cm. The initial potential was –0.10 V. The additions method was used for the quantification, adding 25 µL of ascorbic acid (1 µg/mL) to the sample and recording the polarogram after each addition, obtaining a polarographic curve (Figure 1).

The accuracy of the method was determined by recovery assays. A known quantity of ascorbic acid (200, 100, and 50 μg/mL) was added to each beverage, and the extraction and subsequent polarographic determination were then carried out. The recoveries ranged from 92.7 to 96.8%. The instrumental precision of the method was expressed by means of the coefficient of variation of nine consecutive determinations of a given aliquot and six determinations of independent aliquots of a given sample, the results obtained being 2.8% and 2.9%, respectively.

**Results**

Table 4 shows the physicochemical parameters of each of the samples. It can be seen that the orange–carrot juice mixture had the highest conductivity, while the orange juice–milk beverage had the highest ºBrix.

**Table 4.** Physicochemical characteristics of the beverages analysed: orange juice, orange–carrot juice mixture, and orange juice–milk beverage.

| | Orange juice | Orange–carrot juice | Orange juice–milk beverage |
|---|---|---|---|
| pH | 3.35±0.01 | 3.84±0.04 | 3.92 ± 0.01 |
| ºBrix | 11.8±0.1 | 10.3±0.3 | 14.3 ± 0.1 |
| Conductivity (mS/cm) | 3.42± 0.04 | 4.55± 0.05 | 2.99 ± 0.05 |

The ascorbic acid concentration in the untreated orange juice (47.6±1.4 mg/100 mL) was higher than that of the other two samples (27.1±0.4 mg/100 mL and 25.9±1.4 mg/100 mL for the orange–carrot juice and the orange juice–milk beverage, respectively).

*Heat Treatment*

The ascorbic acid concentration in the orange juice after pasteurisation at 90 ºC for 20 s did not vary significantly ($p$<0.01). The ascorbic acid retention in the pasteurised orange–carrot juice (98 ºC, 21 s) was 83%, while in the orange juice–

milk mixture (90 ºC, 20 s) it was 86%. After pasteurising orange juice (90 ºC, 60 s), Elez-Martínez and co-workers [45] found an ascorbic acid retention of 82.4%, while Sánchez-Moreno and co-workers [46] obtained a higher value (92%) in the same conditions. Min and co-workers [47] applied the same temperature for 90 s and obtained a retention of 81%.

*High-intensity pulsed electric fields (PEF)*

**Table 5.** Ascorbic acid concentration in the orange juice after PEF treatment.

| *E* (kV/cm) | *t* (μs) | Ascorbic acid (mg/100 mL) |
|---|---|---|
| 25 | 40 | 47.60±0.33 |
| | 100 | 45.24±0.72 |
| | 170 | 45.67±1.17 |
| | 240 | 44.73±0.75 |
| | 300 | 43.77±2.50 |
| | 340 | 38.82±1.63 |
| 30 | 40 | 45.31±0.59 |
| | 80 | 45.03±0.56 |
| | 100 | 44.78±1.31 |
| | 150 | 43.74±0.78 |
| | 190 | 42.54±0.67 |
| | 240 | 41.64±0.71 |
| 35 | 40 | 45.42±0.73 |
| | 60 | 44.32±1.78 |
| | 80 | 43.28±1.54 |
| | 100 | 43.17±0.81 |
| | 140 | 42.01±0.97 |
| | 170 | 41.41±1.34 |
| 40 | 30 | 46.19±1.53 |
| | 40 | 45.12±0.97 |
| | 60 | 44.02±0.76 |
| | 80 | 44.23±1.21 |
| | 100 | 43.69±1.20 |
| | 130 | 41.54±0.37 |

Tables 5, 6, and 7 show the results obtained for the PEF treatments applied in the orange juice, the orange–carrot juice, and the orange juice–milk beverage, respectively.

The retention of ascorbic acid after PEF treatment in the orange juice ranged between 81.6%, after a treatment of 25 kV/cm for 340 μs, and 97.0% when the electric field strength applied was 40 kV/cm and the time was 30 μs. Similar values were obtained in the orange–carrot juice, 83.1 and 97.1% for treatments of 35 kV/cm (150 μs) and 40 kV/cm (30 μs), respectively. In the orange juice–milk beverage, however, the ascorbic acid retention was slightly higher, 90.7 and 97.3% for treatments of 15 kV/cm (500 μs) and 25 kV/cm (40 μs), respectively.

**Table 6.** Ascorbic acid concentration in the orange–carrot juice mixture after PEF treatment.

| *E* (kV/cm) | *t* (μs) | Ascorbic acid (mg/100 mL) |
|---|---|---|
| 25 | 60 | 26.16±0.64 |
| | 110 | 25.57±0.32 |
| | 200 | 24.69±0.86 |
| | 280 | 24.40±0.69 |
| | 340 | 23.68±1.50 |
| 30 | 60 | 26.07±0.47 |
| | 110 | 25.83±1.04 |
| | 170 | 25.47±1.03 |
| | 200 | 24.66±0.87 |
| | 220 | 24.60±1.03 |
| 35 | 60 | 25.07±0.68 |
| | 90 | 24.24±0.27 |
| | 110 | 23.50±0.26 |
| | 130 | 23.46±0.44 |
| | 150 | 22.53±1.19 |
| 40 | 30 | 26.32±0.26 |
| | 60 | 24.83±0.39 |
| | 80 | 25.38±0.15 |
| | 90 | 25.27±0.31 |
| | 110 | 24.21±0.29 |

**Table 7.** Ascorbic acid concentration in the orange juice–milk beverage after PEF treatment.

| *E* (kV/cm) | *t* (μs) | Ascorbic acid (mg/100 mL) |
|---|---|---|
| 15 | 0 | 25.28±0.44 |
| | 40 | 24.54±0.32 |
| | 80 | 24.19±0.56 |
| | 130 | 23.93±0.21 |
| | 300 | 23.30±0.48 |
| | 500 | 22.92±0.35 |
| | 700 | 23.08±0.42 |
| 25 | 0 | 22.72±0.34 |
| | 40 | 22.10±0.76 |
| | 80 | 21.69±0.43 |
| | 130 | 21.31±0.91 |
| | 200 | 21.17±0.56 |
| | 280 | 21.00±0.33 |
| | 310 | 20.99±0.23 |
| 35 | 0 | 26.98±0.42 |
| | 40 | 26.03±0.54 |
| | 60 | 25.88±0.36 |
| | 80 | 25.66±0.74 |
| | 100 | 25.28±0.65 |
| | 130 | 25.05±0.41 |
| | 180 | 25.01±0.35 |
| 40 | 0 | 26.40±0.58 |
| | 40 | 25.39±0.44 |
| | 60 | 25.22±0.28 |
| | 80 | 24.98±0.89 |
| | 90 | 24.71±0.39 |
| | 110 | 24.48±0.42 |
| | 130 | 24.39±0.35 |

These results are in agreement with those obtained by Elez-Martínez and co-workers [45], in which vitamin C retention after PEF treatment lay between 87.5 and 98.2%. Sánchez-Moreno and co-workers [46] found an ascorbic acid retention

of 93% in orange juice treated by PEF (35 kV/cm, 750 μs), and Min and co-workers [47] did not observe significant changes in the vitamin C concentration when they applied PEF (40 kV/cm, 97 μs).

A regression analysis was performed to analyse the influence of electric field strength ($E$, kV/cm) and treatment time ($t$, μs) on ascorbic acid retention ($C/C_0$) in each of the beverages studied. The results were fitted to a linear model ($p$<0.01): $C/C_0$=1.118 – 0.004·$E$ (kV/cm) – 0.0005·$t$ (μs) ($R^2$=69.37, standard error 0.023) for orange juice; $C/C_0$=1.079 – 0.004·$E$ (kV/cm) – 0.0004·$t$ (μs) ($R^2$=41.89, standard error 0.030) for orange–carrot juice; and $C/C_0$=0.976 – 0.0006·$E$ (kV/cm) – 0.0001·$t$ (μs) ($R^2$=68.24, standard error 0.011) for the orange juice–milk beverage. The fit was significant in all cases ($p$<0.01). It can be seen that both the electric field strength applied and treatment time influence the degradation of ascorbic acid, and it can be said that the orange juice and the orange–carrot juice show similar behaviour, and that it differs from the behaviour of the orange juice–milk beverage, as deduced from the slopes.

To evaluate the treatments applied, a study was made of the degradation kinetics of ascorbic acid with treatment time in each of the fields applied.

**Orange juice:** When the ascorbic acid retention was plotted against treatment time for each of the fields applied, it could be seen that it follows a zero-order kinetic (Figure 2): $C=C_0 - kt$, where $C$ is the ascorbic acid concentration after treatment (mg/100 mL) and $C_0$ is the initial ascorbic acid concentration (mg/100 mL), $k$ indicates the ascorbic acid degradation rate ($\mu s^{-1}$), and $t$ is treatment time (μs). The degradation constant obtained from the degradation curve calculated by plotting Ln($C/C_0$) against time for each treatment was: $–0.00046±1·10^{–4}$ $\mu s^{-1}$ (correlation coefficient 0.857, standard error 0.039), $–0.00051±5·10^{–5}$ $\mu s^{-1}$ (correlation coefficient 0.980, standard error 0.009), $–0.00074±8·10^{–5}$ $\mu s^{-1}$ (correlation coefficient 0.969, standard error 0.012), and $–0.00095±1·10^{–4}$ $\mu s^{-1}$ (correlation coefficient 0.967, standard error 0.012), for fields of 25, 30, 35, and 40 kV/cm, respectively. The fit was significant at the 99 percent confidence level ($p$<0.01) in all cases except the field of 25 kV/cm, where the fit was significant at the 95 percent level ($p$<0.05).

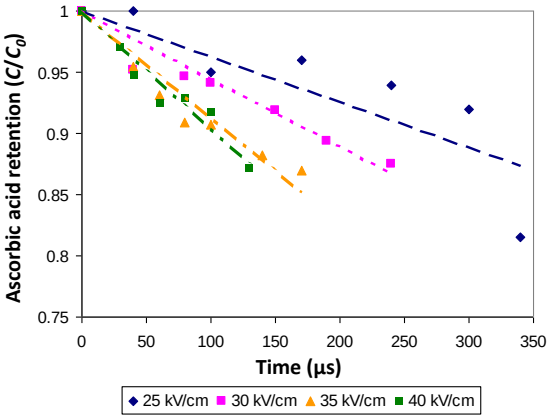


**Figure 2.** Ascorbic acid retention in orange juice after PEF treatment.

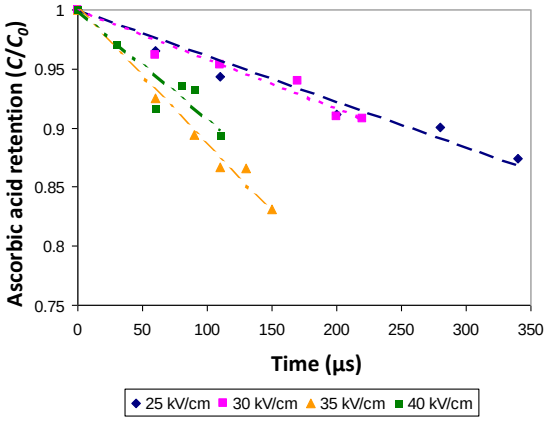


**Figure 3.** Ascorbic acid retention in orange–carrot juice after PEF treatment.

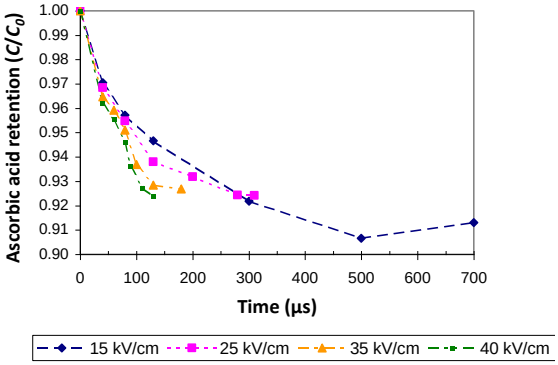


**Figure 4.** Ascorbic acid retention in the orange juice–milk beverage after PEF treatment.

**Orange–carrot juice mixture:** Least squares fitting of ascorbic acid retention versus treatment time in each of the fields showed that it follows a zero-order kinetic (Figure 3). The ascorbic acid degradation rate ($k_E$) obtained was – $0.00037\pm3\cdot10^{-5}$ $\mu s^{-1}$ (correlation coefficient 0.987, standard error 0.009); – $0.00042\pm5\cdot10^{-5}$ $\mu s^{-1}$ (correlation coefficient 0.975, standard error 0.009); – $0.00119\pm7\cdot10^{-5}$ $\mu s^{-1}$ (correlation coefficient 0.993, standard error 0.008), and – $0.00091\pm2\cdot10^{-4}$ $\mu s^{-1}$ (correlation coefficient 0.917, standard error 0.018), for fields of 25, 30, 35, and 40 Kv/cm, respectively. The fit was significant at the 99 percent confidence level ($p<0.01$) in all cases except the field of 40 kV/cm, where the fit was significant at the 95 percent level ($p<0.05$).

**Orange juice–milk beverage:** In this case, when the ascorbic acid retention was plotted against treatment time for each of the fields applied, it was seen that the experimental data for each electrical field fit an exponential model of the type: $C/C_0=a\cdot e^{-bt}$ (Figure 4).

The ascorbic acid degradation rate ($k_E$) obtained was: $-0.00012\pm3\cdot10^{-5}$ $\mu s^{-1}$ (correlation coefficient 0.874, standard error 0.019); $-0.00022\pm5\cdot10^{-5}$ $\mu s^{-1}$ (correlation coefficient 0.910, standard error 0.013); –0.00042±7·10–5 $\mu s^{-1}$ (correlation coefficient 0.934, standard error 0.010), and $-0.00061\pm6\cdot10^{-5}$ $\mu s^{-1}$ (correlation coefficient 0.979, standard error 0.006), for fields of 15, 25, 35, and 40 Kv/cm, respectively. The fit was significant at the 99 percent confidence level ($p<0.01$) in all cases except the field of 15 kV/cm, where the fit was significant at the 95 percent level ($p<0.05$).

To date, various authors have studied the thermal degradation kinetics of ascorbic acid in different kinds of foods, finding that it fits a first-order model; in this case the degradation curves divide clearly into two linear sections that correspond to two types of degradation, one aerobic and the other anaerobic [18, 48-51]. For PEF treatment, Bendicho and co-workers [52] found first-order kinetics for ascorbic acid degradation in skim milk and ultrafiltered skim milk when treated by PEF (18.3–27.1 kV/cm). As Tannenbaum noted [53], ascorbic acid degradation mechanisms are specific for each particular food, depending on different factors that vary according to the composition of the food; hence the importance of conducting studies of nutrient modifications for each food [30].

The differences observed between the degradation constants of the three beverages may be due to the physical and chemical characteristics of the products, which might affect the ascorbic acid degradation kinetics considerably. The initial ascorbic acid concentration in the orange juice (47.56±1.36 mg/100 mL) was higher than in the other two samples, and the pH was lower (3.35±0.01). In the orange–carrot juice mixture the values of ascorbic acid concentration (27.11±0.44) and pH (3.84±0.04) were similar to those of the orange juice–milk beverage (25.92±1.35 mg/100 mL and 3.92±0.01, respectively). The conductivity differed in the three products: 3.42 mS/cm in the orange juice, 4.55 mS/cm in the orange–carrot juice mixture, and 2.99 mS/cm in the orange juice–milk beverage. It is worth noting that when one parameter changes the other parameters are also affected to a greater or lesser extent and they might therefore influence the ascorbic acid degradation. A change in conductivity modifies the energy ($J \cdot L^{-1}$) applied during the treatment. Assuming that the energy formula is $W = \sigma \cdot \tau \cdot E^2$, higher conductivity results in greater energy applied to the product ($J \cdot L^{-1}$) and might therefore have a greater effect on changes in the ascorbic acid content. For the orange juice–milk beverage, Sampedro and co-workers [54] found that the inactivation of *L. plantarum* achieved by pulses was lower than that found by other authors for products with a less complex composition. Bendicho and co-workers [52] found that after PEF treatment vitamin C retention was greater in skim milk than in ultrafiltered skim milk owing to the greater complexity of the former and the protective role that some components of milk, especially caseins, play in vitamin C degradation. The protective effect of casein against the inactivation of microorganisms and enzymes was also described by Goff and Hill [55] and Phelan and co-workers [56]. Moreover, some authors have reported that the fat content in foods might present a barrier to inactivation of microorganisms, protecting them from treatment by electric pulses. The mechanisms of this effect have not yet been elucidated [57] and so far similar works for vitamins have not been found.

It is also worth noting that the addition of 0.1% of citric acid to the product may give the ascorbic acid greater stability. Millán and Roa [58] found that there is a synergy between citric acid and ascorbic acid that works against degradation of

the latter. This can be explained by the acidulant and complexing capacity of the former, that acts as an aid to the chemical stability of ascorbic acid by chelation of metal ions and reduction in the pH of the medium, which is related with greater stability of the vitamin.

The decimal reduction time (*D*), is the time required at a particular field strength to reduce the initial ascorbic acid concentration by 10%, was obtained from the destruction curve. It was calculated by using a procedure analogous to the one used in thermal destruction studies, from the expression $D=2.303/k_E$. Table 8 shows the decimal reduction time values for each of the samples studied.

**Table 8.** Decimal reduction time for each of the treatments applied and samples analysed.

| *E* (kV/cm) | *D* (ms) | | |
|---|---|---|---|
| | Orange juice | Orange–carrot juice | Orange juice–milk beverage |
| 15 | - | - | 19.54 |
| 25 | 5.04 | 6.21 | 10.38 |
| 30 | 4.50 | 5.54 | - |
| 35 | 2.89 | 1.94 | 5.50 |
| 40 | 2.43 | 2.52 | 3.80 |

The *D* values (at each electric field strength) were higher in the orange juice–milk beverage, indicating that to produce the same degradation of ascorbic acid the treatment time would have to be longer than that required for the orange juice and the orange–carrot juice.

The *D* values were used to obtain the $Z_E$ value (parameter of sensitivity to electric field strength), which represents the increase in field strength (kV/cm) required for the ascorbic acid degradation rate to decrease 10 times. It was calculated as the negative inverse of the destruction curve obtained by plotting Log *D* against the field applied (*E*, kV/cm) (Figure 5), giving the following $Z_E$ values: 42.9 kV/cm (correlation coefficient 0.978, standard error 0.040, $p<0.05$), 30.6 kV/cm (correlation coefficient 0.856, standard error 0.156, $p<0.1$), and 35.1 kV/cm (correlation coefficient 0.999, standard error 0.010, $p<0.05$), for the orange juice,

orange–carrot juice mixture, and the orange juice–milk beverage, respectively. The higher value obtained in the orange juice indicates that it is more resistant to changes in electric field strength in PEF treatment.

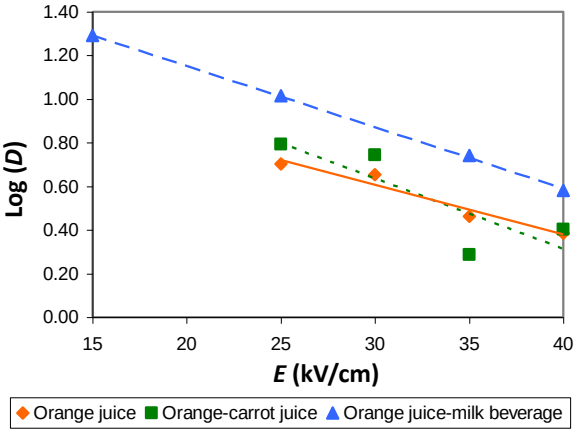


**Figure 5.** Logarithm of *D* value against electric field applied to obtain the $Z_E$ value.

For microorganisms, Álvarez and co-workers [59] found $Z_E$ values of 14.8 kV/cm for *Salmonella enteritidis* and 16.3 kV/cm for *Salmonella typhimurium* suspended in citrate–phosphate buffer when treated by PEF. Gómez and co-workers [60] found a $Z_E$ value of 9.1 kV/cm for *Lactobacillus plantarum* in orange–apple juice mixtures treated by PEF. With regard to inactivation of enzymes, Zhong and co-workers [61] found $Z_E$ values of 36.9 kV/cm for peroxidase and 16.2 kV/cm for polyphenol oxidase in buffer systems, which they suggest may be because the structure of polyphenol oxidase is larger and more complex. All these results show that ascorbic acid and peroxidase are more resistant (lower degradation) to treatment by high-intensity pulsed electric fields than microorganisms, so that they can be used to obtain safe products that retain their nutritional and organoleptic characteristics.

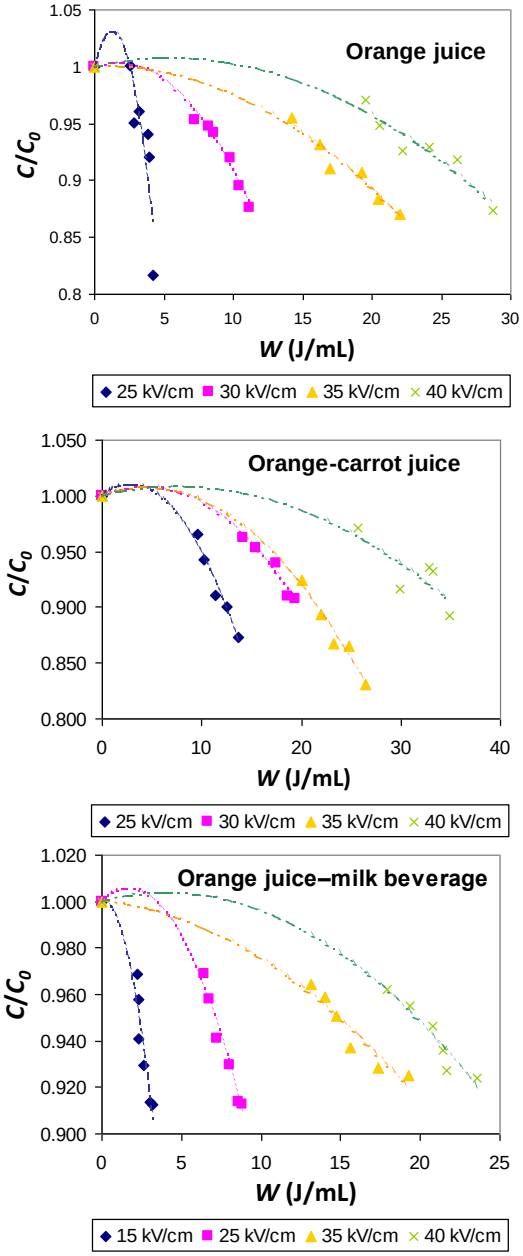


**Figure 6.** Degradation of ascorbic acid ($C/C_0$) with energy ($W$) applied during treatment.

**Table 9.** Variation in ascorbic acid concentration with $W$ (J/mL).

| $E$ (kV/cm) | Equation | $R^2$ (error) |
|---|---|---|
| | Orange juice | |
| 25 | $0.998+0.0508 \cdot W-0.0199 \cdot W^2$ | 77.0 (0.04), $p<0.10$ |
| 30 | $0.999+0.0045 \cdot W-0.0014 \cdot W^2$ | 98.3 (0.01), $p<0.01$ |
| 35 | $1.000+0.0004 \cdot W-0.0003 \cdot W^2$ | 97.4 (0.01), $p<0.01$ |
| 40 | $1.000+0.0026 \cdot W-0.0002 \cdot W^2$ | 94.0 (0.01), $p<0.01$ |
| | Orange–carrot juice | |
| 25 | $1.004+0.0070 \cdot W-0.0012 \cdot W^2$ | 97.6 (0.01), $p<0.01$ |
| 30 | $0.999+0.0038 \cdot W-0.0004 \cdot W^2$ | 97.8 (0.01), $p<0.01$ |
| 35 | $1.000+0.0033 \cdot W-0.0004 \cdot W^2$ | 98.5 (0.01), $p<0.01$ |
| 40 | $1.000+0.0022 \cdot W-0.0001 \cdot W^2$ | 83.2 (0.02), $p<0.10$ |
| | Orange juice–milk beverage | |
| 15 | $1.001+0.0044 \cdot W-0.0111 \cdot W^2$ | 91.0 (0.01), $p<0.01$ |
| 25 | $1.000+0.0008 \cdot W-0.0011 \cdot W^2$ | 95.5 (0.01), $p<0.01$ |
| 35 | $1.000+0.0009 \cdot W-0.0002 \cdot W^2$ | 95.1 (0.01), $p<0.01$ |
| 40 | $1.000+0.0018 \cdot W-0.0002 \cdot W^2$ | 97.2 (0.01), $p<0.01$ |

For a constant pulse energy ($W$), an increase in the electric field applied means an increase in the ascorbic acid degradation constant (Figure 6). The regression analysis performed indicates a degradation of ascorbic acid with pulse energy that fits a quadratic function (Table 9). When the treatment time is constant, the increase in the strength of the electric field applied means an increase in electric conductivity and therefore in the degradation of ascorbic acid.

As commented earlier, the energy applied in any treatment depends on the characteristics of the particular food (mainly conductivity). Therefore, to compare the effect of PEF on different foods it is necessary to calculate the energy applied ($Q$, J·mL$^{-1}$) in each beverage, and thus it is possible to estimate the effect of the matrix when a particular energy is applied. Figure 7 shows the evolution of ascorbic acid in relation to treatment energy. A regression analysis by least squares showed that the curve fits the following model: $C/C_0=1.001 - 0.004 \cdot Q^{1/2}$

($R^2$=0.867, standard error=0.023); $C/C_0$=1.003 – 0.003·$Q^{1/2}$ ($R^2$=0.868, standard error=0.024), and $C/C_0$=0.999 – 0.002 $Q^{1/2}$ ($R^2$=0.900, standard error=0.011), for the orange juice, orange–carrot juice mixture, and the orange juice–milk beverage, respectively.

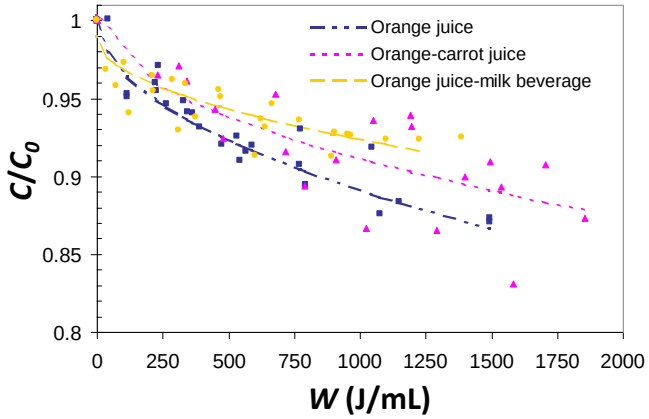


**Figure 7.** Variation in ascorbic acid versus energy applied ($Q$).

The slopes differ in the three cases, showing once again that the matrix influenced the ascorbic acid degradation rate, with the orange juice being more sensitive to the changes in energy applied in each treatment.

## Conclusion

PEF treatment can be put forward as a firm alternative to traditional pasteurisation treatments. The orange juice was more resistant to changes in electric field strength in PEF treatment, while the treatment time required to produce the same degradation of ascorbic acid was greater in the orange juice–milk beverage than for the orange juice or the orange–carrot juice mixture. These results show the need to optimise treatment conditions whenever there is a change in the matrix (food) or some processing factor.

## Acknowledgments

This research project was supported by the Spanish Ministry of Science and Technology and European Regional Development Funds (AGL2010-22206-C02-01).

Carbonell-Capella, J.M. holds an award from the Spanish Ministry of Education (AP2010-2546).

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## 3.6. *Stevia rebaudiana* Bertoni as a natural antioxidant/antimicrobial for pulsed electric field processed fruit juice: Processing parameter optimisation

*Food Control (Under review)*

# *Stevia rebaudiana* Bertoni as a natural antioxidant/antimicrobial for pulsed electric field processed fruit juice: Processing parameter optimisation

*Clara Miracle Belda-Galbis[1], María Nieves Criado[1], Juana María Carbonell-Capella[2], Ana Frígola[2], Dolores Rodrigo[1] **

[1] Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC). Departamento de Conservación y Calidad. C. Catedràtic Agustín Escardino Benlloch, 7, 46980 Paterna, Spain

[2] University of Valencia, Department of Nutrition and Food Science, Av. Vicent Andrés Estellés, s/n, 46100 Burjassot, Spain

* Corresponding author:
Dolores Rodrigo
e-mail: lolesra@iata.csic.es
Phone: (+34) 963 900 022, Fax: (+34) 963 636 301

**ABSTRACT**

Considering pulsed electric fields (PEF) and *Stevia rebaudiana* Bertoni antimicrobial and antioxidant properties, response surface methodology (RSM) was used to define the optimal processing conditions needed to maximise the safety, stability and nutritional quality of a PEF treated juice mixture containing mango, papaya and stevia leaves infusion (1.25 and 2.50% (w/v)). With this aim, PEF and stevia effects on the microbial load, the polyphenoloxidase (PPO) and peroxidase (POD) activities, the total phenolic content (TPC), the non-enzymatic browning index (NEBI) and the total antioxidant capacity (TAC) of the formulated food were assessed. Results obtained show that submitting the fruit mixture to 40 kV/cm for 360 µs, in presence of 1.79% (w/v) of stevia, maximised its TPC and TAC, while minimising the NEBI, as well as the microbial load and the activity of oxidative enzymes that could impair the nutritional and the sensory quality of minimally processed ready-to-eat foods.

**Keywords:** Food preservation, *Stevia rebaudiana* Bertoni, pulsed electric field processing, spoilage and pathogenic microorganisms, oxidative enzymes, phenolic compounds, non-enzymatic browning index, total antioxidant capacity, response surface methodology**.**

## 1. Introduction

Consumers currently know that fruit and vegetables regular intake has beneficial effects on their health. Drinking juices is undoubtedly one of the easiest and tastiest ways to increase these products daily intake and, therefore, juice consumption worldwide increases year on year.

Traditionally, sterilisation and pasteurisation have allowed lengthen juices shelf life by means of different time-temperature combinations (D'Amico, Silk, Wu, & Guo, 2006). If not, just after their obtainment, a fast microbial, enzymatic, chemical and physical deterioration takes place (Bates, Morris, & Crandall, 2001), which makes the product unacceptable both for sale and consumption. Taking into account consumers' preference for minimally processed ready-to-eat foods, in the past 30 years, development and implementation of non-thermal preservation technologies have been simulated as intense heat produces undesirable changes in the nutritional and sensory characteristics of any food (Corbo, Bevilacqua, Campaniello, D'Amato, Speranza, & Sinigaglia, 2009). High hydrostatic pressure (HHP) processing, ultraviolet exposure, membrane filtration and pulsed electric fields (PEF) application (Altuner & Tokuşoğlu, 2013; Buckow, Ng, & Toepfl, 2013; Noci, Riener, Walkling-Ribeiro, Cronin, Morgan, & Lyng, 2008; Zárate-Rodríguez, Ortega-Rivas, & Barbosa-Cánovas, 2001) are some of the existing alternatives nowadays. Among them, PEF processing is one of the most promising options for liquid foods, especially those rich in heat sensitive compounds.

PEF processing is able to inactivate spoilage and/or pathogenic microorganisms as well as oxidative enzymes, such as polyphenoloxidase (PPO, EC 1.14.18.1) and peroxidase (POD, EC 1.11.1.7), maintaining the nutritional quality, the antioxidant content and the freshness of foods (Altuntas, Evrendilek, Sangun, & Zhang, 2010; Buckow, Ng, & Toepfl, 2013; Terefe, Buckow, & Versteeg, 2015), especially if its application is combined with the use of preservatives from animal, vegetal and microbial origin, because they can increase PEF antimicrobial effectiveness (Ait-Ouazzou, Espina, García-Gonzalo, & Pagán, 2013; Pina-Pérez,

Martínez-López, & Rodrigo, 2013), as well as post-processing quality and safety, in absence of synthetic chemicals.

The antimicrobial and antioxidant activity of different *Stevia rebaudiana* Bertoni extracts and its potential use as a natural preservative have recently been studied. In few years, different researchers have shown that stevia leaves contain polyphenols and other antioxidant compounds (Lemus-Mondaca, Vega-Gálvez, Zura-Bravo, & Ah-Hen, 2012; Tadhani, Patel, & Subhash, 2007) which can inhibit enzyme activity and microbial growth (Belda-Galbis, Pina-Pérez, Espinosa, Marco-Celdrán, Martínez, & Rodrigo, 2014; Criado, Barba, Frígola, & Rodrigo, 2014; Muanda, Soulimani, Diop, & Dicko, 2011; Tadhani & Subhash, 2006), increasing, therefore, the value of yoghurts, beverages, chocolates and biscuits in which stevia is added in its formulation as a non-caloric sweetener (Yadav & Guleria, 2012).

Taking into account that stevia use and PEF application could be a good strategy to improve the microbial, nutritional and physicochemical quality of minimally processed ready-to-eat foods, the aim of this work was to evaluate PEF effects on the microbial load, the enzymatic activity (PPO and POD), the total phenolic content (TPC), the non-enzymatic browning index (NEBI) and the total antioxidant capacity (TAC) of a beverage containing mango, papaya and stevia in order to (i) elucidated if stevia increase PEF processing effectiveness, and to (ii) determine, by means of response surface methodology (RSM), which combination of electric field, treatment time and stevia concentration allows maximising the microbiological safety, the sensorial stability and nutritional quality of the matrix under study.

## 2. Material and methods

### *2.1. Samples*

#### 2.1.1. Fruit juice mixture: Obtainment and characterisation

Mango (*Mangifera indica* Linnaeus) and papaya (*Carica papaya* Linnaeus) juices were obtained separately using a household blender. To remove the pulp, before being mixed, the juices were filtered by means of a mesh sieve (pore size:

0.297 mm). The fruit juice mixture was prepared by mixing 50.75% (v/v) of papaya, 19.25% (v/v) of mango and 30% (v/v) of mineral water (in samples without stevia).

2.1.2. Stevia infusion and sample preparation

To prepare a concentrated stock solution of stevia leaves infusion (8.33±0.01% (w/v)), 100 mL of boiling mineral water were added to dried leaves (8.33 g) and the mixture was covered and allowed to infuse for 30 min. After that, before being stored at –40 °C, the water-leaves mix was vacuum filtered using a Kitasato flask, a Büchner funnel, a vacuum pump (VDE 0530, KNF Neuberger GmbH, Germany) and filter paper (Whatman® No. 1, Whatman International Ltd., UK).

From the stock, samples with a 1.25 and 2.50% (w/v) of stevia were prepared, replacing part of the water added to the juices mix by infusion. The highest stevia concentration tested (2.50% (w/v)) was established taking into account the maximum sucrose percentage that can be used to obtain sensorial acceptable foods along with the sweetness equivalence stevia/sucrose (Savita, Sheela, Sunanda, Shankar, Ramakrishna, & Sakey, 2004).

2.1.3. Microorganisms

PEF antimicrobial potential, in presence and in absence of stevia, was evaluated both in non-sterile samples and in sterile samples inoculated with *Listeria monocytogenes* (CECT 4032), with the aim of assessing, respectively, (i) PEF inactivation of moulds, yeasts and mesophiles naturally present in the mix under studied, and (ii) PEF inactivation of a psychotropic foodborne pathogen commonly found in minimally processed ready-to-eat foods (Codex Alimentarius Commision, 2002).

For that, from a lyophilised pure culture provided by the Spanish Type Culture Collection, a stock of vials containing *L. monocytogenes* (CECT 4032) was generated following the method described by Saucedo-Reyes, Marco-Celdrán, Pina-Pérez, Rodrigo, and Martínez-López (2009).

*2.2. Methods*

2.2.1. PEF processing

An OSU-4D bench-scale continuous PEF system designed at the Ohio State University (USA) was used to treat samples at laboratory scale setup. For this purpose, 8 co-field treatment chambers were connected in series, being 0.230 and 0.293 cm, respectively, the chambers diameter and the gap distance. A heat exchanger was used to guarantee that samples and chambers pre-treatment temperature was 25±3 °C. Pre- and post-treatment temperatures at the inlet and the outlet of treatment chambers were monitored by type T thermocouples. A square-wave bipolar pulse was selected. The pulse width was fixed to 2.5 µs. Pulse waveform, voltage and intensity of treatment chambers were recorded with a digital oscilloscope (Tektronix TDS 210, Tektronix Inc., USA). The flow rate was adjusted to 30 mL/min with a peristaltic pump (Cole-Parmer® 75210-25, Cole-Parmer Instruments Co., USA).

Treatment time ranged from 100 to 360 µs and the electric field intensity from 20 to 40 kV/cm. In each experiment, inoculated and un-inoculated samples, with and without stevia, were collected before and after each treatment and stored under refrigeration (3±1 °C) until being analysed. Microbial load, PPO and POD activities, TPC, NEBI and TAC of both untreated (blank) and PEF-treated samples were evaluated.

2.2.2. Assessment of microbial inactivation

The cellular density of treated samples, with and without stevia, was determined in terms of $\log_{10}$ (cfu/mL), before and after different PEF treatments, by viable plate count. The inactivation associated with each of the treatments tested ($\log_{10} S$) was established according to the difference existing between the counts obtained pre- and post-treatment. For that, aliquots of untreated and treated non-sterile and sterile inoculated samples were serially diluted, plated and incubated, at different temperatures.

**Table 1.** Media used and incubation conditions needed for viable plate count with the aim of assessing the antibacterial and antifungal properties of pulsed electric fields (PEF), in presence and in absence of stevia.

| Microorganism | Media used for samples spread[a] | Incubation conditions |
|---|---|---|
| Moulds and yeasts | Potato Dextrose Agar (PDA)[b] | 22–25 °C, 5–7 d |
| Mesophiles | Plate Count Agar (PCA) | 30 °C, 48 h |
| *Listeria monocytogenes* | Tryptic Soy Agar (TSA) | 37 °C, 48 h |

[a] All were supplied by Scharlau Chemie, SA (Spain).[b] Supplemented with sterile 10% (w/v) tartaric acid.

The dilutions were done employing buffered peptone water (Scharlau Chemie SA, Spain). The media used and the incubation conditions needed to assess PEF antibacterial and antifungal properties are shown in Table 1.

2.2.3. PPO and POD activities determination

The enzyme extracts for PPO and POD activities determination were obtained following a modification of the method described by Cano, Hernández, and De Ancos (1997). Ten mL of fruit juice mixture were homogenised with 20 mL of 0.2 M sodium phosphate buffer solution (Panreac Química SLU, Spain) at pH 7.0. The homogenate was centrifuged at 24000 g for 15 min at 4 °C, in polycarbonate tubes. The supernatant constituted the enzyme extract which and was used without delay to determine PPO and POD activities.

PPO activity was determined measuring the increase in absorbance that occurs when 1950 µL of 1,2-dihydroxybenzene (pyrocatechol; Sigma-Aldrich® Co., LLC, USA) in sodium phosphate buffer (0.05 M, pH 7.00) as substrate reacted with 0.1 mL of enzyme extract. For that, samples absorbance was recorded at 410 nm every 1 s, for 2.5 min, using a LAN OPTICS PG1800 UV-VIS scanning spectrophotometer (Labolan SL, Spain).

POD activity was also measured spectrophotometrically by mixing 2.7 mL of 0.05 M sodium phosphate buffer (pH 7.0), 0.2 mL of *p*-phenylenediamine (1% (w/v); Sigma-Aldrich® Co., LLC, USA) as H-donor, 0.1 mL of hydrogen peroxide

(1.5% (w/v); Sigma-Aldrich® Co., LLC, USA) as oxidant, and 0.1 mL of enzyme extract. The oxidation of *p*-phenylendiamine was measured at 485 nm.

Enzyme activity results were obtained at 25 °C. Both activities were calculated from the slope of the linear part of the graph of absorbance vs time. One unit of PPO and POD activities was defined as the change in absorbance at 410 and 485 nm, respectively, per min and mL of enzyme extract. In both cases, before and immediately after applying PEF, PPO and POD activities of the fruit juice extract were measured and compared with the initial activities of untreated samples. The enzyme activity was expressed as a percentage of relative activity (%*RA*), which was calculated using the following formula (Eq. (1)):

$$\% \, RA = 100 \times \left(\frac{A}{A_0}\right) \qquad (1)$$

where $A$ and $A_0$ are PPO and POD enzyme activities of treated and untreated samples, respectively.

2.2.4. TPC determination

The TPC was determined according to the method described by Georgé, Brat, Alter, and Amiot (2005), with some modifications. Ten mL of sample were homogenised with 50 mL of a mixture of acetone-water (70:30 (v/v)) for 30 min. Mixture supernatants were recovered by filtration (Whatman® No. 2, Whatman International Ltd., UK) and constituted the raw extracts (REs). REs (2 mL) were settled on an Oasis® cartridge (Waters SA, Spain). Interfering water-soluble components (reducing sugars and ascorbic acid) were recovered with 2 × 2 mL of distilled water. The recovered volume of the washing extract (WE) was carefully measured.

In order to eliminate vitamin C, heating was carried out on the WE (3 mL) for 2 h at 85 °C and this led to the heated washing extract (HWE). All extracts (RE, WE and HWE) were submitted to the Folin-Ciocalteu method, adapted and optimised (Barba, Esteve, Tedeschi, Brandolini, & Frígola, 2013). Gallic acid calibration standards with concentrations of 0, 100, 300, 500, 700 and 1000 ppm were prepared and 0.1 mL was transferred to borosilicate tubes. Three mL of sodium

carbonate solution (2% (w/v)) (Scharlau Chemie SA, Spain) and 100 µL of Folin-Ciocalteu reagent (1:1 (v/v)) were added to an aliquot of 100 µL from each gallic acid standard or sample tube. The mixture was incubated 1 h at room temperature and absorbance was measured at 765 nm, using a Perkin Elmer UV/VIS LAMBDA 2 spectrophotometer (Perkin-Elmer, Germany). Results were expressed as mg of gallic acid equivalents (GAE) per L.

2.2.5. Determination of the NEBI

To determine the NEBI, samples were centrifuged (824 g, 20 min, 18 ºC) and the supernatant was diluted in ethanol (1:1 (v/v)). The mixture was filtered using filter paper (Whatman® No. 42, Whatman International Ltd., UK) and the absorbance of the filtrate was spectrophotometrically measured at 420 nm (Meydav, Saguy, & Kopelman, 1977).

2.2.6. TAC assessment

Trolox equivalent antioxidant capacity (TEAC). The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was carried out following the method described by Re, Pellegrini, Proteggente, Pannala, Yang, and Rice-Evans (1999), which is based on the capacity of a sample to inhibit the ABTS radical ($ABTS^{\bullet+}$; Sigma-Aldrich® Co., LLC, USA) compared with a reference antioxidant standard (Trolox®; Sigma-Aldrich® Co., LLC, USA). The radical was generated using 440 µL of potassium persulfate (140 Mm) (Sigma-Aldrich® Co., LLC, USA). The solution was diluted with ethanol in order to obtain an absorbance of 0.70±0.02 units, at 734 nm. Once the radical was formed, 2 mL of $ABTS^{\bullet+}$ were mixed with 100 µL of appropriately diluted sample and the absorbance was measured at 734 nm for 20 min, using a Perkin Elmer UV/VIS LAMBDA 2 spectrophotometer (Perkin-Elmer, Germany), in accordance with Barba, Esteve, Tedeschi, Brandolini, and Frígola (2013). Results were expressed as mM Trolox® equivalents (TE).

Oxygen radical absorbance capacity (ORAC). The ORAC assay described by Ou, Hampsch-Woodill, and Prior (2001) was used, employing fluorescein (FL; Sigma-Aldrich® Co., LLC, USA) as "fluorescent probe" and a Wallac 1420 VICTOR$^{2}$™ multilabel counter (Perkin-Elmer, USA) with fluorescence filters, for an excitation

wavelength of 485 nm and an emission wavelength of 535 nm. The measurements were made in plates with 96 white flat-bottom wells (Sero-Wel, Bibby Sterilin Ltd., UK). The reaction was performed at 37 °C as the reaction was started by thermal decomposition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH; Sigma-Aldrich® Co., LLC, USA) in 75 mM phosphate buffer (pH 7.0) because of the sensitivity of FL to pH. The final reaction tested and the concentrations of the different reagents were determined following the methodology used by Barba, Esteve, Tedeschi, Brandolini, and Frígola (2013).

*2.3. Experimental design and statistical analysis*

The experimental design and data analysis were performed using the software Statgraphics® Centurion XV.II, version 11 (Statpoint Technologies Inc., USA). RSM was used to investigate the simultaneous effects of electric field strength, treatment time and stevia concentration on microbial inactivation, PPO and POD enzymatic activities, TPC, NEBI and TAC of the juice under study. Face-centred central composite design was used with 3 levels (maximum, minimum and central) of each independent variable, electric field strength (from 20 to 40 kV/cm), treatment time (from 100 to 300 μs) and stevia concentration (from 0 to 2.50% (w/v)), leading to 16 combinations (Table 2).

Independent variable levels were selected considering the sample characteristics and the operating conditions of PEF equipment. The combinations included different PEF and stevia conditions with an intermediate level (central point) of the 3 variables, replicated 2 times to check the reproducibility and stability of results obtained. The experimental design was performed twice, resulting in 2 blocks of experiments. Accordingly, samples were treated in duplicate and analysed in triplicate. Experiments were randomised to minimise the systematic bias in the observed responses due to extraneous factors and to increase analysis precision.

Regression coefficients for each of the term combinations of the independent variables were obtained and their significance was determined using the *p*-value generated by *t*-test. The quadratic model used for each response was the following (Eq. (2)):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (2)$$

where *Y* represents the predicted response, $X_i$ the independent variables, $\beta_0$ a constant that fixed the response at the experiment central point and $\beta ii$ the regression coefficients for the linear, quadratic and interaction effect terms.

**Table 2.** Experimental design matrix.

| Run | *Electric field* kV/cm ($X_1$) | *Time* µs ($X_2$) | *% Stevia* (w/v) ($X_3$) |
|---|---|---|---|
| 1 | 30 | 230 | 0 |
| 2 | 40 | 100 | 2.50 |
| 3* | 30 | 230 | 1.25 |
| 4 | 40 | 360 | 2.50 |
| 5 | 20 | 100 | 2.50 |
| 6 | 40 | 100 | 0 |
| 7 | 20 | 230 | 1.25 |
| 8 | 30 | 100 | 1.25 |
| 9 | 20 | 100 | 0 |
| 10* | 30 | 230 | 1.25 |
| 11 | 20 | 360 | 2.50 |
| 12 | 20 | 360 | 0 |
| 13 | 30 | 230 | 2.50 |
| 14 | 30 | 360 | 1.25 |
| 15 | 40 | 360 | 0 |
| 16 | 40 | 230 | 1.25 |

* *Design's central point.*

The non-significant terms ($p$>0.05) were deleted from the second-order polynomial model after an ANOVA test and a new ANOVA was performed to obtain the coefficients of the final equation for better accuracy. For this purpose, the program always conducts one search beginning at the centre of the experimental region. Given that the starting point may affect whether a global or local optimum is located, the optimisation was also done using additional searches starting at the best design point (with highest predicted desirability), at

all design points, at the best vertex (combination of low or high level of each factor with highest predicted desirability), and at the all vertices, in order to obtain the best result amongst the set of searches that the program performs.

## 3. Results and discussion

### *3.1 Effect of PEF and stevia on microbial load, oxidative enzymes (PPO and POD), TPC, NEBI and TAC (ORAC and TEAC) of the juice mixture formulated*

Effect of PEF treatments in presence and in absence of stevia on microbial load, PPO and POD activities, TPC, NEBI and TAC (TEAC and ORAC) of the juice fruit formulated are shown in Table 3.

In view of the results obtained, it can be concluded that PEF can reduce the microbial load present in the matrix developed before being treated. The inactivation achieved always increased as the electric field strength and the treatment time increased, regardless the stevia concentration used in the sample formulation (Table 3).

On the other hand, for any electric field and treatment time combination, it was observed that PEF effects on *L. monocytogenes* were enhanced by the addition of stevia in a dose-dependent manner, whereas its addition reduced PEF effects on mesophiles, yeasts and moulds, also in a dose-dependent manner. The addition of stevia only reduced PEF effects on *L. monocytogenes* if the field was 40 kV/cm and the treatment time was 360 μs. So, in general terms, the greater the stevia concentration, the higher the inactivation of *L. monocytogenes* but the lower the inactivation of mesophiles, yeasts and moulds. When food processing strategies are combined, they might act synergistically, provided that each element powered the effect of the others, and vice versa.

**Table 3.** Effect of PEF and stevia on microbial load, PPO and POD activities, TPC, NEBI and TAC of the beverage.

| *E* | *t* | *% Stevia* | Moulds & yeasts | Mesophiles | *L.mono cytogenes* | PPO | POD | TPC | NEBI[b] | TEAC | ORAC |
|---|---|---|---|---|---|---|---|---|---|---|---|
| kV/cm | µs | (w/v) | $\log_{10} S$[a] | $\log_{10} S$[a] | $\log_{10} S$[a] | *% RA* | *% RA* | mg GAE/L | | mM TE | mM TE |
| | 0 | 0 | 0 | 0 | 0 | 100 | 100 | 752.3±62.0 | 1 | 7.05±0.35 | 4.30±0.21 |
| 0 | 0 | 1.25 | 0 | 0 | 0 | 100 | 100 | 2891.8±26.9 | 1 | 13.10±0.15 | 28.47±3.39 |
| | 0 | 2.50 | 0 | 0 | 0 | 100 | 100 | 4267.5±10.8 | 1 | 21.88±1.76 | 35.09±3.38 |
| | 100 | 0 | 0.15±0.06 | 0.14±0.05 | 0.25±0.08 | 100.7±1.6 | 101.9±0.8 | 744.7±35.0 | 0.99±0.06 | 6.40±0.36 | 3.85±0.07 |
| | 100 | 2.50 | 0.12±0.01 | 0.02±0.01 | 0.39±0.02 | ND | 104.3±6.4 | 4378.1±59.3 | 0.96±0.01 | 21.73±1.09 | 33.79±0.35 |
| 20 | 230 | 1.25 | 0.44±0.03 | 0.25±0.05 | 0.58±0.04 | ND | 114.4±5.3 | 2929.9±59.3 | 1.01±0.02 | 13.01±0.78 | 26.55±1.21 |
| | 360 | 0 | 1.02±0.05 | 0.32±0.04 | 0.72±0.07 | 103.1±2.8 | 104.7±1.1 | 786.6±8.1 | 0.97±0.02 | 4.89±0.66 | 3.85±0.27 |
| | 360 | 2.50 | 0.29±0.03 | 0.11±0.06 | 0.96±0.04 | ND | 97.5±2.7 | 4465.7±43.1 | 0.95±0.00 | 22.88±1.50 | 34.92±1.75 |
| | 100 | 1.25 | 0.51±0.03 | 0.36±0.02 | 1.00±0.02 | ND | 100.0±3.2 | 2918.5±32.3 | 0.98±0.01 | 13.66±0.87 | 27.46±1.26 |
| | 230 | 0 | 1.79±0.02 | 0.60±0.08 | 1.62±0.04 | 102.9±0.3 | 101.9±5.9 | 780.9±5.4 | 0.83±0.07 | 5.14±0.51 | 5.00±0.07 |
| 30 | 230 | 1.25 | 1.13±0.10* | 0.52±0.09* | 2.09±0.10* | ND* | 100.4±1.5* | 2954.7±2.7* | 0.97±0.00* | 13.16±0.02* | 29.51±0.30* |
| | 230 | 2.50 | 0.79±0.07 | 0.34±0.05 | 1.99±0.08 | ND | 99.2±7.3 | 4564.8±43.1 | 0.92±0.00 | 23.37±2.89 | 37.04±0.37 |
| | 360 | 1.25 | 1.99±0.01 | 0.79±0.01 | 2.46±0.04 | ND | 51.1±4.8 | 2968.0±27.0 | 0.90±0.02 | 13.14±0.85 | 27.48±1.82 |
| | 100 | 0 | 1.80±0.07 | 0.62±0.05 | 1.88±0.03 | 106.6±2.7 | 107.7±5.7 | 799.9±5.4 | 0.86±0.07 | 6.27±0.10 | 8.13±0.25 |
| | 100 | 2.50 | 0.80±0.04 | 0.49±0.07 | 2.63±0.05 | ND | 98.5±6.1 | 4743.9±27.0 | 0.92±0.00 | 23.77±1.12 | 34.72±0.74 |
| 40 | 230 | 1.25 | 2.71±0.04 | 1.11±0.06 | 2.49±0.04 | ND | 42.6±6.1 | 3013.7±27.0 | 0.91±0.01 | 14.50±0.56 | 27.18±0.34 |
| | 360 | 0 | 3.23±0.12 | 1.76±0.06 | 3.73±0.06 | ND | 77.0±0.8 | 809.5±2.7 | 0.83±0.01 | 4.67±0.70 | 4.45±0.25 |
| | 360 | 2.50 | 2.50±0.65 | 1.20±0.06 | 2.72±0.04 | ND | 28.4±0.8 | 4850.6±27.0 | 0.90±0.00 | 24.21±1.45 | 34.26±1.13 |

* Average of central point. [a] Number of inactivated $\log_{10}$ cycles. [b] Calculated as $C/C_0$. PEF: pulsed electric fields. TAC: total antioxidant capacity. *E*: electric field. *t*: time. log S: decimal logarithm of the survival fraction. *L. monocytogenes: Listeria monocytogenes.* PPO: polyphenoloxidase. POD: peroxidase. %RA: residual activity. TPC: total phenolic content. GAE: gallic acid equivalents. NEBI: non-enzymatic browning index. TEAC: trolox equivalent antioxidant capacity. ORAC: oxygen radical antioxidant capacity. TE: trolox equivalents. ND: Not detected.

Many studies have shown that stevia leaves extracts have antibacterial and antifungal properties (Muanda, Soulimani, Diop, & Dicko, 2011; Puri & Sharma, 2011; Silva, Oliveira, do Prado, de Carvalho, & de Carvalho, 2008), while others have shown that antimicrobial compounds from vegetal origin could enhance the antimicrobial effectiveness of non-thermal preservation technologies (Aymerich, Jofré, Garriga, & Hugas, 2005; Iucci, Patrignani, Vallicelli, Guerzoni, & Lanciotti, 2007; Nguyen & Mittal, 2007). However, additive, antagonist and independent effects are also possible; the effectiveness of a determined combination depends on the microorganism, the matrix and the preservation strategies considered, taking into account that both the antimicrobial compounds concentration and the intensity of any treatment could modify the final result. This would explain the results obtained.

Regarding the quality stability by inactivating oxidative enzymes, in the absence of stevia only the highest PEF treatment tested (40 kV/cm; 360 µs) induced the inactivation of both enzymes; in that case, PPO was completely inactivated while POD activity was reduced by 22.97%. Furthermore, it was observed that the higher the treatment time, the higher the enzymatic inactivation, with the electric field being 40 kV/cm. This could be attributed to the existence of a critical treatment intensity needed to produce enzyme inactivation. For the other PEF treatments assayed in absence of stevia, the relative activity of both enzymes remained equal to the initial value (about 100%), showing resistance to PEF inactivation. In fact, it is generally assumed that PEF has limited effects on enzymes. Based on the available information in literature, depending on enzymes source and PEF process characteristics (electric field strength, treatment time, temperature, pulse frequency and width), different levels of PEF inactivation of PPO and POD have been reported previously. While most enzymes are inactivated in model solutions or extracts (Giner, Ortega, Mesegué, Gimeno, Barbosa-Cánovas, & Martín, 2002; Quintão-Teixeira, Soliva-Fortuny, Mota Ramos, & Martín-Belloso, 2013), others are not affected or are even activated by PEF processing (Van Loey, Verachtert, & Hendrickx, 2001; Yang, Li, & Zhang, 2004).

Table 3 shows the capacity of stevia to inhibit both PPO and POD enzymes, under given experimental conditions. To the best of our knowledge, this is the

first time that the combined effect of PEF and stevia on PPO and POD from fruit juices has been reported. The results obtained show that, for any electric field and treatment time combination, the complete inactivation of juice PPO was achieved when it was PEF processed in presence of stevia, regardless of the percentage added. In the case of POD, however, both electric field and treatment time had influence on its activity. According to this, only in the case of the highest electric field tested (40 kV/cm) stevia enhanced the inactivation percentage achieved by PEF. The addition of 2.50% (w/v) of stevia reduced the POD residual activity from 77.03 to 28.38%, at the same electric field and treatment time (40 kV/cm, 360 µs). Moreover, in the case of POD increasing electric field from 30 to 40 kV/cm, in presence of 1.25% (w/v) of stevia resulted in higher inactivation of POD, when treatment time was 230 µs. When stevia concentration was 1.25% (w/v) and electric field was 30 kV/cm, increasing treatment time from 230 to 360 µs also resulted in a higher POD inactivation, its residual activity being reduced from 100.41% to 51.09%. Increased level of POD and PPO inactivation has been observed with an increase in electric field strength and treatment time by other authors. Similar results were previously reported by Bi, Liu, Rao, Li, Liu, Liao, et al. (2013) in apple juice, and by Marsellés-Fontanet and Martín-Belloso (2007) in grape juice treated by PEF.

As expected, taking into account that stevia leaves contain a great amount of phenolic compounds (Lemus-Mondaca, Vega-Gálvez, Zura-Bravo, & Ah-Hen, 2012; Tadhani, Patel, & Subhash, 2007), the TPC of untreated juice with stevia was considerably higher than the TPC of untreated non-containing stevia juice. The values obtained in absence of infusion as well as in presence of 1.25 and 2.50% (w/v) were, respectively, 752.29±61.98, 2891.77±26.90 and 4267.53±10.78 mg GAE/L. Results are in accordance to those published by other authors (Tavarini & Angelini, 2013; Zayova, Stancheva, Geneva, Petrova, & Dimitrova, 2013). Increasing the electric field also caused an increase in the fruit juice TPC. This could be explained by the fact that PEF processing enhances the extraction of intracellular contents (Agcam, Akyıldız, & Akdemir Evrendilek, 2014). This effect was more pronounced in samples containing stevia, partly due to the complete inactivation of the PPO, while treatment time did not affect significantly juice TPC.

These results are in close agreement with the ones obtained by Grimi, Mamouni, Lebovka, Vorobiev, and Vaxelaire (2011), working with apple juice, and by Puértolas, López, Saldaña, Álvarez, and Raso (2010), working with grape juice.

Both, enzymatic and non-enzymatic browning is related with quality degradation reactions and therefore this parameter must be minimised when a processing technology is applied. NEBI is a measurement commonly used to indicate the browning development in samples. Keeping in mind the brownish colour of the infusion under study to assess PEF effect, the NEBI of treated samples, with or without stevia, was calculated using the following formula: $C/C_0$, where $C$ and $C_0$ are, respectively, post- and pre-processing NEBI values of the sample concerned. According to these data, the higher the electric field and/or the treatment time, the lower the NEBI, probably due to the inactivation of enzymes responsible of browning. Thus, it can be concluded that PEF processing allows the obtainment of fresh-like fruit juices, with a minimum non-enzymatic browning. This was also observed by Aguiló-Aguayo, Soliva-Fortuny, and Martín-Belloso (2009) in strawberry juice and by Altuntas, Akdemir Evrendilek, Sangun, and Zhang (2011) in peach nectar.

According to the results obtained, the addition of stevia increased the TAC of untreated samples, from 7.05±0.35 to 13.10±0.15 and 21.88±1.76 mM TE for 1.25% and 2.5% stevia, respectively, using TEAC method, and from 4.30±0.21 to 28.47±3.39 and 35.09±3.38 mM TE for 1.25% and 2.5% stevia, respectively, when TAC was measured by ORAC method. Results were as expected, taking into account that TAC is closely related to TPC, and given the effect of stevia on juice's TPC. In view of TEAC values, TAC was only dependent on stevia concentration.

In view of ORAC values, TAC was dependent on stevia concentration, but also on electric field and treatment time, for determined processing conditions; in absence of stevia¸ ORAC values increased when increasing the electric field, treatment time being 100 µs, and when decreasing the treatment time, the electric field being 40 kV/cm. Although PEF application is considered a non-thermal processing technology, it is associated with a temperature rise due to the electric current flowing across the liquid food to be processed (Lindgren, Aronsson, Galt, & Ohlsson, 2002).

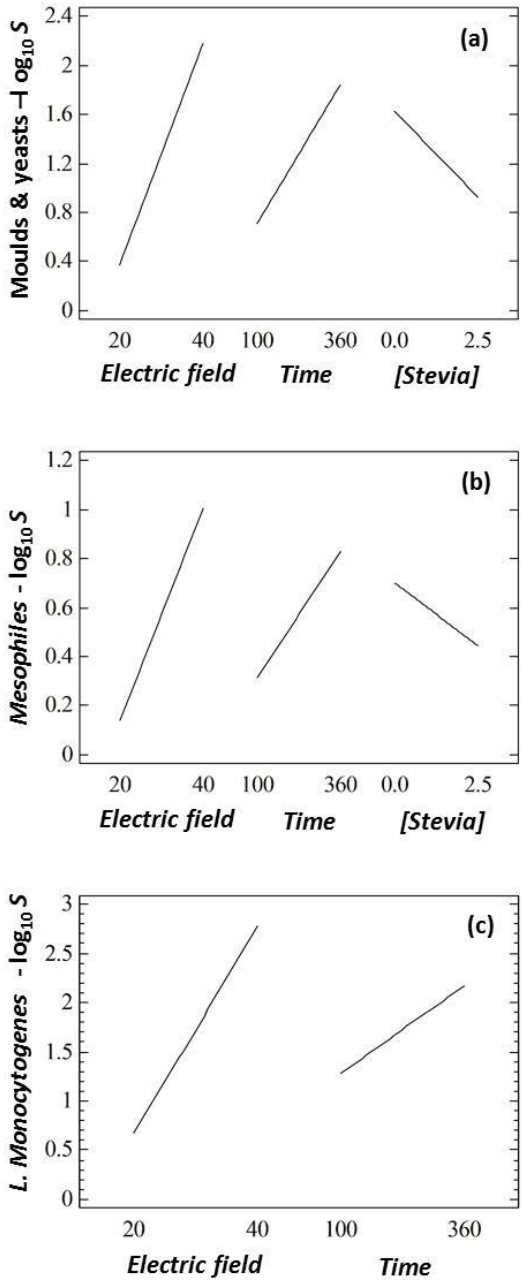


**Figure 1.** Effect of electric field (kV/cm), treatment time (μs) and stevia concentration (% (w/v)) on microbial inactivation ($log_{10}$ S).

It is possible that thermolabile compounds with antioxidant capacity, such as vitamin C, result adversely affected by this increment, especially when treatment intensity is high, as was observed by Cortés, Esteve, and Frígola (2008) in orange juice. This would explain the apparently effect of electric field and treatment time on ORAC values, in absence of stevia. In any case, the ORAC values were always higher than the TEAC ones. That could be due to ORAC method responds to a greater number antioxidants, with greater specificity (Zulueta, Esteve, & Frígola, 2009). Despite the differences, however, the Pearson test showed a positive correlation between those values ($R^2$=0.913), as well as between them and TPC values obtained ($R^2$=0.965).

### *3.2. Processing parameter optimisation based on their effect on the safety and quality of the formulated beverage*

Nowadays, foods are submitted to treatments that guarantee safety and quality, producing minimum changes in the sensory, nutritional and functional characteristics of the product concerned. For this reason, processing parameter optimisation is of outstanding importance from a practical point of view. RSM allows modelling phenomena and establishing which combinations of a certain number of factors (variables) will lead to optimum responses, taking into account the possible existence of interactions between factors (Giovanni, 1983). With this aim, Figures 1, 2 and 3 were constructed to know which relation exists between each of the experimental factors and each of the parameters considered, being relevant only the statistically significant relationships ($p \leq 0.05$).

According to Figure 1, the three experimental factors under study (electric field, treatment time and stevia concentration) had a statistically significant influence on mesophiles, moulds and yeasts inactivation ($p \leq 0.05$). In all cases, the higher the electric field or the treatment time and the lower the stevia concentration, the higher the number of inactivated cycles. In addition, the higher the electric field and the higher the treatment time, the higher the *L. monocytogenes* inactivation ($p \leq 0.05$). According to Figure 2, stevia concentration was the only factor able to reduce PPO activity ($p \leq 0.05$), while POD resulted to be electric field and treatment time inversely dependent ($p \leq 0.05$).

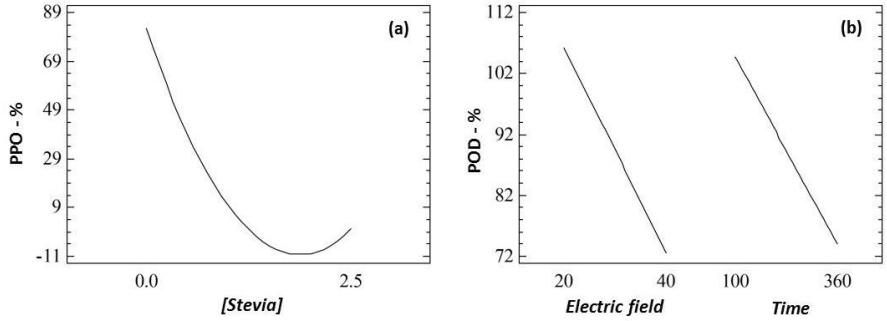


**Figure 2.** Effect of electric field (kV/cm), treatment time (μs) and stevia concentration (% (w/v)) on PPO and POD activities (%).

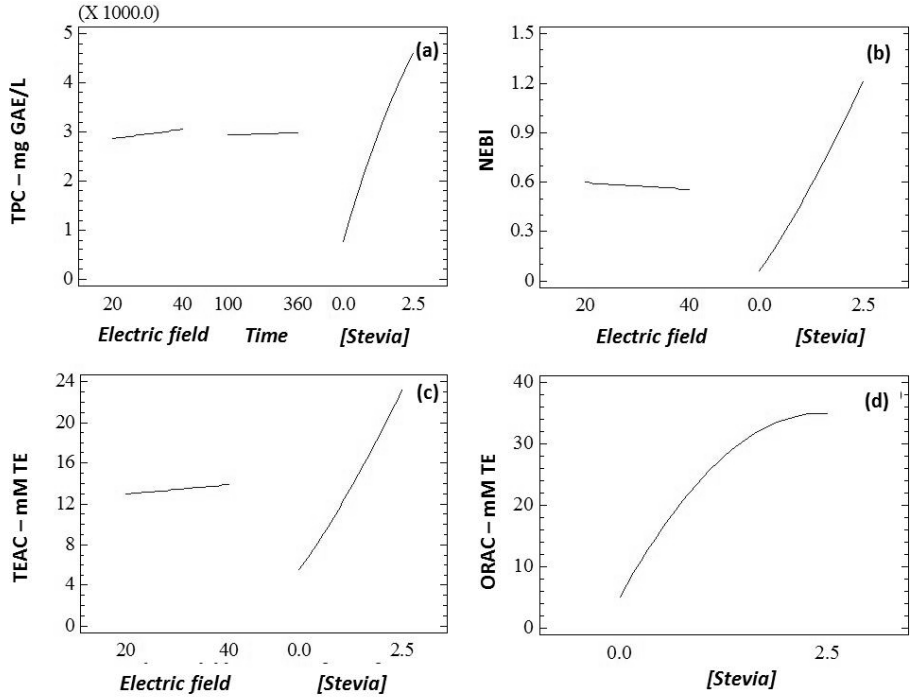


**Figure 3.** Effect of electric field (kV/cm), treatment time (μs) and stevia concentration (% (w/v)) on the total phenolic content (TPC; mg gallic acid equivalents (GAE)/L), the non-enzymatic browning index (NEBI) and the total antioxidant activity (TAC; mM trolox equivalents (TE)), based on the trolox equivalent antioxidant capacity (TEAC) and on the oxygen radical absorbance capacity (ORAC), of the juice mixture under study.

Finally, according to Figure 3, the TPC is mainly influence by stevia concentration, although PEF processing conditions also modify the final content obtained ($p \leq 0.05$). Electric field and stevia concentration had, moreover, a statistical significant effect on the NEBI and the TEAC value, whilst the ORAC one resulted to be only stevia significantly dependent ($p \leq 0.05$).

Based on this data and using the desirability approach to optimise PEF processing taking advantage of the antioxidant, antimicrobial and nutritional properties of stevia, it was determined the optimal processing conditions to maximise microbial inactivation as well as TPC and TAC of the juice formulated, minimising NEBI, PPO and POD activities.

The desirability function is a useful approach to optimise several responses at the same time to obtain the overall desirability, considering each individual response. Thus, to obtain a maximum overall desirability of 0.743, the juice formulated containing 1.79% (w/v) of stevia should be submitted to 40 kV/cm during 360 µs.

**4. Conclusions**

In response to increasingly demand of minimally processed juices, non-thermal preservation technologies development as well as non-synthetic preservatives use have been encouraged. For these reason, PEF and stevia effects on microbial inactivation, enzyme activity, TPC, NEBI and TAC was evaluated, the matrix under study being a beverage containing mango, papaya and stevia leaves infusion.

In view of data obtained, the joint implementation of both strategies can be a useful tool to obtain a safe and stable juice, without added sugars and with enhanced antioxidant properties, in absence of synthetic additives. With this aim, the optimum processing conditions, taking into account which were the variables under study and based on the results obtained, would be 40 kV/cm, 360 µs and 1.79% (w/v) stevia.

**5. Acknowledgements**

The authors thank the Ministry of Economy and Competitiveness for its support through project number AGL2010-22206-C02-01-ALI. Clara Miracle Belda-Galbis and María Nieves Criado are especially grateful to the CSIC for providing their JAE-Predoc grant and JAE-Tec grant, respectively. Juana María Carbonell-Capella is grateful to the Spanish Ministry of Education for providing her predoctoral grant (AP2010-2546). Finally, all the authors also thank the company ANAGALIDE SA (Spain) for providing them with dried stevia leaves.

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## 3.7. Influence of pulsed electric field processing on the quality of fruit juices sweetened with *Stevia rebaudiana*

*Innovative Food Science and Emerging Technologies (Under review*)

# Influence of pulsed electric field processing on the quality of fruit juices sweetened with *Stevia rebaudiana*

Carbonell-Capella, J.M., Buniowska, M., Zulueta, A., Esteve, M.J., Frígola, A.

University of Valencia, Department of Nutrition and Food Science, Av. Vicent Andrés Estellés, s/n, 46100 Burjassot, Spain

*Corresponding author:
Ana Frígola
e-mail: ana.frigola@uv.es.
Phone: +34 963544955 Fax: +34 963544954

**ABSTRACT**

A fruit juice-stevia beverage was processed using pulsed electric fields (PEF), a non-thermal preservation technology, with the purpose of investigating the feasibility of PEF for bioactive compounds and steviol glycosides enhancement and its impact on physicochemical properties. Variable ranges of response surface methodology were 20-40 kV/cm (electric field strength), 100-360 μs (treatment time) and 0-2.5% (w/v) stevia. After PEF, ascorbic acid was retained by more than 74% of the initial content. Some of the analysed PEF treatments resulted in an enhancement of total anthocyanins and carotenoids. The best results for rebaudioside A/stevioside ratio were obtained when PEF was applied at 30 kV/cm for 230 μs. Hydroxymethylfurfural content and total colour differences were maximum at the highest electric field strength assayed (40 kV/cm). PEF conducted at 21 kV/cm during 360 μs with 2.5% stevia led to the beverage with the greatest content in bioactive compounds and sweetening properties with minimal colour changes.

**Industrial Relevance:** Thermal pasteurisation of fruit juices is used to extend their shelf life. However, thermal process may negatively affect sensorial and nutritional properties of juices. In this context, the use of pulsed electric fields (PEF) is a promising technique for the acquisition of beverages with fresh like properties with the potential to be implemented at a commercial scale by the brewing industry. The combination with *Stevia rebaudiana* may constitute a useful tool to provide fruit juices with an increased added value with the improvement of the nutritional, physicochemical and sensorial properties.

**Keywords:** Pulsed electric field processing, *Stevia rebaudiana* Bertoni, Bioactive compounds, Steviol glycosides, Colour.

## 1. Introduction

Increased awareness of the relationship between diet and health has stimulated a trend in nutritional science whereby more attention is given to the health effects of foods. Taking advantage of this awareness, a large quantity of beverages based on exotic fruits have been designed in response to consumer´s demand for highly nutritious foods, as well as innovative tastes and flavours. Between them, mango and papaya juices can be considered dietary sources of bioactive compounds, such as vitamin C, carotenoids and anthocyanins, which have shown to be related to colour of foods. Furthermore, an upsurge of interest in the therapeutic potential of plants and their addition to food products has been observed, with an increased interest in the use of the natural sweetener *Stevia rebaudiana* Bertoni (Korir, Wachira, Wanyoko, Ngure, & Khalid, 2014). Stevia leaves have been shown to exert antioxidant and antimicrobial activity and could be a useful tool to ensure safety and quality of food products (Ramya, Manogaran, Joey, Keong, & Katherasan, 2014).

To prolong shelf life of juices, pasteurisation is the commonest method for inactivating microorganism and enzymes. However, loss of representative flavour compounds, colour and vitamins has been reported (Galaverna & Dall'Asta, 2014). Solving colour alterations of fruit juice-based beverages by preserving their phytochemical composition during processing is a major challenge for the beverage industry. In this sense, PEF (pulsed electric fields) have emerged as a non-thermal technology with potential to pasteurise foods nonthermally via exposure to short high-voltage pulses. Under the effect of PEF with electric field strength of 0.5–40 kV/cm and pulse duration from several microseconds to several milliseconds, cell membranes become electroporated with the following alteration in the transport of ions and changes in enzyme structures (Terefe, Buckow, & Versteeg, 2015).

Retention studies of vitamins and organoleptic characteristics to assess the effects of food processing on the nutritive value of foods are of great importance to food technologists and consumers. For instance, vitamin C is a thermolabile vitamin that is especially affected by heat treatment and has been used as a

quality indicator after application of a preservation process, also because it is related to other parameters, such as colour retention (Zulueta, Barba, Esteve, & Frígola, 2013). Anthocyanin pigments readily degrade during processing of foodstuffs, which can have a dramatic impact on colour quality and may also affect nutritional properties (Guo et al., 2014). Carotenoids content may be also modified by the processing conditions with the consequent effect in the colour characteristics (Sanchez-Vega, Elez-Martínez, & Martín-Belloso, 2015). Furthermore, steviol glycosides related to the sweetening properties of stevia derived products may be affected by processing parameters (Espinoza et al., 2014).

Previous studies suggest that PEF treatment may have no detrimental effect on heat-labile compounds and even more, may increase extractability of some bioactive compounds. This could have not only nutritional consequences, but also colour and flavour modifications, which are recognised as the major factors affecting food product acceptance.

However, compared to the wide range of research investigating enzyme and microorganism inactivation by PEF, there are few studies related to the effect of PEF treatment on sensorial characteristics and bioactive compounds of juices (Barba et al., 2012; Guo et al., 2014; Sanchez-Moreno, De Ancos, Plaza, Elez-Martínez, & Pilar Cano, 2011; Wiktor et al., 2015), and to the best of our knowledge, none about the effect of PEF processing on steviol glycosides content in fruit juices sweetened with stevia. Therefore, taking into account that stevia use and PEF application could be a good strategy to improve nutritional and sensory quality of minimally processed ready-to-eat foods, the aim of the present study was to evaluate the impact of PEF on bioactive compounds, steviol glycosides and physicochemical parameters of a beverage containing mango, papaya and stevia in order to (i) elucidate if stevia influences PEF processing, and to (ii) determine by means of response surface methodology (RSM) which combination of electric field, treatment time and stevia concentration allows maximising bioactive compounds of the matrix under study while minimising colour changes.

