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MINIATURIZED LIQUID CHROMATOGRAPHY: ON-LINE COUPLING TO IN-TUBE SOLID-PHASE MICROEXTRACTION AND PORTABILITY FOR DIFFERENT MATRICES

Tesis presentada para alcanzar el grado de Doctor en Química dentro del Programa de Doctorado en Química con Mención de Excelencia (R.D. 99/2011)

Henry Daniel Ponce Rodríguez

Directores
Profª. Dra. Pilar Campíns Falcó
Prof. Dr. Jorge Verdú Andrés

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VNIVERSITAT DE VALÈNCIA Facultat de Química

Departament de Química Analítica

Dña. Pilar Campíns Falcó, Catedrática de Universidad, y D. Jorge Verdú Andrés, Profesor Titular de Universidad, ambos del Departamento de Química Analítica de la Universitat de València,

CERTIFICAN

Que la presente memoria, titulada "Miniaturized liquid chromatography: on-line coupling to in-tube solid phase microextraction and portability for different matrices", constituye la Tesis Doctoral de Henry Daniel Ponce Rodríguez para optar al grado de Doctor en Química, y que ha sido realizada en los laboratorios del Departamento de Química Analítica de la Universitat de València, bajo su dirección y supervisión.

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Fdo. Dra. Pilar Campíns Falcó	Fdo. Dr. Jorge Verdú Andrés
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A Bessy,

cuyo inmenso amor y apoyo me

han seguido, incluso al otro lado

del mundo, recordándome que su

alma fue tatuada en mi piel.

“Por los sueños, se suspira.

Por las metas, se trabaja.”

(Humberto Ramos)

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Abstract

Recent innovations and trends in analytical chemistry lead to the development and application of new instrumental systems, innovative sample treatment techniques, and application to problem solving in various fields of analysis. Likewise, the growing concern about the reduction of the environmental impact during the analytical process, without impairing benefits in terms of sensitivity and sensitivity, have modulated the main topics in this field of research.

In this context, the continuous application of miniaturized liquid chromatography (LC) systems, including capillary liquid chromatography (CapLC) and nano liquid chromatography (NanoLC), has represented important advances, due to their tangible advantages, related not only to their performance, such as high sensitivity, but also from an environmental point of view, reducing the use of solvents, electricity, involved material, and generated waste. Along these same lines, recent applications of portable liquid chromatography systems have a significant potential for solving analytical challenges in various applications in situ or at-situ monitoring, due to their ability to perform measurements at the point of sampling. These potential applications are focused on the need to obtain results quickly, overcome problems in the preservation of the sample or the remote location of the monitoring site.

Despite the great advances made at the instrumental level, sample treatment continues to be the stage of the analytical process with the greatest investment of time, sources of error and possible losses of the analytes of interest, therefore the replacing of conventional offline techniques by novel techniques, developed under the green chemistry approach, has become one of the main topics on which researchers have put much effort. An important group of these novel techniques is those based on the absorption of the analytes of interest in a solid, known as solid-phase microextraction (SPME), which reduce the consumption of solvents and therefore minimize the generation of waste, as well as achieve higher productivity due to its automation capacity. In-tube solid-phase microextraction (IT-SPME) corresponds to a type of dynamic SPME, with figures of merit related to the reduction of solvent consumption, easy automation, and miniaturization, allowing it to be easily coupled to miniaturized chromatographic systems. Currently, the development and application of new sorbent phases that allow increasing the selectivity and sensitivity of IT-SPME is one of the main lines of investigation of this technique.

In the framework of the development of this thesis, the research has been focused on the application of miniaturized LC systems for the analysis of highly polar and non-polar compounds in various matrices such as biological samples, environmental waters, dietary supplements, and natural resins. Likewise, aspects such as the development of new sorbent phases and the use of new commercial phases for IT-SPME have been evaluated. Furthermore, the evaluation of the portable NanoLC system for the analysis of emergent compounds in environmental waters has been successfully studied.

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CHAPTER 1. INTRODUCTION

1. INTRODUCTION

In the last 30 years, sample treatment techniques have shown an advance in the green chemistry point of view, searching for new methodologies friendly with the environment, with less waste, toxic solvent volumes, energy consumption and timeless development. In this sense, the work of Gałuszka et al. [1] establishes through the mnemonic code SIGNIFICANCE, the 12 principles of green analytical chemistry, as Figure 1 shows.

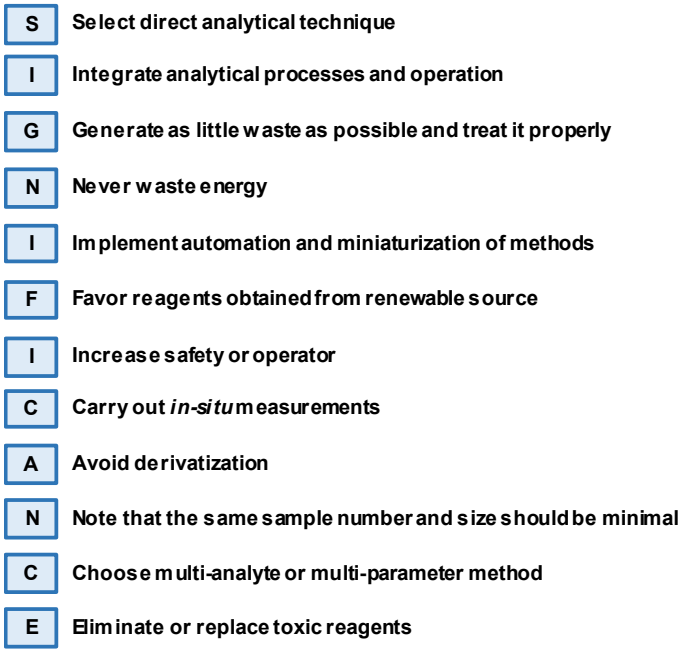


Figure 1. *Mnemonic SIGNIFICANCE of the twelve principles of green analytical chemistry by Gałuszka et al. [1]*

Most of these principles have a great impact on the stage of sample preparation of the analytical process, so the application of microextraction techniques, preferably online, as well as coupled with miniaturized instrumental techniques is of great interest to the green analytical chemistry. The current approach for sample treatment techniques aims to increase the selectivity and sensitivity of the analysis [2], to extract, purify, concentrate and/or derivatize the analytes, without using laborious and tedious procedures, with a high economic and time cost such a case of conventional extraction techniques.

In this context, the greening of the sample treatment process is a current demand of contemporary researchers, so that, classical sample preparation techniques such as solid-phase extraction (SPE) and liquid-liquid extraction (LLE) are being rapidly replaced by microextraction techniques, generally divided into two groups: Liquid-Phase and Solid-Phase extractions [3]. In the

first one group, single-drop microextraction (SDME), dispersive liquid-liquid microextraction (DLLME) or hollow-fiber liquid-phase microextraction (HF-LPME) are some examples included.

Solid-phase microextractions techniques (SPME) are based on sorption processes of the analytes in a solid, where SPME is the most widely used for several different matrices and using alternative sorbent materials [4]. Configurations of SPME can be classified into dynamic and static techniques. In the last one, the extraction is carried out in a stirred sample mode. Examples of these are thin-film, fiber microextraction, and rotating-disk sorptive extraction. On the other hand, methods such as in-tube SPME, in-tip and in-needle microextraction, and capillary microextraction are classified as dynamic techniques [5]. Other denomination employed are microextraction in packed syringes (MEPS), disposable pipette tip extraction (DPX) and stir bar sorption extraction (SBSE). Figure 2 shows a schematic summary of microextraction techniques.

1.1. IN-TUBE SOLID-PHASE MICROEXTRACTION (IT-SPME)

As mentioned above, an important group of microextraction techniques includes those that use the mechanism of sorption of the analytes in a solid, within which is the solid-phase microextraction (SPME), introduced in 1990 by Pawliszyn et al [6], which manages to miniaturize the sample preparation stage, as well as sampling, extraction, and preconcentration in a single stage. Later, in order to avoid the disadvantages of SPME, associated with fiber fragility and long desorption times, Eisert y Pawliszyn introduced the In-tube Solid-phase microextraction (IT-SPME) [7].

IT-SPME has been widely used because of its advantages such as the elimination of solvent extraction, as well as combining the extraction, clean-up and preconcentration in a single step, so that it significantly reduces the analysis time. Likewise, IT-SPME shows special attention for the on-line coupling to liquid chromatography (LC), which facilitates the separation and detection of target analytes.

Also, it is possible to affirm that IT-SPME is an environmentally friendly technique, achieving the minimization of toxic solvents, waste reduction and being energy-efficient and cost-effective, capable of maintaining the reliability of performance parameters, such as sensitivity, precision, and accuracy [8].

The principal topics of this technique are aimed at developing the coupling with new chromatographic modes, such as ultra-high performance liquid chromatography (UHPLC), capillary liquid chromatography (cap-LC) and Nano liquid chromatography (Nano-LC) [9], as well as the synthesis of new extraction phases, off-line development, and applications.

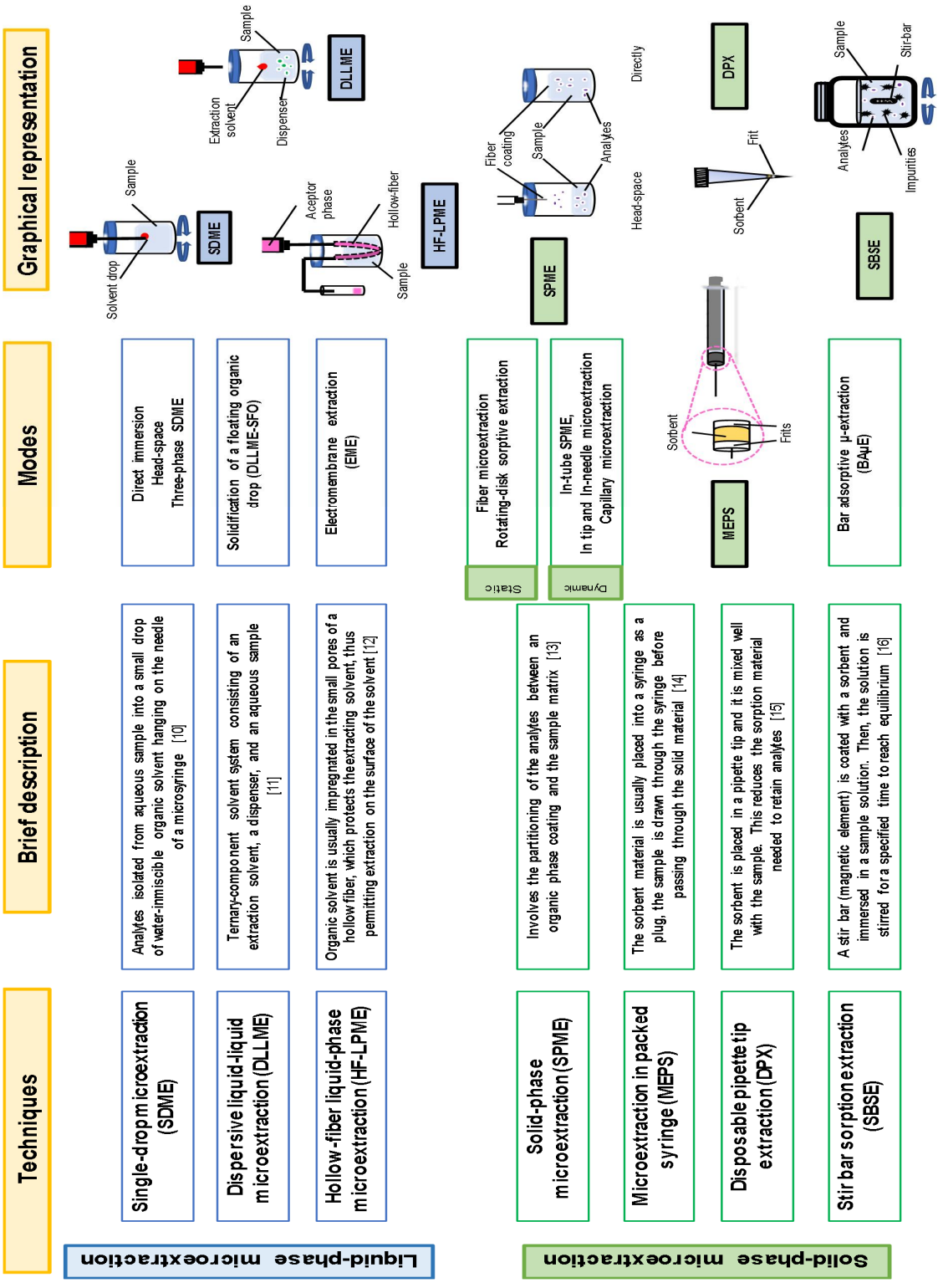


Figure 2. *Schematic summary of microextraction techniques.*

The application of IT-SPME has increased since its appearance, which is supported by the linear increase in papers and citations. The database ISI Web of Science shows more than 430 entries at the end of 2019, with an H index of 69 and more than 15,000 citations (Figure 3) for the research topic “in-tube solid phase microextraction”.

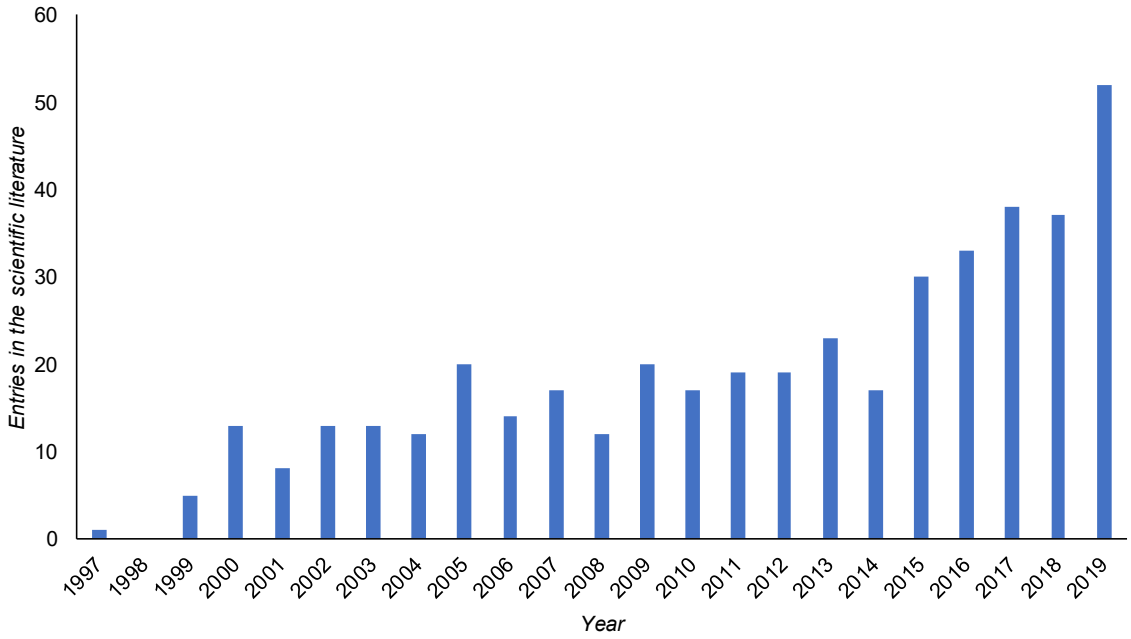


Figure 3. *Evolution in the number of works of the topic "in-tube solid-phase microextraction" since 1997. Source Web of Science (May 2020).*

1.1.1. Theoretical considerations

In IT-SPME the sample is passed throughout a capillary, generally a fused-silica tube coated or packed on its inner surface with a sorbent phase, where analytes are retained and concentrated by a process of sorption. Once the analytes are retained, they are desorbed by filling the capillary with a proper solvent, to be collected for further processing or transferred to the analytical instrument (static desorption), or if the mobile phase was used, dynamic desorption was carried out [17]. Due to the amount of sample processed is generally higher than extracting sorbent, IT-SPME is considered a not exhaustive technique [18].

