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Original

Antioxidant capacity of infant fruit beverages; influence of storage and *in vitro* gastrointestinal digestion

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Abstract

Aim: The total antioxidant capacity of three beverages based on fruit juice, milk and cereals, intended for infants and young children up to 3 years of age was evaluated by two methods Trolox Equivalent Antioxidant Capacity and Oxygen Radical Absorption Capacity.

Results: According to the total antioxidant values obtained by both methods, the beverages can be ranked as follows: grape-orange-banana > peach-apple > pineapple-banana. Ascorbic acid was the main contributor (60%) to the total antioxidant capacity, while the contribution of skimmed milk was less than 1.2%. After one month of storage at -20 ºC, significant losses (p < 0.05) in total antioxidant capacity were found, though these were lower than 3% and therefore lacked nutritional significance. The bioaccessible fractions (maximum soluble fraction in simulated gastrointestinal media) of the beverages, obtained by in vitro gastrointestinal digestion, had antioxidant activities significantly lower (p < 0.05) than the original beverages, though the loss of antioxidant activity was always lower than 19% —thus indicating the stability of the total antioxidant capacity under the applied conditions.

Conclusions: The total antioxidant capacity values of the bioaccessible fraction show that most antioxidants are available for absorption after digestion, and might contribute to the beneficial effects attributed to antioxidants.

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Key words: *Antioxidant capacity. Ascorbic acid. Bioaccessibility. Fruit beverages. Storage.*

CAPACIDAD ANTIOXIDANTE DE LAS BEBIDAS INFANTILES DE FRUTAS; INFLUENCIA DEL ALMACENAMIENTO Y DE LA DIGESTIÓN GASTROINTESTINAL *IN VITRO*

Resumen

Objetivo: Se evaluó la capacidad antioxidante de tres bebidas basadas en zumo de frutas, leche y cereales, indicadas para lactantes y niños de hasta 3 años de edad, mediante dos métodos: Capacidad Antioxidante Equivalente Trolox y Capacidad de Absorción del Oxígeno Reactivo.

Resultados: de acuerdo con los valores antioxidantes totales obtenidos con ambos métodos, se pudo clasificar las bebidas de la siguiente manera: uva-naranja-plátano > melocotón-manzana > piña-plátano. El ácido ascórbico fue el principal contribuyente (60%) a la capacidad antioxidante total, mientras que la contribución de la leche desnatada fue menor del 1,2%. Tras un mes de almacenamiento a -20 ºC, se encontraron unas pérdidas significativas de la capacidad antioxidante total (p < 0,05), aunque éstas fueron menores del 3% y, por lo tanto, carecían de significación nutricional. Las fracciones bioaccesibles (fracción soluble máxima en medios gastrointestinales simulados) de las bebidas, obtenidas mediante digestión gastrointestinal in vitro, tuvieron actividades antioxidantes significativamente menores (p < 0,05) que las bebidas originales, si bien la pérdida de actividad antioxidante fue siempre menor del 19% — indicando así la estabilidad de la capacidad antioxidante bajo las condiciones aplicadas.

Conclusiones: Los valores de la capacidad antioxidante de la fracción bioaccesible muestran que la mayoría de los antioxidantes están disponibles para ser absorbidos tras la digestión y podrían contribuir a los efectos beneficiosos atribuidos a los antioxidantes.

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Palabras clave: *Capacidad antioxidante. Ácido ascórbico. Bioaccesibilidad. Bebidas de frutas. Almacenamiento.*

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Introduction

An increase in the consumption of foods rich in antioxidants during the first years of life favors the nutritional rehabilitation of malnourished children;[1] improves vitamin and antioxidant status and the clinical conditions of children suffering from gastrointestinal disorders;[2] ameliorates respiratory function in pulmonary diseases;[3] and among other effects reduces the risk of cancer[4] and controls blood cholesterol[5] in adulthood.

The intestinal absorption of antioxidant compounds from fruit juices is even better than that of antioxidants coming directly from fruits.[6] In addition, the synergic activity of antioxidants present in fruits and fruits juices can result in increased antioxidant activity.[7]

The first foods introduced in the complementary feeding of infants are usually cereals such as baby foods or paps, and fruits as juices. At present, the market offers beverages intended for young children (1 to 3 years old) that contain fruit juices, milk and cereals. Taking into account the reported benefits of antioxidant intake, the evaluation of the antioxidant activity of beverages of this kind was considered of interest.