## 2. Material and methods

### *2.1. Fruit juice mixture and sample preparation*

Mango (*Mangifera indica* Linnaeus) and papaya (*Carica papaya* Linnaeus) juices were prepared separately using a household blender. The resulting juice was filtered using a mesh sieve with a pore size of 0.297 mm. The fruit juice mixture was prepared by mixing 50.75% (v/v) of papaya, 19.25% (v/v) of mango and 30% (v/v) of mineral water (in samples without stevia).

A concentrated stock solution of stevia leaves infusion (8.33±0.01%, w/v) was prepared by adding 100 mL of boiling mineral water to 8.33 g of dried leaves, allowing it to infuse for 30 min. This infusion was vacuum filtered using a Kitasato flask, a Büchner funnel, a vacuum pump (VDE 0530, KNF Neuberger GmbH, Germany) and filter paper (Whatman® No. 1, Whatman International Ltd., UK). Samples with 1.25 and 2.50% (w/v) of stevia were prepared taking different volumes of stevia stock solution and were added to the fruit juice mixture replacing part of the water. The highest stevia concentration tested (2.50% (w/v)) was selected taking into account the maximum sucrose percentage that can be used to obtain sensorially acceptable foods and the sweetness equivalence stevia/sucrose (Savita, Sheela, Sunanda, Shankar, & Ramakrishna, 2004).

### *2.2. PEF equipment*

PEF treatments were carried out in an OSU-4D continuous-flow bench-scale system (Ohio State University, USA). The system consisted of eight co-field treatment chambers connected in series with a diameter of 0.230 and an electrode gap of 0.293 cm. A heat exchanger was used to guarantee that samples and chambers pre-treatment temperature was 25±3 °C. The inlet and outlet temperatures of treatment chambers were monitored by type T thermocouples. A square-wave bipolar pulse was selected. The pulse width was fixed to 2.5 µs. Pulse waveform, voltage and intensity in the treatment chambers were fed into a digital oscilloscope (Tektronix TDS 210, Tektronix Inc., USA). The flow rate was set at 30 mL/min and controlled by a peristaltic pump (Cole-Parmer® 75210-25, Cole-Parmer Instruments Co., USA). In order to decide the most appropriate PEF

conditions, a number of alternative processing conditions were tested. Treatment time ranged from 100 to 360 µs and the electric field intensity from 20 to 40 kV/cm. Samples were collected after each treatment and stored under refrigeration (3±1 °C) until being analysed. Experiments were performed in duplicate.

*2.3. Ascorbic acid determination*

Ascorbic acid was assayed by polarographic determination using a Metrohm 746 VA Trace Analyser (Herisau, Switzerland) equipped with a Metrohm 747 VA stand. The working electrode was a Metrohm multi-mode electrode operated in the dropping mercury mode. A platinum wire counter electrode and a saturated calomel reference electrode were used. The following instrumental conditions were applied: $DP_{50}$, mode DME, drop size 2, drop time 1 s, scan rate 10 mV/s, initial potential −0.10 V. Samples (5 mL) were diluted to 25 mL with the extraction solution (oxalic acid 1% w/v, trichloroacetic acid 2% w/v, sodium sulfate 1% w/v). After vigorous shaking, the solution was filtered through a folded filter (Whatman® No. 1, Whatman International Ltd., UK). Oxalic acid (9.5 mL) 1% (w/v) and 2 ml of acetic acid/sodium acetate 2 M buffer (pH=4.8) were added to an aliquot of 0.5 mL of filtrate and the solution was transferred to the polarographic cell. Determinations were carried out by using the peak height and standard addition method in accordance to Carbonell-Capella, Barba, Esteve, & Frígola (2013).

*2.4. Total anthocyanins assessment*

Total anthocyanins were determined using a modified method of Mazza, Fukumoto, Delaquis, Girard, & Ewert (1999). A 10-fold diluted sample of 100 µl was mixed with 1700 µl of distilled water and 200 µl of 5% (v/v) HCl. Samples were incubated at room temperature for 20 min before measuring the absorbance at 520 nm. Calculations of total anthocyanins were based on cyanidin-3-glucoside (molar absorptivity 26900 L/mol·cm).

*2.5. Total carotenoids determination*

Extraction of total carotenoid was carried out in accordance with Lee and Castle (2001). An aliquot of sample (2 mL) was homogenised with 5 mL of extracting solvent (hexane/acetone/ethanol, 50:25:25, v/v) and centrifuged for 5 min at 4000 rpm at 5 ºC. The top layer of hexane was recovered and transferred to a 25 mL volumetric flask and volume was adjusted to 25 mL with hexane. Absorbance was measured at 450 nm and total carotenoids were calculated according to Ritter and Purcell (1981) using an extinction coefficient of β-carotene, $E^{1\%}$=2505.

*2.6. Steviol glycosides analysis*

The method of JECFA (2010) with various modifications was used. Samples were filtered through a Sep-Pak® cartridge (a reverse-phase C-18 cartridge; Millipore, MA, USA) which retains steviol glycosides. Cartridges were previously activated with 10 ml of methanol (MeOH) and 10 ml of water. Every 10 ml of previously diluted sample was eluted with 2 ml of MeOH and collected, filtered through a 0.45 µm membrane filter Millex-HV13 (Millipore) and analysed by liquid chromatography. Kromasil 100 C18 precolumn (guard column) (5 µm, 150 x 4.6 mm); Kromasil 100 C18 column (5 µm, 150 x 4.6 mm) (Scharlab, Barcelona, Spain). The mobile phase consisted of two solvents: Solvent A, acetonitrile and Solvent B, 10 mmol/L sodium phosphate buffer (pH=2.6) (32:68, v/v). Steviol glycosides were eluted under 1 mL/min flow rate and the temperature was set at 40 °C with a column thermostat (Prostar 510, Varian). Triplicate analyses were performed for each sample. Chromatograms were recorded at 210 nm. Identification of steviol glycosides were obtained by addition of authentic standards, while quantification was performed by external calibration with standards.

*2.7. Physicochemical analysis*

°Brix (total soluble solid content) were measured in accord to IFU methods (2001). ºBrix was determined with an Atago RX-1000 digital refractometer (Atago Company Ltd., Tokyo, Japan). HMF content was measured using the method described by IFFJP (1984).

Colour analysis was performed using a Hunter Labscan II spectrophotometric colourimeter (Hunter Associates Laboratory Inc., Reston, VA., USA) controlled by a computer that calculates the colour ordinates from the reflectance spectrum. Results were expressed in accordance with the Commission International d'Eclairage LAB (CIELAB) system with reference to illuminant D65 and with a visual angle of 10°. Three consecutive measurements of each sample were taken. The CIE *L** (lightness [0=black, 100=white]), *a** (*–a**=greenness, *+a**=redness) and *b** (*–b**=blueness, *+b**=yellowness) values were used to calculate the total colour differences (*ΔE**=[(*ΔL**)2+(*Δa**)2+(*Δb**)2]1/2), where Δ*L**, *Δa** and *Δb** are the differences between the untreated and the treated (PEF) juice mixture (Calvo, 2004).

*2.8. Experimental design and statistical analysis*

Response surface methodology was used to investigate the effect of electric field strength (20-40 kV/cm), time (100-360 µs) and *Stevia rebaudiana* concentration (0-2.5%, w/v) on the nutritional parameters (ascorbic acid, total carotenoids and total anthocyanins), steviol glycosides and physicochemical parameters (colour, HMF and ºBrix) of the beverage. A 3 level, 3 variable central composite design was used, resulting in 16 combinations (Table 1).

**Table 1.** Independent variables and their levels used for the face-centred central composite analysis.

| Independent variable | Code | Variable level | | |
|---|---|---|---|---|
| | | -1 | 00 | 1 |
| Electric field intensity (kV/cm) | $X_1$ | 20 | 30 | 40 |
| Time treatment (µs) | $X_2$ | 100 | 230 | 360 |
| *Stevia* percentage | $X_3$ | 0 | 1.25 | 2.50 |

The range of independent variables was established based on the conclusions of a previous study (Belda-Galbis et al., 2014), with an intermediate level (central point) of the 3 levels. The experimental design was performed twice, to check the reproducibility and stability of the results obtained. Experiments were randomised to minimise the systematic bias in the observed responses due to

extraneous factors and to increase precision. Experimental data were fitted by a second-order polynomial model (Eq. (1)):

$$Y = b_0 + b_1X_1 + b_2X_2 ++ b_{12}X_1X_2 + b_{11}X_{12} + b_{22}X_{22} \quad (1)$$

where $Y$ represents the predicted response, $X_i$ the independent variables, $b_0$ is the centre point of the system and $b_{ii}$ are coefficients of the linear, quadratic and interactive effect.

The non-significant terms were deleted from the second-order polynomial model after an ANOVA test, and a new ANOVA was performed to obtain the coefficients of the final equation for better accuracy. Experimental design and data analysis was examined using Statgraphics® Centurion XVI (Statpoint Technologies Inc., USA). All the individual desirability functions obtained for each response were combined into an overall expression, which is defined as the geometrical mean of the individual functions. The nearer the desirability value to the unit, the more adequate the system (Ross, 1996). In the present study, desirability functions were developed in order to obtain the beverage with the highest levels of antioxidant compounds and the best physicochemical properties. Subsequently, an ANOVA of three factors (pressure, time, and *Stevia rebaudiana* concentration) was applied, and in the parameters for which significant differences were obtained with more than two levels, Tukey's test was applied to ascertain the range of values in which the differences were located. Finally, a study was conducted with the aim of determining whether there were correlations between a pair of variables (Pearson's test).

## 3. Results and discussion

### *3.1. Effect of PEF and stevia on bioactive compounds content of the juice beverage*

To explore the impact of PEF on the nutritional indicators of the fruit juices, ascorbic acid, total anthocyanins and total carotenoids were analysed (Figures 1-3).

Ascorbic acid content for untreated papaya-mango juice without stevia added was 24.94±0.16 mg/100 mL. Previous studies have shown similar values in a

papaya, mango and orange beverage (Carbonell-Capella et al., 2013). After pulsed electric field treatments, a significant decrease ($p$<0.05) in the ascorbic acid content was observed in comparison with the untreated sample. Higher electric fields strength led to higher decrease in the ascorbic acid content, independently of the treatment time.

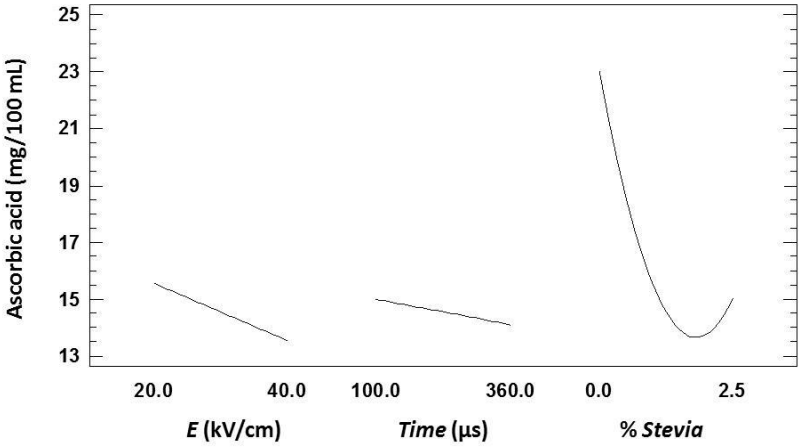


**Figure 1.** Effect of electric field (kV/cm), treatment time (µs) and stevia concentration (%, w/v) on ascorbic acid (mg/100 mL).

Addition of stevia to the fruit juice caused a significant reduction in ascorbic acid content (16.82±0.28 and 16.65±0.22 mg/100 mL for 1.25 and 2.5% stevia (w/v), respectively) in untreated beverages. Although Kim, Yang, Lee, & Kang (2011) detected ascorbic acid in stevia leaves and callus, differences in these results and those found in the present research could be attributed to the preparation of the stevia water extract (weight and temperature submitted). Furthermore, stevia addition may cause a change in the pH of the beverage, reducing the stability of ascorbic acid. PEF treatment caused a reduction of ascorbic acid in the fruit juice-stevia beverage, similarly to the fruit juice without stevia. Higher electric field strengths caused higher ascorbic acid reduction, as shown in Figure 1. This could be explained by the fact that electroporation caused by PEF processing enhances the extraction of intracellular contents, and consequently, ascorbic acid's stability can be comprised. However, when stevia is used in a 2.5%, ascorbic acid seems to be better retained (81.2%) in comparison to

fruit juices sweetened with 1.25% of stevia (71.9%), which may be due to interactions between stevia components and ascorbic acid involved in the kinetics of reaction of ascorbic acid degradation.

A regression analysis was performed to analyse the influence of electric field strength ($E$, kV/cm) and treatment time ($t$, μs) on ascorbic acid retention in each of the beverages studied. Results fitted to a linear model are shown in Eq. (2) ($R^2$=0.946, standard error 0.008) for fruit juice beverage without stevia, Eq. (3) ($R^2$=0.913, standard error 0.023) for fruit juice beverage sweetened with 1.25% stevia (w/v) and Eq. (4) ($R^2$=0.808, standard error 0.024) for fruit juice beverage sweetened with 2.5% stevia (w/v):

$$C/C_0 = 1.027 - 0.003 \cdot E - 0.00004 \cdot t \quad (2)$$

$$C/C_0 = 1.307 - 0.011 \cdot E - 0.00044 \cdot t \quad (3)$$

$$C/C_0 = 1.090 - 0.005 \cdot E - 0.00021 \cdot t \quad (4)$$

The fit was significant in all cases ($p$<0.01). It can be seen that both electric field strength applied and treatment time influence the degradation of ascorbic acid and it can be said that the fruit juice beverage without stevia and with stevia at 1.25 and 2.5% (w/v) show similar behaviour.

Furthermore, experimental data of all three beverages were fitted by a second-order polynomial model. Analysis of variance showed that the regression model was accurate enough ($R^2$=0.980, standard error 0.558). The relation between the independent variables and total ascorbic acid can be described by the Eq. (5):

$$\text{AA (mg/100 mL)} = 26.842 - 0.101 \cdot E - 0.003 \cdot t - 10.371 \cdot \%stevia + 2.865 \cdot \%stevia^2 \quad (5)$$

Differently from ascorbic acid, the PEF processed and unprocessed juice samples had similar concentrations of total anthocyanins, with values from 78.1 to 112.2% in PEF treated compared to untreated fruit juices. This was also observed by Guo et al. (2014) when they treated pomegranate juice at 35 and 38 kV/cm during 281 μs.

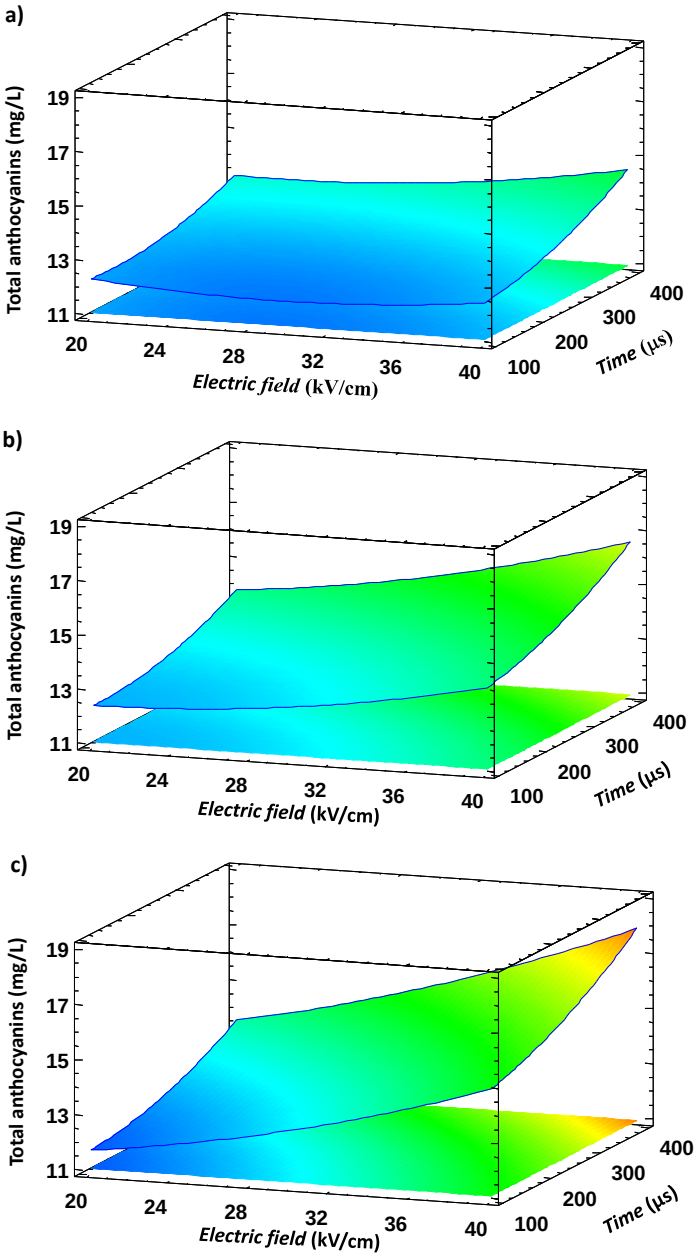


**Figure 2.** Effects of electric field and time on total anthocyanins (mg cyaniding-3-glucoside/L) of a fruit juice sweetened with stevia at: a) 0%, b) 1.25% and c) 2.50%.

When regression analysis was made, electric field was the factor that most influenced anthocyanin content, independently of the stevia concentration. As can be seen in Figure 2, behaviour of anthocyanin content was different after applying PEF depending on the stevia concentration. In the case of fruit juice without stevia, higher TA values were obtained when the beverage was treated at lower electric field strength (20 kV/cm). By contrast, fruit juices sweetened with stevia showed a higher TA content at higher electric field intensity (40 kV/cm).

Electroporation may enhance the extractability of anthocyanins from fruit cells, but in the case of fruit juices without stevia, higher electric fields do not lead to a higher anthocyanin extraction maybe because PEF application at higher electric fields can also promote reactions which cause a decrease in bioactive compounds. This does not occur in fruit juices sweetened with stevia, as stevia may act as an antioxidant ingredient protecting other bioactive compounds from degradation. That is why the highest anthocyanin value (17.9±1.6 mg/L) was obtained in the fruit juice sweetened with stevia treated at 40 kV/cm during 360 µs. These findings promote the combination of two strategies such as PEF technology along with the use of a natural ingredient with antioxidant and antimicrobial activity when processing food products. Previous studies (Carbonell-Capella et al., 2013) also show the combination of stevia and another non-thermal technology (high pressure processing) to influence positively total anthocyanin content and attribute it to the effect that high pressure processing has on the molecules involved in anthocyanin degradation, such as enzymes, which can also occur after PEF treatment. The reduced regression model was as follows (Eq. (6)):

$$TA\ (mg/L) = 10.491 + 0.029 \cdot E + 0.006 \cdot t - 1.320 \cdot \%stevia + 0.062 \cdot E \cdot \%stevia \qquad (6)$$

Untreated fruit juices had a total carotenoid content of 850.8, 898.2 and 815.9 µg/100 mL for 0, 1.25 and 2.5% stevia, respectively, showing that stevia addition does not influence carotenoid content in untreated beverage. However, application of PEF changed total carotenoids (TC) content of fruit juices, as shown in Figure 3. Both electric field and treatment time had a significant positive effect ($p$<0.05) on total carotenoids. As previously observed for anthocyanins, fruit juices

without stevia had a maximum carotenoid content (107.3%) when electric field was of 20 kV/cm for 360 µs.

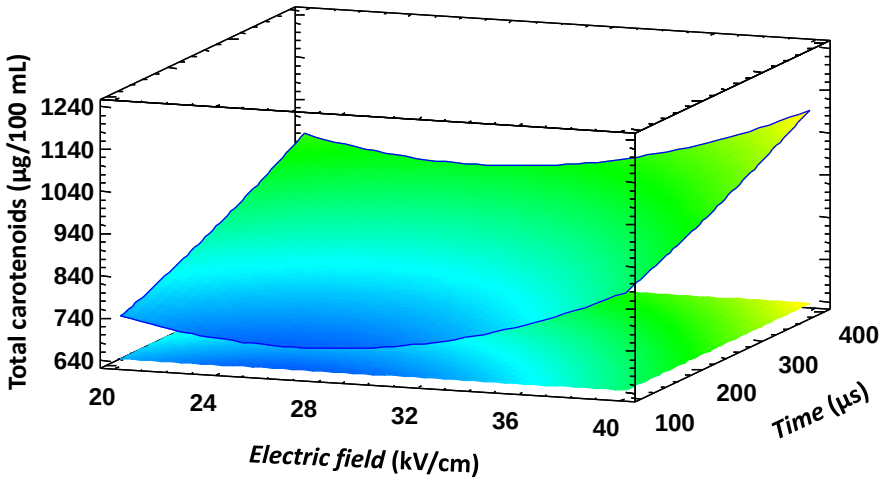


**Figure 3.** Response surface plot for total carotenoids content of the beverage with 2.50% of stevia as affected by PEF at different electric fields (20-40 kV/cm) and times (100-360 µs).

According to Wiktor et al., (2015), PEF can act ambiguously by enhancing extractability of carotenoids from the food matrix on the one hand, but generating ROS (reactive oxygen species) which can promote the oxidation of the β-carotene chain on the other hand. This would explain the enhancement of the carotenoid content at lower electric fields while at higher electric fields, carotenoid content would be lower. However, fruit juices sweetened with stevia reached a maximum (111.9 and 142.5% for 1.25 and 2.5% stevia, respectively) when samples were treated at an electric field of 40 kV/cm for 360 µs. Once more, fruit juices sweetened with stevia increased nutritional quality of fruit juices at higher electric fields and treatment time, which may be attributed to the protective effect of stevia antioxidant compounds. This effect was also observed by Pérez-Ramirez, Castanño-Tostado, Ramirez-De Leon, Rocha-Guzman, & Reynoso-Camacho, (2015) who obtained that the incorporation of stevia to a roselle beverage increased the stability of colour, phenolic compounds and

antioxidant capacity. Experimental data were fitted to a second-order polynomial model (Eq. (7)):

$$TC\ (\mu g/100\ mL) = 1325.940 - 53.642 \cdot E + 0.735 \cdot t + 1.003 \cdot E^2 \qquad (7)$$

*3.2 Effect of PEF and stevia on steviol glycosides of the juice beverage*

In the stevia sweetened beverages, four steviol glycosides were identified and analysed (rebaudioside A (reb A), stevioside (ste), rebaudioside F (reb F) and rebaudioside C (reb C)) in order to study the PEF effect on the sweetening properties of these beverages (Table 2). The major compound found in the samples containing a mixture of steviol glycosides was rebaudioside A followed by stevioside and in minor amounts, rebaudioside C and F. Rebaudioside A in untreated 1.25 and 2.5% stevia beverage was 233.6 and 273.8 mg/100 mL, respectively. Application of electric pulses resulted in mainly unchanged reb A, with the exception of 20 kV/cm for 360 μs and 30 kV/cm for 230 μs in the 2.5% stevia beverage, where reb A increased significantly compared to untreated samples. Although, to the best of our knowledge, no studies regarding PEF processing effect on steviol glycosides content have been carried out up to date, Duval, Grimi, and Vorobiev also observed an enhancement of steviol glycosides extraction yield from stevia after PEF pretreatment (20 kV/cm, 0.5-2 ms).

A similar behaviour was obtained for stevioside. Stevioside content in untreated beverages was of 114.0 and 129.3 mg/100 mL for beverages with 1.25 and 2.5% of stevia. Surprisingly, PEF treatment did not cause any significant change in the stevioside content in the 1.25% stevia beverage.

However, 20 kV/cm-360 μs and 30 kV/cm-230 μs caused a significant enhancement of the stevioside content in the 2.5% stevia beverage. Electroporation caused by PEF may facilitate stevioside extraction, but only at moderate conditions, which may be attributed to degradation of this compound at higher electric fields. These results are in agreement with the findings of Periche, Castello, Heredia, & Escriche (2015) who reported that extraction assisted by ultrasound treatment increased steviol glycosides as long as low temperature and short times were applied.

**Table 2.** Central composite response surface methodology design and response values for steviol glycosides concentration on HIPEF-treated fruit juice.

| Assay no.[a] | Electric field strength (kV/cm) | Time treatment (µs) | *Stevia* (%) (w/v) | Reb A (mg/100 mL) | Ste (mg/100 mL) | Reb F (mg/100 mL) | Reb C (mg/100 mL) |
|---|---|---|---|---|---|---|---|
| 1 | 40 | 100 | 2.50 | 281.6±4.7 | 133.4±1.9 | 14.8±0.3 | 39.5±2.2 |
| 2 | 40 | 100 | 0.00 | - | - | - | - |
| 3 | 20 | 360 | 2.50 | 347.2±28.1 | 169.2±5.5 | 15.3±1.2 | 36.9±0.8 |
| 4 | 20 | 230 | 1.25 | 230.5±4.0 | 111.2±0.7 | 7.2±0.3 | 24.1±0.4 |
| 5[b] | 30 | 230 | 1.25 | 236.8±8.4 | 112.6±8.1 | 7.6±0.7 | 29.3±0.6 |
| 6 | 40 | 360 | 2.50 | 272.7±2.1 | 127.9±2.5 | 16.0±1.4 | 40.0±1.1 |
| 7 | 40 | 360 | 0.00 | - | - | - | - |
| 8 | 20 | 100 | 0.00 | - | - | - | - |
| 9 | 20 | 360 | 0.00 | - | - | - | - |
| 10 | 40 | 230 | 1.25 | 227.9±1.7 | 108.8±0.7 | 9.4±0.5 | 30.9±1.1 |
| 11 | 30 | 230 | 2.50 | 336.2±4.8 | 155.1±3.9 | 14.2±0.3 | 36.3±2.9 |
| 12 | 30 | 230 | 0.00 | - | - | - | - |
| 13 | 30 | 100 | 1.25 | 228.5±3.6 | 113.9±0.5 | 7.1±0.4 | 27.1±0.7 |
| 14[b] | 30 | 230 | 1.25 | 240.4±10.6 | 116.2±5.0 | 7.5±0.4 | 29.4±0.4 |
| 15 | 20 | 100 | 2.50 | 272.5±9.8 | 128.3±2.7 | 12.0±0.5 | 29.6±1.1 |
| 16 | 30 | 360 | 1.25 | 225.0±5.8 | 108.1±2.6 | 9.2±0.5 | 29.0±0.9 |

Reb A: rebaudioside A, Ste. stevioside, Reb F: rebaudioside F, Reb C: rebaudioside.

[a] Order of assays was randomised.

[b] Central points.

Untreated 1.25 and 2.5% stevia beverages had a rebaudioside F content of 6.8 and 11.0 mg/100 mL, respectively. PEF treatment did not lead to significant changes in the 1.25% stevia beverage, but significantly higher values were obtained for PEF treated 2.5% stevia beverage. These differences may be attributed to the higher initial steviol glycosides content found in the 2.5% stevia beverage. Rebaudioside F may be product of the glycosylation of other steviol glycosides such as rebaudioside A and stevioside, although metabolism of these compounds is complex and not yet fully understood. Consequently, higher steviol glycosides content in the initial food product may stimulate the biosynthesis of rebaudioside F after high electric fields cause the electroporation and release of different enzymes involved in the steviol glycosides metabolism pathway.

Regarding rebaudioside C content, it was noteworthy that 1.25% stevia fruit juice had a 24.2 mg/100 mL content, while 2.5% stevia fruit juice had a 28.2 mg/100 mL content, not much higher. Interactions between the compounds present in the different ingredients of the beverage might take place masking their detection and also their biological action, making further studies evaluating steviol glycosides behaviour necessary. As observed for rebaudioside F content, PEF treatment did not result in any significant change in the 1.25% stevia fruit juice. Conversely, a significant increase in rebaudioside C content was obtained after PEF treatments, reaching the maximum (40.0 mg/100 mL) at 40 kV/cm, 360 µs. Once more, this increase may be not only due to the release of these compounds from inside of the cells after electric pulses are applied, but probably also because of the formation of rebaudioside C from dulcoside A through the glycosylation of C-3' of the C-13 glucose observed by Ceunen & Geuns (2013) because of the increase enzyme-substrate contact after the loss of compartmentalisation. Consequently, electric pulses application may favour certain steviol glycosides hydrolysis or glycosylation, altering their content in the different treated food products.

Furthermore, it is important to consider the relative amounts of the two main compounds found in the beverages, observing that the ratio between rebaudioside A and stevioside increased after PEF treatments. This is a very interesting finding, since the rebaudioside A/stevioside ratio represents a good

qualitative measurement of the sweetness (the higher the ratio, the better the taste) (Tavarini & Angelini, 2013). The maximum rebaudioside A/stevioside ratio was reached in the 2.5% stevia fruit juice treated at 30 kV/cm for 230 µs, which must be taken into account when examining the sweetening juice quality. The regression equation (Eq. (8)) describes the following model accurately enough ($R^2$=0.998):

$$\text{Reb A/ste ratio} = 2.467 \cdot \%stevia - 0.648 \cdot \%stevia^2 \qquad (8)$$

*3.3. Effect of PEF and stevia on physicochemical properties of the juice beverage*

ºBrix, hydroxymethylfurfural (HMF) and colour parameters (*a**, *b** and *L**) (Table 3, Figure 4) were used to test the effect of PEF in the physicochemical properties of fruit juices. As can be expected, among the studied parameters, stevia percentage had the greatest effect on the physicochemical parameters of the juice beverage. Overall, no statistically significant changes were observed in ºBrix values of the PEF beverages compared to the untreated beverage with 0 and 1.25% (w/v) stevia. PEF treatments with an electric field of 30 and 40 kV/cm produced a significant increase in ºBrix values in comparison with the unprocessed 2.5% (w/v) stevia beverage. A comparison of the results obtained by the response surface analysis showed that only stevia and electric field strength had a significant influence in the values of °Brix immediately after processing. A second-order response surface function fitted properly the experimental data with a determination coefficient ($R^2$) of 0.970 and a nonsignificant lack of fit. The following model (Eq. (9)) describes ºBrix of PEF treated beverages with stevia added:

$$\text{ºBrix} = 8.890 + 0.001 \cdot E - 0.421 \cdot \%\text{stevia} + 0.309 \cdot \%stevia^2 \qquad (9)$$

A positive correlation was found between ºBrix and rebaudioside A/stevioside ratio in the stevia sweetened fruit juices, confirming the use of this ratio as a measure of the sweetness quality.

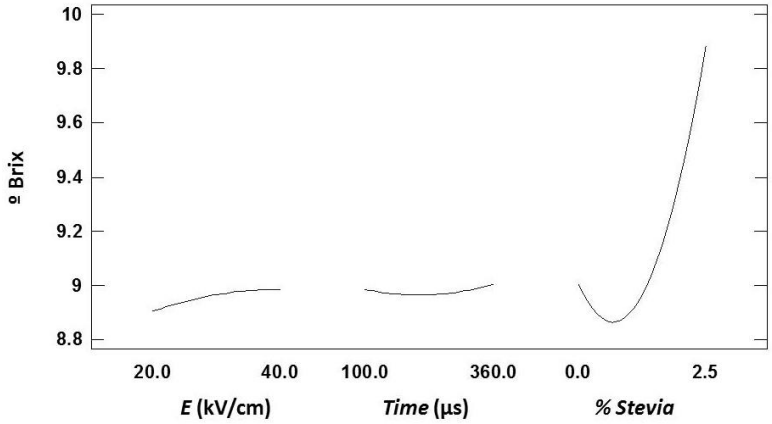


**Figure 4.** Effect of electric field (kV/cm), treatment time (µs) and stevia concentration (%, w/v) ºBrix values.

Quality loss of processed foods can also be determined by the hydroxymethylfurfural (HMF) content, which is one of the final products of non-enzymatic browning. Independently of the stevia concentration, higher electric fields led to significantly higher HMF value. However, HMF value in the presence of stevia was not as high as in the fruit juice without stevia, showing once more the protective effect of the stevia addition to the fruit juice. On the basis of experimental design presented in Table 1, the statistically significant regression equation ($p$<0.05) with $R^2$=0.915 which displays the influence of electric field, treatment time and stevia concentration on the HMF content in terms of actual values was as follows (Eq. (10)):

$$\text{HMF (mg/100 mL)} = 0.601 - 0.052 \cdot E + 0.001 \cdot t - 0.354 \cdot \%stevia + 0.002 \cdot E^2 + 0.098 \cdot \%stevia^2 \quad (10)$$

Colour parameters of stevia-fruit beverages as affected by different PEF treatment conditions are shown in Table 3. Lightness ($L^*$) is the most indicative parameter associated with fruit enzymatic browning (Chaikham, Apichartsrangkoon, & Seesuriyachan, 2014).

**Table 3.** Central composite response surface methodology design and response values for physicochemical parameters on HIPEF-treated fruit juice.

| Assay no.[a] | Electric field strength (kV/cm) | Time treatment (µs) | *Stevia* (%) (w/v) | HMF (mg/100 mL) | *L** | *a** | *b** | *ΔE* |
|---|---|---|---|---|---|---|---|---|
| 1 | 40 | 100 | 2.50 | 0.829±0.029 | 31.1±1.2 | 9.4±0.5 | 22.5±1.8 | 6.69±1.18 |
| 2 | 40 | 100 | 0.00 | 0.933±0.059 | 37.7±1.1 | 16.0±0.1 | 30.9±0.4 | 10.95±0.72 |
| 3 | 20 | 360 | 2.50 | 0.121±0.029 | 26.9±0.1 | 9.9±0.1 | 28.1±0.1 | 0.61±0.09 |
| 4 | 20 | 230 | 1.25 | 0.121±0.029 | 29.8±0.1 | 11.7±0.1 | 30.6±0.1 | 1.34±0.07 |
| 5[b] | 30 | 230 | 1.25 | 0.288±0.029 | 30.4±0.1 | 11.7±0.1 | 31.0±0.1 | 1.80±0.11 |
| 6 | 40 | 360 | 2.50 | 1.058±0.118 | 33.9±0.6 | 9.9±0.2 | 27.6±1.2 | 7.26±0.58 |
| 7 | 40 | 360 | 0.00 | 1.267±0.059 | 39.3±0.1 | 16.2±0.4 | 32.6±0.3 | 10.05±0.28 |
| 8 | 20 | 100 | 0.00 | 0.246±0.029 | 36.9±0.3 | 16.3±0.1 | 32.5±0.7 | 9.20±0.74 |
| 9 | 20 | 360 | 0.00 | 0.329±0.029 | 36.9±0.6 | 16.3±0.4 | 33.0±1.1 | 8.72±1.25 |
| 10 | 40 | 230 | 1.25 | 0.829±0.029 | 32.4±0.1 | 12.1±0.1 | 31.7±0.3 | 3.43±0.21 |
| 11 | 30 | 230 | 2.50 | 0.121±0.029 | 27.1±0.1 | 9.9±0.1 | 28.2±0.1 | 0.78±0.16 |
| 12 | 30 | 230 | 0.00 | 0.829±0.088 | 37.6±0.7 | 16.0±0.4 | 31.6±1.3 | 10.28±1.47 |
| 13 | 30 | 100 | 1.25 | 0.121±0.029 | 30.0±0.1 | 11.8±0.1 | 31.1±0.0 | 1.84±0.04 |
| 14[b] | 30 | 230 | 1.25 | 0.308±0.059 | 30.3±0.1 | 11.7±0.1 | 30.9±0.1 | 1.67±0.02 |
| 15 | 20 | 100 | 2.50 | 0.121±0.029 | 26.8±0.2 | 10.0±0.1 | 28.0±0.2 | 0.45±0.12 |
| 16 | 30 | 360 | 1.25 | 0.496±0.029 | 31.6±0.1 | 12.1±0.1 | 31.7±0.1 | 2.93±0.11 |

HMF: hydroxymethylfurfural, ΔE: total colour differences. Values are expressed as mean±SD of two treatment repetitions. Each assay was performed in duplicate.

[a] Order of the assays was randomised

[b] Central points

Initial *L** values of untreated beverages were 34.2±0.1, 30.0±0.1 and 26.7±0.1 for the 0, 1.25 and 2.5% stevia beverage, respectively, showing a significant decrease in *L** values with the addition of stevia to the beverage. The three way ANOVA showed that electric field strength, time and stevia concentration had a significant influence ($p$<0.05) in this parameter. In the beverage without stevia, higher *L** values were found for samples treated by PEF. However, in the 1.25 and 2.5% stevia beverages, only electric fields of 40 kV/cm caused a significant increase in *L** values.

Azhuvalappil, Fan, Geveke, & Zhang (2010) also reported a significant increase in luminosity after PEF processing apple juice and they attributed it to partial precipitation of insoluble suspended particles in the juice. Furthermore, (Bi et al., 2013) attributed the lightening tendency observed in a PEF-treated apple juice to the inactivation of enzymes responsible for browning. Thus, the maintenance of colour of the fruit juice-stevia beverage might be due to the inactivation of enzymes which catalyze phenolic compounds oxidation and cause enzymatic browning.

Additionally, *a** values were 17.9±0.1, 11.5±0.1 and 10.0±0.1 in the untreated fruit juice beverages with 0, 1.25 and 2.5% stevia beverage, respectively. Only treatment time and stevia percentage had a significant influence in this parameter, but not electric field strength. *a** values diminished when the fruit juice beverage without stevia was treated by PEF, independently of the electric field strength and time treatment applied, while the opposite trend was observed for fruit juice beverages sweetened with 1.25 and 2.5% stevia. In this case, PEF treated at high electric field strengths and time treatments resulted in an increase of *a** values. Regarding *b** values, these were of 41.1±0.1, 29.3±0.1 and 27.6±0.1 in the untreated fruit juice beverages sweetened with 0, 1.25 and 2.5% stevia, respectively. Electric field strength, time treatment and stevia percentage influenced significantly in this parameter. Yellowness was reduced in the PEF treated samples of the fruit juice beverage without stevia, while it increased significantly in the beverage with 1.25% stevia. However, no significant differences were observed after PEF treatments in the fruit juice beverage sweetened with 2.5% stevia. Finally, colour variations (ΔE) were greater in

beverages without stevia than in beverages with 1.25 and 2.5% stevia. Colour modifications may be due to the breakage of cellular membranes which would cause a loss of functional cell compartmentalisation, increasing enzyme-substrate contact with the consequent increase in tissue browning, also related with results obtained for *L** parameter. However, stevia addition may cause inactivation of enzymes and therefore prevent the enzyme-substrate contact. Eq. (11) allowed the prediction of the effects of independent variables on the total colour differences ($R^2$=0.939):

$$\Delta E = 8.183 + 0.053 \cdot E - 12.135 \cdot \%stevia + 0.092 \cdot E \cdot \%stevia + 2.664 \cdot \%stevia^2 \quad (11)$$

It is noteworthy observing that changes in colour were significantly correlated with HMF values ($R^2$=0.947), also related to changes in food products colour, and with ascorbic acid content ($R^2$=0.875), whereas a negative correlation was found between changes of colour and steviol glycosides content (rebaudioside A, stevioside, rebaudioside F and rebaudiosice C).

### *3.4. Processing parameter optimisation based on their effect on the quality of the formulated beverage*

For multi-response optimisation, critical PEF parameters which maximised bioactive compounds (ascorbic acid, total anthocyanins and total carotenoids) and sweetening properties (rebaudioside A/stevioside ratio and ºBrix) while minimising sensorial changes (HMF and total colour differences) were determined. The same priority was assigned to each independent variable in order to obtain a beverage with the maximal nutritional and sensorial quality, being the optimal conditions 21 kV/cm of pulsed electric field for 360 µs with 2.5% of stevia. At this conditions, the greatest retention of bioactive compounds as well as physicochemical properties were achieved, with an overall score of 0.774.

## 4. Conclusions

When applying a processing technology to a food product, special attention must be paid to its nutritional and sensorial quality in terms of bioactive compounds content along with the sweetening properties and colour, crucial for

consumer's acceptance. Application of the experimental design permitted to investigate the optimum amounts of stevia along with processing PEF parameters in order to obtain a fruit juice beverage with the minimal quality and nutritional loss after PEF processing, enhancing its content in bioactive compounds and sweetening properties while minimising changes in colour.

## 5. Acknowledgements

This research project was supported by the Spanish Ministry of Science and Technology and European Regional Development Funds (AGL2010-22206-C02-01). Carbonell-Capella, J.M. thanks the F.P.U. grant (No. AP2010-2546) provided by the Spanish Ministry of Education.

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## 3.8. Changes of antioxidant compounds in a fruit juice-*Stevia rebaudiana* blend processed by pulsed electric technologies and ultrasound

*Food and Bioprocess Technology (2016) DOI: 10.1007/s11947-016-1706-1*

# Changes of antioxidant compounds in a fruit juice-*Stevia rebaudiana* blend processed by pulsed electric technologies and ultrasound

Juana M. Carbonell-Capella[1], Magdalena Buniowska[1], Francisco J. Barba[2], Nabil Grimi[2], Eugène Vorobiev[2], María J. Esteve[1], Ana Frígola[1*]

[1]University of Valencia, Department of Nutrition and Food Science, Av. Vicent Andrés Estellés, s/n, 46100 Burjassot, Spain

[2]Université de Technologie de Compiègne, Laboratoire de Transformations Intégrées de la Matière Renouvelable, EA 4297, Centre de Recherches de Royallieu, BP 20529, 60205 Compiègne Cedex, France

*Corresponding author:
Ana Frígola
e-mail: ana.frigola@uv.es.
Phone: +34 963544955 Fax: +34 963544954

**ABSTRACT**

The purpose of this study was to compare the effects of the non-thermal technologies of pulsed electric fields (PEF), high voltage electrical discharges (HVED) and ultrasounds (USN) on bioactive compounds (ascorbic acid, total carotenoids, total phenolic compounds and total anthocyanins) and antioxidant capacity of a fruit juice (papaya and mango) blend sweetened with *Stevia rebaudiana*. Experiments were carried out at two equivalent energy inputs (32-256 kJ/kg) for each technology. Principal Component Analysis (PCA) was used to understand the contribution of ascorbic acid, total carotenoids and ORAC (oxygen radical absorbance capacity) values. These parameters were better retained with PEF treatments. Nevertheless, the use of HVED and USN technologies cannot be ruled out, as they may enhance the contents of other bioactive compounds such as total phenolic compounds when HVED technology is applied at an energy input of 256 kJ/kg. The obtained data can contribute to the determination of optimum processing conditions for production of high nutritional quality liquid foods.

**Keywords:** Exotic fruit juices · Pulsed electric fields · High voltage electrical discharges · Ultrasounds · Bioactive compounds · Antioxidant capacities.

**Introduction**

In the last two decades, a growing demand for exotic fruits has been observed. Among these, mango and papaya are some of the most important, now ranked second and third of the total tropical fruit production, only behind banana production (Evans and Ballen 2012). Moreover, consumption of several mango- and papaya-derived products such as juices, purées, fresh-cut mango slices has increased in response to consumer´s demand for highly nutritious healthy foods (Rawson et al. 2011a). These products contain a large amount of bioactive compounds, including ascorbic acid, phenolic compounds and carotenoids, that have shown to be good contributors to the total antioxidant capacity of foods (Zulueta et al. 2009; Vijaya et al. 2010) and have been associated with a reduced risk of degenerative diseases such as cancer and coronary heart disease (Abuajah et al. 2014; Pistollato and Battino 2014).

Although thermal treatments have been traditionally used in the preservation of liquid foods due to their ability to inactivate microorganisms and spoilage enzymes, several chemical and physical changes may take place, especially when high temperatures (>100 ºC) are used, impairing organoleptic properties and reducing bioactive compounds content. Consequently, new processing technologies which can avoid microbial contamination and allow the obtainment of high-quality food with “fresh-like” characteristics and improved functionalities are required (Rawson et al. 2011a). In this line, the use of electrotechnologies, such as pulsed electric fields (PEF), high voltage electrical discharges (HVED) and ultrasound (USN) processing, have been shown to be promising for liquid food preservation (Toepfl et al. 2006, 2007; Barba et al. 2012; Zulueta et al. 2013). Additionally, PEF and USN treatment can be combined with conventional preservative techniques, such as the use of green herbs with antimicrobial activity in order to enhance the lethal or inhibitory effect of these technologies on microorganisms (Ross et al. 2003) as well as their preservation of nutritional properties (Wang et al. 2008; Soria and Villamiel 2010; Boussetta and Vorobiev 2014). Such combinations enhance food preservation at lower individual treatment intensities (Ross et al. 2003). Moreover, HVED, which is a pulsed

electric-based technology, has also the potential to be used for food preservation. However, the formation of reactive species (e.g. ozone) generated during the discharges makes necessary deeper studies about the effect of this technology in the nutritional properties of foods (Sarkis et al. 2015).

In two previous studies, Carbonell-Capella et al. (2013) and Barba et al. (2014) combined a non-thermal technology (high pressure processing) with a natural antimicrobial Stevia rebaudiana Bertoni (Siddique et al. 2014), obtaining interesting results regarding Listeria monocytogenes, polyphenol oxidase (PPO) and peroxidase (POD) inactivation. Moreover, a significant increase of bioactive compounds and antioxidant capacity was found. However, there is a need to study if other mild preservation technologies such as PEF, HVED and USN have the same positive effect regarding antioxidant compounds.

This manuscript discusses the effect of pulsed electric fields, high voltage electrical discharges and ultrasound technology on bioactive compounds retention and antioxidant capacity of a fruit juice blend based on mango and papaya sweetened with Stevia rebaudiana. Stevia 2.5% (w/v) was selected as a low-calorie sweetener with antioxidant potential based on a previous study (Barba et al. 2014). Equivalent energy inputs were applied in order to compare the different technologies between them and ascorbic acid, total carotenoids, total phenolic compounds, total anthocyanins and antioxidant capacity were investigated.

## Materials and Methods

### *Sample Preparation*

Mango (*Mangifera indica*), and papaya (*Carica papaya*) were purchased from a local supermarket (Valencia, Spain) at commercial maturity stage. Mango and papaya juices were extracted after appropriate washing of the fruits and filtered by means of a mesh sieve (pore size: 0.297 mm).

Dried *Stevia rebaudiana* leaves were supplied by Anagalide S. A. (Spain) and stored at room temperature. A stock solution of 8.33±0.01% (w/v) was prepared according to (Carbonell-Capella et al. 2015). 100 hundred mL of boiling distilled

water were added to the dried leaves (8.33 g) and the mixture was covered and allowed to infuse for 30 min at 100 ºC. The infusion was vacuum filtered using a Kitasato flask, a Büchner funnel, a vacuum pump (VDE 0530, KNF Neuberger GmbH, Germany) and filter paper (Whatman® No. 1, Whatman International Ltd, UK) and the filtrate obtained was stored at 40 ºC.

The fruit juice blend was prepared by mixing 50.8% (v/v) of papaya juice, 19.3% (v/v) of mango juice and completing volume up to 100% with stevia stock infusion, with a final stevia concentration of 2.5% (w/v). The final stevia concentration (2.5%) was selected according to a previous study (Belda-Galbis et al. 2014) which showed the highest antimicrobial activity at this concentration against *Listeria innocua,* a pathogen of great concern in minimally processed beverages because of its ubiquitous, psychotropic nature, and because of its ability to grow in acidic environments.

*Chemicals*

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylicacid), ABTS (2,2'-azinobis(3-ethylbenzothiazoline 6-sulfonate)), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, fluorescein sodium salt and sodium metabisulfite were purchased from Sigma-Aldrich (Steinheim, Germany). Gallic acid was purchased from UCB (Brussels, Belgium). Hexane (LC grade) and potassium dihydrogen phosphate ($KH_2PO_4$) were purchased from Scharlau (Barcelona, Spain). Oxalic acid, acetic acid, sodium acetate, potassium persulphate ($K_2S_2O_8$), sodium and disodium phosphate and 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) were purchased from Panreac (Barcelona, Spain). Ethanol, methanol, hydrochloric acid, sodium chloride (special grade), sodium carbonate anhydrous ($Na_2CO_3$), trichloroacetic acid and sodium sulphate proceeded from Baker (Deventer, The Netherlands). L(+)-ascorbic acid was obtained from Merck (Darmstadt, Germany).

*PEF, HVED and USN Treatments*

Electrical treatments in PEF and HVED modes were done using a high voltage pulsed power 40 kV–10 kA generator (Tomsk Polytechnic University, Tomsk,

Russia). The initial temperature before PEF or HVED treatments was ≈20 °C and the temperature elevation after electrical treatment never exceeded 35 °C. Beverage temperature was controlled by a K-type thermocouple (±0.1 ºC) connected to a data logger thermometer Centre 305/306 (JDC Electronic SA, Yverdon-les-Bains, Switzerland). PEF treatments were carried out in a cylindrical batch treatment chamber between two plate electrodes of 9.5 $cm^2$. The distance between electrodes was fixed to 2 cm with a corresponding electric field strength, *E,* of 25 kV/cm. Total treatment duration, $t_t$ ($t_t$=$n \times t_i$), was changed by increasing the number of pulses, *n,* from 50 to 400. Time delay between sequential pulses was of $\Delta t$=2 s. Exponential decay of voltage, $U_\infty$ exp (-$t/t_i$), with effective decay time, $t_i$≈10.0±0.1 μs, was observed. Total specific energy input (*W*, kJ/kg) was chosen as a parameter to describe the treatment intensity. Two energy inputs were applied, which corresponded to samples $PEF_1$ (32 kJ/kg) and $PEF_2$ (256 kJ/kg). The energy input (*W*) of PEF treatment was calculated as shown in Eq. (1).

$$W = \frac{\sum_{i=1}^{n} W_{PEF}}{m} \quad (1)$$

where $W_{PEF}$ is the pulse energy (kJ/pulse), *n* is the number of pulses and *m* is the product mass (kg). $W_{PEF}$ was determined from Eq. (2).

$$W_{PEF} = \int_0^t UIdt \quad (2)$$

where *U* is the voltage (V) and *I* is the current strength (A).

For HVED treatments, the 1-L treatment chamber (inner diameter=10 cm, wall thickness=2.5 cm) was equipped with needle-plate geometry electrodes. The diameters of stainless steel needle and the grounded disk electrodes were of 10 and 35 mm, respectively. The distance between the electrodes was of 5 mm. Energy was stored in a set of low-inductance capacitors, which were charged by the high-voltage power supply. Electrical discharges were generated by electrical breakdown in water with a peak pulse voltage (*U*) of 40 kV. Damped oscillations were thus obtained over a total duration $t_i$ of ≈10 μs. The voltage (Ross VD45-8.3-A-K-A, Ross Engineering Corp., Campbell, California, USA) and current (Pearson 3972, Pearson Electronics Inc., Campbell, California, USA) measurement units

were connected with a 108 Hz sampling system via an oscilloscope (Tektronix TDS1002, Beaverton, Oregon, USA). The software HPVEE 4.01 (Hewlett-Packard, Palo Alto, USA) was used for data acquisition. The energy input of HVED treatment was calculated as shown in Eqs. 1 and 2, where $W_{PEF}$ was substituted by $W_{HVED}$. Total treatment duration ($t_t$=$n \times t_i$) was changed by increasing the discharge number $n$ from 50 to 400. The discharge pulse duration $t_{HVED}$ was approximately 10 µs. The discharges were applied with a repetition rate of 0.5 Hz, which was imposed by the generator. In order to compare HVED treatments with PEF, equivalent energy inputs were used, obtaining sample $HVED_1$ (32 kJ/kg) and $HVED_2$ (256 kJ/kg).

For ultrasounds (USN) treatments, an ultrasonic processor UP 400S (Hielscher GmbH, Germany) which operates at 400 W and a frequency of 24 kHz was used. Amplitude, which could be adjusted from 20% to 100%, was set at 100%. The instrument can be used in cycle mode (0 ~ 1), where a cycle setting of 1 means that the solution is sonicated without interruption whereas with a cycle setting, for example, of 0.5 the solution is sonicated for 0.5 s and then sonication stops for 0.5 s. Hence, in cycle mode, the ratio of sound-emission time to cyclic pause time can be adjusted continuously from 0% to 100% per second. In the present study, cycle was fixed at 1. The titanium sonotrode H14 with a diameter of 14 mm and a length of 100 mm was used to transmit ultrasound inside the sample. The sample was submerged in a cooling bath to avoid the heating induced by USN irradiation. The energy input of USN treatment was calculated as follows (Eq. (3)):

$$W_{USN} = \frac{POWER\ x\ t_{USN}}{m} \qquad (3)$$

where $t_{USN}$ is the total treatment duration (s), $m$ is the product mass (kg) and the generator power (400 J/s). In order to obtain equivalent energy inputs to the other treatments applied, total treatment duration was of 20 and 160 s, obtaining $USN_1$ (32kJ/kg) and $USN_2$ (256 kJ/kg) samples.

*Polarographic Determination of Ascorbic Acid*

The beverage (5 mL) was diluted to 25 ml with the extraction solution (oxalic acid 1%, w/v, trichloroacetic acid 2%, w/v, sodium sulphate 1%, w/v). After

constant shaking at 1000 rpm during 30 s in a vortex (VV3, VWR International, Spain), the solution was filtered through a folded filter (Whatman® No. 1). Oxalic acid (9.5 ml) 1% (w/v) and 2 ml of acetic acid/ sodium acetate 2 M buffer (pH=4.8) were added to an aliquot of 0.5 ml of filtrate and the solution was transferred to the polarographic cell. A Metrohm 746 VA Trace Analyser (Herisau, Switzerland) equipped with a Metrohm 747 VA stand was used for the polarographic determination (Carbonell-Capella et al. 2013).

*Total Carotenoids*

Extraction of total carotenoids was carried out in accordance with Lee and Castle (2001). 5 mL of extracting solvent (hexane/acetone/ethanol, 50:25:25, v/v) was added to an aliquot of sample (2 mL) and centrifuged for 5 min at 3220 g at 5 ºC. The top layer of hexane containing the colour was recovered and transferred to a 25-mL volumetric flask. The volume of recovered hexane was then adjusted to 25 mL with hexane. Total carotenoid determination was carried out on an aliquot of the hexane extract by measuring the absorbance at 450 nm. Total carotenoids were calculated according to Ritter and Purcell (1981) using an extinction coefficient of *β*-carotene, $E^{1\%}$=2505.

*Total Phenolic Compounds*

Total phenols were determined according to the method reported by Georgé et al. (2005), with some modifications. Briefly, 50 mL of a mixture of acetone/water (7/3, v/v) were added to 10 mL of sample and centrifuged for 30 min at 3220 g. Mixture supernatants were then recovered by filtration (Whatman® No. 2, England) and constituted the raw extracts (REs). REs (2 mL) were settled on an Oasis cartridge (Waters). Interfering water-soluble components (reducing sugars, ascorbic acid) were recovered with 2 x 2 mL of distilled water. The recovered volume of the washing extract (WE) was carefully measured. In order to eliminate vitamin C, heating was carried out on the washing extract (3 mL) for 2 h at 85 °C and led to the heated washing extract (HWE). All extracts (RE, WE, and HWE) were submitted to the Folin-Ciocalteu method, adapted and optimised (Barba et al. 2014). Gallic acid calibration standards with concentrations of 0, 100,

300, 500, 700 and 1000 ppm were prepared and 0.1 mL was transferred to borosilicate tubes. 3 mL of 2% (w/v) sodium carbonate solution and 100 µL of Folin–Ciocalteau reagent (1:1, v/v) were added to 100 µL of all gallic acid standard and sample tubes. The mixture was incubated for 1 h at room temperature. Absorbance was measured at 765 nm.

*Total Anthocyanins*

Total anthocyanins were determined using a modified method of Mazza et al. (1999). A 10-fold diluted sample of 100 µL was mixed with 1700 µL of distilled water and 200 µL of 5% (v/v) HCl. The sample was hold at room temperature for 20 min before measuring the absorbance at 520 nm. Calculations of total anthocyanins were based on cyanidin-3-glucoside (molar absorptivity 26900 L/mol·cm). All spectrophotometric analyses were performed using a UV–visible spectrophotometer Lambda 20 (Perkin-Elmer, Überlingen, Germany).

*Total Antioxidant Capacity*

TEAC assay: TEAC (Trolox equivalent antioxidant capacity) was measured using the method of Re et al. (1999) based on application of ABTS decolourisation Assay (Sigma-Aldrich, Steinheim, Germany). The ABTS radical ($ABTS^{\bullet+}$) was generated using 440 µL of potassium persulfate (140 mM). The solution was diluted with ethanol (Baker, Deventer, The Netherlands) until an absorbance of 0.70 was reached at 734 nm. Once the radical was formed, 2 mL of $ABTS^{\bullet+}$ was mixed in a vortex (VV3, VWR International, Spain) for 30 s with 100 µL of extract and the sample was incubated for 60 min at 20 ºC. Absorbance, *A,* was measured at the wavelength of 734 nm (Carbonell-Capella et al. 2013) and percentage of inhibition (% I) was calculated using the following formula (Eq. (4)):

$$\% \, \mathrm{I} = \left(\frac{1 - \text{final absorbance}}{\text{Initial absorbance}}\right) \mathrm{x}100 \qquad (4)$$

Results were expressed as Trolox-equivalent values (mM TE) using a standard calibration curve of Trolox in the range of 50-250 µM.

ORAC assay: The oxygen radical absorbance capacity (ORAC) assay used, with fluorescein as the “fluorescent probe”, was that described by Barba et al. (2014).

The automated ORAC assay was carried out on a Wallac 1420 VICTOR$^2$ multilabel counter (Perkin-Elmer, USA) with fluorescence filters, for an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The measurements were made in plates with 96 white flat bottom wells (Sero-Wel, BibbySterilin Ltd., Stone, UK). The reaction was performed at 37 °C, as the reaction was started by thermal decomposition of AAPH in 75 mM phosphate buffer (pH 7.0). Calculations were done using Eq. (5) and results were expressed as mM TE (mM Trolox Equivalent):

$$\text{ORAC (mM TE)} = \frac{C_{TROLOX} \text{ x } (AUC_{SAMPLE} - AUC_{WHITE}) \text{x K}}{(AUC_{TROLOX} - AUC_{WHITE}) \text{x} 1000} \quad (5)$$

where $C_{TROLOX}$ is 20 µM, *AUC* is the area under the curve and *K* is the dilution factor.

DPPH assay: Antioxidant capacity was also measured following the method described by Brand-Williams, Cuvelier, & Berset (1995). The reaction was begun by adding 50 µL of a suitable dilution of sample to 1.45 mL of DPPH (2,2-diphenyl-1-picrylhydrazyl) coloured radical. The sample was incubated for 30 min at room temperature (T=20 ºC). Absorbance was measured at the wavelength of 515 nm and percentage of inhibition (% I) was calculated using the following formula (Eq. (6)):

$$\% \text{ I} = \left(\frac{1 - \text{final absorbance}}{\text{Initial absorbance}}\right) \text{x} 100 \quad (6)$$

Results were expressed as Trolox-equivalent values (mM TE) using a standard calibration curve of Trolox in the range of 0.05-1 mM.

*Statistical Analysis*

All determinations were performed in triplicate. One-way analysis of variance and Pearson test were used for statistical analysis of the data using Statgraphics® Centurion XVI (Statpoint Technologies Inc., USA) (differences at $p<0.05$ were considered significant). A principal component analysis based on the correlation matrix was performed to assess differences among the different treatments. Response variables were autoscaled prior to chemometrics application, in accordance to Granato et al. (2015).

**Results and Discussion**

*Effect of PEF, HVED and USN Treatment on Bioactive Compounds*

To establish the effect of the non-thermal technologies of PEF, HVED and USN, two equivalent energy inputs (32 and 256 kJ/kg) were applied to a fruit juice blend (papaya and mango) sweetened with *Stevia rebaudiana* Bertoni, and in all cases results were compared with the untreated beverage. Ascorbic acid, total carotenoids, total phenolic compounds, anthocyanins and total antioxidant capacity measured by TEAC, ORAC and DPPH method were determined.

**Table 1.** Bioactive compounds and antioxidant capacity of untreated fruit juice blend sweetened with *Stevia rebaudiana.*

| Parameters | Fruit juice-stevia blend |
|---|---|
| Ascorbic acid (mg/100 mL) | 18.5±0.4 |
| Total carotenoids (μg/100 mL) | 676.1±3.5 |
| Total phenolics (mg/L) | 2685.6±18.4 |
| Total Anthocyanins (mg/L) | 11.8±1.4 |
| TEAC (mM TE) | 23.9±0.9 |
| ORAC (mM TE) | 30.8±0.9 |
| DPPH (mM TE) | 24.3±0.5 |

The ascorbic acid content in the fruit juice blend sweetened with stevia was 18.5±0.4 mg/100 mL (Table 1). These results are in close agreement with values of ascorbic acid obtained by Murillo et al. (2012) in mango and papaya. Immediately after treatment by PEF, HVED and USN, ascorbic acid retention was of 17-91%. USN was the processing technique that better retained the ascorbic acid content of the fruit juice-stevia blend (84-91%), followed by PEF (80-83%). However, after HVED treatment, ascorbic acid retention decreased to 17-23%. This fact can be attributed to the formation of gaseous cavitation bubbles, as well as the emission of shock waves of high pressure and of high intensity UV light when electrical discharges are applied in liquids (Boussetta and Vorobiev 2014). In any case, higher energy inputs caused higher ascorbic acid losses, independently of the treatment applied, only significant in the case of HVED treatment (Figure 1), which

may be caused by the increase of temperature at high energy levels, causing the degradation of this thermolabile vitamin. Nevertheless, our results were in accordance to those found by other previous studies which have reported the feasibility of USN and PEF to preserve ascorbic acid when they are used for liquid food preservation (Barba et al. 2012; Tiwari et al. 2008a; Tiwari et al. 2008b; Zulueta et al. 2013).

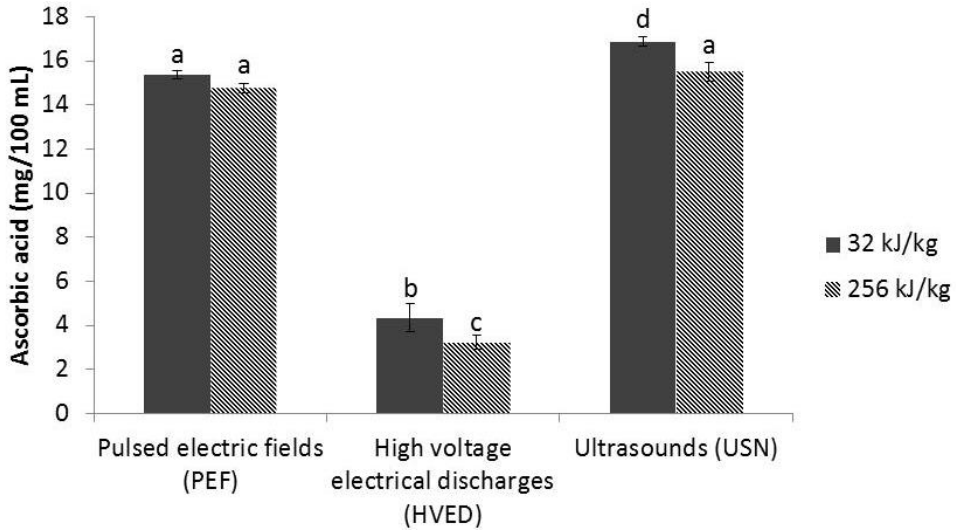


**Figure 1.** Ascorbic acid of a fruit juice blend sweetened with *Stevia rebaudiana* treated by PEF, HVED and USN processing. [a–d] Different letters indicate a significant difference in function of the samples analysed ($p$<0.05).

Regarding total carotenoids, untreated sample exhibited a yield of 676.1±3.5 µg/100 mL, higher than in a previous study of a mango, papaya and orange beverage sweetened with stevia (Carbonell-Capella et al. 2013). After $PEF_1$ processing, total carotenoids were significantly higher ($p$<0.05), with values of 800.9±3.5 µg/100 mL (Figure 2). This may be due to carotenoids being released, or leaching of other minerals or solid substances into the juice as a result of the pulsed electric fields (Roohinejad et al. 2014).

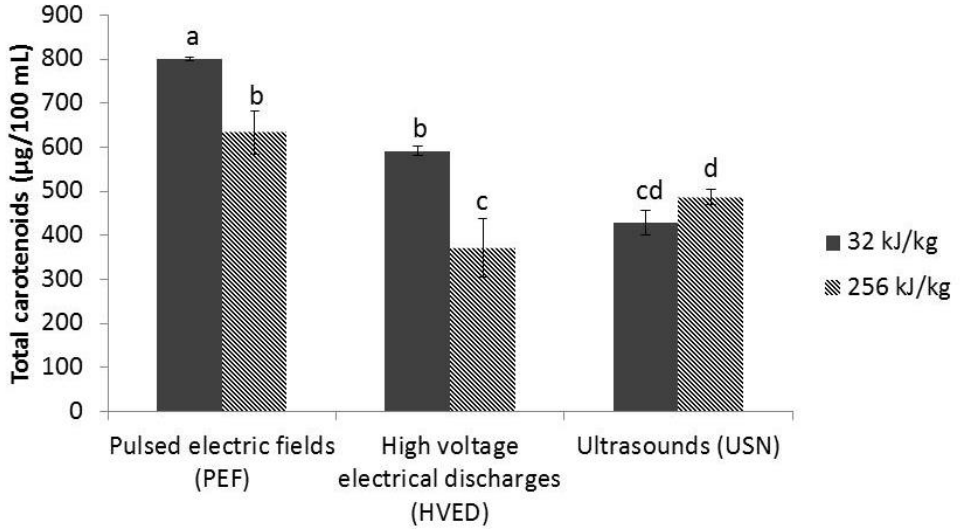


**Figure 2.** Total carotenoids of a fruit juice blend sweetened with *Stevia rebaudiana* treated by PEF, HVED and USN processing. [a–d] Different letters indicate a significant difference in function of the samples analysed ($p$<0.05).

In this line, Torregrosa et al. (2005) found that the application of various PEF treatments in orange–carrot juice produces a significant increase in carotenoid concentrations as treatment time increases. Moreover, Zulueta et al. (2010) also found a slight increase in the concentration of the extracted carotenoids when they applied PEF (25 kV/cm, 80 µs) in an orange juice-milk beverage. However, after $PEF_2$ treatment at an energy input of 256 kJ/kg, total carotenoid diminished significantly ($p$<0.05).

Behaviour of carotenoids after pulsed electric treatments is complex and although other authors have also seen that at higher field intensities, carotenoid content is reduced, this is not yet well understood. For instance, Torregrosa et al. (2005) applied PEF treatment at different field intensities (25, 30, 35 and 40 kV/cm) and only with electric fields of 25 and 30 kV/cm, vitamin A content was higher than in the pasteurised juice. When using HVED technology, total carotenoids decreased, possibly because of the formation of reactive species generated during the treatment. In this case, $HVED_2$ treatment led to the beverage with the lowest content in total carotenoids (46%). Boussetta et al. (2011) also observed a negative effect of HVED in antioxidant compounds above an energy

value of 80 kJ/kg. Carotenoids content after ultrasounds processing was of 429.1-486.5 µg/100 mL. Differently from other technologies, higher treatment time of ultrasounds led to a higher carotenoid content. A possible explanation for this increase may be: 1) the ability of ultrasounds to enhance disruption of cell walls, which might have facilitated the release of bound carotenoid contents or 2) the rupture of carotenoid-proteins binding, thus facilitating the extractability of carotenoids. These results are in close agreement to those obtained by Abid et al. (2014), who found a significant improvement in carotenoid content of USN-treated apple juices and Rawson et al. (2011b), who found a slight increase in lycopene content in USN-treated watermelon juice at low amplitude level.

The contents of total phenolic compounds (TPC) in the untreated fruit juice-stevia blend were 2685.6±3.5 mg/L, in accord with literature data in an exotic fruit-oat beverage with stevia (Carbonell-Capella et al. 2015). Phenolic concentration after $PEF_2$ and $HVED_2$ treatments were significantly higher than that of control (Figure 3), which indicated that extractability of TPC may be increased by the release of solutes into the solvent because of the irreversible pores in cell membranes caused by the use of electrical fields.

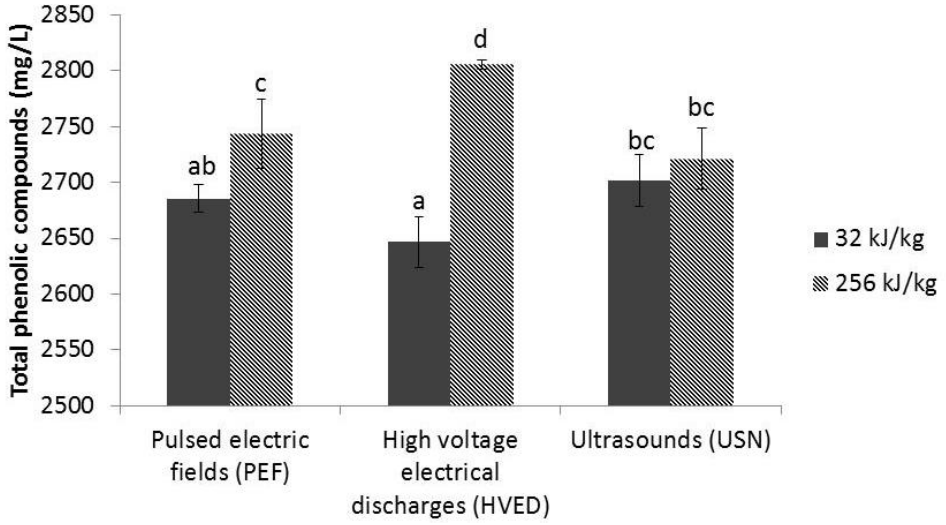


**Figure 3.** Total phenolic compounds of a fruit juice blend sweetened with *Stevia rebaudiana* treated by PEF, HVED and USN processing. [a–d] Different letters indicate a significant difference in function of the samples analysed ($p$<0.05).

In support to these results, Hsieh and Ko (2008) obtained an increase in the amount of TPC when carrot juice was treated at 100 kV/m with high-voltage electrostatic field. On the other hand, although there is no clear trend regarding TPC behaviour after PEF, Morales-de la Peña et al. (2010) demonstrated the ability of PEF to increase the concentration of TPC in fruit juice–soymilk beverages (35 kV/cm, 800-1400 μs). These authors attributed this phenomenon to biochemical reactions during the PEF processing, which led to the formation of new phenolic compounds; significant effects on cell membranes or in phenolic complexes with other compounds, releasing some free phenolic compounds after PEF processing and a possible inactivation of PPO after PEF treatment, preventing further loss of phenolic compounds. Nevertheless, after $PEF_1$ and $HVED_1$ treatments, non-significant changes ($p$>0.05) in total phenolic compounds were found, confirming results obtained by Chen et al. (2014) in blueberry juice. After the fruit juice blend sweetened with stevia was treated by USN technology, non-significant differences were obtained when compared with untreated fruit juice-stevia, independently of the energy input applied. Results are in accord with those obtained by Martínez-Flores et al. (2014), who did not find significant differences in phenolic compounds between control and thermo-sonicated carrot juice.

In the untreated fruit juice-stevia blend, the concentration of total anthocyanins was 11.8±1.4 mg/L, which can be compared with previous results in an exotic fruit-oat beverage sweetened with stevia (Carbonell-Capella et al. 2015). A high retention of anthocyanin content was obtained immediately after PEF, HVED and USN treatments (94-110%). Total anthocyanins increased after applying PEF, HVED and USN treatments at an energy input of 256 kJ/kg with respect to fruit juice-stevia blends treated at an energy input of 32 kJ/kg, although differences were not significant ($p$>0.05) (Figure 4). Results indicate a high stability of anthocyanins to processing conditions. This was also observed in a study carried out by Guo et al. (2014), where PEF processed and unprocessed pomegranate juice had similar concentrations of total anthocyanins. Furthermore, Pérez-Ramírez et al. (2015) found that stevia addition decreased the degradation rate of anthocyanins in a roselle beverage. By contrast, Barba et al. (2012) did observe a statistically significant increase of total anthocyanin immediately after

blueberry juice was treated with pulsed electric fields at 36 kV/cm during 100 µs. Odriozola-Serrano et al. (2009) also reported greater anthocyanin retention in strawberry juice during PEF treatment in bipolar pulses mode compared to monopolar mode, showing that anthocyanin retention during PEF processing is influenced by polarity, treatment time and frequency employed. Meanwhile, total anthocyanins did not undergo any change when apple juice was sonicated at different treatment times in the study carried out by Abid et al. (2014). Similarly, significant retention of anthocyanin content (>94%) was observed by Tiwari et al. (2009) in blackberry juice at the same amplitude conditions of our study although with a treatment duration of 10 min, indicating stability of anthocyanin during sonication.

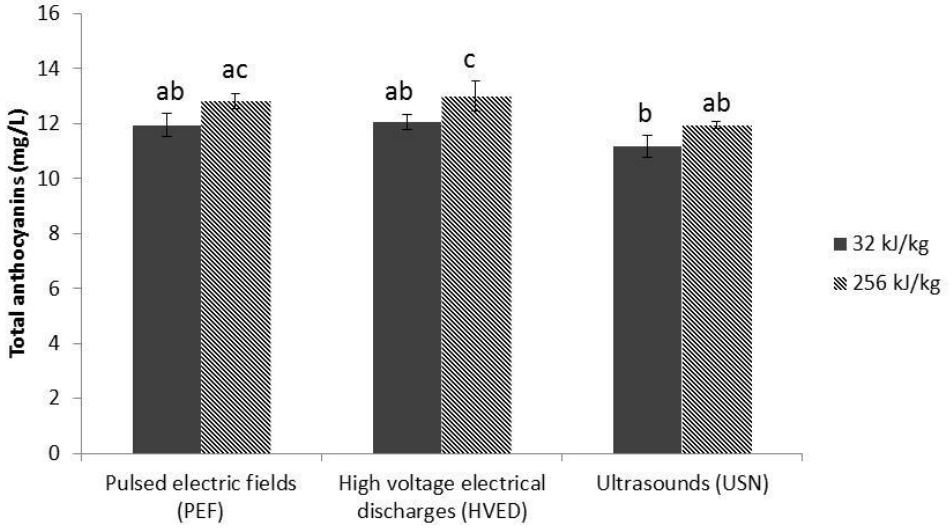


**Figure 4.** Total anthocyanins of a fruit juice blend sweetened with *Stevia rebaudiana* treated by PEF, HVED and USN processing. [a–c] Different letters indicate a significant difference in function of the samples analysed ($p$<0.05).

*Effect of PEF, HVED and USN Treatment on Total Antioxidant Capacity*

Total antioxidant capacity (TAC) measured as TEAC and DPPH values in the untreated beverage were 23.9±0.9 mM TE and 24.3±0.5 mM TE, respectively, in the range of previous studies in mixture of fruits with stevia (Criado et al. 2014). A

good ABTS scavenging was already cited for a fruit juice mixture (mango, papaya and orange) sweetened with stevia *rebaudiana* (Carbonell-Capella et al. 2013).