For those analytes with strong interactions with the extractive phase, the static mode is preferred, as well as in the case of an off-line technique application. In other case, analytes are desorbed and transferred simultaneously to the analytical instrument by the mobile phase (dynamic desorption).

As well as in other sorptive techniques, some experimental parameters, such as phase thickness, capillary length and diameter, processed sample volume, pH, ionic strength and washing solvent, must be optimized in order to achieve the greatest benefits of this technique. The coupling with a suitable chromatographic system is important for decreasing the LODs. Although IT-SPME has been used in combination with gas chromatography (GC), liquid chromatography (LC) is by far the most used system in assays.

Commercial GC columns have been used to perform IT-SPME, typically with two class of sorbents: Si-based (polysiloxane coating) and C-based (divinylbenzene, polyethylene glycol, and carbon molecular sieves). More than 70% of the studies use Si-based sorbents [19]. Although the use of conventional capillary coatings has allowed the popularity of this technique, it represents its main limitation, the low extraction efficiency that can be achieved, attributed to the low sorbent loading capacity, stability, and in some cases, the long extraction times involved in the extraction due to the slow diffusion of the analytes from the sample to the capillary coating.

Due to this, in recent years the research works have been focused on finding more efficient capillary coatings, as well as exploring new extraction phases of IT-SPME. These capillary coatings can tune the interaction, obtaining substantial improvements and counteracting the aforementioned limitations.

1.1.2. Settings for IT-SPME

The coupling of IT-SPME with LC has been established through two types of configurations, which require specific configurations as well as different levels of instrumentation. These are referred to as draw/eject IT-SPME and flow-through IT-SPME, each one with its advantages and limitations.

In draw/eject mode, the sample is repeatedly passed through the extractive capillary, that is placed between the needle and the injection loop (or the metering pump) of the autosampler, by cyclic reversing of the flow direction of the sample (draw/eject) by means of a programmable autosampler. When the sample is aspirated from the sample vial, it becomes into contact with the extractive capillary. As a result, a fraction of the analyte molecules migrates from the sample to the extractive phase. After a number of cycles, the analytes are desorbed and injected (Figure 4.A).

Draw/eject IT-SPME additional solvents (water or buffers) can be placed in different vials and flushed though the capillary in order to eliminate selectively matrix components before the desorption stage, or to clean and condition the capillary before loading the next sample [20]. This mode of IT-SPME has been especially used in the analysis of biological samples, where the sample volume is limited, typically a few millilitres are used [21].

The distribution equilibrium of the extraction is achieved with a number of cycles, generally greater than 20: however, it is not always possible to achieve it, since the analytes are partially desorbed with each loading cycle. Likewise, the times involved in the extraction stage can be much longer than those required for the chromatographic separation.

In the flow-through IT-SPME setting the sample is passed through the extractive capillary once without reversing the flow direction. The extractive capillary is directly or indirectly connected to the chromatographic column so that the compounds are desorbed by filling the capillary with a desorption solvent (off-line) before transferring them to the chromatographic column, or by flushing through the capillary the mobile phase (on-line). This configuration has versatility because it is possible to use a variable combination of pumps, switching valves or external accessories (such as those intended to create magnetic or electric fields or control the temperature) to implement the flow of IT-SPME, making it more useful.

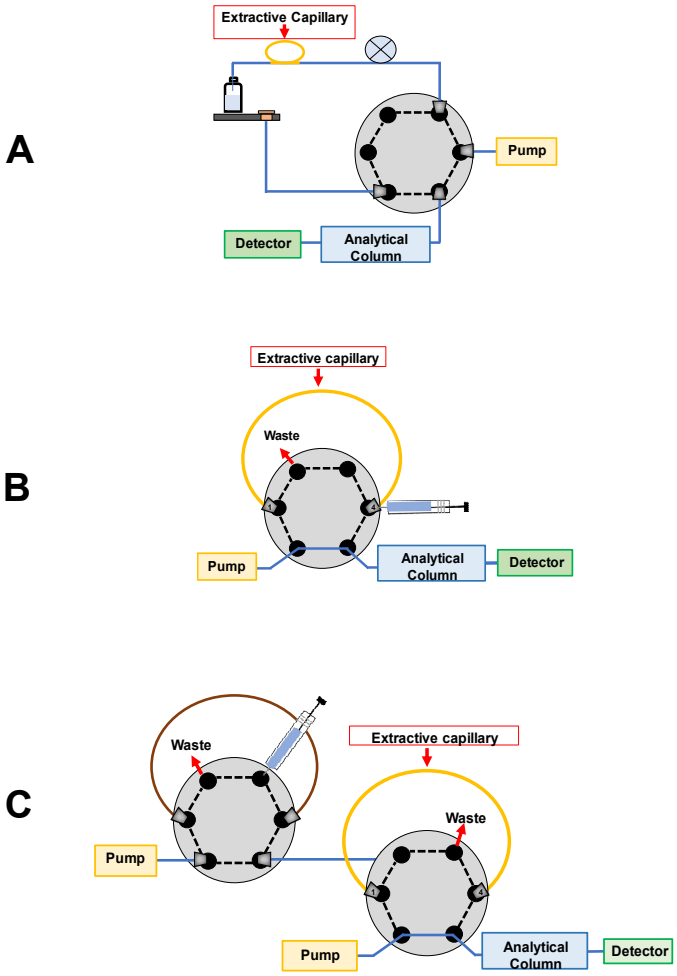


Figure 4. *Schematic representation of configurations in IT-SPME-LC: A) draw/eject mode; B) in valve with one pump and C) in valve with two pumps.*

In the simplest configuration (Figure 4.B) the extractive capillary is used in replacement of the inner loop of the injection valve (in-valve IT-SPME) so that the analytes are retained in the extractive phase during the sample loading, with the valve in the load position. Other steps as clean-up and derivatization can be implemented, if necessary. When the valve is switched to the inject position, the mobile phase desorbs and transfers the analytes to the chromatographic analysis. This configuration permits to process higher amounts of sample volume (with multiple filling of the loop), which represents a great advantage when a higher sensitivity is required, as it is the case of the environmental analysis, regardless of the possible low extraction efficiency. Obviously, a precise control of the volume of sample introduced in the capillary is needed to obtain adequate analytical responses. Other assemblies involve the incorporation of 2 valves and 2 pumps [22-24], which leads to more powerful systems, with which it is possible to process high volumes of samples, combine two (or more) IT-SPME capillaries or even attach systems of different dimensions in reference to liquid chromatography (LC) (Figure 4.C).

Although the absolute recoveries in IT-SPME are generally low, there are three ways to achieve the required sensitivity. In the first instance, processing higher sample volumes, until the amount of retained analyte is adequate [25]. On the other hand, since efficiency depends directly on the affinity of the analytes for the microextraction phase, the development and application of new microextraction phases are essential [26]. Finally, the coupling with miniaturized chromatographic systems (CapLC and NanoLC) influence the sensitivity significantly [27].

1.1.3. Coupling with liquid chromatography

Although there are applications in the literature with other instrumental techniques such as capillary electrophoresis [28], capillary electrochromatography [29], or atomic absorption spectrometry (AAS) [30], IT-SPME coupling has been used mainly to LC with UV-vis [31], fluorescence [32] or mass spectroscopy (MS) detection [33] applied to environmental, food, industrial and biological samples.

The coupling of the IT-SPME with LC facilitates also the derivatization of the analytes, if necessary, being able to be carried out online and in a simple way by successively adding the derivatizing agent and the sample, allowing the reaction to be carried out, and finally, the formed derivative is transported to the analytical column [34].

This coupling offers significant advantages related to enhancing sensitivity or easy hyphenation with MS detector [35]. The coupling of this technique with miniaturized chromatographic methods has been the subject of specialization of the research group “Miniaturization and Total Methods of Analysis” (MINTOTA) where this thesis has been developed,

contributing since 2006, in particular with miniaturized LC techniques such as CapLC and NanoLC. Unlike NanoLC, the coupling to CapLC can be carried out with minor modifications concerning the conventional LC, therefore the IT-SPME-CapLC coupling is sufficiently resolved, achieving its application in several fields of analysis such are the environmental, food, biological , industrial and forensic [36-40], being mainly the one employed in this work.

The coupling IT-SPME to NanoLC has recently been introduced [41], so studies are needed to elucidate and demonstrate the benefits and possibilities of this configuration. There are two practical aspects to overcome in this coupling. On the one hand, the low mobile-phase flow rates used in NanoLC makes it essential to use capillaries with low inner diameters (≤ 100 μm). On the other, as the number of coatings of commercially capillaries compatible with these systems is still rather limited, new sorbents with improved extraction capabilities are needed to be developed [23].

1.1.4. Extraction phases for IT-SPME

Initially in IT-SPME, sorbent phase available in the capillary columns of gas chromatography, called conventional phases, were used. However, as mentioned above, the development of new sorbent phases is one of the main topics in which researchers and users of the technique have made continuous efforts. It is known that the nature of the extraction phase will directly affect the sensitivity of the technique since the ability of the analytes to interact with it, plays an important role in retention and preconcentration in the sorption stage. Therefore, in the following lines, the main extraction phases used in IT-SPME are discussed, with special attention to those used in the development of this work.

1.1.4.1. Conventional capillary columns

The use of this type of capillaries makes it possible to have a variety of sorbent phases with different polarity and thickness or sorbent layer. As mentioned before, the sorbents can be made of polymeric material of carbon or silicon, which can be modified with other molecules, which allows increasing the selectivity for the analytes of interest.

To date, columns based on polydimethylsiloxane (PDMS) are the most used for this technique. PDMS is a polymer of the silicon family consisting of flexible chains of Si-O$(CH_3)_2$ units, which has a transparent appearance that due to its marked hydrophobic character does not react with most chemical products, including hydrophilic solvents, and with the advantages of not being toxic or bioaccumulative [42]. When IT-SPME is coupled to LC, the use of PDMS based sorbents is extremely convenient, since its hydrophobic character will favor the adsorption of the hydrophobic compounds present in the sample, being an ideal phase for chromatographic separations in reverse phase.

The use of conventional columns of modified PDMS, through the incorporation of functional groups, mainly variable percentages of polydiphenylsiloxane and others, has been very useful in the application of IT-SPME. With these modifications, it is possible to reduce the hydrophobicity of the extractant phase, as well as to establish new interactions of π-π type of the analytes with the aromatic groups, which will lead to greater retention [43]. To date, the results achieved with PDMS capillaries modified with diphenyl groups have been reflected in works in the literature that report the use of these, in various fields of analysis, such as environmental pollutants [44], pharmaceuticals [39], industrial products [45], biologicals [46] and study of nanoparticles [23].

A few applications with commercial GC columns based on other silicon derivatives, such as cyanopropylmethylsilicone or dimethylsiloxane, have also been reported for the study of biological matrices [47]. In relation to conventional columns with C-based sorbents, mainly those that use polymeric sorbents of divinylbenzene type (Supel-Q PLOT and/or Valco PLOT) or molecular carbon (Carboxen 1006) have shown excellent results. The sorbent in these columns, PLOT type (open porous layer tubular columns), has a larger surface area which leads to an increase in the retention of the extracted target compounds. Some papers of the literature have demonstrated the applicability of these sorbents for the determination of compounds of relative polarity [48]; and mainly for the determination of non-polar compounds in environmental [49], food [50] or bioanalysis [51].

It is important to point out the performance of the CP-Pora PLOT amines column, which due to dipole-dipole interactions, hydrogen bonds or ion exchange, has a higher extraction efficiency for relatively polar compounds, such as medicines for influenza treatment [52]. Table 1 shows selected examples of different methods of IT-SPME coupled to LC in which commercial capillary columns of gas chromatography are used. In the present thesis, commercial columns have been used for the determination of trimethylxanthines and triazine pesticides in biological and environmental samples, including those of fused-silica coated with PDMS, with different percentages of diphenyl groups (5, 20, 35 and 50%), as well as commercial Carbowax columns coated with polyethylene glycol (PEG), FFAP columns coated with PEG modified with nitroterephthalic groups, and two polystyrene-divinylbenzene (PS-DVB) columns with different coating thickness.

Table 1. *Examples of different procedures specified in LC systems with capillary columns methods used in IT-SPME.*

Extraction Phase	Application field	Analytes	System	Reference
TRB-5	Environmental	DEHP	CapLC-DAD	[53]
TRB-5	Food	DEHP	CapLC-DAD	[54]
TRB-5	Environmental	Ethylenediamine	HPLC-FLD	[55]
TRB-35	Biological	Meropenem	CapLC-DAD	[39]
Supel-Q PLOT	Biological	Anabolic steroids	LC-MS	[56]
Supel-Q PLOT	Biological	Heterocyclic amines	LC-MS/MS	[57]
Carboxen 1006 PLOT	Food	Patulin	LC-MS	[58]
CP-Pora PLOT amine	Environmental	Perfluorooctanoic acid, Perfluorooctane, sulfonate	LC-MS	[59]

1.1.4.2. Development of new sorbent phases for IT-SPME

Despite the good results achieved with commercial capillary columns, treated in the previous section, the variety of sorbents available is still limited, especially for smaller diameter columns typically used in miniaturized LC systems. In addition, the phase thickness of these columns becomes insufficient in those determinations where it is essential to reach lower detection limits. In this way, the functionalization of the capillary columns, as well as the synthesis of new sorbent materials, capable of tune the interaction, thereby, improving the sensitivity, selectivity, stability and extraction time are an important subject matter in IT-SPME. The sorbent extraction phases recently developed for use in IT-SPME are discussed below.

Metal nanoparticles and metal oxides. In recent years the use of nanomaterials has extended to numerous fields of knowledge, such as medicine, electronics, agriculture, food industry or biotechnology. In the specific case of Analytical Chemistry, its application as sorbents in different extraction techniques has been proposed [60, 61]. In that sense, the increase in nanomaterial extraction capacity is due to two different and complementary effects. In the first place, its presence in the extraction phase modifies the morphology of the sorbent, thereby increasing its porosity [62]. On the other hand, new interactions, as well as an increase in the number and intensity of existing ones, are obtained by immobilizing the materials in the extraction phase, which will also favor the useful life of the capillary, reducing its fragility. Castillo-García et al., point out that the main

advantages of the use of nanomaterials as extraction sorbents include increased adsorption and preconcentration capacity, easy functionalization and possibility of reuse [63].