Total antioxidant capacity (TAC) cannot be measured directly, though it can be assessed through analysis of the effect of the antioxidant substances in response to a given form of oxidation.[8] Several methods have been proposed to measure the TAC of foods —the most widely used options being the Trolox Equivalent Antioxidant Capacity (TEAC) and Oxygen Radical Absorption Capacity (ORAC) assays, due to their operational simplicity.[8]

The original TEAC assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) to produce the cation radical.[9-10] However, this has been criticized because the reaction shows interferences with substances that possess peroxidase activity, and the antioxidants present in the sample might also contribute to the reduction of the ferryl myoglobin radical.[11] The improved technique for the generation of $ABTS^{+\Sigma}$, prior to reaction with putative antioxidants, involves direct production of the radical through the reaction between ABTS and potassium persulfate.[11-12]

The ORAC assay in turn is based on the detection of chemical damage to phycoerythrin through the decrease in its fluorescence. The principal drawback of this assay is phycoerythrin itself, since its varies from batch to batch, is not photostable, can be photobleached after exposure to excitation light, and can interact with polyphenols causing erroneous ORAC values.[13] Considering these disadvantages, Ou et al., 2001[13] developed and validated an improved assay using fluorescein as the fluorescent probe. Fluorescein shows excellent photostability, does not interact with antioxidants, and moreover reduces the cost of experiments.

Because different antioxidant compounds may act *in vivo* through different mechanisms, no single method can fully evaluate the TAC of foods. Moreover, TAC measured by an individual assay only reflects chemical reactivity under the specific conditions applied in that assay; as a result, the information obtained varies according to the method used. Therefore, several methods for assessing TAC are needed. The greatest problem with these methods is the lack of a validated assay that can reliably measure the TAC of foods. As a result, the use of different methods affords more robust data.[14-16]

The TAC of fruit and fruit beverages have been measured by several methods such as TEAC in apple juice,[10,17] orange juice,[17-22] pomegranate juice[23] and other fruit beverages,[7,15,24-28] and ORAC in fruit beverages —including the fruits analyzed in the present study.[29-30] However, these studies do not necessarily relate to the bioavailable antioxidant that can be released during passage through the gastrointestinal tract, where can modify the antioxidant activity of food.[31]

The first step towards bioavailability is represented by solubility within the intestinal tract (bioaccessibility) for subsequent absorption.[32] Studies of the effect of

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Table I
Fruit juices containing milk and cereals (mM Trolox)

Sample	Storage temperature	TEAC	ORAC
FMC1	Room temperature	2.96 ± 0.04^a	4.43 ± 0.18^a
	-20 ºC	$2.87 \pm 0.02^*$	4.31 ± 0.02
FMC2	Room temperature	3.12 ± 0.03^b	4.73 ± 0.14^b
	-20 ºC	$3.03 \pm 0.02^*$	4.59 ± 0.01
FMC3	Room temperature	3.37 ± 0.04^c	4.94 ± 0.15^c
	-20 ºC	$3.28 \pm 0.02^*$	4.78 ± 0.05

Mean values ± standard deviation (n = 3).
Non-coincidence of superscripts (a, b, c) denotes statistically significant differences (p < 0.05) between values in the same column.
* Statistically significant differences (p < 0.05) between FMC at room temperature and stored at -20 ºC.
FMC: juice + cereals + milk (1 pineapple and banana, 2 peach and apple, 3 grape, orange and banana).
ORAC = Oxygen Radical Absorption Capacity.
TEAC = Trolox Equivalent Antioxidant Activity.

		FMC 1	FMC 2	FMC 3
TEAC	Ascorbic Acid	60.9 ± 0.9	61.5 ± 0.9	63.2 ± 0.9
	Milk	0.9 ± 0.1	0.9 ± 0.3	0.8 ± 0.3
ORAC	Ascorbic Acid	73.0 ± 0.9	73.6 ± 0.9	75.2 ± 0.9
	Milk	1.2 ± 0.2	1.1 ± 0.1	1.0 ± 0.1

Mean values ± standard deviation (n = 3).
FMC: juice + cereals + milk (1 pineapple and banana, 2 peach and apple, 3 grape, orange and banana).
ORAC = Oxygen Radical Absorption Capacity.
TEAC = Trolox Equivalent Antioxidant Activity.

in vitro gastrointestinal digestion on the stability of compounds from fruits and/or fruit juices contributing to TAC are scarce —though the stability of anthocyanins and phenols from raspberry,[33] phenolic compounds, anthocyanins and vitamin C from pomegranate juice[34] and phenolic compounds (flavanones, flavones and hydroxycinnamic acid derivates) from orange juice[35] in an *in vitro* dialysis system have been evaluated.