**Table 2.** Effect of PEF, HVED and USN on total antioxidant capacity (mM TE) of a fruit juice blend sweetened with *Stevia rebaudiana.*

| | TEAC | ORAC | DPPH |
|---|---|---|---|
| **Untreated** | 23.92±0.95$^{a}$ | 30.78±0.93$^{ab}$ | 24.27±0.55$^{ab}$ |
| **$PEF_1$** | 23.64±0.18$^{a}$ | 37.44±1.90$^{c}$ | 24.35±0.40$^{ab}$ |
| **$PEF_2$** | 25.63±0.33$^{ab}$ | 36.79±1.45$^{c}$ | 25.55±0.75$^{ac}$ |
| **$HVED_1$** | 23.73±1.67$^{a}$ | 29.06±1.32$^{a}$ | 24.45±0.75$^{ab}$ |
| **$HVED_2$** | 26.42±0.55$^{b}$ | 31.65±1.28$^{b}$ | 26.06±0.03$^{c}$ |
| **$USN_1$** | 24.92±0.31$^{ab}$ | 35.82±0.12$^{c}$ | 23.30±0.75$^{b}$ |
| **$USN_2$** | 25.53±0.67$^{ab}$ | 37.12±0.14$^{c}$ | 24.72±0.42$^{abc}$ |

PEF: pulsed electric fields. HVED: high voltage electrical discharges. USN: ultrasounds. TEAC: trolox equivalent antioxidant capacity. ORAC: oxygen radical antioxidant capacity. DPPH: 2,2-diphenyl-1-picrylhydrazyl. $_1$: 32 kJ/kg. $_2$: 256 kJ/kg. $^{a–c}$ Different letters in the same column indicate a significant difference in function of the samples analysed ($p$<0.05)

As shown in Table 2, the increase in the ABTS and DPPH scavenging capacity of the fruit juice-stevia blend was only significant in HVED treated samples. This may be due to the ability of these techniques to enhance disruption of cell walls and thus facilitate the release of antioxidant compounds, increasing total antioxidant capacity measured with these methods. A positive impact of HVED on TEAC and DPPH values was observed in fruit juices-stevia blends treated at an energy input of 256 kJ/kg, while non-significant changes ($p$>0.05) were found immediately after PEF and USN treatment with regard to the control sample. Grimi et al. (2014) observed that HVED provided a more powerful disintegration of *Nannochloropsis* sp. microalgae suspensions than application of PEF, while Rajha et al. (2014) obtained that HVED lead to higher cell damage than PEF and USN for the same energy input due to the cavitation phenomena and pressure shock waves induced by HVED. Consequently, the beverage treated by $HVED_2$ would be better homogenised, which could explain the higher TAC values obtained. However, its applications in the food and pharmaceutical oriented

industry can be reduced because of possible contamination of the treated product by chemical products of electrolysis, free reactive radicals, etc. (Sarkis et al. 2015). In accordance with the present study, TEAC values were not significantly modified in PEF-treated orange juice-milk beverage in the research carried out by Zulueta et al. (2013). Moreover, Morales-de la Peña et al. (2010), immediately after PEF processing of fruit juice–soymilk beverage, did not observe significant changes in antioxidant capacity (TEAC and DPPH, respectively) in comparison with the untreated beverage. By contrast, Martínez-Flores et al. (2014) obtained an increase in the antioxidant capacity measured with DPPH assay due to the effect of ultrasound in carrot juice. It is noteworthy that although TEAC and DPPH are two different procedures used to measure the free radical-scavenging of food products, values obtained in the fruit juice-stevia blend were similar.

ORAC value in the control fruit juice-stevia blend was 30.8±0.9 mM. This value is consistent with previous studies of a fruit juice mixture (papaya, mango and orange) sweetened with *Stevia rebaudiana* (Carbonell-Capella et al. 2013) and comparable to the results obtained in different fruit formulations (smoothies, fruit purees, concentrates and juices) by Müller et al. (2010). The ANOVA analysis confirmed an increase of TAC when the fruit juice-stevia blend was treated by PEF and USN (116.4-121.7%), independently of the energy input and time treatment, in comparison with the untreated fruit juice-stevia blend. This increase in TAC may be related to an increased extractability of some of the antioxidant components following PEF and USN processing. HVED treatment did not result in an increase of ORAC values in comparison with the untreated fruit juice-stevia blend, independently of the energy input applied. The different TAC levels obtained from the assays may reflect a relative difference in the ability of antioxidant compounds in the beverage to quench aqueous peroxyl radicals and to reduce ABTS and DPPH free radical in *in vitro* systems (Zulueta et al. 2009).

When the possible correlation (Pearson test) between the various parameters that contribute to antioxidant capacity (ascorbic acid, total carotenoids, total phenolic compounds and total anthocyanins) was studied for the different non-thermally treated fruit juice-stevia blends, a positive correlation between total carotenoids and total phenolic compounds with TEAC values ($p$=0.0496 and

*p*=0.0172, respectively) and between anthocyanins and DPPH values (*p*=0.0006) was observed, reflecting the importance of using different methods to measure total antioxidant capacity in foodstuff as different bioactive compounds may be better represented by one or another assay. In this line, Bishi et al. (2015) found a significant correlation between phenol content and antioxidant capacity measured with TEAC assay in forty-one Indian peanut cultivars. In addition, Paz et al. (2014) observed a positive correlation between total phenolic compounds and antioxidant capacity measured with DPPH method in mango pulp. Moreover, in model juices prepared exclusively with purified plum extract, a high correlation between total anthocyanins and TEAC was found (Hernández-Herrero and Frutos 2015).

*Principal Component Analysis (PCA)*

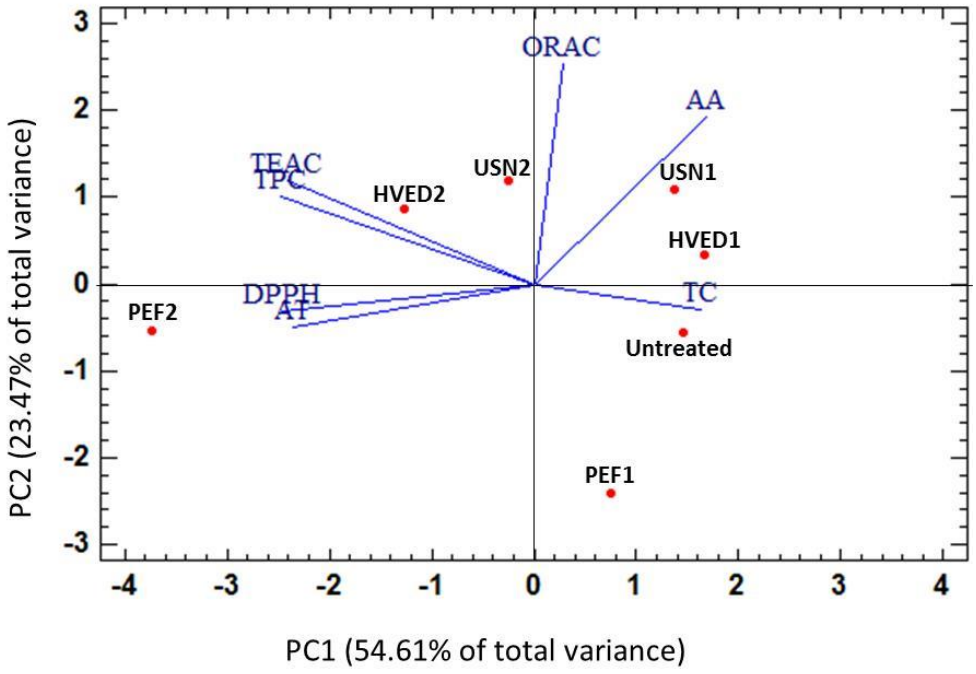


**Figure 5.** PCA plot for bioactive compounds and antioxidant capacity in untreated and treated fruit juice blend sweetened with *Stevia rebaudiana.* PEF:

pulsed electric fields. HVED: high voltage electrical discharges. USN: ultrasounds. 1: 32 kJ/kg. 2: 256 kJ/kg.

In a way of comparing the different treatments applied to the fruit juice-stevia beverage, a principal component analysis was effectuated. The biplot for the first two principal components (accounting for 78% of the total variance, with an eigenvalue>1) is shown in Figure 5. $PEF_1$ and $PEF_2$ samples are separated from the others, whereas $HVED_1$ and $USN_1$ treated samples, and $HVED_2$ and $USN_2$ treated samples are clustered together. The PCA allowed discovering which parameters seemed to drive the whole distribution. Carotenoids and ascorbic acid seemed to be the key elements influencing the bioactive compound and antioxidant capacity distribution. Furthermore, PCA indicated that ORAC might also be an important component for antioxidant interactions, as it has been well-known (Zulueta et al. 2009).

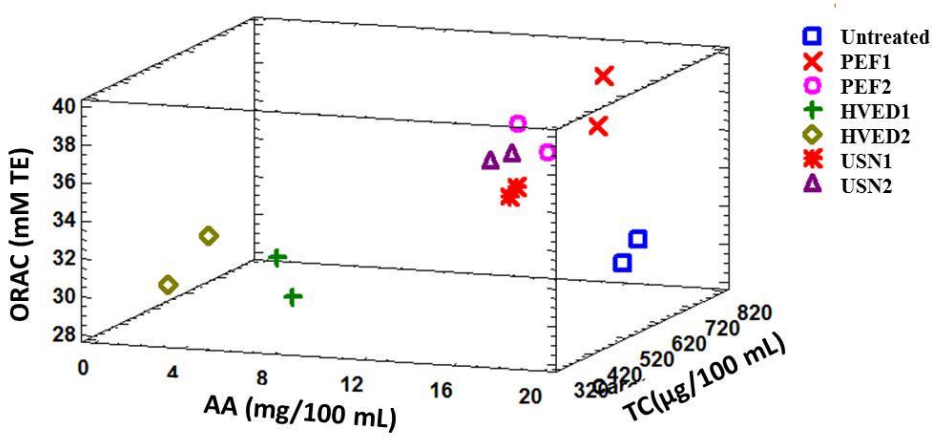


**Figure 6.** Ascorbic acid (AA), total carotenoids (TC) and ORAC values of untreated and treated fruit juice blend sweetened with *Stevia rebaudiana.* PEF: pulsed electric fields. HVED: high voltage electrical discharges. USN: ultrasounds. 1: 32 kJ/kg. 2: 256 kJ/kg.

Focusing on these three parameters (total carotenoids, ascorbic acid and ORAC values), PEF treated fruit juice-stevia beverage at an energy input of 32 kJ/kg was the treatment that better enhanced TC, AA and ORAC values, clearly separated from the rest of the samples and from untreated beverage and

followed by PEF treatment at an energy input of 256 kJ/kg (Figure 6). Consequently, it could be concluded that of all treatments, PEF technology resulted in the highest retention of bioactive compounds and antioxidant capacity with regard to the quality of the fruit juice-stevia beverage, always taking into account that the use of one or other technology will highly depend on which compound is pretended to be enhanced.

**Conclusions**

The non-thermal technologies discussed in the present study (PEF, HVED and USN) are processing technologies which can enhance bioactive compound retention and antioxidant capacity in the analysed fruit juice (papaya and mango)-stevia blend. A high recovery of ascorbic acid, total carotenoids and ORAC values was obtained after PEF treatments. Nevertheless, HVED and USN processing technologies were also found to be useful for the retention of certain bioactive compounds. The results obtained in the present work may be used by food producers in order to obtain new functional foods rich in bioactive compounds and antioxidant capacity. However, further studies evaluating the impact of PEF, HVED and USN technology on microbiological safety, sensorial parameters and storage stability of liquid foods are required.

**Acknowledgements**

This research project was supported by the Spanish Ministry of Science and Technology and European Regional Development Funds (AGL2010-22206-C02-01) in the framework of the COST Action TD1104: European network for development of electroporation-based technologies and treatments (EP4Bio2Med). Carbonell-Capella, J.M. holds an award from the Spanish Ministry of Education (AP2010-2546). Buniowska, M. thanks to Podkarpacie Marshal's Office for Ph.D. scholarship number 8.2.2/IV.26/217/11/U/155/12 of RSI Project for Podkarpacie Region, Poland. Barba, F.J. wishes to thank the Valencian Autonomous Government (Consellería d´Educació, Cultura i Esport. Generalitat Valenciana) for the postdoctoral fellowship of the VALi+d program (APOSTD/2013/092).

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## 3.9. “Ice” juice from apples obtained by pressing at subzero temperatures of apples pretreated by pulsed electric fields

*Innovative Food Science and Emerging Technologies (2016)*
*DOI:10.1016/j.ifset.2015.12.016*

# "Ice" juice from apples obtained by pressing at subzero temperatures of apples pretreated by pulsed electric fields

Juana M. Carbonell-Capella[1,2], Oleksii Parniakov[1], Francisco J. Barba[2], Nabil Grimi[1], Olivier Bals[1], Nikolai Lebovka[1,3], Eugene Vorobiev[1]

[1]Sorbonne Universités, Université de Technologie de Compiègne, Laboratoire de Transformations Intégrées de la Matière Renouvelable, EA 4297, Centre de Recherches de Royallieu, BP 20529, 60205 Compiègne Cedex, France

[2] University of Valencia, Department of Nutrition and Food Science, Av. Vicent Andrés Estellés, s/n, 46100 Burjassot, Spain

[3]Institute of Biocolloidal Chemistry named after F. D. Ovcharenko, NAS of Ukraine, 42, blvr. Vernadskogo, Kyiv 03142, Ukraine

*Corresponding Author:
Oleksii Parniakov
Phone number: +33 3 44 23 44 42
E-mail address: oleksii.parniakov@utc.fr

**ABSTRACT**

The impact of apple pretreatment by pulsed electric field (PEF) on juice extraction using the freezing-assisted pressing was studied. Apple discs were PEF pretreated at electric field strength of *E*=800 V/cm and then air blast frozen inside the freezer (-40 ºC). Then, pressing experiments in a laboratory-pressing chamber (2-5 bars) were started at subzero temperature (-5 °C). Time evolution of juice yield and its nutritional qualities were compared for PEF and untreated apple samples. High improvements of juice yield were obtained for freeze-thawed (FT) and PEF+FT samples. The combination of PEF + pressing (5 bar) at subzero temperature gave optimum results for juice extraction with high levels of carbohydrates, and antioxidant bioactive compounds. At fixed value of extraction yield, *Y*, PEF pretreatment improved nutritional parameters. E.g., at *Y*=0.6, an increase in °Brix (by ≈1.27), carbohydrates (by ≈1.42), total phenolic compounds (by ≈1.16), flavonoids (by ≈1.09) and antioxidant capacity (by ≈1.29) was observed after PEF pretreatment.

**Industrial Relevance:** Pressing constitutes one of the most commonly used technologies at industrial scale to obtain fruit juices. However, during the pressing some undesirable chemical, physical and biological changes may occur in juices, thus reducing their nutritional and sensorial properties. For instance, the use of freezing-assisted pressing is a promising technique for the production of juice concentrates rich in sugars and other solids as the low temperature operation prevents undesirable modifications. But this method is rather expensive and requires strong control of the quality of “ice” wines, their sensory and compositional profiles. Thus, there is an increased search for obtaining new efficient methodologies for producing high quality juices. In this line, PEF-assisted pressing has been shown as a useful technology to increase juice yield. Therefore, the combination of PEF-assisted “ice” juice extraction by pressing of fruits at subzero temperatures may be a useful tool to improve the extraction yield of juices, thus improving their nutritional, physicochemical and sensorial properties.

**Keywords:** “Ice” juice, Apple, Pulsed electric fields, Freezing-assisted pressing.

## 1. Introduction

A high juice yield is desirable in terms of economics, but organoleptic, nutritional properties and beneficial health effects of juices, partly attributed to the presence of antioxidants, especially phenolic compounds are also very important (Krawitzky et al., 2014). In this line, the potential of several juice extraction methods to obtain high juice yields with improved nutritional properties has been evaluated by both food researchers and food industry, concluding that the quality of juices is highly dependent of the production process.

Moreover, it has been observed that low-temperature assisted processing of foods (e.g., freeze concentration, pressing, etc.) is rather preferable in application for products with very delicate flavors (fruit juices, coffee, tea, and alcoholic beverages) (Deshpande, Cheryan, Sathe, & Salunkhe, 1984; Sánchez, Ruiz, Auleda, Hernández, & Raventós, 2009).

Freeze concentration is considered as a method for producing high quality juices. This method is based on a selective separation of water in the form of ice from the frozen solution. Freeze concentration has been applied in the production of concentrated "ice" juices from apple (Bayindirli, Özilgen, & Ungan, 1993; Hernández, Raventós, Auleda, & Ibarz, 2009; Olowofoyeku, Gil, & Kramer, 1980), pear (Hernández et al., 2009; Miyawaki, Kato, & Watabe, 2012; Tobitsuka, Ajiki, Nouchi, & Miyawaki, 2010), blueberry (Petzold, Moreno, Lastra, Rojas, & Orellana, 2015), sugarcane (Rane & Jabade, 2005; Sahasrabudhe, Desai, & Jabade, 2012), pineapple (Bonilla-Zavaleta, Vernon-Carter, & Beristain, 2006; Petzold et al., 2015), orange (Fang, Chen, Tang, & Wang, 2008; Sánchez, Ruiz, Raventós, Auleda, & Hernández, 2010), pomegranate (Khajehei, Niakousari, Eskandari, & Sarshar, 2015), tomato (Liu, Miyawaki, & Hayakawa, 1999; Miyawaki et al., 2012) concentration of grape must (Hernández, Raventós, Auleda, & Ibarz, 2010), fresh tea juice (Feng, Tang, & Ning, 2006) and preparation of coffee extract (Gunathilake, Shimmura, Dozen, & Miyawaki, 2014).

Among techniques of juice production, pressing is one of the most used at industrial scale (Markowski, Baron, Le Quéré, & Płocharski, 2015). Freezing

assisted pressing can be also used as a promising technique for the production of juice concentrates rich in sugars and other solids (Petzold et al., 2015). Concentrated aqueous solutions do not freeze, while pure water does, allowing a more concentrated juice product. The low temperature operation in freeze-concentration prevents undesirable chemical, physical and biological changes that may occur in other types of processing.

This procedure has already been employed in the production of the so-called “ice” wine, “ice” cider or “ice” juice by pressing of frozen fruits (Alessandria et al., 2013; Bowen, 2010; Crandles, Reynolds, Khairallah, & Bowen, 2015; Kirkey & Braden, 2014; Motluk, 2003; Musabelliu, 2013). Such extreme processing allows the production of smaller amounts of concentrated and sweeter wines, e.g., high quality dessert wines. Application of cold pressing is rather popular in those countries (e.g., Canada and Germany), where fruit varieties are not harvested before the first frosts. However, this processing is rather expensive, risky and requires strong control of the quality of “ice” wines, their sensory and compositional profiles (Bowen, 2010). E.g., these characteristics may be significantly different for British Columbia, Ontario and German ice wines (Cliff, Yuksel, Girard, & King, 2002; Nurgel, Pickering, & Inglis, 2004). However, application of low-temperature assisted pressing is still very limited (Petzold et al., 2015).

The extraction efficiency by pressing may be noticeably enhanced using pulsed electric fields (PEF) pre-treatment (Lebovka & Vorobiev, 2010). Different examples of PEF-assisted dehydration of fruit and vegetable tissues have already been demonstrated (Donsi, Ferrari, & Pataro, 2010; Vorobiev & Lebovka, 2011). These techniques allow avoidance of undesirable changes typical of other techniques, such as thermal, chemical and enzymatic ones (Donsi, Ferrari, Maresca, & Pataro, 2011; Jaeger, Reineke, Schoessler, & Knorr, 2012; Jaeger, Schulz, Lu, & Knorr, 2012; Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2013; Raso & Heinz, 2006)

The positive effects of PEF pre-treatment on drying, freezing, freeze-drying processes, freezing tolerance and texture of biomaterials have been demonstrated (Ben Ammar, Lanoiselle, Lebovka, Van Hecke, & Vorobiev, 2010;

Jalte, Lanoiselle, Lebovka, & Vorobiev, 2009; Parniakov, Lebovka, Bals, & Vorobiev, 2015; Phoon, Galindo, Vicente, & Dejmek, 2008; Shayanfar, Chauhan, Toepfl, & Heinz, 2013, 2014; Shynkaryk, Lebovka, & Vorobiev, 2008; Wiktor, Schulz, Voigt, Witrowa-Rajchert, & Knorr, 2015). However, the effect of PEF pre-treatment on freezing assisted pressing and in the production of "ice" juice has not yet been studied.

This manuscript discusses the PEF-assisted "ice" juice extraction by pressing of apple at subzero temperatures. Apples were initially pretreated by PEF to a high level of electroporation, frozen and pressed during their thawing at 2-5 bars. Extraction yield and nutritional qualities of the extracted "ice" juice were analysed.

## 2. Material and methods

### *2.1. Raw material and sample preparation*

Apples (*Malus domestica* var. *Jonagold*) were purchased at the local supermarket and stored at 4 ºC until analysis. Wet basis moisture content was measured by drying 20 g of the fresh apple tissue at 105 °C to constant weight. It was found between 82-85% w. b. Apple discs ($d$=50 mm and $h$=5 mm) were prepared using a vegetable cutter (Robot Cupe CL 50, Montceau-en-Bourgogne-Cedex, France).

### *2.2. PEF pretreatment*

PEF pretreatment was carried out using a pulse generator, 400 V- 38 A (Service Electronique UTC, Compiegne, France). Apple sample was placed in the PEF-treatment cell between two electrodes (Figure 1). Electrical treatment cell consisted of a Teflon cylindrical tube (Atelier Genie des Procédés Industriels, UTC, Compiegne, France) with ≈110 mm inner diameter and an electrode (stainless steel 316 L) at the bottom. The apple disc-shaped sample was placed inside the cell on the bottom electrode and covered with fresh apple juice. After that, the second electrode was put on top of the samples. The distance between the electrodes, 5 mm, was determined by the height of the sample. Temperature was

controlled by a Teflon-coated thermocouple Thermocoax type 2-AB 25 NN (Thermocoax, Suresnes, France) inserted into the geometrical centre of the sample with a temperature measurement precision of ±0.1 ºC. The PEF generator provided bipolar pulses of near-rectangular shape with an electric field of $E$=800 V/cm and series of $N$=10 were applied. Each separate series consisted of $n$=10 pulses with pulse duration $t_i$=100 µs, distance between pulses $\Delta t$=1000 µs and pause $\Delta t_t$=10 s after each series. Total time of PEF treatment was calculated as $t_p = nNt_i$. The chosen protocol of successive trains with long pause after each train allowed a fine control of the plant tissue permeabilisation without any significant temperature elevation (ΔT≤3 °C) during PEF treatment. These conditions were chosen according to a previous study (Parniakov et al., 2015) where a high level of tissue electroporation (conductivity disintegration index $Z$ was ≈0.98) was obtained. Electrical conductivity of the sample was measured during the pause period $\Delta t_t$ between two consecutive series of pulses. All the output data (current, voltage, electrical conductivity and temperature) were collected using a data logger and special software adapted by Service Electronique UTC.

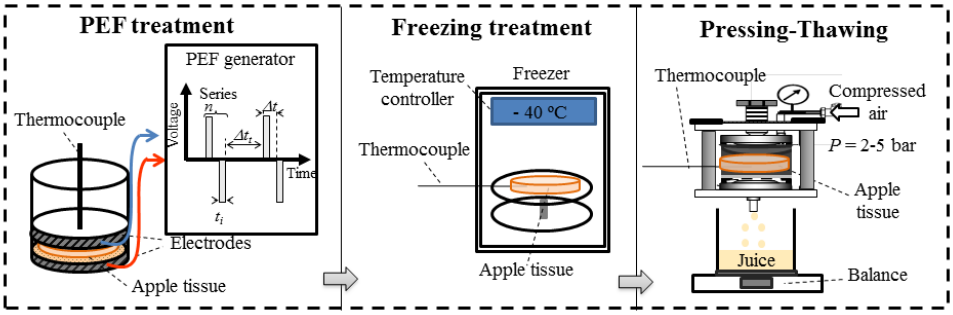


**Figure 1.** Schematic presentation of experimental procedures including PEF pretreatment, freezing, and pressing-thawing experiments.

### *2.3. Freezing*

Apple samples were frozen in an ultra-low-temperature air-blast freezer MDF-U2086S (Sanyo, Gunma, Japan), supplied with a modular-type temperature controller SR Mini System (TC Ltd., Dardilly, France) and the software Spec-View Plus (SpecView Corporation, Gig Harbor, USA). Untreated and PEF pretreated

samples were placed inside the freezer at –40 ºC with an air velocity of 2 m/s controlled by an electronic device VEAT 2.5 A (Air-technic, Firminy, France). Sample temperature was measured with a T type thermocouple of 0.5 mm diameter (TC, Ltd., Dardilly, France) with an accuracy of ±0.1 ºC that was introduced in the geometrical centre of the sample. Initial temperature before freezing was uniform and constant at 20 ºC for the whole sample. Total freezing time, *t*, from the beginning of the cooling was 50 minutes, the final temperature of the sample was ≈-35 ºC.

*2.4. Pressing-thawing experiments*

Immediately after the end of freezing the apple samples were placed into a pressing chamber maintained at 20 ºC. The temperature inside the tissue was measured with a Teflon-coated thermocouple Thermocoax type 2-AB 25 NN (Thermocoax, Suresnes, France) with precision of ±0.1 ºC. Samples were compressed using a laboratory pressing chamber (hemispherical shape with a radius of 28 mm) equipped with an elastic diaphragm (Figure 1). The compression at 2, 3 and 5 bars was started when the temperature inside the tissue attained –5 °C and continued for 100 min. The extracted juice was collected and weighted continuously by an electronic balance.

In present experiments the maximum quantity of recovered juice, mm, was obtained for long time of pressing of PEF pretreated tissue, $t \approx 5000$ s, at $P=5$ bars. Extraction yield, *Y*, was calculated as

$$Y=m/m_m \qquad (1)$$

In order to follow the pressing kinetic, an acquisition computer system was used to record the mass of recovered juice *m* every 5 s.

*2.5. Nutritional characteristics*

Concentration of total soluble matter was measured in accordance to IFU methods (IFU, 2001) with a digital refractometer (Atago, USA) at room temperature. Results were expressed in °Brix (g of total soluble solid content /100 g solution).

Total carbohydrates concentration, $C_c$, was determined using the phenol–sulphuric acid method (Du Bois, Gilles, Hamitton, Reders, & Smith, 1956), with some modifications (Parniakov, Lebovka, Van Hecke, & Vorobiev, 2014). 0.4 ml of sample were mixed with 0.2 ml of 5% (w/v) phenol solution and 1 ml of concentrated sulphuric acid (Sigma-Aldrich, France). Then the reaction mixture was kept at 25 °C for 30 min. Absorbance of the mixture was measured at 490 nm and the polysaccharide content was calculated using D-glucose (VWR International, Belgium) as a standard. Results were expressed in mg of glucose equivalent/L of extract.

Adapted and optimised assay for total polyphenols determination was used (Singleton, Orthofer, & Lamuela-Raventos, 1999). Initially, 200 µL of diluted extract and 1000 µL of the Folin–Ciocalteau reagent (diluted 10 folds in distilled water, w/w) were mixed and left at room temperature for 5 min. Then, 800 µL of $Na_2CO_3$ solution (7.5 g of Na2CO3 and 100 g of water) were added. The mixture was kept for 1 hour at room temperature and absorbance was measured at 750 nm using a UV–vis spectrophotometer (Milton Roy Company, Spectronic 20 Genesys, United States). Gallic acid calibration standards with concentrations of 0, 20, 40, 60, 80, 100 and 120 ppm were prepared and results were expressed as milligrams of gallic acid equivalents (GAE) per litre of apple juice (mg GAE/L).

Total flavonoids content was determined using the method as described in Zhishen, Mengcheng, & Jianming (1999). 100 µL of sample were mixed with 1.088 mL of ethanol (30%, v/v) and 48 µL of sodium nitrite solution (0.5 mol/L). After 5 min, 48 µL of aluminium chloride (0.3 mol/L) were added. The mixture was stirred and allowed to react for 5 min. Then, 320 µL of sodium hydroxide (1 mol/L) were added and absorbance was measured at 510 nm using a UV-Vis spectrophotometer (Milton Roy Company, Spectronic 20 Genesys, United States). Catechin was used as standard with concentrations in the range of 0-75 µg/L and results were expressed as milligrams of catechin equivalents (CTE) per litre of apple juice [mg CTE/L].

Total antioxidant capacity was measured using the TEAC (Trolox Equivalent Antioxidant Capacity) assay according to Carbonell-Capella et al. (2015). ABTS radical ($ABTS^{\bullet+}$) was generated using 440 µL of potassium persulfate

(140 mM). The solution was diluted with ethanol until an absorbance of 0.70 was reached at 734 nm. Once the radical was formed, 2 mL of $ABTS^{\bullet+}$ were mixed with 100 µL of extract and incubated for 60 min at 20 ºC and absorbance was measured.

The browning index (BI) was measured accordingly to (Meydav, Saguy, & Kopelman, 1977). For this purpose samples were centrifuged (824 g, 20 min, 20 ºC) and supernatant was diluted in ethanol (1:1 (v/v)). The mixture was filtered using filter paper (Whatman® No. 2, Whatman International Ltd., UK) and absorbance was measured at 420 nm.

*2.6. Statistical Analysis*

Each experiment was repeated at least three times. Error bars presented on the figures correspond to the standard deviations. One-way analysis of variance was used for statistical analysis of the data using the Statgraphics plus (version 5.1, Statpoint Technologies Inc., Warrenton, VA). Turkey tests were also performed on data for all pairwise comparisons of the mean responses to the different treatment groups. This test allows determination of treatments which are statistically different from the other at a probability level of $p$=0.05.

**3. Results and discussion**

Figure 2 presents typical examples of temperature evolution inside the apple discs during the cooling of untreated and PEF pretreated apples. The temperature decrease was initially rather slow. The first crystallisation stage corresponds to a phase transformation of water into ice inside the sample (Chevalier, Le Bail, & Ghoul, 2000). However, after some time, the temperature began to decrease rapidly to the storage value (-40 °C). The second cooling stage is started when the most freezable water is converted to ice. The effective freezing time, $t_f$, was determined as a crosspoint of tangent lines of freezing and cooling part of *T(t)* curve (Figure 2).

PEF pretreatment resulted in a significant acceleration of the freezing process. E.g., values of $t_f$ were ≈1400 s and ≈850 s for untreated and PEF-treated apple discs, respectively. Similar effects of PEF-pretreatment were previously reported

for potato (Ben Ammar et al., 2010; Jalte et al., 2009) and apple (Parniakov et al., 2015). The different possible mechanisms of PEF-pretreatment in the increase of the freezing rate were earlier discussed (Jalte et al., 2009). In general, the resultant faster freezing is desirable, leading to smaller size of formed ice crystals and better quality of processed products (Delgado & Sun, 2001).

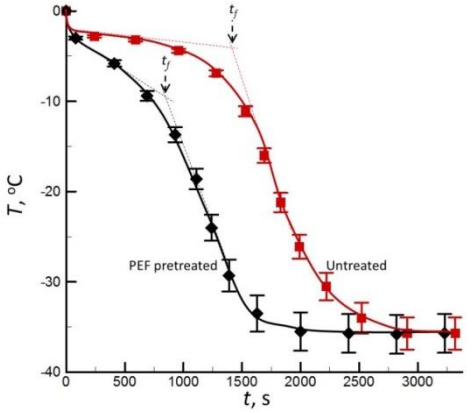


**Figure 2.** Evolution of temperature inside the geometrical centre of the apple disc during freezing for untreated and PEF treated samples. Here, $t_f$ is an effective freezing time.

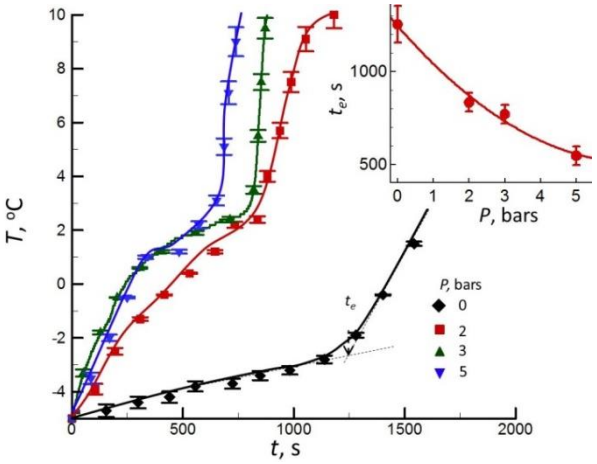


**Figure 3.** Evolution of temperature inside the geometrical centre of the apple tissue during pressing-thawing experiments for untreated ($P$=0 bars) and PEF pretreated ($P$=2, 3 and 5 bars) apple tissue. The case of $P$=0 bars corresponds to the thawing without applied pressure. Break of the curve $T(t)$ at $t=t_e$ for $P$=0 is shown. Inset shows transition time, $t_e$, versus applied pressure $P$.

Figure 3 presents the temperature evolution inside apple samples during the pressing-thawing experiments for PEF pretreated samples at different pressures $P$=0, 2, 3 and 5 bars. The evolution of the temperature can be divided into two phases with the break of the curve $T(t)$ at $t=t_e$ (Figure 3). Transition time, $t_e$, decreased significantly with increase of the applied pressure $P$ (see inset to Figure 3).

Figure 4 presents extraction yield, $Y$, and concentration of soluble solids in local portions of juice, °Brix$^l$, versus extraction time, $t$, in pressing-thawing experiments at different pressures $P$. Data are presented for PEF pretreated apples. Extraction yield, $Y$, increased with the pressing time, $t$, and reached a maximum level, $Y_m$, after a long time of pressing, $t\geq$2400 s. Values of $Y_m(P)$ were ≈0.6, ≈0.8 and =1 for $P$=2, 3 and 5 bars, respectively. Note that similar values of $Y_m(P)$ were also obtained in pressing-thawing experiments with untreated samples (data are not presented). However, the rate of juice release for PEF pretreated apples was significantly higher compared to untreated ones and the maximum level, $Y_m$, for untreated samples was only obtained at t≥6000 s. So, electroporation facilitates the rate of juice release during thawing of frozen apple tissue, but does not change the amount of juice expressed. It can be speculated that this phenomenon reflects the changes in spatial distribution of unfrozen content inside the tissue that facilitates the juice flow when pressure is applied. The observed behaviour was also in accordance with significant decreasing of the transition time, $t_e$, with increase of the applied pressure $P$ (see inset to Figure 3).

Concentration of soluble solids in the first local fractions of apple juice, °Brix$^l$, was rather high (Figure 4). During the pressing-thawing experiments, °Brix$^l$ value reached a maximum at $t=t_{max}$. At $t\geq t_{max}$, values of °Brix$^l$ decreased significantly and reached approximately the same minimum value, ≈5.9, independently of the pressure applied. Initial expression of the most concentrated juice with the highest content of sugars and other solids was expected, as it reflects the typical process of freezing-assisted pressing. When time is increased, the thawing of the ice crystals found inside the apple tissue takes place and consequently, the concentration of soluble solids in the expressed juice drops gradually. The

observed time evolution of $Y(t)$ and °Brix$^{l}(t)$ (Figure 4) is in qualitative correspondence with the temperature evolution presented in Figure 3.

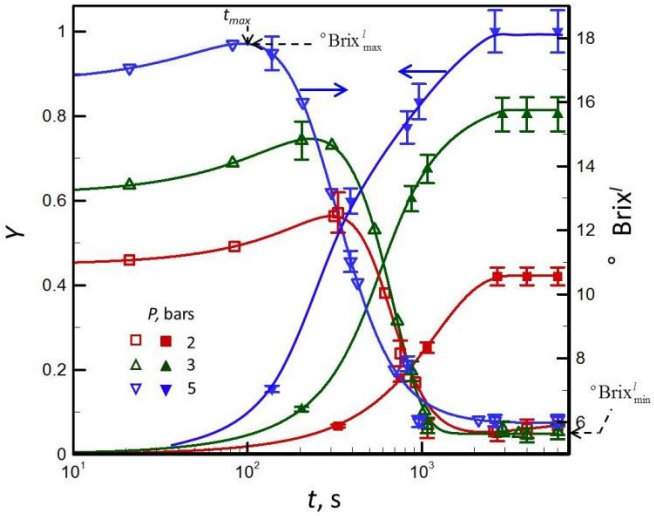


**Figure 4.** Extraction yield, *Y*, and concentration of soluble solids in local portion of juice, °Brix$^{l}$, versus extraction time, *t*, in pressing-thawing experiments at different pressures *P* (2, 3 and 5 bars). Data are presented for PEF pretreated apples.

However, the origin of °Brix$^{l}$ maximum at $t=t_{max}$ is not completely clear. In the applied experimental schemes of freezing and pressing-thawing, the spatial distributions of the temperature and concentrations of the different components found inside the sample are highly heterogeneous. Freezing is started from the sample surface and can result in heterogeneous spatial distributions of both segregated ice crystals and unfreezable portions of juice inside the apple sample. From the other hand, during pressing-thawing, temperature is highest on the surface of the sample and initial portions of juice are expressed from the surface layer. Electroporation may have a supplementary effect on the heat and mass transfer processes in the sample. That's why the maximum of °Brix$^{l}$ at $t=t_{max}$ can be the reflection of the above-mentioned heterogeneities in temperature and component concentrations.

Figure 5 presents the concentration of soluble solids in local portion of juice, °Brix$^{/}$, versus the extraction yield, *Y*, in pressing-thawing experiments at different pressures *P*. Note that the extraction yield of the most concentrated local portions of juice at $t=t_{max}$ was rather small, $Y\approx0.1\text{-}0.15$. Maximum values decreased whereas $t_{max}$ values increased with increase of pressure, *P* (see inset to Figure 5).

From a practical point of view, it is interesting to compare the characteristics of the total portion of accumulated juice at different values of extraction yield, *Y*. Figure 6 presents the concentration of soluble solids, °Brix, (a) and total phenolic compounds, $C_{TPC}$, (b) versus juice yield, *Y*, at different pressures *P*. Data are presented for PEF pretreated (filled symbols, dashed lines) and untreated (open symbols, solid lines) apple samples.

Results show that freezing assisted pressing is rather effective at high pressure, *P*=5 bars, and ineffective at small pressure, *P*=2 bars. At high pressure (*P*=5 bars), final values of ºBrix (*Y*=1) were ≈12.2 and ≈11.5 for PEF pretreated and untreated samples, respectively. However, at small pressure, *P*=2 bars, final values of ºBrix ($Y\approx0.4$) were ≈8.8 for both PEF pretreated and untreated samples. This may reflect the capture of more concentrated juice inside the apple sample for the given mode of freezing-assisted pressing at *P*=2 bars.

In further discussion, we will only evolve the data obtained at the highest pressure, *P*=5 bars. It is remarkable that in PEF-assisted pressing-thawing experiments, ºBrix values (Figure 6a) and $C_{TPC}$ (Figure 6b) noticeably exceeded those values obtained for untreated apple samples.

It evidently reflects the impact of electroporation on the freezing-assisted pressing. Noticeably, both electroporation and freezing cause cell damage in tissues. However, electroporation can also affect heat and mass transfer processes during freezing, which was found to be important for enhancement of the pressing-thawing process.

The different nutritional characteristics of apple juice obtained in pressing-thawing experiments at 5 bars for untreated and PEF pretreated apples are compared in Table 1.

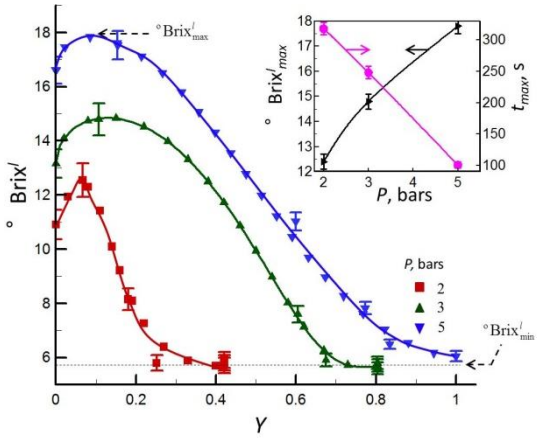


**Figure 5.** Concentration of soluble solids in local portion of juice, °Brix$^{l}$, versus extraction yield, *Y*, in pressing-thawing experiments at different pressures *P* (2, 3 and 5 bars). Data are presented for PEF pretreated apples. Insert shows the maximum values of °Brix$^{l}_{max}$ in local portion of juice and corresponding time of extraction, $t_{max}$, versus the applied pressure *P*. Data are presented for PEF pretreated apples.

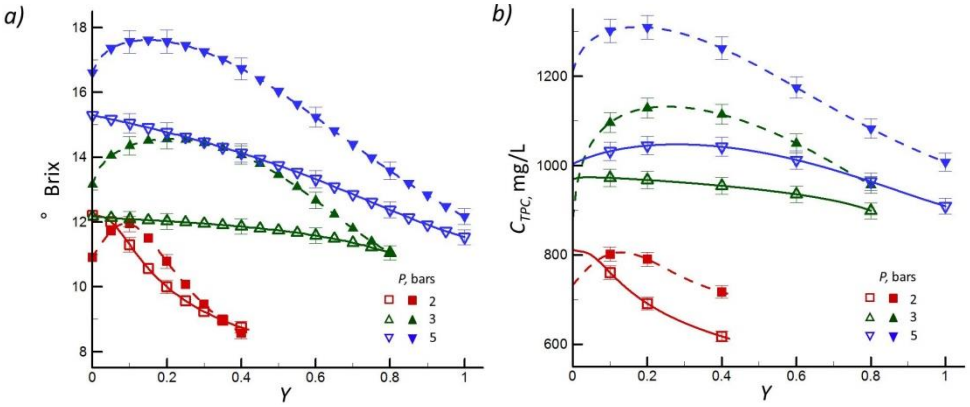


**Figure 6.** Concentration of soluble solids, °Brix, (a) and total phenolic compounds, $C_{TPC}$, (b) versus juice yield, *Y*, at different pressures, *P*=2, 3 and 5 bars. Data are presented for PEF pretreated (filled symbols, dashed lines) and untreated (open symbols, solid lines) apple samples.

At fixed value of extraction yield, *Y*, PEF pretreatment always allowed noticeable enhancement of nutritional parameters. E.g., at *Y*=0.6 an increase in

°Brix of ≈1.27, total carbohydrates of ≈1.42, total phenolic compounds of ≈1.16, flavonoids of ≈1.09 and total antioxidant capacity of ≈1.29 was observed after PEF pretreatment. The highest concentrations of total carbohydrates ($C_C$≈281.5 g/L), total phenolic compounds ($C_{TPC}$≈1310.4 mg GAE/L), flavonoids ($C_{TF}$≈292.7 mg CTE/L) and total antioxidant capacity value (TAC≈9.2 mM trolox equivalent) were obtained with juice yields of ≈13%, ≈18%, ≈14% and ≈18%, respectively.

**Table 1.** Different nutritional characteristics of apple juice obtained in pressing-thawing experiments at 5 bars for untreated and PEF pretreated apples.

| | Nutritional parameters | *Y* | | | | |
|---|---|---|---|---|---|---|
| | | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
| Untreated | °Brix | 13.5±0.1 | 12.8±0.1 | 12.0±0.1 | 11.1±0.1 | 11.5±0.1 |
| | $C_C$ (g/L) | 164.7±1.6 | 168.1±0.5 | 161.1±3.4 | 147.2±3.2 | 131.2±3.9 |
| | $C_{TPC}$ (mg/L) | 1045.0±5.2 | 1042.2±3.2 | 1012.9±4.1 | 964.2±1.0 | 909.4±4.1 |
| | $C_{TF}$ (mg/L) | 247.5±2.5 | 248.3±1.9 | 237.0±1.9 | 223.0±4.3 | 211.7±1.2 |
| | TAC (mM TE) | 7.46±0.09 | 7.14±0.14 | 6.53±0.11 | 6.03±0.05 | 5.92±0.07 |
| PEF treated | °Brix | 17.6±0.1 | 16.7±0.1 | 15.2±0.1 | 13.6±0.1 | 12.2±0.1 |
| | $C_C$ (g/L) | 278.4±4.3 | 255.8±2.7 | 227.8±1.8 | 200.3±1.6 | 176.3±0.5 |
| | $C_{TPC}$ (mg/L) | 1309.7±7.2 | 1262.8±6.6 | 1174.8±2.1 | 1083.0±1.9 | 1007.8±1.2 |
| | $C_{TF}$ (mg/L) | 291.7±0.6 | 278.8±2.5 | 259.5±2.5 | 238.7±3.1 | 220.6±1.9 |
| | TAC (mM TE) | 9.07±0.18 | 8.84±0.15 | 8.39±0.13 | 7.88±0.09 | 7.42±0.06 |

Data are presented as mean ± standard deviation. °Brix: Total soluble solids. $C_C$: Concentration of carbohydrates. $C_{TPC:}$ Concentration of total phenolic compounds. $C_{TF:}$ Concentration of total flavonoids. TAC: Total antioxidant capacity.

Thus, freezing-assisted pressing of PEF pretreated samples has a positive effect on all nutritional parameters of the extracted apple juice and allowed obtaining an ice juice rich in bioactive compounds. Likewise, it was observed that browning index of juice obtained by freezing-assisted pressing of PEF pretreated samples was rather lower (BI≈0.101) than that of juice obtained by traditional method (BI≈0.306).

## 4. Conclusions

Freezing-assisted pressing at subzero temperatures is an effective tool in order to obtain an apple juice rich in bioactive compounds. The efficiency of this process can be noticeably improved by the application of PEF pretreatment of apple tissue before freezing. PEF pretreatment resulted in a reduction of both freezing and thawing time of apple tissue and that pressing was more effective at high pressure, *P*=5 bars. Furthermore, PEF pretreatment facilitated the rate of juice release but did not change the total amount of juice expressed. The observed effects can reflect the impact of electroporation on heat and mass transfer at low temperature processes inside the apple sample. In PEF-assisted pressing-thawing experiments, ºBrix values and other nutritionally important parameters of apple juice noticeably exceeded those values obtained for untreated samples. It is remarkable that both electroporation and freezing can cause cell damage in tissues. However, electroporation can also affect the process of freezing, which was found to be important for the enhancement of the pressing-thawing. Thus, freezing-assisted pressing of PEF pretreated samples has a positive effect on all investigated nutritional parameters of the extracted apple juice. The proposed scheme of freezing-assisted pressing of PEF pretreated samples at subzero temperatures is applicable for different fruit and vegetable tissues and may be used by food producers in order to obtain a high quality ice juice rich in bioactive compounds. However, further investigations are required with regard to the adjustment and optimisation of pressing protocols, further improvement of beneficial PEF effects and checking the “ice” juice storage stability.

## Acknowledgements

The authors appreciate the support from the COST Action TD1104 (EP4Bio2Med — European network for development of electroporation based technologies and treatments). Carbonell-Capella, J.M. thanks the F.P.U. grant (No. AP2010-2546) provided by the Spanish Ministry of Education. Parniakov O. thanks thesis grant provided by the French Ministry of Education.

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## 3.10. Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: A review

*Comprehensive Reviews in Food Science and Food Safety*
*13(2) (2014) 155–171*

# Analytical Methods for Determining Bioavailability and Bioaccessibility of Bioactive Compounds from Fruits and Vegetables: A Review

Juana M. Carbonell-Capella · Magdalena Buniowska · Francisco J. Barba · María J. Esteve · Ana. Frígola*

University of Valencia , Department of Nutrition and Food Science, Av. Vicent Andrés Estellés, s/n. 46100 Burjassot, Spain

*Corresponding author:
Ana Frígola
e-mail: ana.frigola@uv.es.
Phone: +34 963544955 Fax: +34 963544954

**ABSTRACT**

Determination of bioactive compounds content directly from foodstuff is not enough for the prediction of potential *in vivo* effects, as metabolites reaching the blood system may be different from the original compounds found in food, as a result of an intensive metabolism that takes place during absorption. Nutritional efficacy of food products may be ensured by the determination of bioaccessibility, which provides valuable information in order to select the appropriate dosage and source of food matrices. However, between all the methods available, there is a need to establish the best approach for the assessment of specific compounds. Comparison between *in vivo* and *in vitro* procedures used to determine bioaccessibility and bioavailability is carried out, taking into account the strengths and limitations of each experimental technique, along with an intensive description of actual approaches applied to assess bioaccessibility of bioactive compounds. Applications of these methods for specific bioactive compound's bioaccessibility or bioavailability are also discussed, considering studies regarding the bioavailability of carotenoids, polyphenolic compounds, glucosinolates, vitamin E, and phytosterols.

**Keywords:** Bioaccessibility, bioavailability, bioactive compounds, *in vitro* methods, *in vivo* methods.

**Introduction**

Nowadays, consumers are more and more aware of the benefits beyond basic nutrition provided by food and food compounds. Between these, plant foods including fruits and vegetables have been demonstrated to exhibit multiple health benefits, closely related to their high contents in vitamins and other bioactive compounds (vitamin C, carotenoids, phenolic compounds, vitamin E, glucosinolates) with antioxidant properties (Nehir and Simsek 2012; Barba and others 2013; Carbonell-Capella and others 2013a). However, when studying the role of bioactive compounds in human health, their bioavailability is not always well known. Before becoming bioavailable, they must be released from the food matrix and modified in the gastrointestinal tract. Therefore, it is important before concluding on any potential health effect, to analyse whether the digestion process affects bioactive compounds and their stability, as this, in turn, will affect their bioavailability and their possible beneficial effects.

Different digestion models have been developed by the scientific community that accurately mimic the complex physicochemical and physiological conditions of the human gastrointestinal (GI) tract, along with *in vivo* models in living organisms (Hur and others 2011). However, comparison of results between different studies is difficult to accomplish, as there is no defined experimental model for studying bioaccessibility and bioavailability. Analysis of the procedures for measuring or predicting bioactive compounds bioavailability is therefore required, particularly as a result of continuous developments of new products by food industries considered “functional” because of their specific antioxidant or phytochemical contents.

The aim of the present article is to critically review different approaches used in the estimation of bioaccessibility and bioavailability of food compounds, focusing on bioactive compounds, as these are of major interest in current functional food development. Furthermore, results of studies in which bioaccessibility and bioavailability of bioactive compounds were investigated are also discussed.

**Bioaccessibility, Bioavailability, and Bioactivity**

The concept of bioaccessibility can be defined as the quantity or fraction which is released from the food matrix in the gastrointestinal tract and becomes available for absorption (Heaney 2001). This includes digestive transformations of food into material ready for assimilation, the absorption/assimilation into intestinal epithelium cells, and lastly, the presystemic metabolism (both intestinal and hepatic). For some nutrients, beneficial effects of unabsorbed nutrients (such as binding of bile salts by calcium in the tract) would be missed by absorption-based definitions. Bioaccessibility is usually evaluated by *in vitro* digestion procedures, generally simulating gastric and small intestinal digestion, sometimes followed by Caco-2 cells uptake (Courraud and others 2013).

Differently, the term bioavailability includes also in its definition the utilisation of a nutrient and therefore can be defined as the fraction of ingested nutrient or compound that reaches the systemic circulation and is utilised (Wood and others 2005). Overall, bioavailability includes gastrointestinal digestion, absorption, metabolism, tissue distribution, and bioactivity. Consequently, in terms of bioavailability, when a claim is made, it must be demonstrated that the component analysed is efficiently digested and assimilated and then, once absorbed, exerts a positive effect in human health. However, practical and ethical difficulties are found when measuring bioactivity, so the term “bioavailability” is usually defined as the fraction of a given compound or its metabolite that reaches the systemic circulation (Holst and others 2008), without considering bioactivity. According to this definition, bioavailability of a compound is determined *in vivo* in animals or humans as the area under the curve (AUC) (plasma-concentration) of the compound obtained after administration of an acute or chronic dose of an isolated compound or a compound-containing food (Rein and others 2013).

Bioactivity is the specific effect upon exposure to a substance. It includes tissue uptake and the consequent physiological response (such as antioxidant, anti-inflammatory). It can be evaluated *in vivo*, ex vivo, and *in vitro* (Figure 1) (Fernández-García and others 2009).

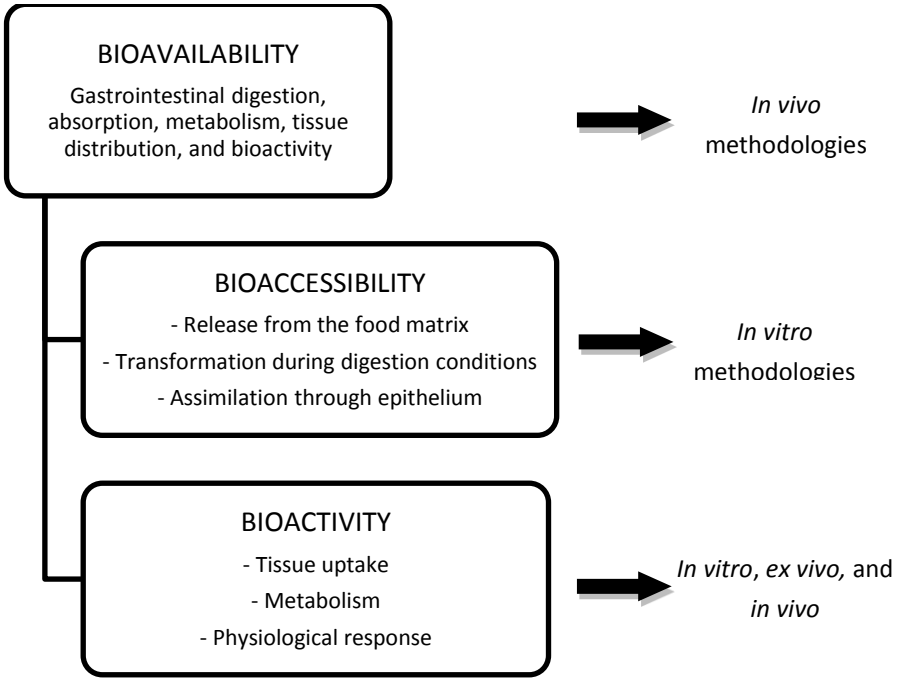


**Figure 1.** Definition of bioavailability, bioaccessibility, and bioactivity and their potential assessment methodologies.

Meanwhile, digestibility applies specifically to the fraction of food components that is transformed by digestion into potentially accessible matter through all physical–chemical processes that take place in the lumen. Assimilation, meanwhile, refers to the uptake of bioaccessible material through the epithelium by some mechanism of transepithelial absorption (Etcheverry and others 2012).

**Bioavailability of Bioactive Compounds**

Bioactive compounds are phytochemicals that are present in foods and are capable of modulating metabolic processes, resulting in the promotion of better health. In general, these compounds are mainly found in plant foods such as fruit, vegetables, and whole grains (Gil-Chávez and others 2013; Carbonell-Capella and others 2013b) and typically occur in small amounts. These compounds exhibit

beneficial effects such as antioxidant action, inhibition or induction of enzymes, inhibition of receptor activities, and induction and inhibition of gene expression (Correia and others 2012). They can be considered an extremely heterogeneous class of compounds with different chemical structures (hydrophilic/lipophilic), distribution in nature (specific to vegetable species/ubiquitous), range of concentrations both in foods and in the human body, possible site of action, effectiveness against oxidative species, and specificity and biological action (Porrini and Riso 2008). Among them, polyphenolic compounds, carotenoids, tocopherols, phytosterols, and organosulfur compounds constitute important groups in the human diet.

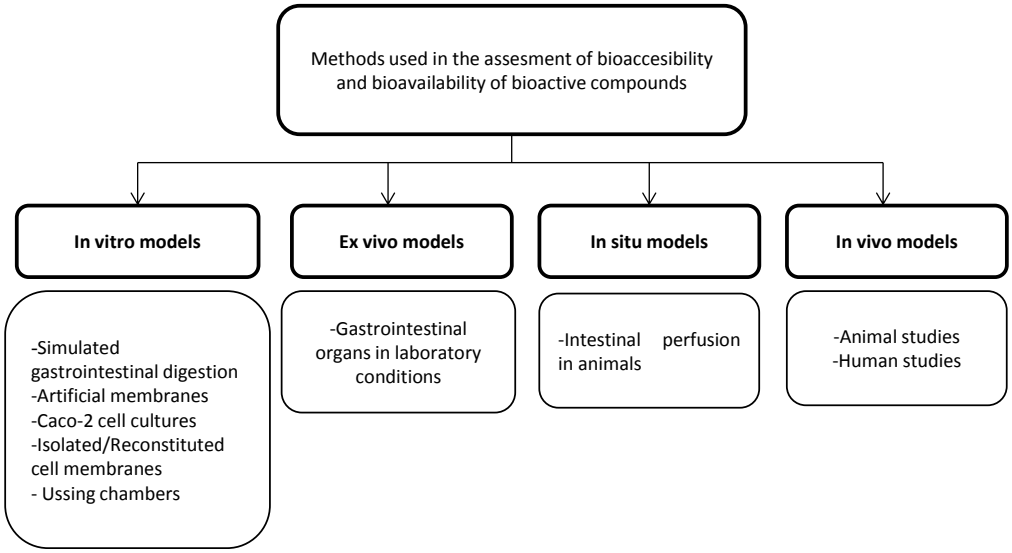


**Figure 2.** Methodologies used in the assessment of bioavailability and bioaccessibility of bioactive compounds.

Indeed, bioavailability of bioactive compounds may be modified because of interactions with other macronutrients such as fibre in low-processed foods and beverages or proteins and polysaccharides in processed food products (Dupas and others 2006). Furthermore, when different foods come in contact in the mouth or digestive tract, various interactions may take place affecting phytochemical bioavailability (for example fat enhances quercetine bioavailabilty in meals) (Lesser and others 2006). On that basis, significant research effort has recently

focused on achieving optimal uptake of phytochemicals to mantain body funcions and health and, consequently, carefully controlled studies are necessary in order to determine phytochemical bioavailability.

As shown in Figure 2, different approaches to study bioaccessibility and bioavailability of bioactive compounds include *in vitro* methods, ex vivo techniques, in situ assays, and *in vivo* models. Advantages of each procedure are summarised in Table 1. However, comparisons between different approaches are difficult as conditions differ between them and only *in vivo* studies provide accurate values (Oomen and others 2002).

## Approaches in the Assesment of Bioaccessibility and Bioavailability of Bioactive Compounds

### Carotenoids

Carotenoids are found in fruits and vegetables as carotenes (unsatured hydrocarbons) and xanthophylls (oxygenated derivatives). Generally, the main carotenoids in vegetables are lutein, β-carotene, violaxanthin, and neoxanthin, whereas in fruits xanthophylls are usually found in a greater proportion. They are prone to isomerisation and/or oxidation due to their unsaturation (Hill and others 2012).

Only a very low proportion of carotenoids has been reported to become bioaccesible (Courraud and others 2013). In some fruits (such as mango, papaya) carotenoids are found in oil droplets in chromoplast and hydroxycarotenoids are mostly esterified with fatty acids, being more easily extracted during digestion. Carotenoids bioavailability from foods varies greatly depending on endogenous (product-related) and exogenous (process-related) factors. Amount and type of fat present in the vicinity is a key factor that affects bioaccessibility. A minimum amount of fat is neccessary for absorption (Fernández-García and others 2012), so formulation of carotenoids in an oily matrix may enhance higher bioaccessibility. Important steps in carotenoid absorption are release from the food matrix, micelle formation, uptake into mucosal cells, packing into chylomicrons, and transport within the lymphatic system.

**Table 1.** Strengths and drawbacks of *in vivo* and *in vitro* procedures used to assess bioaccessibility and bioavailability of bioactive compounds.

| | Advantages | Disadvantages |
|---|---|---|
| ***In vitro* digestion** | | |
| *Simulated gastrointestinal digestion* | Relatively inexpensive and technically simple<br>Screening of numerous samples is possible<br>Focus on small number of components<br>Specific mechanisms of action can be tested<br>Validation with reference material<br>Efficiency of each digestion, absorption or transport mechanism can be studied | Extrapolation to *in vivo*<br>Homeostatic mechanisms are not present<br>Dynamic conditions of gastrointestinal tract are not fully reproduced with biochemical and cell culture models<br>Intestinal bacteria and hepatic metabolism is not always considered<br>Oral and large intestinal phases are often not included although can readily be added<br>Closed system not responsive to composition and quantity of foods<br>Exocrine pancreas secretions not only contains pancreatin |
| *Caco-2 cells* | Phenotype is similar to normal absorptive epithelial cells<br>Grow on dish surface and on membrane inserts<br>Secretion of chylomicrons is posible | Original from human colonic adencarcinoma<br>Mucin, biofilms, and other epithelial cell types are not present |
| ***In vivo* digestion** | *In vivo* conditions<br>Selection of specific subjects<br>Pharmacokinetic studies can be performed | Lower throughput<br>Extremely complex functional systems<br>Influence of different factors<br>Extrapolation from animal studies to human<br>Certified reference standards lack<br>High cost of equipment and labor<br>Ethical constraints |

Moreover, carotenoids content might be affected by oxidative reactions during analytical procedures, so incubation time should be kept to a minimum

without affecting sensitivity. Garret and others (2000) added α-tocopherol in order to ensure protection against oxidation and thus improve carotenoids stability.

Different *in vitro* methods used in the assessment of carotenoid bioaccessibility comprise simulated gastrointestinal digestion, intestinal segments, brush-border and basolateral membrane vesicles, enterocytes, and transformed intestinal cell lines, mainly Caco-2 human cells (Table 2). Garret and others (1999) may be considered the pioneeers in the development of the Caco-2 procedure for the assesment of carotenoid bioaccessibility. The method consists of an *in vitro* digestion including a gastric and small intestinal step based on that described by Miller and others (1981) to estimate iron availability from foods. Subsequently, the digestate is filtered (which would be representative of micellarised carotenoids) and added to Caco-2 cells. To ensure that carotenoids were found in micelles, these authors filtered the aqueus fraction. They ascertained that lycopene was poorly micellarised and thus its quantity decreased after filtration, but lutein, α-carotene, and β-carotene did not change in their quantities. They also observed that hydrophobic species were efficiently micellarised when bile salts and pancreatic enzymes were combined. Furthermore, these authors found out that differentiated Caco-2 cells were able to accumulate carotenoids from mixed micelles. Further modifications were made to this method by Thakkar and others (2007) who included an oral digestion phase because of high starch content, and by Chitchumroonchokchai and Failla (2006) who added lipase and carboxyl ester lipase. These latter authors observed that xanthophyll esters were hydrolysed by carboxyl ester lipase before xanthophylls are transported into enterocytes, resulting in an enhanced cellular accumulation of zeaxanthin.

Hedrén and others (2002) also developed an *in vitro* digestion method for the estimation of carotenoid bioaccessibility (called *in vitro* accessibility) in raw and cooked carrots, which was further used in several different studies assessing carotenoid bioaccessibility (Lemmens and others 2009; Colle and others 2013). The groups carefully examined critical steps in the digestion procedure, such as the impact of added pancreatic enzymes and different bile salts amounts, along with shaking conditions used in the micellarisation step, so as to validate the

method. When bile salts were not added, β-carotene bioaccessibility decreased by about 80%, but duplicating the amount of bile salts (from 25 to 50 g/L) resulted in no additional increment of carotenoid bioaccessibility. Moreover, orbital shaking gave more reproducible results in comparison with reciprocal shaking. In contrast to data by Garret and others (1999), they estimated not only the micellarised fraction, but total carotenoids released, as the intestinal phase was not achieved by centrifugation and filtration. Courraud and others (2013) introduced an oral phase to Hedrén's method, without α-amylase as most of the matrices were nonstarchy. They obtained a significant loss of *β*-carotene only during the gastric phase and of retinyl palmitate in the oral and in the gastric phases, confirming that sensitivity of carotenoids to acidic conditions is higher than to alkaline conditions.

Reboul and others (2006) made some modifications to the method established by Garret and others (1999). BHT used as antioxidant was replaced by pyrogallol, more water soluble. Gastric pH was set at 4 instead of 2, simulating the pH in the human stomach after vegetable-rich meals ingestion. Moreover, duodenal pH was adjusted to 6 instead of 7.5 as this is the pH measured in human duodenum during digestion. Instead of 2 h of incubation time, duodenal conditions were adjusted to 30 min to approach the digestive transit time, and amount of bile salts were increased. They observed that carotenoid bioaccessibility was dependent of the different food matrix, being more bioaccessible in carrot juice and processed tomato in comparison with crude tomato and watermelon sources, which had very low accessibility. Werner and Böhm (2011) employed this procedure in the assessment of carotenoid bioaccessibility in durum wheat and egg pasta. Durum wheat pasta exhibited higher carotenoid bioaccessibility. The authors also observed that results were highly dependent on bile extract concentration and to a lesser extent on gastric pH and incubation time with digestive enzymes.

**Table 2.** Comparison of *in vitro* methods for carotenoid bioaccessibility determination.

| Step | Method | | | | | |
|---|---|---|---|---|---|---|
| | **Failla and others (2008) (adapted from Garret and others 1999)** | **Hedrén and others (2002)** | **Reboul and others (2006)** | **Granado-Lorencio and others (2007)** | **Colle and others (2010)** | **Cilla and others (2012)** |
| Food sample preparation | Homogenisation | Finely ground or cut into small pieces, with nitrogen blown | Homogenisation in saline + pyrogallol | Homogenisation with kitchen blender, 15 s, to simulate mastication | Homogenisation in saline | Homogenisation |
| Oral phase | α-Amylase, pH 6.8, 10 min, 37 ºC | | | α-Amylase, pH 6.5, 5 min, 37 ºC | | |
| Gastric phase | Porcine pepsin, pH 2.5, 1 h, 37 ºC | Porcine pepsin, pH 2, 1 h, 37 ºC | Porcine pepsin, pH 4, 30 min, 37 ºC | Mucin, bovine serum albumin, porcine pepsin, pH 1.1, 1 h, 37 ºC | Porcine pepsin, pH 4, 30 min, 37 ºC + pH 2, 30 min, 37 ºC | Porcine pepsin, pH 2, 2h, 37 ºC |
| Small intestine phase | Porcine bile extract, carboxyl ester lipase, porcine pancreatin, lipase, pH 6.5, 2 h, 37 ºC | Porcine pancreatin, porcine bile salt, pH 7.5, 30 min, 37 ºC. | Porcine bile extract, porcine pancreatin, pH 6, 30 min, 37 ºC | Bovine bile, porcine pancreatin, human pancreatic lipase, colipase, cholesterol esterase, phospolipase $A_2$, taurocholate salts, pH 7.8, 2 h, 37 ºC | Porcine pancreatin, porcine bile salt, pH 6.9, 2 h, 37 ºC. | Porcine pancreatin, porcine bile, pH 6.5, 2 h, 37 ºC. |
| Isolation of micellar fraction | Centrifugation 5000 x g, 45 min, 4 ºC, filtration | Centrifugation 5000 x g, 20 min | Centrifugation 20000 rpm, 18 h, 10 ºC, filtration | Overnight sedimentation/ Centrifugation 5000 rpm, 20 min | Centrifugation 16500 x g, 65 min, 4 ºC, filtration | Centrifugation 3300 x g, 1 h, 4 ºC |
| Cell uptake | Caco-2 cells, 4h, 37 ºC | | | | | |

As previous methods were found unsuitable for xanthophyll ester hydrolysis, Granado-Lorencio and others (2007) adapted a method originally applied to the evaluation of soil contaminants. Compared to previous *in vitro* models, these authors included the use of human pancreatic lipase, phospholipase $A_2$, cholesterol esterase, and taurocholate salts. They obtained a remainder of over 70% of carotenoids in the final digesta and observed that cholesterol esterase hydrolysed xanthophyll esters, and human pancreatic lipase did not.

The *in vitro* digestion procedure for carotenoids followed by Wright and others (2008), as adapted from Garret and others (1999) and Hedrén and others (2002) consisted of dissolving the carotenoids in the oily phase, considering thus exclusively the intestinal digestion phase, as previous research had not shown significant changes when eliminating the gastric step (Garret and others 1999). They observed that β-carotene transfer increased as did bile (from 0 up to 20 mg/mL) and pancreatin concentration (from 0 up to 4.8 mg/mL) and with pH from 3.5 to 9.

Colle and others (2010) also introduced several modifications to the method established by Hedrén and others (2002). Both the pH and transit times were adapted to closely simulate human conditions. A certain amount of lipid (0-10%) was added to tomatoes prior to the *in vitro* digestion. A significant increase of lycopene bioaccessibility was observed when 5% of lipid was additioned.

Cilla and others (2012) adapted a method used for iron bioaccessibility to determine carotenoid bioaccessibility of fruit juice-milk beverages, along with other bioactive compounds. Bioaccessibility of carotenoids was dependent of the type of milk used. Whole milk-fruit beverage led to a higher carotenoid extraction (11%) in comparison to the skimmed milk-fruit beverage.

As reported in the review by Rodríguez-Amaya and others (2010), although these models simulate human digestion closely, a better description of the food sample preparation should be carried out. Furthermore, carotenoid extraction efficiency from food and micelles should be similar, so no overestimation or underestimation of micellarisation is done. Results obtained in the different studies are shown in Table 3.

**Table 3.** Carotenoid bioaccessibility and bioavailability (%) of plant-derived products.

| Sample | Carotenoids bioavailability (%) | Method employed in the determination | Reference |
|---|---|---|---|
| Baby food meal | Lutein (24); β-carotene (13.8); α-carotene (10.4) | Simulated gastric and small intestinal digestion coupled with Caco-2 cells | Garret and others (1999) |
| Spinach purée | β-Carotene (29); lutein (27) | Simulated gastric and small intestinal digestion (Garret and others 1999) | Ferruzi and others (2001) |
| Raw pulped carrot | β-Carotene (21) | Simulated gastric and small intestinal digestion | Hedrén and others (2002) |
| Carrot puree | β-Carotene (8.9); α-carotene (4.4) | Simulated gastric and small intestinal digestion | Reboul and others (2006) |
| Spinach | Lutein (37.6); α-carotene (2.4) | Simulated gastric and small intestinal digestion | Reboul and others (2006) |
| Orange | β-Cryptoxanthin (45); zeaxanthin (43); lutein (26) | Simulated oral, gastric, and small intestinal digestion | Granado-Lorencio and others (2007) |
| Salad (tomato, spinach, carrot, lettuce and orange pepper) | Lutein (+zeaxanthin) (45.6); β-carotene (2.8); α-carotene (2.0); lycopene (1.1) | Simulated gastric and small intestinal digestion | Huo and others (2007) |
| Boiled cassava | β-Carotene (30) | Simulated oral, gastric and small intestinal digestion coupled with Caco-2 (Garret and others 1999). | Failla and others (2008) |
| Lycopene from tomato extract | Lycopene | *In vivo* single dose design | Riso and others (2010) |
| Orange fleshed melons | β-Carotene (3.2) | Simulated gastric and small intestinal digestion | Fleshman and others (2011) |
| Tomato pulp | Lycopene (2) | Simulated gastric and small intestinal digestion (Colle and others 2012) | Colle and others (2012) |
| Butternut squash | α-Carotene (17.9); β-carotene (16.5); lutein (15.9); violaxanthin (4.3) | Simulated gastric and small intestinal digestión (Garret and others 1999) | Jeffery and others (2012a) |
| Carrot | Lycopene (38.9); α-carotene (20.2); β-carotene (21.6); lutein (40.5); phytoene (64.2) | Simulated gastric and small intestinal digestion (Garret and others 1999) | Jeffery and others (2012a) |
| Grapefruit | Lycopene (4.5); β-carotene (7.9); lutein (8.7); violaxanthin (8.4); phytoene (47.1) | Simulated gastric and small intestinal digestion (Garret and others 1999) | Jeffery and others (2012a) |

| | | | |
|---|---|---|---|
| Mango | β-Carotene (31.8); lutein (13.5); violaxanthin (19.4) | Simulated gastric and small intestinal digestion (Garret and others 1999) | Jeffery and others (2012a) |
| Papaya | β-Carotene (48.5); lutein (37.3); violaxanthin (21.6); phytoene (67.8) | Simulated gastric and small intestinal digestion (Garret and others 1999) | Jeffery and others (2012a) |
| Tomato | Lycopene (1.4); β-carotene (15.5); lutein (58.6); phytoene (96.2) | Simulated gastric and small intestinal digestion (Garret and others 1999) | Jeffery and others (2012a) |
| Whole milk- fruit beverage | Neoxanthin + 9-cis-violaxanthin (47.3); zeaxanthin (14.7); lutein (13.9) | Simulated gastric and small intestinal digestion | Cilla and others (2012) |
| Soy milk-fruit beverage | Neoxanthin + 9-cis-violaxanthin (18.5); zeaxanthin (4.4); lutein (3.7) | Simulated gastric and small intestinal digestion | Cilla and others (2012) |
| Carrot juice | Lutein (22); α-carotene (1.5), β-carotene (1.5) | Simulated gastric and small intestinal digestion (Hedrén and others 2012) | Courraud and others (2013) |

The dynamic digestion TIM® system that more closely mimics *in vivo* conditions was also employed in the assessment of lycopene bioaccessibility by Déat and others (2009), followed with Caco-2 cells. At the end of the dynamic experiment (300 min), lycopene decreased by 25%, in accordance with static *in vitro* models.

Animal studies have also been designed in the assesment of carotenoid bioavailability (Zuniga and Erdman 2011; Sy and others 2012). They obtained more accurate results than with *in vitro* methods. Despite this, human carotenoid absorption and metabolism is not accurately mimicked by any animal model (Lee and others 1999). For instance, in enterocytes, β-carotene is converted to vitamin A in rodents much more efficiently than in humans. Likewise, Failla and others (2008) observed that gerbils and preruminant calves, but not ferrets, hydrolysed the ingested β-carotene to vitamin A with an efficiency similar to humans.

Many studies have examined carotenoid bioavailability in humans (Micozzi and others 1992; Castenmiller and others 1999; Tyssandier and others 2003). The most frequently used *in vivo* approach to study bioavailability of carotenoids involves the single-dose design. An increase in β-cryptoxanthin, β-carotene, and zeaxanthin plasma concentrations was observed after supplementation of blood orange juice in a long-term human study (Riso and others 2005), although this did

not exert significant effects on several markers of oxidative stress. Meanwhile, Riso and others (2010) found a low increase of lycopene, along with inter-individual variability. Interestingly, Ross and others (2011) demonstrated the fate of oral lycopene in humans in plasma, with the detection in skin for up to 42 days of lycopene and its metabolites. Goltz and others (2013) observed that carotenoid absorption increased when vegetables were consumed in a single meal rather than over multiple meals.

Borel and others (1998) demonstrated a high correlation between *in vitro* carotenoid bioaccessibility, *in vivo* observations and with results from bioavailability trials with human subjects. Therefore, *in vitro* models may constitute a less tedious and less costly alternative to *in vivo* studies in the assessment of carotenoid bioaccessibility.

Studies show that percentages of bioaccessibility and bioavailability of the different carotenoids vary widely. Lutein was more readily solubilised than α-carotene, β-carotene, and lycopene (Garret and others 1999), probably because oxycarotenoids are more hydrophilic than hydrocarbon carotenoids and to different subcellular location and molecular interactions in plant foods. Sy and others (2012) also obtained a high recovery of lutein and astaxanthin, whereas lycopene was the least abundantly recovered. However, Jeffery and others (2012a) reported for the first time a high phytoene bioaccessibility, several times that of other carotenoids, followed by lutein in carrot and tomato and β-carotene in papaya and mango. In human studies, Tyssiander and others (2003) reported greater bioavailability of lutein and β-carotene compared to lycopene.