Metal nanoparticles and metal oxides are nanomaterials formed by one, two or three metals and/or metal oxides. These materials have a large surface area and high absorption efficiency, hence the special interest in their application as a sorbent phase [64]. In the case of metal oxides, the active sites contain hydroxyl groups. These nanoparticles have the characteristic of establishing ion exchange interactions, depending on the pH they can act as cation or anion. Likewise, both the metal oxide NPs and the metal NPs can act as Lewis acids, presenting a strong interaction with species that can act as Lewis bases [65]. To date, the most used nanomaterials of this type are those of Au, Ag, Al_2O_3, Fe_3O_4, CeO_2, SiO_2, TiO_2, ZnO and ZrO_2. However, despite its wide use in fiber microextraction techniques and other extraction formats, its use in IT-SPME is still reduced [66, 67].

Interesting applications have been carried out with NP's of TiO_2 and SiO_2, the first ones for the extraction of phosphopeptides [68], and the latter's, due to their high hydrophobic grade, for the extraction of PAHs and endocrine disruptors by IT-SPME-UV [26]. The NPs of Fe_3O_4 are a superparamagnetic material that can be used in magnetic IT-SPME. By applying a magnetic field to the extraction phase, it creates a magnetic order with regions of a different magnetic gradient. Upon entering the sample through the extraction capillary, subjected to the magnetic field, a retention effect is achieved for diamagnetic analytes in regions where the magnetic field is minimal, which leads to an improvement in the extraction capacity. In the elution part, the external magnetic field is eliminated, making the analytes easily eluted with the appropriate solvent or the mobile phase [69, 70].

The influence on the extraction capacity of a polymeric material of tetraethylorthosilicate (TEOS) and trimethoxyethylsilane (MTEOS) fortified with NPs of SiO_2, TiO_2, ZrO_2, CuO and ZnO has been an object of study in this work, for the determination of trimethylxanthines in biological and environmental samples.

Ionic liquids (IL). Ionic liquids are defined as ionic salts that are in a liquid state at room temperature, formed by an organic cation (for example, imidazolium, pyridinium or quaternary ammonium) and an inorganic or organic anion (Cl-, Br-, PF6-, trifluoromethylsulfonate) [71]. The application of ionic liquids to increase the extraction capacity in SPME techniques, including IT-SPME, has been gaining popularity in the last years. This is due to its retention capacity of analytes through different interaction mechanisms, such as hydrophobic and hydrophilic interactions, ion exchange, π-π type or hydrogen bonds [72].

Initially, ionic liquids were linked to the extraction phase by covalent bonds. However, at present, they are being part of the polymer network that forms the extraction phase, as one monomer. Some works of literature apply ionic liquids for determination of PAHs in water, based on the monolithic polymer 1-dodecyl-3-vinylimidazolium, which was used to coat steel fibers that were subsequently packaged in a PEEK capillary for this purpose [73]. The sensitivity achieved by this sorbent phase improved 200 times the extraction capacity than other SPME techniques, and the detection limits were lower than those obtained when conventional phases were used.

Monoliths. One of the most active areas of research efforts for IT-SPME is the development of monolithic capillary columns. Monolithic materials have special structural characteristics since they have macropores and mesopores. The first ones facilitate rapid dynamic transport, which allows increasing the flow of the mobile phase, this reduces the analysis time without a significant increase in pressure compared to the use of particulate capillary columns. Other advantages include a rapid mass transfer, high stability, and loading capacity, as well as a wide variety of surface chemistry[74].

According to the nature of the monomers that form the monolith sorbent, this can be classified into organic, silicon-based, and hybrid monoliths. The two most common procedures for the synthesis of monolith capillary columns are the thermal and UV-polymerization, typically using as support capillaries fused silica, PEEK and PTFE. The modification of the capillary surface can be done in-situ if the molecule, material or nanomaterial to be immobilized is added to the monomer mixture or carried out in another stage different from the synthesis.

For fabrication of these monoliths, generally, five steps are required, include activation, modification of the capillary surface, deposition of the polymeric mixture, polymerization, and washing and drying. Activation of capillary columns is necessary to activate silanol groups and normally is carried out by a rinse with methanol, then the modification of the surface is needed to achieve the covalent binding of the capillary inner wall and polymeric material [75].

A mixture composed of monomers, such as choline-chloride, acrylamide, divinylbenzene or methacrylic acid, a crosslinking agent, commonly azobisisobutyronitrile (AIBN), and a solvent mixture such as isopropanol, toluene, methanol, and isooctane, called *porogen*, whose function is to form the pores, are needed to obtain the monolith, which is passed through the capillary column. Finally, if a thermal treatment is applied, typically temperatures up to 80°C with times between 3 and 24 hours are required. In UV-treatment, UV radiation for less than 30 minutes is necessary.

The application of organic monoliths as sorbent phases for IT-SPME to resolve different analytical problems has been reported in the literature. In food analysis, Wu et al. [76] reported the use of poly (octadecyl methacrylate-co-ethylene dimethacrylate) monolith for the analysis of trans

fatty acids in samples of instant coffee. In environmental analysis, the determination of thiazoles in water samples by the application of a poly (4-vinylpyridine-co-ethylenedimethacrylate) monolith has been described by Pang et al., [77]. Likewise, the analysis of alkaloids derived from tobacco in urine samples has been carried using IT-SPME coupled to LC-MS with a poly (N-isopropylacylamide-co-divinylbenzene-co-N, N`-methylenediacrylamide) monolith [78].

Molecularly Imprinted Polymers (MIPs). In MIPs the specific gaps of these three-dimensional polymers allow a selective interaction with those compounds whose structure is similar to that molecule. These specific gaps are induced by a template molecule. The application of these sorbent phases has been studied extensively for SPE applications, as a mechanism to improve the selectivity for target analytes, which can be used too for capillary columns. The application of these sorbents represents advantages such as high thermal, mechanical and chemical resistance, easy synthesis and low cost, and mainly, their selectivity can result in an improvement in the extraction efficiency [79].

The preparation of this type of polymers is quite similar to that described in monoliths synthesis, with the difference that the template molecule, with a similar structure to analytes, must be added to the monomer mixture and not polymerize with them. After the polymerization process is done, the polymer chains grow around the template molecule in all three dimensions, and finally, the template molecule is removed, and the sorbent phase obtained has a size, shape, and chemical interactions specifics for the analytes of interest [80].

Recently, the preparation of a MIPS as an extraction phase in IT-SPME-LC for the determination of citrin in food matrices such as cereals and food supplements has been reported by Lhotská et al. They used 1-hydroxy-2-naphthalic acid as the template molecule, acrylamide as the functional monomer, ethylene glycol dimethacrylate as the crosslinker, and acetonitrile as the pore generator, improving the sensitivity and selectivity compared with traditional sorbents as a C18 phase [81].

Restricted Access Materials (RAMs). Restricted access materials are sorbent materials with a capacity for the retention of different types of analytes regardless of the presence of proteins in the analyzed sample, based on a process of molecular exclusion, which increases the selectivity. The use of these materials has an important application for analysis of biological samples, where the presence of macromolecules as proteins affects the sensitivity and selectivity of the extraction, as well as, they can obstruct tubing and columns of the chromatographic system.

In RAMs preparation, the sorbent's surface is modified by the addition of hydrophilic compounds that prevent the interaction of proteins with the sorbent, that means a protective layer is created on the sorbent that acts as a filter, and only the smallest molecules can be retained in the

sorbent [82]. The resulted material can be easily combined with other materials to produce improved sorbents. Souza et al. [83] reported the determination of parabens in breast milk samples using IT-SPME-UHPLC-MS/MS without the need for any prior treatment of the sample. Likewise, Huang et al. synthesized a RAM based on an inorganic monolith of methyltrimethoxysilane and APTES for the determination of antibiotic residues in the same matrix [82].

1.1.5. Off-line development

It is possible to hold out the IT-SPME procedure offline, although only 20% of the literature reports its use [84], due to the loss of some advantages of the technique, as not all the extracted analytes are introduced within the determination system, whereby a brand new step is introduced, and automation is not achieved. Nevertheless, the best advantage of the operation is that, once the target analytes are extracted, cleaning operation are often administrated that facilitate the compatibility with chromatographic or detection systems, as an example, to derivatize the extracted compounds [85].

Normally IT-SPME offline has been used with electrophoretic techniques, HPLC, UHPLC and mass spectrometry detectors [86, 87]. This work is pioneering to develop a method based on IT-SPME offline combined with a hand-portable LC for the determination of trimethylxanthines in environmental samples.

1.2. MINIATURIZED LIQUID-CHROMATOGRAPHY

The first work of the literature using a column with an internal diameter (i.d.) of 1 mm to obtain the separation of ribonucleotides was developed by Horváth et al. at the end of the ´60s [88]. Ten years later, Tsuda and Novotny established the required equipment for working with capillary LC, implementing modifications on conventional injection and detector systems aiming to reduce the band-broadening effects [89]. In the following decades, some publications continued to use columns below that internal diameter. Nowadays, it is possible to find columns with an internal diameter below 0.1 mm, as it is the case with columns for NanoLC [90].

Among the main advantages of miniaturized systems, we can point out the decrease in the consumption of the mobile phases that promotes a reduction in both solvent consumption and analysis waste. It is also possible to achieve a reduction in sample consumption, which may be important in some fields of analysis. On the other hand, the use of analytical columns with low internal diameter allows reducing the dispersion of the analytes by decreasing the chromatographic dilution, which translates into an improvement in the signal-to-noise ratio, resulting in a significant increase in sensitivity with respect to conventional LC systems. Additionally, the low flow rate allows

direct hyphenation with mass spectrometry (MS) and flame ionization detector (FID) [91, 92]. It is known that the use of capillary columns with small i.d. values improve chromatographic efficiency, as well as minimizes band broadening [93].

Despite the advantages obtained with the decrease of the i.d. in miniaturization of the LC systems, another parameter of interest is the particle size of the stationary phase. In that sense, these particles have undergone modifications, such as the decrease of the diameters, superficially porous particles and a more diverse range that has contributed to a better separation performance [94]. Other instrumental components of miniaturized systems must be modified, so, in the following lines, the main components of miniaturized LC instrumentation are briefly discussed.

The solvent delivery for miniaturized LC systems involves important challenges to ensure the generation of precise, accurate and pulseless pumping at low flow rates, achieving µL or nL per min. The first commercial devices developed included a mobile phase split valve coupled in the pump outlet, to achieve a nano and capillary flow rate. However, this system has some drawbacks, such as not achieve a significant decrease in waste generation, irreproducible results due to variable split ratios, and flow fluctuations due to the varying viscosity of the gradient solvent mixtures [95].

Nowadays, piston and syringe pumps continue being the pump of choice in new miniaturized HPLC systems. The miniaturized dual-piston reciprocating pump with an electronic controller is capable of assuring reproducible flow rate under isocratic and gradient elution without splitters. However, most commercial systems still use a splitter after the mixer chamber, so a high amount of the mobile phase goes to waste. On the other hand, a syringe pump may be thought of as a type of piston pump without pulsation. Nevertheless, they find limited to a finite volume of solvent in the inner reservoir for the separation. A way to overcome this limitation is the configuration of LC miniaturized with two or more syringe pumps, it allows a continuous mobile phase flow in isocratic mode or performing gradient mode with a finite volume of solvent [96].

In the last years, the application of electroosmotic pumps (EOPs) has been popularized, due to the growing trend in microanalytical systems. These pumping systems use electroosmosis through charged porous media (pumping elements) to generate pressure and flow pulse-free, offering a cost-effective and simplistic method, readily miniaturized and integrated [97]. Despite their advantages, the EOPs show some limitations, as pump-solution incompatibility with high organic contents, flow rate fluctuations, unstable voltage sources, and/or chemical breakdown within the pumping element itself [98]. However, more efforts in research are needed to overcome its limitations before they will ever reach the market.

Injector choice influences at void volume, flow disturbance, and precision to reduce band broadening and increase resolution. However, due to their inherent small size, injectors not undergone big transformations over the years. Until now, two categories are available: (1) stop-flow and (2) continuous-flow injectors. The last one has been widely used in prototypes and commercial systems; nevertheless, they typically cause an increased dead-volume. On the other hand, Stop-flow injectors allow for much lower dead-volume, so in the last few years they have been used with either manual control or an actuated switch [99]. The extra-column dispersion in miniaturized LC must be avoided by the use of connections tubing and fittings of reduced inner diameter (25-75 μm).

The miniaturization of the rest of the components of the chromatographic system involves the resizes the detectors, in order to maximize the detectability, resolution, and efficiency. The simplest class of detectors is absorbance detectors, such as UV-Vis absorption detector, which cell volume in miniaturized LC is reduced in comparison with conventional instruments [100]. Recently, the development and application of the small size LED-UV absorption detector has shown good results in portable miniaturized LC [101]. Likewise, miniaturized mass spectrometry development and commercialization are progressing rapidly [102], and many applications are anticipated since MS presents good selectivity, detectability, and can generate additional chemical structural information.

In general, liquid chromatography systems can be classified according to the size of the internal diameter of the analytical column or the flow used in the mobile phase, both criteria being dependent on each other, as well as critical when defining their performance, coupling and applications. Figure 5 shows the currently accepted classification for liquid chromatography systems and their typical values.

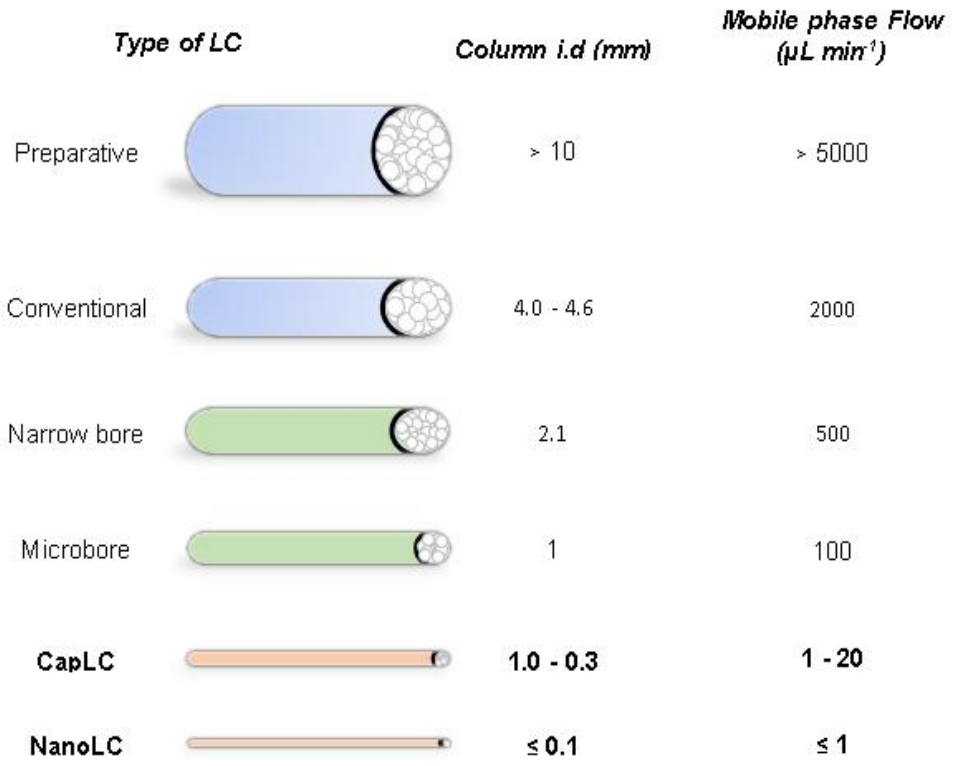


Figure 5. *Classification of liquid chromatography systems and operational parameters.*

The application of LC miniaturized systems has been important for the resolution of analytical problems, becoming effective and versatile tools for the study of various matrices and analytes. Table 2 presents some selected examples of the application of miniaturized liquid chromatography found in the literature.