The aim of the present study was to apply TEAC and ORAC assays to evaluate the TAC of different infant fruit beverages formulated with milk and cereals, and their bioaccessible fractions. To our knowledge, TAC of the bioaccessible fraction of infant fruit beverages, has not been investigated to date.

Materials and methods

Samples

Three fruit beverages containing different juice proportions (51-55%), skimmed milk (6%) and cereals (1%) (FMC), enriched with ascorbic acid (0.5 mg/ml), and intended for infants and young children were studied (FMC1 = pineapple-banana, FMC2 = peach-apple, and FMC3 = grape-orange-banana).

Samples were kept in their unopened vacuum commercial package (N_2/CO_2, < 3% O_2, modified atmosphere) at 25 ºC and protected from exposure to light until analysis. Aliquots of the same samples were stored at -20 ºC, protected from light, during one month.

Material and reagents

Enzymes and bile salts were purchased from Sigma Chemical Co (St. Louis MO, USA): pepsin (Porcine: cat no. P-7000), pancreatin (Porcine: cat. no. P-1750) and bile extract (Porcine: cat. no. B-8631). The working dissolutions of these enzymes were prepared immediately before use.

For TAC, the reagents used were dipotassium peroxodisulfate, ascorbic acid, acetic acid, 2,6-dichlorophenolindophenol, ethanol, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydrogen carbonate from Merck (Barcelona, Spain); 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), fluorescein disodium and metaphosphoric acid from Sigma Chemical Co; and 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) from Wako Chemicals (Richmond, VA, USA). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), purchased from Sigma Chemical Co, was used as antioxidant standard.

Millipore-MilliQ distilled-deionized water (Millipore Ibérica S.A., Barcelona, Spain) was used throughout the experiments.

In vitro *digestion*

The *in vitro* procedure described by Perales et al., 2005,[36] was used.

Briefly, for gastric digestion 80 g of FMC were used. The pH was adjusted to 2.0 and an amount of freshly prepared demineralized pepsin solution sufficient to yield 0.02 g pepsin/sample was added. The sample was incubated in a shaking water bath at 37 ºC/120 strokes/min. for 2 h.

To facilitate the intestinal digestion stage, the pH of the gastric digests was raised to pH 5.0. Then, an amount of freshly prepared and previously demineralized pancreatin/bile solution sufficient to provide 0.005 g of pancreatin and 0.03 g of bile salts/sample was added, and incubation was continued for an additional 2 h. To stop intestinal digestion, the sample was kept for 10 minutes in an ice bath. The pH was adjusted to 7.2, and the digests were centrifuged at 3,500 g for 1 h at 4 ºC. The resulting supernatant constituted the bioaccessible fraction.

TEAC method (Trolox Equivalent Antioxidant Activity)

The method proposed by Pellegrini et al., 2003a[15] was used, and the dilution sample and analytical time

were optimized. ABTS solution (7 mM) was oxidized by treatment with potassium persulfate (molar ratio = 1:0.35) for 12-16 hours in the dark, at room temperature. Oxidation of ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 hours had elapsed. The radical was stable in this form for two days when stored in the dark at room temperature. Prior to assay, the ABTS$^+$ solution was diluted in ethanol to give an absorbance at 734 nm of 0.70 ± 0.02 nm in a 1 cm cuvette, with equilibration at 30 ºC, the temperature at which all assays were performed.

Samples were diluted 1/25 v/v in distilled water to obtain a linear response.[15] The diluted sample (0.1 ml) was added to the ethanolic ABTS$^+$ solution (2 ml) and incubated at 30 ºC. Scavenging of the ABTS$^+$ radical was measured by the absorbance decrease at 734 nm using a Perkin-Elmer (Boston, USA) Lambda 2 spectrophotometer. The absorbance reading was taken exactly 3 min. after initial mixing. All determinations were performed in triplicate. An ethanolic blank was run in each assay.

The percentage inhibition of absorbance at 734 nm was calculated as follows: inhibition % = [1- (A_f/A_o)] x 100, where A_f = absorbance at 3 min and A_o = absorbance at 0 min. A solution of Trolox (0-200 µM) in ethanol was used for preparing a standard curve to which all data were referred.