Furthermore, dietary fat appears to be necessary for the efficient solubilisation of lipophilic compounds. In this line, Failla and others (2008) demonstrated an increase in carotenes when triglycerides were added to a carotenoid-rich salad, in accordance with Hedrén and others (2002), who observed a significant increase in β-carotene bioaccessibility after oil addition. Qian and others (2012) found the lowest bioaccessibility of β-carotene (0%) when orange oil was used as the carrier lipid, probably because flavored oils do not contain triacylglycerol components and thus cannot be digested into free fatty acids. Moreover, Borel and others (1998) demonstrated that β-carotene

incorporated into chylomicrons higher in meals with long-chain rather than medium-chain triglycerides. For this reason, Jeffery and others (2012b) used yogurt as a lipid source with long-chain triglycerides. Human studies have also prooved the importance of lipid in the absorption of dietary lutein. On this subject, Mamatha and others (2011) obtained a higher plasma lutein level in rats when lutein was solubilised in mixed micelles with fat. Brown and others (2004) also observed that consumption in humans of full-fat salad dressing enhanced a higher carotenoid bioavailability than reduced-fat salad dressing.

Interestingly, not only lipid amount, but also qualitative lipid profile has its influence in carotenoid bioavailability (Goltz and Ferruzzi, 2013). Monounsatured fatty acids promote a higher carotenoid bioavailability than polyunsatured fatty acids, as demonstrated by Clark and others (2000) in mesenteric lymph duct cannulated rats. This was further observed by Gleize and others (2013), who found that bioaccessibility of the xanthophylls lutein and zeaxanthin was higher with saturated fatty acids than with monounsaturated and polyunsatured fatty acids both in an *in vitro* digestion model followed by Caco-2 cell study and *in vivo* in orally administered rats. Furthermore, long-chain triglyceride increased the β-carotene bioaccessibility in comparison with medium-chain triglyceride in a simulated intestinal digestion (Salvia-Trujillo and others, 2013).

Effect of pH on the transfer efficiency of carotenoids is also of importance, as suggested by *in vitro* results. Wright and others (2008) demonstrated an increase in the β-carotene transfer to the aqueous phase under higher pH conditions, while Jeffery and others (2012b) obtained a positive correlation of β-carotene and phytoene with food pH. However, this hypothesis can be rejected in *in vivo* methods, because there is no significant meal effect on stomach pH (Tyssandier and others 2003).

Contrary to expectation, several authors (Parada and others 2007; Courraud and others 2013) have demonstrated that technological processes such as cooking of vegetables increase carotenoid bioavailability by disruption of the natural food matrix during food processing. However, severe thermal treatment or inadequate storage may cause isomerisation during the formation of by-products that can, in turn, reduce the absorption of desirable bioactive compounds.

**Polyphenolic compounds**

Phenolic compounds or polyphenols form a large group of chemical substances considered as secondary metabolites of plants. They have an aromatic ring and a benzene ring with one or more hydroxide groups, including phenolic acids (hydroxy-benzoic acids and hydroxy-trans-cinnamic acids), coumarins, flavonoids (flavones, flavonols, flavanones, flavanolols, flavanols, and anthocyanidins), isoflavonoids, lignans, stilbenes, and phenolic polymers (proanthocyanidins and hydrolysable tanins) (Craft and others 2012). Among the various phenolic compounds, bioavailability appears to differ greatly and the most abundant ones in our diet don't necessarily correspond to those with best bioavailability profile. Absorption and metabolism of polyphenolic compounds are determined primarily by their physicochemical characteristics. For example, molecular size, their basic structure, degree of polymerisation or glycosylation, solubility, and conjugation with other phenolics can be considered critical factors. Phenolic acids with small-molecular weight such as gallic acid and isoflavones are easily absorbed through the tract, as well as flavones, catechins, and quercetin glucosides (Martin and Apple 2010). On the contrary, large polyphenols such as proanthocyanidins are poorly absorbed. Most proanthocyanidins are degraded into monomer or dimer units before being absorbed (Hackman and others 2008).

In plant products, most of the phenolic compounds are found as glycosylated forms or as esters or polymers that must be hydrolysed by intestinal enzymes or microflora before the released aglycones can be absorbed. However, anthocyanins can be absorbed as glycosides and appear as such in blood (D'archivio and others 2007). Metabolism is another factor, strongly affecting their bioavailability. Generally, after absorption, polyphenols undergo biotransformations of phase I and II into 3 main O-sulfated, O-glucuronidated, and O-methylated forms. Despite this, anthocyanins do not appear to undergo extensive metabolism. Neither do galloylated monomeric flavonols such as epigallocatechin and epicatechin gallate, which may appear unconjugated, at least to a large extent, in the systemic circulation (Cermak and others 2009). Thus, the structure of the resulting metabolites could be totally different from the parent

compounds, and they may or not exert their biological action (Denev and others 2012). Results published by Vitaglione and others (2007) suggested that protocatechuic acid, which can be absorbed both from the small and large intestine, may be the metabolite involved in the activity observed after the intake of cyanidin-3-glucoside, whose absorption and excretion are reported to be below 1% of intake. Therefore, evaluation of polyphenol bioavailability should include the analysis not only of native compounds, but also their metabolic products. Technological processes may also affect bioavailability of phenolic compounds, showing a significant increase of chlorogenic acid and naringenin in plasma levels when consuming cooked tomato in comparison with the fresh product (Bugianesi and others 2004).

Despite the great variability of this group of substances, along with their occurrence in plant materials as a complex mixture, experiments reported in the literature have analysed bioavailability of polyphenolic compounds with different chemical structures and solubility through *in vitro* and *in vivo* assays, as shown in Table 4. The most widely used procedure for screening polyphenolic compound bioaccessibility is the *in vitro* static gastrointestinal method. Gil-Izquierdo and others (2001) may be considered the piooneers in adapting the method established by Miller and others (1981) to simulate human digestion and absorption of dietary iron in the study of phenolic compound release. During the intestinal phase, a cellulose dialysis tubing is used to simulate intestinal absorption. The main modification introduced by Gil-Izquierdo and others (2002) was the placement of the food and cellulose dialysis tubing in a polyethylene tube to assure close contact between food and membrane, reaching faster equilibration of pH values and thus a faster liquid exchange. They observed that phenolic composition was not affected by pepsin digestion in any of the assayed food products.

**Table 4.** Comparison of *in vitro* methods for polyphenolic bioaccessibility determination.

| Step | Method | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Gil-Izquierdo and others (2001)** | **Bouayed and others (2011)** | **Dupas and others (2006)** | **Bermúdez-Soto and others (2007)** | **Gawlik-Dziki (2012)** | **Shim and others (2012)** | **Chen and others (2013)** |
| Food sample preparation | Homogenisation | Homogenisation | | | Homogenisation and centrifugation | Homogenisation in saline | Homogenisation |
| Oral phase | | | | | Mucin, α-amylase, 10 min, 37 ºC | Amylase, pH 6.9 5 min, 37 ºC | |
| Gastric phase | Porcine pepsin, pH 2, 2 h, 37 ºC | Porcine pepsin, pH 2-2.5, 1 h, 37 ºC | Porcine pepsin, pH 2, 1 h, 37 ºC | Porcine pepsin, pH 2, 2 h, 37 ºC | Porcine pepsin, pH 1.2, 1 h, 37 ºC | Porcine pepsin pH 2, 1 h, 37 ºC | Porcine pepsin, pH 2, 1 h, 37 ºC |
| Transition step | | pH 6.5, 45 min, 37 ºC. | Cooling in ice for 10 min | | pH 6 | pH 5.3 | pH 5.3 |
| Small intestine phase | Porcine bile extract, porcine pancreatin, lipase, pH 7, 2 h, 37 ºC | Porcine bile extract, porcine pancreatin, pH 7-7.5, 2 h, 37 ºC | Porcine bile extract, porcine pancreatin, pH 6, 2 h, 37 ºC | Porcine bile extract, porcine pancreatin, pH 7, 2 h, 37 ºC | Porcine bile extract, porcine pancreatin, pH 7, 2 h, 37 ºC | Porcine bile extract, porcine pancreatin, lipase pH 7, 2 h, 37 ºC | Porcine bile extract, porcine pancreatin, taurodeoxycholate, taurocholate, pH 7.4, 2.5h, 37ºC |
| Separation | Dialysis in a semipermeable cellulose membrane simultaneously with intestinal phase | Dialysis in a semipermeable cellulose membrane simultaneously with intestinal phase | | Filtration | Dialysis in a semipermeable cellulose membrane 2 x 2h, 37 ºC | Centrifugation a 3000 rpm, 30 min 4 ºC. | |
| Cell uptake | | | Caco-2 cell | | | | |

This method has been employed in the screening of multiple foods, including orange juice (Gil-Izquierdo and others 2002), pomegranate juice (Pérez-Vicente and others 2002), broccoli (Vallejo and others 2004), soymilk (Rodríguez-Roque and others 2013) and gooseberry (Chiang and others 2013) among other foods. These authors found that gastric digestion increased polyphenolic concentration, whereas the duodenal fraction significantly diminished polyphenolic content and even more so in the dialysed fraction. Results are shown in Table 5.

**Table 5.** Polyphenolic bioaccessibility and bioavailability (%) of plant-derived products.

| Sample | Polyphenolic bioavailability (%) | Method employed in the determination | Reference |
|---|---|---|---|
| Orange juice (soluble fraction) | Narirutin (10.5); hesperidin (16.2); total flavanones (12.0), vicenin-2 (18.6) | Simulated gastric and small intestinal digestion with cellulose dialysis tubing | Gil-Izquierdo and others (2001) |
| Orange juice (soluble fraction) | Narirutin (23.4); hesperidin (24.0); hesperetin (21.1); total flavanones (23.5), vicenin-2 (24.5) | Simulated gastric and small intestinal digestion with cellulose dialysis tubing | Gil-Izquierdo and others (2002) |
| Strawberry | Cyanidin-3-glucoside (6.6), pelargonidin-3-glucoside (12.6); pelargonidin-rutinoside (11.7); ellagic acid-arabinoside (20.6); ellagic acid (172.8); quercetin-3-glucoside (28.3); kaempferol-3-glucoside (27.4) | Simulated gastric and small intestinal digestion with cellulose dialysis tubing | Gil-Izquierdo and others (2002) |
| Strawberry jam | Cyanidin-3-glucoside (2.3), pelargonidin-3-glucoside (3.7); pelargonidin-rutinoside (3.8); ellagic acid-arabinoside (6.1); ellagic acid (9.7); quercetin-3-glucoside (6.1); kaempferol-3-glucoside (12.0) | Simulated gastric and small intestinal digestion with cellulose dialysis tubing | Gil-Izquierdo and others (2002) |
| Coffee | Chlorogenic acid (traces); benzoic acid (traces) | *In vivo* digestion in rats | Dupas and others |

| | | | |
|---|---|---|---|
| | | | (2006) |
| Chokeberry | Cyanidin-3-glucoside (56.7), cyanidin (0), quercetin 3-glucoside (81.2), quercetin (0), neochlorogenic acid (71.6), chlorogenic acid (123.6). | Simulated gastric and small intestinal digestion followed by filtration | Bermúdez-Soto and others (2007) |
| Tixia gooseberry | Caffeic acid (44.1), epigallocatechin gallate (28.1), kaempferol (100.2), *ρ*-coumaric (61.0), pelargonidin chloride (96.6), quercetin hydrate (516.3), resveratrol (58.1), rutin (95.3). | Simulated gastric and small intestinal digestion according to Gil-Izquierdo and others (2001) | Chiang and others (2013) |
| Invicta gooseberry | Caffeic acid (59.5), kaempferol (82.8), *ρ*-coumaric (73.4), quercetin hydrate (154.7), resveratrol (94.6), rutin (101.0). | Simulated gastric and small intestinal digestion according to Gil-Izquierdo and others (2001) | Chiang and others (2013) |

Further modifications (Villanueva-Carvajal and others 2013) included the use of crushed ice after each digestion phase to ensure the end of enzymatic activity. These authors also studied particle size and concluded that this was inversely proportional to phenolic release, so the enlargement of the contact area could improve digestion efficiency with an absorption increase of polyphenols.

Bermúdez-Soto and others (2007) removed the employment of the dialysis membrane during intestinal digestion as substantial losses were observed of some of the phenolic compounds. They simply determined polyphenolic compounds after separation by filtration. During gastric digestion no significant changes were observed in the stability of polyphenols in chokeberry, but anthocyanins were increased, due to the low pH after the gastric step. During intestinal digestion a significant decrease in anthocyanins (43%) and flavonols (26%) was observed, whereas chlorogenic acid increased (24%). This method was further employed by Tagliazucchi and others (2010) in the assessment of grape polyphenols, with the addition of an oral phase. Furthermore, once the pancreatic digestion was finalised, samples were taken to pH 2 to ensure the stability of phenolic

compounds. Differently, these authors observed an increase in the bioaccessibility of total polyphenols, flavonoids, and anthocyanins during the gastric digestion in grape, while intestinal digestion caused a decrease in all classes of polyphenols.

Bouayed and others (2011) also developed a method to assess free soluble polyphenols potentially available for further uptake than from Miller's method. They found out that after simulated gastrointestinal digestion of apples, polyphenols release was mainly achieved during the gastric phase. Subsequently, a further increase (<10%) in total phenolics and flavonoids was obtained after the intestinal phase. This increase may be due to the additional time of extraction along with the effect of intestinal enzyme on the complex food matrix, which facilitates the release of phenolics bound to the matrix. Results showed a dialysability of 40% for flavonoids and 55% for free soluble phenolics, respectively, in comparisson with their undigested counterparts in apples. Regarding anthocyanins, these authors could not measure them after gastric and intestinal digestion, probably because they degradate in alkaline intestinal environment. Further studies carried out by Bouayed and others (2012) found that phenolic compounds in the gastric or intestinal medium were approximately similar (chlorogenic acid), higher (phloridzin and quercetin 3-*O*-glucoside) or lower (*ρ*-coumaric acid) compared to those found in fresh apples, in accord with results obtained by Chiang and others (2013). Polyphenol concentration decreased during dialysis through the semipermeable cellulose membrane, although all polyphenols in the intestinal medium were dialysable, which could be indicative of passive diffusion, an important mechanisms for cellular polyphenol uptake, at least for several aglycones.

Gawlik-Dziki and others (2012) carried out an *in vitro* digestion including an oral phase. Dialysis sacks were added after 2 h of intestinal digestion, for a total time of 4 h. They also obtained a decrease of phenolic compounds in the dialysate. Shim and others (2012) also included an oral phase. However, they did not use dialysis sacks but centrifugation. They used the *in vitro* method to compare phenolic bioaccessibility in different parts of *Smilax china* and obtained 36.4, 17.8, and 9.9% of the remaining total polyphenols after digestion of leaf, root, and stem, respectively.

Chen and others (2013) carried out an *in vitro* digestion model according to the method established for carotenoid bioavailablity in the assessment of 9 commercially available tea juices. After the gastric phase there was a significant decrease in total polyphenol content of 5 of the juices. After the duodenal phase, a further increase in the total polyphenol content was obtained in 4 of the juices, possibly due to structural transformation of polyphenols.

However, these methods did not include a colonic phase and polyphenols may be metabolised by the colonic microflora. For this reason, Saura-Calixto and others (2007) estimated the bioaccessibility of dietary polyphenols with the isolated indigestible fraction (small intestine bioaccessibility) and a colonic fermentation of this fraction (large intestine bioaccessibility). Bioaccessibility of polyphenols in the large intestine was calculated by the difference of polyphenol contents between the total indigestile fraction and the residue after the fermentation; and 48% of dietary polyphenols were estimated bioaccessible in the small intestine, while 42% became bioaccesible in the large intestine. Only 10% was not accessible and remained in the food matrix after the entire digestion process. Furthermore, Nordlund and others (2012) used an *in vitro* colon model to study the formation of phenolic microbial metabolites from rye, wheat, and oat bran. The major metabolites found were hydroxylated phenylpropionic acid metabolites, closely related to the ferulic acid content in the cereal samples.

The dynamic gastrointestinal model (TIM®) has also been extensively used to measure phenolic bioaccessibility. Colonic fermentation experiments may be incorporated in this model, so the assessment of polyphenol bioaccessibility may be more reliable. This model, which mimicks the biological environment through the duodenum, jejunum, and ileum was employed in the monitorisation of anthocyanins stability and bioaccessibility in maqui berry and wild blueberry (Lila and others 2012). These authors observed that after intake, most anthocyanins were bioaccessible between the second and third hours. López de Lacey and others (2012) also used a dynamic gastrointestinal model to study the bioaccessibility of green tea polyphenols incorporated into agar. Their results revealed that the polyphenols incorporated in the agar were bioaccessible, and consequently available for absorption. Furthermore, the gelatin used to simulate

the presence of protein during the digestion partly reduced green tea flavonols bioaccessibility.

However, certain transport mechanisms such as unidentified stomach active transport or the transport in the small intestine of flavonoids through interaction with the sodium-dependent glucose transporter are not considered with the *in vitro* digestion method (Bermúdez-Soto and others 2007). For this reason, other methods have been developed.

Bioaccessibility studies using Caco-2 cells have been conducted (Dupas and others 2006; Fernandes and others 2012). Glucuronidation, sulfation and methylation processes carried out by polyphenols can be studied using these cells. Yi and others (2006) found that by growing on Transwell membranes Caco-2 cells, anthocyanins could be degraded and demethylated during absorption and transport. Epigallocatechin was minimaly uptaken in the human intestinal Caco-2 cell model (Vaidyanathan and Walle 2003), in accordance with Hong and others (2002), who observed a poor uptake of epigallocatechin gallate by HT-29 human colon adenocarcinoma cells. Neilson and others (2010) used this method to compare the efficiency of dimer absorption compared to monomers of catechin. In adittion, an assesment of proanthocyanidin transport showed that oligomers of 6 units were transported approximately 10-fold less across a layer of Caco-2 cells than radiolabeled monomers, dimers, and trimers (Déprez and others 2001). A deeper study was carried out by Wang and others (2013) in which grape seed phenolic extract was subjected to *in vitro* gastrointestinal digestion and ileal fermentation, followed by Caco-2 cells assay. Only microbial metabolites, but not original phenolic compounds passed through the Caco-2 cell layer.

Some polyphenols may be metabolised by Caco-2 cells, which must be taken into account. Ferulic acid-sulfate, synaptic acid-sulfate, *ρ*-coumaric acid-sulfate, and methyl ferulate-sulfate were generated after 24-h exposure of hydroxycinnamates to differentiated Caco-2 cells according to Kern and others (2003). Meanwhile, Yi and others (2006) suggested a degradation and demethylation of anthocyanins from blueberries during absorption and transport by Caco-2 cells.

Further assays include the use of the Ussing chamber, where a small section of intestinal mucosa is situated between two chambers with buffer solution, preserving the epithelial polarity (Clarke, 2009). Not only passive diffusion but transporter-mediated, transcellular, paracellular, and endocytosis transport can be measured. Bergmann and others (2009) employed the Ussing chamber in order to study the intestinal transport of polyphenols in apples. They used monolayers of the T84 colon carcinoma cell line and found that the transport of various hydroxycinnamic acids and flavonoids depended on the polarity. Cardinali and others (2013) also used colonic cells in a Ussing chamber and obtained a bioaccessibility of 0.1% of the polyphenol verbascoside. Moreover, Erk and others (2013) observed that the absorption of cofee polyphenols in the jejunum is governed by their physicochemical properties when they used pig jejunal mucosa in the Ussing chamber.

*In situ* studies have also been carried out in the assessment of polyphenolic bioavailability. Wang and others (2011) followed this procedure in the study of total flavonoid extracts, with the inclusion of liver perfusion, in order to determine flavonoid metabolism. This method was also used by Fong and others (2012) to study the metabolism and absorption of flavones from herbs using rat intestines. This way they found out that acetaminophen, (-)-epicatechin, piperine, and mainly curcumin could significantly inhibit the intestinal metabolism of the flavone baicalein and subsequently increase its absorption.

Meanwhile, *in vivo* studies were carried out to test the bioaccessibility of polyphenols in rats (Dupas and others 2006; Mateos-Martín and others 2012), pigs (Lesser and others 2006; Walton and others 2006), and dogs (Reinboth and others 2010). This way, Gonthier and others (2003) did not detect parent compounds or catechin derivatives in the plasma of rats given purified procyanidins. Crespy and others (2002) also used Wister rats to determine that quercetin, but not its glycosides, was absorbed from the rat stomach. Disparity in the results between *in vitro* data and epidemiological studies are likely atributed to the physicochemical characteristics of polyphenols. Bioavailability in rodent studies has been estimated to be over 10% of ingested dose, ranging from 2 to 20%. Interestingly, quantification of the flavonol quercetin and its main

methylated metabolites (isorhamnetin and tamarixetin) by Surco-Laos and others (2011) in a *Caenorhabditis elegans* model revealed that higher levels of quercetin plus metabolites were present in the worm's organism than those of isorhamnetin or tamarixetin plus their respective metabolites. This observation suggests that greater capacity of quercetin uptake than of methylated derivatives by the nematode exists, although quercetin is further transformed by *Caenorhabditis elegans* to a greater extent than isorhamnetin or tamarixetin.

With reference to human studies, these are limited as large population sizes are necessary. Nevertheless, Manach and others (2005) reported plasma concentrations of phenolic metabolites of 0-4 μmol/L after 97 human volunteers ingested 50 mg aglycone equivalents. Russell and others (2009) recovered in the urine 26-27% of the major free benzoic acids (gentisic, protocatechuic, and ρ-hydroxybenzoic) and the major conjugated acid (syringic acid), detected in plasma within 5 h after consumption of a single dose of a portion of strawberries. Research carried out by Hackman and others (2008) showed a rapid transport into blood of metabolites, in a dose-dependent manner, with peak plasma concentrations at 1 to 2.5 h after ingesting a flavanol-rich food, reaching baseline levels within 8 h. Colonic microflora metabolised most of the flavanols not absorbed in the small intestine to a variety of derivatives of phenolic acid and valerolactone, able to be absorbed. After 48 h of incubation with human colonic microflora, procyanidins of 6 units were degraded into low-molecular-weight aromatic acids (Deprez and others 2000). The wide variability of results obtained by Suárez and others (2011) indicated a high dependence on the individual in the absorption and metabolism of olive oil phenols.

Moreover, although some *in vitro* studies suggest the degradation of anthocyanins in the intestinal phase, under *in vivo* conditions direct absorption of anthocyanins may take place in the stomach (Manach and others 2004). Bioavailability of anthocyanins has been demonstrated to be lower than that of other flavonoids, and according to Yang and others (2011), generally less than 1% of the consumed amounts (180-215 mg/day) is absorbed. They are absorbed by different mechanisms in the stomach and small intestine involving specific enzymes, such as bilitranslocase (Passamonti 2002). They subsequently enter the

circulatory system within 15-60 min, after passing through the liver, and are distributed to different tissues, with a maximum concentration of nanomolar levels. Mostly, anthocyanins reach the colon and are extensively metabolised there by bacteria, contributing therefore to their bioavailability (Hidalgo and others 2012).

Among the isoflavones, genistein, daidzein, and glycitein are the most active compounds found in soybeans. Equol is a highly bioavailable metabolite that comes from diadzein and exhibits higher activity than the original isoflavone (Kanazawa 2011). Using a Caco-2 cell model, Simmons and others (2012) found that the lipid source and amount did not affect bioaccessibility of isoflavones. However, transport across the monolayer was greater with shorter molecules. The *in vivo* human study carried out by Shinkaruk and others (2012) revealed that the bioavailability of glycitein from soy-based food was similar to that of daidzein and its urinary excretion was significantly higher than that of genistein.

**Glucosinolates**

Glucosinolates have gained much attention as food compounds of high dietary value due to its alleged beneficial effect in cancer prevention (Fimognari and others 2002). Nearly all of the biological activities of these compounds may be attributed to their hydrolytic products, of which the isothiocyanates are prominent examples. Glucosinolates are hydrolysed into isothiocyanates mediated by myrosinase, which is still active in fresh vegetable products, and by the bacterial microflora of the gastrointestinal tract. Antibiotic treatment along with inactivation of the plant myrosinase (after cooking, for example) causes a decrease in bioavailability, as indicated by the fact that bioavailability is greater following ingestion of myrosinase-containing versus myrosinase-lacking preparations (Dinkova-Kostova and others 2012). One of the most extensively studied isothiocyanates is sulforaphane whose glucosinolate precursor is glucoraphanin, abundant in broccoli. In humans, metabolisation of isothiocyanates occurs via the mercapturic acid pathway.

Simulated static gastrointestinal digestion, dynamic gastrointestinal digestion, Caco-2 uptake, transport assays, and/or *in vivo* studies with animals and with

humans have all been used as glucosinolate bioaccessibility and bioavailability screening methods. However, colonic fermentation is essential for the absorption of isothiocyanates, which must be taken into account. Recent evidence (Peñas and others 2012) suggests that certain strains of *Lactobacillus spp., L. mesenteroides* and *L. plantarum,* were capable of digesting *in vitro* glucosinolates. As a result, in human studies, degradation of glucosinolates to isothiocyanates exhibited high inter-individual variation beacuse of colonic microflora differences (Rungapamestryi and others 2007). It is important to note that urinary isothiocyanate metabolite (dithiocarbamate) excretion decreases from 47% to a neglible amount when bowel microflora is reduced by mechanical and antibiotics.

As a result of the importance of colonic fermentation, few *in vitro* studies have been carried out towards the assessment of glucosinolate bioaccessibility. Despite the fact that mastication of cooked vegetables liberates glucosinolates, and mastication of fresh plants additionally causes enzymatic hydrolysis of glucosinolates, no *in vitro* research has yet been conducted on the impact of these process. Vallejo and others (2004) carried out a simulated *in vitro* digestion which consisted in a gastric phase followed by an intestinal phase that included a cellulose dialysis tubing, as described previously by Gil-Izquierdo and others (2001) for determinig phenolic bioaccessibility. These authors reported a high loss of glucosinolates (69%) under gastric conditions of homogenised fresh broccoli inflorescence. However, Iori and others (2004) suggested that the previous article had underestimated the degradative activity of myrosinase, still active in the uncooked broccoli. Consequently, stability of glucosinolates under pepsin digestion is considered quite high, as reported by Maskell and others (1994) who obtained after simulated gastric digestion an overall drop of total glucosinolates of only 14%. Progoitrin and gluconapoleiferin showed greater susceptibility to peptic digestion than gluconapin or glucobrassicin, and 4-hydroxyglucobrassicin became undetectable. Differences between the results obtained with the different inocula employed (Table 6) were minor. After 4 h of small intestine simulated digestion, the loss of the total glucosinolates was 32%. Lai and others (2010) effectuated an *in vitro* simulated digestion of glucoraphanin in the upper gastrointestinal tract, along with an *ex vivo* study using rat cecal microbiota and

an *in situ* rat cecum assay. The *in vitro* study confirmed that glucoraphanin was not degraded by upper gastrointestinal digestive enzymes, consequently reaching the rat cecum intact. Meanwhile, in both *in situ* and *ex vivo* procedures, glucoraphanin was hydrolysed to sulforaphane by F344 rat cecal microbiota and able to cross the cecal enterocyte for systemic absorption.

A dynamic computer-controlled *in vitro* large-intestinal model was designed by Krul and others (2002), with the inoculation of complex microflora of human origin, a semi-permeable membrane, and pH continously adjusted to 5.8. They observed peak levels of allyl isothiocyanate 9-12 h after the addition of sinigrin, which accounts for 1% of the degraded sinegrin. Slightly higher values were obtained by Getahun and Chung (1999) who incubated human feces with cooked watercress juice for 2 h. They found that 18% of total glucosinolates were hydrolysed into isothiocyanates.

**Table 6.** Comparison of *in vitro* methods for glucosinolates bioaccessibility determination.

| Step | Method | | |
|---|---|---|---|
| | **Maskell and others (1994)** | **Vallejo and others (2004)** | **Lai and others (2010)** |
| Food sample preparation | | Homogenisation | |
| Oral phase | - | - | Amylase, 3 min, 37 ºC |
| Gastric phase | Porcine pepsin, pH 2, 4 h, 37 ºC. | Porcine pepsin, pH 2, 2 h, 37 ºC | Porcine pepsin, pH 2, 2 h, 37 ºC |
| Transition step | Centrifugation, 1000 rpm, 20 min | | |
| Small intestine phase | Innocula of small intestine of pig fed with rapeseed meal/ soya-bean meal or commercial diet/Porcine pancreatin, pH 6, 1-4 h, 37 ºC | Porcine bile extract, porcine pancreatin, lipase, pH 7, 2 h, 37 ºC | Porcine bile extract, porcine pancreatin, pH 7.5, 2 h, 37 ºC |
| Separation | Centrifugation, 1000 rpm, 20 min | Dialysis in a semipermeable cellulose membrane simultaneously with intestinal phase | Hydrolysation with myrosinase |

**Table 7.** *In vivo* studies regarding glucosinolate bioavailability (%) of plant-derived products.

| | Study desing | Participants | Sample preparation | Measurements | Major findings |
|---|---|---|---|---|---|
| Shapiro and others (2001) | Single dose study | Inpatient and outpatient volunteers of a hospital. | Uncooked fresh sprouts and homogenates of boiled sprouts devoid of myrosinase activity with either glucosinolates only or isothiocyanates only. | Urine was collected throughout the entire study in 8-h collection intervals | -No isothiocyanates were found in urine.<br>-Dithiocarbamates were the major metabolites in urine.<br>-Myrosinase activity in intact sprouts contributed significantly to bioavailability by boosting conversion to isothiocyanate. |
| Gasper and others (2005) | Single oral doses of 16 or 52 mmol of isothiocyanates (standard broccoli and high-glucosinolate broccoli). | 16 Healthy subjects | Individual soup portions of 2 cultivars prepared by cooking 100 g florets with 150 mL water for 90 s on high power in a 700-W microwave oven followed by homogenisation. | Liquid chromatography linked to tandem mass spectrometry to quantify sulforaphane and thiol conjugates in plasma and urine. | -GSTM1 (glutathione S-transferase M1 allele) genotypes had a significant effect on the metabolism of sulforaphane. |
| Clarke and others (2011) | Single oral doses of 161-221 mmol of glucosinolates, with or without active myrosinase. | 12 Healthy subjects | Samples studied were fresh broccoli sprouts with active myosine and commercially available broccoli supplement and were designed to be indistinguishable from each other. | Blood and urine samples were collected for 48 h during each phase and analysed for sulforaphane and erucin metabolites using LC–MS/MS. | -Bioavailability of sulforaphane and erucin is dramatically lower and delayed in time when subjects consume broccoli supplements compared to fresh broccoli sprouts.<br>-Broccoli supplements devoid of myrosinase activity did not produce equivalent plasma concentrations of the bioactive isothiocyanate metabolites compared to broccoli sprouts. |
| Egner and others (2012) | Multiple dose study of 800 mmol of glucoraphanin or 150 mmol of sulforaphane for 7 days. | 50 Healthy subjects | Re-hydrated, previously lyophilised broccoli sprout powders rich in either glucoraphanin or sulforaphane. | Sulforaphane and sulforaphane metabolites in overnight (roughly 12 h) urine samples. | -70% of the administered sulforaphane was eliminated in 24 hours.<br>-Only 5% of the administered glucoraphanin was recovered as sulforaphane metabolites.<br>-Bioavailability of sulforaphane was far superior to glucoraphanin extracts. |

Intact glucosinolate and its metabolites in feces were lowly recovered in animal studies, using different species, suggesting substantial absorption and metabolism of these compounds (Slominski and others 1988; Conaway and others 1999). In an *in vivo* animal study published by Hanlon and others (2008), rats were administered sulforaphane in either a single intravenous dose (2.8 mmol/kg) or single oral doses of 2.8, 5.6, and 28 mmol/kg. This compound was well and rapidly absorbed, with an absolute bioavailability of 82%, which decreased at higher doses, indicating a dose-dependent pharmacokinetic behaviour.

In an *in vivo* human study, Rouzaud and others (2004) observed that isothiocyanates release was delayed when ingesting cooked cabbage and, therefore, suggested that glucosinolates passed through the upper digestive tract without modification. Furthermore, Riso and others (2009) carried out an *in vivo* human cross-over intervention study (broccoli diet versus cruciferous-free diet). They observed an increase of isothiocyanate plasma concentrations, while the intervention did not affect plasma glucosinolate activity. Other *in vivo* studies discussed in Table 7 firmly established that, compared to isothiocyanates, intake of glucosinolates is associated with lower bioavailability, slower elimination, and greater inter-individual variation in excretion. Overall, the large inter-individual variability of conversion of glucosinolates to urinary dithiocarbamates is evident following administration of either single or multiple doses of glucosinolates, and ranges between 1% and more than 40% of the dose. Interestingly, there are also diurnal variations: conversion of glucosinolates to dithiocarbamates is greater during the day, whereas conversion of isothiocyanates to dithiocarbamates is more efficient during the night (Fajey and others 2012).

**Vitamin E**

Vitamin E is actually a family of molecules, which include the tocopherols and the tocotrienols, all of them with important antioxidant properties and health benefits.

Alpha-tocopherol exhibits the highest biological activity and molar concentration of lipid-soluble antioxidants in the human.

A handful of *in vitro* and *in vivo* assays have been conducted on the determination of vitamin E bioaccessibility and bioavailability, as shown in Table 8. It is important to note that during digestion, vitamin E must be packaged into micelles to facilitate absorption, the same as carotenoids. Therefore, Reboul's simulated gastrointestinal digestion procedure (Reboul and others 2006) employed in the assessment of carotenoids is also used to study vitamin E bioaccessibility, with subsequent centrifugation and filtration steps.

**Table 8.** Comparison of *in vitro* methods for vitamin E bioaccessibility determination.

| Step | Method | | | |
|---|---|---|---|---|
| | **Reboul and others (2006)** | **O'Callaghan and others (2010)** | **Werner and Böhm (2011)** | **Mandalari and others (2013)** |
| Food sample preparation | Homogenisation in saline + pyrogallol | Homogenisation | Homogenisation in saline + pyrogallol | |
| Oral phase | | | Amylase, pH 6.5, 5 min, 37 ºC | Amylase, pH 6.9 |
| Gastric phase | Porcine pepsin, pH 4, 30 min, 37 ºC | Porcine pepsin, lipase, pH 4, 1 h, 37 ºC | Porcine pepsin, pH 3.5-4.5, 0.5 h, 37 ºC | Porcine pepsin, 37 ºC |
| Transition step | | pH 5.4 | | |
| Small intestine phase | Porcine bile extract, porcine pancreatin, pH 6, 30 min, 37 ºC | Porcine pancreatin, pH 7.8, 2 h, 37 ºC | Porcine bile extract, porcine pancreatin, pH 6.5-7, 0.5 h, 37 ºC | Porcine bile extract, porcine pancreatin, lecithin, cholesterol, sodium taurocholate pH 7, 2 h, 37 ºC |
| Separation | Centrifugation 20000 rpm, 18 h, 10 ºC + Filtration | Ultracentrifugation 200 000 g, 95 min + Filtration | Centrifugation 4000 rpm, 20 min, 10 ºC + Centrifugation 14000 rpm, 5min, 22 ºC + Filtration | Centrifugation, 3700 rpm, 15 min, 7 ºC. |
| Cell uptake | | Caco-2 cell | | |

Desmarchelier and others (2013) followed Reboul´s *in vitro* digestion, with palm oil as added fat. These authors showed that α-tocopheryl acetate was distributed between mixed micelles (36%), liposomes (9%), and nonsolubilised food debris (52%). Furthermore, they followed the *in vitro* digestion by uptake studies using Caco-2 cells. These cells were able to hydrolyse α-tocopheryl acetate and to uptake α-tocopherol when α-tocopheryl acetate was incorporated into mixed micelles but not into emulsions. Werner and Böhm (2011) extended Reboul´s method by an oral phase. Overall, results obtained by these authors were highly dependent on the amount of bile extract present in the digestive medium and to a lesser extent on the simulated gastric pH and the incubation time with digestive enzymes. Bioaccessibility of β-tocotrienol was found to be higher than that of α-tocotrienol.

Depending on the dietary source, the bioaccessibility of vitamin E has been shown to vary widely. O'Callaghan and others (2010), who used *in vitro* simulated gastrointestinal digestion coupled with Caco-2 cells, obtained bioaccessibility values of α-tocopherol ranging from 11% in apple sauce to 86% in beef. Likewise, Reboul and others (2006) reported a 100% bioaccessibility of α-tocopherol in bananas and bread, 29% and 22% in cheese and milk, respectively, and as low as 0.5% in apples. These differences between different food sources may be due to different sites and physicochemical states of α-tocopherol, along with the presence of fibre, fat and phytosterols in the food source.

A dynamic gastric digestion model with nonhomogeneous gastric mixing, shearing, and rate of delivery to the duodenum was employed by Mandalari and others (2013) in the assessment of tocopherols bioaccessibility of pistachios. They obtained a bioaccessibility of almost 100% of tocopherols after duodenal digestion. Déat and others (2009) employed the TIM procedure coupled to Caco-2 cells. These authors showed that the absorption of α-tocopherol from a vitamin E containing meal was significantly lower when compared to the pure compound. This finding reveals that other components present in a meal may change the uptake behaviour of vitamin E or compete in the absorption through the SR-BI transporter.

*In vivo* studies have also been used in the assessment of vitamin E bioavailability. Nagy and others (2012) carried out a human study with healthy volunteers under maldigestion conditions. They found out that the acetylated form of α-tocopherol exhibited the same bioavailability as free α-tocopherol. A long-term human study was also carried out by Novotny and others (2012). They observed that ingesting diary 9.2 mmol (4 mg) of α-tocopherol maintained plasma concentrations of α-tocopherol at 23 mmol/L, suggesting that the dietary requirement for vitamin E may be less than that currently recommended. Johnson and others (2012), employing mouse and human *in vivo* assays, discovered novel urinary metabolites: α-carboxyethylhydroxychroman (α-CEHC) glycine, α-CEHC glycine glucuronide, and α-CEHC taurine.

Correlation between *in vitro* bioaccessibility data with bioavailability determined by *in vivo* human assays was studied by Granado and others (2006). They observed no measurable difference in the case of broccoli in the plasma levels of α-tocopherol after a 7-day feeding intervention.

Therefore, the great variety of methods employed in the assessment of tocopherol bioavailability provides different findings that will be important for future updates of intake recommendations and will aid in understanding the disposition and roles of vitamin E *in vivo*.

**Phytosterols**

Phytosterols have attracted much attention in recent years due to their health benefits, such as cholesterol lowering, anti-inflammatory, anti-atherogenicity, and anti-cancer potential. β-sitosterol is the most common phytosterol found in leaf vegetable natural products followed by campesterol, stigmasterol and sitostanol.

Granado-Lorencio and others (2011) applied the same *in vitro* method as the one used to study polyphenols bioaccesibility (Granado-Lorencio and others 2007) in the assessment of phytosterol bioaccesibility. Mandak and others (2012) also used an *in vitro* digestion. These authors observed that bioaccessibility of steryl ferulates (various plant sterols esterified to ferulic acid) was found to be almost negligible. These findings suggest that intestinal enzymes immediately hydrolyse steryl ferulates and thus they are practically unavailable for absorption in the

small intestine, possibly being bioactive in the gut. This was also shown in a further study (Mandak and Nyström, 2013), where the low bioaccessibility of steryl ferulates (0.01-0.25%) was independent of the cereal matrix. A similar analytical method was applied by Alemany and others (2013). These authors obtained a sterol bioaccessibility of 2 to 6% in fruit-based milk beverages. However, a higher bioaccessibility was observed for oxides of β-sitosterol, suggesting differences in tha solubilisation and absorption mechanism between plant sterols and their oxides.

Yi and others (2012) carried out an *in vivo* rat study where oral bioavailability of sterols enhanced by *Flammulina velutipes* was demonstrated. Although *in vitro* and *in vivo* methods have been used to measure sterols bioaccesibility and bioavailabilty respectively, *in vitro* procedures have yet to be validated against human absorption data.

**Conclusions**

The wide range of options available to evaluate digestion and uptake in *in vitro* and model organisms has guaranteed a role for them in bioaccessibility and bioavailability studies for years to come. Both *in vitro* and *in vivo* approaches are increasing our understanding of uptake of bioactive compounds from food products. Nevertheless, more validation studies are needed which compare *in vivo* with *in vitro* results. It is noteworthy that none of the methods presented in this report will absolutely predict how much of a specific bioactive compound a human will absorb and utilise. In addition, the low bioavailability of the bioactive compounds (in particular polyphenols), could imply the activation of some alternative mechanisms that can justify their possible beneficial effect. Nonetheles, results obtained with *in vivo* assays enable the prediction of the situation in humans quite accurately and may help accelerate the study of phytochemical absorption for better comprehension of their possible beneficial effects.

**Acknowledgments**

This research project was supported by the Spanish Ministry of Science and Technology and European Regional Development Funds (AGL2010-22206-C02-01). J.M. Carbonell-Capella holds an award from the Spanish Ministry of Education (AP2010-2546). M. Buniowska thanks the Podkarpacie Marshal's Office for Ph.D. scholarship number 8.2.2/IV.26/217/11/U/155/12 of RSI Project for Podkarpacie Region, Poland. F.J. Barba thanks the Valencian Autonomous Government (Consellería d´Educació, Cultura i Esport. Generalitat Valenciana) for the postdoctoral fellowship of the VALi+d program ‘‘Programa VALi+d per a investigadors en fase postdoctoral 2013” (APOSTD/2013/092).

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## 3.11. Effect of *Stevia rebaudiana* addition on bioaccessibility of bioactive compounds and antioxidant activity of beverages based on exotic fruits mixed with oat following simulated human digestion

*Food Chemistry 184 (2015) 122–130*

# Effect of *Stevia rebaudiana* addition on bioaccessibility of bioactive compounds and antioxidant activity of beverages based on exotic fruits mixed with oat following simulated human digestion

Juana M. Carbonell-Capella, Magdalena Buniowska, María J. Esteve, Ana Frígola*

Department of Nutrition and Food Chemistry, Universitat de València, Avda Vicent Andrés Estellés, s/n, 46100 Burjassot, Spain

*Corresponding author:
Ana Frígola
e-mail: ana.frigola@uv.es
Phone: +34 963544955, Fax: +34 963544954

**ABSTRACT**

In order to determine the impact of *Stevia rebaudiana (SR)* addition on bioactive compounds bioaccessibility of a new developed functional beverage based on exotic fruits (mango juice, papaya juice and açaí) mixed with orange juice and oat, an *in vitro* gastrointestinal digestion was performed. Ascorbic acid, total carotenoids, total phenolics, total anthocyanins, total antioxidant capacity and steviol glycosides were evaluated before and after a simulated gastrointestinal digestion. Salivary and gastric digestion had no substantial effect on any of the major phenolic compounds, ascorbic acid, total antioxidant capacity and steviol glycosides, whereas carotenoids and anthocyanins diminished significantly during the gastric step. All analysed compounds were significantly altered during the pancreatic-bile digestion and this effect was more marked for carotenoids and total anthocyanins. However, phenolic compounds, anthocyanins, total antioxidant capacity and steviol glycosides bioaccessibility increased as did *SR* concentration. Ascorbic acid bioaccessibility was negatively affected by the *SR* addition.

**Keywords**: *Stevia rebaudiana* · Exotic fruits · Bioaccessibility · Bioactive compounds · Antioxidant capacity

## 1. Introduction

Current trends and worldwide developments on new food products with functionality aim to demonstrate a significant bioactivity of exotic fruits with positive impact in several chronic disorders (Costa, García-Díaz, Jimenez, & Silva, 2013). In this sense, research has focused on combinations of exotic fruits with other ingredients in beverages (Carbonell-Capella, Barba, Esteve, & Frígola, 2013). Fruit juice blends with other ingredients are gaining importance in the market probably due to public perception of juices as a healthy natural source of nutrients and increased public interest in health issues.

Additionally, the use of *Stevia rebaudiana (SR)* leaves is increasing as a natural sweetener 300 times sweeter than sucrose without caloric value, allowing consumers to enjoy sweet taste without concerns about weight gain. They do not replace the sugar naturally present in foods, but they can be an excellent substitute for added sugars and thus an effective aid in weight management. The European Commission granted final regulatory approval for the use of stevia extracts in foods and beverages on 11 November 2011. Stevia leaves contain a mixture of diterpene glycosides (steviosides) and is considered a good source dietary fibre, minerals and essential amino acids (Kim, Yang, Lee, & Kang, 2011). Stevia leaf extract shows a high level of antioxidant activity, as well as a variety of phytochemicals such as phenolic compounds, directly associated with the removal of free electrons and superoxide radicals (Geuns, Hajihashemi, & Claes, 2012). Due to its chemical structure and health-promoting phytochemical components, stevia is suitable as a replacement for sucrose in beverages and for the production of functional food ingredients (Šic Žlabur et al., 2013). The sweetening power of steviol glycosides differ between them, with rebaudioside A being 400 times sweeter than sugar and stevioside about 300 times sweeter (Ceunen & Geuns, 2013). As a result, determination of the steviol glycoside profile is of great interest to industry.

Despite the enormous research on antioxidant properties of fruit beverages, studies investigating the effect of gastrointestinal digestion on dietary antioxidants are scarce. Only phytochemicals released from matrices become

bioaccessible and are potentially available for absorption by the gastro-intestinal tract, and, therefore, able to exert their beneficial effects in the human body. Under gastrointestinal conditions, transformations (degradation, epimerisation, hydrolysis and oxidation) and interactions between phytochemicals and food components may also occur, modifying therefore the biological activity of the bioactive compounds (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014). Therefore, it is important, before concluding on any potential health effect, to assess how the digestion process affects bioactive compounds and their stability, as this, in turn, will affect their bioavailability for uptake, as well as their possible beneficial effects.

Previous studies have confirmed that an *in vitro* digestion model system simulating human digestion could support reliable prediction of bioaccessibility of bioactive compounds and total antioxidant capacity in plant products (Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso, 2013). However, the effect of *SR* extracts on the stability and bioaccessibility of phytochemicals in beverages typically consumed with adjuncts or as formulated products has not yet been reported in the literature data so far. The extent to which formulation may modify the bioactive compound profile of exotic fruit-oat beverages or influence their bioavailability is critical to understanding ultimate physiological effects elicited by these beverages. Furthermore, available knowledge on the digestibility of steviol glycosides is limited. Therefore, at this stage of development, it is necessary to study the impact of digestive conditions when a new specific formulation of commercial ready-to-drink matrix is designed in order to better design future studies focused on assessment of specific biological outcomes.

The objective of the current study was to investigate the bioaccessibility of phenolic compounds, anthocyanins, carotenoids, ascorbic acid, steviol glycosides and antioxidative effect in exotic fruit-oat beverages with (1.25% and 2.5%) and without *SR*. The effect of *SR* extract addition on the bioaccessibility of bioactive compounds and total antioxidant capacity was evaluated with an *in vitro* physiological approach simulating human digestion in the upper gastrointestinal tract, with the inclusion of a salivary, gastric and duodenal step with a dialysis membrane. The release of bioactive compounds as well as the total antioxidant

capacity of the beverages were determined in aliquots collected at the end of each digestion step.

## 2. Materials and methods

### *2.1. Samples*

Cultivars of papaya (*Carica papaya*), mango (*Mangifera indica*), oranges (*Citrus sinensis,* cultivar Navel) and oat beverage (Santiveri, Lérida, Spain) were purchased from a local supermarket. Papaya, mango and orange juices were extracted after appropriate washing of the fruits and the pulp was removed. Açaí provided by Nature's Way Products Inc. (Utah, USA) (containing 450 mg of açaí berries extract, with 10% of polyphenols) was added to the beverage.

*Stevia rebaudiana* leaves were supplied by Anagalide, S.A. (Barbastro, Huesca, Spain) and stored at room temperature. A stock solution (8.33%, w/v) of *Stevia rebaudiana* was prepared in order to formulate the beverage (Carbonell-Capella et al., 2013). For this purpose, 100 mL of bottled water at 100 ºC were added on the dried leaves (8.33 g) and were kept for 30 min. The infusion was vacuum filtered using filter paper (Whatman No. 1) and the filtrate obtained was stored for the duration of the experiment at –40 ºC.

The fruit juice mixture was prepared by mixing 32.5% (v/v) of papaya juice, 10% (v/v) of mango juice, 7.5% (v/v) of orange juice, 20% of oat beverage, 1% of açaí powder (w/v) and water to 100%. To obtain final stevia concentrations of 1.25% and 2.5% (w/v), different volumes of stevia stock solution (30 mL and 60 mL) were added to prepare 200 mL of beverage instead of water. The higher stevia concentration (2.5%, w/v) was selected, taking into account the sucrose concentration of commercial fruit-based beverages and the sweetness equivalence of stevia and sucrose.

### *2.2. Chemicals and reagents*

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), as a standard substance (2 mM) to measure TEAC, 2,2´-azobis(2-methylpropionamidina)dihydrochloride (ABTS), 2,2-Diphenyl-1-picrylhydrazyl

(DPPH), fluorescein sodium salt, 2,2´-azobis(2-amidinopropane)dihydrochloride (AAPH), disodium metabisulfite, Folin-Ciocalteu (ammonium molibdotugstat) reagent, rebaudioside A, stevioside, steviol hydrate, α-amylase from *Bacillus*, mucin from porcine stomach, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, bile extract porcine and EDTA $Na_2$ were purchased from Sigma (Steinheim, Germany). Gallic acid 1-hydrate in distilled water, as a standard (10 mg/mL) for phenolic compounds, was purchased from UCB (Brussels, Germany). Oxalic acid, acetic acid, chlorhidric acid, acetone, sodium acetate, potassium persulphate ($K_2S_2O_8$), sodium di-hydrogen phosphate (anhydrous) ($NaH_2PO_4$) and di-potassium hydrogen phosphate ($K_2HPO_4$) were purchased from Panreac (Barcelona, Spain), while di-sodium hydrogen phosphate anhydrous ($Na_2HPO_4$) and potassium dihydrogen phosphate ($KH_2PO_4$) from Scharlau (Barcelona, Spain). Ethanol, methanol, acetonitrile, hexane, sodium chlorure, sodium carbonate anhydrous ($Na_2CO_3$), trichloroacetic acid and sodium sulphate proceeded from Baker (Deventer, The Netherlands). Ascorbic acid and sodium dodecyl sulfate were obtained from Merck (Darmstadt, Germany) and rebaudioside C and rebaudioside F from Wako (Osaka, Japan).

*2.3. Simulated digestion*

A three-stage *in vitro* digestion model was performed based on the previously described procedure by Rodríguez-Roque et al. (2013), with the addition of a salivary step. Briefly, 50 mL of each beverage (in triplicate) was transferred to an Erlenmeyer flask, and a saliva solution (5 mL, pH 6.75±0.2) containing 2.38 g $Na_2HPO_4$, 0.19 g $KH_2PO_4$, 8 g NaCl, 100 mg of mucin and α-amylase (200 U/L of enzyme activity) in 1 L of distilled water was added. This mixture was kept in a shaking water bath (37 °C, 90 rpm) for 10 min. Salivary digested aliquots were taken for analysis. Afterwards, 13,08 mg of pepsin from porcine stomach was added and pH was adjusted to 2 by addition of HCl (12 M). This mixture was incubated in darkness in a water bath at 37 °C with continuous stirring (90 rpm) for 2 hours. At the end of the gastric digestion, aliquots were taken for analysis and 20 mL were used for titration with NaOH (0.5 M) to pH 7.5 after adding 5 ml of pancreatin (4 g/L) – bile (25 g/L) mixture.

Dialysis membrane was prepared by soaking it with 0.01 M EDTA $Na_2$, 2% $NaHCO_3$ and 0.1% sodium dodecyl sulfate at boiling point, rinsing it with distilled water and cutting it into segments of 30 cm. Dialysis membrane segments were filled with 25 mL of water-$NaHCO_3$ mixture, with the amount of $NaHCO_3$ (0.5 N) used in the previous titration. 20 mL of the gastric digest were placed into a beaker and the dialysis membrane was immersed in that digest until reaching pH 5.0. This process allows gradual pH adjustment, mimicking intestinal conditions. After 30 min, 5 mL of pancreatin (4 g/L) - bile (25 g/L) mixture was added and the incubation continued for further 2 h (37 °C, 90 rpm). The dialysate (fraction inside the dialysis sac), consisting of soluble compounds of low molecular weight, and the retentate (fraction outside the dialysis sac), consisting of soluble and insoluble compounds of low and high molecular weight, were collected and placed in a cold water bath for 10 min.

*2.4. Bioactive compounds analysis*

2.4.1. Polarographic determination of ascorbic acid

The method used was in accordance to Barba, Cortés, Esteve, & Frígola (2012). Beverage (5 mL) was diluted to 25 ml with the extraction solution (1% w/v oxalic acid, 2% w/v trichloroacetic acid and 1% w/v sodium sulphate). After vigorous shaking, the solution was filtered through a folded filter (Whatman No. 1). 1% (w/v) oxalic acid (9.5 ml) and 2 ml of 2 M acetic acid/ sodium acetate buffer (pH=4.8) were added to an aliquot of 0.5 ml of filtrate and the solution was transferred to the polarographic cell. A Metrohm 746 VA Trace Analyser (Herisau, Switzerland) equipped with a Metrohm 747 VA stand was used for the polarographic determination. The working electrode was a Metrohm multi-mode electrode operated in the dropping mercury mode. A platinum wire counter electrode and a saturated calomel reference electrode were used. The following instrumental conditions were applied: $DP_{50}$, mode DME, drop size 2, drop time 1 s, scan rate 10 mV/s, initial potential -0.10 V. Determinations were carried out by using the peak heights and standard additions method.

2.4.2. Total carotenoids

Extraction of total carotenoid was carried out in accordance with Barba et al. (2012). An aliquot of sample (2 mL) was homogenised with 5 mL of extracting solvent (hexane/acetone/ethanol, 50:25:25, v/v) and centrifuged for 5 min at 4000 rpm at 5 ºC. The top layer of hexane containing the colour was recovered and transferred to a 25-mL volumetric flask. The volume of recovered hexane was then adjusted to 25 mL with hexane. Total carotenoid determination was carried out on an aliquot of the hexane extract by measuring the absorbance at 450 nm. Total carotenoids were calculated using an extinction coefficient of *β*-carotene, $E^{1\%}$=2505.

2.4.3. Total phenolic compounds

Total phenols were determined according to the method reported by Georgé, Brat, Alter, & Amiot (2005), with some modifications. Briefly, 10 mL of sample were homogenised with 50 mL of a mixture of acetone/water (7/3, v/v) for 30 min. Mixture supernatants were then recovered by filtration (Whatman No. 2, England) and constituted the raw extracts (REs). REs (2 mL) were settled on an Oasis cartridge (Waters). Interfering water-soluble components (reducing sugars, ascorbic acid) were recovered with 2 x 2 mL of distilled water. The recovered volume of the washing extract (WE) was carefully measured. In order to eliminate vitamin C, heating was carried out on the washing extract (3 mL) for 2 h at 85 °C and led to the heated washing extract (HWE). All extracts (RE, WE, and HWE) were submitted to the Folin-Ciocalteu method, adapted, and optimised (Barba et al., 2012): 2 % (w/v) sodium carbonate solution (3 mL) and 100 µL of Folin–Ciocalteau reagent were added to an aliquot of 100 µL of sample. The mixture was incubated for 1 h at room temperature. Absorbance was measured at 765 nm.

2.4.4. Total anthocyanins

Total anthocyanins were determined using a modified method of Mazza, Fukumoto, Delaquis, Girard, & Ewert (1999). A 10-fold diluted sample of 100 µL was mixed with 1700 µL of distilled water and 200 µL of 5% (v/v) HCl. The sample was hold at room temperature for 20 min before measuring the absorbance at

520 nm in a 10 mm cuvette. Calculations of total anthocyanins were based on cyanidin-3-glucoside (molar absorptivity 26900 L/mol·cm). All spectrophotometric analyses were performed using a UV–visible spectrophotometer Lambda 20 (Perkin-Elmer, Überlingen, Germany).

2.4.5. Total antioxidant capacity

2.4.5.1. Trolox Equivalent Antioxidant Capacity (TEAC) assay

The Trolox Equivalent Antioxidant Capacity (TEAC) test was determined according to the method reported by Barba et al. (2012), based on the capacity of antioxidants to inhibit the radical cation 2,2-azino-bis(3-ethylbenzothiazoline6-sulphonate) (ABTS), which has a characteristic long-wavelength absorption spectrum, showing a maximal peak at 734 nm. The ABTS radical cation is formed by the interaction of ABTS (7 mM) with $K_2S_2O_8$ (2.45 mM).

2.4.5.2. Oxygen Radical Absorbance Capacity (ORAC) Assay

The oxygen radical absorbance capacity (ORAC) assay used, with fluorescein as the "fluorescent probe", was that described by Barba et al. (2012). The automated ORAC assay was carried out on a Wallac 1420 VICTOR$^2$ multilabel counter (Perkin-Elmer, USA) with fluorescence filters, for an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The measurements were made in plates with 96 white flat bottom wells (Sero-Wel, BibbySterilin Ltd., Stone, UK). The reaction was performed at 37 °C, as the reaction was started by thermal decomposition of AAPH in 75 mM phosphate buffer (pH 7.0).

2.4.5.3. DPPH Assay

The value of DPPH (millimolar Trolox equivalents, mMTE) measures the antioxidant capacity of a given substance, as compared to the standard (Trolox). The method used was as described by Brand-Williams, Cuvelier, & Berset (1995). The reaction was begun by adding 50 µL of a suitable dilution of sample to 1.45 mL of DPPH coloured radical. The sample was incubated for 30 min at room temperature (20 ºC). Absorbance, A, was measured at the wavelength of 515 nm.

*2.5. Liquid chromatographic analysis of steviol glycosides*

The method of Joint FAO/WHO Expert Committee on Food Additives (JECFA) (2010) with various modifications was used. Samples were filtered through a Sep-Pak® cartridge (a reverse-phase C-18 cartridge; Millipore, MA, USA) which retains steviol glycosides. The cartridges were previously activated with 10 ml of methanol (MeOH) and 10 ml of water. Every 10 ml of sample was eluted with 2 ml of MeOH, and all methanolic fractions were collected, filtered through a 0.45 µm membrane filter Millex-HV13 (Millipore) and then analysed by liquid chromatography using a Kromasil 100 C18 precolumn (guard column) and Kromasil 100 C18 column (5 µm, 250 x 4.6 mm) (Scharlab, Barcelona, Spain). The mobile phase consisted of two solvents: Solvent A, acetonitrile and Solvent B, 10 mmol/L sodium phosphate buffer (pH=2.6) (32:68, v/v). Steviol glycosides were eluted under 1 mL/min flow rate and the temperature was set at 40 °C. Chromatograms were recorded at 210 nm. The identification of steviol glycosides were obtained out by using standards and by comparing the retention times, while quantification was performed by external calibration with standards.

*2.6. Statistical analysis*

All determinations were performed in triplicate. An analysis of variance (ANOVA) was applied to the results obtained in order to verify whether there were significant differences in the parameters studied in relation to sample analysed, and to ascertain possible interactions between factors (differences at $p<0.05$ were considered significant). Where there were differences, an LSD test was applied to indicate the samples in which differences were observed. A multiple regression analysis was performed to study the influence of bioactive compounds to antioxidant capacity (the results are shown in the significant cases, $p<0.05$). Finally, a study was conducted with the aim of determining whether there were correlations between a pair of variables (Pearson´s test). All statistical analyses were performed using Statgraphics® Centurion XVI (Statpoint Technologies Inc., USA).

## 3. Results and Discussion

### *3.1. Bioactive compounds*

Ascorbic acid

The effect of gastrointestinal digestion on the ascorbic acid recovery was distinct and affected by different *SR* content in the exotic fruit-oat beverages (Figure 1).

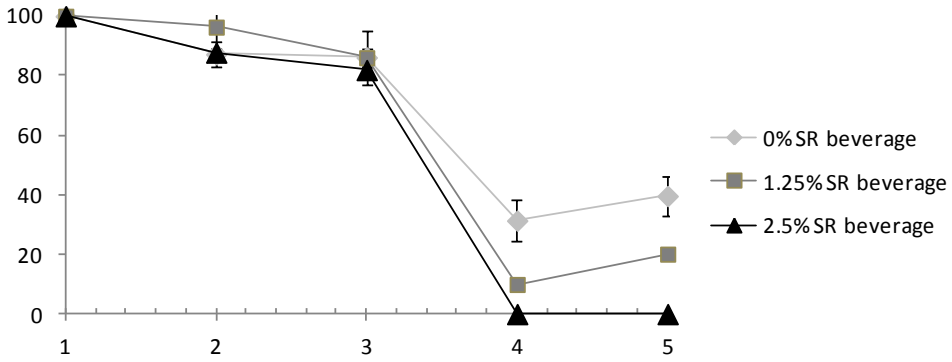



**Figure 1.** Ascorbic acid content of 0%, 1.25% and 2.5% (w/v) *Stevia rebaudiana* (*SR*) beverage during *in vitro* gastrointestinal digestion, expressed as percentage. 1: Non-digested sample. 2: Salivary digesta. 3: Gastric digesta. 4: Non-dialysed intestinal fraction. 5: Dialysed intestinal fraction.

The three nondigested beverages (0, 1.25 and 2.5% *SR*) had similar ascorbic acid values (28-33 mg/100 mL). Although Kim et al. (2011) detected ascorbic acid in stevia leaves and callus, differences in these results and those found in the present research could be attributed to the preparation of the *SR* water extract (weight and temperature submitted). Ascorbic acid diminished just over 5-13% (corrected by the varying volumes of digesta) in the salivary phase, as pH is not substantially changed and duration is of only 10 minutes. Zulueta, Esteve, Frasquet, & Frígola (2007) found out that pH had a significant influence and correlated negatively with the ascorbic acid concentrations, as acid media contribute to the stability of the vitamin. This explains why the ascorbic acid did not diminish significantly ($p$>0.05) during the gastric digestion (pH 2.20±0.01) of

the three beverages (14-19%). These results are in agreement with previous findings, which proved that *in vitro* gastric conditions (pH 2 or 3) had very little effect on ascorbic acid stability. Only a slight loss (6.7%) was observed by Vallejo, Gil-Izquierdo, Pérez-Vicente, & García-Viguera (2004) in broccoli inflorescences after pepsin digestion. Rodríguez-Roque et al. (2013) also demonstrated that gastric digestion had little effect on ascorbic acid stability, recovering 83% of this bioactive compound in a blended fruit juice containing orange, pineapple and kiwi. However, neither of these authors submitted their sample to a salivary step before the gastric digestion, so bioaccessibility might be overestimated.

Nevertheless, after *in vitro* intestinal digestion, there were significant decreases in ascorbic acid concentration in the non-dialysed fraction (54.8 and 76.1 in the 0 and 1.25% *SR* beverage respectively) with regard to gastric digesta due to the low stability of this compound at high pH, and in the 2.5% *SR* beverage, ascorbic acid was not detected. In the dialysed fraction, an increase in the ascorbic acid content was obtained (8.3 and 10.2% in the 0 and 1.25% *SR* beverage, respectively) with regard to the non-dialysed fraction. These results are in agreement with previous results for broccoli inflorescences (91% loss) (Vallejo et al., 2004), pomegranate juice (80%) (Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002) and for orange, pineapple and kiwi blended fruit juice (75%) (Rodríguez-Roque et al., 2013). Differences in the composition of the samples and the use of dialysis membrane instead of solubility assays to obtain the bioaccessible fraction, could explain the differences found in comparison with our study. However, in an *in vivo* study, Davey et al. (2000) obtained values of biavailability of ascorbic acid between 80-100% at doses normally ingested (≤ 180 mg). Aside from the passive transport mechanism which in humans is only predominant at high intake levels, ascorbic acid is also absorbed by an active transport system located in the gut (Stahl et al., 2002), which must be taken into account.

When evaluating the effect of *SR* addition on ascorbic acid bioaccessibility, it is noteworthy that this compound remained undetected in the dialysed fraction of the 2.5% *SR* beverage. In the intestinal phase, *SR* may exert a negative effect over the ascorbic acid stability. This can be due to interferences between the steviol

glycosides with the ascorbic acid structure at basic conditions, as in previous steps no significant differences were detected with respect to the formulation involved. No study has been published up to date showing these possible interferences. Interaction between stevioside and ascorbic acid was investigated by Kroyer (2010), but he observed a protective effect of stevioside on the degradation of ascorbic acid. However, the pH used in his study is not specified. Interestingly, Šic Žlabur et al. (2013) obtained a decrease in the ascorbic acid content when apple purees were sweetened with stevia and pasteurised. However, they attribute this loss to the heat treatment and not to the stevia addition.

According to Vallejo et al. (2004), ascorbic acid was the metabolite that showed the greater decrease (91% loss) after intestinal digestion. As ascorbic acid is a thermosensitive compound, in fruit and vegetables it has been used as an indicator of the loss of other vitamins (Zulueta et al., 2007). Consequently, the decrease in bioaccessibility observed for this bioactive compound may also be observed in other similarly alike thermosensitive vitamins, such as vitamin B group, although further studies are necessary.

Total carotenoids

The amounts of bioaccessible carotenoids after simulated gastrointestinal digestion expressed as µg/100 mL are presented in Table 1. As carotenoids are highly hydrophobic compounds, a micellisation step is needed in order to evaluate their bioaccessibility. With this purpose, digested samples were centrifuged during 20 min at 4000 rpm and 20ºC (Courraud, Berger, Cristol, & Avallone, 2013), but no statistical differences were found after the centrifugation step (data not shown). A statistically significant ($p$<0.05) decrease of 94–99.4% in the dialysed fraction with respect to the initial carotenoids in the original fruit-oat beverage was found. Although carotenoids are considered to be absorbed in a relatively non-specific way by passive diffusion of the micelles in the mucosa cells (Stahl et al., 2002), this low bioaccessibility of carotenoids is mainly caused by their limited solubilisation to the aqueous phase, which hinders their ability to be taken up by the intestines. Other authors have included the use of human pancreatic lipase, cholesterol esterase, phospholipase $A_2$ and taurocholate salts in order to

reproduce more physiological conditions, as these enzymes may hydrolyse ester forms and provide optimum conditions for carotenoid hydrolysis and micellisation (Granado-Lorencio et al., 2007).

The highest carotenoid recovery (6%) was achieved in the formulation with 2.5% *SR*, showing a positive correlation between the content of *SR* and the bioaccessibility of total carotenoids. Results suggest that the addition of the natural sweetener *SR* may enhance somehow dialysability of carotenoids through the semipermeable membrane. Amongst the factors that affect carotenoid bioaccessibility, other authors have shown that the matrix in which carotenoids are embedded can play a significant role on bioaccessibility, along with effectors of absorption and bioconversion (West & Castenmiller, 1998). However, the use of a dialysis membrane does not take into account active transport via membrane transporters through which carotenoids are absorbed (Reboul & Borel, 2011), leading to an underestimation of the real bioaccessibility of carotenoids.

Regarding the different steps through which the beverages pass in the digestion process, salivary addition enhances the release of carotenoids independently of the *SR* concentration. However, gastric and intestinal digestions lead to a decrease in the carotenoid content of around 15% and 21% respectively with regard to the undigested sample, without significant differences between the three formulations. Indeed, carotenoids are more sensitive to acidic than alkaline conditions (Rodríguez-Amaya, 2010). This is why the decrease was higher during the gastric phase. This was also confirmed by Wright, Pietrangelo, & MacNaughton (2008), who showed increased *β*-carotene transfer to the aqueous phase under higher pH conditions.

Similar results were reported by Courraud et al. (2013) in carrot, but Granado-Lorencio et al. (2007) reported a higher carotenoid stability of about 70% in the final digesta of loquat, orange and broccoli. As mentioned before, these authors included the use of human pancreatic lipase, cholesterol esterase, phospholipase $A_2$ and taurocholate salts during the intestinal digestion and also a previous homogenisation to simulate mastication and did not include a semipermeable membrane. In a different study, Granado et al. (2006) concluded that behaviour of carotenoids under *in vitro* gastrointestinal conditions does not fully explain

changes observed *in vivo,* limiting the use of *in vitro* models for screening relative bioaccessibility of carotenoids.

Phenolic compounds

Absorption of polyphenolics from fruit beverages follows similar multistep pathways to other bioactive compounds that generally require (a) release of the specific phenolic from the beverage matrix, (b) solubilisation in the gut lumen, (c) stability of the polyphenolic to digestive conditions, (d) uptake by small intestinal absorptive epithelial cells and (d) potential for intracellular metabolism and secretion into blood stream. Nevertheless, phenolic compounds can also be metabolised by colonic microflora to simple phenolic, organic acids and several other products which are subsequently absorbed and distributed to tissues (Ferruzzi, 2010). However, for the purpose of this paper, only small intestinal absorption will be discussed.

Total phenolic contents of the formulated beverages before and after *in vitro* simulated gastrointestinal digestion is shown in Table 1. Total soluble phenolic compounds of the three beverages before digestion ranged from 876 to 4896 mg/L, measured as gallic acid equivalents. Recoveries of total polyphenols in the dialysed fraction were 30-33% compared to their non-digested counterparts. These results compare well with those reported of recoveries of total phenolic compounds from 29 to 62% in pomegranate juice (29%) (Pérez-Vicente et al., 2002) and apples (Bouayed, Hoffmann, & Bohn, 2011) (44-62%).

The effect of *SR* upon the soluble extractable phenolic content of exotic fruit-oat beverages before and after digestion is depicted in Table 1. Prior to digestion, the 2.5% *SR* beverage exhibited the highest total phenolic content, indicating that *Stevia rebaudiana* could be useful as a potential source of natural polyphenols. After digestion, the 2.5% *SR* beverage still had six times more total soluble extractable polyphenols than the 0% *SR* beverage, confirming that the addition of *SR* extracts in formulated beverages is a way of enhancing the consumption of these beneficial components.

**Table 1.** Total carotenoids, total phenolic compounds and total anthocyanins of a beverage mixture of exotic fruit juices with oat beverage and sweetened with 0%, 1.25% and 2.5% (w/v) *Stevia rebaudiana* (*SR*) during *in vitro* gastrointestinal digestion.

| *SR* | | Total carotenoids | | Total phenolic compounds | | Total anthocyanins | |
|---|---|---|---|---|---|---|---|
| | | (µg/100 mL) | % | (mg GAE/L) | % | (mg cyanidin-3-glucoside/L) | % |
| 0% | 1 | 1629.2±10.6 | | 876.1±53.9 | | 40.4±1.1 | |
| | 2 | 1778.4±10.9 | 109.2 | 930.2±11.9 | 106.2 | 28.6±1.9 | 70.7 |
| | 3 | 1386.0±66.0 | 85.1 | 919.7±8.9 | 105.0 | 25.4±1.4 | 63.0 |
| | 4 | 1262.0±38.8 | 77.5 | 522.4±3.0 | 59.6 | 14.9±1.0 | 36.8 |
| | 5 | 10.0±1.4 | 0.6 | 262.9±4.5 | 30.0 | 0.1±0.1 | 0.2 |
| 1.25% | 1 | 1719.0±24.7 | | 2994.7±12.9 | | 44.5±0.8 | |
| | 2 | 1901.9±50.5 | 110.6 | 3227.1±11.9 | 107.8 | 39.6±1.1 | 89.1 |
| | 3 | 1501.2±42.7 | 87.3 | 3160.0±11.9 | 105.5 | 38.9±1.1 | 87.3 |
| | 4 | 1420.3±9.7 | 82.6 | 1818.4±29.6 | 60.7 | 14.3±1.0 | 32.1 |
| | 5 | 34.3±9.7 | 2.0 | 942.7±22.2 | 31.5 | 2.1±1.4 | 4.8 |
| 2.5% | 1 | 1671.7±7.1 | | 4896.3±26.4 | | 49.3±1.1 | |
| | 2 | 1841.6±27.2 | 110.2 | 5293.8±53.3 | 108.1 | 45.3±0.8 | 91.7 |
| | 3 | 1388.7±46.6 | 83.1 | 5205.7±94.9 | 106.3 | 45.1±1.6 | 91.3 |
| | 4 | 1317.4±97.0 | 78.8 | 3070.8±22.2 | 62.7 | 14.0±1.4 | 28.3 |
| | 5 | 99.5±14.6 | 6.0 | 1608.2±29.6 | 32.8 | 4.7±0.8 | 9.4 |

1: Non-digested beverage. 2: Salivary digestion. 3: Gastric digestion. 4: Non-dialysed intestinal fraction. 5: Dialysed intestinal fraction. GAE: Gallic acid equivalents.

An increase in the total extractable phenolic content was observed (6-8%) after the salivary step, possibly by enhancement of solubilisation of phenolic compounds, as high molecular weight phenols may be insoluble and the enzyme activity or agitation conditions could facilitate the breakage of large molecules. After the gastric digestion, there was a slight loss in the total phenolic contents, although this decrease was not significant ($p$>0.05) and recoveries continued to be

higher than their non-digested counterparts, possibly because the low pH reached may reduce oxidised species back to the native compounds (Stahl et al., 2002). Similarly, Pérez-Vicente et al. (2002) did not obtain differences in total phenolics content before and after pepsin digestion. However, the mild alkaline conditions reached during the intestinal digestion, along with possible interactions between polyphenols and other components such as enzymes, could explain the significant decrease ($p$<0.05) observed during the intestinal digestion. Polyphenol concentration decreased during dialysis through the semipermeable cellulose membrane, although all polyphenols found in the intestinal medium were also found to be dialysable, which could be regarded as indicative of passive diffusion, one of the most important mechanisms for cellular polyphenol uptake, at least for aglycones. A large portion of the phenolic compounds found in the non-dialysed fraction is likely to reach the colonic lumen where they can be metabolised by the microflora and hydrolysed (Ferruzzi, 2010). In this line, Saura-Calixto, Serrano, & Goñi (2007) estimated that about 48% of total phenolics are bioaccessible in the small intestine, whereas 42% become bioaccessible in the large intestine. Interestingly, Coates et al. (2013) demonstrated that polyphenols likely to reach the colon are capable of inhibiting several important stages in colon carcinogenesis *in vitro*. Furthermore, when Brown et al. (2014) compared *in vivo* and *in vitro* digestion in lingonberries, they observed notable differences in the phenolic composition between the *in vitro* digested extract and the ileal fluid, reinforcing the need of bioactivity studies when investigating dietary phytochemicals.

The impact of gastrointestinal digestion on total anthocyanins is shown in Table 1. In general, the recovery of total anthocyanins diminished stepwise from salivary to dialysed digesta for all three beverages. Amounts of total anthocyanins detected after the salivary and intestinal phase were significantly lower than those determined in the non-digested 0% *SR* beverage. In general, no significant differences ($p$<0.05) were observed in the total anthocyanins recovery after gastric digestion. Similar results were obtained by Bouayed et al. (2011) with a 91.2% of total anthocyanins gastric recovery in Jonaprinz apples. In the dialysed fractions, total anthocyanins were poorly recovered, with similar patterns

obtained by Pérez-Vicente et al. (2002) (2.4%) in pomegranate juice and Gil-Izquierdo, Zafrilla, & Tomás-Barberán (2002) (2.3-3.8%) in strawberries. Bouayed et al. (2011) however, did not detect anthocyanins following intestinal digestion. At this stage of the digestion, part of the anthocyanins could be metabolised to some non-coloured forms, oxidised or degraded into other chemicals, escaping this way from the detection under present conditions. According to numerous studies, low bioaccessibility of anthocyanins can be attributed to their low stability in the alkaline conditions of small intestine, as it is generally accepted that anthocyanins are stable at low pH values (between 1 and 3) (Kosinska-Cagnazz, Diering, Prim, & Andlauer, 2014). However, although *in vitro* studies suggest the degradation of anthocyanins in the intestinal phase, under *in vivo* conditions direct absorption may take place in the stomach (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). Furthermore, the addition of *SR* led to a significant increase in the bioaccessibility of total anthocyanins, suggesting a higher harnessing of these bioactive compounds when *SR* is present in the digestive tract.