1.3. PORTABLE LIQUID-CHROMATOGRAPHY

The development of miniaturized systems has opened the possibility of the introduction of hand-portable LC instruments, which have significant potential for solving analytical challenges in various applications in situ or at-situ monitoring, due to their ability to perform measurements at the point of sampling. The potential applications of portable LC systems focus on the need to obtain results quickly, overcome problems in the preservation of the sample or remote location of the monitoring site.

Table 2. *Examples of application of different procedures based on miniaturized LC.*

LC system-Detector	Application	Column specification			Stationary phase or coated material	Reference
		i.d. (µm)	Length (cm)	Flow rate (µL/min)		
CapLC-UV	Pharmaceuticals in biological sample	300	15	4	C18, 2 µm	[103]
CapLC-DAD	Sulfonylurea herbicides in environmental water	300	15	10	C18, 5 µm	[104]
CapLC-MS	Antidepressants in human blood drop	500	25	20	C12, 4 µm	[105]
NanoLC-UV	Pharmaceutical drugs in commercial preparations	75	20	0.23	Poly (glycidyl methacrylate-coethylene dimethacrylate) monolith	[106]
NanoLC-DAD	Δ9-tetrahydrocannabinol, cannabidiol and cannabinol residues on different surfaces	75	5	0.80	C18, 3.5	[107]
NanoLC-MS	Pesticides in food	75	15	0.30	C18, 3	[108]

DAD: diode array detection, MS: mass spectrometry, UV: ultraviolet absorption.

Like any other LC instrument, the main components that a portable LC system must include are (a) an eluent pump; (b) an injector assembly to introduce the sample; (c) a column that separates the analytes, and (d) a detector to record and, ideally, quantify the individual components that leave the column [109]. At this point, it is important to establish that there can be three types of portable instruments, based on their size: handheld, person-portable, and transportable [110]. In that sense, until a few years ago the use of these systems has involved an important challenge, mainly due to the inconveniences associated with the reduction of solvent consumption and requirements for size, weight and system power [111].

According to Sharma et al [112], there are at least ten essential requirements to consider an LC system as portable, related to aspects of size, performance, operation and functionality:

(1) weighs <7 kg and measures < 16,387 cm^3;

(2) contains all necessary electronics, digital interface and software integrated;

(3) allow at least 8 hours of operation;

(4) is easily operable with minimal supervision;

(5) is rugged enough to withstand changes in temperature and humidity;

(6) needs short instrument warm-up time;

(7) uses low amounts of toxic organics;

(8) is customized for capillary column use with a non-splitting flow arrangement, non-splitting injector, and low extra column volume to minimize dispersion;

(9) is integrated with a small detector that has excellent sensitivity; and

(10) is capable of binary gradient generation, competitive in performance to benchtop instruments.

To date, some works have reported on the development of hand-portable LC systems, the first of which was developed by Baram et al. [113] in 1996, with a power requirement similar to conventional LC systems and was used for the analysis of various compounds of environmental interest. In the following years other systems appeared, however some presented operational problems or limited applications [111]. Some other commercial systems were deprecated, while certain works that attribute the term of portability do not complete the requirements to be considered as a portable.

Recently, Lam et al. published the results of the development and application of a homemade miniature capillary LC system for the determination of small pharmaceutical molecules by UV detection and capable of coupling to a mass detector, with excellent performance [114]. Axcend® company has introduced in 2019, a new portable nano liquid chromatograph with an on-column UV-absorption detector [115]. Flow rate of the mobile phase in the order of 2 µL in gradient elution is achieved. Here, the performance of this instrument was checked and evaluate what variables are needed to optimize for obtaining suitable results. Table 3 includes a comparison of current and historical portable LC systems reported in the literature, with special attention to their applications and compliance with the portability criteria established by Sharma.

Table 3. *Summary of modern and historical portable LC systems reported in the literature.*

Portable LC systems	Application field / analytes	Size and weight specifications		Essential requirements [a]			
		Total weight (kg)	Total dimension (cm^3)	Physical characteristics	Performance	Operation	Functionality
Baram (1996) [113]	Environmental / Pesticides, phenols and phthalates	14	31800	0	+	++	+
Tulchinsky (1998) [116]	Environmental / Organic and inorganic compounds	9.5	23575	0	+	++	++
Ishida (2012) [117]	Environmental / Alquilphenols	2.0	9828	+++	+	+++	++
Sharma (2014) [111]	Standards / Uracil and alkyl-substituted benzenes	4.4	7812	+++	++	+++	+++
Lam (2019) [115]	Environmental / Pesticides, phenols and phthalates	2.7	7252	+++	+++	++	++
Chatzimichail (2019) [118]	Environmental / Pesticides, phenols and phthalates	6.7	13398	++	++	++	+++
Abonamah (2019) [110]	Forensic / Fentanyl and its derivatives	37	61560	0	+++	+	+
Current work (2020)	Environmental / Trimethylxanthines	7.8	14720	++	+++	+++	+++

[a] The essential requirements are evaluated on a scale of *0* for noncompliance and *+++* as a higher grade of compliance.

1.4. MATRIX AND ANALYZED COMPOUNDS

The study of this thesis has focused on the applications of the analytical methodologies described above, in biological, environmental, food and natural product matrices, to study a series of compounds belonging to several families and with different polarities. Therefore, the approach of this chapter will be based on the matrices analyzed.

1.4.1. Biological samples: trimethylxanthines

Analysis of biological samples represents an important challenge to overcome in the development of analytical methods due to very demanding requirements in terms of method reliability, sensitivity, speed of analysis and sample throughput. The terms “specimen” and “sample” are accustomed denote a portion of a body fluid, tissue, incubation medium, etc., collected under defined conditions [119]. Biological samples usually encompass fluids like whole blood, serum, plasma, urine, saliva, breast milk, sweat, cerebrospinal fluid, gastric fluid; exhaled breath (gas sample); and solid samples include a different kind of tissue (i.e., hair, nail, skin, bone, muscle).

Despite this large number of specimens, it is possible to indicate as those of greatest analytical interest the blood, including serum and plasma, urine and, recently, saliva, for the analysis of drugs, metabolites, xenobiotics or biomarkers. Plasma, serum and blood are normally used if quantitative measurements are needed. In urine the concentrations of target compounds, and their metabolites, tend to be higher than the ones found in blood, thereby facilitating detection [120]. In recent years, saliva has attracted attention due to advantages for its collection as it is a non-invasive procedure.

Serious complications for analysis of biological samples include the presence of endogenous or exogenous macromolecules, small molecules, and salts that interfere with analysis; the low analyte concentration and, generally, the incompatibility of these kind of samples with analytical instruments [121]. In that sense, sample preparation is of paramount importance to obtain the target analytes, meeting at least four requirements: (a) reduce or eliminate matrix or unwanted interference endogenous compounds; (b) increase selectivity for interest analytes; (c) pre-concentration effect to improve sensitivity; and, (d) stabilize the sample by reconstituting it in an inert solvent [122].

In general, analysis of biological samples involves the application of some pretreatment procedures, which can facilitate instrumental analysis (dilution), minimize the presence of endogenous substances (centrifugation, filtration and saponification), reduce quantitative errors due to the extraction procedure (internal standards) and/or release metabolized analytes as conjugated substances (enzymatic hydrolysis) [123].

Other aspects no less important, related to the analysis of biological samples, are several ethical, legal and social issues co-exist. The confidentiality, ownership, export, storage and secondary use of samples (individual good) with specific consent, regulations and policies must be established in accordance with the guidelines of the Research Policy Ethics Committees (REC). [124].

In the following lines of this section, aspects related to the collection, storage, preservation and important issues of the biological samples studied during the development of this thesis will be discussed. In the final part, a description of the analyzed compounds is presented.

1.4.1.1. Blood, plasma and serum

Whole blood is probably the most widely used complex biological matrix in bioanalysis and shows the best correlation between the pharmacological effect and the concentration of the compound [125], so is preferentially selected for quantitative analyzes. However, it has some well-known disadvantages, such as invasive collection, the trained required personnel and the need for special storage conditions. Whole blood is a complex but relatively homogeneous matrix, composed of blood cells suspended in blood plasma. Plasma constitutes more than 50% of blood fluid, is constituted by water, proteins, glucose, mineral ions, hormones, and erythrocytes, leukocytes, and platelets.

Plasma and serum are derived from whole blood that undergone different biochemical processes after blood collection, by refrigerated centrifuging. The serum is obtained from blood that has coagulated and centrifuged to separate blood cells and coagulation factors. To obtain plasma, an anticoagulant is added before the removal of blood cells. So, the major difference between both, that no anticoagulants are used in the collection of serum and all the fibrinogen and associated proteins are removed through the clotting process [126].

Although plasma and serum are generally considered to have similar compositions and properties, some analytes, as in the case of metabolomic studies, show differences in both. However, in some cases, it is preferred to work with serum, because it produces less precipitate by freezing and thawing cycles, and additionally, there is an absence of additives that may interfere. This has special attention in miniaturized techniques.

Normally, for the routine collection of plasma and serum samples, venous blood is immediately divided into two tubes, one is a tube containing anticoagulant for obtaining plasma, and the other a blank tube, for serum. Then, both tubes are isolated by centrifugation and then stored until analysis. Whole blood, plasma or serum samples are recommended to be stored at -20°C, and thawed at least twice if possible using a warm bath to prevent the protein unfolding [127].

Recently, dried blood spot (DBS) has gained a great interest in the bioanalysis field. Here, a blood sample is deposited onto a filter paper, followed by drying in the air for several hours. Subsequently, a disk is punched out from the blood spot and this disk is extracted for the target analytes. Some advantages of this technique include the ease of sampling (less invasive), small sampling volume, long-term analyte stability, and low solvent volumes needed in the extraction [128].

1.4.1.2. Urine

Normal and healthy urine is mainly composed of water, plus ions such as Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^- and NH_4^+, urea, creatinine, proteins, and products processed by the kidney and liver, including drugs and metabolites [129]. Urine sample has its own advantages, compared with serum and plasma: a large volume of non-invasive sampling can be obtained, sampling repetition is not a problem, requires less complex sample preparation due to the lower amounts of protein, lipids, and other substances of high molecular-weight that it contains. However, the lack of proteins and lipids can cause issues in bioanalysis, as these compounds perform a number of useful functions, prevents adsorption to containers, binds analytes and helps to solubilize them [130].

From the bioanalytical point of view, urine analysis has the advantage of the high concentrations of analytes and their metabolites, as it represents one of the main routes of elimination of the body, which facilitates the detection of the compounds of interest. Urine analysis is mainly used as a screening test for the determination of drugs of abuse and prescription drugs. The absence of circulating serum proteins, lipids and other related large-molecular-weight compounds greatly simplify the preparation of the specimen for bioanalysis, enabling the application of immunoassays or non-instrumental spot tests.

According to Fernandez-Peralbo et al. [129], urine samples are collected as random samples, timed samples or 24-h samples. The first is done at any time of the day, instead, while timed samples are necessary to study time-related trends to catalog metabolites with high diurnal variation in different species. However, a 24 h sample collection is preferred to eliminate the great variability in metabolite profiles. Midstream portions or clean-catch urine of first-morning samples are the most common and appropriate obtained specimens because the presence of contaminating elements is minimized.

Urine samples for clinical practice are usually collected in a sterile container, which is not endowed with special characteristics or reagents. The storage of urine samples is critical for the reliability of analysis results, due to changes in the concentration of analytes or the formation of some endogenous urine reactants that can appear [127]. Freeze-thaw cycles are related to sample handling and are another pivotal aspect, as it determines the exposition to degrading environmental

conditions, so it is recommended to avoid them and rapidly freeze and store pre-aliquoted samples to minimize potential degradation as much as possible.

An important issue related to the reported results in urine analysis is the variability of urine volume due to water consumption and other physiological factors. This makes that the concentration of targeted and untargeted analytes in urine varies widely. So, volume correction is necessary. Although there is no universally accepted procedure for this correction, some approaches based on urine volume, creatinine concentration, osmolality, and components that are common to all samples are strategies successfully used [131].

1.4.1.3. Saliva (oral fluid)

Saliva is an “ultra-filtrate” of blood, very dilute, and composed of about 99% water. Saliva contains compounds produced within the salivary glands (immunoglobulin A [IgA] and α-amylase) moreover as compounds diffused within the plasma (electrolytes, proteins, metabolites, and hormones) [132]. As this excretion product is actually a fluid mixture, the term “oral fluid” seems more appropriate, instead of “saliva” or “whole saliva”. This is of particular interest to define the recollection site of the sample: samples collected directly from the saliva glands (mainly the parotid glands) are defined as "whole saliva", and “oral fluid“ are the ones collected in the oral cavity.

Saliva represents a potential source of clinical information since salivary biomarkers can virtually reflect the state of a pathology such as oncological, cardiovascular, autoimmune, viral and bacterial diseases, as well as the presence of drugs [133-135]. Some studies have demonstrated the correlation between plasma or serum concentrations with those found in oral fluid for some substances, which facilitate the analysis without the need to obtain blood samples. As with urine, saliva can be collected through noninvasive means, performed by the patient himself or untrained caregivers. Actually, analysis of saliva is a potential substitute for blood, especially for long-term therapeutic drug monitoring or for screening a large number of patients, as well as for developing salivary point-of-care technology [136].

There are two modes of saliva sampling, unstimulated and stimulated collection. For stimulated collection, stimulants like paraffin, unflavored chewing gum base, cotton puff, and rubber bands will be accustomed to sample saliva by masticatory stimulation, because saliva is physiologically secreted in response to those stimulations. In the unstimulated collection, the secretions in the mouth are collected in the absence of exogenous stimuli and depend on the daily basal salivary flow rate in the oral cavity and is often preferred because it minimizes the dilution of analytes. For this, there are different ways to collect oral fluid including passive drooling and draining, spitting, and swab-based sampling [137].

The latter is particularly suitable for patients with less or no collaboration, such as disabled people, children, the elderly and newborns, which is why it was used in this work. These devices have a disposable non-cellulosic absorbent pad, which is placed under the tongue or near the cheeks and can take samples up to about 1.2 mL in a few minutes. Finally, the oral fluid sample is recovered by squeezing the pad or centrifuging into a container like an Eppendorf tube. Until now, there is no agreement on the storage temperature of oral fluid samples. A normal recommendation is that samples should be refrigerated (4°C) if they are processed within 3-6 h after collection. For long periods of storage, they should be kept at -20°C to prevent bacterial growth.

Recently Abdel-Rehim et al. have proposed a method called dried saliva spot (DSS), to see the quantity of lidocaine in saliva [138]. Some drops of saliva are spotted onto a group card and dry at room conditions, using 50 µL of the sample, and allowing easier transportation, storage, and pre-treatment of samples. Another application of DSS was reported for analysis of D- and L-lactic acid in diabetic patients, pre-diabetic and nominally healthy persons [139].

Table 4 shows a comparison of characteristics, advantages, and disadvantages of the biological samples addressed in the previous sections.