ORAC method (Oxygen Radical Absorption Capacity)

The method proposed by Ou et al., 2001[13] was used, with minor modifications. The reaction was carried out in 75 mM phosphate buffer (pH 7.4). Prior to assay, samples were diluted 1/150 v/v in phosphate buffer to obtain (after 70 minutes) fluorescence in the test < 5% of initial fluorescence.

Diluted sample (0.075 ml) and fluorescein solution 0.075 ml (30 mg/l) were placed in a microplate. AAPH solution 0.037 ml (60 g/l) was added rapidly to each well. The microplate was immediately placed in the reader at 37 ºC, and the fluorescence was recorded every 5 min for 70 min using a Perkin-Elmer VICTOR^{2}V 1420-040 fluorescence spectrophotometer (excitation 485 nm, emission 535 nm). A phosphate buffer (0.075 ml) was used as blank, and a Trolox 20 µM (0.075 ml) solution was used as a standard, both added in a manner similar to the samples. Blank and standard were performed in each assay. All the assays were prepared in triplicate.

The final results were expressed using Trolox equivalents: ORAC value (mM) = 20 x K x (S_{sample}-S_{blank})/(S_{Trolox}-S_{blank}), where K is the sample dilution factor and S is the area under the fluorescence decay curve of the sample. Trolox or blank was calculated as follows: $S = (0.5 + F_5/F_0 + F_{10}/F_0 + F_{15}/F_0 + \ldots + F_{70}/F_0)$ x 5, where F_0 is the initial fluorescence at 0 min and F_5-F_{70} is the fluorescence measured every 5 min.

Ascorbic acid determination

The AOAC method (967.21) (37) was used to determine the ascorbic acid content in samples. Sample and acetic acid-metaphosphoric acid were mixed 1:1 v/v. The ascorbic acid content was measured by titrimetry using 2,6-dichlorophenolindophenol. A blank (distilled water) and standard (ascorbic acid solution, 1 mg/ml) were also measured. All measurement were carried out in triplicate.

A determination is made of the contribution to TAC of the ascorbic acid present in the samples analyzed. To this effect, standard ascorbic acid solutions are prepared and TAC is determined via ORAC and TEAC. These methods are also applied to a milk solution (at a concentration equal to that found in the samples), to assess its contribution to the TAC of the study samples.

Statistical analysis

The paired data Student t-test was applied to assess significant differences between TAC from FMC stored at room temperature and at -20 ºC, or their bioaccessible fraction. Statistical significance was accepted for $p < 0.05$.

The Statgraphics Plus version 5.0 statistical package (Rockville, Maryland, USA) was used throughout.

Results and discussion

TAC and ascorbic acid content

The TEAC and ORAC values obtained for the three FMC at room temperature and stored for a month at -20 ºC are reported in table I. The values corresponding to the beverages at room temperature are in the ranges of 2.96-3.37 mM and 4.43-4.94 mM of Trolox for TEAC and ORAC, respectively. Statistically significant differences ($p < 0.05$) among the three FMC were found. Given that all FMC had the same milk and cereals contents, the differences should be ascribed to the different fruit compositions.

With the TEAC method, statistically significant differences ($p < 0.05$) were detected between the TAC of beverages stored at room temperature and those stored at -20 ºC, though the losses of TAC in samples stored at -20 ºC were less than 3%, and thus meaningless from a nutritional point of view. These differences were not detected when the ORAC method was applied. Therefore, storage at -20 ºC for a month did not affect the TAC of the studied beverages.

According to their TAC as measured by the TEAC and ORAC assays, the beverages can be ranked as follows: grape-orange-banana (FMC3) > peach-apple (FMC2) > pineapple-banana (FMC1), independently of the method used and of whether storage is at room temperature or at -20 ºC.

Comparison between the values obtained with TEAC and ORAC and the values reported by other aut-

hors cannot be direct, due to the lack of consensus regarding the methodology used in the TEAC and ORAC assays (reagent compound/type and concentration, wavelength and time of analysis), and differences in the composition of the assayed samples.[14,38] It is only possible to compare the observed trends in TAC with those reported in other studies.