### *3.2. Antioxidant capacity*

Due to the complex mechanism of antioxidant compounds, there is not an official method to determine total antioxidant capacity (TAC), so trolox equivalent antioxidant capacity (TEAC), DPPH (α,α-diphenyl-β-picrylhydrazyl) scavenging activity and oxygen radical antioxidant capacity (ORAC) were used in the determination of total antioxidant capacity (TAC) after the simulated gastrointestinal procedure of the fruit juice beverages. TAC values of undigested samples without *SR* were 11.4±0.9, 11.1±1.6 and 10.5±0.1 mM TE (Trolox Equivalent) for TEAC, DPPH and ORAC methods, respectively (Table 2). The ANOVA analysis confirmed an increase in TEAC, DPPH and ORAC values when *SR* concentration was increased, independently of the digestion step analysed. These results are in agreement with previous studies carried out by different authors who have shown a high antioxidant capacity of *SR* products (Šic Žlabur et al., 2013). Furthermore, no significant differences ($p$>0.05) were observed between TEAC and DPPH values, albeit TAC values measured with ORAC assay were

significantly higher ($p$<0.05) for samples with *SR* at 1.25% (24.1±0.2 mM TE) and 2.5% (35.5±0.6 mM TE) than those obtained with TEAC (22.0±2.1 and 32.2±1.9 mM TE for 1.25 and 2.5% *SR* beverages, respectively) and DPPH method (21.1±1.1 and 30.0±0.8 mM TE for the beverages with 1.25 and 2.5% *SR*, respectively). Differences may be explained because ORAC assay is based on the transfer of H atoms, whereas TEAC and DPPH assays are based on a redox reaction.

**Table 2.** Antioxidant capacity values of a of a beverage mixture of exotic fruit juices with oat beverage and sweetened with 0%, 1.25% and 2.5% (w/v) *Stevia rebaudiana* (*SR*) during *in vitro* gastrointestinal digestion.

| *SR* | | TEAC | | DPPH | | ORAC | |
|---|---|---|---|---|---|---|---|
| | | (mM TE) | % | (mM TE) | % | (mM TE) | % |
| 0% | 1 | 11.4±0.9 | | 11.1±1.6 | | 10.5±0.1 | |
| | 2 | 10.7±1.1 | 94.3 | 10.4±1.3 | 94.0 | 11.5±0.1 | 109.6 |
| | 3 | 10.6±1.2 | 93.2 | 10.2±2.2 | 92.1 | 11.1±0.2 | 106.2 |
| | 4 | 6.1±0.4 | 53.4 | 5.6±1..5 | 50.8 | 7.9±0.2 | 75.8 |
| | 5 | 3.9±0.4 | 34.7 | 3.7±1.5 | 33.7 | 3.5±0.4 | 33.6 |
| 1.25% | 1 | 22.0±2.1 | | 21.1±1.1 | | 24.1±0.2 | |
| | 2 | 21.5±0.1 | 97.7 | 20.1±1.6 | 95.3 | 26.3±0.9 | 109.0 |
| | 3 | 21.2±0.5 | 96.2 | 19.9±0.8 | 94.2 | 25.7±0.6 | 106.8 |
| | 4 | 12.0±0.4 | 54.3 | 11.5±1.7 | 54.7 | 18.4±0.6 | 76.4 |
| | 5 | 7.9±0.6 | 36.0 | 7.5±1.1 | 35.7 | 8.5±0.5 | 35.5 |
| 2.5% | 1 | 32.2±1.9 | | 30.0±0.8 | | 35.5±0.6 | |
| | 2 | 31.6±0.9 | 98.2 | 29.5±4.8 | 98.5 | 39.0±1.0 | 109.7 |
| | 3 | 31.3±0.6 | 97.3 | 29.2±4.8 | 97.4 | 37.4±0.5 | 105.3 |
| | 4 | 17.9±1.5 | 55.7 | 16.5±3.5 | 55.1 | 26.5±0.4 | 74.7 |
| | 5 | 12.2±0.6 | 38.0 | 11.3±1.5 | 37.7 | 11.3±0.3 | 31.8 |

*SR*: *Stevia rebaudiana*. TEAC: trolox equivalent antioxidant capacity. DPPH: α,α-diphenyl-β-picrylhydrazyl. ORAC: oxygen radical antioxidant capacity. TE: Trolox Equivalent. 1: Non-digested sample. 2: Salivary digesta. 3: Gastric digesta. 4: Non-dialysed intestinal fraction. 5: Dialysed intestinal fraction.

A multivariate regression analysis test was effectuated in order to study the contribution of the different bioactive compounds to the TEAC, DDPH and ORAC values (Eq. (1-3)). Coefficients of the equations were $R^2$=0.939, $p$<0.05, standard error=0.836, $R^2$=0.921, $p$<0.05, standard error=2.578 and $R^2$=0.996, $p$<0.05, standard error=0.807 for TEAC, DPPH and ORAC methods, respectively. For TEAC and DPPH methods, only water-soluble components contributed to the total antioxidant capacity. Zulueta, Esteve, & Frígola (2009) compared the TEAC and ORAC methods, noting that the TEAC method had greater specificity for water-soluble antioxidants.

$$TEAC = 0.818145 + 0.010975 \cdot (TPC) \quad (1)$$

$$DPPH = 2.36701 + 0.121547 \cdot (AA) + 0.00470771 \cdot (TPC) \quad (2)$$

$$ORAC = 2.34997 + 0.003246 \cdot (TC) - 0.074694 \cdot (AA) + 0.006221 \cdot (TPC) \quad (3)$$

High correlation coefficients were found between the total phenolic content and TEAC, DDPH and ORAC assay ($R^2$=0.9804, $R^2$=0.9471 and $R^2$=0.9896, respectively). Kim et al. (2011) also reported total phenolics to be responsible for the antioxidant activities of *Stevia rebaudiana* water extracts.

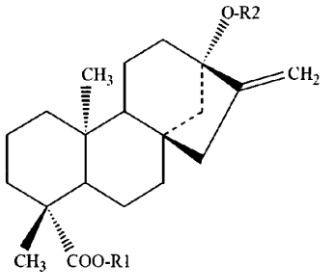
Moreover, a strong correlation was found between total antioxidant capacity measured by TEAC and DPPH ($R^2$=0.9656, $p$<0.05), TEAC and ORAC ($R^2$=0.9743, $p$<0.05), and by DPPH and ORAC method ($R^2$=0.9398, $p$<0.05). Zulueta et al. (2009) also obtained good correlations for ORAC and TEAC assays in orange juice ($R^2$=0.955, $p$<0.05).

### *3.3. Steviol glycosides*

Using high-performance liquid chromatography (HPLC), four different steviol glycosides were identified as rebaudioside A (reb A), stevioside (ste), rebaudioside F (reb F) and rebaudioside C (reb C) (Table 3, Figures 2-3), albeit the actual JECFA analytical method (JECFA, 2010) lists nine different steviol glycosides.

Reb A content in the non-digested beverage sweetened with 1.25 and 2.5% (v/v) of *SR* was 46.5±0.1 and 85.1±0.6 mg/100 mL, respectively. The stevioside concentration was 38.7±0.8 and 73.7±1.6 mg/100 mL in the 1.25 and 2.5% *SR*

beverage, respectively. These two glycosides (reb A and ste), which are present in the highest concentration, show the highest sweetness activity and minor toxicity (Montoro et al 2013).




| Compound name | R1 | R2 |
|---|---|---|
| Stevioside | *β*-Glc | *β*-Glc- *β*-Glc(2→1) |
| Rebaudioside A | *β*-Glc | *β*-Glc- *β*-Glc(2→1)<br>\|<br>*β*-Glc(3→1) |
| Rebaudioside C | *β*-Glc | *β*-Glc- *α*-Rha(2→1)<br>\|<br>*β*-Glc(3→1) |
| Rebaudioside F | *β*-Glc | *β*-Glc- *β*-Xyl(2→1)<br>\|<br>*β*-Glc(3→1) |

**Figure 2.** Steviol glycoside structures found in the beverage mixtures of exotic fruit juice and oat sweetened with *Stevia rebaudiana* water extracts.

**Table 3.** Steviol glycosides of a beverage mixture of exotic fruit juices with oat beverage and sweetened with 0%, 1.25% and 2.5% (w/v) *Stevia rebaudiana* (*SR*) during *in vitro* gastrointestinal digestion.

| *SR* | | Reb A | | Ste | | Reb F | | Reb C | |
|---|---|---|---|---|---|---|---|---|---|
| | | (mg/100 mL) | % | (mg/100 mL) | % | (mg/100 mL) | % | (mg/100 mL) | % |
| | 1 | 46.5±0.1 | | 38.7±0.8 | | 1.6±0.1 | | 6.5±0.1 | |
| | 2 | 51.4±0.3 | 110.5 | 40.3±0.3 | 104.3 | 1.7±0.1 | 110.8 | 7.4±0.2 | 114.1 |
| 1.25% | 3 | 55.7±0.6 | 119.8 | 44.8±1.3 | 115.8 | 2.2±0.2 | 139.7 | 9.4±0.1 | 145.6 |
| | 4 | 42.5±1.1 | 91.4 | 34.5±2.8 | 89.3 | 4.5±0.2 | 289.0 | 5.9±0.1 | 90.5 |
| | 5 | 11.1±0.2 | 23.9 | 10.3±0.2 | 26.7 | 2.7±0.5 | 172.9 | 1.9±0.3 | 29.0 |
| | 1 | 85.1±0.6 | | 73.7±1.6 | | 3.5±0.1 | | 13.4±0.5 | |
| | 2 | 93.9±0.4 | 110.4 | 78.7±0.3 | 106.8 | 3.9±0.3 | 111.5 | 15.4±0.2 | 115.0 |
| 2.5% | 3 | 122.9±4.6 | 144.5 | 104.3±2.4 | 141.5 | 5.1±0.1 | 146.4 | 20.1±2.4 | 149.5 |
| | 4 | 79.8±2.3 | 93.7 | 66.8±1.3 | 90.6 | 7.3±0.2 | 209.5 | 12.3±0.1 | 91.5 |
| | 5 | 21.0±1.6 | 24.6 | 19.8±2.6 | 26.9 | 5.6±0.1 | 160.3 | 3.8±0.3 | 28.0 |

Reb A: rebaudioside A. Ste: stevioside. Reb F: rebaudioside F. Reb C: rebaudioside C. 1: Non-digested sample. 2: Salivary digesta. 3: Gastric digesta. 4: Non-dialysed intestinal fraction. 5: Dialysed intestinal fraction.

Followingly, reb C content in the original 1.25 and 2.5% *SR* beverage was 6.5±0.1 and 13.4±0.5 mg/100 mL, respectively. Finally, reb F was present in a lower concentration of 1.6±0.1 and 3.5±0.1 mg/100 mL in the beverage sweetened with 1.25 and 2.5% (v/v) of *SR*, respectively. No significant influence in the stevioside content was observed by Kroyer (2010) when stevioside was mixed with coffee or tea beverage, although minimal losses could be noticed after 4 h at high temperature. The contents of reb A, ste, reb F and reb C recorded in the present study are in agreement with and sometimes higher than those reported in literature (Montoro et al., 2013), notwithstanding that as is the case of most secondary metabolites, glycosides profiles of stevia are subjected to considerable variability according to geographic area, state of plant maturity, environment, harvesting and processing conditions.

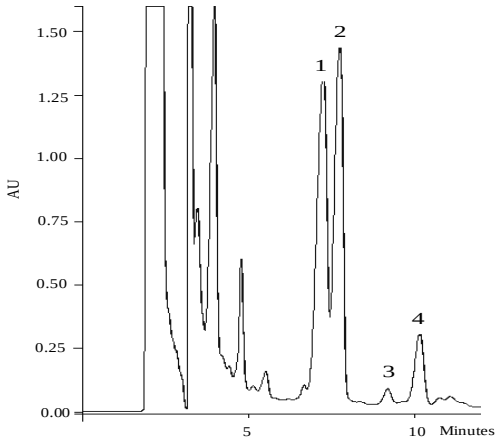



**Figure 3.** Chromatogram HPLC analysis of steviol glycosides 1: rebaudioside A, 2: stevioside, 3: rebaudioside F, 4: rebaudioside C in a beverage mixture of exotic fruit juice and oat sweetened with *Stevia rebaudiana* (*SR*) Bertoni at 2.5% (w/v).

The analysis of variance indicated an increase (4-15%) in reb A, ste, reb F and reb C values after salivary digestion, independently of the *SR* percentage used. Steviol glycosides have glycosidic bonds which may be possible sites of action of α-amylase enzyme, and thus result in an increase of these compounds. Additionally, gastric digestion increased the reb A, ste, reb F and reb C content in about 9-35% with respect to salivary digesta, with independence of the *SR*

percentage. These compounds are diterpenoid glycosides and thus pepsin is not able to attack them. Therefore, the increase at this stage of digestion may be due to interactions at acidic pH. Our results differ from previous studies found in published literature. Hutapea, Toskulkao, Buddhasukh, Wilairat, & Glinsukon (1997) obtained no significant changes after *in vitro* digestion of stevioside with saliva, α-amylase, pepsin and pancreatin. By contrast, Kroyer (2010) detected a significant decrease in the stevioside concentration under strong acidic conditions (pH 1), but not at our gastric pH 2, where stevioside was remarkable stable.

After intestinal digestion, reb A, ste, and reb C concentration diminished significantly ($p$<0.05). Reb F however, became highly bioaccessible. This could be attributed to important changes occurring during *in vivo* and *in vitro* metabolism of steviol glycosides. Ceunen & Geuns (2013) suggest a spatial separation of glycosidases from the steviol glycosides. Consequently, after cell disruption by enzymatic degradation, further metabolism might take place, reflecting a complex and dynamic process not yet fully understood, despite the known biosynthetic relationship between individual steviol glycosides. Dialysed fraction of reb A, ste and reb C was of 24-29% with respect to their undigested counterparts, suggesting an important loss of these compounds during digestion and dialysis process. Surprisingly, bioaccessibility of reb F was of 172.9 and 160.3% with regard to the undigested 1.25 and 2.5% *SR* beverages, respectively, showing the complex metabolism of steviol glycosides. Ceunen & Geuns (2013) explain that although stevioside and rebaudioside A are the most common steviol glycosides, they may not be the final product of the pathway, as further glycosilations are likely to take place, and although biosynthesis of rebaudioside F is not completely elucidated, it is believed that the enzymes UGT76G1 and UGT74G1 might be involved in it, explaining the high bioaccessibility of rebaudioside F. However, this hypothesis has not yet been characterised *in vitro* nor *in vivo*. Koyama et al. (2003) observed in an *in vivo* study that stevia mixture components were first degraded and then absorbed as steviol in the rat intestine. Geuns, Augustijns, Mols, Buyse, & Bert (2003) obtained a minor fraction of ste and reb A transported through Caco-2 cell layers, suggesting a carrier-mediated transport. However, they could not detect stevioside or steviol in the blood of pigs, probably because

in the Caco-2 study, steviol is applied as a solution facilitating the uptake, whereas in the colon, steviol is probably adsorbed to other compounds. Further studies have found out that ste and reb A are completely hydrolysed to the aglycon steviol when incubated with intestinal bacteria (Renwick & Tarka, 2008).

There appears to be a positive correlation ($p$<0.05) between the amounts of reb A, ste and reb C and the total antioxidant capacity measured by TEAC, DPPH and ORAC assay. This correlation was stronger when the ORAC method was employed. However, a negative correlation was obtained between antioxidant capacity measured with TEAC and DPPH method and reb F content, suggesting that reb F could be the result of the degradation of any other steviol glycoside with potential antioxidant capacity. Previous studies have suggest that although phenolic compounds are the major responsible of the antioxidant capacity shown by stevia extracts, steviol glycosides are known to be potent ROS (reactive oxygen species) scavengers (Geuns et al., 2012). Toward hydroxyl radicals, they observed that stevioside and rebaudioside A had similar scavenging activities indicating that their antioxidant activity is mostly related to their common diterpene skeleton, but stevioside demonstrated a stronger scavenger activity than rebaudioside A for superoxide radicals.

**4. Conclusions**

The addition of 1.25% and 2.5% of *SR* in an exotic fruit-orange-oat beverage contributes to increase the concentration and bioaccessibility of total carotenoids, total phenolic compounds, total anthocyanins and total antioxidant capacity of the beverage. Nevertheless, ascorbic acid was not detected after the *in vitro* simulated digestion when *SR* was found in a 2.5% (w/v). Investigation of the specific interactions between ascorbic acid and *SR* extracts will be critical for understanding how the formulation can be used to optimise circulating and tissue levels of these phytochemical constituents. Despite these results, *in vitro* limitations should be taken into account, as these methods enable an approach, but will not absolutely predict how much of a specific bioactive compound a human will absorb and utilise. *In vivo* assays of beverages sweetened with *SR*

must be carried out in order to verify the relevance of the increase in the bioaccessibility of bioactive compounds in blood.

**Acknowledgments**

This research project was supported by the Spanish Ministry of Science and Technology and European Regional Development Funds (AGL2010-22206-C02-01). Carbonell-Capella, J.M. holds an award from the Spanish Ministry of Education (AP2010-2546). Buniowska, M. holds an award from the European Union (8.2.2/IV.26/217/11/U/155/12).

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# 4. General discussion

*Discusión general*

## 4. DISCUSIÓN GENERAL

En la realización del presente trabajo de investigación se ha formulado una bebida a base de frutas exóticas (papaya, mango y açaí) y zumo de naranja, con un alto contenido en compuestos bioactivos y con nuevos sabores para el consumidor. Se ha adicionado stevia como edulcorante acalórico natural que además se considera un ingrediente natural antioxidante. Con el objetivo de obtener un zumo con un elevado contenido en compuestos bioactivos y con las características organolépticas más parecidas al fresco, se ha estudiado el efecto de la aplicación de tecnologías no térmicas (altas presiones hidrostáticas, pulsos eléctricos, descargas eléctricas de alto voltaje y ultrasonidos) en su conservación. Se ha evaluado la aplicación de pulsos eléctricos como pretratamiento para obtener un zumo de manzana rico en compuestos bioactivos. Finalmente se ha estudiado la bioaccesibilidad de los compuestos bioactivos en la bebida a base de zumos de frutas adicionada de stevia.

### 4.1. Determinación de compuestos bioactivos y capacidad antioxidante de muestras de stevia y bebidas a base de zumo de frutas con bebida de avena y stevia adicionada

*Selección de un ingrediente natural con capacidad antioxidante*

En primer lugar se caracterizan diferentes muestras de *Stevia rebaudiana* de varias marcas comerciales y con distintos formatos de presentación. Entre ellas se incluyen glucósidos de esteviol purificados y extractos de hojas de stevia. Tras múltiples estudios químicos, toxicológicos y clínicos, los glucósidos de esteviol están permitidos como aditivo alimentario desde el 2 de diciembre del 2011 con el código E960 (EC, 2011), aunque no ocurre los extractos de hojas de *Stevia rebaudiana* (EC, 2000), que se consideran nuevos alimentos y están pendiente de autorización para su uso como aditivo en la Unión Europea. Sin embargo, se considera interesante su análisis con el fin de contribuir a una investigación científica más profunda previa a su aprobación en Europa, pues son ampliamente usados y están autorizados en otros países como Japón.

Los resultados muestran que los extractos de *Stevia rebaudiana* pueden utilizarse como edulcorantes acalóricos y además son fuente de compuestos bioactivos con una alta capacidad antioxidante. Tanto los compuestos bioactivos como la capacidad antioxidante total de los extractos acuosos de stevia dependen en gran medida del origen de sus hojas, coincidiendo con resultados observados en estudios previos (González y col., 2014).

Los extractos acuosos preparados a partir de hojas de stevia junto con el extracto crudo de stevia (Glycostevia-EP®) se caracterizan por ser una buena fuente de glucósidos de esteviol. El mayor contenido en glucósidos de esteviol se obtiene en uno de los extractos acuosos de hojas de stevia, con un contenido mayoritario de steviósido, seguido por el rebaudiósido F y A. En los glucósidos de esteviol purificados disponibles comercialmente sólo se detecta el rebaudiósido A, y en el caso de Truvia®, además el esteviósido.

En la literatura científica se observa que los glucósidos de esteviol, además de ser edulcorantes acalóricos de origen natural, presentan capacidad antioxidante (Stoyanova y col., 2011; Wozniak y col., 2014). Sin embargo, en el presente estudio, los resultados muestran que los extractos purificados de glucósidos de esteviol presentan capacidad antioxidante cuando se analizan con el método ORAC pero no con el método TEAC. Con el fin de verificar el distinto comportamiento de los glucósidos de esteviol con los dos métodos de análisis, se preparan y analizan distintas concentraciones de patrones de glucósidos de esteviol (10-50 mg/100 mL). Un incremento en la concentración de rebaudiósido A, C, F, esteviósido y esteviol da lugar a un incremento en la capacidad antioxidante con el método ORAC, pero no se detecta capacidad antioxidante con el TEAC, corraborando los resultados obtenidos previamente. Prakash & Chaturvedula (2014) describen la presencia de protones anoméricos en las estructuras de los glucósidos de esteviol y dado que el método ORAC se basa en la transferencia de átomos de hidrógeno (Zulueta y col., 2009), dichos compuestos pueden determinarse con este método de análisis, lo que se debe tener en cuenta cuando se establecen conclusiones acerca de la capacidad antioxidante de los glucósidos de esteviol.

*Formulación de una bebida a base de zumo de frutas, bebida de avena y extracto acuoso de stevia*

Cuando se preparan tres formulaciones a base de zumo de frutas (papaya, mango y naranja), bebida de avena y açaí deshidratado con distintas concentraciones de extracto acuoso de stevia (0, 1,25 y 2,5%, p/v), se observa un aumento significativo de los compuestos fenólicos (unas 3 y 4 veces mayor) y de la capacidad antioxidante cuando se endulza la bebida con 1,25 y 2,5% (p/v) de stevia, respectivamente.

Asimismo, se observan interacciones sinérgicas entre los distintos componentes de las matrices formuladas (0, 1,25 y 2,5% de stevia, p/v) en la capacidad antioxidante total medida con el método TEAC, posiblemente atribuido a la mejora de la solubilidad o estabilidad de los compuestos con capacidad antioxidante, tal como indican estudios previos (Sęczyk y col., 2016). Sin embargo, en la capacidad antioxidante medida con el método ORAC en las bebidas con stevia (1,25 y 2,5%, p/v) no se observan interacciones sinérgicas sino aditivas, lo que muestra que la presencia de stevia en la matriz alimentaria es determinante en la modulación de la capacidad antioxidante potencial de la bebida objeto de estudio.

*Puesta a punto de un método de determinación de lisina disponible en alimentos líquidos*

En ocasiones, las bebidas funcionales incluyen entre sus ingredientes alimentos que son fuente de proteínas, como es el caso de la bebida de avena. Entre sus aminoácidos, la lisina se considera un aminoácido limitante en cereales, con un requerimiento diario de 30 mg/kg/día de acuerdo con la OMS (WHO/FAO/UNU, 2007). Sin embargo, cuando los alimentos se someten a altas temperaturas se puede producir la destrucción de este aminoácido y la lisina queda inutilizada por unirse a la glucosa (reacción de Maillard). El resto de la lisina que se encuentra disponible puede ser usada como indicador de la calidad proteica y ser una herramienta para predecir cambios nutricionales (Lizarazo y col., 2015). Por este motivo, se pone a punto un método fluorimétrico

automatizado para el análisis de la lisina disponible en alimentos, para evaluar distintos parámetros nutritivos y de calidad de estas bebidas, sometidas o no a tecnologías no térmicas de conservación.

Se automatiza el método fluorimétrico propuesto por Ferrer y col. (2003) empleando placas Well de 96 pocillos y se analiza el contenido de lisina en alimentos tanto de origen animal como vegetal incluyendo alimentos líquidos (bebida de avena, bebida de soja, bebida de quinoa y leche esterilizada UHT) y alimentos en polvo (fórmulas infantiles de inicio, continuación y crecimiento).

Los parámetros analíticos muestran que el método es sensible, preciso y exacto, además de sencillo, lo que le hace adecuado para determinaciones rutinarias del contenido de lisina disponible en alimentos.

## 4.2. Aplicación de tecnologías no térmicas en la elaboración y procesado de bebidas a base de frutas

A continuación se estudia el uso de tecnologías no térmicas (altas presiones hidrostáticas (APH), pulsos eléctricos de alta intensidad (PEAI), descargas eléctricas de alto voltaje (DEAV) y ultrasonidos (USN)) en la elaboración y procesado de las bebidas formuladas para disminuir los cambios sensoriales y nutricionales que tienen lugar con la aplicación de calor.

*Procesado de zumos de frutas por APH adicionados de Stevia rebaudiana como ingrediente antioxidante*

Para estudiar el efecto de las APH en la bebida a base de zumo de papaya, mango y naranja, así como su combinación con el uso de stevia como ingrediente con capacidad antioxidante, se realiza un análisis de respuesta superficie con las siguientes variables: presión de 300-500 MPa, tiempo de tratamiento de 5-15 minutos y porcentaje de stevia de 0-2,5% (p/v).

Se obtiene un alto porcentaje de retención de ácido ascórbico (≥ 92%) al aplicar APH junto con el uso de stevia, lo que resulta prometedor por la elevada labilidad de esta vitamina y su especial interés cuando se ingieren bebidas a base de zumos de frutas. También se observa una relativa resistencia por parte de los

compuestos fenólicos a las APH, efecto que se ve potenciado en bebidas formuladas con stevia e incluso se obtiene un incremento en el contenido de compuestos fenólicos al aplicar una presión de 300 MPa durante 10 minutos en una bebida con 1,25% (p/v) de stevia y al aplicar la misma presión durante 5 minutos con 2,5% (p/v) de stevia. Este incremento puede atribuirse a la extracción de algunos compuestos intracelulares tras la aplicación de APH. Asimismo, el contenido mayor de antocianinas se observa al aplicar el máximo nivel de presión (500 MPa), tiempo (15 minutos) y concentración de stevia (2,5%, p/v) por el motivo comentado anteriormente y la ruptura de enlaces entre antocianinas y distintas moléculas a las que se encuentran unidas, aumentando su contenido en la bebida a base de zumo de frutas y evitando su degradación, que cabría esperar tras el tratamiento térmico (Sui y col., 2016). La presión es el parámetro que más afecta a los carotenoides totales, con un aumento de su contenido al aumentar la presión.

Como es de esperar, la capacidad antioxidante total de la bebida aumenta al aplicar de manera conjunta APH junto con la adición de stevia, obteniendo la máxima capacidad antioxidante al aplicar una presión de 500 MPa durante 5-15 minutos y 2,5% (p/v) de stevia, lo que muestra la idoneidad de la combinación de estrategias de conservación en el procesado de bebidas a base de zumo de frutas.

Sin embargo, las diferencias de color son superiores al aumentar la presión o el tiempo de tratamiento de APH. Las condiciones óptimas para obtener la bebida con el máximo contenido de compuestos bioactivos y capacidad antioxidante y el mínimo cambio de color son de 300 MPa, 14 minutos y 1,73% (p/v) de stevia.

*Estudio del efecto de los PEAI en el contenido de ácido ascórbico en bebidas a base de zumo de naranja*

Antes de proceder al estudio del efecto de los PEAI sobre la bebida a base de zumos de frutas con stevia, se estudia su efecto sobre el contenido en ácido ascórbico, vitamina termolábil fácilmente degradable con el tratamiento térmico que se emplea como indicador de calidad nutricional tras la aplicación de un proceso de conservación. Se evalua la cinética de degradación del ácido ascórbico tras la aplicación de PEAI en distintas bebidas a base de zumo de naranja (zumo

de naranja, mezcla de zumo de naranja y zanahoria y bebida a base de zumo de naranja y leche UHT desnatada), considerado el de mayor consumo a nivel mundial (AIJN, 2015) y se compara con las muestras tratadas térmicamente.

La retención de ácido ascórbico en las muestras tratadas térmicamente es de 92,5, 83,2 y 86,1% para el zumo de naranja, mezcla de zumo de naranja y zanahoria y bebida a base de zumo de naranja y leche, respectivamente. La aplicación de PEAI con un campo eléctrico de 15-40 kV/cm y un tiempo de tratamiento de 30-700 µs en las distintas muestras de naranja da lugar a una retención de ácido ascórbico superior al 97% en todas las muestras. Estos resultados se obtienen tras la aplicación de un campo eléctrico de 25 kV/cm durante 40 µs, 40 kV/cm durante 30 µs, y 25 kV/cm durante 40 µs para el zumo de naranja, mezcla de zumo de naranja y zanahoria y bebida a base de zumo de naranja y leche, respectivamente.

El tiempo de reducción decimal de la bebida zumo-leche es superior al de las otras matrices, es decir, es necesario más tiempo de tratamiento para obtener una reducción del 10% de ácido ascórbico. En cambio, el zumo de naranja es el más resistente a los cambios de campo eléctrico. Este distinto comportamiento observado en la bebida a base de zumo de naranja y leche al estudiar la influencia del campo eléctrico y tiempo de tratamiento en la degradación del ácido ascórbico puede deberse a sus distintas características fisicoquímicas (pH y conductividad eléctrica), al efecto protector que pueden ejercer componentes de la leche, principalmente caseínas (Sobrino-López & Martín-Belloso, 2010), y/o a la adición de ácido cítrico como conservante en la bebida zumo-leche.

*Procesado de zumos de frutas por PEAI adicionados de Stevia rebaudiana como ingrediente natural con capacidad antioxidante/antimicrobiana*

Para evaluar el efecto del tratamiento de PEAI con la adición de *Stevia rebaudiana* como ingrediente natural con actividad antioxidante y antimicrobiana, se realiza un análisis de respuesta superficie (campo eléctrico de 20-40 kV/cm, tiempo de 100-360 µs y porcentaje de stevia de 0-2,5% (p/v)) a una bebida a base de zumo de papaya y mango, factible de ser tratada por PEAI, con una conductividad de 0,245 S/m. Además de demostrar la eficacia de stevia en la

inactivación de *Listeria monocytogenes* y de la actividad enzimática en combinación con la aplicación de PEAI, se observa un incremento de los compuestos fenólicos totales en esta bebida con stevia tratada por PEAI, que puede atribuirse a la extracción del contenido intracelular causado por la electroporación tras la aplicación de puslsos eléctricos junto con la demostrada inactivación de la polifenoloxidasa en presencia de stevia.

Sin embargo, el tratamiento por PEAI da lugar a una disminución del contenido de ácido ascórbico (a mayor campo eléctrico, mayor degradación), posiblemente causado por la liberación de vitamina C tras la electroporación, susceptible de ser oxidada y degradada, como señalan otros autores (Leong y col., 2016). El mayor contenido de antocianinas totales se obtiene al aplicar el mayor campo eléctrico (40 kV/cm) en presencia de stevia, de manera similar a los resultados obtenidos tras el tratamiento con APH. Este mismo efecto se observa con el contenido de carotenoides totales, ya que en presencia de stevia se alcanzan los máximos niveles con el tratamiento más intenso de PEAI. Los PEAI pueden actuar de modo ambiguo favoreciendo la extracción de carotenoides a partir de la matriz alimentaria pero generando especies reactivas que pueden dar lugar a la oxidación de los carotenoides, por lo que la stevia puede desempeñar un efecto protector dado su contenido en compuestos antioxidantes.

En la bebida sin stevia tratada con un campo eléctrico de 40 kV/cm, un aumento del tiempo de tratamiento repercute negativamente en la capacidad antioxidante. Aunque los PEAI son una técnica de conservación no térmica, puede tener lugar un ligero aumento de la temperatura con campos eléctricos elevados, como han visto otros autores (Zhang y col., 2015), con lo que los compuestos termolábiles pueden verse afectados y disminuir la capacidad antioxidante. Sin embargo, no ocurre cuando se incluye stevia entre los ingredientes de la bebida, mostrando su efecto protector en la conservación de compuestos bioactivos y capacidad antioxidante de la bebida objeto de estudio.

Como no sólo se pretende asegurar el aporte de compuestos bioactivos sino ofrecer un producto similar al fresco, se analizan también algunos parámetros fisicoquímicos tras los tratamientos de PEAI. Se obtiene una disminución del índice de pardeamiento al aumentar la concentración de stevia, el campo

eléctrico y el tiempo de tratamiento. El contenido de hidroximetilfurfural, relacionado con la pérdida de calidad de productos procesados, es menor en bebidas con adición de stevia, mostrando nuevamente el efecto protector de este ingrediente. La luminosidad (*L**) aumenta tras la aplicación de PEAI en la bebida sin stevia, posiblemente en relación con la inactivación de enzimas responsables del pardeamiento tras la aplicación de pulsos eléctricos, tal y como señalan otros estudios (Bi y col., 2013). Las mayores variaciones de color observadas en bebidas sin stevia pueden deberse a la ruptura de membranas celulares con la consiguiente pérdida de funcionalidad de la compartimentalización celular y el aumento del contacto enzima-sustrato.

Por lo que respecta a los glucósidos de esteviol, la aplicación de PEAI no modifica el contenido de rebaudiósido A y esteviósido de la bebida, salvo cuando se aplica 20 kV/cm durante 360 µs y 30 kV/cm durante 230 µs, dando lugar a un incremento de estos compuestos. Los PEAI pueden facilitar la extracción de los glucósidos de esteviol pero sólo aplicando condiciones moderadas, posiblemente por la degradación de estos compuestos a campos eléctricos altos, coincidiendo con observaciones de otros autores al emplear otras tecnologías no térmicas (Periche y col., 2015), que obtienen un incremento de la extracción de glucósidos de esteviol con ultrasonidos siempre que se empleen bajas temperaturas y tiempos de tratamiento. El contenido de rebaudiósido F y C aumenta tras el tratamiento por PEAI pero sólo en el caso de stevia al 2,5%, mostrando que hay una liberación de estos compuestos de la matriz alimentaria, y que además pueden tener lugar reacciones de síntesis y degradación como consecuencia de la pérdida de compartimentalización tras la electroporación, favoreciendo ciertas reacciones de hidrólisis o glicosilación de los glucósidos de esteviol y modificando su contenido en los productos alimentarios. Sin embargo, lo realmente interesante es el ratio rebaudiósido A/esteviósido, que representa un buen indicador cualitativo de la dulzura (a mayor ratio, mejor sabor) (Tavarini & Angelini, 2013). El mayor ratio se alcanza en la bebida adicionada de 2,5% de stevia tratada a 30 kV/cm durante 230 µs, correlacionado positivamente con los valores de sólidos solubles totales, por lo que puede valorarse su uso como medida de calidad edulcorante.

Teniendo en cuenta los resultados anteriores y como cabe esperar del mayor contenido de stevia (2,5%), la aplicación de un campo eléctrico de 21 kV/cm durante 360 µs permite la obtención del máximo contenido de compuestos bioactivos y propiedades edulcorantes y el mínimo cambio sensorial (hidroximetilfurfural y cambios de color). Sin embargo, cuando el objetivo es conocer las condiciones para obtener una bebida con la mínima carga microbiana, actividad enzimática y pardeamiento no enzimático y el máximo contenido de compuestos fenólicos y capacidad antioxidante con el fin de ofrecer al consumidor una bebida segura con una alta capacidad antioxidante, éstas son 40 kV/cm de campo eléctrico, 360 µs de tiempo y 1,79%, (p/v) de stevia. Esta concentración de stevia coincide prácticamente con la obtenida en el estudio de optimización de APH aplicado a la bebida a base de zumo de papaya, mango y naranja.

*Comparación del procesado de zumos de frutas por tecnologías de pulsos eléctricos y ultrasonidos*

Asimismo, es importante comparar el efecto de las distintas tecnologías no térmicas, por lo que se trata la bebida a base de zumo de papaya y mango edulcorada con un 2,5% (p/v) de stevia, la de mayor contenido en compuestos bioactivos, a dos energías equivalentes (32 y 256 kJ/kg) con pulsos eléctricos de alta intensidad, descargas eléctricas de alto voltaje y ultrasonidos.

En función del compuesto bioactivo analizado, una tecnología resulta mejor que otra. Así, los USN son la tecnología que permite la mayor concentración remanente de ácido ascórbico, mientras que en el caso de los carotenoides son los PEAI y en el caso de los compuestos fenólicos las DEAV con una energía de 256 kJ/kg. Teniendo en cuenta estos resultados, se realiza un estudio de componente principal para poder ofrecer una conclusión sobre qué tecnología puede ser mejor para asegurar la presencia de compuestos bioactivos y capacidad antioxidante en la bebida. Los principales compuestos influyentes en la distribución de compuestos bioactivos son los carotenoides y el ácido ascórbico, seguido por los valores de capacidad antioxidante analizados por ORAC. Teniendo en cuenta esto, se puede concluir que el tratamiento de PEAI es el que permite obtener la bebida

a base de zumo de papaya, mango y stevia con mayor contenido de compuestos bioactivos.

*Obtención de zumo de "hielo" por prensado de manzanas pretratadas por PEAI*

La congelación del tejido vegetal y su posterior descongelación a la vez que se prensa permite la producción de un zumo más concentrado en las primeras etapas del proceso, cuando todavía existen cristales de agua en el tejido vegetal, mientras que se extraen los solutos no congelados, conocido como zumo de "hielo". Buscando obtener un zumo con el mayor contenido en compuestos bioactivos, se estudia el prensado de tejidos de manzana (congelados y tratados por PEAI + congelados) a distintas presiones (2, 3 y 5 bares) para la obtención de zumo de manzana, el tercero en orden de consumo tras el de naranja y el multisabor (AIJN, 2015).

En este caso se combina con el pretratamiento por PEAI, que acelera el proceso de congelación del tejido de manzana, posiblemente causado por los poros creados por los PEAI, lo que resulta beneficioso para la formación de cristales de hielo más pequeños y una mejor calidad del producto final. A pesar de que ambos procesos (congelación y aplicación de PEAI) causan daño celular, aumentando el rendimiento del zumo obtenido, la aplicación de PEAI puede dar lugar a cambios en la distribución espacial del contenido no congelado en el interior del tejido que facilita el flujo de zumo al aplicar una presión externa y por tanto explica la mejora en la cinética de producción del zumo de manzana.

Las primeras fracciones de zumo de manzana obtenido cuando la temperatura en el interior del tejido es inferior a 0 ºC tienen un contenido de sólidos solubles (ºBrix) superior al de las últimas fracciones, alcanzando el máximo en el intervalo de temperatura entre 0 y 5 ºC, independientemente de la presión aplicada, como cabe esperar, dado que refleja el típico proceso de concentración por congelación, donde inicialmente se obtiene el zumo más concentrado y conforme pasa el tiempo, los cristales de hielo en el interior del tejido se descongelan y la concentración de sólidos solubles disminuye gradualmente. La aplicación de PEAI supone un aumento de la concentración de sólidos solubles en las primeras fracciones, lo que se puede explicar teniendo en cuenta que la descongelación

comienza en la superficie y la electroporación puede ejercer un efecto suplementario en el proceso de transferencia de materia en el tejido.

Cuando se analiza la concentración de sólidos solubles en las porciones de zumo acumulado, se obtiene una mayor concentración de sólidos solubles en el zumo de manzana pretratado y sin pretratar (12,2 y 11,5 ºBrix, respectivamente) con el prensado a 5 bares en comparación con el prensado a 2 bares (8,8 ºBrix en ambos casos y un rendimiento de un 40% con respecto al zumo obtenido a 5 bares).

El pretratamiento por PEAI también da lugar a una mayor extracción de sólidos solubles, hidratos de carbono, compuestos fenólicos, flavonoides y capacidad antioxidante total en todos los casos. Por tanto, el proceso propuesto de PEAI + congelación + descongelado/prensado no sólo permite una reducción del tiempo de congelado, descongelado y prensado desde el punto de vista de ahorro de tiempo, sino también la obtención de un zumo de elevada calidad nutricional con un alto contenido en compuestos bioactivos.

### 4.3. Evaluación *in vitro* de la bioaccesibilidad de compuestos bioactivos en bebidas a base de zumo de frutas y stevia

Dada la importancia que los compuestos bioactivos se liberen de la matriz intestinal y se encuentren disponibles para ser absorbidos en el tracto gastrointestinal, se realiza un estudio de su bioaccesibilidad mediante una simulación gastrointestinal de las bebidas a base de zumo de papaya, mango, naranja, bebida de avena y açaí, edulcoradas con distintos porcentajes de stevia (0, 1,25 y 2,5%, p/v). Entre los métodos propuestos, se selecciona la simulación gastrointestinal descrita por Rodríguez-Roque y col. (2013) con algunas modificaciones, consistente en tres fases secuenciales: salivar (α-amylasa, mucina, $Na_2HPO_4$, $KH_2PO_4$ y NaCl, pH 6,75), gástrica (pepsina, pH 2) e intestinal incluyendo diálisis (pancreatina y bilis, pH 7,5).

El contenido inicial de ácido ascórbico en las bebidas es de 28-33 mg/100 mL, mientras que en la fracción bioaccesible es inferior (0-13 mg/100 mL). No se detecta ácido ascórbico en la fracción bioaccesible de la bebida con stevia al 2,5%

(p/v), posiblemente porque la presencia de extracto acuoso de stevia puede interferir en la estabilidad del ácido ascórbico al aumentar el pH resultante. Con la ingesta de zumos a base de frutas se busca un aporte de vitaminas, siendo una de las principales la vitamina C que sólo está presente en alimentos de origen vegetal, lo que condiciona el uso del extracto acuoso de stevia como ingrediente en esta bebida, o al menos de su concentración, ya que en un porcentaje de 1,25% (p/v), sí que se detecta ácido ascórbico en la fracción bioaccesible (6,1 mg/100 mL), aunque inferior al contenido en la bebida sin stevia.

Sin embargo, la adición de stevia supone una mejora en el contenido de carotenoides, compuestos fenólicos y antocianinas en la fracción bioaccesible. Además, la adición de extracto acuoso de stevia aumenta la capacidad antioxidante total en la fracción bioaccesible, por ser mayor ésta en la bebida inicial, independientemente del método de análisis empleado (TEAC, DPPH y ORAC), y por el aumento del porcentaje bioaccesible en el caso de TEAC y DPPH, posiblemente relacionado con el contenido en compuestos fenólicos como muestra el análisis de regresión múltiple.

Los bajos valores de bioaccesibilidad de los carotenoides (0,6-6,0%) y antocianinas (0,2-9,4%) que se observan pueden atribuirse a su escasa incorporación en la fracción bioaccessible, pero debe tenerse en consideración que los resultados aquí presentados pueden estar subestimados, ya que en la absorción intestinal tienen lugar otros mecanismos de transporte (transporte facilitado y transporte activo) que no se analizan en el presente estudio. Además, en el caso de los compuestos fenólicos, la microflora intestinal también desempeña un papel crucial en la absorción de estos compuestos, no evaluado con el método descrito. Por ello, aunque estos datos puedan servir para establecer conclusiones preliminares, serían necesarios estudios adicionales *in vivo* evaluando la idoneidad de la adición de stevia en la bebida a base de zumo de papaya, mango, naranja, bebida de avena y açaí.

A pesar del aumento del contenido de glucósidos de esteviol en la bebida tras el proceso de digestión al aumentar la concentración de stevia, su porcentaje de bioaccesibilidad no se ve modificado. Éste es de 23,9-29,0% en el caso del rebaudiósido A, esteviósido y rebaudiósido C, mientras que la bioaccesibilidad del

rebaudiósido F es muy elevada (160,3-172,9%), pudiéndose atribuir a una degradación enzimática a partir de otros glucósidos de esteviol (Pande & Gupta, 2013), aunque el mecanismo de biosíntesis de los glucósidos de esteviol aún no está completamente elucidado.

### 4.4. Referencias

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# 5. Conclusions

*Conclusiones*

## 5. CONCLUSIONES

Del estudio realizado se pueden establecer las siguientes conclusiones:

1. Los extractos acuosos de *Stevia rebaudiana* se pueden considerar una fuente natural de glucósidos de esteviol y compuestos antioxidantes, principalemente compuestos fenólicos.
2. El empleo de un 2,5% (p/v) de extracto acuoso de stevia en una bebida a base de zumo de papaya, mango, naranja, bebida de avena y açaí aumenta el contenido en compuestos bioactivos y su capacidad antioxidante total, mostrando efectos sinérgicos cuando se emplea el método TEAC para determinar la capacidad antioxidante.
3. El método fluorimétrico puesto a punto permite la cuantificación y automatización de la determinación de lisina disponible en alimentos líquidos y en polvo. Los parámetros analíticos indican que es un método sensible, preciso y exacto.
4. Las APH, aplicando una presión de 300 MPa durante 14 minutos, junto con el empleo de stevia al 1,73% (p/v) maximiza el contenido de compuestos bioactivos (ácido ascórbico, compuestos fenólicos, antocianinas y carotenoides) y capacidad antioxidante mientras que minimiza las diferencias de color de una bebida a base de zumo de papaya, mango y naranja.
5. Al aplicar los PEAI en distintos zumos a base de naranja se obtiene que el zumo de naranja es el más resistente a los cambios de intensidad de campo eléctrico mientras que para producir la misma degradación del ácido ascórbico, el tiempo de tratamiento es superior en la bebida de zumo de naranja-leche, mostrando la necesidad de optimizar las condiciones de tratamiento cada vez que se cambia la matriz o algún factor del procesado.
6. La aplicación de dos estrategias de conservación (PEAI y stevia) a una bebida a base de zumo de papaya y mango permite obtener:
   a) Una bebida segura con la menor carga microbiana, actividad de enzimas oxidativas y pardeamiento no enzimático así como un alto contenido en compuestos fenólicos y capacidad antioxidante, con un campo eléctrico de 40 kV/cm durante 360 µs y la adición de 1,79% (p/v) de stevia.

b) Una bebida rica en compuestos bioactivos (ácido ascórbico, antocianinas y carotenoides), glucósidos de esteviol y mínimos cambios sensoriales (hidroximetilfurfural y color) con un campo eléctrico de 21 kV/cm durante 360 μs y un porcentaje de stevia de 2,5% (p/v).

7. El efecto de las tecnologías no térmicas estudiadas (PEAI, DEAV y USN) en la bebida a base de zumo de papaya, mango y stevia (2,5%, p/v) al aplicar energías de 32 y 256 kJ/kg depende del compuesto bioactivo estudiado, de modo que:
   a) Los USN permiten la obtención de la bebida con el mayor contenido de ácido ascórbico.
   b) Los PEAI son la tecnología que permiten un mayor contenido de carotenoides y capacidad antioxidante medida con el método ORAC.
   c) El contenido en compuestos fenólicos y capacidad antioxidante medida con el método TEAC y DPPH es superior en la bebida tratada por DEAV con una energía de 256 kJ/kg.
   d) Los PEAI son, en general, la tecnología que mejor retiene los compuestos bioactivos y capacidad antioxidante.
8. Los PEAI constituyen una técnica que, aplicada como pretratamiento al prensado a temperaturas bajo cero, permite obtener un zumo de manzana con un mayor contenido de sólidos solubles, hidratos de carbono, compuestos fenólicos, flavonoides y capacidad antioxidante en las condiciones empleadas.
9. El estudio de la bioaccesibilidad de la bebida a base de zumo de papaya, mango, naranja, bebida de avena y açaí, adicionada o no de stevia, indica que:
   a) No se detecta ácido ascórbico en la bebida con la máxima concentración (2,5%, p/v) de stevia tras el proceso de digestión.
   b) La adición de stevia supone un aumento de la bioaccesibilidad de carotenoides, compuestos fenólicos y antocianinas.
   c) La bioaccesibilidad de la capacidad antioxidante aumenta a medida que lo hace el contenido de stevia.
   d) A pesar del aumento del contenido de glucósidos de esteviol en la bebida tras el proceso de digestión al aumentar la concentración de stevia, su porcentaje de bioaccesibilidad no se ve modificado.

**CONCLUSIONS**

The following conclusions can be established from the present study:

1. Aqueous extracts of *Stevia rebaudiana* can be considered a natural source of steviol glycosides and antioxidant compounds, mainly phenolic compounds.
2. The use of 2.5% (w/v) aqueous stevia extracts in a beverage based on papaya, mango and orange juice, oat beverage and açaí increases bioactive compounds and total antioxidant capacity, showing synergistic effects when TEAC method is used to determine total antioxidant capacity.
3. The fluorimetric method developed allows quantification and automatisation of available lysine determination in liquid and powdered foods. Analytical parameters indicate it is a sensitive, precise and exact method.
4. Application of HPP at a pressure of 300 MPa during 14 minutes, along with the use of 1.73% (w/v) of stevia maximises bioactive compounds content (ascorbic acid, phenolic compounds, anthocyanins and carotenoids) and antioxidant capacity while minimising the differences in colour of a drink made from papaya, mango and orange juice.
5. When applying PEF treatment to different orange juice based beverages, orange juice is the most resistant to electric field strength changes while orange juice-milk beverage needs a greater treatment time in order to achieve the same ascorbic acid degradation. These results highlight the need to optimise processing conditions when food matrix or a processing factor is changed.
6. Application of two conservation strategies (PEF and stevia) to a beverage based on papaya and mango juice allows the obteinment of:
    a) A safe beverage with the lowest microbial load, oxidative enzymes activity and non-enzymatic browning along with a high content of phenolic compounds and antioxidant capacity with an electric field of 40 kV/cm during 360 μs and 1.79% (w/v) of stevia.
    b) A beverage rich in bioactive compounds (ascorbic acid, anthocyanins, carotenoids), steviol glycosides and the minimum sensory changes

(hydroxymethylfurfural and colour) with an electric field of 21 kV/cm during 360 μs and 2.5% (w/v) of stevia.

7. The effect of the studied non-thermal technologies (PEF, HVED and USN) in the papaya, mango juice and stevia (2.5%, w/v) based beverage when applying energy inputs of 32 and 256 kJ/kg depends on the bioactive compound studied, so:
   a) USN allows the obteinment of the beverage with the highest content of ascorbic acid.
   b) PEF technology allows the highest content of carotenoids and antioxidant capacity measured with the ORAC method.
   c) Phenolic content and antioxidant capacity measured with DPPH and TEAC method are highest in the beverage treated by HVED with an energy input of 256 kJ/kg.
   d) PEF is, in general, the technology which better retains bioactive compounds and antioxidant capacity.
8. PEF treatment is a technique which, applied as pretreatment to pressing at subzero temperatures, allows the obteinment of an apple juice with a higher soluble solid content, carbohydrates, phenolic compounds, flavonoids and total antioxidant capacity under the assayed conditions.
9. The bioaccessibility study of the beverage based on papaya, mango, orange, açaí and oat beverage, with or without stevia, indicates that:
   a) No ascorbic acid is detected in the beverage with the maximum stevia concentration (2.5%, w/v) after the digestion process.
   b) Stevia addition results in an increase in the carotenoid, anthocyanin and phenolic compound bioaccessibility.
   c) Bioaccessibility of antioxidant capacity increases as does stevia concentration.
   d) Despite the increase in the steviol glycosides content in the beverage after the digestion process when increasing the stevia concentration, bioaccessibility percentage does not modify.

# 6. Annex

*Anexo*

In: Leaf Sweeteners
Editor: Wenbiao Wu
ISBN: 978-1-63463-072-6


*Chapter 4*

# EMERGING ROLE OF *STEVIA REBAUDIANA* BERTONI AS SOURCE OF NATURAL FOOD ADDITIVES

***Juana M. Carbonell-Capella, María J. Esteve and Ana Frígola****
Department of Nutrition and Food Chemistry,
Universitat de València, Burjassot, Spain

## ABSTRACT

*Stevia rebaudiana* (Stevia) leaf extract, used as a vegetable-based sweetening additive in drinks and other foods due to steviol glycosides content, has been demonstrated to exhibit extremely high antioxidant capacity due to its high content in potential antioxidant food compounds such as phenolic compounds. However, concentration of bioactive compounds and total antioxidant capacity in stevia products may depend on the origin of the product. For this reason, Stevia leaves direct infusions, *Stevia* crude extract (Glycostevia-EP®), purified steviol glycosides (Glycostevia-R60®), and commercialized Stevia powdered samples in different countries (PureVia, TruVia and Stevia Raw) were evaluated for their content in ascorbic acid (AA), total carotenoids (TC), total phenolic content (TPC), phenolic profile, total anthocyanins (TA), steviol glycosides profile, and antioxidant capacity (trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC)). Eleven phenolic compounds, including hydroxybenzoic acids (2), hydroxycinnamic acids (5), flavones (1), flavonols (2) and flavanols (1) compounds, were identified in *Stevia*-derived products. Of these, chlorogenic acid was the major phenolic acid. Rebaudioside A and stevioside were the most abundant sweet-tasting diterpenoid glycosides. Total antioxidant capacity (TEAC and ORAC) was shown to be correlated with TPC. From all of the analysed samples, Stevia leaves direct infusions and *Stevia* crude extract (Glycostevia-EP®) were found to be a good source of sweeteners with potential antioxidant capacity.

**Keywords:** *Stevia rebaudiana*, food additives, steviol glycosides, phenolic compounds



* E-mail address: ana.frigola@uv.es. Phone: +34 963544955, Fax: +34 963544954.

Food Anal. Methods
DOI 10.1007/s12161-012-9535-y

# Automating a 96-Well Microtiter Plate Assay for Quick Analysis of Chemically Available Lysine in Foods

**Francisco J. Barba · Juana M. Carbonell-Capella · María J. Esteve · Ana Frígola**

Received: 7 September 2012 / Accepted: 15 November 2012


**Abstract** A new method for quick analysis of available lysine content in different food products has been developed by automating a 96-well microtiter plate assay. Although manual fluorometric methods validated in order to determinate available lysine content already existed for this compound, the benefits of applying appropriate automation should provide continuous operation, increased precision, an affordable electronic audit trail, and significantly reduced time and reagent consumption. The objective of this work was to adapt the *ortho*-phthaldialdehyde (OPA) fluorometric method to an automated workstation. Considerable effort went into developing and validating an automated method. The analytical parameters of linearity ($r$=0.999), the precision of the method (relative standard deviations=2.8–3.0 % for the different samples), and the results of the comparison with the corresponding OPA manual fluorometric method show that the studied method is useful for the measurement of available lysine in several food products from different natural origins such as liquid foods (soy, oat, quinoa beverages, and ultra-high temperature/sterilized milk) and powdered samples (powdered adapted, powdered follow-up, and junior milk infant formulas) with reduced time and reagent consumption.

**Keywords** Lysine · 96-Well microtiter plate assay · Automation · Maillard reaction · Fluorometric determination

F. J. Barba
Instituto de Agroquímica y Tecnología de Alimentos,
Consejo Superior de Investigaciones Cientificas (CSIC),
Avda. Agustín Escardino, 7.,
46980 Paterna, Spain

J. M. Carbonell-Capella · M. J. Esteve · A. Frígola (✉)
Department of Nutrition and Food Chemistry,
Universitat de València, Avda. Vicent Andrés Estellés, s/n.,
46100 Burjassot, Spain
e-mail: ana.frigola@uv.es

## Introduction

Maillard browning is one of the main chemical reaction causing deterioration of proteins during processing and storage of foods. This reaction between free amino groups and reducing sugars reduces protein digestibility and amino acid availability (Malec et al. 2002).

Available lysine content is an indicator of early and advanced Maillard reaction phases (Ferrer et al. 1999) and can be a useful tool in order to predict nutritional losses. The quantitative analysis of available lysine content together with its degradation products has been used as a chemical marker of protein quality (Meade et al. 2005).

Different methods like spectrophotometric (Carpenter 1960; Kakade and Liener 1969; Vigo et al. 1992), chromatographic (Albalá-Hurtado et al. 1997; Fernández-Artigas et al. 1999; McEwen et al. 2010), and fluorometric (Ferrer et al. 2003; Goodno et al. 1981; Morales et al. 1995) have been proposed for the determination of available lysine content in food products. Conventionally, the fluoro-2,4-dinitrobenzene assay has been the most extensively used method (Smith 2010). However, Vigo et al. (1992) and Morales et al. (1995) demonstrated that this method was time consuming and special precautions were necessary. Dialysis of carbohydrate-rich samples is recommended since it avoids the uncertainty inherent in applying correction factors for reaction interference, but this adds 2 or 3 days to each assay (Tomarelli et al. 1985). In order to eliminate possible interferences by spectrophotometric methods, Goodno et al. (1981) established a fluorometric analysis using *ortho*-phthaldialdehyde (OPA) for estimating reactive lysine in proteins, which has been used widely in the published literature (Morales et al. 1995; Swaisgood and Ctagnani 1985; Vigo et al. 1992). The use of OPA does not require hydrolysis or amino acid analysis of the sample and does not require heating or solvent extraction that can release lysine from modified forms (Ferrer et al. 2003). The

Published online: 29 November 2012

OPA reaction is rapid and complete at room temperature, and the complex formed by lysine and OPA is fluorescent, while side-products are not fluorescent. Furthermore, the amount of sample needed is small, sugars do not interfere, and the assay is reproducible and easy to perform. The main disadvantage is the instability of the fluorescent complex (Goodno et al. 1981). More recently, chromatography has been used for determination of available lysine. Chromatographic methods offer good linearity and reproducibility, but they are time and solvent consuming. The aim of the current study was to develop and validate a new method (sensitive, economical, and with minimal solvent consumption) for quick analysis of available lysine content in different food products by automating a 96-well microtiter plate assay.

## Materials and Methods

### Samples

Three units from each of two batches of different food products marketed in Spain were purchased from a local supermarket (Valencia, Spain) and were analyzed: liquid foods (soy, oat, quinoa beverages, and ultra-high temperature/sterilized milk) and powdered samples (powdered adapted, powdered follow-up, and junior milk infant formulas) were used. The powdered samples were rehydrated in accordance with the manufacturer's instructions (130 g/L). Table 1 gives details (as indicated on the label) of each of the samples analyzed.

### Materials and Reagents

All reagents were of analytical reagent grade. Anhydrous ethanol, sodium tetraborate, and sodium hydroxide were from Panreac (Barcelona, Spain). Casein from bovine milk was from Sigma-Aldrich (Steinheim, Germany). Intermediate (10 mg/mL casein) and working standard solutions were prepared in sodium tetraborate buffer (pH=9). Trichloroacetic acid (TCA) was from Fluka (Buchs, Switzerland). Hydrochloric acid, β-mercaptoethanol, sodium dodecyl sulfate (SDS), and ethanol were from Merck (Darmstadt, Germany). OPA reagent was prepared daily according to Goodno et al. (1981) as follows: 80 mg OPA 99 % (Merck) in 2 mL ethanol, 50 mL 0.1 M sodium tetraborate buffer (pH9.7–10.0), 5 mL SDS (200 g/L), and 0.2 mL β-mercaptoethanol. Ninety-six-well black bottom plates were from Sero-Wel, Bibby Sterilin Ltd. (Stone, UK).

### Instrumentation

All analyses were performed on a spectrofluorophotometer (RF-5000 Shimadzu Corporation, Kyoto, Japan) for the OPA manual method and on a microplate spectrofluorometer Wallac 1420 VICTOR2 multilabel counter from Perkin-Elmer (CA, USA) for the OPA automated method. Setting was: $\lambda_{excitation}$=340 nm and $\lambda_{emission}$=455 nm.

### Procedure for Analytical Optimization of the Automated Method

Preparation of samples, interferences determination, preparation of blanks, calibration, and calculations for available lysine determination were already optimized during the previous development of the manual method of the OPA assay (Ferrer et al. 2003). The amount of sample and the volume of water needed to obtain a solution containing 0.6–3 % of proteins were used. Then, 1 mL SDS solution (120 g/L) was added to 950 μL of water and 50 μL of liquid food or powdered sample (0.3–1.5 mg of proteins). In order to eliminate possible interferences caused by small peptides, 2 mL of TCA was added to 2 mL of liquid samples or powdered samples and then centrifuged at 3,000 rpm for 15 min. Nine hundred microliters of water and 1 mL SDS solution (120 g/L) were added to 100 μL of supernatant. For the preparation of the blanks, 1 mL SDS solution (120 g/L) was added to 1 mL of water. The tubes from samples, interferences, and blanks were allowed to cool at 4 °C for 12 h and the sonicated for 15 min at 25 °C. Figure 1 shows a

**Table 1** Nutritional composition of the commercial samples analyzed as indicated on the labels

| | Sample | Proteins | Carbohydrates | Fat |
|---|---|---|---|---|
| Liquid samples (g/100 mL) | Soy beverage | 3.4 | 3.7 | 0.9 |
| | Oat beverage | 1.0 | 6.1 | 1.0 |
| | Quinoa beverage | 3.0 | 3.7 | 2.8 |
| | Liquid UHT milk/liquid sterilized milk | 3.0 | 4.9 | 1.6 |
| | | 3.0 | 4.6 | 1.5 |
| Powdered samples (g/100 g) | Powdered adapted IF | 9 | 58 | 26 |
| | Powdered follow-up IF | 10 | 62 | 19 |
| | Junior milk IF | 13 | 56 | 25 |

*IF* infant formulas, *UHT* ultra-high temperature

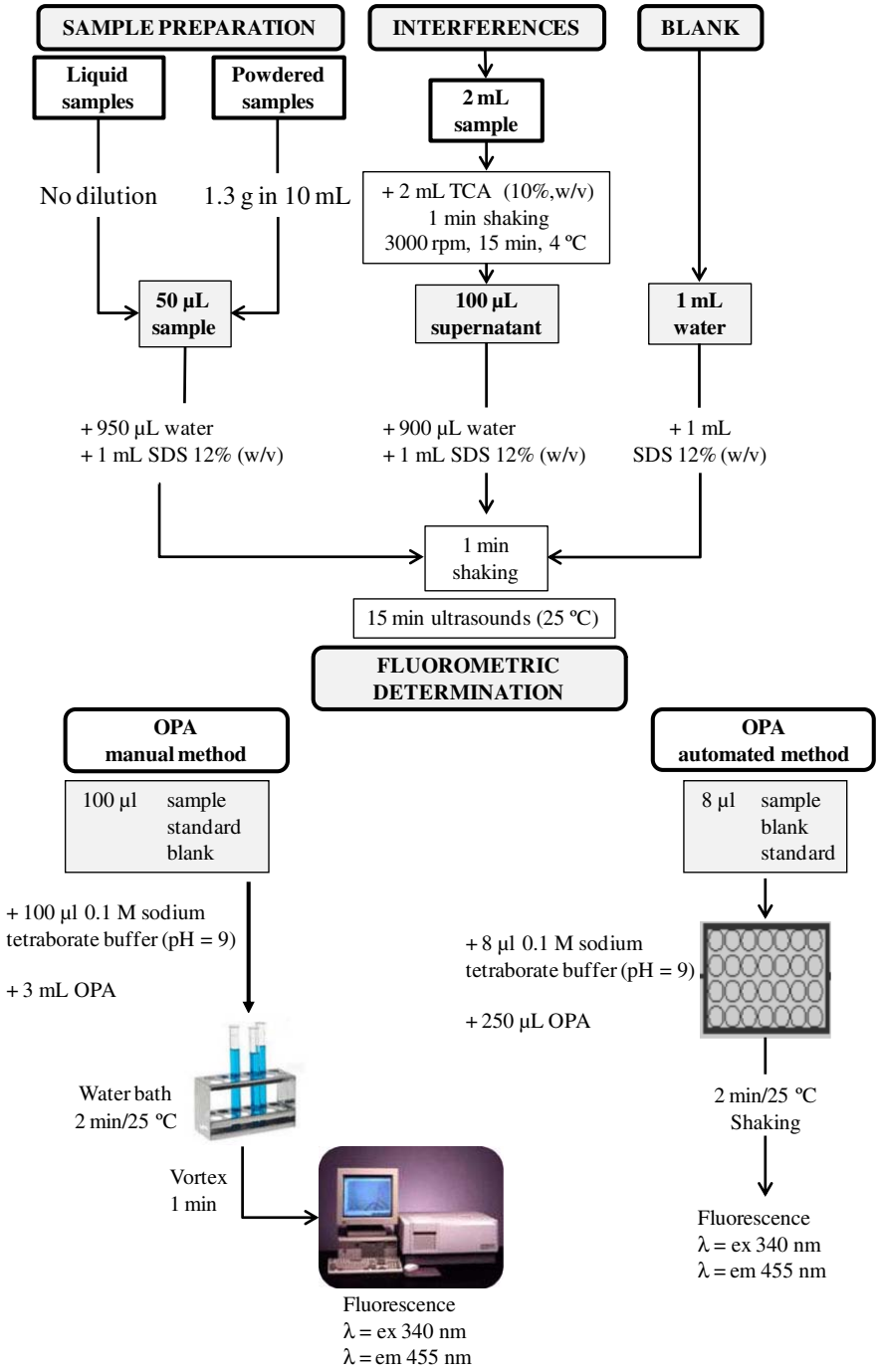


**Fig. 1** Schematic of the experimental procedure for the determination of available lysine

diagram of the preparation process. A standard of casein bovine milk was used to prepare a calibration curve. A set of casein working standards (0.1–1 mg/mL casein in assay solution), with lysine contents ranging from 0.0085 to 0.0850 mg lysine $mL^{-1}$ assay solution, was prepared using 0.1 M sodium tetraborate buffer (pH=9) as solvent. The conversion factor of casein to lysine was calculated considering that $a_{s1}$-casein, $a_{s2}$-casein, β-casein, and κ-casein have, respectively, 14, 24, 11, and 9 residues of lysine/protein molecule, that the $\alpha_{s1}$-casein:$\alpha_{s2}$-casein:β-casein:κ-casein ratio was 0.45:0.12:0.33:0.10, the molecular weights were 23,615, 25,230, 23,983, and 19,007 Da, respectively, and the molecular weight of lysine was 146.1 Da (Eigel et al. 1984; Modler 1985). Thus, milligram lysine/milligram casein ($F$)=$\Sigma$ ($R_L \times W_L / W_c \times R_p$)=0.08484, where $R_L$ are the residues of lysine in each casein; $W_L$ is the molecular weight of lysine; $W_c$ is the molecular weight of each casein; and $R_p$ is the protein ratio in each casein.

## Results and Discussion

### Adaptation and Optimization of the OPA Method by Automating a 96-Well Microtiter Plate Assay

There is no methodology available to analyze available lysine using 96-well microtiter plates; the use of which would allow a rapid determination of available lysine in different samples after processing or storage at the same time. The described method is an adaptation of the OPA fluorometric assay for the determination of available lysine proposed by Ferrer et al. (2003) with modifications. After applying the method described, it was seen that with the conditions applied, it was not possible to determinate available lysine content due to the reduced volume of wells. Sample quantity was decreased (5–20 μL). When 8 μL of sample was used, the best reproducibility of the results was found. The plate allows the analysis of a high number of samples at the same time. The spectrofluorometer Wallac 1420 VICTOR2 multilabel was programmed to use a two-reagent system. The reaction mode pipetted and transferred the sample (8 μL), 0.1 M sodium tetraborate buffer (pH=9) (8 μL), and main reagent (250 μL OPA) into the main reagent wells of their respective cuvette rotor positions. With spinning of the rotor, the reagents were mixed and incubated for 2 min at 25 °C and fluorescence was measured. Figure 1 shows a schematic of the experimental arrangement for automated determination of available lysine. The 0.1-M sodium tetraborate buffer (pH=9) was used as a blank, and 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mg/mL casein were used as standards. A sample of 0.3 mg/mL casein was used as quality control (QC). Samples and standard calibration solutions were always analyzed in duplicate in a "forward-then-reverse" order as follows: blank, 0.1 mg/mL casein, 0.2 mg/mL casein, 0.4 mg/mL casein, 0.6 mg/mL casein, 0.8 mg/mL casein, and 1 mg/mL casein, QC, sample 1… sample 1, QC, 1 mg/mL casein, 0.8 mg/mL casein, 0.6 mg/mL casein, 0.4 mg/mL casein, 0.2 mg/mL casein, 0.1 mg/mL casein, and blank. This arrangement can correct possible errors due to the signal drifting associated with the different positions of the same sample. Determinations were carried out in quadruplicate. The absorbance of the sample was corrected for the absorbance of the blank and of the interferences. Lysine content (in milligrams) was obtained by interpolating in the calibration curve.

### Validation of the OPA Automated Using a 96-Well Microtiter Plate Assay

To verify the quality and usefulness of the method, the analytical parameters linearity, sensitivity, precision, and percentage of recovery were determined for all the matrices mentioned in Section 2.1. Linearity was checked in the range of 0.1–1 mg standard casein/mL, corresponding to 0.0085–0.085 mg available lysine per milliliter. Good linearity was obtained for the studied range of available lysine contents for the OPA fluorometric automated ($y=7 \cdot 10^6 x+6135.9$; $r^2=0.999$) and manual ($y=217.890 x+0.554$; $r^2=0.999$) methods.

LOD was evaluated on the standard deviation of the response of the blank and the slope using the ratio $3 \cdot S_{n-1}/m$, where $S_{n-1}$ is the standard deviation of the response of the blank and $m$ is the slope of the calibration curve of the analyte. The $S_{n-1}$ value estimated on the standard deviation of the blank was obtained by analyzing $n=10$ blanks. The values of the detection limit for the automated method were calculated in the cuvette and in the samples (liquid and powdered). The detection limits in cuvette were 0.00008 and 0.00264 mg for automated and manual methods,

**Table 2** $F$ test for comparison of the precision of the OPA automated and manual fluorometric methods

| Samples | $S'_1$ | $S'_2$ | $F$ test[a] |
|---|---|---|---|
| Soy beverage | 0.0013 | 0.0027 | 0.4316 |
| Oat beverage | 0.0007 | 0.0009 | 0.7576 |
| Quinoa beverage | 0.0038 | 0.0065 | 0.5787 |
| Liquid UHT and sterilized milk | 0.0026 | 0.0031 | 0.8321 |
| Powdered adapted IF | 0.0103 | 0.0214 | 0.4800 |
| Powdered follow-up IF | 0.0293 | 0.1098 | 0.2665 |
| Junior milk IF | 0.0481 | 0.0720 | 0.6679 |

$n=6$

*IF* infant formulas, $S'^2$ corrected sample variance, *UHT* ultra-high temperature

[a] $F=S_1'^2/S_2'^2$; tabulated $F$ values: $F_{0.05\ (5.5)}=5.05$ ($p=0.05$), $F_{0.01\ (5.5)}=10.97$ ($p=0.01$)

**Table 3** *T* test for comparison of the accuracy of the OPA automated and manual fluorometric methods

| | Paired data *t* test | | |
|---|---|---|---|
| Samples | $\overline{d}$ | $S_{n-1}$ | $\frac{\lvert\overline{d}\rvert}{S_{n-1}} \cdot \sqrt{n}$ |
| Soy beverage | −0.04 | −0.06 | 1.60 |
| Oat beverage | 0.02 | 0.04 | 1.45 |
| Quinoa beverage | 0.10 | 0.09 | 2.30 |
| Liquid UHT and sterilized milk | 0.08 | 0.07 | 2.52 |
| Powdered adapted IF | −0.04 | 0.16 | 0.61 |
| Powdered follow-up IF | −0.19 | 0.34 | 1.23 |
| Junior milk IF | 0.03 | 0.32 | 0.19 |

$n=6$

*IF* infant formulas, *UHT* ultra-high temperature, $\overline{d}$ mean value of differences (OPA automated method−OPA manual method), $S_{n-1}$ standard deviation of differences; ${t_{n-1}}^{0.05/2}=2.571$

respectively. In addition, the detection limits for liquid samples were 0.0090 and 0.0331 g/L for automated and manual methods, respectively. Meanwhile, the values of detection limit for powdered samples were 0.2544 and 0.6881 g/kg for automated and manual methods, respectively. These results showed that the OPA automated method was more sensitive than manual method for liquid and powdered samples, although in all the cases, the values obtained allowed the detection of available lysine in the studied samples. In addition, the OPA automated method was more sensitive for liquid foods than for the powdered samples.

Limit of quantification (LOQ) corresponds to the minimum quantity with which it is possible to quantify without uncertainty ($LOQ=10\cdot S_{n-1}/m$). The values of the detection limit for the automated method were calculated in the cuvette and in the samples (liquid and powdered). The quantification limits in cuvette were 0.00026 and 0.00879 mg for automated and manual methods, respectively. In addition, the quantification limits for liquid samples were 0.1102 and 0.2982 g/L for automated and manual methods, respectively. Meanwhile, the values of quantification limit for powdered samples were higher in automated (0.8480 g/kg) and manual (2.2937 g/kg) method. These results showed that the OPA automated and manual methods allowed the quantification of all the samples analyzed, in the present study, without problems.

Instrumental precision was checked from six consecutive analysis of a sample extract and was expressed as relative standard deviations (RSD%). The instrumental precision values of liquid foods were 2.5 and 3.1 % for automated and manual methods, respectively. With regard to powdered samples, the instrumental precision values given by automated (2.1 %) and manual method (2.9 %) were in the range of those previously reported in liquid samples.

The precision of the method was determined by preparing six aliquots of the sample and was expressed as RSD%. The RSDs for six replicates of the liquid samples given by automated and manual methods were 3.0 and 3.8 %, respectively. With regard to powdered samples, method precision values were 2.8 and 4.3 %, for automated and manual methods, respectively. Due to the difficulty in finding certified samples similar to those used in this study for evaluating method conditions, a recovery assay was carried out. A known quantity of casein standard (1 mg/mL casein assay; 0.085 mg/mL lysine of assay) was added to each of the samples analyzed in this study and the method described was applied. The recovery percentages obtained in liquid samples were 100.3±1.9 and 101.0±1.2 % for automated and manual methods, respectively. The results obtained when the recovery assays were performed in powdered samples were in accord to those obtained for liquid samples. In this case, automated and manual methods quantified the 101.2±1.0 and 103.3±3.4 % of available lysine, respectively.

In order to estimate if the studied methods can be used with the same confidence, the precision and accuracy of

**Table 4** Chemically available lysine content by OPA manual and automated fluorometric methods

| Samples | | Manual | | Automated | |
|---|---|---|---|---|---|
| | | g/L | mg lys/g ref protein | g/L | mg lys/g ref protein |
| Liquid samples | Soy beverage | 1.09±0.04 | 31.91±1.04 | 1.07±0.03 | 31.47±0.83 |
| | Oat beverage | 0.67±0.02 | 67.50±1.41 | 0.66±0.02 | 66.00±2.83 |
| | Quinoa beverage | 2.08±0.07 | 69.17±2.59 | 2.22±0.05 | 74.33±1.41 |
| | Liquid UHT and sterilized milk | 2.02±0.04 | 68.00±1.89 | 2.12±0.04 | 71.00±2.36 |
| | | g/kg | mg lys/g ref protein | g/kg | mg lys/g ref protein |
| Powdered samples | Powdered adapted IF | 3.72±0.09 | 41.22±1.41 | 3.45±0.07 | 38.33±0.79 |
| | Powdered follow-up IF | 5.97±0.22 | 59.45±2.47 | 5.42±0.13 | 54.25±1.06 |
| | Junior milk IF | 8.56±0.20 | 65.81±1.03 | 8.54±0.17 | 65.58±0.82 |

$n=6$

*IF* infant formulas, *Lys* lysine, *Ref* reference, *UHT* ultra-high temperature

these for the different samples were compared (Tables 2 and 3). In the comparison of precision, six batches of a sample were analyzed for the studied methods. A comparison of variance by an $F$ test showed that the methods were similar in precision ($p>0.05$; Table 2). In the comparison of accuracy ($t$ test), six batches of the different samples were analyzed by each method, twice and on different days (Table 3). From these results, it should be noted that non-significant differences ($p<0.05$) among the OPA manual and automated fluorometric assays were obtained for the samples analyzed.