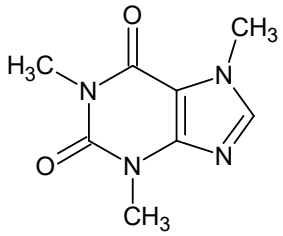
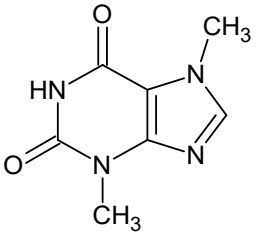
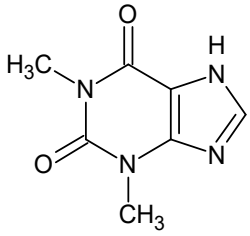
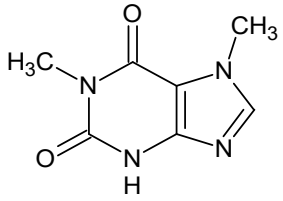
Table 4. *Comparison of the characteristics, advantages, and disadvantages of biological samples discussed. Adapted from [140]*

Characteristics	Blood (Plasma and serum)	Urine	Saliva (Oral fluid)
Sample collection	Requires medically trained personal	Privacy concerns; not easily field collected	Noninvasive
Amount of sample typically available	1–5 mL	>50 mL	1–5 mL
Speed of collection	Minutes	Minutes	Minutes
Drug concentration	High	Moderate-to high	Low
Window of detection average	Narrow	Moderate, usually wider than for blood	Narrow, similar to blood
Risk of infection	Higher than others	Low	Low

1.4.1.4. Determination of trimethylxanthines

Trimethylxanthines are a group of substances naturally present in beans, leaves, seeds, and fruits of more than 60 plants. The most common sources are coffee and cocoa beans, as well as tea leaves and guarana berries [141]. These compounds are alkaloids produced in plants as secondary metabolites, with high polar characteristics and low log Kow values (Table 5).

Table 5. *Chemical structure and log Kow of trimethylxanthines studied.*

Compounds	Structure	log Kow
Caffeine		-0.10
Theobromine		-0.80
Theophylline		-0.02
Paraxanthine		-0.22

Caffeine is the most consumed stimulant of the central nervous system, due to its presence in beverages such as coffee, tea, energy drinks, and cola drinks. The metabolic pathway of caffeine

(Figure 6) generates three main metabolites, in descending order of their metabolic concentrations, paraxanthine, theobromine, and theophylline, which produce different effects in the human body, including an increased concentration of adrenaline which then acts in increasing energy consumption and fat oxidation. Particularly, caffeine stimulates the respiratory center, increasing mean respiratory rate and improve pulmonary blood flow and enhanced diaphragmatic function and breathing pattern [142].

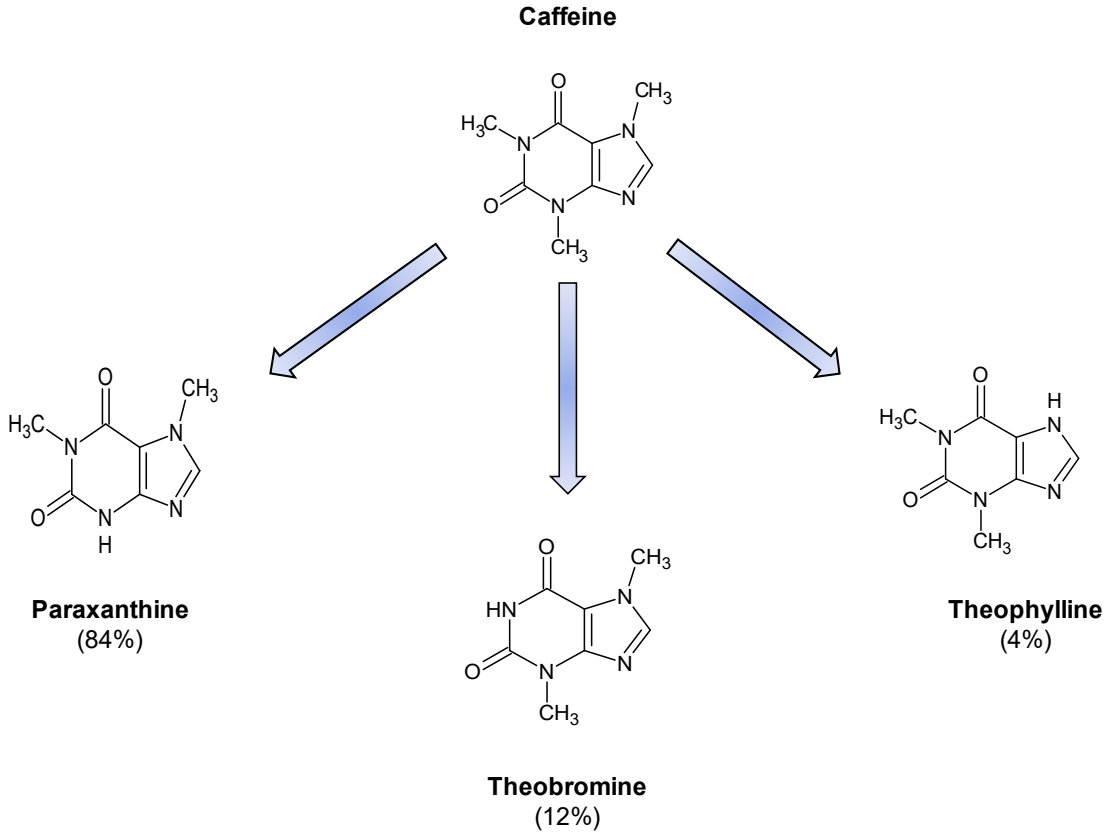


Figure 6. *Metabolic pathway of caffeine.*

These effects allow the use of caffeine in the treatment of apnea of prematurity in neonates to stimulate breathing efforts [143]. Apnea of prematurity (AOP) affects the majority of infants born prematurely, before 34 weeks of gestational age, and is defined under the following parameters: a recurrent cessation of breathing (>20 seconds) or respiratory pauses of shorter duration and/or oxygen desaturations (<90%) [144]. Some studies concluded that methylxanthines, and particularly caffeine [145], are effective in reducing the frequency of apnea of prematurity and the use of mechanical ventilation in two to seven days after starting treatment. The mechanism of action of methylxanthines involves the stimulation of the medullary respiratory centers, causing an increase in the detection of CO_2 and O_2, improving diaphragmatic function and bronchodilation [146].

Over other therapeutic guides for AOP, caffeine has important advantages related to its long half-life and wide therapeutic range [147]. The loading and maintenance dosage of caffeine in the treatment of AOP is 10 mg kg^{-1} of caffeine (intravenously) and 5–10 mg kg^{-1} every 24 hours (orally or intravenously) [148]. Dose between 5 and 30 mg L^{-1} is commonly used and under this therapeutic scheme, serum levels below 50 mg L^{-1} are considered safe. In clinical practice, caffeine citrate instead of caffeine is administrated, so the doses prepared from this compound are doubled to the aforesaid values.

In all therapeutic schemes, it is necessary to control the concentrations of the drugs administered by determining the blood levels of caffeine throughout the treatment, to ensure the expected clinical response, as well as possible signs of toxicity. For this, when therapeutic failure is suspected, blood samples should be obtained just before the administration of the next dose, and between 2 and 4 h after the previous dose, when toxicity is suspected. Perera et al., found an adequate correlation between plasma and salivary concentrations of caffeine when 100 mg of caffeine was administered to healthy male non-smoking volunteers, which may serve as a measure in future pharmacokinetic trials [149].

In the literature, many methods have been used for the determination of caffeine and its metabolites in biological samples, using traditional sample treatment techniques like liquid-liquid extraction and solid-phase extraction (SPE) [150-152]. These procedures involve many stages, high consumption of organic solvents and a high quantity of sample volume. This thesis proposes the determination of trimethylxanthines in serum, urine and oral fluid using IT-SPME-CapLC, which provides a simple and fast analytical methodology using micro volumes of samples.

1.4.2. Environmental samples: trimethylxanthines in waters

Pollution represents a significant public health issue in the world, which according to Landrigan et al, is responsible for approximately 25% of deaths in the most polluted countries and 16% of deaths worldwide [153]. The control and monitor of pollutants that can be found in water, air, soils, sediments, and biota require the study of environmental matrices. The different environmental pollutants can be found in their source of emission (places, objects, activities, or entities) and can be transported in the environment through their receiving environmental compartment, which in turn can act in some cases as a source of emission and reach human beings, through many processes. In this cycle, human beings are at risk of exposure to these contaminants in their daily activities.

From all these matrices, water has a particular interest as an environmental compartment, for its role in life and constant mobility on the planet by, so pollutants that reach the water cycle could eventually travel to other compartments. Factors like population growth and development can alter

certain stages of the water cycle, causing increasing contamination in soils, groundwater, air, and, other environmental compartments [154].

The Directive 2013/39/EU of the European Parliament and of the Council [155] classify the waters as continental and coastal waters. Inland waters are understood as all surface or underground waters, located towards the ground from the line that serves as the basis for measuring the width of territorial waters. They can be classified in groundwater or surface water (rivers, lakes, transition, etc.). Waters that extend between this line and a nautical mile inland are the so-called coastal waters. Another important type of water is wastewater, defined as any type of water whose quality has been damaged by anthropogenic activities.

In the last ten years, the presence of so-called "emerging" or "new" contaminants such as pharmaceutical compounds and their metabolites in the aquatic environment, both water and wastewater, has emerged as a major concern for the international scientific community on the need to establish controls for their regulation [156]. According to Mackulak et al. [157], more than 200 active pharmaceutical substances have been identified in the environment, which enters in it according to its pattern of usage, as well as the mode of application, and for those coming from human use and/or excretion, sewage discharge is a very important source for the aquatic environment.

Until today, the number and variety of these compounds are constantly increasing, as well as their metabolites, which are detected even in natural compartments, which makes its control and elimination difficult. Likewise, some of these compounds are considered as indicators of anthropogenic activity, since their presence in environmental matrices come mainly for human use and elimination [158, 159]. In that sense, wastewater has a wide range of chemical and biological markers of human activity, whose study has been used for decades to monitor removal efficiencies of wastewater treatment processes and to evaluate wastewater effluent as a point source for environmental contamination [160].

Trimethylxanthines are an important group of compounds used as biomarkers of human activity on wastewater under an epidemiological approach [161], and also as an indicator of lifestyle [162] and for the evaluation of their intake [163]. The total daily intakes of these compounds varies throughout the world, although coffee usually contributes more significantly more than other drinks to overall caffeine consumption (coffee 71%, soft drinks 16% and tea 12%), particularly among adults [164].

High stability and high consumption of these products have made it common to detect them in considerable concentrations in surface water [165], seawater [166], stormwater [167], drinking

water [168] and groundwater [169]. Recently, some studies have reported high concentrations of caffeine and paraxanthine in wastewater treatment plants (WWTP) [170]. Likewise, low concentrations downstream of WWTPs demonstrate the efficiency in the degradation and elimination of these molecules [171]. Figure 7 shows the growing interest related to the study of trimethylxanthines in environmental issues, provided by the increase in the evolution of the number of citations in the last thirty years.

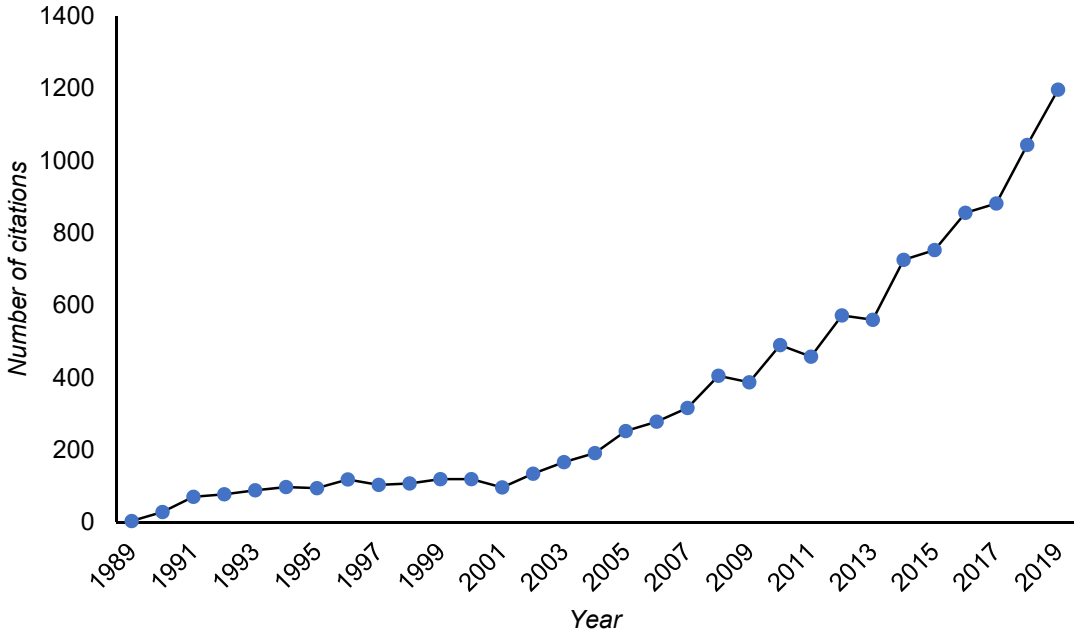


Figure 7. *Evolution in the number of citations on caffeine and trimethylxanthines in the topic environment in the last 30 years. Source Web of Science (May 2020).*

In general, for environmental analysis, pollutant concentrations may be below instrumental sensitivity, so nowadays the need for reliable and sensitive analytical methods is highly desirable. Likewise, sample treatment techniques with high preconcentration capacity and selectivity are necessary [172]. Along these lines, SPE has been widely used with adequate results [173], applying new polymeric sorbents, which improve retention by hydrophilic-hydrophobic mechanism, especially for molecules with high polarity characteristics such as trimethylxanthines [174]. Despite the advantages of SPE, some drawbacks have been indicated including a large sample volume, the use of organic solvents, and time required [175].

Table 6 shows some of the analytical methods developed in the last five years for the determination of trimethylxanthines in environmental water samples by liquid chromatography with mass spectrometry detection. In terms of extraction techniques, predominate the application of SPE.

In those studies, concentrations for caffeine were reported, mainly in upstream of WWTP´s. In this thesis, a method with portable NanoLC-UV has been developed, which has been compared with IT-SPME CapLC method, for the analysis of caffeine, theobromine, and theophylline in seawater and river samples.

1.4.3. Dietary supplements: Multiclass analysis

Dietary supplements, also called food or health supplements, are defined as products that are intended to supplement the diet, which may include, some substances as vitamins, minerals, botanicals (herbs), amino acids, or concentrates, and extracts of these [181]. According to the European Directive 2002/46/EC, are 'foodstuffs to supplement the normal diet, with a nutritional or physiological effect, alone or in combination, marketed in various dosage forms, such as liquids and powders, designed to be taken in measured small unit quantities' [182].

The rising popularity of these products, especially those offered as natural food supplements and alternative herbal medicines, is due in part to be conceived as free of adverse effects. A wide variety of these products, administered as tablets, capsules, concentrated extracts, and infusion bags, are extensively promoted in marketing campaigns to influence the idea to have "magical results" for weight loss, to be anti-carcinogenic, to improved sports performance, and to be stimulant, revitalizing and "healthier" than conventional pharmaceutical drugs [183].