Evaluations of different fruit juices show orange and grape juices to have greater TAC than peach and apple juices.[7,15,25,29] This agrees with the greater TAC of FMC3, containing grape and orange, when compared to the other two beverages. Hesperidin, naringenin, carotenoids and ascorbic acid stand out as the main antioxidant compounds of orange juice, while grape juice contains benzoic acid and its derivates, anthocyans and malic acid, as well as tannins. In turn, chlorogenic acid, cumaric acid, epicatechin and tannins, among others, are found in apple juice.[17,39]

There is no information available on the effect of freezing upon the TAC of fruit juices, though there are studies reporting that refrigeration does not affect TAC. In this sense, no changes were found in the TAC as measured by TEAC, in apple juice stored at 4 ºC for 10 days;[10] orange juice at 0 ºC for 1-2 months;[20] at 2 ºC for 2 months;[18] and at 4 ºC for 12-15 days;[20] or in tangerine juice stored at 5 ºC for 3 months.[28]

The contribution of ascorbic acid to the TAC of the analyzed beverages is reported in table II. The ascorbic acid contents of the beverages are in a narrow range 52.4-53.8 mg/100 ml. This is to be expected, since the beverages contain added ascorbic acid. This acid contributes to the TAC by more than 60%, in coincidence with the observations of other authors. Thus, in orange juice[17-18] and in several fruit beverages including apple, orange, grapefruit and pineapple[40] with added ascorbic acid contents (50-61 mg/100 ml) at levels similar to those of the studied beverages, ascorbic acid was seen to contribute more than 60% of the TAC.

The contribution to TAC of the 6% of skimmed milk in the beverages was less than 1.2%. In milk, TAC is ascribed to proteins (lactoferrin, whey and casein) with a high content of potentially antioxidant amino acids such as tyrosine, tryptophan, histidine, lysine and methionine.[41]

Other compounds contributing to the TAC of the beverages are polyphenols and carotenoids from fruit juices,[15] and proteins from cereals.[42]

Antioxidant activity of the bioaccessible fraction

Percentages of the TAC (TEAC and ORAC values) of the beverages corresponding to the bioaccessible fractions are shown in figure 1. No significant differences ($p < 0.05$) were found in TAC among the bioaccessible fractions of the beverages.

Bioaccessible fractions have TAC significantly lower ($p < 0.05$) than whole beverages. The loss of TAC was always less than 19% (FMC1: 18.6 ± 1.9 and 12.4 ± 1.5, FMC2: 16.6 ± 1.7 and 14.0 ± 3.1, and FMC3: 17.8 ± 3.0 and 16.1 ± 1.2, estimated by TEAC and ORAC, respectively).

Bioaccessible fractions have ascorbic acid contents (0.490-0.505 mg/ml) 5.9-6.5% lower than the corresponding original FMC. In studies of preservation under mild oxidative conditions, it has been shown that the phenolics in fruit juices exert an ascorbate-sparing effect[17] that could explain the low ascorbic acid losses.

Several authors have reported the effect of gastrointestinal digestion and dialysis upon antioxidant compounds from fruits and/or fruit juices. Only 2.4% of the total anthocyanin content of pomegranate juice dialyses could be ascribed to their transformation into non-red forms and/or degradation as a consequence of the pH increase after pancreatin-bile salt digestion. Only 29% of phenolic compounds were present in the dialyzed fraction. Ascorbic acid losses of 29% and 80% after pepsin and intestinal digestion, respectively, have been reported. These could be partly explained by pH changes and the presence of oxygen.[34]

During pancreatin digestion, flavonones from orange juice are partly converted into less soluble chalcones (50-60%), which would not be found in the dialyzed fraction.[35] At basic pH (pancreatin-bile salt), anthocyanins from raspberry suffer degradation and/or insolubilization, secondary to binding to compounds of the pancreatin-bile salt mixture, yielding insoluble complexes.[33]

The reported studies show that during gastric digestion, antioxidant compounds from fruits and fruit juices remain stable, most of the changes occurring in intestinal digestion of the *in vitro* digestion dialysis systems. Though the reported studies evaluated different antioxidant compounds, not TAC, their results could justify the lower antioxidant activity of the bioaccessible fraction when compared to that of the infant fruit beverages obtained in this study.

The digestion of native milk proteins yields physiologically important bioactive peptides. Thus, caseinophosphopeptides and casein hydrolysates in the bioaccessible fractions can prevent oxidation reactions through mechanisms that involve direct free radical scavenging and sequestering of potential metal prooxidants such as iron.[43-44]

In addition, most of the phenolic acids from cereals are not available, because they are in insoluble bound forms associated with cell wall polysaccharides. However, the conditions applied in simulated digestion may release the bulk of the cereal-bound phytochemicals, making them available for absorption and contributing to TAC.[42]

Though an *in vitro* digestion method cannot mimic the *in vivo* process, the stability of antioxidant compounds under gastrointestinal conditions is crucial for their potential bioavailability. Thus, *in vitro* systems will allow the screening of samples and may provide data on the potential relative bioavailability of different compounds.