### Comparison of the Available Lysine Contents After Determination with OPA Method Using Automated 96-Well Microtiter Plate Assay and Traditional OPA Method

The available lysine contents in the samples analyzed are shown in Table 4 (expressed as grams per liter for liquid foods and grams per kilogram for powdered samples). In general, the average available lysine value in dairy protein-based formulas was in the range of the standard values of amino acid requirements established by Institute of Medicine (2005) (children ≤2 years 58, 10–12 years 44, and adults 16 mg lysine/g reference protein) and the mean value accepted for human milk (66 mg/g) established by World Health Organization (2000). Available lysine contents of these formulas were lower than that for powdered milk prepared in laboratory studied by Pereyra-Gonzáles et al. (2003) (80.4 mg/g) or those reported in the literature (76.6–85.4 mg/g protein) by different authors (Erbersdobler and Hupe 1991; Ferrer et al. 2003; van Mil and Jans 1991; Vigo et al. 1992). However, powdered adapted IF, containing soy protein, had a lower content in available lysine ($p<0.05$) in comparison to the other formulas. Pereyra-Gonzáles et al. (2003) also observed significantly lower values in formulas containing soy protein with regard to dairy protein-based formulas, obtaining similar values of 45.0±8.3 mg/g protein to those found in the present study. In addition, powdered infant formulas had lower contents of available lysine than those reported for the corresponding protein sources: soy protein (63.4–64.1 mg/g protein); casein (79.8–85.0 mg/g protein); milk (76.6–85.4 mg/g protein); and whey protein (80–97.5 mg/g protein) (Erbersdobler and Hupe 1991; Friedman and Brandon 2001; Souci et al. 2000; Vigo et al. 1992). The losses were probably a consequence of the combined effects of the type of protein, the treatments during the manufacture, and the different time and conditions of storage.

Amigo-Benavent et al. (2008) found available lysine values ranging from 1.54 to 9.76 mg/g of protein in soy products. When they studied a liquid soymilk beverage similar to the soymilk studied in the present study, they reported available lysine content (29.2 mg/g) very closed to that found in this study. In addition, data on lysine content of the soymilk studied in this work compared well with previously published data (Kwok et al. 1998; Souci et al. 2000).

In the published literature, it was not possible to find any studies of chemically available lysine contents in oat or in quinoa-based beverages in order to make a comparison with the results obtained in this study. However, bearing in mind the nutritional purpose of these foods in some population groups, it should be noted that compliance in available lysine content is essential to ensure nutritional quality and to avoid potential harmful effects in target groups. Moreover, taking into account the lysine content of oat and quinoa beverages, it can be concluded that generally the protein quality of these beverages was comparable to that of cow's milk (74.3 mg/g of protein) and egg whites (64.9 mg/g of protein) (Souci et al. 2000).

## Conclusion

The analytical parameters linearity, detection limit, precision, and accuracy of the assay showed that the automated method studied was useful for measuring the available lysine content in food products. The method was easy to perform, and the fact that sample preparation was the same for all the samples makes it suitable as a method for routine determinations. This method was valid in order to quantify the changes in the available lysine content of liquid foods and powdered samples after and during processing/preservation treatment and subsequent storage.

**Acknowledgments** This research project was supported by the Spanish Ministry of Science and Technology and European Regional Development Funds (AGL2010-22206-C02-01). Barba, F.J received a contract employment from this project to carry out the study. Carbonell-Capella, J.M. holds an award from the Spanish Ministry of Education (AP2010-2546).

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Innovative Food Science and Emerging Technologies 18 (2013) 48–56

Contents lists available at SciVerse ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

# High pressure processing of fruit juice mixture sweetened with *Stevia rebaudiana* Bertoni: Optimal retention of physical and nutritional quality

Juana M. Carbonell-Capella [a], Francisco J. Barba [b], María J. Esteve [a], Ana. Frígola [a,*]

[a] Department of Nutrition and Food Chemistry, Universitat de València, Avda. Vicent Andrés Estellés, s/n. 46100 Burjassot, Spain
[b] Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas (CSIC), Avda. Agustín Escardino, 7. 46980 Paterna, Spain

ARTICLE INFO

*Article history:*
Received 5 December 2012
Accepted 29 January 2013

Editor Proof Receive Date 28 February 2013

*Keywords:*
*Stevia rebaudiana* Bertoni
Exotic fruits
Orange
Bioactive compounds
Total antioxidant capacity
High pressure processing

ABSTRACT

The impact of high pressure processing (HPP) technology on physicochemical properties (color, browning index, turbidity index), bioactive compounds (ascorbic acid, total phenolic compounds, total anthocyanins, total carotenoids) and antioxidant capacity of a fruit juice mixture (papaya (32.5%, v/v), mango (10%, v/v) and orange (7.5%, v/v)) sweetened with *Stevia rebaudiana* Bertoni at different percentages was studied. The experimental design comprised a response surface methodology according to a central composite face-centered design. The variable ranges were 300–500 MPa (pressure), 5–15 min (time), 0–2.5% *Stevia* percentage. This design was used to determine the optimal high pressure-*Stevia* concentration in order to obtain the best retention of physicochemical and nutritional quality in the beverage following high pressure. HPP conducted at 300 MPa for 14 min led to a beverage with the greatest presence of antioxidant compounds and total color differences lower than 3.

*Industrial relevance:* There has been increasing interest in the use of non-caloric sweeteners from plant sources, among them is *Stevia rebaudiana* Bertoni, due to the growing evidence of its health benefits. Combined mixtures of *S. rebaudiana* water extracts and fruit juice can be a useful tool in order to provide new food products with increased nutritional properties. Moreover, high pressure processing (HPP) allows the acquisition of drinks that keep their characteristics similar to the fresh product. A deeper knowledge of the effect of HPP on the nutritional and physicochemical characteristics of these new beverages processed by HPP with regard to unprocessed juices is necessary.



## 1. Introduction

In the last years, new functional juices without sugar and/or obtained from exotic fruits are becoming common in Japanese, US and EU markets and have been receiving considerable attention as their market potential grows (Perumalla & Hettiarachchy, 2011; Puri, Sharma, & Tiwari, 2011). In addition, food industry has shown increased interest in plant extracts from *Stevia rebaudiana* (*Stevia*) Bertoni, because it can be a nutritional strategy in order to replace or substitute sugar energy content due to its high content in non-nutritive sweeteners (Nehir El & Simsek, 2012). Currently, *Stevia* in leaf or extracted forms was approved by FDA as a dietary supplement in the US, and under similar classifications in several other countries. In November 2011, the European Commission approved steviol glycosides as food additives (European Commission, 2011), which will probably lead to wide-scale use in Europe (Stoyanova, Geuns, Hideg, & Van den Ende, 2011). So far, little data has been available regarding the practical applications in foods and stability under different processing and storage conditions (Nehir El & Simsek, 2012). The leaves of *Stevia* have functional and sensory properties superior to those of many other high-potency sweeteners, and is likely to become a major source of high-potency sweetener for the growing natural food market (Goyal, Samsher, & Goyal, 2010). Moreover, it has been reported that *Stevia* is nutrient-rich, containing substantial amounts of minerals, vitamins, polyphenols and other antioxidant compounds. In some countries, *Stevia* has been consumed as a food and medicine (ethnobotanical) for many years, including most notably Japan and Paraguay (Lemus-Mondaca, Vega-Gálvez, Zura-Bravo, & Ah-Hen, 2012). In addition, *Stevia* sweetener extractives are suggested to exert beneficial effects on human health, as they have anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumor, anti-diarrheal, diuretic, and immunomodulatory effects (Chatsudthipong & Muanprasat, 2009).

On the other hand, orange and different exotic fruits such as mango and papaya are a good source of bioactive compounds like ascorbic acid, polyphenols, and carotenoids. These compounds have been shown to be good contributors to the total antioxidant capacity of foods (Vijaya, Sreeramulu, & Raghunath, 2010; Zulueta, Esteve, & Frígola, 2009) and have been involved in the prevention of some degenerative diseases (Devalaraja, Jain, & Yadav, 2011).

The development of non-thermal processing technologies combined with natural additives to obtain healthier and safer food products is one of the major challenges facing the food industry in the new century

* Corresponding author. Tel.: +34 963544955; fax: +34 963544954.
*E-mail address:* ana.frigola@uv.es (A. Frígola).

1466-8564/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ifset.2013.01.011

(Barros-Velazquez, 2011; Norton & Sun, 2008). So, high pressure processing (HPP) can be a useful tool in order to achieve this goal. With this kind of treatment it is possible to inactivate and inhibit microorganisms, and it can activate or inactivate enzymes at low temperatures (Saucedo-Reyes, Marco-Celdrán, Pina-Pérez, Rodrigo, & Martínez-López, 2009; USFDA, 2000), while compounds of low molecular weight, such as vitamins and compounds related to pigmentation and aroma, remain unaltered (Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007). In fluid foods, pressure is transmitted uniformly and instantly, that is, there are no gradients (it follows the so-called isostatic rule) (Thakur & Nelson, 1998; Toepfl, Mathys, Heinz, & Knorr, 2006). Unlike what happens with heat processes, HPP is independent of the size and geometry of the product, which reduces the time required to process large quantities of food (Rastogi et al., 2007).

Response surface methodology (RSM) has been used in the optimization of food processes to define the relationships between the responses and independent variables. RSM has important application in the design, development, and formulation of new products (Bas & Boyaci, 2007). Different authors have used RSM to evaluate the effect of the HPP conditions such as the pressure, temperature and time on antioxidant compounds and physicochemical parameters of food products (Roldán-Marín, Sánchez-Moreno, Lloría, De Ancos, & Cano, 2009; Terefe, Matthies, Simons, & Versteeg, 2009). These authors concluded that all the factors, pressure, temperature, and time, significantly influenced different nutritional and quality parameters.

The aims of the present study were (1) to investigate the effects of the HPP conditions, specifically pressure and time, as well as the influence of *Stevia* concentrations on bioactive compounds (ascorbic acid, total phenolics, total anthocyanins, total carotenoids), antioxidant capacity, and physicochemical properties (turbidity, browning, color) of a fruit juice mixture sweetened with *Stevia* and (2) to determine optimum conditions in order to obtain a fruit juice mixture beverage sweetened with *Stevia* with the highest levels of health-related compounds and the best physicochemical properties.

## 2. Materials and methods

### 2.1. Samples

#### 2.1.1. Fruit juice mixture

Oranges (*Citrus aurantium*, cultivar Salustiana), mango (*Mangifera indica*), and papaya (*Carica papaya*) were purchased from a local supermarket (Valencia, Spain). Orange, mango and papaya juices were extracted after appropriate washing and hygienization of the fruits, then the pulp was removed. The fruit juice mixture was prepared, based on sensory evaluations of color and appearance of 25 assessors from the University of Valencia and Agro-Chemistry and Food Technology Inst., by mixing 32.5% (v/v) of papaya juice, 10% (v/v) of mango juice, 7.5% (v/v) of orange juice, and water to 100%.

#### 2.1.2. Stevia infusion

A stock solution of 8.33% (w/v) was prepared from dried leaves. One hundred mL of boiling distilled water was added to the dried leaves (8.33 g), the mix was covered and let infuse for 30 min. The infusion was vacuum filtered using filter paper (Whatman No. 1) and the filtrate obtained was stored at −40 °C.

#### 2.1.3. Fruit juice-Stevia mixture (FJ-Stevia)

Different volumes of *Stevia* stock solution (3 and 6 mL) were added to 14 mL of fruit juice mixture to obtain *Stevia* concentrations of 1.25 and 2.50%, respectively. Water was added when necessary to complete a final matrix volume of 20 mL. In parallel, a blank sample without *Stevia* in its composition (0% *Stevia*) was formulated with 14 mL of fruit mixture juice and 6 mL of water. The maximum *Stevia* concentration (2.5%) was selected taking into account the sucrose concentration of commercial fruit based beverages and the sweetness equivalence *Stevia*/sucrose (Savita et al., 2004). Under these conditions the samples were rated as excellent and were characterized by higher acceptability.

### 2.2. Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylicacid), ABTS (2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonate)), Folin–Ciocalteu reagent, and fluorescein sodium salt were purchased from Sigma-Aldrich (Steinheim, Germany). Gallic acid was purchased from UCB (Brussels, Belgium). Hexane (LC grade), potassium hydroxide, and hydrogen peroxide were purchased from Scharlau (Barcelona, Spain). Sodium and disodium phosphate, L(+)-ascorbic acid, acetonitrile (special grade), magnesium hydroxide carbonate (40–45%), and 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) were purchased from Panreac (Barcelona, Spain). Ethanol, diethyl ether, methanol, hydrochloric acid, and sodium chloride (special grade) from Baker (Deventer, The Netherlands). Chloroform was obtained from Merck (Darmstadt, Germany).

### 2.3. HPP equipment

The samples, inserted in PE-LD bottles, were placed in polyethylene bags filled with water and heat-sealed (MULTIVAC Thermosealer, Hünenberg, Switzerland) before being placed in the HPP unit (High-Pressure Food Processor; EPSI NV, Belgium). The equipment consists on a vessel with an internal diameter of 100 mm and 300 mm high, with an operation pressure vessel of 689 MPa and an operation temperature vessel of −20 to 100 °C and a volume of 2.35 L. The pressure medium was a water–ethylenglycol mixture (70:30). The samples were pressurized at 300, 400 and 500 MPa for specific times in a range of 5, 10, and 15 min. Pressure level, pressurization time, and temperature were controlled automatically. Pressure increase rate was 300 MPa/min and the depressurization time was less than 1 min. The initial temperature was 15 °C, the final temperature after pressurization at highest pressure was 32 °C, the final temperature after holding time at highest pressure was 26.6 °C and final temperature after decompression at highest pressure was 12.5 °C. Come-up time was 90 s and decompression time was 15 s. All the treatments were applied in duplicate, with three bottles per treatment. Immediately after pressurization, samples were analyzed. For HPP, literature reports 5–15 min at 300–500 MPa to achieve 5-log reduction of different foodborne pathogens in different liquid foods (Alpas, Kalchayanaud, Bozoglu, & Ray, 2000; Donsì, Ferrari, Di Matteo, & Bruno, 1998).

### 2.4. Determination of ascorbic acid

A Metrohm 746 VA Trace Analyzer (Herisau, Switzerland) equipped with a Metrohm 747 VA stand was used for the polarographic determination. The working electrode was a Metrohm multi-mode electrode operated in the dropping mercury mode. A platinum wire counter electrode and a saturated calomel reference electrode were used. The following instrumental conditions were applied: DP50, mode DME, drop size 2, drop time 1 s, scan rate 10 mV/s, and initial potential −0.10 V. Beverage (5 mL) was diluted to 25 mL with the extraction solution (oxalic acid 1% w/v, trichloroacetic acid 2% w/v, sodium sulfate 1% w/v). After vigorous shaking, the solution was filtered through a folded filter (Whatman no. 1). Oxalic acid (9.5 mL) 1% (w/v) and 2 mL of acetic acid/sodium acetate 2 M buffer (pH = 4.8) were added to an aliquot of 0.5 mL of filtrate and the solution was transferred to the polarographic cell. Determinations were carried out by using the peak height and standard addition method in accordance to Barba, Esteve, Tedeschi, Brandolini, and Frigola (2013).

### 2.5. Total phenolic compounds

Total phenols were determined according to the method reported by Georgé, Brat, Alter, and Amiot (2005), with some modifications. Briefly, 10 mL of sample was homogenized with 50 mL of a mixture of acetone/water (7/3, v/v) for 30 min. Mixture supernatants were then recovered by filtration (Whatman no. 2, England) and constituted the raw extracts (REs). REs (2 mL) were settled on an Oasis cartridge (Waters). Interfering water-soluble components (steviol glycosides, reducing sugars, ascorbic acid) were recovered with 2 × 2 mL of distillated water. The recovered volume of the washing extract (WE) was carefully measured. In order to eliminate vitamin C, heating was carried out on the washing extract (3 mL) for 2 h at 85 °C and led to the heated washing extract (HWE). All extracts (RE, WE, and HWE) were submitted to the Folin–Ciocalteu method, adapted, and optimized (Barba et al., 2013). Gallic acid calibration standards with concentrations of 0, 100, 300, 500, 700 and 1000 ppm were prepared and 0.1 mL was transferred to borosilicate tubes. 3 mL of sodium carbonate solution (2%, w/v) and 0.1 mL of Folin–Ciocalteau reagent (1:1, v/v) were added to 0.1 mL of all gallic acid standard and sample tubes. The mixture was incubated for 1 h at room temperature and absorbance was measured at 765 nm.

### 2.6. Total anthocyanins

Total anthocyanins were determined using a modified method of Mazza, Fukumoto, Delaquis, Girard, and Ewert (1999). A 10-fold diluted sample of 100 μL was mixed with 1700 μL of distilled water and 200 μL of 5% (v/v) HCl. The sample was hold at room temperature for 20 min before measuring the absorbance at 520 nm in a 10 mm cuvette. This reading corresponds to the total anthocyanin content after considering the relevant dilution. Calculations of total anthocyanins were based on malvidin-3-glucosid (molar absorptivity 28,000).

### 2.7. Total carotenoids

Extraction of total carotenoid was carried out in accordance with Lee and Castle (2001). An aliquot of sample (2 mL) was homogenized with 5 mL of extracting solvent (hexane/acetone/ethanol, 50:25:25, v/v) and centrifuged for 5 min at 4000 rpm at 5 °C. The top layer of hexane containing the color was recovered and transferred to a 25-mL volumetric flask and the volume was then adjusted to 25 mL with hexane. Total carotenoid determination was carried out on an aliquot of the hexane extract by measuring the absorbance at 450 nm. Total carotenoids were calculated according to Ritter and Purcell (1981) using an extinction coefficient of β-carotene, $E^{1\%} = 2505$.

### 2.8. Total antioxidant capacity

#### 2.8.1. $ABTS^{\bullet+}$ test

The method used was described by Re et al. (1999), based on the capacity of a sample to inhibit the ABTS radical ($ABTS^{\bullet+}$) (Sigma-Aldrich, Steinheim, Germany) compared with a reference antioxidant standard (Trolox®) (Sigma-Aldrich, Steinheim, Germany). The radical was generated using 440 μL of potassium persulfate (140 mM). The solution was diluted with ethanol (Baker, Deventer, The Netherlands) until an absorbance of 0.70 was reached at 734 nm. Once the radical was formed, 2 mL of $ABTS^{\bullet+}$ was mixed with 100 μL of appropriately diluted sample and the absorbance was measured at 734 nm for 20 min in accordance with Zulueta et al. (2009). The results, obtained from duplicate analyses, were expressed as: mM TE (millimolar Trolox equivalents).

#### 2.8.2. ORAC (Oxygen Radical Absorbance Capacity) assay

The ORAC assay used, with fluorescein (FL) (Sigma-Aldrich, Steinheim, Germany) as the "fluorescent probe," was that described by Ou, Hampsch-Woodill, and Prior (2001). The automated ORAC assay was carried out on a Wallac 1420 VICTOR$^2$ multilabel counter (Perkin–Elmer, Valencia, Spain) with fluorescence filters, for an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The measurements were made in plates with 96 white flat-bottom wells (Sero-Wel, Bibby Sterilin Ltd., Stone, UK). The reaction was performed at 37 °C as the reaction was started by thermal decomposition of AAPH in 75 mM phosphate buffer (pH 7.0) because of the sensitivity of FL to pH. The final reaction tested and the concentrations of the different reagents were determined following Zulueta et al. (2009).

### 2.9. Physicochemical properties

To measure the turbidity index (TI), a sample was centrifuged (618 ×*g*, 10 min, 20 °C), the supernatant was taken, and the absorbance at 660 nm was measured (Krop & Pilnik, 1974). To determine the browning index (BI), a sample was centrifuged (824 ×*g*, 20 min, 18 °C), and the supernatant was taken and diluted with ethanol (1:1, v/v). The mixture was filtered with Whatman no. 42 filters and the absorbance of the filtrate was measured at 420 nm (Meydav, Saguy, & Kopelman, 1977). The color analysis was performed using a Hunter Labscan II spectrophotometric colorimeter (Hunter Associates Laboratory Inc., Reston, VA., U.S.A.) controlled by a computer that calculates color ordinates from the reflectance spectrum. The results were expressed in accordance with the Commission International d'Eclairage LAB (CIELAB) system with reference to illuminant D65 and with a visual angle of 10°. Three consecutive measurements of each sample were taken. The CIE L* (lightness [0 = black, 100 = white]), a* ($-a^*$ = greenness, $+a^*$ = redness) and b* ($-b^*$ = blueness, $+b^*$ = yellowness) values were used to calculate the total color differences ($\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$), where ΔL*, Δa*, and Δb* are differences between the untreated HP-treated beverage (Calvo, 2004).

### 2.10. Experimental design and statistical analysis

A face-centered central composite response surface analysis was used to determine the effect of pressure (MPa) (*P*), time (minutes) (*t*) and *S. rebaudiana* concentration (*Stevia*) (%, w/v) (*% Stevia*) on the health-related compounds (ascorbic acid, total phenolic compounds, total anthocyanins, total carotenoids), antioxidant capacity (TEAC and ORAC methods), and physicochemical properties (color, turbidity and browning index) of the beverage. Response surface methodology (RSM) consists of a set of mathematical and statistical methods developed for modeling phenomena and finding combinations of a number of experimental factors (variables) that will lead to optimum responses. With RSM, several variables are tested simultaneously with a minimum number of trials, according to special experimental designs, which elucidates interactions between variables. This is not an option with classical approaches. In addition, RSM has the advantage of being less expensive and less time-consuming than the classical methods. The independent variables of the RSM were: pressure (from 300 to 500 MPa), time (from 5 to 15 min) and *Stevia* concentration (from 0 to 2.5%, w/v). The levels for each independent parameter were chosen considering sample and equipment limitations. Three (maximum, minimum and central) values of each factor were considered, leading to 26 experiments (Table 1). The experimental design was performed twice, resulting in two blocks of experiments. The combinations included HPP-*Stevia* conditions with an intermediate level (central point) of the three variables replicated 4 times, which was used to determine inherent variance in the technique. Experiments were randomized to minimize the systematic bias in the observed responses due to extraneous factors and to increase precision. Experimental data were fitted to a polynomial response surface. The second-order response function was predicted using the following equation:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{i=i+1}^{3} b_{ij} X_i X_j$$

**Table 1**
Experimental design matrix in terms of actual variables and the average values of the response for experiments on the effect of combined high pressure-*Stevia rebaudiana* concentration on the antioxidant activities of a fruit juice mixture.

| Run[a] | Pressure (MPa) | Time (min) | Stevia (%) | TC[b] (μg/100 mL) | TPC[b] (mg GAE/L) | TA[b] (mg/100 mL) | TEAC[b] (mM TE) | ORAC[b] (mM TE) |
|---|---|---|---|---|---|---|---|---|
| | ($X_1$) | ($X_2$) | ($X_3$) | ($Y_1$) | ($Y_2$) | ($Y_3$) | ($Y_4$) | ($Y_5$) |
| 1 | 500 | 15 | 0 | 364.3 ± 15.1 | 165.7 ± 9.8 | 21.4 ± 1.0 | 2.91 ± 0.21 | 3.41 ± 0.41 |
| 2 | 300 | 5 | 0 | 294.6 ± 17.3 | 184.5 ± 10.2 | 23.2 ± 0.4 | 2.68 ± 0.12 | 4.67 ± 0.50 |
| 3 | 500 | 15 | 0 | 349.3 ± 15.4 | 143.7 ± 8.6 | 22.9 ± 0.5 | 2.92 ± 0.31 | 3.20 ± 0.25 |
| 4 | 500 | 15 | 2.5 | 329.3 ± 18.3 | 3683.1 ± 151.5 | 46.4 ± 1.1 | 29.54 ± 1.01 | 40.95 ± 0.71 |
| 5 | 300 | 15 | 0 | 279.4 ± 15.3 | 164.2 ± 9.6 | 22.3 ± 0.3 | 2.34 ± 0.32 | 4.87 ± 0.43 |
| 6 | 300 | 5 | 2.5 | 304.6 ± 20.4 | 4494.6 ± 201.5 | 30.7 ± 0.6 | 24.73 ± 0.81 | 39.33 ± 0.32 |
| 7 | 400 | 10 | 1.25 | 319.4 ± 16.1 | 2756.5 ± 156.4 | 26.3 ± 0.4 | 18.47 ± 0.72 | 19.22 ± 0.63 |
| 8 | 300 | 15 | 2.5 | 304.4 ± 15.6 | 4146.5 ± 210.3 | 28.9 ± 0.3 | 32.32 ± 1.01 | 36.73 ± 0.81 |
| 9 | 500 | 5 | 2.5 | 330.3 ± 12.7 | 3815.8 ± 198.4 | 39.1 ± 1.0 | 40.03 ± 1.03 | 43.68 ± 0.85 |
| 10 | 400 | 10 | 1.25 | 300.6 ± 18.3 | 2869.2 ± 156.1 | 26.2 ± 0.5 | 19.24 ± 0.81 | 20.81 ± 0.75 |
| 11 | 400 | 5 | 1.25 | 289.4 ± 20.1 | 2837.1 ± 149.3 | 27.1 ± 0.6 | 17.35 ± 0.92 | 24.13 ± 0.67 |
| 12 | 400 | 10 | 2.5 | 305.3 ± 21.1 | 3901.0 ± 205.6 | 36.6 ± 0.8 | 35.64 ± 0.71 | 35.81 ± 0.65 |
| 13 | 300 | 15 | 2.5 | 284.4 ± 13.6 | 4220.6 ± 210.9 | 33.2 ± 1.1 | 32.32 ± 0.81 | 36.73 ± 0.81 |
| 14 | 500 | 5 | 2.5 | 325.6 ± 12.5 | 4000.2 ± 208.7 | 39.6 ± 1.0 | 40.05 ± 0.83 | 42.09 ± 1.00 |
| 15 | 300 | 10 | 1.25 | 289.5 ± 14.6 | 3057.9 ± 132.6 | 27.5 ± 0.9 | 16.21 ± 0.61 | 24.09 ± 0.71 |
| 16 | 500 | 5 | 0 | 295.4 ± 17.0 | 170.7 ± 9.6 | 23.0 ± 1.0 | 2.80 ± 0.24 | 4.33 ± 0.21 |
| 17 | 500 | 15 | 2.5 | 314.4 ± 17.2 | 4060.4 ± 263.4 | 45.6 ± 2.0 | 29.54 ± 0.71 | 40.95 ± 0.86 |
| 18 | 500 | 5 | 0 | 300.7 ± 13.6 | 173.8 ± 10.0 | 23.3 ± 1.8 | 2.93 ± 0.10 | 4.07 ± 0.15 |
| 19 | 300 | 15 | 0 | 289.4 ± 15.3 | 184.8 ± 7.6 | 23.6 ± 1.0 | 2.72 ± 0.31 | 4.64 ± 0.16 |
| 20 | 400 | 15 | 1.25 | 315.4 ± 14.4 | 2919.3 ± 123.6 | 28.1 ± 0.9 | 20.07 ± 0.72 | 21.63 ± 0.51 |
| 21 | 400 | 10 | 0 | 279.9 ± 16.6 | 169.5 ± 8.3 | 24.0 ± 0.7 | 3.67 ± 0.21 | 3.95 ± 0.17 |
| 22 | 300 | 5 | 2.5 | 248.6 ± 15.3 | 4355.5 ± 213.1 | 29.3 ± 0.8 | 18.92 ± 0.80 | 35.43 ± 0.61 |
| 23 | 400 | 10 | 1.25 | 339.3 ± 16.1 | 2638.8 ± 160.5 | 26.7 ± 0.9 | 19.22 ± 1.01 | 20.47 ± 0.55 |
| 24 | 400 | 10 | 1.25 | 349.3 ± 15.2 | 2728.9 ± 149.7 | 27.9 ± 0.7 | 18.89 ± 0.92 | 19.26 ± 0.43 |
| 25 | 300 | 5 | 0 | 304.6 ± 15.2 | 177.9 ± 7.3 | 22.3 ± 0.8 | 2.37 ± 0.34 | 3.78 ± 0.12 |
| 26 | 500 | 10 | 1.25 | 315.8 ± 12.3 | 2818.5 ± 162.5 | 25.5 ± 0.6 | 20.43 ± 0.81 | 22.05 ± 0.46 |

TC: total carotenoids. TPC: total phenolic compounds. TA: total anthocyanins. TEAC: trolox equivalent antioxidant capacity. ORAC: oxygen radical antioxidant capacity.
[a] Order of the assays was randomized.
[b] Data shown are the mean ± SD of two treatment repetitions, each assay was performed in triplicate.

where $Y$ represents a response variable, $b_0$ is the center point of the system, $b_i$, $b_{ii}$, and $b_{ij}$, are coefficients of the linear, quadratic and interactive effect, respectively; $X_i$, $X_i^2$, and $X_iX_j$ represent linear, quadratic and interactive effects of the independent variables, respectively. The non-significant terms were deleted from the second-order polynomial model after an ANOVA test, and a new ANOVA was performed to obtain the coefficients of the final equation for better accuracy. The experimental design and the data analysis were performed using SPSS® (Statistical Package for the Social Sciences) v.19.0 for Windows (SPSS Inc., Chicago, USA). The optimization was done following the method proposed by Derringer and Suich (1980). All the individual desirability functions obtained for each response were combined into an overall expression, which is defined as the geometrical mean of the individual functions. The nearer the desirability value to the unit, the more adequate the system (Ross, 1996). In the present study, desirability functions were developed in order to obtain the beverage with the highest levels of antioxidant compounds and the best physicochemical properties. Subsequently, an ANOVA of three factors (pressure, time, and *S. rebaudiana* concentration) was applied, and in the parameters for which significant differences were obtained with more than two levels, Tukey's test was applied to ascertain the range of values in which the differences were located. Finally, a study was conducted with the aim of determining whether there were correlations between a pair of variables (Pearson's test).

## 3. Results and discussion

### 3.1. Effect of HPP and Stevia concentration on nutritional qualities and antioxidant capacity of the beverages

The ascorbic acid concentration in the fruit juice blend (papaya, mango and orange) without *Stevia* was 25.5 ± 0.3 mg/100 mL (Table 2). These results were in close agreement with the values obtained by other authors in papaya, mango and orange (Beserra-Almeida et al., 2011; Burdulu, Koca, & Karandeniz, 2006; U.S. Department of Agriculture (USDA) & Agricultural Research Service, 2012). In addition, similar results were found for the fruit juice mixtures sweetened with *Stevia* at 1.25% (w/v) (25.3 ± 0.2 mg/100 mL), and 2.5% (w/v) (25.3 ± 0.1 mg/100 mL), respectively. Immediately after HPP, ascorbic acid retention was higher than 92% in all cases (data not shown). Several authors have reported that ascorbic acid of fruit and vegetable juices was minimally affected by HPP at mild temperatures (Barba, Esteve, & Frigola, 2010; Barba, Esteve, & Frígola, 2012; Bull et al., 2004).

The results obtained for total phenolic compounds, total anthocyanins, total carotenoids and antioxidant capacity in the untreated and HPP samples are shown in Tables 1–2. Total phenolic content (TPC) of untreated fruit juice mixture without *Stevia* sweetened was 166.9 ± 11.7 mg GAE/L. However, TPC values were 15 and 23-fold

**Table 2**
Physicochemical and nutritional characteristics of untreated fruit juice mixture sweetened with *Stevia rebaudiana* (*Stevia*) Bertoni.

| Parameters | Beverage with *Stevia* (%) | | |
|---|---|---|---|
| | 0 | 1.25 | 2.50 |
| Ascorbic acid (mg/100 mL) | 25.5 ± 0.3[a] | 25.3 ± 0.2[a] | 25.3 ± 0.1[a] |
| Total phenolics (mg GAE/100 mL) | 166.9 ± 11.7[a] | 2509.5 ± 142.5[b] | 3824.4 ± 100.0[c] |
| Total anthocyanins (mg/100 mL) | 21.8 ± 0.1[a] | 24.6 ± 0.5[b] | 28.8 ± 0.3[c] |
| Total carotenoids (μg/100 mL) | 329.3 ± 14.1[a] | 337.6 ± 10.1[a] | 324.4 ± 7.1[a] |
| ORAC(mM TE) | 4.5 ± 0.5[a] | 22.2 ± 0.7[b] | 38.8 ± 1.7[c] |
| TEAC(mM TE) | 2.3 ± 0.1[a] | 17.9 ± 1.4[b] | 26.5 ± 0.8[c] |
| Browning index | 0.097 ± 0.003[a] | 2.313 ± 0.033[b] | 1.581 ± 0.004[c] |
| Turbidity index | 0.075 ± 0.001[a] | 0.613 ± 0.004[b] | 0.316 ± 0.003[c] |
| Lightness (L*) | 72.4 ± 0.2[a] | 39.3 ± 0.1[b] | 36.7 ± 0.1[c] |
| Redness (a*) | −1.9 ± 0.1[a] | 10.8 ± 0.2[b] | 10.1 ± 0.1[c] |
| Blueness (b*) | 8.5 ± 0.2[a] | 50.4 ± 0.2[b] | 39.3 ± 0.1[c] |

[a–c]Different letters in the same file indicate significant statistical differences in function of the *Stevia* percentage.

higher when *Stevia* at 1.25% and 2.5% *Stevia* (w/v) was used as a sweetener, respectively. These results were in close agreement with some previous studies that have reported high levels of phenolic compounds in *S. rebaudiana* products. Tadhani, Patel, and Subhash (2007) and Abou-Arab and Abu-Salem (2010) obtained that total phenolic compounds in *Stevia* water extracts were 25.18 and 24.01 mg gallic acid equivalents (GAE)/g dry weight basis, respectively. In addition, Shukla, Mehta, Mehta, and Bajpai (2011) found 56.74 mg GAE in 1 g of aqueous leaf extract while ethanolic leaf extract of *S. rebaudiana* has been reported to show 61.50 mg GAE of phenols (Shukla, Mehta, Bajpai, & Shukla, 2009).

Three-way ANOVA showed that the preservation treatment applied (pressure, time) and the percentage of *Stevia* had a significant influence ($p<0.05$) on the values of total phenolics. As can be seen in Fig. 1, the behavior of TPC was different after applying HPP depending on *Stevia* concentration used in the formulation of the beverages. In addition, a multiple linear regression equation of a second order polynomial model was generated in order to elucidate the effects of % *Stevia* and HPP (pressure and time) on the beverages. The reduced regression model presented in the Eq. (1) allowed for prediction of the effects of independent variables on total phenolic compounds.

$$\begin{aligned}\mathrm{TPC(mg\ GAE/L)} = {} & -14240.5 + 91.9 \cdot P - 557.7 \cdot t + 2432.2 \cdot \%Stevia \\ & - 0.1P^2 + 1.3 \cdot P * t - 1.1 \cdot P * \%Stevia \\ & + 24.4 \cdot t * \%Stevia - 286.0 \cdot \%Stevia^2\end{aligned} \quad (1)$$

The statistical analysis indicates that the quadratic model proposed for TPC was adequate ($p<0.05$) in order to evaluate the changes after applying HPP, with satisfactory determination coefficients ($R^2=0.961$, $p<0.05$, standard error = 1.761). No significant lack of fit of the model was found, showing that it fits properly within the range of HPP-*Stevia* assayed conditions. Phenols appeared to be relatively resistant to HPP, even they were significantly increased (22%) after HPP (300 MPa/10 min) when the fruit juice mixture was sweetened with *Stevia* (1.25%, w/v) and after 300 MPa for 5 min (18%) when the highest concentration of *Stevia* (2.5%, w/v) was used. This increase in total phenolic content may be related to an increased extractability of some of the antioxidant components following high pressure processing. These results were in accord with those found by Plaza et al. (2011) in orange juice processed at 400 MPa/40 °C/1 min, Barba, Esteve, et al. (2012) in orange juice mixed with milk processed at 100–400 MPa/20–42 °C/2–9 min, and Barba, Esteve, and Frígola (2011) in blueberry juice after HPP at 200–600 MPa/20–42 °C/5–15 min. They reported an increase in phenolic content following HPP.

Total anthocyanin concentration in the fruit juice blend without *Stevia* was $21.8 \pm 0.1$ mg/100 mL. However, total anthocyanin concentration was higher when the fruit mixture was sweetened with 1.25% (w/v) *Stevia* ($24.6 \pm 0.5$ mg/100 mL) and 2.5% (w/v) *Stevia* ($28.8 \pm 0.3$ mg/100 mL), respectively. Muanda, Soulimani, Diop, and Dicko (2011) reported values of total anthocyanins (measured as Cyaniding-3-glucoside) of $0.35 \pm 0.01$ and $0.67 \pm 0.09$ when they studied *Stevia* water extracts and methanol–water extracts, respectively. In addition, the anthocyanin content, expressed as relative retention, ranged between 98% and 161% in HPP samples under the studied experimental conditions. The reduced regression model for total anthocyanins is presented in the Eq. (2):

$$\begin{aligned}\mathrm{TA(mg/100\,mL)} = {} & 197.736 + 1.337 \cdot P + 15.983 \cdot t - 1.854 \cdot \%Stevia \\ & + 0.002 \cdot P^2 - 0.030 \cdot P * t + 0.010 \cdot P * \%Stevia \\ & - 0.348 \cdot t^2.\end{aligned} \quad (2)$$

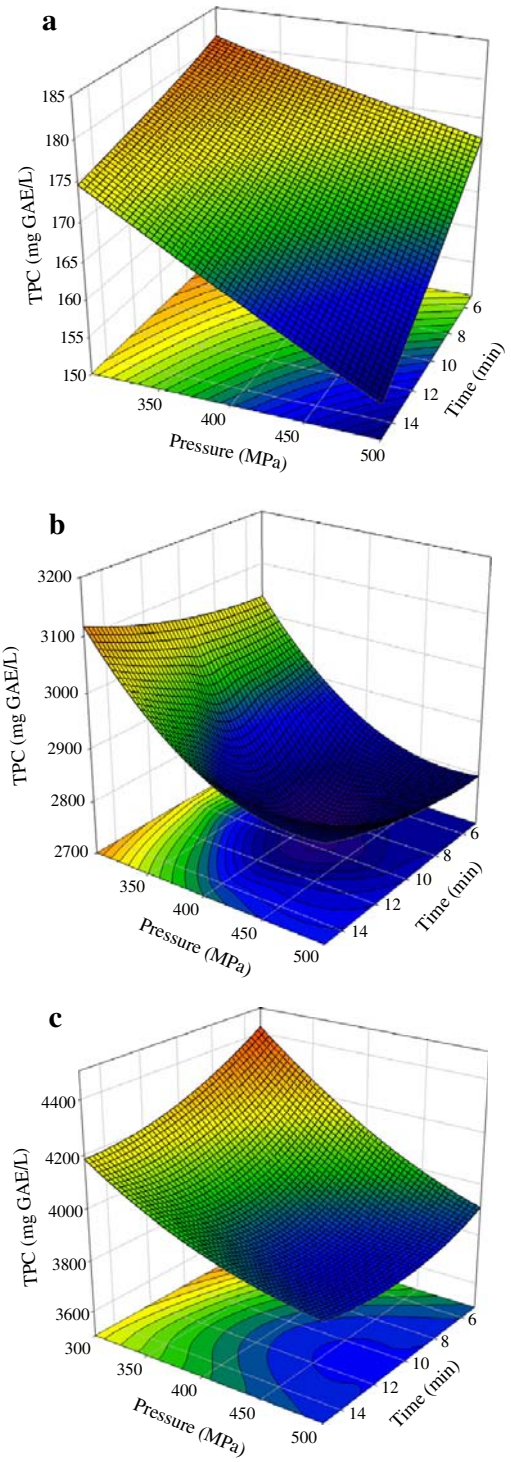


**Fig. 1.** Effects of pressure and time on total phenolic compounds (mg GAE/100 mL) of a fruit juice sweetened with *Stevia rebaudiana* at: a) 0%, b) 1.25%, and c) 2.50%.

Fig. 2 indicates a positive influence of the pressure on the total anthocyanin content. The highest anthocyanin content was observed at the highest levels of both pressure and time (500 MPa/15 min) as well as *Stevia* concentration (2.5%, w/v). This result indicates that in this particular range of processing conditions the HPP mainly modifies the mechanism of anthocyanin degradation by affecting the molecules involved in the kinetics of reaction, such as enzymes. Barba et al. (2011) and Ferrari, Maresca, and Ciccarone (2010) observed similar results in HPP (200–600 MPa/15 min) blueberry juice and HPP (400–600 MPa/5–10 min) pomegranate juice, respectively.

Total carotenoid (TC) content in the untreated beverage sweetened with 0, 1.25 and 2.5% (w/v) were 329.3 ± 14.1, 337.6 ± 10.1, and 324.4 ± 7.1 μg/100 mL, respectively. The analysis of variance showed that the regression model was accurate enough ($R^2 = 0.902$, $p < 0.05$, standard error = 18.905). The relationship between the independent variables and total carotenoids can be described by the Eq. (3):

$$TC(\mu g/100\ mL) = 245.650 + 0.161 \cdot P. \quad (3)$$

Pressure had a significant positive effect ($p < 0.05$) on the total carotenoids of the beverage independently of the *Stevia* concentration used. Overall, at higher pressures, the values of total carotenoids were higher, reaching a maximum (6–10% increase) when sample without *Stevia* was HP-treated at 500 MPa/15 min (Fig. 3), indicating that the beverages treated at higher pressure had an increased nutritional value.

Trolox equivalent antioxidant capacity (TEAC) and oxygen radical antioxidant capacity (ORAC) methods have been used widely for the determination of total antioxidant capacity (TAC) after applying HPP in fruit juice complex mixtures (Barba, Esteve, et al., 2012). In the present study, TAC values of untreated samples without *Stevia* were 4.5 ± 0.5 and 2.3 ± 0.1 mM TE for ORAC and TEAC assays, respectively. The ANOVA results indicated an increase in ORAC and TEAC values when *Stevia* percentage was increased, independently of the preservation treatment applied. These results were in accordance to those found by different authors who have demonstrated the high antioxidant capacity of *S. rebaudiana* products (Muanda et al., 2011; Shukla et al., 2009, 2011; Tadhani et al., 2007). In addition antioxidant capacity values measured with ORAC assay were significantly higher ($p < 0.05$) for the samples with *Stevia* at 1.25% (22.2 ± 0.7 mM TE) and 2.5% (38.8 ± 1.7 mM TE) than those obtained with TEAC method (17.9 ± 1.4 and 26.5 ± 0.8 mM TE for the beverages sweetened with 1.25 and 2.5% *Stevia*, respectively). The antioxidant activity of some *Stevia*-derived products has been attributed to the scavenging of free radical electrons and superoxides (Thomas & Glade, 2010). As the ORAC method is a reaction based on the transfer of H atoms, these compounds present in *S. rebaudiana* may be better represented by this assay.

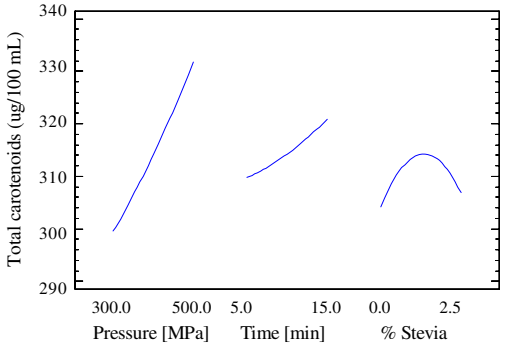


**Fig. 3.** Effect of % *Stevia rebaudiana* and HPP (pressure and time) on total carotenoids (μg/100 mL): interactions.

The results obtained for the three-way ANOVA showed that pressure, time and *Stevia* percentage had a significant influence ($p < 0.05$) on the total antioxidant capacity of the beverages measured as TEAC values. However, when ORAC assay was used, only *Stevia* percentage had a significant effect. Moreover, the regression analysis test showed that a second-order model fits well the antioxidant capacity (ORAC and TEAC) after applying HPP. The determination coefficients were ($R^2 = 0.960$, $p < 0.05$, standard error = 1.823) and ($R^2 = 0.970$, $p < 0.05$, standard error = 2.220) for TEAC and ORAC methods, respectively. Experimental data were fitted by a second-order polynomial model (Eqs. (4)–(5)):

$$TEAC(mM\ TE) = -24.048 + 0.013 \cdot P + 6.504 \cdot t + 5.270 \cdot \%Stevia - 0.007 \cdot P * t + 0.005 \cdot P * \%Stevia - 0.218 \cdot t^2 + 0.075 \cdot t * Stevia^2 \quad (4)$$

$$ORAC(mM\ TE) = 33.38820 + 15.61100 \cdot \%Stevia - 1.99497 \cdot \%Stevia^2. \quad (5)$$

When the possible correlation (Pearson test) between the various parameters that contribute to antioxidant capacity (ascorbic acid, total carotenoids, total phenolics and total anthocyanins) was studied for the different *Stevia* concentrations, it was found that there was a positive correlation between total phenolics and TEAC ($r = 0.920$, $p < 0.05$) and total phenolics with ORAC ($r = 0.961$). In this line, Cai, Luo, Sun, and Corke (2004) and Silva, Souza, Rogez, Rees, and Larondelle (2007) obtained a high correlation between TEAC and TPC when they studied 112 traditional Chinese medicinal plants and 15 Brazilian plants from Amazonian region, respectively. In addition, Ehlenfeldt and Prior (2001) and Bisby, Brooke, and Navaratnam (2008) also found a positive correlation between TPC and ORAC assays when they studied different plant food materials. Moreover, in the present study a positive correlation was found between ORAC and TEAC methods ($r = 0.905$, $p < 0.05$). These results were in accord to those found by Barba et al. (2010) when they treated a vegetables beverage with HPP.

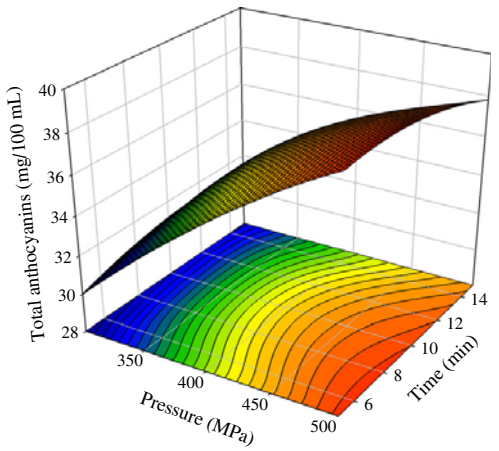


**Fig. 2.** Response surface plots for total anthocyanin content of the beverage with 2.5% *Stevia rebaudiana* as affected by HPP at different pressures (300–500 MPa) and times (5–15 min).

### 3.2. Effect of HPP and Stevia concentration on physicochemical properties of the beverages

The results obtained for turbidity index (TI), browning index (BI) and color parameters (a*, b* and L*) in the untreated and HPP samples are shown in Tables 2–3 and Fig. 4. As can be expected, among the studied parameters, *Stevia* percentage had the greatest effect on the quality parameters of the beverages. Compared to the untreated samples, lower browning index values were obtained for the HPP samples when 0 and 1.25% (w/v) *Stevia* percentages were used, however a significant increase in BI was observed after applying HPP in the samples with 2.5% (w/v) *Stevia*. The regression model was accurate enough ($R^2 = 0.962$, $p<0.05$, standard error = 0.181). The regression equation describes the following model (Eq. (6)):

$$BI = 8.066 - 0.0551 \cdot P + 0.4856 \cdot t + 0.6965 \cdot \%Stevia + 0.0001 \cdot P^2 - 0.0009 \cdot P * t + 0.0009 \cdot P * \%Stevia - 0.0108 \cdot t^2 - 0.0092 \cdot t * \%Stevia. \quad (6)$$

Overall, no statistically significant changes were observed in TI values of the HPP samples in comparison with the untreated beverage when *Stevia* was not added. However, there was a statistically significant ($p<0.05$) decrease in the HPP samples with 1.25% (w/v) *Stevia* in comparison with the unprocessed beverage. In addition, the opposite trend was obtained after applying HPP when *Stevia* percentage was 2.5% (w/v) (Eq. (7)).

$$TI = -0.4166 + 0.0141 \cdot \%Stevia + 0.0004 \cdot P * \%Stevia - 0.0152 \cdot t^2 \quad (7)$$

With regard to lightness (L*), the three-way ANOVA showed that pressure, time and *Stevia* concentration had a significant influence ($p<0.05$) on this parameter. Compared to the untreated beverages, lower L* values were found for samples treated by HPP independently of the *Stevia* used in the formulation of the beverages. In addition, the response surface equation obtained in the present study described the experimental data adequately ($R^2 = 0.921$, $p<0.05$, standard error = 5.324), which also was confirmed by the insignificant lack of fit ($p = 0.810$). The Eq. (8) was as follows:

$$L* = -82.361 - 0.963 \cdot P - 6.430 \cdot t - 34.891 \cdot \%Stevia - 0.001 \cdot P^2 + 0.013 \cdot P * t - 0.020 \cdot P * \%Stevia + 0.238 \cdot t * \%Stevia + 9.834 \cdot \%Stevia^2. \quad (8)$$

As can be observed, L* value was decreased for all the HPP treatments applied, obtaining a higher decrease at the higher pressure and longer time range. These results were in accord to those previously reported by Barba, Cortés, Esteve and Frígola (2012) in HPP orange juice mixed with milk. These authors attributed it to partial precipitation of unstable particles in the juices after processing.

With regard to a* values, the behavior was different depending on *Stevia* concentration of the untreated samples. The reduced regression model presented in the Eq. (9) allowed for prediction of the effects of independent variables on the a* values:

$$a^* = 41.967 - 0.304 \cdot P + 3.046 \cdot t + 11.683 \cdot \%Stevia + 0.001 \cdot P^2 - 0.006 \cdot P * t + 0.002 \cdot P * \%Stevia - 0.067 \cdot P^2 - 3.313 \cdot \%Stevia^2. \quad (9)$$

There were significant differences ($p<0.05$) in the a* values at different HPP conditions. Overall, the a* value changed toward a more positive direction for the HPP beverage with no *Stevia* added for all high pressure treatments and the beverage with 2.5% (w/v) of *Stevia* when HP treatment at 500 MPa/15 min was applied. The increase in CIE a* values was similar to the results found by Barba et al. (2010) when they studied the effects of HPP in a vegetable beverage and Patras, Brunton, Da Pieve, Butler, and Downey (2009) for high pressure processed tomato and carrot purées. However, the opposite trend was obtained when *Stevia* at 1.25% (w/v) was added for all HPP conditions. The highest decrease in CIE a* values was observed

**Table 3**
Experimental design matrix in terms of actual variables and the average values of the response for experiments on the effect of combined high pressure-*Stevia rebaudiana* concentration on the physicochemical parameters of a fruit juice mixture.

| Run[a] | Pressure (MPa) | Time (min) | Stevia (%) | BI[b] | TI[b] | L*[b] | a*[b] | b*[b] | ΔE |
|---|---|---|---|---|---|---|---|---|---|
| | ($X_1$) | ($X_2$) | ($X_3$) | ($Y_1$) | ($Y_2$) | ($Y_3$) | ($Y_4$) | ($Y_5$) | ($Y_6$) |
| 1 | 500 | 15 | 0 | 0.083 ± 0.003 | 0.078 ± 0.004 | 70.4 ± 0.1 | −1.5 ± 0.1 | 11.3 ± 0.2 | 3.5 ± 0.2 |
| 2 | 300 | 5 | 0 | 0.054 ± 0.006 | 0.076 ± 0.008 | 71.9 ± 0.2 | −1.8 ± 0.1 | 8.3 ± 0.2 | 0.5 ± 0.1 |
| 3 | 500 | 15 | 0 | 0.086 ± 0.005 | 0.082 ± 0.004 | 70.4 ± 0.3 | −1.5 ± 0.1 | 11.3 ± 0.3 | 3.5 ± 0.3 |
| 4 | 500 | 15 | 2.5 | 2.256 ± 0.010 | 0.700 ± 0.005 | 31.7 ± 0.2 | 11.1 ± 0.2 | 37.7 ± 0.3 | 5.3 ± 0.3 |
| 5 | 300 | 15 | 0 | 0.056 ± 0.004 | 0.070 ± 0.004 | 72.1 ± 0.1 | −1.6 ± 0.1 | 5.5 ± 0.2 | 3.0 ± 0.2 |
| 6 | 300 | 5 | 2.5 | 1.890 ± 0.009 | 0.429 ± 0.008 | 36.0 ± 0.2 | 9.1 ± 0.2 | 38.8 ± 0.3 | 1.3 ± 0.1 |
| 7 | 400 | 10 | 1.25 | 1.460 ± 0.011 | 0.327 ± 0.005 | 33.9 ± 0.1 | 9.4 ± 0.1 | 37.1 ± 0.4 | 14.4 ± 0.4 |
| 8 | 300 | 15 | 2.5 | 1.641 ± 0.012 | 0.414 ± 0.006 | 37.2 ± 0.2 | 10.4 ± 0.1 | 44.5 ± 0.4 | 5.2 ± 0.2 |
| 9 | 500 | 5 | 2.5 | 1.458 ± 0.010 | 0.452 ± 0.004 | 35.7 ± 0.1 | 10.3 ± 0.2 | 40.7 ± 0.5 | 1.7 ± 0.1 |
| 10 | 400 | 10 | 1.25 | 1.468 ± 0.009 | 0.333 ± 0.006 | 35.0 ± 0.2 | 9.0 ± 0.1 | 33.0 ± 0.2 | 18.0 ± 0.5 |
| 11 | 400 | 5 | 1.25 | 1.467 ± 0.007 | 0.319 ± 0.007 | 35.5 ± 0.2 | 8.9 ± 0.1 | 34.1 ± 0.3 | 16.8 ± 0.3 |
| 12 | 400 | 10 | 2.5 | 1.990 ± 0.009 | 0.445 ± 0.006 | 33.8 ± 0.2 | 9.5 ± 0.1 | 37.0 ± 0.2 | 3.7 ± 0.3 |
| 13 | 300 | 15 | 2.5 | 1.648 ± 0.010 | 0.416 ± 0.008 | 37.6 ± 0.4 | 10.4 ± 0.2 | 44.3 ± 0.1 | 5.1 ± 0.3 |
| 14 | 500 | 5 | 2.5 | 1.472 ± 0.011 | 0.466 ± 0.009 | 35.8 ± 0.3 | 10.3 ± 0.1 | 40.3 ± 0.2 | 1.4 ± 0.1 |
| 15 | 300 | 10 | 1.25 | 1.388 ± 0.015 | 0.345 ± 0.010 | 35.1 ± 0.2 | 9.1 ± 0.1 | 35.5 ± 0.3 | 15.6 ± 0.4 |
| 16 | 500 | 5 | 0 | 0.083 ± 0.003 | 0.076 ± 0.003 | 70.6 ± 0.3 | −1.5 ± 0.1 | 9.1 ± 0.4 | 1.9 ± 0.1 |
| 17 | 500 | 15 | 2.5 | 2.235 ± 0.010 | 0.697 ± 0.011 | 31.7 ± 0.2 | 11.1 ± 0.2 | 37.4 ± 0.2 | 5.4 ± 0.2 |
| 18 | 500 | 5 | 0 | 0.086 ± 0.004 | 0.078 ± 0.003 | 70.6 ± 0.4 | −1.5 ± 0.1 | 9.1 ± 0.2 | 1.9 ± 0.1 |
| 19 | 300 | 15 | 0 | 0.059 ± 0.005 | 0.076 ± 0.004 | 72.1 ± 0.4 | −1.6 ± 0.1 | 5.5 ± 0.1 | 3.0 ± 0.1 |
| 20 | 400 | 15 | 1.25 | 1.465 ± 0.008 | 0.337 ± 0.009 | 34.8 ± 0.3 | 8.8 ± 0.1 | 32.8 ± 0.3 | 18.3 ± 0.4 |
| 21 | 400 | 10 | 0 | 0.201 ± 0.006 | 0.115 ± 0.009 | 71.0 ± 0.5 | −1.1 ± 0.1 | 11.4 ± 0.2 | 3.3 ± 0.2 |
| 22 | 300 | 5 | 2.5 | 1.910 ± 0.010 | 0.426 ± 0.011 | 36.0 ± 0.3 | 9.0 ± 0.2 | 38.5 ± 0.2 | 1.5 ± 0.2 |
| 23 | 400 | 10 | 1.25 | 1.456 ± 0.013 | 0.330 ± 0.010 | 35.0 ± 0.2 | 9.0 ± 0.1 | 33.1 ± 0.3 | 17.9 ± 0.5 |
| 24 | 400 | 10 | 1.25 | 1.463 ± 0.012 | 0.335 ± 0.010 | 35.0 ± 0.2 | 9.0 ± 0.1 | 33.0 ± 0.4 | 18.0 ± 0.6 |
| 25 | 300 | 5 | 0 | 0.057 ± 0.006 | 0.078 ± 0.003 | 71.9 ± 0.2 | −1.9 ± 0.1 | 8.3 ± 0.1 | 0.5 ± 0.1 |
| 26 | 500 | 10 | 1.25 | 1.470 ± 0.010 | 0.342 ± 0.011 | 34.8 ± 0.4 | 8.6 ± 0.2 | 32.8 ± 0.3 | 18.3 ± 0.6 |

BI: browning index. TI: turbidity index. ΔE: total color differences.
[a] Order of the assays was randomized.
[b] Data shown are the mean ± SD of two treatment repetitions, each assay was performed in triplicate.

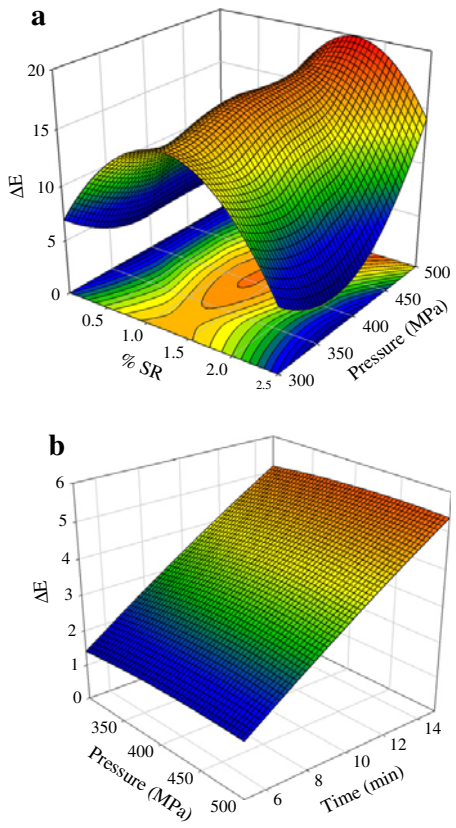


**Fig. 4.** The effect of combined high pressure–time and % of *Stevia rebaudiana* (*Stevia*) Bertoni on the color of a beverage mixture of fruit juice sweetened with *Stevia*. a) Effects of pressure and % *Stevia* on total color differences (ΔE) of the beverage for 10 min treatment time. b) Effects of pressure and time on total color differences (ΔE) of the beverage for 2.5% *Stevia*.

at 500 MPa/10 min. The decrease in a* values was similar to the results found by Patras, Brunton, Da Pieve, and Butler (2009) in HPP strawberry and blackberry purées and by Barba, Cortés, et al. (2012) in HPP orange juice mixed with milk samples.

The yellowness (b* values) of untreated samples was 8.5 ± 0.2, 50.4 ± 0.2 and 39.3 ± 0.1 when 0, 1.25 and 2.5% (w/v) of *Stevia* was added. Overall, longer treatment times and processing at the highest pressure resulted in the highest decrease in b* values (see Table 3). These results were in accord with those found by Saldo, Suárez-Jacobo, Gervilla, Guamis, and Roig-Sagués (2009) in apple juice processed at 300 MPa/4 °C, Daoudi et al. (2002) in white grape juice processed at 500 MPa/600 s/2 °C, and Barba, Cortés, et al. (2012) in orange juice–milk samples after HPP. Furthermore, statistical analyses showed a positive correlation between browning index and b* value ($p = 0.809$). In addition, relatively low correlation coefficient was observed ($R^2 = 0.873$) with a non-significant lack of fit ($p = 0.85$). The regression Eq. (10) describes the following model:

$$b^* = -48.276 + 29.040 \cdot \%Stevia - 0.026 \cdot P * \%Stevia + 0.435 \cdot t * \%Stevia - 6.242 \cdot \%Stevia^2. \quad (10)$$

As can be expected, the linear and quadratic effects of *Stevia* percentage were very significant for this model. In addition, the combined effect of time and *Stevia* also had a significant effect.

The total color difference (ΔE*) indicates the magnitude of the color difference. Depending on the value of ΔE, the color difference between the treated and untreated samples can be estimated such as not noticeable (0–0.5), slightly noticeable (0.5–1.5), noticeable (1.5–3.0), well visible (3.0–6.0) and great (6.0–12.0) (Cserhalmi, Sass-Kiss, Tóth-Markus, & Lechner, 2006). As can be seen in Table 2, *Stevia* addition had a strong effect on the beverage color, increasing significantly ($p < 0.05$) the browning of the juices. Moreover, in the present study, ΔE values were found to be different in behavior, depending on treatment time or HPP intensity level (Table 3, Fig. 4). Color changes increased when pressure and treatment times were higher independently of the *Stevia* percentage used, with the highest differences appearing at 500 MPa (3.3, 18.3 and 5.3 for 0, 1.25 and 2.5% (w/v) *Stevia*). The regression Eq. (11) describes the following model:

$$\Delta E = 141.297 - 0.858 \cdot P + 5.299 \cdot t + 13.257 \cdot \%Stevia + 0.001 \cdot P^2 + 0.019 \cdot P * \%Stevia - 0.218 \cdot t * \%Stevia - 6.603 \cdot \%Stevia^2. \quad (11)$$

### 3.3. Optimization and validation of the HPP and Stevia concentration conditions

The combination of HPP critical parameters that lead to a beverage (fruit juice mixture sweetened with *Stevia*) with the highest nutritional and physicochemical quality was determined. The same priority was assigned to each dependent variable in order to obtain a beverage with maximal retention of bioactive compounds (ascorbic acid, total phenolic compounds, total anthocyanins, and total carotenoids), and antioxidant capacity. Likewise, the conditions that lead to a beverage with total color differences lower than 3 were selected. Therefore, the optimal conditions of HPP in the present study were as follows: 1.7% (w/v) of *Stevia* concentration, and 300 MPa of high hydrostatic pressure for 14 min. Under such conditions the greatest retention of bioactive compounds, antioxidant capacity as well as physicochemical properties were achieved, with an overall score of 0.626.

## 4. Conclusion

The results obtained in the present research suggested that optimizing pressure–time conditions after HPP can be considered as a factor of great interest in order to obtain a better retention of bioactive compounds and physicochemical characteristics. HPP combined with *S. rebaudiana* water extracts can be a useful tool in order to provide new functional foods of proven physical and nutritional quality, thus increasing added value. In any case, more studies on the combined effect of pressure and time are required to elucidate the effects of HPP parameters on bioactive compounds and color in foods, and further studies dealing with the effects of HPP in liquid foods during storage are needed.

## Acknowledgments

This research project was supported by the Spanish Ministry of Science and Technology and European Regional Development Funds (AGL2010-22206-C02-01). Carbonell-Capella, J.M. holds an award from the Spanish Ministry of Education (AP2010-2546). Barba, F.J. received a contract employment from this project to carry out the study.

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In: Fruit Juices
Editor: Katherlyn Elizabeth Elder
ISBN: 978-1-63321-134-6


*Chapter 6*

# ASCORBIC ACID IN ORANGE JUICE-BASED BEVERAGES PROCESSED BY PULSED ELECTRIC FIELDS

***Juana M. Carbonell-Capella, Francisco J. Barba, Ana Zulueta, María J. Esteve and Ana. Frígola****
Department of Nutrition and Food Chemistry, Universitat de València, Avda Vicent Andrés Estellés, s/n, Burjassot, Spain

## ABSTRACT

Citrus fruits have been widely used as ingredients in fruit juices and beverages. However, vitamin C content may be reduced by thermal processing, thus reducing beneficial health effects. For this reason, the degradation kinetics of ascorbic acid were determined in orange juice based beverages after treatment by pulsed electric field, a novel emerging technology with increasing interest in the conservation of juices. The orange juice and orange-carrot juice mixture fitted a linear model, while the orange juice–milk beverage followed an exponential model. The degradation constants obtained in the orange juice were $0.00046\pm1\cdot10^{-4}$, $-0.00051\pm5\cdot10^{-5}$, $-0.00074\pm8\cdot10^{-5}$, and $-0.00095\pm1\cdot10^{-4}$ $\mu s^{-1}$ for fields of 25, 30, 35, and 40 kV/cm, respectively. In the case of the orange–carrot juice they were $-0.00037\pm3\cdot10^{-5}$, $-0.00042\pm5\cdot10^{-5}$, $-0.00119\pm7\cdot10^{-5}$, and $-0.00091\pm2\cdot10^{-4}$ $\mu s^{-1}$ for fields of 25, 30, 35, and 40 kV/cm, respectively, and for the orange juice–milk beverage they were $-0.00012\pm3\cdot10^{-5}$, $-0.00022\pm5\cdot10^{-5}$, $-0.00042\pm7\cdot10^{-5}$, and $-0.00061\pm6\cdot10^{-5}$ $\mu s^{-1}$ for fields of 15, 25, 35, and 40 kV/cm, respectively, demonstrating the use of PEF as an alternative to pasteurization treatments. The D value increased with electric field strength, and the $Z_E$ values were 42.9, 30.6, and 35.1 kV/cm for the orange juice, orange–carrot mixture, and orange juice–milk beverage, respectively. Therefore, the orange juice was more resistant to changes in electric field strength in PEF treatment, while the treatment time required to produce the same degradation of ascorbic acid was greater in the orange juice–milk beverage than for the orange juice or the orange–carrot juice mixture, showing the need to optimize treatment conditions whenever there is a change in the food matrix.

* Corresponding author: Email: ana.frigola@uv.es. Phone: +34 963544955, Fax: +34 963544954.

Innovative Food Science and Emerging Technologies xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

# "Ice" juice from apples obtained by pressing at subzero temperatures of apples pretreated by pulsed electric fields

Juana M. Carbonell-Capella [a,b], Oleksii Parniakov [a,*], Francisco J. Barba [b], Nabil Grimi [a], Olivier Bals [a], Nikolai Lebovka [a,c], Eugene Vorobiev [a]

[a] Sorbonne Universités, Université de Technologie de Compiègne, Laboratoire de Transformations Intégrées de la Matière Renouvelable, EA 4297, Centre de Recherches de Royallieu, BP 20529, 60205 Compiègne Cedex, France
[b] Universitat de València, Faculty of Pharmacy, Nutrition and Food Science Area, Avda. Vicent Andrés Estellés, s/n, 46100, Burjassot, València, Spain
[c] Institute of Biocolloidal Chemistry named after F. D. Ovcharenko, NAS of Ukraine, 42, blvr. Vernadskogo, Kyiv 03142, Ukraine

ARTICLE INFO

*Article history:*
Received 2 September 2015
Received in revised form 12 October 2015
Accepted 12 December 2015
Available online xxxx

ABSTRACT

The impact of apple pretreatment by pulsed electric field (PEF) on juice extraction using the freezing-assisted pressing was studied. Apple discs were PEF pretreated at electric field strength of $E = 800$ V/cm and then air blast frozen inside the freezer (−40 °C). Then, pressing experiments in a laboratory-pressing chamber (2–5 bars) were started at sub-zero temperature (−5 °C). Time evolution of juice yield and its nutritional qualities were compared for PEF and untreated apple samples. High improvements of juice yield were obtained for freeze-thawed (FT) and PEF + FT samples. The combination of PEF + pressing (5 bar) at sub-zero temperature gave optimum results for juice extraction with high levels of carbohydrates, and antioxidant bioactive compounds. At fixed value of extraction yield, *Y*, PEF pretreatment improved nutritional parameters. E.g., at $Y = 0.6$, an increase in °Brix (by ≈1.27), carbohydrates (by ≈1.42), total phenolic compounds (by ≈1.16), flavonoids (by ≈1.09) and antioxidant capacity (by ≈1.29) was observed after PEF pretreatment.

*Industrial relevance:* Pressing constitutes one of the most commonly used technologies at industrial scale to obtain fruit juices. However, during the pressing some undesirable chemical, physical and biological changes may occur in juices, thus reducing their nutritional and sensorial properties. For instance, the use of freezing-assisted pressing is a promising technique for the production of juice concentrates rich in sugars and other solids as the low temperature operation prevents undesirable modifications. But this method is rather expensive and requires strong control of the quality of "ice" juices, their sensory and compositional profiles. Thus, there is an increased search for obtaining new efficient methodologies for producing high quality juices. In this line, PEF-assisted pressing has been shown as a useful technology to increase juice yield. Therefore, the combination of PEF-assisted "ice" juice extraction by pressing of fruits at subzero temperatures may be a useful tool to improve the extraction yield of juices, thus improving their nutritional, physicochemical and sensorial properties.

Keywords: "Ice" juice, Apple, Pulsed electric fields, Freezing-assisted pressing



## 1. Introduction

A high juice yield is desirable in terms of economics, but organoleptic, nutritional properties and beneficial health effects of juices, partly attributed to the presence of antioxidants, especially phenolic compounds are also very important (Krawitzky et al., 2014). In this line, the potential of several juice extraction methods to obtain high juice yields with improved nutritional properties has been evaluated by both food researchers and food industry, concluding that the quality of juices is highly dependent of the production process.

Moreover, it has been observed that low-temperature assisted processing of foods (e.g., freeze concentration, pressing, etc.) is rather preferable in application for products with very delicate flavors (fruit juices, coffee, tea, and alcoholic beverages) (Deshpande, Cheryan, Sathe, & Salunkhe, 1984; Sánchez, Ruiz, Auleda, Hernández, & Raventós, 2009).

Freeze concentration is considered as a method for producing high quality juices. This method is based on a selective separation of water in the form of ice from the frozen solution. Freeze concentration has been applied in the production of concentrated "ice" juices from apple (Bayindirli, Özilgen, & Ungan, 1993; Hernández, Raventós, Auleda, & Ibarz, 2009; Olowofoyeku, Gil, & Kramer, 1980), pear (Hernández et al., 2009; Miyawaki, Kato, & Watabe, 2012; Tobitsuka, Ajiki, Nouchi, & Miyawaki, 2010), blueberry (Petzold, Moreno, Lastra, Rojas, & Orellana, 2015), sugarcane (Rane & Jabade, 2005; Sahasrabudhe, Desai, & Jabade, 2012), pineapple (Bonilla-Zavaleta, Vernon-Carter, & Beristain, 2006; Petzold et al., 2015), orange (Fang, Chen, Tang, &

* Corresponding author. Tel.: +33 3 44 23 44 42.
*E-mail address:* oleksii.parniakov@utc.fr (O. Parniakov).

http://dx.doi.org/10.1016/j.ifset.2015.12.016
1466-8564/

Wang, 2008; Sánchez, Ruiz, Raventós, Auleda, & Hernández, 2010), pomegranate (Khajehei, Niakousari, Eskandari, & Sarshar, 2015), tomato (Liu, Miyawaki, & Hayakawa, 1999; Miyawaki et al., 2012) concentration of grape must (Hernández, Raventós, Auleda, & Ibarz, 2010), fresh tea juice (Feng, Tang, & Ning, 2006) and preparation of coffee extract (Gunathilake, Shimmura, Dozen, & Miyawaki, 2014).

Among techniques of juice production, pressing is one of the most used at industrial scale (Markowski, Baron, Le Quéré, & Płocharski, 2015). Freezing assisted pressing can be also used as a promising technique for the production of juice concentrates rich in sugars and other solids (Petzold et al., 2015). Concentrated aqueous solutions do not freeze, while pure water does, allowing a more concentrated juice product. The low temperature operation in freeze-concentration prevents undesirable chemical, physical and biological changes that may occur in other types of processing.

This procedure has already been employed in the production of the so-called "ice" wine, "ice" cider or "ice" juice by pressing of frozen fruits (Alessandria et al., 2013; Bowen, 2010; Crandles, Reynolds, Khairallah, & Bowen, 2015; Kirkey & Braden, 2014; Motluk, 2003; Musabelliu, 2013). Such extreme processing allows the production of smaller amounts of concentrated and sweeter wines, e.g., high quality dessert wines. Application of cold pressing is rather popular in those countries (e.g., Canada and Germany), where fruit varieties are not harvested before the first frosts. However, this processing is rather expensive, risky and requires strong control of the quality of "ice" wines, their sensory and compositional profiles (Bowen, 2010). E.g., these characteristics may be significantly different for British Columbia, Ontario and German ice wines (Cliff, Yuksel, Girard, & King, 2002; Nurgel, Pickering, & Inglis, 2004). However, application of low-temperature assisted pressing is still very limited (Petzold et al., 2015).

The extraction efficiency by pressing may be noticeably enhanced using pulsed electric fields (PEF) pre-treatment (Lebovka & Vorobiev, 2010). Different examples of PEF-assisted dehydration of fruit and vegetable tissues have already been demonstrated (Donsi, Ferrari, & Pataro, 2010; Vorobiev & Lebovka, 2011). These techniques allow avoidance of undesirable changes typical of other techniques, such as thermal, chemical and enzymatic ones (Donsi, Ferrari, Maresca, & Pataro, 2011; Jaeger, Reineke, Schoessler, & Knorr, 2012; Jaeger, Schulz, Lu, & Knorr, 2012; Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2013; Raso & Heinz, 2006)

The positive effects of PEF pre-treatment on drying, freezing, freeze-drying processes, freezing tolerance and texture of biomaterials have been demonstrated (Ben Ammar, Lanoiselle, Lebovka, Van Hecke, & Vorobiev, 2010; Jalte, Lanoiselle, Lebovka, & Vorobiev, 2009; Parniakov, Lebovka, Bals, & Vorobiev, 2015; Phoon, Galindo, Vicente, & Dejmek, 2008; Shayanfar, Chauhan, Toepfl, & Heinz, 2013, 2014; Shynkaryk, Lebovka, & Vorobiev, 2008; Wiktor, Schulz, Voigt, Witrowa-Rajchert, & Knorr, 2015). However, the effect of PEF pre-treatment on freezing assisted pressing and in the production of "ice" juice has not yet been studied.

This manuscript discusses the PEF-assisted "ice" juice extraction by pressing of apple at sub-zero temperatures. Apples were initially pretreated by PEF to a high level of electroporation, frozen and pressed during their thawing at 2–5 bars. Extraction yield and nutritional qualities of the extracted "ice" juice were analysed.

## 2. Material and methods

### 2.1. Raw material and sample preparation

Apples (*Malus domestica* var. *Jonagold*) were purchased at the local supermarket and stored at 4 °C until analysis. Wet basis moisture content was measured by drying 20 g of the fresh apple tissue at 105 °C to constant weight. It was found between 82 and 85% w. b. Apple discs ($d = 50$ mm and $h = 5$ mm) were prepared using a vegetable cutter (Robot Cupe CL 50, Montceau-en-Bourgogne-Cedex, France).