Some researchers have estimated that dietary supplements represent an industry with sales gains of more than $ 37 billion annually, only in the USA [184]. Particularly, weight loss products constitute a fast-growing segment of this field which increases annually by 10-20% [185]. This can be explained due to the high availability of these products in grocery stores, supermarkets, and e-commerce platforms. Dietary supplements are products registered as food, which in terms of requirements for production, quality control and commerce are much more lenient than guidelines for pharmaceutical products [186, 187].

Although the main components of dietary supplements have a natural origin, a trending topic in recent years is the adulteration with pharmaceutical active ingredients as some works have shown [188]. These adulterants are added and not reported on the composition of the package label, even in almost all cases they are present at potentially dangerous doses. This fact means that consumers are not aware that they are taking other compounds, which at high doses can have toxic effects and/or drug interactions with other medical treatments [189]. Adulteration of dietary supplements with anabolic steroids [190], erectile dysfunction drugs [191], pharmaceuticals and plant toxins [192], synthetic drugs [193], antidiabetic [194] and abuse drugs [195] has been reported in the literature.

Additionally, the control in the composition of these products is made even more difficult since new formulations are constantly introduced.

Table 6. *Analytical methods in last five years for the determination of trimethylxanthines in environmental water samples*

Analytes	***Matrix***	***Analytical technique***	***Sample Preparation***	***Sample Volume (mL)***	***Concentration found (μg mL^{-1})***	***Reference***
Caffeine and diverse markers	Environmental waters	LC-MS-MS	SPE (HLB)	1000	0.2 - 0.5	*[176]*
Caffeine and metabolites	Wastewater	LC-MS-MS	SPE (HLB)	3	17	*[177]*
Caffeine and emerging contaminants	Tap water	LC-MS-MS	SPE (polymeric)	5	0.5	*[169]*
Caffeine and emerging contaminants	Wastewater	UPLC/TQD-MS	SPE (HLB)	50	0.8 (Upstream) 2.0 (Downstream)	*[178]*
Caffeine and biomarkers compounds	Wastewater	LC-MS-MS	SPE (polymeric)	250	36 - 77	*[179]*
Caffeine	Wastewater	LC-MS	SPE (polymeric)	100	0.8 - 9.5 (Upstream) 0.1 - 1.9 (Downstream)	*[180]*

LC-MS/MS liquid chromatography-mass spectrometry in tandem, SPE solid-phase extraction, HLB hydrophilic-lipophilic balance, UPLC/TQD Ultra performance liquid chromatography/triple quadrupole-mass spectrometry.

In recent years, there is a growing concern about the composition, quality, labeling, and safety of dietary supplements, which has resulted in the establishment of new regulations [196, 197]. The presence of botanical extracts makes that dietary supplements have a diverse and complex chemical composition, and many compounds that may be present, some of them considered as major compounds, as well as trace compounds. The following lines present a description of major and trace compounds commonly found in dietary products.

1.4.3.1. Major compounds

Nowadays, dietary supplements with different functions and compositions are widely available, such as slimming dietary supplements, also called fat burning for weight loss, multivitamin formulations, natural extracts, and sexual enhancement. The main compounds responsible for these desirable effects are the following.

Chlorogenic acids

Phenolic compounds are widely found in nature, among which chlorogenic acids (CGA) are a family of esters formed between certain phenolic acids (trans-cinnamic acids) and quinic acid. Chemically, chlorogenic acids can be classified according to the type, number, and position of acyl residues, where the most common isomer is 5-caffeoquinine acid [198]. Another related compound to chlorogenic acids in caffeic acid, which has structure and properties quite similar to these. The main subgroups of CGA are caffeoylquinic acids (CQA), dicaffeoylquinic acids (diCQA) and feruloyilquinic acids (FQA), each one of these groups have, at least, three isomers.

Although these compounds can be found in a variety of plants, mainly green (or raw) coffee is a major source of CGA in nature, with an estimated percentage between 5 and 12% [199]. Green coffee has a mild aroma similar to the characteristic aroma of coffee during the roasting process. According to the literature, the scientific evidence is abundant to support the great health benefits of CGA to humans. Recent studies demonstrated that they exhibit antimutagenic, anticarcinogenic and antioxidant activities. Additionally, these compounds act as protective agents, reducing the oxidative stress of cells, and they are also being investigated for their positive effect on blood pressure and glucose regulation [200].

Roshan et al. [201], reported that the ingestion of the green coffee extract could feasibly be an effective approach for the management of some of the metabolic syndrome features, as well as insulin resistance, and abdominal obesity. Likewise, the ingestion of green coffee seems to facilitate weight loss and show a tendency to reduce visceral fat and body weight, that is why green coffee extracts are common components of dietary supplements.

Caffeine

Caffeine, as well as theobromine, has a potential thermogenic effect and reduces energy intake. Although these compounds are naturally present in coffee, green coffee or tea extracts, powdered pure caffeine is added intentionally as an adulterant in dietary supplements for physical fitness and weight loss. This is of special attention for those fortified products with high quantities marketed in bulk containers since they can present a significant or unreasonable risk of disease, injury or even toxic doses [202]. Furthermore, the combination of trimethylxanthines with other thermogenic ingredients, such as adrenergic amines, in these products can represent a major problem due to the adverse drug interactions that may occur.

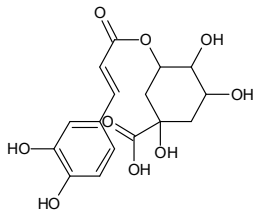
Synephrine

Synephrine is a biogenic protoalkaloid, chemically similar to ephedrine and catecholamines, commonly present in various citrus species (*C. sinensis, C. limon, C. limoni, etc.*), especially in bitter oranges (*Citrus Aurantium*). It has been used in Asian traditional medicine and in the Mediterranean region as a cardiac and vascular stimulant, and in the treatment of digestive and gastric problems [203]. Normally this molecule can be found in the human body at very low plasma concentrations, which is why it is considered as a trace amine. Citrus aurantium extract contains para-synephrine at a 4-6% level [204].

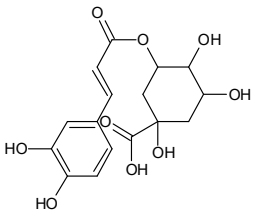
Both synephrine species have an affinity for adrenergic receptors present in adipose tissue, therefore they stimulate lipolysis. Zheng et al. recently reported that this molecule modulates NMUR2 receptors in the hypothalamus, achieving appetite suppression in humans [205]. Due to these effects, Citrus aurantium extract has been extensively used in dietary supplements as a principal constituent in fat-burning weight-loss products. The FDA's ban on the use of ephedra alkaloids in dietary supplements in 2004, meant that synephrine quickly replaced them, making their use popular.

The effectiveness, as well as the safety of the intake of products containing synephrine, is frequently questioned. For some authors, there is no solid evidence to support the beneficial effects of synephrine as a lipolytic agent [206]; on the other hand, Stohs et al. concluded that modest increases in weight loss were observed with bitter orange extract in a study of three months [207]. According to Jordan et al. [208], severe cardiovascular symptoms associated with the intake of dietary supplements containing synephrine were reported between 1998 and 2004. In 2010 a new guide to restrict the daily limit of 30 mg of synephrine as a value for maximum intake was published [209]. This makes it necessary to quantify synephrine concentrations in dietary supplements. Recently, some studies have denied the risk of the cardiovascular effects of p-synephrine [210, 211].

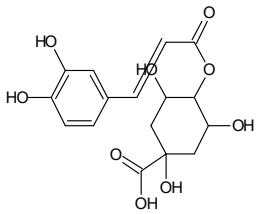
Figure 8 shows the chemical structure and log Kow for major compounds found in dietary supplements.



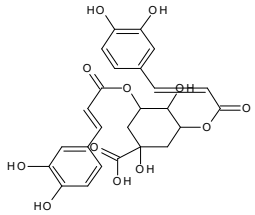
5-caffeoylquinic acid
(5-CQA)
log Kow = -0.36



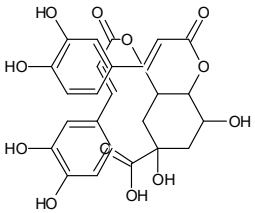
Neochlorogenic acid
(3-CQA)
log Kow = -0.4



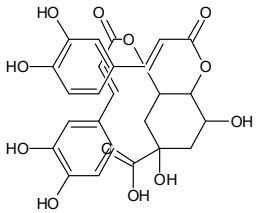
Cryptochlorogenic acid
(4-CQA)
log Kow = -0.4



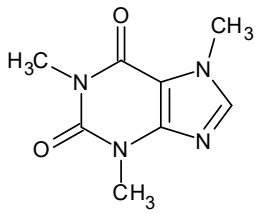
Isochlorogenic acid A
(3,5-diCQA)
log Kow = 1.5



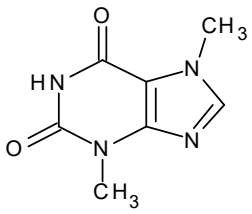
Isochlorogenic acid B
(3,4-diCQA)
log Kow = 1.5



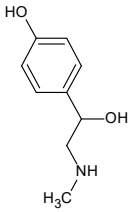
Isochlorogenic acid C
(4,5-diCQA)
log Kow = 1.5



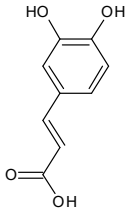
Caffeine
log Kow = -0.1



Theobromine
log Kow = -0.8



Synephrine
log Kow = -0.45



Caffeic acid
log Kow = 1.15

Figure 8. *Chemical structure and log Kow for major compounds found in dietary supplements.*

1.4.3.2. Minor compounds

Due to the natural origin of dietary supplements, numerous compounds in low concentrations, considered as minor compounds, are present. Although they are not normally reported, they can have physiological effects that can cause synergistic or antagonistic effects.

Riboflavin

Riboflavin, also called vitamin B2, is a water-soluble vitamin, normally found in a wide variety of foods, including milk, cereals, fatty fish, meats and certain fruits and vegetables. Chemically it is a nucleoside with the presence of flavin and ribitol. The deficiency of this vitamin has been linked to

some diseases like anemia, cancer, and cardiovascular disease. Likewise, riboflavin is also thought to protect tissues from ischemia [212]. Some sanitary guidelines establish the estimated average requirements for riboflavin in 0.4 mg per day [213], so the consumption of dietary supplements containing this compound may be beneficial.

Kaempferol

Flavonoids are polyphenolic compounds, which have commonly been incorporated into the human diet through vegetal foods. Kaempferol is a flavonoid abundantly found in tea, broccoli, apples, strawberries, and beans. The presence of these aliments in all food guide models around the world [214] ensures a high consumption in all populations. This molecule shows antioxidant and anti-inflammatory properties and activates thermogenic processes, which is why it is found in several dietary products. Additionally, it has been shown to invoke several different mechanisms in the regulation of cancer cells [215].

Terpenic compounds

Terpenes are organic compounds derivatives of isoprene. They are produced in a high variety of plants and are the most representative molecules in essential oils (> 90%). Terpenes have a great variety and diversity of structures, the most common in nature being monoterpenes and sesquiterpenes. The basic structure of monoterpenes consists of two linked isoprene units, which are formed by a 5-carbon-base (C5) each [216]. There are different types of monoterpenes in nature, including acyclic ones (such as linalool and myrcene), monocyclic monoterpenes (for example, limonene among others), and bicyclic monoterpenes (as alpha and beta-pinene). Sesquiterpenes present three isoprene units in its structure, and such as monoterpenes they may be acyclic or contain rings. Farnesene is an acyclic sesquiterpene.

Numerous studies have attributed the biological activity to terpenic compounds, which is why they are used as antioxidants, antifungals, and antibacterial [217]. Likewise, all of these compounds present important anesthetic and sedative effects [218-220]. Historically terpenic compounds have been applied in flavorings and fragrance industry, but recently, there is an increasing interest in its application in the pharmaceutical and nutraceutical industry. Due to the plant nature of some dietary supplements, terpenic substances are expected to be present as minor components. Chemical structure and log Kow for trace compounds found in dietary supplements are given in Figure 9.

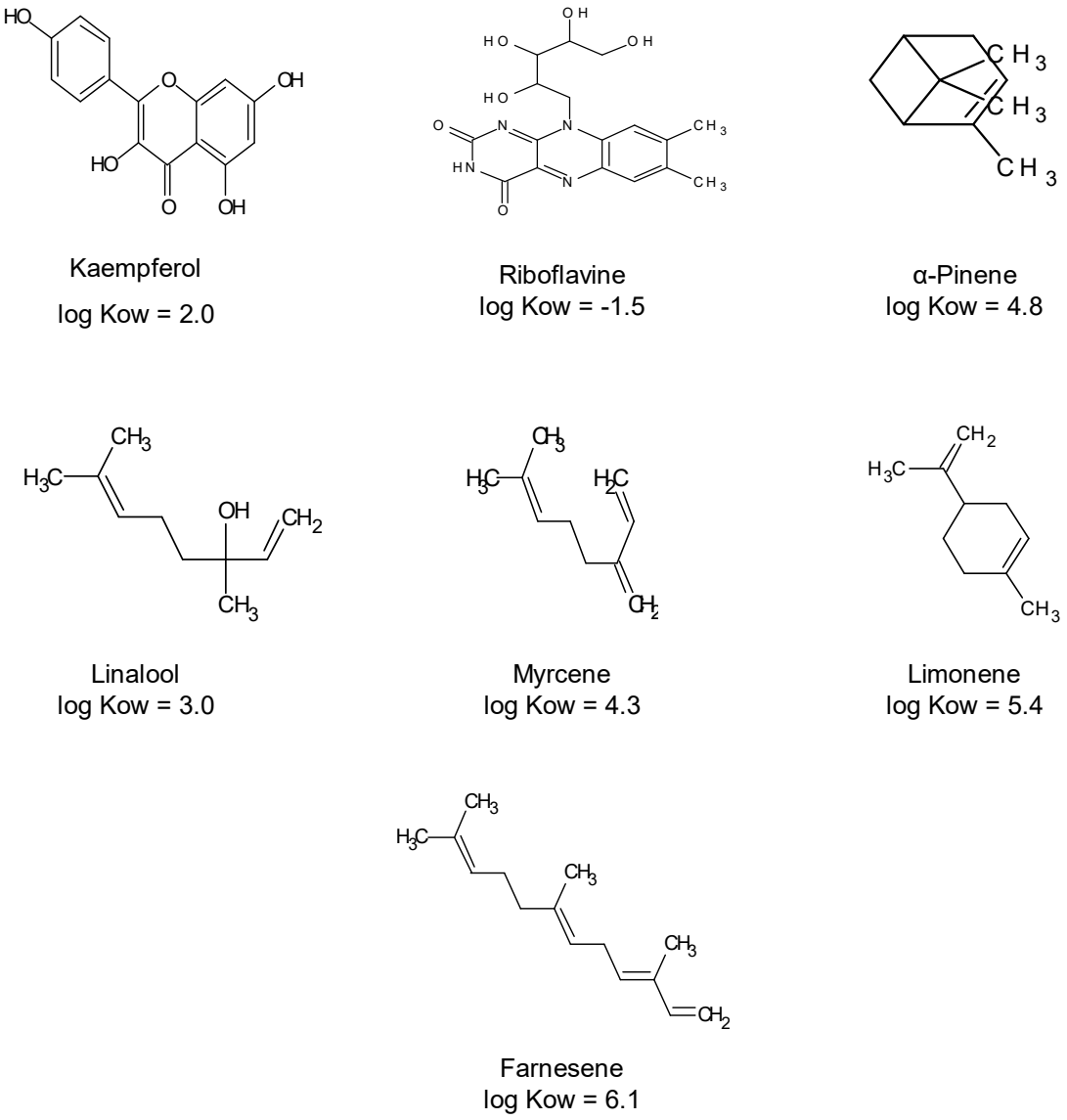


Figure 9. *Chemical structure and log Kow for trace compounds found in dietary supplements.*

1.4.3.3. Analysis of dietary supplements

The analysis of dietary products can be carried out by two approaches, by the determination of specific components or by multi-class analysis methods, the latter being preferred according to the principles of green analytical chemistry. However, multiclass methods involved sophisticated and expensive instrumentation such as UHPLC and high resolution mass detectors. Thus, simple and rapid methods for the qualitative and quantitative determination of major and trace compounds in dietary supplements are highly desirable for improved sample characterization with a minimum of experimental effort

Table 7 shows summarized the different methods found in the literature for the analysis of dietary supplements by LC, some for individual determination of chlorogenic acids, synephrine, caffeine, minerals or vitamins, and others for multi-class analysis. Due to the increasing concern about the composition of dietary supplements, in this thesis, a multi-class method was developed, based on capillary liquid chromatography for the simultaneous analysis of seventeen compounds that can be found in those supplements at different concentration levels.