The TAC values of the bioaccessible fraction show that most antioxidants are available for absorption after digestion, and might contribute to the beneficial effects attributed to antioxidants.
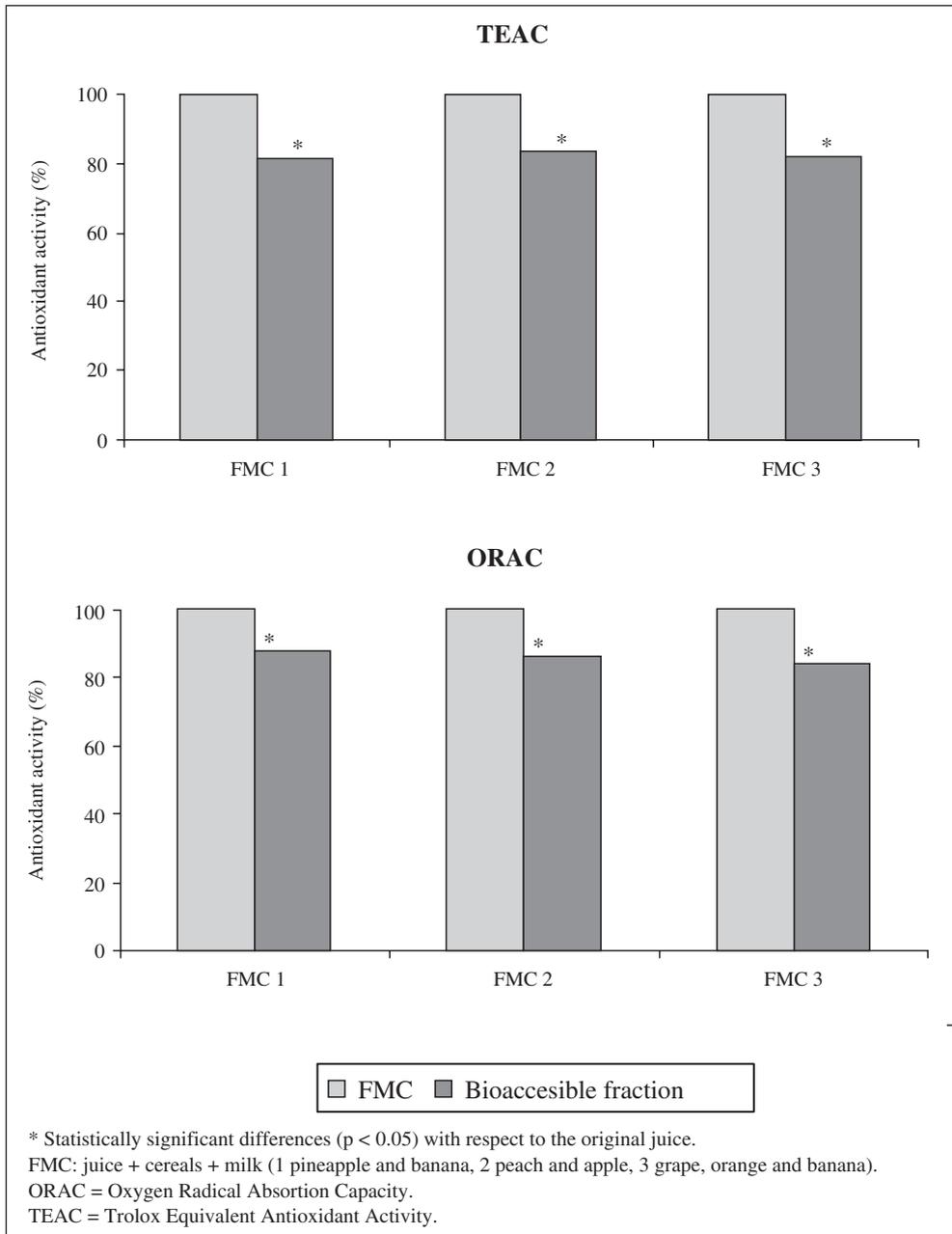
TEAC

ORAC

☐ FMC ■ Bioaccesible fraction

* Statistically significant differences (p < 0.05) with respect to the original juice.
FMC: juice + cereals + milk (1 pineapple and banana, 2 peach and apple, 3 grape, orange and banana).
ORAC = Oxygen Radical Absortion Capacity.
TEAC = Trolox Equivalent Antioxidant Activity.

Fig. 1.—TAC of bioaccessible fraction from fruit juices containing milk and cereals.

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