### 2.2. PEF pretreatment

PEF pretreatment was carried out using a pulse generator, 400 V-38 A (Service Electronique UTC, Compiegne, France). Apple sample was placed in the PEF-treatment cell between two electrodes (Fig. 1). Electrical treatment cell consisted of a Teflon cylindrical tube (Atelier Genie des Procédés Industriels, UTC, Compiegne, France) with ≈110 mm inner diameter and an electrode (stainless steel 316 L) at the bottom. The apple disc-shaped sample was placed inside the cell on the bottom electrode and covered with fresh apple juice. After that, the second electrode was put on top of the samples. The distance between the electrodes, 5 mm, was determined by the height of the sample. Temperature was controlled by a Teflon-coated thermocouple Thermocoax type 2-AB 25 NN (Thermocoax, Suresnes, France) inserted into the geometrical centre of the sample with a temperature measurement precision of ±0.1 °C. The PEF generator provided bipolar pulses of near-rectangular shape with an electric field of $E = 800$ V/cm and series of $N = 10$ were applied. Each separate series consisted of $n = 10$ pulses with pulse duration $t_i = 100$ μs, distance between pulses $\Delta t = 1000$ ms and pause $\Delta t_t = 10$ s after each series. Total time of PEF treatment was calculated as $t_p = nNt_i$. The chosen protocol of successive trains with long pause after each train allowed a fine control of the plant tissue permeabilization without any significant temperature elevation (ΔT ≤ 3 °C) during PEF treatment. These conditions were chosen according to a previous study (Parniakov et al., 2015) where a high level of tissue electroporation (conductivity disintegration index $Z$ was ≈0.98) was obtained. Electrical conductivity of the sample was measured during the pause period $\Delta t_t$ between two consecutive series of pulses. All the output data (current, voltage, electrical conductivity and temperature) were collected using a data logger and special software adapted by Service Electronique UTC.

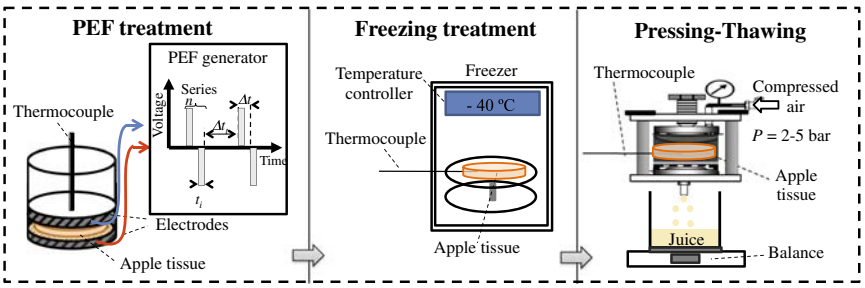


**Fig. 1.** Schematic presentation of experimental procedures including PEF pretreatment, freezing, and pressing–thawing experiments.

Please cite this article as: Carbonell-Capella, J.M., et al., "Ice" juice from apples obtained by pressing at subzero temperatures of apples pretreated by pulsed electric fields, *Innovative Food Science and Emerging Technologies* (2016), http://dx.doi.org/10.1016/j.ifset.2015.12.016

### 2.3. Freezing

Apple samples were frozen in an ultra-low-temperature air-blast freezer MDF-U2086S (Sanyo, Gunma, Japan), supplied with a modular-type temperature controller SR Mini System (TC Ltd., Dardilly, France) and the software Spec-View Plus (SpecView Corporation, Gig Harbor, USA). Untreated and PEF pretreated samples were placed inside the freezer at −40 °C with an air velocity of 2 m/s controlled by an electronic device VEAT 2.5 A (Air-technic, Firminy, France). Sample temperature was measured with a T type thermocouple of 0.5 mm diameter (TC, Ltd., Dardilly, France) with an accuracy of ±0.1 °C that was introduced in the geometrical centre of the sample. Initial temperature before freezing was uniform and constant at 20 °C for the whole sample. Total freezing time, $t$, from the beginning of the cooling was 50 min, the final temperature of the sample was ≈−35 °C.

### 2.4. Pressing–thawing experiments

Immediately after the end of freezing the apple samples were placed into a pressing chamber maintained at 20 °C. The temperature inside the tissue was measured with a Teflon-coated thermocouple Thermocoax type 2-AB 25 NN (Thermocoax, Suresnes, France) with precision of ±0.1 °C. Samples were compressed using a laboratory pressing chamber (hemispherical shape with a radius $R = 28$ mm) equipped with an elastic diaphragm (Fig. 1). The compression at 2, 3 and 5 bars was started when the temperature inside the tissue attained −5 °C and continued for 100 min. The extracted juice was collected and weighted continuously by an electronic balance.

In present experiments the maximum quantity of recovered juice, $m_m$, was obtained for long time of pressing of PEF pretreated tissue, $t \approx 5000$ s, at $P = 5$ bars. Extraction yield, $Y$, was calculated as

$$Y = m/m_m. \quad (1)$$

In order to follow the pressing kinetic, an acquisition computer system was used to record the mass of recovered juice $m$ every 5 s.

### 2.5. Nutritional characteristics

Concentration of total soluble matter was measured in accordance to IFU methods (IFU, 2001) with a digital refractometer (Atago, USA) at room temperature. Results were expressed in °Brix (g of total soluble solid content/100 g solution).

Total carbohydrates concentration, $C_c$, was determined using the phenol–sulphuric acid method (Du Bois, Gilles, Hamitton, Reders, & Smith, 1956), with some modifications (Parniakov, Lebovka, Van Hecke, & Vorobiev, 2014). 0.4 mL of sample were mixed with 0.2 mL of 5% ($w/v$) phenol solution and 1 mL of concentrated sulphuric acid (Sigma-Aldrich, France). Then the reaction mixture was kept at 25 °C for 30 min. Absorbance of the mixture was measured at 490 nm and the polysaccharide content was calculated using D-glucose (VWR International, Belgium) as a standard. Results were expressed in mg of glucose equivalent per liter of apple juice (mg/L).

Adapted and optimised assay for total polyphenols determination was used (Singleton, Orthofer, & Lamuela-Raventos, 1999). Initially, 200 μL of diluted extract and 1000 μL of the Folin–Ciocalteau reagent (diluted 10 folds in distilled water, $w/w$) were mixed and left at room temperature for 5 min. Then, 800 μL of $Na_2CO_3$ solution (7.5 g of $Na_2CO_3$ and 100 g of water) was added. The mixture was kept for 1 h at room temperature and absorbance was measured at 750 nm using a UV–vis spectrophotometer (Milton Roy Company, Spectronic 20 Genesys, United States). Gallic acid calibration standards with concentrations of 0, 20, 40, 60, 80, 100 and 120 ppm were prepared and results were expressed as milligrams of gallic acid equivalents (GAE) per liter of apple juice (mg/L).

Total flavonoid content was determined using the method as described in (Zhishen, Mengcheng, & Jianming, 1999). 100 μL of sample was mixed with 1.088 mL of ethanol (30%, $v/v$) and 48 μL of sodium nitrite solution (0.5 mol/L). After 5 min, 48 μL of aluminium chloride (0.3 mol/L) was added. The mixture was stirred and allowed to react for 5 min. Then, 320 μL of sodium hydroxide (1 mol/L) was added and absorbance was measured at 510 nm using a UV–Vis spectrophotometer (Milton Roy Company, Spectronic 20 Genesys, United States). Catechin was used as standard with concentrations in the range of 0–75 μg/L and results were expressed as milligrams of catechin equivalents (CTE) per litre of apple juice [mg C/L].

Total antioxidant capacity was measured using the TEAC (Trolox Equivalent Antioxidant Capacity) assay according to Carbonell-Capella, Buniowska, Esteve, and Frigola (2015). ABTS radical ($ABTS^{\bullet+}$) was generated using 440 μL of potassium persulfate (140 mM). The solution was diluted with ethanol until an absorbance of 0.70 was reached at 734 nm. Once the radical was formed, 2 mL of $ABTS^{\bullet+}$ was mixed with 100 μL of extract and incubated for 60 min at 20 °C and absorbance was measured.

The browning index (BI) was measured accordingly to (Meydav, Saguy, & Kopelman, 1977). For this purpose samples were centrifuged (824 g, 20 min, 20 °C) and supernatant was diluted in ethanol (1:1 ($v/v$)). The mixture was filtered using filter paper (Whatman® No. 42, Whatman International Ltd., UK) and absorbance was measured at 420 nm.

### 2.6. Statistical analysis

Each experiment was repeated at least three times. Error bars presented on the figures correspond to the standard deviations. One-way analysis of variance was used for statistical analysis of the data using the Statgraphics plus (version 5.1, Statpoint Technologies Inc., Warrenton, VA). Tukey´s test was also performed on data for all pairwise comparisons of the mean responses to the different treatment groups. This test allows determination of treatments which are statistically different from the other at a probability level of $P = 0.05$.

## 3. Results and discussion

Fig. 2 presents typical examples of temperature evolution inside the apple discs during the cooling of untreated and PEF pretreated apples.

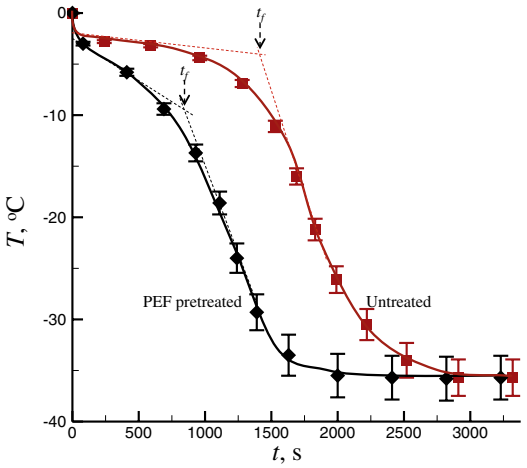


**Fig. 2.** Evolution of temperature inside the geometrical centre of the apple disc during freezing for untreated and PEF treated samples. Here, $t_f$ is an effective freezing time. The symbols are the experimental data. The curves were drawn for the guidance of eye.

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The temperature decrease was initially rather slow. The first crystallization stage corresponds to a phase transformation of water into ice inside the sample (Chevalier, Le Bail, & Ghoul, 2000). However, after some time, the temperature began to decrease rapidly to the storage value (−40 °C). The second cooling stage is started when the most freezable water is converted to ice. The effective freezing time, $t_f$, was determined as a crosspoint of tangent lines of freezing and cooling part of $T(t)$ curve (Fig. 2).

PEF pretreatment resulted in a significant acceleration of the freezing process. E.g., values of $t_f$ were ≈1400 s and ≈850 s for untreated and PEF-treated apple discs, respectively. Similar effects of PEF-pretreatment were previously reported for potato (Ben Ammar et al., 2010; Jalte et al., 2009) and apple (Parniakov et al., 2015). The different possible mechanisms of PEF-pretreatment in the increase of the freezing rate were earlier discussed (Jalte et al., 2009). In general, the resultant faster freezing is desirable, leading to smaller size of formed ice crystals and better quality of processed products (Delgado & Sun, 2001).

Fig. 3 presents the temperature evolution inside apple samples during the pressing–thawing experiments for untreated ($P = 0$ bars) and PEF pretreated samples at different pressures $P = 0$, 2, 3 and 5 bars. The case of $P = 0$ bars corresponds to the thawing without applied pressure. During the thawing, the temperature increased to reach the temperature plateau or break of the curve $T(t)$ in the vicinity of melting point at some transition time $t = t_e$ (Fig. 3). The temperature plateaus were practically absent for the thawing without applied pressure ($P = 0$ bars) and were present at $P > 0$ bars. For PEF pretreated samples at $P > 0$ the transition time, $t_e$, characterises the starting point of intensive releasing of juice from sample. Note, that transition time decreased significantly with increase of the applied pressure $P$ (see inset to Fig. 3).

Fig. 4 presents extraction yield, $Y$, and concentration of soluble solids in local portions of juice, °Brix$^l$, versus extraction time, $t$, in pressing–thawing experiments at different pressures $P$. Data are presented for PEF pretreated apples. Extraction yield, $Y$, increased with the pressing time, $t$, and reached a maximum level, $Y_m$, after a long time of pressing, $t \geq 2400$ s. Values of $Y_m(P)$ were ≈0.6, ≈0.8 and = 1 for $P = 2$, 3 and 5 bars, respectively. Note that similar values of $Y_m(P)$ were also obtained in pressing–thawing experiments with untreated samples (data are not presented). However, the rate of juice release for PEF pretreated apples was significantly higher compared to untreated ones and the maximum level, $Y_m$, for untreated samples was only obtained at $t \geq 6000$ s. So, electroporation facilitates the rate of juice release during thawing of frozen apple tissue, but does not change the amount of juice expressed. It can be speculated that this phenomenon reflects the changes in spatial distribution of unfrozen content inside the tissue that facilitates the juice flow when pressure is applied. The observed behaviour was also in accordance with significant decreasing of the transition time, $t_e$, with increase of the applied pressure $P$ (see inset to Fig. 3).

Concentration of soluble solids in the first local fractions of apple juice, °Brix$^l$, was rather high (Fig. 4). During the pressing–thawing experiments, °Brix$^l$ value reached a maximum at $t = t_{max}$. At $t \geq t_{max}$, values of °Brix$^l$ decreased significantly and reached approximately the same minimum value, $^{o}\text{Brix}_{\min}{}^{l} \approx 5.9$, independently of the pressure applied. Initial expression of the most concentrated juice with the highest content of sugars and other solids was expected, as it reflects the typical process of freezing-assisted pressing. When time is increased, the thawing of the ice crystals found inside the apple tissue takes place and consequently, the concentration of soluble solids in the expressed juice drops gradually. The observed time evolution of $Y(t)$ and °Brix$^l(t)$ (Fig. 4) is in qualitative correspondence with the temperature evolution presented in Fig. 3.

However, the origin of °Brix$^l$ maximum at $t = t_{max}$ is not completely clear. In the applied experimental schemes of freezing and pressing–thawing, the spatial distributions of the temperature and concentrations of the different components found inside the sample are highly heterogeneous. Freezing is started from the sample surface and can result in heterogeneous spatial distributions of both segregated ice crystals and unfreezable portions of juice inside the apple sample. From the other hand, during pressing–thawing, temperature is higher on the surface of the sample and initial portions of juice are expressed from the surface layer. Electroporation may have a supplementary effect on the heat and mass transfer processes in the sample. That is why the maximum of °Brix$^l$ at $t = t_{max}$ can be the reflection of the

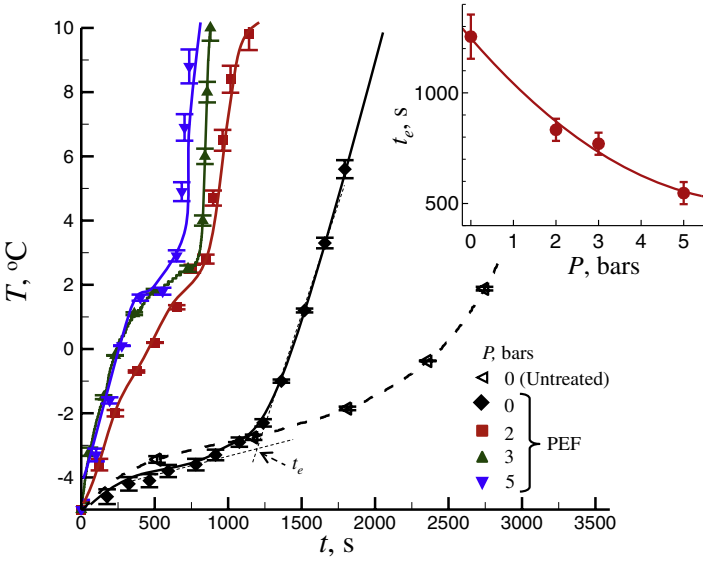


**Fig. 3.** Evolution of temperature inside the geometrical centre of the apple tissue during pressing–thawing experiments for untreated ($P = 0$ bars) and PEF pretreated ($P = 0$, 2, 3 and 5 bars) apple tissue. The case of $P = 0$ bars corresponds to the thawing without applied pressure. Break of the curve $T(t)$ at $t = t_e$ for $P = 0$ is shown. Inset shows transition time, $t_e$, versus applied pressure $P$. The symbols are the experimental data. The curves were drawn for the guidance of eye.

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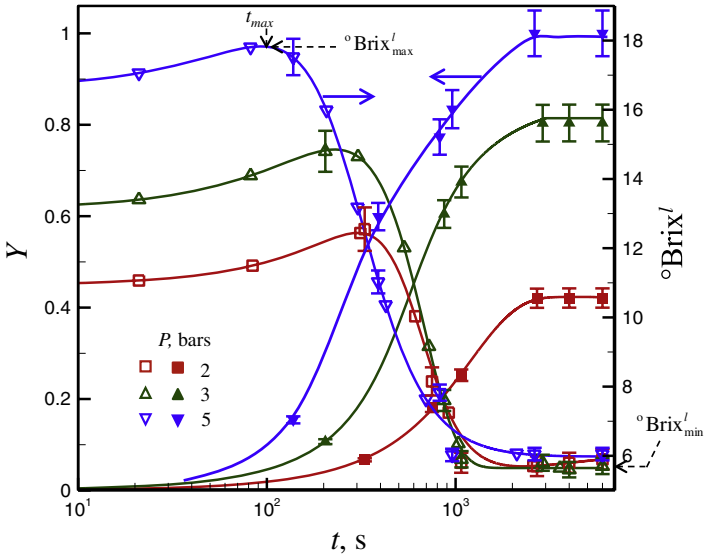


**Fig. 4.** Extraction yield, *Y*, and concentration of soluble solids in local portion of juice, °Brix$^{l}$, versus extraction time, *t*, in pressing–thawing experiments at different pressures *P* (=2, 3 and 5 bars). Data are presented for PEF pretreated apples. The symbols are the experimental data. The curves were drawn for the guidance of eye.

above-mentioned heterogeneities in temperature and component concentrations.

Fig. 5 presents the concentration of soluble solids in local portion of juice, °Brix$^{l}$, versus the extraction yield, *Y*, in pressing–thawing experiments at different pressures *P*. Note that the extraction yield of the most concentrated local portions of juice at $t = t_{max}$ was rather small, $Y \approx 0.1$–$0.15$. Maximum values, °Brix$^{l}_{max}$, decreased whereas $t_{max}$ values increased with increase of a pressure, *P* (See, insert to Fig.5).

From a practical point of view, it is interesting to compare the characteristics of the total portion of accumulated juice at different values of extraction yield, *Y*. Fig. 6 presents the concentration of soluble solids, °Brix, (a) and total phenolic compounds, $C_{TPC}$, (b) versus juice yield, *Y*,

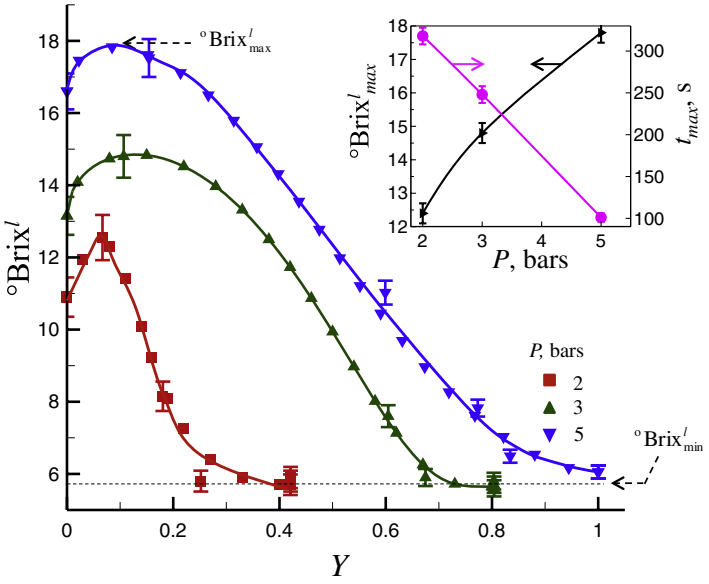


**Fig. 5.** Concentration of soluble solids in local portion of juice, °Brix$^{l}$, versus extraction yield, *Y*, in pressing–thawing experiments at different pressures *P* (=2, 3 and 5 bars). Data are presented for PEF pretreated apples. Insert shows the maximum values of °Brix$_{max}{}^{l}$ in local portion of juice and corresponding time of extraction, $t_{max}$, versus the applied pressure *P*. Data are presented for PEF pretreated apples. The symbols are the experimental data. The curves were drawn for the guidance of eye.

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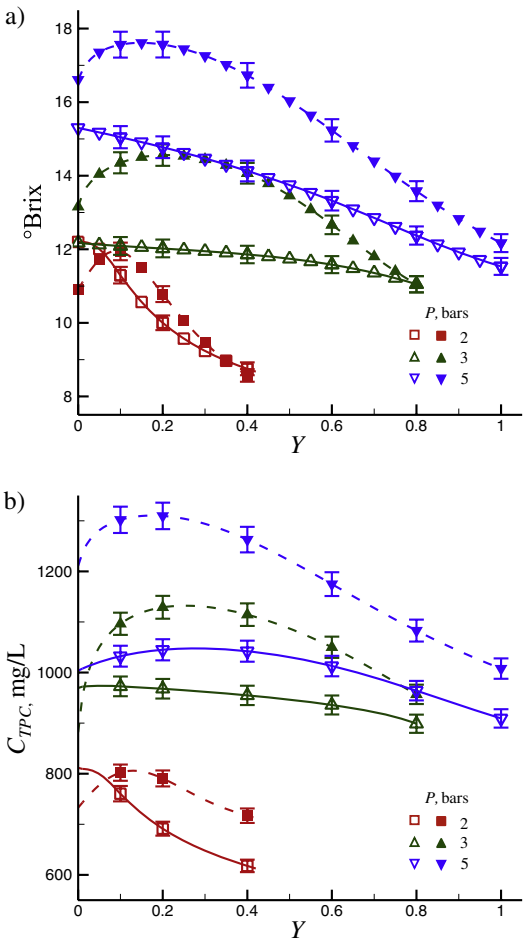


**Fig. 6.** Concentration of soluble solids, °Brix, (a) and total phenolic compounds, $C_{TPC}$, (b) versus juice yield, $Y$, at different pressures, $P = 2$, 3 and 5 bars. Data are presented for PEF pretreated (filled symbols, dashed lines) and untreated (open symbols, solid lines) apple samples. The symbols are the experimental data. The curves were drawn for the guidance of eye.

at different pressures $P$. Data are presented for PEF pretreated (filled symbols, dashed lines) and untreated (open symbols, solid lines) apple samples.

Results show that freezing assisted pressing is rather effective at high pressure, $P = 5$ bars, and ineffective at small pressure, $P = 2$ bars. At high pressure ($P = 5$ bars), final values of °Brix ($Y = 1$) were $\approx$12.2 and $\approx$11.5 for PEF pretreated and untreated samples, respectively. However, at small pressure, $P = 2$ bars, final values of °Brix ($Y \approx 0.4$) were $\approx$8.8 for both PEF pretreated and untreated samples. This may reflect the capture of more concentrated juice inside the apple sample for the given mode of freezing-assisted pressing at $P = 2$ bars.

In further discussion, we will only evolve the data obtained at the highest pressure, $P = 5$ bars. It is remarkable that in PEF-assisted pressing–thawing experiments, °Brix values (Fig. 6a) and $C_{TPC}$ (Fig. 6b) noticeably exceeded those values obtained for untreated apple samples. It evidently reflects the impact of electroporation on the freezing-assisted pressing. Noticeably, both electroporation and freezing cause cell damage in tissues. However, electroporation can also affect heat and mass transfer processes during freezing, which was found to be important for enhancement of the pressing–thawing process.

The different nutritional characteristics of apple juice obtained in pressing–thawing experiments at 5 bars for untreated and PEF pretreated apples are compared in Table 1. At fixed value of extraction yield, $Y$, PEF pretreatment always allowed noticeable enhancement of nutritional parameters. E.g., at $Y = 0.6$ an increase in °Brix of $\approx$1.27, total carbohydrates of $\approx$1.42, total phenolic compounds of $\approx$1.16, flavonoids of $\approx$1.09 and total antioxidant capacity of $\approx$1.29 was observed after PEF pretreatment. The highest concentrations of total carbohydrates ($C_C \approx 281.5$ g/L), total phenolic compounds ($C_{TPC} \approx 1310.4$ mg GAE/L), flavonoids (292.7 mg CTE/L) and total antioxidant capacity value (TAC $\approx$ 9.2 mM trolox equivalent) were obtained with juice yields of $\approx$13%, $\approx$18%, $\approx$14% and $\approx$18%, respectively. Thus, freezing-assisted pressing of PEF pretreated samples has a positive effect on all nutritional parameters of the extracted apple juice and allowed obtaining an ice juice rich in bioactive compounds. Likewise, it was observed that browning index of juice obtained by freezing-assisted pressing of PEF pretreated samples was rather lower (BI $\approx$ 0.101) than that of juice obtained by traditional method (BI $\approx$ 0.306).

## 4. Conclusions

Freezing-assisted pressing at sub-zero temperatures is an effective tool in order to obtain an apple juice rich in bioactive compounds. The efficiency of this process can be noticeably improved by the application of PEF pretreatment of apple tissue before freezing. PEF pretreatment resulted in a reduction of both freezing and thawing time of apple tissue and that pressing was more effective at high pressure, $P = 5$ bars.

**Table 1**
Different nutritional characteristics of apple juice obtained in pressing–thawing experiments at 5 bars for untreated and pulsed electric fields (PEF) pretreated apples.

| Nutritional parameters | | $Y$ | | | | |
|---|---|---|---|---|---|---|
| | | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
| Untreated | °Brix | $14.8 \pm 0.1^{a}$ | $14.1 \pm 0.1^{ab}$ | $13.2 \pm 0.1^{c}$ | $12.2 \pm 0.1^{d}$ | $11.5 \pm 0.1^{e}$ |
| | $C_C$ (g/L) | $164.7 \pm 1.6^{a}$ | $168.1 \pm 0.5^{a}$ | $161.1 \pm 3.4^{a}$ | $147.2 \pm 3.2^{b}$ | $131.2 \pm 3.9^{c}$ |
| | $C_{TPC}$ (mg/L) | $1045.0 \pm 5.2^{a}$ | $1042.2 \pm 3.2^{a}$ | $1012.9 \pm 4.1^{b}$ | $964.2 \pm 1.0^{c}$ | $909.4 \pm 4.1^{d}$ |
| | $C_{TF}$ (mg/L) | $247.5 \pm 2.5^{a}$ | $248.3 \pm 1.9^{a}$ | $237.0 \pm 1.9^{b}$ | $223.0 \pm 4.3^{c}$ | $211.7 \pm 1.2^{d}$ |
| | TAC (mM TE) | $7.46 \pm 0.09^{a}$ | $7.14 \pm 0.14^{a}$ | $6.53 \pm 0.11^{b}$ | $6.03 \pm 0.05^{c}$ | $5.92 \pm 0.07^{d}$ |
| PEF treated | °Brix | $17.6 \pm 0.1^{a}$ | $16.7 \pm 0.1^{b}$ | $15.2 \pm 0.1^{c}$ | $14.1 \pm 0.1^{d}$ | $12.2 \pm 0.1^{e}$ |
| | $C_C$ (g/L) | $278.4 \pm 4.3^{a}$ | $255.8 \pm 2.7^{b}$ | $227.8 \pm 1.8^{c}$ | $200.3 \pm 1.6^{d}$ | $176.3 \pm 0.5^{e}$ |
| | $C_{TPC}$ (mg/L) | $1309.7 \pm 7.2^{a}$ | $1262.8 \pm 6.6^{b}$ | $1174.8 \pm 2.1^{c}$ | $1083.0 \pm 1.9^{d}$ | $1007.8 \pm 1.2^{e}$ |
| | $C_{TF}$ (mg/L) | $291.7 \pm 0.6^{a}$ | $278.8 \pm 2.5^{ab}$ | $259.5 \pm 2.5^{b}$ | $238.7 \pm 3.1^{c}$ | $220.6 \pm 1.9^{d}$ |
| | TAC (mM TE) | $9.07 \pm 0.18^{a}$ | $8.84 \pm 0.15^{ab}$ | $8.39 \pm 0.13^{b}$ | $7.88 \pm 0.09^{c}$ | $7.42 \pm 0.06^{c}$ |

$C_C$: Concentration of carbohydrates. $C_{TPC}$: Concentration of total phenolic compounds. $C_{TF}$: Concentration of total flavonoids. TAC: Total antioxidant capacity. TE: Trolox equivalent.
[a–e]For each row, means followed by the same letter are not significantly different ($P > 0.05$).

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Furthermore, PEF pretreatment facilitated the rate of juice release but did not change the total amount of juice expressed. The observed effects can reflect the impact of electroporation on heat and mass transfer at low temperature processes inside the apple sample. In PEF-assisted pressing–thawing experiments, °Brix values and other nutritionally important parameters of apple juice noticeably exceeded those values obtained for untreated samples. It is remarkable that both electroporation and freezing can cause cell damage in tissues. However, electroporation can also affect the process of freezing, which was found to be important for the enhancement of the pressing–thawing. Thus, freezing-assisted pressing of PEF pretreated samples has a positive effect on all investigated nutritional parameters of the extracted apple juice. The proposed scheme of freezing-assisted pressing of PEF pretreated samples at subzero temperatures is applicable for different fruit and vegetable tissues and may be used by food producers in order to obtain a high quality ice juice rich in bioactive compounds. However, further investigations are required with regard to the adjustment and optimization of pressing protocols, further improvement of beneficial PEF effects and checking the "ice" juice storage stability.

## Acknowledgements

The authors appreciate the support from the COST Action TD1104 (EP4Bio2Med — European network for development of electroporation based technologies and treatments). Carbonell-Capella, J.M. thanks the F.P.U. grant (no. AP2010-2546) provided by the Spanish Ministry of Education. Parniakov O. thanks thesis grant provided by the French Ministry of Education.

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Please cite this article as: Carbonell-Capella, J.M., et al., "Ice" juice from apples obtained by pressing at subzero temperatures of apples pretreated by pulsed electric fields, *Innovative Food Science and Emerging Technologies* (2016), http://dx.doi.org/10.1016/j.ifset.2015.12.016

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Comprehensive REVIEWS in Food Science and Food Safety

# Analytical Methods for Determining Bioavailability and Bioaccessibility of Bioactive Compounds from Fruits and Vegetables: A Review

Juana M. Carbonell-Capella, Magdalena Buniowska, Francisco J. Barba, María J. Esteve, and Ana. Frígola

**Abstract:** Determination of bioactive compounds content directly from foodstuff is not enough for the prediction of potential *in vivo* effects, as metabolites reaching the blood system may be different from the original compounds found in food, as a result of an intensive metabolism that takes place during absorption. Nutritional efficacy of food products may be ensured by the determination of bioaccessibility, which provides valuable information in order to select the appropriate dosage and source of food matrices. However, between all the methods available, there is a need to establish the best approach for the assessment of specific compounds. Comparison between *in vivo* and *in vitro* procedures used to determine bioaccessibility and bioavailability is carried out, taking into account the strengths and limitations of each experimental technique, along with an intensive description of actual approaches applied to assess bioaccessibility of bioactive compounds. Applications of these methods for specific bioactive compound's bioaccessibility or bioavailability are also discussed, considering studies regarding the bioavailability of carotenoids, polyphenolic compounds, glucosinolates, vitamin E, and phytosterols.

**Keywords:** bioaccessibility, bioactive compounds, bioavailability, *in vitro* methods, *in vivo* methods

## Introduction

Nowadays, consumers are more and more aware of the benefits beyond basic nutrition provided by food and food compounds. Between these, plant foods including fruits and vegetables have been demonstrated to exhibit multiple health benefits, closely related to their high contents in vitamins and other bioactive compounds (vitamin C, carotenoids, phenolic compounds, vitamin E, glucosinolates) with antioxidant properties (Nehir and Simsek 2012; Barba and others 2013; Carbonell-Capella and others 2013a). However, when studying the role of bioactive compounds in human health, their bioavailability is not always well known. Before becoming bioavailable, they must be released from the food matrix and modified in the gastrointestinal (GI) tract. Therefore, it is important before concluding on any potential health effect, to analyze whether the digestion process affects bioactive compounds and their stability, as this, in turn, will affect their bioavailability and their possible beneficial effects.

Different digestion models have been developed by the scientific community that accurately mimic the complex physicochemical and physiological conditions of the human GI tract, along with *in vivo* models in living organisms (Hur and others 2011). However, comparison of results between different studies is difficult to accomplish, as there is no defined experimental model for studying bioaccessibility and bioavailability. Analysis of the procedures for measuring or predicting bioactive compounds bioavailability is therefore required, particularly as a result of continuous developments of new products by food industries considered "functional" because of their specific antioxidant or phytochemical contents.

The aim of the present article is to critically review different approaches used in the estimation of bioaccessibility and bioavailability of food compounds, focusing on bioactive compounds, as these are of major interest in current functional food development. Furthermore, results of studies in which bioaccessibility and bioavailability of bioactive compounds were investigated are also discussed.

## Bioaccessibility, Bioavailability, and Bioactivity

The concept of bioaccessibility can be defined as the quantity or fraction which is released from the food matrix in the GI tract and becomes available for absorption (Heaney 2001). This includes digestive transformations of food into material ready for assimilation, the absorption/assimilation into intestinal epithelium cells, and lastly, the presystemic metabolism (both intestinal and hepatic). For some nutrients, beneficial effects of unabsorbed nutrients (such as binding of bile salts by calcium in the tract) would be missed by absorption-based definitions. Bioaccessibility is usually evaluated by *in vitro* digestion procedures, generally simulating gastric and small intestinal digestion, sometimes followed by Caco-2 cells uptake (Courraud and others 2013).

*MS 20131126 Submitted 08/9/2013, Accepted 10/17/2013.* *Authors are with Dept. of Nutrition and Food Chemistry, Univ. de València, Avda, Vicent Andrés Estellés, s/n. 46100 Burjassot, Spain. Direct inquiries to author Frígola (E-mail: ana.frigola@uv.es).*


doi: 10.1111/1541-4337.12049

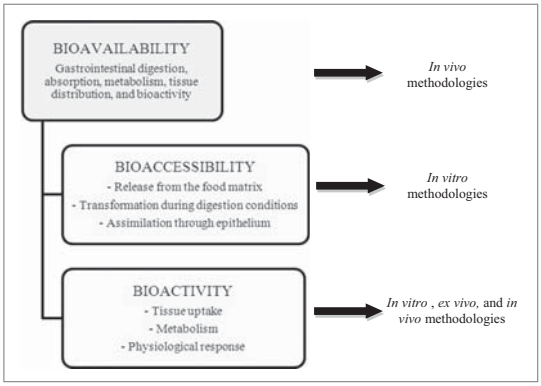


**Figure 1–Definition of bioavailability, bioaccessibility, and bioactivity and their potential assessment methodologies.**

Differently, the term bioavailability includes also in its definition the utilization of a nutrient and therefore can be defined as the fraction of ingested nutrient or compound that reaches the systemic circulation and is utilized (Wood 2005). Overall, bioavailability includes GI digestion, absorption, metabolism, tissue distribution, and bioactivity. Consequently, in terms of bioavailability, when a claim is made, it must be demonstrated that the component analyzed is efficiently digested and assimilated and then, once absorbed, exerts a positive effect in human health. However, practical and ethical difficulties are found when measuring bioactivity, so the term "bioavailability" is usually defined as the fraction of a given compound or its metabolite that reaches the systemic circulation (Holst and Williamson 2008), without considering bioactivity. According to this definition, bioavailability of a compound is determined *in vivo* in animals or humans as the area under the curve (plasma-concentration) of the compound obtained after administration of an acute or chronic dose of an isolated compound or a compound-containing food (Rein and others 2013).

Bioactivity is the specific effect upon exposure to a substance. It includes tissue uptake and the consequent physiological response (such as antioxidant, anti-inflammatory). It can be evaluated *in vivo*, *ex vivo*, and *in vitro* (Figure 1) (Fernández-García and others 2009).

Meanwhile, digestibility applies specifically to the fraction of food components that is transformed by digestion into potentially accessible matter through all physical–chemical processes that take place in the lumen. Assimilation, meanwhile, refers to the uptake of bioaccessible material through the epithelium by some mechanism of transepithelial absorption (Etcheverry and others 2012).

## Bioavailability of Bioactive Compounds

Bioactive compounds are phytochemicals that are present in foods and are capable of modulating metabolic processes, resulting in the promotion of better health. In general, these compounds are mainly found in plant foods such as fruit, vegetables, and whole grains (Carbonell-Capella and others 2013b; Gil-Chávez and others 2013) and typically occur in small amounts. These compounds exhibit beneficial effects such as antioxidant action, inhibition or induction of enzymes, inhibition of receptor activities, and induction and inhibition of gene expression (Correia and others 2012). They can be considered an extremely heterogeneous class of compounds with different chemical structures (hydrophilic/lipophilic), distribution in nature (specific to vegetable species/ubiquitous), range of concentrations both in foods and in the human body, possible site of action, effectiveness against oxidative species, and specificity and biological action (Porrini and Riso 2008). Among them, polyphenolic compounds, carotenoids, tocopherols, phytosterols, and organosulfur compounds constitute important groups in the human diet.

Indeed, bioavailability of bioactive compounds may be modified because of interactions with other macronutrients such as fiber in low-processed foods and beverages or proteins and polysaccharides in processed food products (Dupas and others 2006). Furthermore, when different foods come in contact in the mouth or digestive tract, various interactions may take place affecting phytochemical bioavailability (for example, fat enhances quercetine bioavailabilty in meals) (Lesser and others 2006). On that basis, significant research effort has recently focused on achieving optimal uptake of phytochemicals to maintain body functions and health and, consequently, carefully controlled studies are necessary in order to determine phytochemical bioavailability.

As shown in Figure 2, different approaches to study bioaccessibility and bioavailability of bioactive compounds include *in vitro* methods, *ex vivo* techniques, *in situ* assays, and *in vivo* models. Advantages of each procedure are summarized in Table 1. However, comparisons between different approaches are difficult as conditions differ between them and only *in vivo* studies provide accurate values (Oomen and others 2002).

## Approaches in the Assessment of Bioaccessibility and Bioavailability of Bioactive Compounds

### Carotenoids

Carotenoids are found in fruits and vegetables as carotenes (unsatured hydrocarbons) and xanthophylls (oxygenated derivatives). Generally, the main carotenoids in vegetables are lutein, $\beta$-carotene, violaxanthin, and neoxanthin, whereas in fruits xanthophylls are usually found in a greater proportion. They are prone to isomerization and/or oxidation due to their unsaturation (Hill and Johnson 2012).

Only a very low proportion of carotenoids has been reported to become bioaccesible (Courraud and others 2013). In some fruits (such as mango, papaya) carotenoids are found in oil droplets in chromoplast and hydroxycarotenoids are mostly esterified with fatty acids, being more easily extracted during digestion. Carotenoids bioavailability from foods varies greatly depending on endogenous (product-related) and exogenous (process-related) factors. Amount and type of fat present in the vicinity is a key factor that affects bioaccessibility. A minimum amount of fat is necessary for absorption (Fernández-García and others 2012), so formulation of carotenoids in an oily matrix may enhance higher bioaccessibility. Important steps in carotenoid absorption are release from the food matrix, micelle formation, uptake into mucosal cells, packing into chylomicrons, and transport within the lymphatic system. Moreover, carotenoids content might be affected by oxidative reactions during analytical procedures, so incubation time should be kept to a minimum without affecting sensitivity. Garret and others (2000) added $\alpha$-tocopherol in order to ensure protection against oxidation and thus improve carotenoids stability.

Different *in vitro* methods used in the assessment of carotenoid bioaccessibility comprise simulated GI digestion, intestinal segments, brush-border and basolateral membrane vesicles, enterocytes, and transformed intestinal cell lines, mainly Caco-2 human cells (Table 2). Garret and others (1999) may be considered the pioneers in the development of the Caco-2 procedure for the assessment of carotenoid bioaccessibility. The method consists of

 

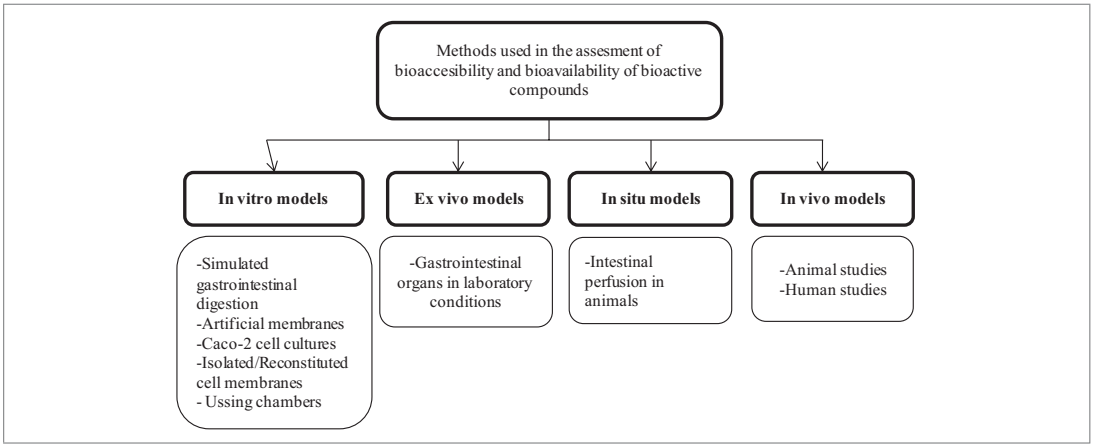


**Figure 2–Methodologies used in the assessment of bioavailability and bioaccessibility of bioactive compounds.**

**Table 1–Strengths and drawbacks of *in vivo* and *in vitro* procedures used to assess bioaccessibility and bioavailability of bioactive compounds.**

| | Advantages | Disadvantages |
|---|---|---|
| ***In vitro* digestion** | | |
| *Simulated gastrointestina digestion* | Relatively inexpensive and technically simple | Extrapolation to *in vivo* |
| | Screening of numerous samples is possible | Homeostatic mechanisms are not present |
| | Focus on small number of components | Dynamic conditions of gastrointestinal tract are not fully reproduced with biochemical and cell culture models |
| | Specific mechanisms of action can be tested | Intestinal bacteria and hepatic metabolism is not always considered |
| | Validation with reference material | Oral and large intestinal phases are often not included although can readily be added |
| | Efficiency of each digestion, absorption or transport mechanism can be studied | Closed system not responsive to composition and quantity of foods |
| | | Exocrine pancreas secretions not only contains pancreatin |
| *Caco-2 cells* | Phenotype is similar to normal absorptive epithelial cells | Original from human colonic adencarcinoma |
| | Grow on dish surface and on membrane inserts | Mucin, biofilms, and other epithelial cell types are not present |
| | Secretion of chylomicrons is possible | |
| ***In vivo* digestion** | *In vivo* conditions | Lower throughput |
| | Selection of specific subjects | Extremely complex functional systems |
| | Pharmacokinetic studies can be performed | Influence of different factors |
| | | Extrapolation from animal studies to human |
| | | Certified reference standards lack |
| | | High cost of equipment and labor |
| | | Ethical constraints |

an *in vitro* digestion including a gastric and small intestinal step based on that described by Miller and others (1981) to estimate iron availability from foods. Subsequently, the digestate is filtered (which would be representative of micellarized carotenoids) and added to Caco-2 cells. To ensure that carotenoids were found in micelles, these authors filtered the aqueus fraction. They ascertained that lycopene was poorly micellarized and thus its quantity decreased after filtration, but lutein, $\alpha$-carotene, and $\beta$-carotene did not change in their quantities. They also observed that hydrophobic species were efficiently micellarized when bile salts and pancreatic enzymes were combined. Furthermore, these authors found out that differentiated Caco-2 cells were able to accumulate carotenoids from mixed micelles. Further modifications were made to this method by Thakkar and others (2007) who included an oral digestion phase because of high starch content, and by Chitchumroonchokchai and Failla (2006) who added lipase and carboxyl ester lipase. These latter authors observed that xanthophyll esters were hydrolyzed by carboxyl ester lipase before xanthophylls are transported into enterocytes, resulting in an enhanced cellular accumulation of zeaxanthin.

Hedrén and others (2002) also developed an *in vitro* digestion method for the estimation of carotenoid bioaccessibility (called *in vitro* accessibility) in raw and cooked carrots, which was further used in several different studies assessing carotenoid bioaccessibility (Lemmens and others 2009; Colle and others 2013). The groups carefully examined critical steps in the digestion procedure, such as the impact of added pancreatic enzymes and different bile salts amounts, along with shaking conditions used in the micellarization step, so as to validate the method. When bile salts were not added, $\beta$-carotene bioaccessibility decreased by about 80%, but duplicating the amount of bile salts (from 25 to 50 g/L) resulted in no additional increment of carotenoid bioaccessibility. Moreover, orbital shaking gave more reproducible results in comparison with reciprocal shaking. In contrast to data by Garret and others (1999), they estimated not only the micellarized fraction, but total carotenoids released, as the intestinal phase was not achieved by centrifugation and filtration. Courraud and others (2013) introduced an oral phase to Hedrén's method, without $\alpha$-amylase as most of the matrices were nonstarchy. They obtained a significant loss of $\beta$-carotene only during the gastric phase and of retinyl

Table 2–Comparison of *in vitro* methods for carotenoid bioaccessibility determination.

| | Method | | | | | |
|---|---|---|---|---|---|---|
| Step | Failla and others (2008) (adapted from Garret and others 1999) | Hedrén and others (2002) | Reboul and others (2006) | Granado-Lorencio and others (2007) | Colle and others (2010) | Cilla and others (2012) |
| Food sample preparation | Homogenization | Finely ground or cut into small pieces, with nitrogen blown | Homogenization in saline + pyrogallol | Homogenization with kitchen blender, 15 s, to simulate mastication | Homogenization in saline | Homogenization |
| Oral phase | α-Amylase, pH 6.8, 10 min, 37 °C | | | α-Amylase, pH 6.5, 5 min, 37 °C | | |
| Gastric phase | Porcine pepsin, pH 2.5, 1 h, 37 °C | Porcine pepsin, pH 2, 1 h, 37 °C | Porcine pepsin, pH 4, 30 min, 37 °C | Mucin, bovine serum albumin, porcine pepsin, pH 1.1, 1 h, 37 °C | Porcine pepsin, pH 4, 30 min, 37 °C + pH 2, 30 min, 37 °C | Porcine pepsin, pH 2, 2h, 37 °C |
| Small intestine phase | Porcine bile extract, carboxyl ester lipase, porcine pancreatin, lipase, pH 6.5, 2 h, 37 °C | Porcine pancreatin, porcine bile salt, pH 7.5, 30 min, 37 °C. | Porcine bile extract, porcine pancreatin, pH 6, 30 min, 37 °C | Bovine bile, porcine pancreatin, human pancreatic lipase, colipase, cholesterol esterase, phospolipase $A_2$, taurocholate salts, pH 7.8, 2 h, 37 °C | Porcine pancreatin, porcine bile salt, pH 6.9, 2 h, 37 °C. | Porcine pancreatin, porcine bile, pH 6.5, 2 h, 37 °C. |
| Isolation of micellar fraction | Centrifugation 5000 × *g*, 45 min, 4 °C, filtration | Centrifugation 5000 × *g*, 20 min | Centrifugation 20000 rpm, 18 h, 10 °C, filtration | Overnight sedimentation / Centrifugation 5000 rpm, 20 min | Centrifugation 16500 × *g*, 65 min, 4 °C, filtration | Centrifugation 3300 × *g*, 1 h, 4 °C |
| Cell uptake | Caco-2 cells, 4h, 37 °C | | | | | |

palmitate in the oral and in the gastric phases, confirming that sensitivity of carotenoids to acidic conditions is higher than to alkaline conditions.

Reboul and others (2006) made some modifications to the method established by Garret and others (1999). BHT used as antioxidant was replaced by pyrogallol, more water soluble. Gastric pH was set at 4 instead of 2, simulating the pH in the human stomach after vegetable-rich meals ingestion. Moreover, duodenal pH was adjusted to 6 instead of 7.5 as this is the pH measured in human duodenum during digestion. Instead of 2 h of incubation time, duodenal conditions were adjusted to 30 min to approach the digestive transit time, and amount of bile salts were increased. They observed that carotenoid bioaccessibility was dependent of the different food matrix, being more bioaccessible in carrot juice and processed tomato in comparison with crude tomato and watermelon sources, which had very low accessibility. Werner and Böhm (2011) employed this procedure in the assessment of carotenoid bioaccessibility in durum wheat and egg pasta. Durum wheat pasta exhibited higher carotenoid bioaccessibility. The authors also observed that results were highly dependent on bile extract concentration and to a lesser extent on gastric pH and incubation time with digestive enzymes.

As previous methods were found unsuitable for xanthophyll ester hydrolysis, Granado-Lorencio and others (2007) adapted a method originally applied to the evaluation of soil contaminants. Compared to previous *in vitro* models, these authors included the use of human pancreatic lipase, phospholipase $A_2$, cholesterol esterase, and taurocholate salts. They obtained a remainder of over 70% of carotenoids in the final digesta and observed that cholesterol esterase hydrolyzed xanthophyll esters, and human pancreatic lipase did not.

The *in vitro* digestion procedure for carotenoids followed by Wright and others (2008), as adapted from Garret and others (1999) and Hedrén and others (2002) consisted of dissolving the carotenoids in the oily phase, considering thus exclusively the intestinal digestion phase, as previous research had not shown significant changes when eliminating the gastric step (Garret and others 1999). They observed that β-carotene transfer increased as did bile (from 0 up to 20 mg/mL) and pancreatin concentration (from 0 up to 4.8 mg/mL) and with pH from 3.5 to 9.

Colle and others (2010) also introduced several modifications to the method established by Hedrén and others (2002). Both the pH and transit times were adapted to closely simulate human conditions. A certain amount of lipid (0% to 10%) was added to tomatoes prior to the *in vitro* digestion. A significant increase of lycopene bioaccessibility was observed when 5% of lipid was additioned.

Cilla and others (2012) adapted a method used for iron bioaccessibility to determine carotenoid bioaccessibility of fruit juice-milk beverages, along with other bioactive compounds. Bioaccessibility of carotenoids was dependent of the type of milk used. Whole milk-fruit beverage led to a higher carotenoid extraction (11%) in comparison to the skimmed milk-fruit beverage.

As reported in the review by Rodriguez-Amaya (2010), although these models simulate human digestion closely, a better description of the food sample preparation should be carried out. Furthermore, carotenoid extraction efficiency from food and micelles should be similar, so no overestimation or underestimation of micellarization is done. Results obtained in the different studies are shown in Table 3.

The dynamic digestion TIM® system that more closely mimics *in vivo* conditions was also employed in the assessment of

Table 3–Carotenoid bioaccessibility and bioavailability (%) of plant-derived products.

| Sample | Carotenoids bioavailability (%) | Method employed in the determination | Reference |
|---|---|---|---|
| Baby food meal | Lutein (24); β-carotene (13.8); α-carotene (10.4) | Simulated gastric and small intestinal digestion coupled with Caco-2 cells | Garret and others (1999) |
| Spinach purée | β-Carotene (29); lutein (27) | Simulated gastric and small intestinal digestion (Garret and others 1999) | Ferruzzi and others (2001) |
| Raw pulped carrot | β-Carotene (21) | Simulated gastric and small intestinal digestion | Hedrén and others (2002) |
| Carrot puree | β-Carotene (8.9); α-carotene (4.4) | Simulated gastric and small intestinal digestion | Reboul and others (2006) |
| Spinach | Lutein (37.6); α-carotene (2.4) | Simulated gastric and small intestinal digestion | Reboul and others (2006) |
| Orange | β-Cryptoxanthin (45); zeaxanthin (43); lutein (26) | Simulated oral, gastric, and small intestinal digestion | Granado-Lorencio and others (2007) |
| Salad (tomato, spinach, carrot, romaine lettuce, and orange pepper) | Lutein (+zeaxanthin) (45.6); β-carotene (2.8); α-carotene (2.0); lycopene (1.1) | Simulated gastric and small intestinal digestion | Huo and others (2007) |
| Boiled cassava | β-Carotene (30) | Simulated oral, gastric, and small intestinal digestion coupled with Caco-2 (Garret and others 1999). | Failla and others (2008) |
| Lycopene from tomato extract | Lycopene | *In vivo* single dose design | Riso and others (2010) |
| Orange fleshed melons | β-Carotene (3.2) | Simulated gastric and small intestinal digestion | Fleshman and others (2011) |
| Tomato pulp | Lycopene (2) | Simulated gastric and small intestinal digestion (Colle and others 2012) | Colle and others (2012) |
| Butternut squash | α-Carotene (17.9); β-carotene (16.5); lutein (15.9); violaxanthin (4.3) | Simulated gastric and small intestinal digestion (Garret and others 1999) | Jeffery and others (2012a) |
| Carrot | Lycopene (38.9); α-carotene (20.2); β-carotene (21.6); lutein (40.5); phytoene (64.2) | Simulated gastric and small intestinal digestion (Garret and others 1999) | Jeffery and others (2012a) |
| Grapefruit | Lycopene (4.5); β-carotene (7.9); lutein (8.7); violaxanthin (8.4); phytoene (47.1) | Simulated gastric and small intestinal digestion (Garret and others 1999) | Jeffery and others (2012a) |
| Mango | β-Carotene (31.8); lutein (13.5); violaxanthin (19.4) | Simulated gastric and small intestinal digestion (Garret and others 1999) | Jeffery and others (2012a) |
| Papaya | β-Carotene (48.5); lutein (37.3); violaxanthin (21.6); phytoene (67.8) | Simulated gastric and small intestinal digestion (Garret and others 1999) | Jeffery and others (2012a) |
| Tomato | Lycopene (1.4); β-carotene (15.5); lutein (58.6); phytoene (96.2) | Simulated gastric and small intestinal digestion (Garret and others 1999) | Jeffery and others (2012a) |
| Whole milk-fruit beverage | Neoxanthin + 9-cis-violaxanthin (47.3); zeaxanthin (14.7); lutein (13.9) | Simulated gastric and small intestinal digestion | Cilla and others (2012) |
| Soy milk-fruit beverage | Neoxanthin + 9-cis-violaxanthin (18.5); zeaxanthin (4.4); lutein (3.7) | Simulated gastric and small intestinal digestion | Cilla and others (2012) |
| Carrot juice | Lutein (22); α-carotene (1.5), β-carotene (1.5) | Simulated gastric and small intestinal digestion (Hedrén and others 2002) | Courraud and others (2013) |

lycopene bioaccessibility by Déat and others (2009), followed with Caco-2 cells. At the end of the dynamic experiment (300 min), lycopene decreased by 25%, in accordance with static *in vitro* models.

Animal studies have also been designed in the assessment of carotenoid bioavailability (Zuniga and Erdman 2011; Sy and others 2012). They obtained more accurate results than with *in vitro* methods. Despite this, human carotenoid absorption and metabolism is not accurately mimicked by any animal model (Lee and others 1999). For instance, in enterocytes, β-carotene is converted to vitamin A in rodents much more efficiently than in humans. Likewise, Failla and others (2008) observed that gerbils and preruminant calves, but not ferrets, hydrolyzed the ingested β-carotene to vitamin A with an efficiency similar to humans.

Many studies have examined carotenoid bioavailability in humans (Micozzi and others 1992; Castenmiller and others 1999; Tyssandier and others 2003). The most frequently used *in vivo* approach to study bioavailability of carotenoids involves the single-dose design. An increase in β-cryptoxanthin, β-carotene, and zeaxanthin plasma concentrations was observed after supplementation of blood orange juice in a long-term human study (Riso and others 2005), although this did not exert significant effects on several markers of oxidative stress. Meanwhile, Riso and others (2010) found a low increase of lycopene, along with interindividual variability. Interestingly, Ross and others (2011) demonstrated the fate of oral lycopene in humans in plasma, with the detection in skin for up to 42 d of lycopene and its metabolites. Goltz and others (2013) observed that carotenoid absorption increased when vegetables were consumed in a single meal rather than over multiple meals.

Borel and others (1998) demonstrated a high correlation between *in vitro* carotenoid bioaccessibility, *in vivo* observations

and with results from bioavailability trials with human subjects. Therefore, *in vitro* models may constitute a less tedious and less costly alternative to *in vivo* studies in the assessment of carotenoid bioaccessibility.

Studies show that percentages of bioaccessibility and bioavailability of the different carotenoids vary widely. Lutein was more readily solubilized than $\alpha$-carotene, $\beta$-carotene, and lycopene (Garret and others 1999), probably because oxycarotenoids are more hydrophilic than hydrocarbon carotenoids and to different subcellular location and molecular interactions in plant foods. Sy and others (2012) also obtained a high recovery of lutein and astaxanthin, whereas lycopene was the least abundantly recovered. However, Jeffery and others (2012a) reported for the 1st time a high phytoene bioaccessibility, several times that of other carotenoids, followed by lutein in carrot and tomato and $\beta$-carotene in papaya and mango. In human studies, Tyssiander and others (2003) reported greater bioavailability of lutein and $\beta$-carotene compared to lycopene.

Furthermore, dietary fat appears to be necessary for the efficient solubilization of lipophilic compounds. In this line, Failla and others (2008) demonstrated an increase in carotenes when triglycerides were added to a carotenoid-rich salad, in accordance with Hedrén and others (2002), who observed a significant increase in $\beta$-carotene bioaccessibility after oil addition. Qian and others (2012) found the lowest bioaccessibility of $\beta$-carotene (0%) when orange oil was used as the carrier lipid, probably because flavored oils do not contain triacylglycerol components and thus cannot be digested into free fatty acids. Moreover, Borel and others (1998) demonstrated that $\beta$-carotene incorporated into chylomicrons higher in meals with long-chain rather than medium-chain triglycerides. For this reason, Jeffery and others (2012b) used yogurt as a lipid source with long-chain triglycerides. Human studies have also proved the importance of lipid in the absorption of dietary lutein. On this subject, Mamatha and Baskaran (2011) obtained a higher plasma lutein level in rats when lutein was solubilized in mixed micelles with fat. Brown and others (2004) also observed that consumption in humans of full-fat salad dressing enhanced a higher carotenoid bioavailability than reduced-fat salad dressing.

Interestingly, not only lipid amount, but also qualitative lipid profile has its influence in carotenoid bioavailability (Goltz and Ferruzi 2013). Monounsatured fatty acids promote a higher carotenoid bioavailability than polyunsatured fatty acids, as demonstrated by Clark and others (2000) in mesenteric lymph duct cannulated rats. This was further observed by Gleize and others (2013), who found that bioaccessibility of the xanthophylls lutein and zeaxanthin was higher with saturated fatty acids than with monounsaturated and polyunsatured fatty acids both in an *in vitro* digestion model followed by Caco-2 cell study and *in vivo* in orally administered rats. Furthermore, long-chain triglyceride increased the $\beta$-carotene bioaccessibility in comparison with medium-chain triglyceride in a simulated intestinal digestion (Salvia-Trujillo and others 2013).

Effect of pH on the transfer efficiency of carotenoids is also of importance, as suggested by *in vitro* results. Wright and others (2008) demonstrated an increase in the $\beta$-carotene transfer to the aqueous phase under higher pH conditions, while Jeffery and others (2012b) obtained a positive correlation of $\beta$-carotene and phytoene with food pH. However, this hypothesis can be rejected in *in vivo* methods, because there is no significant meal effect on stomach pH (Tyssandier and others 2003).

Contrary to expectation, several authors (Parada and Aguilera 2007; Courraud and others 2013) have demonstrated that technological processes such as cooking of vegetables increase carotenoid bioavailability by disruption of the natural food matrix during food processing. However, severe thermal treatment or inadequate storage may cause isomerization during the formation of by-products that can, in turn, reduce the absorption of desirable bioactive compounds.

## Polyphenolic compounds

Phenolic compounds or polyphenols form a large group of chemical substances considered as secondary metabolites of plants. They have an aromatic ring and a benzene ring with one or more hydroxide groups, including phenolic acids (hydroxy-benzoic acids and hydroxy-trans-cinnamic acids), coumarins, flavonoids (flavones, flavonols, flavanones, flavanolols, flavanols, and anthocyanidins), isoflavonoids, lignans, stilbenes, and phenolic polymers (proanthocyanidins and hydrolyzable tanins) (Craft and others 2012). Among the various phenolic compounds, bioavailability appears to differ greatly and the most abundant ones in our diet do not necessarily correspond to those with best bioavailability profile. Absorption and metabolism of polyphenolic compounds are determined primarily by their physicochemical characteristics. For example, molecular size, their basic structure, degree of polymerization or glycosylation, solubility, and conjugation with other phenolics can be considered critical factors. Phenolic acids with small-molecular weight such as gallic acid and isoflavones are easily absorbed through the tract, as well as flavones, catechins, and quercetin glucosides (Martin and Apple 2010). On the contrary, large polyphenols such as proanthocyanidins are poorly absorbed. Most proanthocyanidins are degraded into monomer or dimer units before being absorbed (Hackman and others 2008).

In plant products, most of the phenolic compounds are found as glycosylated forms or as esters or polymers that must be hydrolyzed by intestinal enzymes or microflora before the released aglycones can be absorbed. However, anthocyanins can be absorbed as glycosides and appear as such in blood (D'archivio and others 2007). Metabolism is another factor, strongly affecting their bioavailability. Generally, after absorption, polyphenols undergo biotransformations of phase I and II into 3 main O-sulfated, O-glucuronidated, and O-methylated forms. Despite this, anthocyanins do not appear to undergo extensive metabolism. Neither do galloylated monomeric flavonols such as epigallocatechin and epicatechin gallate, which may appear unconjugated, at least to a large extent, in the systemic circulation (Cermak and others 2009). Thus, the structure of the resulting metabolites could be totally different from the parent compounds, and they may or not exert their biological action (Denev and others 2012). Results published by Vitaglione and others (2007) suggested that protocatechuic acid, which can be absorbed both from the small and large intestine, may be the metabolite involved in the activity observed after the intake of cyanidin-3-glucoside, whose absorption and excretion are reported to be below 1% of intake. Therefore, evaluation of polyphenol bioavailability should include the analysis not only of native compounds, but also their metabolic products. Technological processes may also affect bioavailability of phenolic compounds, showing a significant increase of chlorogenic acid and naringenin in plasma levels when consuming cooked tomato in comparison with the fresh product (Bugianesi and others 2004).

Despite the great variability of this group of substances, along with their occurrence in plant materials as a complex mixture, experiments reported in the literature have analyzed bioavailability

 

of polyphenolic compounds with different chemical structures and solubility through *in vitro* and *in vivo* assays, as shown in Table 4. The most widely used procedure for screening polyphenolic compound bioaccessibility is the *in vitro* static GI method. Gil-Izquierdo and others (2001) may be considered the pioneers in adapting the method established by Miller and others (1981) to simulate human digestion and absorption of dietary iron in the study of phenolic compound release. During the intestinal phase, a cellulose dialysis tubing is used to simulate intestinal absorption. The main modification introduced by Gil-Izquierdo and others (2002) was the placement of the food and cellulose dialysis tubing in a polyethylene tube to assure close contact between food and membrane, reaching faster equilibration of pH values and thus a faster liquid exchange. They observed that phenolic composition was not affected by pepsin digestion in any of the assayed food products. This method has been employed in the screening of multiple foods, including orange juice (Gil-Izquierdo and others 2002), pomegranate juice (Pérez-Vicente and others 2002), broccoli (Vallejo and others 2004), soymilk (Rodriguez-Roque and others 2013) and gooseberry (Chiang and others 2013) among other foods. These authors found that gastric digestion increased polyphenolic concentration, whereas the duodenal fraction significantly diminished polyphenolic content and even more so in the dialyzed fraction. Results are shown in Table 5.

Further modifications (Villanueva-Carvajal and others 2013) included the use of crushed ice after each digestion phase to ensure the end of enzymatic activity. These authors also studied particle size and concluded that this was inversely proportional to phenolic release, so the enlargement of the contact area could improve digestion efficiency with an absorption increase of polyphenols.

Bermúdez-Soto and others (2007) removed the employment of the dialysis membrane during intestinal digestion as substantial losses were observed of some of the phenolic compounds. They simply determined polyphenolic compounds after separation by filtration. During gastric digestion no significant changes were observed in the stability of polyphenols in chokeberry, but anthocyanins were increased, due to the low pH after the gastric step. During intestinal digestion a significant decrease in anthocyanins (43%) and flavonols (26%) was observed, whereas chlorogenic acid increased (24%). This method was further employed by Tagliazucchi and others (2010) in the assessment of grape polyphenols, with the addition of an oral phase. Furthermore, once the pancreatic digestion was finalized, samples were taken to pH 2 to ensure the stability of phenolic compounds. Differently, these authors observed an increase in the bioaccessibility of total polyphenols, flavonoids, and anthocyanins during the gastric digestion in grape, while intestinal digestion caused a decrease in all classes of polyphenols.

Bouayed and others (2011) also developed a method to assess free soluble polyphenols potentially available for further uptake than from Miller's method. They found out that after simulated GI digestion of apples, polyphenols release was mainly achieved during the gastric phase. Subsequently, a further increase (<10%) in total phenolics and flavonoids was obtained after the intestinal phase. This increase may be due to the additional time of extraction along with the effect of intestinal enzyme on the complex food matrix, which facilitates the release of phenolics bound to the matrix. Results showed a dialyzability of 40% for flavonoids and 55% for free soluble phenolics, respectively, in comparison with their undigested counterparts in apples. Regarding anthocyanins, these authors could not measure them after gastric and intestinal digestion, probably because they degradate in alkaline intestinal

**Table 4–Comparison of *in vitro* methods for polyphenolic bioaccessibility determination.**

| | Method | | | | | | |
|---|---|---|---|---|---|---|---|
| **Step** | **Gil-Izquierdo and others (2001)** | **Bouayed and others (2011)** | **Dupas and others (2006)** | **Bermúdez-Soto and others (2007)** | **Gawlik-Dziki (2012)** | **Shim and others (2012)** | **Chen and others (2013)** |
| Food sample preparation | Homogenization | Homogenization | | | Homogenization and centrifugation | Homogenization in saline | Homogenization |
| Oral phase | | | | | Mucin, $\alpha$-amylase, 10 min, 37 °C | Amylase, pH 6.9, 5 min, 37 °C | |
| Gastric phase | Porcine pepsin, pH 2, 2 h, 37 °C | Porcine pepsin, pH 2 to 2.5, 1 h, 37 °C | Porcine pepsin, pH 2, 1 h, 37 °C | Porcine pepsin, pH 2, 2 h, 37 °C | Porcine pepsin, pH 1.2, 1 h, 37 °C | Porcine pepsin, pH 2, 1 h, 37 °C | Porcine pepsin, pH 2, 1 h, 37 °C |
| Transition step | | pH 6.5, 45 min, 37 °C. | Cooling in ice for 10 min | | pH 6 | pH 5.3 | pH 5.3 |
| Small intestine phase | Porcine bile extract, porcine pancreatin, lipase, pH 7, 2 h, 37 °C | Porcine bile extract, porcine pancreatin, pH 7 to 7.5, 2 h, 37 °C | Porcine bile extract, porcine pancreatin, pH 6, 2 h, 37 °C | Porcine bile extract, porcine pancreatin, pH 7, 2 h, 37 °C | Porcine bile extract, porcine pancreatin, pH 7, 2 h, 37 °C | Porcine bile extract, porcine pancreatin, lipase, pH 7, 2 h, 37 °C | Porcine bile extract, porcine pancreatin, taurodeoxycholate, taurocholate, pH 7.4, 2.5 h, 37 °C |
| Separation | Dialysis in a semipermeable cellulose membrane simultaneously with intestinal phase | Dialysis in a semipermeable cellulose membrane simultaneously with intestinal phase | | Filtration | Dialysis in a semipermeable cellulose membrane 2 × 2h, 37 °C | Centrifugation at 3000 rpm, 30 min, 4 °C. | |
| Cell uptake | | | Caco-2 cell | | | | |

**Table 5–Polyphenolic bioaccessibility and bioavailability (%) of plant-derived products.**

| Sample | Polyphenolic bioavailability (%) | Method employed in the determination | Reference |
|---|---|---|---|
| Orange juice (soluble fraction) | Narirutin (10.5); hesperidin (16.2); total flavanones (12.0), vicenin-2 (18.6) | Simulated gastric and small intestinal digestion with cellulose dialysis tubing | Gil-Izquierdo and others (2001) |
| Orange juice (soluble fraction) | Narirutin (23.4); hesperidin (24.0); hesperetin (21.1); total flavanones (23.5), vicenin-2 (24.5) | Simulated gastric and small intestinal digestion with cellulose dialysis tubing | Gil-Izquierdo and others (2002) |
| Strawberry | Cyanidin-3-glucoside (6.6), pelargonidin-3-glucoside (12.6); pelargonidin-rutinoside (11.7); ellagic acid-arabinoside (20.6); ellagic acid (172.8); quercetin-3-glucoside (28.3); kaempferol-3-glucoside (27.4) | Simulated gastric and small intestinal digestion with cellulose dialysis tubing | Gil-Izquierdo and others (2002) |
| Strawberry jam | Cyanidin-3-glucoside (2.3), pelargonidin-3-glucoside (3.7); pelargonidin-rutinoside (3.8); ellagic acid-arabinoside (6.1); ellagic acid (9.7); quercetin-3-glucoside (6.1); kaempferol-3-glucoside (12.0) | Simulated gastric and small intestinal digestion with cellulose dialysis tubing | Gil-Izquierdo and others (2002) |
| Coffee | Chlorogenic acid (traces); benzoic acid (traces) | *In vivo* digestion in rats | Dupas and others (2006) |
| Chokeberry | Cyanidin-3-glucoside (56.7), cyanidin (0), quercetin 3-glucoside (81.2), quercetin (0), neochlorogenic acid (71.6), chlorogenic acid (123.6). | Simulated gastric and small intestinal digestion followed by filtration | Bermúdez-Soto and others (2007) |
| Tixia gooseberry | Caffeic acid (44.1), epigallocatechin gallate (28.1), kaempferol (100.2), *ρ*-coumaric (61.0), pelargonidin chloride (96.6), quercetin hydrate (516.3), resveratrol (58.1), rutin (95.3). | Simulated gastric and small intestinal digestion according to Gil-Izquierdo and others (2001) | Chiang and others (2013) |
| Invicta gooseberry | Caffeic acid (59.5), kaempferol (82.8), *ρ*-coumaric (73.4), quercetin hydrate (154.7), resveratrol (94.6), rutin (101.0). | Simulated gastric and small intestinal digestion according to Gil-Izquierdo and others (2001) | Chiang and others (2013) |

environment. Further studies carried out by Bouayed and others (2012) found that phenolic compounds in the gastric or intestinal medium were approximately similar (chlorogenic acid), higher (phloridzin and quercetin 3-*O*-glucoside) or lower (*ρ*-coumaric acid) compared to those found in fresh apples, in accord with results obtained by Chiang and others (2013). Polyphenol concentration decreased during dialysis through the semipermeable cellulose membrane, although all polyphenols in the intestinal medium were dialyzable, which could be indicative of passive diffusion, an important mechanisms for cellular polyphenol uptake, at least for several aglycones.

Gawlik-Dziki (2012) carried out an *in vitro* digestion including an oral phase. Dialysis sacks were added after 2 h of intestinal digestion, for a total time of 4 h. They also obtained a decrease of phenolic compounds in the dialysate. Shim (2012) also included an oral phase. However, they did not use dialysis sacks but centrifugation. They used the in vitro method to compare phenolic bioaccessibility in different parts of *Smilax china* and obtained 36.4%, 17.8%, and 9.9% of the remaining total polyphenols after digestion of leaf, root, and stem, respectively.

Chen and others (2013) carried out an *in vitro* digestion model according to the method established for carotenoid bioavailability in the assessment of 9 commercially available tea juices. After the gastric phase there was a significant decrease in total polyphenol content of 5 of the juices. After the duodenal phase, a further increase in the total polyphenol content was obtained in 4 of the juices, possibly due to structural transformation of polyphenols.

However, these methods did not include a colonic phase and polyphenols may be metabolized by the colonic microflora. For this reason, Saura-Calixto and others (2007) estimated the bioaccessibility of dietary polyphenols with the isolated indigestible fraction (small intestine bioaccessibility) and a colonic fermentation of this fraction (large intestine bioaccessibility). Bioaccessibility of polyphenols in the large intestine was calculated by the difference of polyphenol contents between the total indigestile fraction and the residue after the fermentation; and 48% of dietary polyphenols were estimated bioaccessible in the small intestine, while 42% became bioaccesible in the large intestine. Only 10% was not accessible and remained in the food matrix after the entire digestion process. Furthermore, Nordlund and others (2012) used an *in vitro* colon model to study the formation of phenolic microbial metabolites from rye, wheat, and oat bran. The major metabolites found were hydroxylated phenylpropionic acid metabolites, closely related to the ferulic acid content in the cereal samples.

The dynamic GI model (TIM®) has also been extensively used to measure phenolic bioaccessibility. Colonic fermentation experiments may be incorporated in this model, so the assessment of polyphenol bioaccessibility may be more reliable. This model, which mimics the biological environment through the duodenum, jejunum, and ileum, was employed in the monitorization of anthocyanins stability and bioaccessibility in maqui berry and wild blueberry (Lila and others 2012). These authors observed that after intake, most anthocyanins were bioaccessible between the 2nd and 3rd hours. López de Lacey and others (2012) also used a dynamic GI model to study the bioaccessibility of green tea polyphenols incorporated into agar. Their results revealed that the polyphenols incorporated in the agar were bioaccessible, and consequently available for absorption. Furthermore, the gelatin used to simulate the presence of protein during the digestion partly reduced green tea flavonols bioaccessibility.

 

However, certain transport mechanisms such as unidentified stomach active transport or the transport in the small intestine of flavonoids through interaction with the sodium-dependent glucose transporter are not considered with the *in vitro* digestion method (Bermúdez-Soto and others 2007). For this reason, other methods have been developed.

Bioaccessibility studies using Caco-2 cells have been conducted (Dupas and others 2006; Fernandes and others 2012). Glucuronidation, sulfation, and methylation processes carried out by polyphenols can be studied using these cells. Yi and others (2006) found that by growing on Transwell membranes Caco-2 cells, anthocyanins could be degraded and demethylated during absorption and transport. Epigallocatechin was minimally uptaken in the human intestinal Caco-2 cell model (Vaidyanathan and Walle 2003), in accordance with Hong and others (2002), who observed a poor uptake of epigallocatechin gallate by HT-29 human colon adenocarcinoma cells. Neilson and others (2010) used this method to compare the efficiency of dimer absorption compared to monomers of catechin. In addition, an assessment of proanthocyanidin transport showed that oligomers of 6 units were transported approximately 10-fold less across a layer of Caco-2 cells than radiolabeled monomers, dimers, and trimers (Déprez and others 2000). A deeper study was carried out by Wang and others (2013) in which grape seed phenolic extract was subjected to *in vitro* GI digestion and ileal fermentation, followed by Caco-2 cells assay. Only microbial metabolites, but not original phenolic compounds passed through the Caco-2 cell layer.