1.4.4. Natural resins: terpenic compounds

According to Langenheim [226] in general terms, resins are a sap or exudate, different from other vegetable exudates such as gums, mucilage, oils, waxes, and latex. Natural resins are defined as plant secretions formed by a lipid-soluble mixture of organic compounds, including a volatile and non-volatile terpenoid fraction, and/or secondary phenolic compounds, secreted in specialized structures, that can be located internally or on the surface of the same, and that show potential importance in ecological interactions.

The composition of resins consists primarily of secondary metabolites, which apparently play no role in the fundamental physiology of the plant, with two fundamental fractions. In the first place, a volatile fragrant fraction called essential oil, and on the other hand, a non-volatile fraction, usually consisting of long-chain terpenoids [227]. The percentages and quantity of these fractions highly depend on two aspects, the botanical origin and the age of the resins. In fresh resins, the presence of the essential oil fraction makes the physical aspect of the material to be as translucent liquids, but by lossing of this fraction, the material change into brown, yellow, or white solids that, by polymerization and oxidation reactions, looks like an amber material.

Regarding botanical origin, the family of the *Burseraceae*, which includes over 20 genera and more than 600 species of trees and shrubs from tropical and subtropical regions, three important genera have been studied widely, *Boswellia*, *Commiphora*, and *Bursera*. The first one is commonly called frankincense, myrrh is the resin of *Commiphora*, and *Bursera* resin is often called copal, all of them with important use in perfumery and particularly as an incense [228]. The Bursera genus grows widely in the Mesoamerica region, from the southwestern United States to the northern part of Brazil and includes between 90 and 100 species of trees and shrubs [229], historically has been the source of resin for the Aztec and Mayan civilizations.

Table 7. *Analytical methods found in the literature for the analysis of dietary supplements by LC*

Approach	*Analytes*	*Matrix*	*Analytical technique*	*Analytical column*	*Chromatographic conditions*	*Detector*	*Reference*
Specific components	5-CQA, 4-CQA, 3-CQA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA	Green coffee beans extracts	LC-UV	C18 (100 x 4.8 mm; 5 µm)	Mobile phase: 0.1% TFA in water (A) and acetonitrile (B). Gradient elution Flow rate: 1.5 mL/min	330 nm	*[198]*
	Antioxidant markers and caffeine	Commercial teas and dietary supplements	LC-DAD	Inertsil ODS-3v (250 x 4.6 mm; 5 µm)	Mobile phase: water containing 0.05% orthophosphoric acid: acetonitrile (85:15 v/v). Isocratic elution. Flow rate: 1.5 mL/min	200 - 400 nm	*[199]*
	Synephrine and amines	Food supplements	LC-FLD-UV	C-18 (250 x 4 mm, 5 µm)	2.9 g/L SDS in water (pH = 4.2, ortho-phosphoric acid) (A): 2.9 g/L SDS in acetonitrile (62:38, v/v) (pH = 4.2, ortho-phosphoric acid) (B). Gradient elution. Flow rate: 1.0 mL/min	FLD: 270/305nm (Ex/Em). UV: 224 nm	*[200]*

Table 7. *Continuation.*

Approach	*Analytes*	*Matrix*	*Analytical technique*	*Analytical column*	*Chromatographic conditions*	*Detector*	*Reference*
	13 Slimming drugs (caffeine, synephrine, ephedrine, sibutramine)	Dietary supplements	LC/TOF-MS	ODP2-HP 2D column (150 × 2.0 mm, 5 µm)	Mobile phase: 10 mmol/L ammonium formate buffer (pH 5.0) (A): methanol (B): 10 mmol/L formic acid (C). Gradient elution. Flow rate: 0.2 mL/min	ESI +, capillary 2500 V	*[201]*
Multi-class							
	111 amine-based (p-synephrine, trimethylxanthines, ephedrine,	Weight loss and ergogenic supplements	LC-QToF-MS	YMC Triart (100 × 2.0 mm, 3 µm)	Mobile phase: water (0.1% formic acid) (A): acetonitrile (0.1% formic acid) (B). Gradient elution Flow rate: 0.21 mL/min	ESI +, capillary 3000 V	*[202]*

LC-UV liquid chromatography-ultraviolet detector; LC-DAD liquid chromatography-diode array detector; LC-FLD liquid chromatography-fluorescence detector; LC/ToF-MS liquid chromatography/Time-of-Flight Mass Spectrometry; LC-QToF-MS liquid chromatography-quadrupole time of flight mass spectrometry; ESI + electrospray ionization positive mode

In Maya languages, the term "pom" was assigned to the resin obtained of diverse trees. Copal word derives from "copalli", the term used by Aztecs in Náhuatl language, which means resin or incense. This term was export to the rest of the world when the Spanish arrived in America [230]. Nowadays, a variety of copal types can be found in marketplaces, mainly in Mexico and Guatemala, includes copal blanco, copal oro, copal negro, copal lágrima, copal incienso and copal de piedra. According to Stacey et al [231], “Copal blanco”, most commonly found, is exuded directly from incisions made in the tree bark, and “Copal lágrima” (copal in tears) is the product remaining in the recipient of collection and the incision in the bark.

Natural resins have important industrial applications, which involves the synthesis of flavors and fragrances, as painting varnishes, and also in pharmaceutical and cosmetic preparations [232]. Specifically, copal has been appreciated and used from ancient times because of their variety of purposes including religious ceremonies [233], beauty [234], dental care [235], as well as the decoration of artworks [236]. In the last years, some constituents of copal have been an object of study for researchers, due to their pharmacological effects as an anti-inflammatory, antipruritic, anti-fungal and others [237–240].

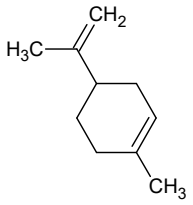
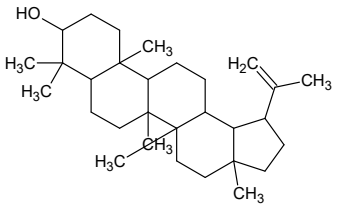
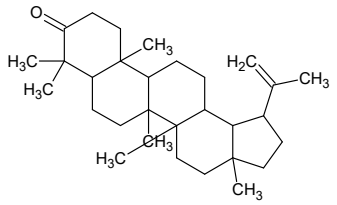
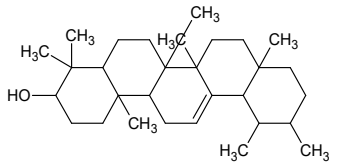
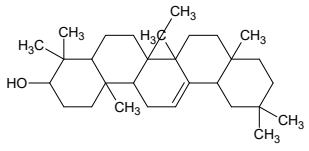
In the study of copal composition, some works have reported the presence of monoterpene compounds such as pinene and limonene as the most abundant in volatile fraction, whereas triterpenoids such as ursane and oleanane are predominant in the non-volatile fraction of copal [238,241]. As mentioned in the previous section, terpenic compounds are organic derivatives of isoprene with general formula $(C_5H_8)n$, which are the most numerous and structurally diverse secondary metabolites among various natural products (>50%) [242]. According to the number of carbons atoms in their structure can be classified in monoterpenes (C = 10), Sesquiterpenes (C= 15), Diterpenes (C =20), Sesterpenes (C =25),Triterpenes (C=30) and Carotenoids (C=40).

Triterpenes are widely found in the plant kingdom and have aroused interest from both an evolutionary and a functional perspective [243]. Some of them, like the ursane (α-amyrin) and oleanane (β-amyrin) skeletons, present properties of significant pharmacological importance. Table 8 shows the chemical structure and log Kow values of terpenic compounds commonly found in copal resins, in which low polarity is observed especially for triterpenes compounds.

Quantitative analysis of copal resins could be of interest to discriminate resins by their botanical origin as well as to explore the age and storage conditions of the samples [244]. In that sense, methods capable to provide a better knowledge of the amounts of the major components of these natural resins are a point of interest for researchers of natural products. A method for the determination of triterpenes as lupeol, α and β-amyrin, in copal resins used in folk ceremonies was

described by Merali et al. [245]. Liquid chromatography with UV detection is applied, in which 0.5 grams of resins are needed; although the analytical performance of the method applied was not reported, the method may not be suitable for those cases in which the quantity of available samples is restricted, as is the case of the study of archaeological pieces and works of art.

Table 8. *Chemical structure and log Kow values of terpenic compounds*

Compounds	Structure	log Kow
Limonene ($C_{10}H_{16}$)		4.57
Lupeol ($C_{30}H_{50}O$)		9.23
Lupenone ($C_{30}H_{48}O$)		8.72
α-Amyrin ($C_{30}H_{50}O$)		9.16
β-Amyrin ($C_{30}H_{50}O$)		9.19

In this line, some works have been described in the literature for the chemical analysis of resins in archeological pieces, by the application of gas chromatography (GC) with mass spectrometry (MS) detection [230, 246]. However, those studies were aimed to define the chemical fingerprinting in combination with chemometric tools, and none reported the quantitative composition of the target compounds, which may be probably explained by the lack of reliable quantitative methods that can be applied to micro samples, as the low amount of sample available is a major limitation in such studies.

The determination of triterpenic compounds in different vegetal materials has been reported, applying traditional and exhaustive sample treatments for large amounts of the samples, and multiple steps of extractions, purification, solvent evaporation, and redissolution since the amount of sample is not limited [247-249]. Chromatographic analysis of terpenic compounds in natural resins arises several difficulties, such as these samples contain numerous compounds with very wide chemical properties. For example, the most abundant high molecular triterpenes are highly nonpolar with high octanol-water partition coefficients (Kow > $10^{9.0}$), so, application of reverse-phase mechanism represents a challenge because the choice of the mobile phase is rather limited [243]. Additionally, the lack of chromophores in those molecules maybe also a limitation when a UV detection is applied, especially in the analysis of micro samples.

On the other hand, for GC-based methods, the application of previous derivatization step is required, especially for the determination of low-volatile high-molecular triterpenes. The complexity of resin samples makes that reported assays have been focused only on one family of compounds, typically the triterpene fraction. Alternatively, portions of the sample extract can be analyzed under two or more different chromatographic conditions to obtain more exhaustive sample characterization, which moves away from the principles of the green analytical chemistry.

In this thesis, a method was developed for the quantification of representative components of copal resins, both volatile (limonene) and non-volatile (lupeol, lupenone, and amyrins) fractions, using capillary chromatography with UV detection, taking the advantages of high sensitivity attainable with this miniaturized LC systems, which make them better suited for the analysis of micro samples.

CHAPTER 2. OBJECTIVES

As has been discussed in the introductory part of this thesis, the development and applicability of miniaturized liquid chromatography systems in recent years have facilitated the resolution of analytical problems in fields such as clinical, foodstuff, forensic, omics, and pharmaceutical. The emergence and commercialization of these new instruments capable of meeting the needs of these fields, and others on the rise, have offered significant advantages like the possibility to work with a small sample volume, reduce solvent consumption, thus maintain lower analysis costs, as well as an improve the detection as a result of the lower chromatographic dilution. Besides, the development of these systems has opened the introduction of hand-portable LC instruments, which special ability to offer results quickly, overcome obstacles related to the preservation of the sample, and allow applications in situ or at-situ monitoring.

The main characteristics of miniaturized and portable systems are tuned with the environmental approach, in the recent context of green analytical chemistry, which demands the need to develop new and sustainable analytical techniques from an ecological point of view, to expand the applicability of liquid chromatography. In this sense, the development of new sample treatment techniques, alternatives to liquid-liquid extraction and solid-phase extraction, traditionally used, represents one of the most important topics that researchers address. As noted above, a preponderant group of these alternative techniques are the SPME techniques, within which IT-SPME stands out for its characteristics in reducing analysis time and solvent consumption, decrease in waste generation, as well as the easy implementation in line with the chromatographic system, making the coupling of IT-SPME with miniaturized and portable systems an ideal option for the development of methodologies with high analytical performance and low environmental impact.

In this scenario, the use of commercially available extractive phases, as well as the development of new sorbent phases for IT-SPME, and the application of analytical procedures using miniaturized and portable LC systems for the study of substances of interest in biological, environmental matrices, food and natural products is eminently required. Therefore, the main objective of this doctoral thesis is the study of miniaturized and portable liquid chromatography, and its on-line coupling to in-tube solid-phase microextraction for the analysis of different matrices. To fulfill it, the following specific objectives are set:

- Develop a methodology based on IT-SPME coupled with Cap LC for the determination of caffeine and its main metabolites in serum, urine, and oral fluid, with characteristics of simple and green chemistry sample treatment technique, simple analytical procedure, and use of a minimum amount of sample.

- Explore the basic characteristics of the merit and operability of a portable NanoLC for the determination of trimethylxanthines in environmental waters.
- Evaluate possible strategies for water sample treatment for on-site analysis, including the off-line IT-SPME and solid-phase extraction (SPE) with cartridges.
- Developed chromatographic strategies for the simultaneous determination of major and trace compounds commonly found in dietary supplements based on capillary liquid chromatography.
- Developed a method for the quantification of volatile and non-volatile representative terpenic compounds in micro samples for the characterization of natural resins.