Some polyphenols may be metabolized by Caco-2 cells, which must be taken into account. Ferulic acid-sulfate, synaptic acid-sulfate, $\rho$-coumaric acid-sulfate, and methyl ferulate-sulfate were generated after 24-h exposure of hydroxycinnamates to differentiated Caco-2 cells according to Kern and others (2003). Meanwhile, Yi and others (2006) suggested a degradation and demethylation of anthocyanins from blueberries during absorption and transport by Caco-2 cells.

Further assays include the use of the Ussing chamber, where a small section of intestinal mucosa is situated between 2 chambers with buffer solution, preserving the epithelial polarity (Clarke 2009). Not only passive diffusion but transporter-mediated, transcellular, paracellular, and endocytosis transport can be measured. Bergmann and others (2009) employed the Ussing chamber in order to study the intestinal transport of polyphenols in apples. They used monolayers of the T84 colon carcinoma cell line and found that the transport of various hydroxycinnamic acids and flavonoids depended on the polarity. Cardinali and others (2013) also used colonic cells in a Ussing chamber and obtained a bioaccessibility of 0.1% of the polyphenol verbascoside. Moreover, Erk and others (2013) observed that the absorption of coffee polyphenols in the jejunum is governed by their physicochemical properties when they used pig jejunal mucosa in the Ussing chamber.

*In situ* studies have also been carried out in the assessment of polyphenolic bioavailability. Wang and others (2011) followed this procedure in the study of total flavonoid extracts, with the inclusion of liver perfusion, in order to determine flavonoid metabolism. This method was also used by Fong and others (2012) to study the metabolism and absorption of flavones from herbs using rat intestines. This way they found out that acetaminophen, (-)-epicatechin, piperine, and mainly curcumin could significantly inhibit the intestinal metabolism of the flavone baicalein and subsequently increase its absorption.

Meanwhile, *in vivo* studies were carried out to test the bioaccessibility of polyphenols in rats (Dupas and others 2006; Mateos-Martín and others 2012), pigs (Lesser and others 2006; Walton and others 2006), and dogs (Reinboth and others 2010). This way, Gonthier and others (2003) did not detect parent compounds or catechin derivatives in the plasma of rats given purified procyanidins. Crespy and others (2002) also used Wister rats to determine that quercetin, but not its glycosides, was absorbed from the rat stomach. Disparity in the results between *in vitro* data and epidemiological studies are likely attributed to the physicochemical characteristics of polyphenols. Bioavailability in rodent studies has been estimated to be over 10% of ingested dose, ranging from 2% to 20%. Interestingly, quantification of the flavonol quercetin and its main methylated metabolites (isorhamnetin and tamarixetin) by Surco-Laos and others (2011) in a *Caenorhabditis elegans* model revealed that higher levels of quercetin plus metabolites were present in the worm's organism than those of isorhamnetin or tamarixetin plus their respective metabolites. This observation suggests that greater capacity of quercetin uptake than of methylated derivatives by the nematode exists, although quercetin is further transformed by *C. elegans* to a greater extent than isorhamnetin or tamarixetin.

With reference to human studies, these are limited as large population sizes are necessary. Nevertheless, Manach and others (2005) reported plasma concentrations of phenolic metabolites of 0 to 4 $\mu$mol/L after 97 human volunteers ingested 50 mg aglycone equivalents. Russell and others (2009) recovered in the urine 26% to 27% of the major free benzoic acids (gentisic, protocatechuic, and $\rho$-hydroxybenzoic) and the major conjugated acid (syringic acid), detected in plasma within 5 h after consumption of a single dose of a portion of strawberries. Research carried out by Hackman and others (2008) showed a rapid transport into blood of metabolites, in a dose-dependent manner, with peak plasma concentrations at 1 to 2.5 h after ingesting a flavanol-rich food, reaching baseline levels within 8 h. Colonic microflora metabolized most of the flavanols not absorbed in the small intestine to a variety of derivatives of phenolic acid and valerolactone, able to be absorbed. After 48 h of incubation with human colonic microflora, procyanidins of 6 units were degraded into low-molecular-weight aromatic acids (Deprez and others 2000). The wide variability of results obtained by Suárez and others (2011) indicated a high dependence on the individual in the absorption and metabolism of olive oil phenols.

Moreover, although some *in vitro* studies suggest the degradation of anthocyanins in the intestinal phase, under *in vivo* conditions direct absorption of anthocyanins may take place in the stomach (Manach and others 2004). Bioavailability of anthocyanins has been demonstrated to be lower than that of other flavonoids, and according to Yang and others (2011), generally less than 1% of the consumed amounts (180 to 215 mg/day) is absorbed. They are absorbed by different mechanisms in the stomach and small intestine involving specific enzymes, such as bilitranslocase (Passamontia and others 2002). They subsequently enter the circulatory system within 15 to 60 min, after passing through the liver, and are distributed to different tissues, with a maximum concentration of nanomolar levels. Mostly, anthocyanins reach the colon and are extensively metabolized there by bacteria, contributing therefore to their bioavailability (Hidalgo and others 2012).

Among the isoflavones, genistein, daidzein, and glycitein are the most active compounds found in soybeans. Equol is a highly bioavailable metabolite that comes from diadzein and exhibits higher activity than the original isoflavone (Kanazawa 2011). Using a Caco-2 cell model, Simmons and others (2012) found that the lipid source and amount did not affect bioaccessibility of isoflavones. However, transport across the monolayer was greater

with shorter molecules. The *in vivo* human study carried out by Shinkaruk and others (2012) revealed that the bioavailability of glycitein from soy-based food was similar to that of daidzein and its urinary excretion was significantly higher than that of genistein.

## Glucosinolates

Glucosinolates have gained much attention as food compounds of high dietary value due to its alleged beneficial effect in cancer prevention (Fimognari and others 2002). Nearly all of the biological activities of these compounds may be attributed to their hydrolytic products, of which the isothiocyanates are prominent examples. Glucosinolates are hydrolyzed into isothiocyanates mediated by myrosinase, which is still active in fresh vegetable products, and by the bacterial microflora of the GI tract. Antibiotic treatment along with inactivation of the plant myrosinase (after cooking, for example) causes a decrease in bioavailability, as indicated by the fact that bioavailability is greater following ingestion of myrosinase-containing compared with myrosinase-lacking preparations (Dinkova-Kostova and Kostov 2012). One of the most extensively studied isothiocyanates is sulforaphane whose glucosinolate precursor is glucoraphanin, abundant in broccoli. In humans, metabolization of isothiocyanates occurs via the mercapturic acid pathway.

Simulated static GI digestion, dynamic GI digestion, Caco-2 uptake, transport assays, and/or *in vivo* studies with animals and with humans have all been used as glucosinolate bioaccessibility and bioavailability screening methods. However, colonic fermentation is essential for the absorption of isothiocyanates, which must be taken into account. Recent evidence (Peñas and others 2012) suggests that certain strains of *Lactobacillus spp., L. mesenteroides* and *L. plantarum,* were capable of digesting *in vitro* glucosinolates. As a result, in human studies, degradation of glucosinolates to isothiocyanates exhibited high interindividual variation because of colonic microflora differences (Rungapamestryi and others 2007). It is important to note that urinary isothiocyanate metabolite (dithiocarbamate) excretion decreases from 47% to a negligible amount when bowel microflora is reduced by mechanical and antibiotics.

As a result of the importance of colonic fermentation, few *in vitro* studies have been carried out toward the assessment of glucosinolate bioaccessibility. Despite the fact that mastication of cooked vegetables liberates glucosinolates, and mastication of fresh plants additionally causes enzymatic hydrolysis of glucosinolates, no *in vitro* research has yet been conducted on the impact of these process. Vallejo and others (2004) carried out a simulated *in vitro* digestion which consisted in a gastric phase followed by an intestinal phase that included a cellulose dialysis tubing, as described previously by Gil-Izquierdo and others (2001) for determining phenolic bioaccessibility. These authors reported a high loss of glucosinolates (69%) under gastric conditions of homogenized fresh broccoli inflorescence. However, Iori and others (2004) suggested that the previous article had underestimated the degradative activity of myrosinase, still active in the uncooked broccoli. Consequently, stability of glucosinolates under pepsin digestion is considered quite high, as reported by Maskell and Smithardt (1994) who obtained after simulated gastric digestion an overall drop of total glucosinolates of only 14%. Progoitrin and gluconapoleiferin showed greater susceptibility to peptic digestion than gluconapin or glucobrassicin, and 4-hydroxyglucobrassicin became undetectable. Differences between the results obtained with the different inocula employed (Table 6) were minor. After 4 h of small intestine simulated digestion, the loss of the total glucosinolates was 32%. Lai and others (2010) effectuated an *in vitro* simulated digestion of glucoraphanin in the upper GI tract, along with an *ex vivo* study using rat cecal microbiota and an *in situ* rat cecum assay. The *in vitro* study confirmed that glucoraphanin was not degraded by upper GI digestive enzymes, consequently reaching the rat cecum intact. Meanwhile, in both *in situ* and *ex vivo* procedures, glucoraphanin was hydrolyzed to sulforaphane by F344 rat cecal microbiota and able to cross the cecal enterocyte for systemic absorption.

A dynamic computercontrolled *in vitro* large-intestinal model was designed by Krul and others (2002), with the inoculation of complex microflora of human origin, a semipermeable membrane, and pH continuously adjusted to 5.8. They observed peak levels of allyl isothiocyanate 9 to 12 h after the addition of sinigrin, which accounts for 1% of the degraded sinigrin. Slightly higher values were obtained by Getahun and Chung (1999) who incubated human feces with cooked watercress juice for 2 h. They found that 18% of total glucosinolates were hydrolyzed into isothiocyanates.

Intact glucosinolate and its metabolites in feces were lowly recovered in animal studies, using different species, suggesting substantial absorption and metabolism of these compounds (Slominski and others 1988; Conaway and others 1999). In an *in vivo* animal study published by Hanlon and others (2008), rats were administered sulforaphane in either a single intravenous dose (2.8 mmol/kg) or single oral doses of 2.8, 5.6, and 28 mmol/kg. This compound was well and rapidly absorbed, with an absolute bioavailability of 82%, which decreased at higher doses, indicating a dose-dependent pharmacokinetic behavior.

In an *in vivo* human study, Rouzaud and others (2004) observed that isothiocyanates release was delayed when ingesting cooked cabbage and, therefore, suggested that glucosinolates passed through the upper digestive tract without modification. Furthermore, Riso and others (2009) carried out an *in vivo* human crossover intervention study (broccoli diet compared with cruciferous-free diet). They observed an increase of isothiocyanate plasma concentrations, while the intervention did not affect plasma glucosinolate activity. Other *in vivo* studies discussed in Table 7 firmly established that, compared to isothiocyanates, intake of glucosinolates is associated with lower bioavailability, slower elimination, and greater interindividual variation in excretion. Overall, the large interindividual variability of conversion of glucosinolates to urinary dithiocarbamates is evident following administration of either single or multiple doses of glucosinolates, and ranges between 1% and more than 40% of the dose. Interestingly, there are also diurnal variations: conversion of glucosinolates to dithiocarbamates is greater during the day, whereas conversion of isothiocyanates to dithiocarbamates is more efficient during the night (Fajey and others 2012).

## Vitamin E

Vitamin E is actually a family of molecules, which include the tocopherols and the tocotrienols, all of them with important antioxidant properties and health benefits.

Alpha-tocopherol exhibits the highest biological activity and molar concentration of lipid-soluble antioxidants in the human.

A handful of *in vitro* and *in vivo* assays have been conducted on the determination of vitamin E bioaccessibility and bioavailability, as shown in Table 8. It is important to note that during digestion, vitamin E must be packaged into micelles to facilitate absorption, the same as carotenoids. Therefore, Reboul's simulated GI digestion procedure (Reboul and others 2006) employed

 

Table 6–Comparison of *in vitro* methods for glucosinolates bioaccessibility determination.

| Step | Method | | |
|---|---|---|---|
| | Maskell and Smithardt (1994) | Vallejo and others (2004) | Lai and others (2010) |
| Food sample preparation | | Homogenization | |
| Oral phase | – | – | Amylase, 3 min, 37 °C |
| Gastric phase | Porcine pepsin, pH 2, 4 h, 37 °C. | Porcine pepsin, pH 2, 2 h, 37 °C | Porcine pepsin, pH 2, 2 h, 37 °C |
| Transition step | Centrifugation, 1000 rpm, 20 min | | |
| Small intestine phase | Innocula of small intestine of pig fed with rapeseed meal/soyabean meal or commercial diet/Porcine pancreatin, pH 6, 1 to 4 h, 37 °C | Porcine bile extract, porcine pancreatin, lipase, pH 7, 2 h, 37 °C | Porcine bile extract, porcine pancreatin, pH 7.5, 2 h, 37 °C |
| Separation | Centrifugation, 1000 rpm, 20 min | Dialysis in a semipermeable cellulose membrane simultaneously with intestinal phase | Hydrolyzation with myrosinase |

in the assessment of carotenoids is also used to study vitamin E bioaccessibility, with subsequent centrifugation and filtration steps.

Desmarchelier and others (2013) followed Reboul's *in vitro* digestion, with palm oil as added fat. These authors showed that α-tocopheryl acetate was distributed between mixed micelles (36%), liposomes (9%), and nonsolubilized food debris (52%). Furthermore, they followed the *in vitro* digestion by uptake studies using Caco-2 cells. These cells were able to hydrolyze α-tocopheryl acetate and to uptake α-tocopherol when α-tocopheryl acetate was incorporated into mixed micelles but not into emulsions. Werner and Böhm (2011) extended Reboul's method by an oral phase. Overall, results obtained by these authors were highly dependent on the amount of bile extract present in the digestive medium and to a lesser extent on the simulated gastric pH and the incubation time with digestive enzymes. Bioaccessibility of β-tocotrienol was found to be higher than that of α-tocotrienol.

Depending on the dietary source, the bioaccessibility of vitamin E has been shown to vary widely. O'Callaghan and O'Brien (2010), who used *in vitro* simulated GI digestion coupled with Caco-2 cells, obtained bioaccessibility values of α-tocopherol ranging from 11% in apple sauce to 86% in beef. Likewise, Reboul and others (2006) reported a 100% bioaccessibility of α-tocopherol in bananas and bread, 29% and 22% in cheese and milk, respectively, and as low as 0.5% in apples. These differences between different food sources may be due to different sites and physicochemical states of α-tocopherol, along with the presence of fiber, fat, and phytosterols in the food source.

A dynamic gastric digestion model with nonhomogeneous gastric mixing, shearing, and rate of delivery to the duodenum was employed by Mandalari and others (2013) in the assessment of tocopherols bioaccessibility of pistachios. They obtained a bioaccessibility of almost 100% of tocopherols after duodenal digestion. Déat and others (2009) employed the TIM® procedure coupled to Caco-2 cells. These authors showed that the absorption of α-tocopherol from a vitamin E-containing meal was significantly lower when compared to the pure compound. This finding reveals that other components present in a meal may change the uptake behavior of vitamin E or compete in the absorption through the SR-BI transporter.

*In vivo* studies have also been used in the assessment of vitamin E bioavailability. Nagy and others (2013) carried out a human study with healthy volunteers under maldigestion conditions. They found out that the acetylated form of α-tocopherol exhibited the same bioavailability as free α-tocopherol. A long-term human study was also carried out by Novotny and others (2012). They observed that ingesting diary 9.2 mmol (4 mg) of α-tocopherol maintained plasma concentrations of α-tocopherol at 23 mmol/L, suggesting that the dietary requirement for vitamin E may be less than that currently recommended. Johnson and others (2012), employing mouse and human *in vivo* assays, discovered novel urinary metabolites: α-carboxyethylhydroxychroman (α-CEHC) glycine, α-CEHC glycine glucuronide, and α-CEHC taurine.

Correlation between *in vitro* bioaccessibility data with bioavailability determined by *in vivo* human assays was studied by Granado and others (2006). They observed no measurable difference in the case of broccoli in the plasma levels of α-tocopherol after a 7-d feeding intervention.

Therefore, the great variety of methods employed in the assessment of tocopherol bioavailability provides different findings that will be important for future updates of intake recommendations and will aid in understanding the disposition and roles of vitamin E *in vivo*.

## Phytosterols

Phytosterols have attracted much attention in recent years due to their health benefits, such as cholesterol lowering, antiinflammatory, antiatherogenicity, and anticancer potential. β-Sitosterol is the most common phytosterol found in leaf vegetable natural products followed by campesterol, stigmasterol, and sitostanol.

Granado-Lorencio and others (2011) applied the same *in vitro* method as the one used to study polyphenols bioaccesibility (Granado-Lorencio and others 2007) in the assessment of phytosterol bioaccesibility. Mandak and Nyström (2012) also used an *in vitro* digestion. These authors observed that bioaccessibility of steryl ferulates (various plant sterols esterified to ferulic acid) was found to be almost negligible. These findings suggest that intestinal enzymes immediately hydrolyze steryl ferulates and thus they are practically unavailable for absorption in the small intestine, possibly being bioactive in the gut. This was also shown in a further study (Mandak and Nyström 2013), where the low bioaccessibility of steryl ferulates (0.01% to 0.25%) was independent of the cereal matrix. A similar analytical method was applied by Alemany and others (2013). These authors obtained a sterol bioaccessibility of 2% to 6% in fruit-based milk beverages. However, a higher bioaccessibility was observed for oxides of β-sitosterol, suggesting differences in the solubilization and absorption mechanism between plant sterols and their oxides.

Yi and others (2012) carried out an *in vivo* rat study where oral bioavailability of sterols enhanced by *Flammulina velutipes* was

Table 7–*In vivo* studies regarding glucosinolate bioavailability (%) of plant-derived products.

| | Study desing | Participants | Sample preparation | Measurements | Major findings |
|---|---|---|---|---|---|
| Shapiro and others (2001) | Single dose study | Inpatient and outpatient volunteers of a hospital | Uncooked fresh sprouts (with active myrosinase) and homogenates of boiled sprouts devoid of myrosinase activity with either glucosinolates only or isothiocyanates only. | Urine was collected throughout the entire study in 8-h collection intervals. | -No isothiocyanates were found in urine.<br>-Dithiocarbamates were the major metabolites in urine.<br>-Myrosinase activity in intact sprouts contributed significantly to bioavailability by boosting the glucosinolate-to-isothiocyanate conversion. |
| Gasper and others (2005) | Single oral doses of 16 or 52 mmol of isothiocyanates (standard broccoli and high-glucosinolate broccoli). | 16 Healthy subjects | Individual soup portions of the 2 cultivars were prepared by cooking 100 g florets with 150 mL water for 90 s on high power in a 700-W microwave oven followed by homogenization. | Liquid chromatography linked to tandem mass spectrometry was used to quantify sulforaphane and its thiol conjugates in plasma and urine. | -GSTM1 (glutathione S-transferase M1 allele) genotypes had a significant effect on the metabolism of sulforaphane. |
| Clarke and others (2011) | Single oral doses of 161 to 221 mmol of glucosinolates, with or without active myrosinase. | 12 Healthy subjects | Samples studied were fresh broccoli sprouts with active myosine and commercially available broccoli supplement and were designed to be indistinguishable from each other. | Blood and urine samples were collected for 48 h during each phase and analyzed for sulforaphane and erucin metabolites using LC–MS/MS. | -Bioavailability of sulforaphane and erucin is dramatically lower and delayed in time when subjects consume broccoli supplements compared to fresh broccoli sprouts.<br>-Broccoli supplements devoid of myrosinase activity did not produce equivalent plasma concentrations of the bioactive isothiocyanate metabolites compared to broccoli sprouts. |
| Egner and others (2011) | Multiple dose study of 800 mmol of glucoraphanin or 150 mmol of sulforaphane for 7 d. | 50 Healthy subjects | Rehydrated, previously lyophilized broccoli sprout powders rich in either glucoraphanin or sulforaphane | Sulforaphane and sulforaphane metabolites in overnight (roughly 12 h) urine samples | -70% of the administered sulforaphane was eliminated in 24 h.<br>-Only 5% of the administered glucoraphanin was recovered as sulforaphane metabolites.<br>-Bioavailability of sulforaphane was far superior to glucoraphanin extracts. |

**Table 8–Comparison of *in vitro* methods for vitamin E bioaccessibility determination.**

| Step | Method | | | |
|---|---|---|---|---|
| | Reboul and others (2006) | O'Callaghan and others (2010) | Werner and Böhm (2011) | Mandalari and others (2013) |
| Food sample preparation | Homogenization in saline + pyrogallol | Homogenization | Homogenization in saline + pyrogallol | |
| Oral phase | | | Amylase, pH 6.5, 5 min, 37 °C | Amylase, pH 6.9 |
| Gastric phase | Porcine pepsin, pH 4, 30 min, 37 °C | Porcine pepsin, lipase, pH 4, 1 h, 37 °C | Porcine pepsin, pH 3.5 to 4.5, 0.5 h, 37 °C | Porcine pepsin, 37 °C |
| Transition step | | pH 5.4 | | |
| Small intestine phase | Porcine bile extract, porcine pancreatin, pH 6, 30 min, 37 °C | Porcine pancreatin, pH 7.8, 2 h, 37 °C | Porcine bile extract, porcine pancreatin, pH 6.5 to 7, 0.5 h, 37 °C | Porcine bile extract, porcine pancreatin, lecithin, cholesterol, sodium taurocholate pH 7, 2 h, 37 °C |
| Separation | Centrifugation 20000 rpm, 18 h, 10 °C + Filtration | Ultracentrifugation 200000 *g*, 95 min + Filtration | Centrifugation 4000 rpm, 20 min, 10 °C + Centrifugation 14000 rpm, 5 min, 22 °C + Filtration | Centrifugation, 3700 rpm, 15 min, 7 °C. |
| Cell uptake | | Caco-2 cell | | |

demonstrated. Although *in vitro* and *in vivo* methods have been used to measure sterols bioaccesibility and bioavailabilty respectively, *in vitro* procedures have yet to be validated against human absorption data.

## Conclusions

The wide range of options available to evaluate digestion and uptake in *in vitro* and model organisms has guaranteed a role for them in bioaccessibility and bioavailability studies for years to come. Both *in vitro* and *in vivo* approaches are increasing our understanding of uptake of bioactive compounds from food products. Nevertheless, more validation studies are needed which compare *in vivo* with *in vitro* results. It is noteworthy that none of the methods presented in this report will absolutely predict how much of a specific bioactive compound a human will absorb and utilize. In addition, the low bioavailability of the bioactive compounds (in particular polyphenols), could imply the activation of some alternative mechanisms that can justify their possible beneficial effect. Nonetheless, results obtained with *in vivo* assays enable the prediction of the situation in humans quite accurately and may help accelerate the study of phytochemical absorption for better comprehension of their possible beneficial effects.

## Acknowledgments

This research project was supported by the Spanish Ministry of Science and Technology and European Regional Development Funds (AGL2010-22206-C02-01). J.M. Carbonell-Capella holds an award from the Spanish Ministry of Education (AP2010-2546). M. Buniowska thanks the Podkarpacie Marshal's Office for Ph.D. scholarship nr 8.2.2/IV.26/217/11/U/155/12 of RSI Project for Podkarpacie Region, Poland. F.J. Barba thanks the Valencian Autonomous Government (Conselleria d´Educació, Cultura i Esport. Generalitat Valenciana) for the postdoctoral fellowship of the VALi+d program "Programa VALi+d per a investigadors en fase postdoctoral 2013" (APOSTD/2013/092).

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Food Chemistry 184 (2015) 122–130

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

# Effect of *Stevia rebaudiana* addition on bioaccessibility of bioactive compounds and antioxidant activity of beverages based on exotic fruits mixed with oat following simulated human digestion

Juana M. Carbonell-Capella, Magdalena Buniowska, María J. Esteve, Ana Frígola *

*Department of Nutrition and Food Chemistry, Universitat de València, Avda. Vicent Andrés Estellés, s/n, 46100 Burjassot, Spain*

ARTICLE INFO

*Article history:*
Received 3 October 2014
Received in revised form 17 March 2015
Accepted 19 March 2015
Available online 28 March 2015

*Keywords:*
*Stevia rebaudiana*
Exotic fruits
Bioaccessibility
Bioactive compounds
Antioxidant capacity

ABSTRACT

In order to determine the impact of *Stevia rebaudiana* (*SR*) addition on bioactive compounds bioaccessibility of a new developed functional beverage based on exotic fruits (mango juice, papaya juice and açaí) mixed with orange juice and oat, an *in vitro* gastrointestinal digestion was performed. Ascorbic acid, total carotenoids, total phenolics, total anthocyanins, total antioxidant capacity and steviol glycosides were evaluated before and after a simulated gastrointestinal digestion. Salivary and gastric digestion had no substantial effect on any of the major phenolic compounds, ascorbic acid, total antioxidant capacity and steviol glycosides, whereas carotenoids and anthocyanins diminished significantly during the gastric step. All analysed compounds were significantly altered during the pancreatic-bile digestion and this effect was more marked for carotenoids and total anthocyanins. However, phenolic compounds, anthocyanins, total antioxidant capacity and steviol glycosides bioaccessibility increased as did *SR* concentration. Ascorbic acid bioaccessibility was negatively affected by the *SR* addition.



## 1. Introduction

Current trends and worldwide developments on new food products with functionality aim to demonstrate a significant bioactivity of exotic fruits with positive impact in several chronic disorders (Costa, García-Díaz, Jimenez, & Silva, 2013). In this sense, research has focused on combinations of exotic fruits with other ingredients in beverages (Carbonell-Capella, Barba, Esteve, & Frígola, 2013). Fruit juice blends with other ingredients are gaining importance in the market probably due to public perception of juices as a healthy natural source of nutrients and increased public interest in health issues.

Additionally, the use of *Stevia rebaudiana* (*SR*) leaves is increasing as a natural sweetener 300 times sweeter than sucrose without caloric value, allowing consumers to enjoy sweet taste without concerns about weight gain. They do not replace the sugar naturally present in foods, but they can be an excellent substitute for added sugars and thus an effective aid in weight management. The European Commission granted final regulatory approval for the use of stevia extracts in foods and beverages on 11 November 2011. Stevia leaves contain a mixture of diterpene glycosides (steviosides) and is considered a good source dietary fibre, minerals and essential amino acids (Kim, Yang, Lee, & Kang, 2011). Stevia leaf extract shows a high level of antioxidant activity, as well as a variety of phytochemicals such as phenolic compounds, directly associated with the removal of free electrons and superoxide radicals (Geuns, Hajihashemi, & Claes, 2012). Due to its chemical structure and health-promoting phytochemical components, stevia is suitable as a replacement for sucrose in beverages and for the production of functional food ingredients (Šic Žlabur et al., 2013). The sweetening power of steviol glycosides differ between them, with rebaudioside A being 400 times sweeter than sugar and stevioside about 300 times sweeter (Ceunen & Geuns, 2013). As a result, determination of the steviol glycoside profile is of great interest to industry.

Despite the enormous research on antioxidant properties of fruit beverages, studies investigating the effect of gastrointestinal digestion on dietary antioxidants are scarce. Only phytochemicals released from matrices become bioaccessible and are potentially available for absorption by the gastro-intestinal tract, and, therefore, able to exert their beneficial effects in the human body. Under gastrointestinal conditions, transformations (degradation, epimerisation, hydrolysis and oxidation) and interactions between phytochemicals and food components may also occur, modifying therefore the biological activity of the bioactive compounds (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014). Therefore, it is important, before concluding on any potential health effect, to assess how the digestion process affects bioactive compounds and their stability, as this, in turn, will affect their

* Corresponding author. Tel.: +34 963544955; fax: +34 963544954.
*E-mail address:* ana.frigola@uv.es (A. Frígola).

http://dx.doi.org/10.1016/j.foodchem.2015.03.095
0308-8146/

bioavailability for uptake, as well as their possible beneficial effects.

Previous studies have confirmed that an *in vitro* digestion model system simulating human digestion could support reliable prediction of bioaccessibility of bioactive compounds and total antioxidant capacity in plant products (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2013). However, the effect of *SR* extracts on the stability and bioaccessibility of phytochemicals in beverages typically consumed with adjuncts or as formulated products has not yet been reported in the literature data so far. The extent to which formulation may modify the bioactive compound profile of exotic fruit-oat beverages or influence their bioavailability is critical to understanding ultimate physiological effects elicited by these beverages. Furthermore, available knowledge on the digestibility of steviol glycosides is limited. Therefore, at this stage of development, it is necessary to study the impact of digestive conditions when a new specific formulation of commercial ready-to-drink matrix is designed in order to better design future studies focused on assessment of specific biological outcomes.

The objective of the current study was to investigate the bioaccessibility of phenolic compounds, anthocyanins, carotenoids, ascorbic acid, steviol glycosides and antioxidative effect in exotic fruit-oat beverages with (1.25% and 2.5%) and without *SR*. The effect of *SR* extract addition on the bioaccessibility of bioactive compounds and total antioxidant capacity was evaluated with an *in vitro* physiological approach simulating human digestion in the upper gastrointestinal tract, with the inclusion of a salivary, gastric and duodenal step with a dialysis membrane. The release of bioactive compounds as well as the total antioxidant capacity of the beverages were determined in aliquots collected at the end of each digestion step.

## 2. Materials and methods

### 2.1. Samples

Cultivars of papaya (*Carica papaya*), mango (*Mangifera indica*), oranges (*Citrus sinensis*, cultivar Navel) and oat beverage (Santiveri, Lérida, Spain) were purchased from a local supermarket. Papaya, mango and orange juices were extracted after appropriate washing of the fruits and the pulp was removed. Açaí provided by Nature's Way Products Inc. (Utah, USA) (containing 450 mg of açaí berries extract, with 10% of polyphenols) was added to the beverage.

*S. rebaudiana* leaves were supplied by company Anagalide, S.A. (Barbastro, Huesca, Spain) and stored at room temperature. A stock solution (8.33%, w/v) of *S. rebaudiana* was prepared in order to formulate the beverage (Carbonell-Capella et al., 2013). For this purpose, 100 mL of bottled water at 100 °C were added on the dried leaves (8.33 g) and were kept for 30 min. The infusion was vacuum filtered using filter paper (Whatman No. 1) and the filtrate obtained was stored for the duration of the experiment at −40 °C.

The fruit juice mixture was prepared by mixing 32.5% (v/v) of papaya juice, 10% (v/v) of mango juice, 7.5% (v/v) of orange juice, 20% of oat beverage, 1% of açaí powder (w/v) and water to 100%. To obtain final stevia concentrations of 1.25% and 2.5% (w/v), different volumes of stevia stock solution (30 and 60 mL) were added to prepare 200 mL of beverage instead of water. The higher stevia concentration (2.5%, w/v) was selected, taking into account the sucrose concentration of commercial fruit-based beverages and the sweetness equivalence of stevia and sucrose.

### 2.2. Chemicals and reagents

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), as a standard substance (2 mM) to measure TEAC, 2,2′-azobis (2-methylpropionamidina)dihydrochloride (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein sodium salt, 2,2′-azobis (2-amidinopropane)dihydrochloride (AAPH), disodium metabisulfite, Folin–Ciocalteu (ammonium molibdotugstat) reagent, rebaudioside A, stevioside hydrate, steviol hydrate, α-amylase from *Bacillus*, mucin from porcine stomach, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, bile extract porcine and EDTA $Na_2$ were purchased from Sigma (Steinheim, Germany). Gallic acid 1-hydrate in distilled water, as a standard (10 mg/mL) for phenolic compounds, was purchased from UCB (Brussels, Germany). Oxalic acid, acetic acid, chlorhidric acid, acetone, sodium acetate, potassium persulphate ($K_2S_2O_8$), sodium di-hydrogen phosphate (anhydrous) ($NaH_2PO_4$) and di-potassium hydrogen phosphate ($K_2HPO_4$) were purchased from Panreac (Barcelona, Spain), while di-sodium hydrogen phosphate anhydrous ($Na_2HPO_4$) and potassium dihydrogen phosphate ($KH_2PO_4$) from Scharlau (Barcelona, Spain). Ethanol, methanol, acetonitrile, hexane, sodium chlorure, sodium carbonate anhydrous ($Na_2CO_3$), trichloroacetic acid and sodium sulphate proceeded from Baker (Deventer, The Netherlands). Ascorbic acid and sodium dodecyl sulphate were obtained from Merck (Darmstadt, Germany) and rebaudioside C and rebaudioside F from Wako (Osaka, Japan).

### 2.3. Simulated digestion

A three-stage *in vitro* digestion model was performed based on the previously described procedure by Rodríguez-Roque et al. (2013), with the addition of a salivary step. Briefly, 50 mL of each beverage (in triplicate) was transferred to an Erlenmeyer flask, and a saliva solution (5 mL, pH 6.75 ± 0.2) containing 2.38 g $Na_2HPO_4$, 0.19 g $KH_2PO_4$, 8 g NaCl, 100 mg of mucin and α-amylase (200 U/L of enzyme activity) in 1 L of distilled water was added. This mixture was kept in a shaking water bath (37 °C, 90 rpm) for 10 min. Salivary digested aliquots were taken for analysis. Afterwards, 13.08 mg of pepsin from porcine stomach was added and pH was adjusted to 2 by addition of HCl (12 M). This mixture was incubated in darkness in a water bath at 37 °C with continuous stirring (90 rpm) for 2 h. At the end of the gastric digestion, aliquots were taken for analysis and 20 mL were used for titration with NaOH (0.5 M) to pH 7.5 after adding 5 mL of pancreatin (4 g/L) – bile (25 g/L) mixture.

Dialysis membrane was prepared by soaking it with 0.01 M EDTA $Na_2$, 2% $NaHCO_3$ and 0.1% sodium dodecyl sulphate at boiling point, rinsing it with distilled water and cutting it into segments of 30 cm. Dialysis membrane segments were filled with 25 mL of water–$NaHCO_3$ mixture, with the amount of $NaHCO_3$ (0.5 N) used in the previous titration. 20 mL of the gastric digest were placed into a beaker and the dialysis membrane was immersed in that digest until reaching pH 5.0. This process allows gradual pH adjustment, mimicking intestinal conditions. After 30 min, 5 mL of pancreatin (4 g/L) – bile (25 g/L) mixture was added and the incubation continued for further 2 h (37 °C, 90 rpm). The dialysate (fraction inside the dialysis sac), consisting of soluble compounds of low molecular weight, and the retentate (fraction outside the dialysis sac), consisting of soluble and insoluble compounds of low and high molecular weight, were collected and placed in a cold water bath for 10 min.

### 2.4. Bioactive compounds analysis

#### 2.4.1. Polarographic determination of ascorbic acid

The method used was in accordance to Barba, Cortés, Esteve, and Frígola (2012). Beverage (5 mL) was diluted to 25 mL with the extraction solution (1% w/v oxalic acid, 2% w/v trichloroacetic acid and 1% w/v sodium sulphate). After vigorous shaking, the solution was filtered through a folded filter (Whatman No. 1). 1%

(w/v) oxalic acid (9.5 mL) and 2 mL of 2 M acetic acid/sodium acetate buffer (pH = 4.8) were added to an aliquot of 0.5 mL of filtrate and the solution was transferred to the polarographic cell. A Metrohm 746 VA Trace Analyzer (Herisau, Switzerland) equipped with a Metrohm 747 VA stand was used for the polarographic determination. The working electrode was a Metrohm multi-mode electrode operated in the dropping mercury mode. A platinum wire counter electrode and a saturated calomel reference electrode were used. The following instrumental conditions were applied: $DP_{50}$, mode DME, drop size 2, drop time 1 s, scan rate 10 mV/s, initial potential −0.10 V. Determinations were carried out by using the peak heights and standard additions method.

*2.4.2. Total carotenoids*

Extraction of total carotenoid was carried out in accordance with Barba et al. (2012). An aliquot of sample (2 mL) was homogenised with 5 mL of extracting solvent (hexane/acetone/ethanol, 50:25:25, v/v) and centrifuged for 5 min at 4000 rpm at 5 °C. The top layer of hexane containing the colour was recovered and transferred to a 25-mL volumetric flask. The volume of recovered hexane was then adjusted to 25 mL with hexane. Total carotenoid determination was carried out on an aliquot of the hexane extract by measuring the absorbance at 450 nm. Total carotenoids were calculated using an extinction coefficient of $\beta$-carotene, $E^{1\%} = 2505$.

*2.4.3. Total phenolic compounds*

Total phenols were determined according to the method reported by Georgé, Brat, Alter, and Amiot (2005), with some modifications. Briefly, 10 mL of sample were homogenised with 50 mL of a mixture of acetone/water (7/3, v/v) for 30 min. Mixture supernatants were then recovered by filtration (Whatman No. 2, England) and constituted the raw extracts (REs). REs (2 mL) were settled on an Oasis cartridge (Waters). Interfering water-soluble components (reducing sugars, ascorbic acid) were recovered with 2 × 2 mL of distilled water. The recovered volume of the washing extract (WE) was carefully measured. In order to eliminate vitamin C, heating was carried out on the washing extract (3 mL) for 2 h at 85 °C and led to the heated washing extract (HWE). All extracts (RE, WE, and HWE) were submitted to the Folin–Ciocalteu method, adapted, and optimised (Barba et al., 2012): 2% (w/v) sodium carbonate solution (3 mL) and 100 μL of Folin–Ciocalteu reagent were added to an aliquot of 100 μL of sample. The mixture was incubated for 1 h at room temperature. Absorbance was measured at 765 nm.

*2.4.4. Total anthocyanins*

Total anthocyanins were determined using a modified method of Mazza, Fukumoto, Delaquis, Girard, and Ewert (1999). A 10-fold diluted sample of 100 μL was mixed with 1700 μL of distilled water and 200 μL of 5% (v/v) HCl. The sample was hold at room temperature for 20 min before measuring the absorbance at 520 nm in a 10 mm cuvette. Calculations of total anthocyanins were based on cyanidin-3-glucoside (molar absorptivity 25,740). All spectrophotometric analyses were performed using a UV–visible spectrophotometer Lambda 20 (Perkin–Elmer, Überlingen, Germany).

*2.4.5. Total antioxidant capacity*

*2.4.5.1. Trolox equivalent antioxidant capacity (TEAC) assay.* The trolox equivalent antioxidant capacity (TEAC) test was determined according to the method reported by Barba et al. (2012), based on the capacity of antioxidants to inhibit the radical cation 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS), which has a characteristic long-wavelength absorption spectrum, showing a maximal peak at 734 nm. The ABTS radical cation is formed by the interaction of ABTS (7 mM) with $K_2S_2O_8$ (2.45 mM).

*2.4.5.2. Oxygen radical absorbance capacity (ORAC) assay.* The oxygen radical absorbance capacity (ORAC) assay used, with fluorescein as the "fluorescent probe", was that described by Barba et al. (2012). The automated ORAC assay was carried out on a Wallac 1420 VICTOR$^2$ multilabel counter (Perkin–Elmer, USA) with fluorescence filters, for an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The measurements were made in plates with 96 white flat bottom wells (Sero-Wel, BibbySterilin Ltd., Stone, UK). The reaction was performed at 37 °C, as the reaction was started by thermal decomposition of AAPH in 75 mM phosphate buffer (pH 7.0).

*2.4.5.3. DPPH assay.* The value of DPPH (millimolar Trolox equivalents, mMTE) measures the antioxidant capacity of a given substance, as compared to the standard (Trolox). The method used was as described by Brand-Williams, Cuvelier, and Berset (1995). The reaction was begun by adding 50 μL of a suitable dilution of sample to 1.45 mL of DPPH coloured radical. The sample was incubated for 30 min at room temperature (20 °C). Absorbance, A, was measured at the wavelength of 515 nm.

*2.5. Liquid chromatographic analysis of steviol glycosides*

The method of Joint FAO/WHO Expert Committee on Food Additives (JECFA) (2010) with various modifications was used. Samples were filtered through a Sep-Pak® cartridge (a reverse-phase C-18 cartridge; Millipore, MA, USA) which retains steviol glycosides. The cartridges were previously activated with 10 mL of methanol (MeOH) and 10 mL of water. Every 10 mL of sample was eluted with 2 mL of MeOH, and all methanolic fractions were collected, filtered through a 0.45 μm membrane filter Millex-HV13 (Millipore) and then analysed by liquid chromatography using a Kromasil 100 C18 precolumn (guard column) and Kromasil 100 C18 column (5 μm, 250 × 4.6 mm) (Scharlab, Barcelona, Spain). The mobile phase consisted of two solvents: Solvent A, acetonitrile and Solvent B, 10 mmol/L sodium phosphate buffer (pH = 2.6) (32:68, v/v). Steviol glycosides were eluted under 1 mL/min flow rate and the temperature was set at 40 °C. Chromatograms were recorded at 210 nm. The identification of steviol glycosides were obtained out by using standards and by comparing the retention times, while quantification was performed by external calibration with standards.

*2.6. Statistical analysis*

All determinations were performed in triplicate. An analysis of variance (ANOVA) was applied to the results obtained in order to verify whether there were significant differences in the parameters studied in relation to sample analysed, and to ascertain possible interactions between factors (differences at $p < 0.05$ were considered significant). Where there were differences, an LSD test was applied to indicate the samples in which differences were observed. A multiple regression analysis was performed to study the influence of bioactive compounds to antioxidant capacity (the results are shown in the significant cases, $p < 0.05$). Finally, a study was conducted with the aim of determining whether there were correlations between a pair of variables (Pearson's test). All statistical analyses were performed using Statgraphics® Centurion XVI (Statpoint Technologies Inc., USA).

## 3. Results and discussion

### 3.1. Bioactive compounds

#### 3.1.1. Ascorbic acid

The effect of gastrointestinal digestion on the ascorbic acid recovery was distinct and affected by different *SR* content in the exotic fruit-oat beverages (Fig. 1). The three nondigested beverages (0%, 1.25% and 2.5% *SR*) had similar ascorbic acid values (28–33 mg/100 mL). Although Kim et al. (2011) detected ascorbic acid in stevia leaves and callus, differences in these results and those found in the present research could be attributed to the preparation of the *SR* water extract (weight and temperature submitted). Ascorbic acid diminished just over 5–13% (corrected by the varying volumes of digesta) in the salivary phase, as pH is not substantially changed and duration is of only 10 min. Zulueta, Esteve, Frasquet, and Frígola (2007) found out that pH had a significant influence and correlated negatively with the ascorbic acid concentrations, as acid media contribute to the stability of the vitamin. This explains why the ascorbic acid did not diminish significantly ($p > 0.05$) during the gastric digestion (pH 2.20 ± 0.01) of the three beverages (14–19%). These results are in agreement with previous findings, which proved that *in vitro* gastric conditions (pH 2 or 3) had very little effect on ascorbic acid stability. Only a slight loss (6.7%) was observed by Vallejo, Gil-Izquierdo, Pérez-Vicente, and García-Viguera (2004) in broccoli inflorescences after pepsin digestion. Rodríguez-Roque et al. (2013) also demonstrated that gastric digestion had little effect on ascorbic acid stability, recovering 83% of this bioactive compound in a blended fruit juice containing orange, pineapple and kiwi. However, neither of these authors submitted their sample to a salivary step before the gastric digestion, so bioaccessibility might be overestimated.

Nevertheless, after *in vitro* intestinal digestion, there were significant decreases in ascorbic acid concentration in the non-dialysed fraction (54.8% and 76.1% in the 0% and 1.25% *SR* beverage respectively) with regard to gastric digesta due to the low stability of this compound at high pH, and in the 2.5% *SR* beverage, ascorbic acid was not detected. In the dialysed fraction, an increase in the ascorbic acid content was obtained (8.3% and 10.2% in the 0% and 1.25% *SR* beverage, respectively) with regard to the non-dialysed fraction. These results are in agreement with previous results for broccoli inflorescences (91% loss) (Vallejo et al., 2004), pomegranate juice (80%) (Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002) and for orange, pineapple and kiwi blended fruit juice (75%) (Rodríguez-Roque et al., 2013). Differences in the composition of the samples and the use of dialysis membrane instead of solubility assays to obtain the bioaccessible fraction, could explain the differences found in comparison with our study. However, in an *in vivo* study, Davey et al. (2000) obtained values of bioavailability of ascorbic acid between 80 and 100% at doses normally ingested (⩽180 mg). Aside from the passive transport mechanism which in humans is only predominant at high intake levels, ascorbic acid is also absorbed by an active transport system located in the gut (Stahl et al., 2002), which must be taken into account.

When evaluating the effect of *SR* addition on ascorbic acid bioaccessibility, it is noteworthy that this compound remained undetected in the dialysed fraction of the 2.5% *SR* beverage. In the intestinal phase, *SR* may exert a negative effect over the ascorbic acid stability. This can be due to interferences between the steviol glycosides with the ascorbic acid structure at basic conditions, as in previous steps no significant differences were detected with respect to the formulation involved. No study has been published up to date showing these possible interferences. Interaction between stevioside and ascorbic acid was investigated by Kroyer (2010), but he observed a protective effect of stevioside on the degradation of ascorbic acid. However, the pH used in his study is not specified. Interestingly, Šic Žlabur et al. (2013) obtained a decrease in the ascorbic acid content when apple purees were sweetened with stevia and pasteurised. However, they attribute this loss to the heat treatment and not to the stevia addition.

According to Vallejo et al. (2004), ascorbic acid was the metabolite that showed the greater decrease (91% loss) after intestinal digestion. As ascorbic acid is a thermosensitive compound, in fruit and vegetables it has been used as an indicator of the loss of other vitamins (Zulueta et al., 2007). Consequently, the decrease in bioaccessibility observed for this bioactive compound may also be observed in other similarly alike thermosensitive vitamins, such as vitamin B group, although further studies are necessary.

#### 3.1.2. Total carotenoids

The amounts of bioaccessible carotenoids after simulated gastrointestinal digestion expressed as μg/100 mL are presented in Table 1. As carotenoids are highly hydrophobic compounds, a micellisation step is needed in order to evaluate their bioaccessibility. With this purpose, digested samples were centrifuged during 20 min at 4000 rpm and 20 °C (Courraud, Berger, Cristol, & Avallone, 2013), but no statistical differences were found after the centrifugation step (data not shown). A statistically significant ($p < 0.05$) decrease of 94–99.4% in the dialysed fraction with respect to the initial carotenoids in the original fruit-oat beverage was found. Although carotenoids are considered to be absorbed in a relatively non-specific way by passive diffusion of the micelles in the mucosa cells (Stahl et al., 2002), this low bioaccessibility of carotenoids is mainly caused by their limited solubilisation to the aqueous phase, which hinders their ability to be taken up by the intestines. Other authors have included the use of human pancreatic lipase, cholesterol esterase, phospholipase $A_2$ and taurocholate salts in order to reproduce more physiological conditions, as these enzymes may hydrolyse ester forms and provide optimum conditions for carotenoid hydrolysis and micellisation (Granado-Lorencio et al., 2007).

The highest carotenoid recovery (6%) was achieved in the formulation with 2.5% *SR*, showing a positive correlation between

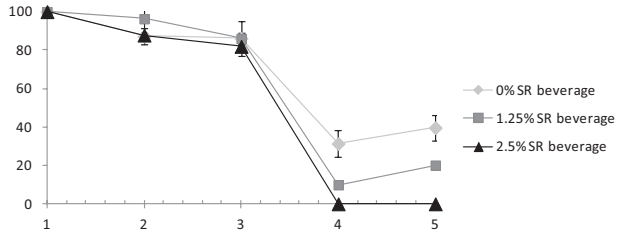



**Fig. 1.** Ascorbic acid content of 0%, 1.25% and 2.5% (w/v) *Stevia rebaudiana* (*SR*) beverage during *in vitro* gastrointestinal digestion, expressed as percentage. 1: Non-digested sample, 2: salivary digesta, 3: gastric digesta, 4: non-dialysed intestinal fraction, 5: dialysed intestinal fraction.

**Table 1**
Total carotenoids, total phenolic compounds and total anthocyanins of a beverage mixture of exotic fruit juices with oat beverage and sweetened with 0%, 1.25% and 2.5% (w/v) *Stevia rebaudiana* (*SR*) during *in vitro* gastrointestinal digestion.

| *SR* (%) | | Total carotenoids | | Total phenolic compounds | | Total anthocyanins | |
|---|---|---|---|---|---|---|---|
| | | (μg/100 mL) | % | (mg GAE/L) | % | (mg cyanidin-3-glucoside/L) | % |
| 0 | 1 | 1629.2 ± 10.6 | | 876.1 ± 53.9 | | 40.4 ± 1.1 | |
| | 2 | 1778.4 ± 10.9 | 109.2 | 930.2 ± 11.9 | 106.2 | 28.6 ± 1.9 | 70.7 |
| | 3 | 1386.0 ± 66.0 | 85.1 | 919.7 ± 8.9 | 105.0 | 25.4 ± 1.4 | 63.0 |
| | 4 | 1262.0 ± 38.8 | 77.5 | 522.4 ± 3.0 | 59.6 | 14.9 ± 1.0 | 36.8 |
| | 5 | 10.0 ± 1.4 | 0.6 | 262.9 ± 4.5 | 30.0 | 0.1 ± 0.1 | 0.2 |
| 1.25 | 1 | 1719.0 ± 24.7 | | 2994.7 ± 12.9 | | 44.5 ± 0.8 | |
| | 2 | 1901.9 ± 50.5 | 110.6 | 3227.1 ± 11.9 | 107.8 | 39.6 ± 1.1 | 89.1 |
| | 3 | 1501.2 ± 42.7 | 87.3 | 3160.0 ± 11.9 | 105.5 | 38.9 ± 1.1 | 87.3 |
| | 4 | 1420.3 ± 9.7 | 82.6 | 1818.4 ± 29.6 | 60.7 | 14.3 ± 1.0 | 32.1 |
| | 5 | 34.3 ± 9.7 | 2.0 | 942.7 ± 22.2 | 31.5 | 2.1 ± 1.4 | 4.8 |
| 2.5 | 1 | 1671.7 ± 7.1 | | 4896.3 ± 26.4 | | 49.3 ± 1.1 | |
| | 2 | 1841.6 ± 27.2 | 110.2 | 5293.8 ± 53.3 | 108.1 | 45.3 ± 0.8 | 91.7 |
| | 3 | 1388.7 ± 46.6 | 83.1 | 5205.7 ± 94.9 | 106.3 | 45.1 ± 1.6 | 91.3 |
| | 4 | 1317.4 ± 97.0 | 78.8 | 3070.8 ± 22.2 | 62.7 | 14.0 ± 1.4 | 28.3 |
| | 5 | 99.5 ± 14.6 | 6.0 | 1608.2 ± 29.6 | 32.8 | 4.7 ± 0.8 | 9.4 |

1: Non-digested beverage, 2: salivary digestion, 3: gastric digestion, 4: non-dialysed intestinal fraction, 5: dialysed intestinal fraction. GAE: gallic acid equivalents.

the content of *SR* and the bioaccessibility of total carotenoids. Results suggest that the addition of the natural sweetener *SR* may enhance somehow dialysability of carotenoids through the semipermeable membrane. Amongst the factors that affect carotenoid bioaccessibility, other authors have shown that the matrix in which carotenoids are embedded can play a significant role on bioaccessibility, along with effectors of absorption and bioconversion (West & Castenmiller, 1998). However, the use of a dialysis membrane does not take into account active transport via membrane transporters through which carotenoids are absorbed (Reboul & Borel, 2011), leading to an underestimation of the real bioaccessibility of carotenoids.

Regarding the different steps through which the beverages pass in the digestion process, salivary addition enhances the release of carotenoids independently of the *SR* concentration. However, gastric and intestinal digestions lead to a decrease in the carotenoid content of around 15% and 21% respectively with regard to the undigested sample, without significant differences between the three formulations. Indeed, carotenoids are more sensitive to acidic than alkaline conditions (Rodríguez-Amaya, 2010). This is why the decrease was higher during the gastric phase. This was also confirmed by Wright, Pietrangelo, and MacNaughton (2008), who showed increased $\beta$-carotene transfer to the aqueous phase under higher pH conditions.

Similar results were reported by Courraud et al. (2013) in carrot, but Granado-Lorencio et al. (2007) reported a higher carotenoid stability of about 70% in the final digesta of loquat, orange and broccoli. As mentioned before, these authors included the use of human pancreatic lipase, cholesterol esterase, phospholipase $A_2$ and taurocholate salts during the intestinal digestion and also a previous homogenisation to simulate mastication and did not include a semipermeable membrane. In a different study, Granado et al. (2006) concluded that behaviour of carotenoids under *in vitro* gastrointestinal conditions does not fully explain changes observed *in vivo*, limiting the use of *in vitro* models for screening relative bioaccessibility of carotenoids.

### 3.1.3. Phenolic compounds

Absorption of polyphenolics from fruit beverages follows similar multistep pathways to other bioactive compounds that generally require (a) release of the specific phenolic from the beverage matrix, (b) solubilisation in the gut lumen, (c) stability of the polyphenolic to digestive conditions, (d) uptake by small intestinal absorptive epithelial cells and (d) potential for intracellular metabolism and secretion into blood stream. Nevertheless, phenolic compounds can also be metabolised by colonic microflora to simple phenolic, organic acids and several other products which are subsequently absorbed and distributed to tissues (Ferruzzi, 2010). However, for the purpose of this paper, only small intestinal absorption will be discussed.

Total phenolic contents of the formulated beverages before and after *in vitro* simulated gastrointestinal digestion is shown in Table 1. Total soluble phenolic compounds of the three beverages before digestion ranged from 876 to 4896 mg/L, measured as gallic acid equivalents. Recoveries of total polyphenols in the dialysed fraction were 30–33% compared to their non-digested counterparts. These results compare well with those reported of recoveries of total phenolic compounds from 29% to 62% in pomegranate juice (29%) (Pérez-Vicente et al., 2002) and apples (Bouayed, Hoffmann, & Bohn, 2011) (44–62%).

The effect of *SR* upon the soluble extractable phenolic content of exotic fruit-oat beverages before and after digestion is depicted in Table 1. Prior to digestion, the 2.5% *SR* beverage exhibited the highest total phenolic content, indicating that *S. rebaudiana* could be useful as a potential source of natural polyphenols. After digestion, the 2.5% *SR* beverage still had six times more total soluble extractable polyphenols than the 0% *SR* beverage, confirming that the addition of *SR* extracts in formulated beverages is a way of enhancing the consumption of these beneficial components.

An increase in the total extractable phenolic content was observed (6–8%) after the salivary step, possibly by enhancement of solubilisation of phenolic compounds, as high molecular weight phenols may be insoluble and the enzyme activity or agitation conditions could facilitate the breakage of large molecules. After the gastric digestion, there was a slight loss in the total phenolic contents, although this decrease was not significant ($p > 0.05$) and recoveries continued to be higher than their non-digested counterparts, possibly because the low pH reached may reduce oxidised species back to the native compounds (Stahl et al., 2002). Similarly, Pérez-Vicente et al. (2002) did not obtain differences in total phenolics content before and after pepsin digestion. However, the mild alkaline conditions reached during the intestinal digestion, along with possible interactions between polyphenols and other components such as enzymes, could explain the significant decrease ($p < 0.05$) observed during the intestinal digestion. Polyphenol concentration decreased during dialysis through the semipermeable cellulose membrane, although all polyphenols found in the intestinal medium were also found to be dialyzable, which could be regarded as indicative of passive diffusion, one of the most important mechanisms for cellular

polyphenol uptake, at least for aglycones. A large portion of the phenolic compounds found in the non-dialysed fraction is likely to reach the colonic lumen where they can be metabolised by the microflora and hydrolysed (Ferruzzi, 2010). In this line, Saura-Calixto, Serrano, and Goñi (2007) estimated that about 48% of total phenolics are bioaccessible in the small intestine, whereas 42% become bioaccessible in the large intestine. Interestingly, Coates et al. (2013) demonstrated that polyphenols likely to reach the colon are capable of inhibiting several important stages in colon carcinogenesis *in vitro*. Furthermore, when Brown et al. (2014) compared *in vivo* and *in vitro* digestion in lingonberries, they observed notable differences in the phenolic composition between the *in vitro* digested extract and the ileal fluid, reinforcing the need of bioactivity studies when investigating dietary phytochemicals.

The impact of gastrointestinal digestion on total anthocyanins is shown in Table 1. In general, the recovery of total anthocyanins diminished stepwise from salivary to dialysed digesta for all three beverages. Amounts of total anthocyanins detected after the salivary and intestinal phase were significantly lower than those determined in the non-digested 0% *SR* beverage. In general, no significant differences ($p < 0.05$) were observed in the total anthocyanins recovery after gastric digestion. Similar results were obtained by Bouayed et al. (2011) with a 91.2% of total anthocyanins gastric recovery in Jonaprinz apples. In the dialysed fractions, total anthocyanins were poorly recovered, with similar patterns obtained by Pérez-Vicente et al. (2002) (2.4%) in pomegranate juice and Gil-Izquierdo, Zafrilla, and Tomás-Barberán (2002) (2.3–3.8%) in strawberries. Bouayed et al. (2011) however, did not detect anthocyanins following intestinal digestion. At this stage of the digestion, part of the anthocyanins could be metabolised to some non-coloured forms, oxidised or degraded into other chemicals, escaping this way from the detection under present conditions. According to numerous studies, low bioaccessibility of anthocyanins can be attributed to their low stability in the alkaline conditions of small intestine, as it is generally accepted that anthocyanins are stable at low pH values (between 1 and 3) (Kosinska-Cagnazz, Diering, Prim, & Andlauer, 2014). However, although *in vitro* studies suggest the degradation of anthocyanins in the intestinal phase, under *in vivo* conditions direct absorption may take place in the stomach (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). Furthermore, the addition of *SR* led to a significant increase in the bioaccessibility of total anthocyanins, suggesting a higher harnessing of these bioactive compounds when *SR* is present in the digestive tract.

### 3.2. Antioxidant capacity

Due to the complex mechanism of antioxidant compounds, there is not an official method to determine total antioxidant capacity (TAC), so trolox equivalent antioxidant capacity (TEAC), DPPH (α,α-diphenyl-β-picrylhydrazyl) scavenging activity and oxygen radical antioxidant capacity (ORAC) were used in the determination of total antioxidant capacity (TAC) after the simulated gastrointestinal procedure of the fruit juice beverages. TAC values of undigested samples without *SR* were 11.4 ± 0.9, 11.1 ± 1.6 and 10.5 ± 0.1 mM TE (Trolox Equivalent) for TEAC, DPPH and ORAC methods, respectively (Table 2). The ANOVA analysis confirmed an increase in TEAC, DPPH and ORAC values when *SR* concentration was increased, independently of the digestion step analysed. These results are in agreement with previous studies carried out by different authors who have shown a high antioxidant capacity of *SR* products (Šic Žlabur et al., 2013). Furthermore, no significant differences ($p > 0.05$) were observed between TEAC and DPPH values, albeit TAC values measured with ORAC assay were significantly higher ($p < 0.05$) for samples with *SR*

**Table 2**
Antioxidant capacity values of a of a beverage mixture of exotic fruit juices with oat beverage and sweetened with 0%, 1.25% and 2.5% (w/v) *Stevia rebaudiana* (*SR*) during *in vitro* gastrointestinal digestion.

| *SR* (%) | | TEAC | | DPPH | | ORAC | |
|---|---|---|---|---|---|---|---|
| | | (mM TE) | % | (mM TE) | % | (mM TE) | % |
| 0 | 1 | 11.4 ± 0.9 | | 11.1 ± 1.6 | | 10.5 ± 0.1 | |
| | 2 | 10.7 ± 1.1 | 94.3 | 10.4 ± 1.3 | 94.0 | 11.5 ± 0.1 | 109.6 |
| | 3 | 10.6 ± 1.2 | 93.2 | 10.2 ± 2.2 | 92.1 | 11.1 ± 0.2 | 106.2 |
| | 4 | 6.1 ± 0.4 | 53.4 | 5.6 ± 1..5 | 50.8 | 7.9 ± 0.2 | 75.8 |
| | 5 | 3.9 ± 0.4 | 34.7 | 3.7 ± 1.5 | 33.7 | 3.5 ± 0.4 | 33.6 |
| 1.25 | 1 | 22.0 ± 2.1 | | 21.1 ± 1.1 | | 24.1 ± 0.2 | |
| | 2 | 21.5 ± 0.1 | 97.7 | 20.1 ± 1.6 | 95.3 | 26.3 ± 0.9 | 109.0 |
| | 3 | 21.2 ± 0.5 | 96.2 | 19.9 ± 0.8 | 94.2 | 25.7 ± 0.6 | 106.8 |
| | 4 | 12.0 ± 0.4 | 54.3 | 11.5 ± 1.7 | 54.7 | 18.4 ± 0.6 | 76.4 |
| | 5 | 7.9 ± 0.6 | 36.0 | 7.5 ± 1.1 | 35.7 | 8.5 ± 0.5 | 35.5 |
| 2.5 | 1 | 32.2 ± 1.9 | | 30.0 ± 0.8 | | 35.5 ± 0.6 | |
| | 2 | 31.6 ± 0.9 | 98.2 | 29.5 ± 4.8 | 98.5 | 39.0 ± 1.0 | 109.7 |
| | 3 | 31.3 ± 0.6 | 97.3 | 29.2 ± 4.8 | 97.4 | 37.4 ± 0.5 | 105.3 |
| | 4 | 17.9 ± 1.5 | 55.7 | 16.5 ± 3.5 | 55.1 | 26.5 ± 0.4 | 74.7 |
| | 5 | 12.2 ± 0.6 | 38.0 | 11.3 ± 1.5 | 37.7 | 11.3 ± 0.3 | 31.8 |

*SR: Stevia rebaudiana*, TEAC: trolox equivalent antioxidant capacity, DPPH: α,α-diphenyl-β-picrylhydrazyl, ORAC: oxygen radical antioxidant capacity, TE: trolox equivalent. 1: Non-digested sample, 2: salivary digesta, 3: gastric digesta, 4: non-dialysed intestinal fraction, 5: dialysed intestinal fraction.

at 1.25% (24.1 ± 0.2 mM TE) and 2.5% (35.5 ± 0.6 mM TE) than those obtained with TEAC (22.0 ± 2.1 and 32.2 ± 1.9 mM TE for 1.25% and 2.5% *SR* beverages, respectively) and DPPH method (21.1 ± 1.1 and 30.0 ± 0.8 mM TE for the beverages with 1.25% and 2.5% *SR*, respectively). Differences may be explained because ORAC assay is based on the transfer of H atoms, whereas TEAC and DPPH assays are based on a redox reaction.

A multivariate regression analysis test was effectuated in order to study the contribution of the different bioactive compounds to the TEAC, DDPH and ORAC values (Eqs. (1)–(3)). Coefficients of the equations were $R^2 = 0.939$, $p < 0.05$, standard error = 0.836, $R^2 = 0.921$, $p < 0.05$, standard error = 2.578 and $R^2 = 0.996$, $p < 0.05$, standard error = 0.807 for TEAC, DPPH and ORAC methods, respectively. For TEAC and DPPH methods, only water-soluble components contributed to the total antioxidant capacity. Zulueta, Esteve, and Frígola (2009) compared the TEAC and ORAC methods, noting that the TEAC method had greater specificity for water-soluble antioxidants.

$$\mathrm{TEAC} = 0.818145 + 0.010975 \times (\mathrm{TPC}) \quad (1)$$

$$\mathrm{DPPH} = 2.36701 + 0.121547 \times (\mathrm{AA}) + 0.00470771 \times (\mathrm{TPC}) \quad (2)$$

$$\mathrm{ORAC} = 2.34997 + 0.003246 \times (\mathrm{TC}) - 0.074694 \times (\mathrm{AA}) + 0.006221 \times (\mathrm{TPC}) \quad (3)$$

High correlation coefficients were found between the total phenolic content and TEAC, DDPH and ORAC assay ($r = 0.9804$, $r = 0.9471$ and $r = 0.9896$, respectively). Kim et al. (2011) also reported total phenolics to be responsible for the antioxidant activities of *S. rebaudiana* water extracts.

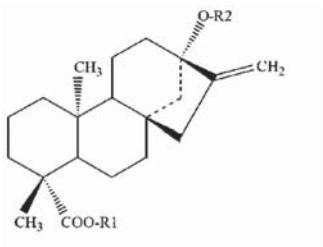
Moreover, a strong correlation was found between total antioxidant capacity measured by TEAC and DPPH ($r = 0.9656$, $p < 0.05$), TEAC and ORAC ($r = 0.9743$, $p < 0.05$), and by DPPH and ORAC method ($r = 0.9398$, $p < 0.05$). Zulueta et al. (2009) also obtained good correlations for ORAC and TEAC assays in orange juice ($r = 0.955$, $p < 0.05$).

### 3.3. Steviol glycosides

Using high-performance liquid chromatography (HPLC), four different steviol glycosides were identified as rebaudioside A (reb

A), stevioside (ste), rebaudioside F (reb F) and rebaudioside C (reb C) (Table 3 and Figs. 2 and 3), albeit the actual JECFA analytical method ((JECFA), 2010) lists nine different steviol glycosides.

Reb A content in the non-digested beverage sweetened with 1.25% and 2.5% (v/v) of *SR* was 46.5 ± 0.1 and 85.1 ± 0.6 mg/100 mL, respectively. The stevioside concentration was 38.7 ± 0.8 and 73.7 ± 1.6 mg/100 mL in the 1.25% and 2.5% *SR* beverage, respectively. These two glycosides (reb A and ste), which are present in the highest concentration, show the highest sweetness activity and minor toxicity (Montoro et al., 2013). Followingly, reb C content in the original 1.25% and 2.5% *SR* beverage was 6.5 ± 0.1 and 13.4 ± 0.5 mg/100 mL, respectively. Finally, reb F was present in a lower concentration of 1.6 ± 0.1 and 3.5 ± 0.1 mg/100 mL in the beverage sweetened with 1.25% and 2.5% (v/v) of *SR*, respectively. No significant influence in the stevioside content was observed by Kroyer (2010) when stevioside was mixed with coffee or tea beverage, although minimal losses could be noticed after 4 h at high temperature. The contents of reb A, ste, reb F and reb C recorded in the present study are in agreement with and sometimes higher than those reported in literature (Montoro et al., 2013), notwithstanding that as is the case of most




| Compound name | R1 | R2 |
|---|---|---|
| Stevioside | β-Glc | β-Glc- β-Glc(2→1) |
| Rebaudioside A | β-Glc | β-Glc- β-Glc(2→1)<br>\|<br>β-Glc(3→1) |
| Rebaudioside C | β-Glc | β-Glc- α-Rha(2→1)<br>\|<br>β-Glc(3→1) |
| Rebaudioside F | β-Glc | β-Glc- β-Xyl(2→1)<br>\|<br>β-Glc(3→1) |

**Fig. 2.** Steviol glycoside structures found in the beverage mixtures of exotic fruit juice and oat sweetened with *Stevia rebaudiana* water extracts.

secondary metabolites, glycosides profiles of stevia are subjected to considerable variability according to geographic area, state of plant maturity, environment, harvesting and processing conditions.

The analysis of variance indicated an increase (4–15%) in reb A, ste, reb F and reb C values after salivary digestion, independently of the *SR* percentage used. Steviol glycosides have glycosidic bonds which may be possible sites of action of α-amylase enzyme, and thus result in an increase of these compounds. Additionally, gastric digestion increased the reb A, ste, reb F and reb C content in about 9–35% with respect to salivary digesta, with independence of the *SR* percentage. These compounds are diterpenoid glycosides and thus pepsin is not able to attack them. Therefore, the increase at this stage of digestion may be due to interactions at acidic pH. Our results differ from previous studies found in published literature. Hutapea, Toskulkao, Buddhasukh, Wilairat, and Glinsukon (1997) obtained no significant changes after *in vitro* digestion of stevioside with saliva, α-amylase, pepsin and pancreatin. By contrast, Kroyer (2010) detected a significant decrease in the stevioside concentration under strong acidic conditions (pH 1), but not at our gastric pH 2, where stevioside was remarkable stable.

After intestinal digestion, reb A, ste, and reb C concentration diminished significantly ($p < 0.05$). Reb F however, became highly bioaccessible. This could be attributed to important changes occurring during *in vivo* and *in vitro* metabolism of steviol glycosides. Ceunen and Geuns (2013) suggest a spatial separation of glycosidases from the steviol glycosides. Consequently, after cell disruption by enzymatic degradation, further metabolism might take place, reflecting a complex and dynamic process not yet fully understood, despite the known biosynthetic relationship between individual steviol glycosides. Dialysed fraction of reb A, ste and reb C was of 24–29% with respect to their undigested counterparts, suggesting an important loss of these compounds during digestion and dialysis process. Surprisingly, bioaccessibility of reb F was of 172.9% and 160.3% with regard to the undigested 1.25% and 2.5% *SR* beverages, respectively, showing the complex metabolism of steviol glycosides. Ceunen and Geuns (2013) explain that although stevioside and rebaudioside A are the most common steviol glycosides, they may not be the final product of the pathway, as further glycosilations are likely to take place, and although biosynthesis of rebaudioside F is not completely elucidated, it is believed that the enzymes UGT76G1 and UGT74G1 might be involved in it, explaining the high bioaccessibility of rebaudioside F. However, this hypothesis has not yet been characterised *in vitro* nor *in vivo*. Koyama et al. (2003) observed in an *in vivo* study that stevia mixture components were first degraded and then absorbed as steviol in the rat intestine. Geuns, Augustijns, Mols, Buyse, and Bert (2003)

**Table 3**
Steviol glycosides of a beverage mixture of exotic fruit juices with oat beverage and sweetened with 0%, 1.25% and 2.5% (w/v) *Stevia rebaudiana* (*SR*) during *in vitro* gastrointestinal digestion.

| *SR* (%) | | Reb A | | Ste | | Reb F | | Reb C | |
|---|---|---|---|---|---|---|---|---|---|
| | | (mg/100 mL) | % | (mg/100 mL) | % | (mg/100 mL) | % | (mg/100 mL) | % |
| 1.25 | 1 | 46.5 ± 0.1 | | 38.7 ± 0.8 | | 1.6 ± 0.1 | | 6.5 ± 0.1 | |
| | 2 | 51.4 ± 0.3 | 110.5 | 40.3 ± 0.3 | 104.3 | 1.7 ± 0.1 | 110.8 | 7.4 ± 0.2 | 114.1 |
| | 3 | 55.7 ± 0.6 | 119.8 | 44.8 ± 1.3 | 115.8 | 2.2 ± 0.2 | 139.7 | 9.4 ± 0.1 | 145.6 |
| | 4 | 42.5 ± 1.1 | 91.4 | 34.5 ± 2.8 | 89.3 | 4.5 ± 0.2 | 289.0 | 5.9 ± 0.1 | 90.5 |
| | 5 | 11.1 ± 0.2 | 23.9 | 10.3 ± 0.2 | 26.7 | 2.7 ± 0.5 | 172.9 | 1.9 ± 0.3 | 29.0 |
| 2.5 | 1 | 85.1 ± 0.6 | | 73.7 ± 1.6 | | 3.5 ± 0.1 | | 13.4 ± 0.5 | |
| | 2 | 93.9 ± 0.4 | 110.4 | 78.7 ± 0.3 | 106.8 | 3.9 ± 0.3 | 111.5 | 15.4 ± 0.2 | 115.0 |
| | 3 | 122.9 ± 4.6 | 144.5 | 104.3 ± 2.4 | 141.5 | 5.1 ± 0.1 | 146.4 | 20.1 ± 2.4 | 149.5 |
| | 4 | 79.8 ± 2.3 | 93.7 | 66.8 ± 1.3 | 90.6 | 7.3 ± 0.2 | 209.5 | 12.3 ± 0.1 | 91.5 |
| | 5 | 21.0 ± 1.6 | 24.6 | 19.8 ± 2.6 | 26.9 | 5.6 ± 0.1 | 160.3 | 3.8 ± 0.3 | 28.0 |

Reb A: rebaudioside A, Ste: stevioside, Reb F: rebaudioside F, Reb C: rebaudioside C. 1: Non-digested sample, 2: salivary digesta, 3: gastric digesta, 4: non-dialysed intestinal fraction, 5: dialysed intestinal fraction.

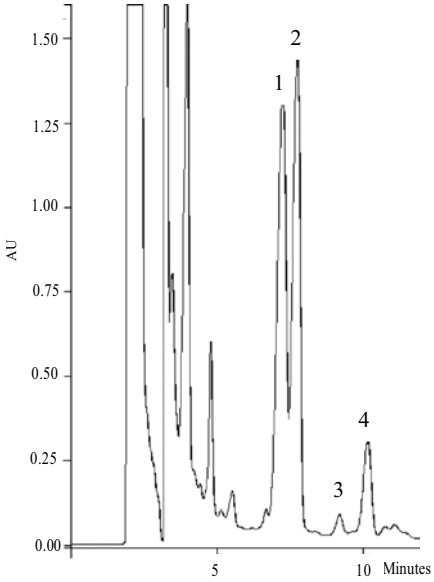



**Fig. 3.** Chromatogram HPLC analysis of steviol glycosides 1: rebaudioside A, 2: stevioside hydrate, 3: rebaudioside F, 4: rebaudioside C in a beverage mixture of exotic fruit juice and oat sweetened with *Stevia rebaudiana* (*SR*) Bertoni at 2.5% (w/v).

obtained a minor fraction of ste and reb A transported through Caco-2 cell layers, suggesting a carrier-mediated transport. However, they could not detect stevioside or steviol in the blood of pigs, probably because in the Caco-2 study, steviol is applied as a solution facilitating the uptake, whereas in the colon, steviol is probably adsorbed to other compounds. Further studies have found out that ste and reb A are completely hydrolysed to the aglycon steviol when incubated with intestinal bacteria (Renwick & Tarka, 2008).

There appears to be a positive correlation ($p < 0.05$) between the amounts of reb A, ste and reb C and the total antioxidant capacity measured by TEAC, DPPH and ORAC assay. This correlation was stronger when the ORAC method was employed. However, a negative correlation was obtained between antioxidant capacity measured with TEAC and DPPH method and reb F content, suggesting that reb F could be the result of the degradation of any other steviol glycoside with potential antioxidant capacity. Previous studies have suggest that although phenolic compounds are the major responsible of the antioxidant capacity shown by stevia extracts, steviol glycosides are known to be potent ROS (reactive oxygen species) scavengers (Geuns et al., 2012). Toward hydroxyl radicals, they observed that stevioside and rebaudioside A had similar scavenging activities indicating that their antioxidant activity is mostly related to their common diterpene skeleton, but stevioside demonstrated a stronger scavenger activity than rebaudioside A for superoxide radicals.

## 4. Conclusions

The addition of 1.25% and 2.5% of *SR* in an exotic fruit-orange-oat beverage contributes to increase the concentration and bioaccessibility of total carotenoids, total phenolic compounds, total anthocyanins and total antioxidant capacity of the beverage. Nevertheless, ascorbic acid was not detected after the *in vitro* simulated digestion when *SR* was found in a 2.5% (w/v). Investigation of the specific interactions between ascorbic acid and *SR* extracts will be critical for understanding how the formulation can be used to optimise circulating and tissue levels of these phytochemical constituents. Despite these results, *in vitro* limitations should be taken into account, as these methods enable an approach, but will not absolutely predict how much of a specific bioactive compound a human will absorb and utilise. *In vivo* assays of beverages sweetened with *SR* must be carried out in order to verify the relevance of the increase in the bioaccessibility of bioactive compounds in blood.

## Acknowledgments

This research project was supported by the Spanish Ministry of Science and Technology and European Regional Development Funds (AGL2010-22206-C02-01). Carbonell-Capella J.M. holds an award from the Spanish Ministry of Education (AP2010-2546). Buniowska M. holds an award from the European Union (8.2.2/IV.26/217/11/U/155/12).

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