The development of this thesis has been possible thanks to doctoral grant “Programa de Relevo Docente” from the Universidad Nacional Autónoma de Honduras (Honduras) and the research projects awarded to the MINTOTA group, which are detailed below:

- P. Campíns (PI), R. Herráez, C. Molins, J. Verdú, Y. Moliner. Ref .: CTQ2017-90082-P TITLE: MICROEXTRATION IN SOLID PHASE IN TUBE COUPLED IN LINE TO LIQUID NANOCROMATOGRAPHY: NEW OPPORTUNITIES FOR / FROM NANOESCALA AND LIQUID CHROMATOGRAPHY ”. Financing: MCIU / FEDER, 01/01/2018 to 12/31/2021, € 134,310.00 and FPI contract.
- Development of new strategies for the design of an in situ analysis device: nano and biomaterials. Prometheus Program for research groups of Excellence PROMETEO / 2016/109 2016-€ 54,298; 2017- € 63,000; 2018- € 63,000; 2019- € 63,000. Responsible researcher: Pilar Campíns Falcó.
- VLC-BIOMED PROGRAM: 2016 grants to carry out joint projects between researchers from the University of Valencia and researchers / professionals from the Hospital Universitario y Politécnico / Instituto de Investigación Sanitària La Fe. Non-invasive pharmacokinetic study of caffeine administered in newborn infants with a diagnosis apnea in prematurity. Financing: € 4,000. IP: Jorge Verdú Andrés.

The knowledge acquired during the development of this doctoral thesis has allowed us to obtain as a result publication of 4 scientific articles published or send to high-impact journals, and one pending submissions for publication.

- **Ponce-Rodríguez, H.D.**, García-Robles, A.A., Sáenz-González, P., Verdú-Andrés, J. and Campíns-Falcó, P. (2020). On-line in-tube solid phase microextraction coupled to capillary liquid chromatography-diode array detection for the analysis of caffeine and its metabolites

in small amounts of biological samples. Journal of Pharmaceutical and Biomedical Analysis, 178, 112914. Impact Factor (JCR 2018): 2.983. Contribution 100%.

- **Ponce-Rodríguez, H.D.**, Herráez-Hernández, R., Verdú-Andrés, J. and Campíns-Falcó, P. (2019). Quantitative Analysis of Terpenic Compounds in Microsamples of Resins by Capillary Liquid Chromatography. Molecules, 24, 4068. Impact Factor (JCR 2018): 3.060. Contribution 100%.

- **Ponce-Rodríguez, H.D.**, Verdú-Andrés, J., Herráez-Hernández, R. and Campíns-Falcó, P. (2020). Innovations in extractive phases for in-tube solid-phase microextraction coupled to miniaturized liquid chromatography: A critical review. Molecules, 25, 2460. Impact Factor (JCR 2018): 3.060. Contribution 100%.

- **Ponce-Rodríguez, H.D.**, Verdú-Andrés, J., Herráez-Hernández, R. and Campíns-Falcó, P. (2020). Exploring hand-portable nano-liquid chromatography for in place water analysis: determination of trimethylxanthines as a use case. Science of the Total Environment (In revision). Contribution 100 %.

- **Ponce-Rodríguez, H.D.**, Verdú-Andrés, J., Herráez-Hernández, R. and Campíns-Falcó, P. (2020). Multi-class analysis of botanical dietary supplements by capillary liquid chromatography. Pending submit. Contribution 100 %.

Likewise, the results obtained in the development of this thesis have been disseminated through its presentation as a poster or oral presentation in different national and international conferences. The works presented include:

- **Ponce-Rodríguez, H.D.**, Verdú-Andrés, J., Herráez-Hernández, R. and Campíns-Falcó, P. (2019). Analysis of terpenic compounds in microsamples of natural resins by capillary liquid chromatography. XXII Reunión de la Sociedad Española de Química Analítica. Valladolid, España, National congress. Póster.

- Verdú-Andrés, J., **Ponce-Rodríguez, H.D.** and Campíns-Falcó, P. (2019). Determination of trimethylxanthines as anthropogenic contaminants in drinking and wastewater by In-tube Solid-phase microextraction - CapLC. XXII Reunión de la Sociedad Española de Química Analítica. Valladolid, España, National congress. Oral presentation.

- **Ponce-Rodríguez, H.D.**, Verdú-Andrés, J., Herráez-Hernández, R. and Campíns-Falcó, P. (2019). Chemical Composition of Dietary Supplements by Capillary Liquid Chromatography-Diode Array Detection. 25th International Symposium on Separation Sciences. Lodz, Polonia, International congress. Oral presentation.

- Verdú-Andrés, J., **Ponce-Rodríguez, H.D.**, García-Robles, A.A., Sáenz-González, P. and Campíns-Falcó, P. (2019). On-line IT-SPME coupled to CapLC-DAD for the Analysis of Small Amounts of whole Biological samples. 25th International Symposium on Separation Sciences. Lodz, Polonia, International congress. Oral presentation.

CHAPTER 3. EXPERIMENTAL METHODOLOGY

3.1 CHEMICALS

For the development of this thesis, analytical grade reagents were used, which are presented in the Table 9. Likewise, the chemical supply company and the safety pictograms are shown, according to the guidelines R (CE) n ° 1272/2008 of the Parliament and of the Council, regarding the classification, labeling, and packaging of substances and mixtures.

***Table 9**. List of reagents, chemical supply company and safety pictograms* *Flammable,* *corrosive,* *toxic,* *high-dose side effects,* *dangerous for health,* *Dangerous for the environment. 1) Sigma: Sigma-Aldrich; Dr. Ehrenst: Dr Ehrenstorfer; Cayman: Cayman Chemical; Fisher: Fisher Scientific.*

Reagent	Chemical supply company[1]						
3-CQA (Neochlorogenic acid)	Phytopurify						
3,4-diCQA (Isochlorogenic acid B)	Phytopurify						
3,5-diCQA (Isochlorogenic acid A)	Phytopurify						
4-CQA (Cryptochlorogenic acid)	Phytopurify						
4,5-diCQA (Isochlorogenic acid C)	Phytopurify						
5-CQA (5-caffeoylquinic acid)	Sigma						
α-amyrin	Sigma						
α-pinene	Sigma	x			x	x	x
β-amyrin	Sigma						
Acetic acid	VWR	x	x				
Acetone	Panreac	x			x		
Acetonitrilo	VWR	x			x		
Ammonia	Scharlab		x	x			x
APTS	Sigma		x		x		
Bovine serum albumin	Sigma						
Brij 35 P	Merck		x				
Caffeic acid	Sigma						
Caffeine	Sigma						
Cetyltrimethylammonium chloride solution (CTAC)	Sigma		x				x
Chloroform	Romil			x			
Copper(II) sulfate pentahydrate	Merck		x				
Ethanol	VWR	x			x		
Ethyl acetate	Romil	x					
Farnesene	Sigma				x		
Formic acid 85%	VWR	x	x	x			
Hydrochloric acid	Scharlab		x		x		
Isopropanol	Scharlab	x					

Reagent	Chemical supply company[1]						
Kaempferol	Fisher						
Limonene	Sigma	x			x	x	x
Linalool	Sigma				x		
Lupenone	Sigma						
Lupeol	Cayman						
Methanol	VWR	x		x		x	
MTEOS	Sigma				x		
Myrcene	Sigma	x			x	x	
Paraxanthine	Sigma						
Phosphoric acid (85%)	Scharlab		x				
Poly(vinyl alcohol)	Sigma						
Polyethylene glycol (PEG)	Sigma						
Riboflavin	Guinama						
SiO_2 NPs (20 nm)	Sigma				x		
Sodium carbonate	Prolabo				x		
Sodium chloride	Scharlab						
Sodium citrate tribasic dihydrate	Sigma						
Sodium dodecyl sulfate	Panreac	x	x		x		
Sodium dodecylbenzenesulfonate	Sigma		x		x		
Sodium hydroxide	Sigma		x				
Sodium phosphate dibasic	Sigma						
Synephrine	Sigma				x		
TEOS	Sigma	x			x		
Tetrahydrofuran	Scharlab	x					
Theobromine	Sigma						
Theophylline	Sigma						
TiO_2 NPs (21 nm)	Sigma				x		
ZnO NPs (44 nm)	Sigma						x
ZrO_2 NPs (20 nm)	Sigma						

3.2 INSTRUMENTATION

In the framework of this thesis, different analytical instruments were used, the specifications of them are detailed below, including spectroscopy, microscopic and chromatographic techniques.

3.2.1 Spectroscopic techniques

3.2.1.1 UV-vis spectrophotometry

For the acquisition of UV-vis molecular absorption spectra, a Carry-60 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) (Figure 10. A) was used. A quartz or plastic cuvette with an optical path of 1 cm and a range of measures between 190 and 1000 nm was used in all experiments. Acquisition of signals, as well as data processing, was performed with Carry WinUV software (Agilent Technologies). Other spectrophotometric measurements were also made with an optical fiber device (3.5 mm diameter and 10 mm optical path) coupled to the same instrument (Figure 10. B).

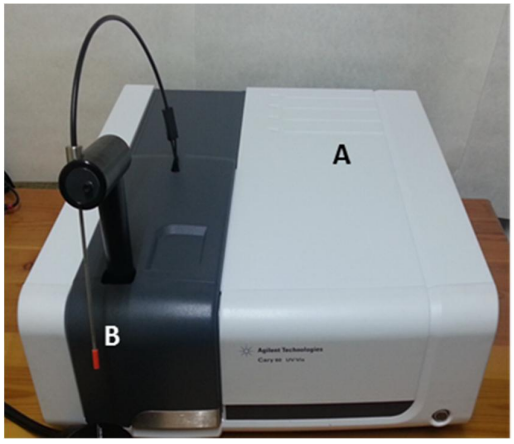


Figure 10. *A) Cary-60 UV-vis spectrophotometer; B) Optical fiber.*

3.2.2 Microscopic techniques

3.2.2.1 Optical microscope

A microscope ECLIPSE E200LED MV Series (Nikon, Tokyo, Japan) under bright and darkfield illumination was used. For images acquisition and processing a Nis-Elements 4.20.02 software (Nikon) was required. Figure 11 shows the microscope, equipped with 3 objective lenses of different magnifications (10x, 50x, and 100x).

Figure 11. *Microscopic optical ECLIPSE E200LED MV.*

3.2.3 Chromatographic techniques

Two chromatographic systems were applied to carry out the studies of this work. A CapLC miniaturized liquid chromatography system with a DAD detector and coupled to IT-SPME with in-valve configuration and, a hand-portable NanoLC with a UV detector. Data acquisition and subsequent processing have been done with HP ChemStation software (Agilent Technologies) and Axcend focus LC software (Provo, UT. USA).

3.2.3.1 Capillary liquid chromatography

Agilent 1200 Series (Agilent Technologies), with a conventional binary pump with a flow split that allows working with flows no higher than 20 µL min^{-1}. For sample introduction, a 6-port manual valve Rheodyne 7721-i (IDEX Health and Science, Rohnert Park, CA. USA) was used, in which the injection loop contained the extraction phase (details in section 3.3). Signals were recorded in a wavelength range of 180 and 400 nm with a diode array detector (DAD) equipped with an 80 nL cell (Agilent Technologies). Figure 12 shows the system with the configuration described above. Chromatographic separations were carried-out with C18 particulate columns (Section 3.5.1) from Agilent and Phenomenex (Torrance, CA, USA).

Figure 12. *Cap LC-DAD system, Agilent 1200 Series with binary pump.*

3.2.3.2 Portable Nano liquid chromatography

Portable Nano LC Focus LC (Axcend Corp, UT.USA) system was used (Figure 13), consisted of two high-pressure syringe pumps capable of delivering flow rates of 0.8-50 µL min^{-1} at pressures up to 600 bar, connected to a mixing valve capable of work in isocratic or gradient mode and, manual injector port with internal sample loop of 40 nL. The chromatograph is equipped with a 100 mm × 150 µm i.d. column packed with ODS, 1.7 µm particle size (Section 3.5.1), and On-capillary UV absorbance was measured at 255 nm using a LED. For data acquisition and calculation, the Axcend Focus v2.0 software was used.

Figure 13. *Portable Nano LC, Focus LC Axcend Corp.*

3.3 SAMPLE TREATMENT

Sample treatments applied in the development of this thesis, which include IT-SPME online with CapLC, IT-SPME off-line and traditional solid-phase extraction (SPE), are described in detail in the next lines. Other pre-treatment procedures, like dilution, acidification, centrifugation, sonication, and, filtration which were necessary to apply, are too explained. IT-SPME online was used for the analysis of water and biological samples, instead, IT-SPME off-line and SPE were apply for water samples. For solid samples, like dietary supplements and natural resins, simple procedures as sonication and filtration were applied.

3.3.1 IT-SPME online

IT-SPME online coupled to CapLC was used under in valve setting. Here, the conventional steel injection loop of the 6-port manual injection valve (Rheodyne) is replaced by a capillary, which contains the sorbent phase, as Figure 14 shows. Conventional GC capillary columns with two sorbents class, Si-based (polysiloxane coating) and C-based (divinylbenzene and polyethylene glycol) type were used. Additionally, some capillaries with homemade sorbents synthesized with metallic nanoparticles, have been used (for more details see Section 3.4). All of these capillaries with an internal diameter of 320 µm.

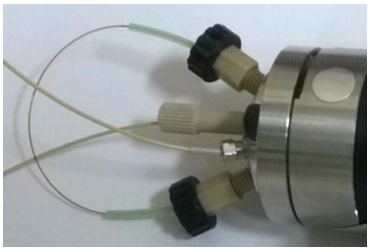


Figure 14. *IT-SPME assembly with 1 capillary.*

Then, a subsequent washing step is required, in which a certain volume, usually the internal volume of the capillary, of nanopure water is passed without changing the valve position. This process removes the impurities and compounds from the matrix that were not absorbed in the extraction phase and avoid its entrance in the chromatographic system. After that, the valve was rotated to the injection position, whereby the mobile phase passed through the extraction capillary, desorbing the previously retained analytes and transferring them to the analytical column.

Biological samples

For serum samples, 25 µL were diluted and acidified with 75 µL of 1% aqueous formic acid solution and then mixed in a vortex for 30 seconds. 10 µL of saliva and 100 µL of urine samples were acidified with 10 µL and 300 µL of the same solution used for serum samples (1% aqueous formic acid) respectively. A step of dilution was required for saliva, diluted to 100 µL, and urine, diluted to 1 mL, both with ultrapure water and mixed for 30 seconds. Finally, an aliquot of diluted samples was processed by IT-SPME coupled to CapLC.

Water samples

For water samples, include river, sea, and tap water, only if necessary a filtration procedure using a 0.22 µm pore size PTFE filter before the analysis was carried out.

Dietary supplements samples

Accurately weighted homogenized portions of the solid dietary supplements were placed in 5 mL glass vials and extracted with a proper volume of methanol. Then, the mixture was subjected to mechanical agitation for 1 minute. After this time, the solution was placed in an ultrasonic bath for 5 minutes at 30°C. The supernatants were removed by filtration with 0.22 µm nylon membranes. Finally, the extracts were acidified and mixed with 0.1 % hydrochloric acid solution in a proportion 1:10 (v/v) and chromatographed.

Natural resins samples

Portions of resins previously homogenized mechanically in a mortar with a pestle were accurately weighed (≈1-15 mg) in 2 mL glass vials. Then, the samples were treated with 1 mL of methanol and the mixture was vortexed for 1 minute. Subsequently, the result solutions were filtered through 0.22 µm nylon membranes to remove any particulate that could be present. Finally, aliquots of filtrated were chromatographed.

3.3.2 IT-SPME offline

For IT-SPME off-line a C-based capillary column (VP-HayeSep P 50 cm long) with an internal diameter of 320 µm was connected to a 6-port manual injection valve (Rheodyne). In the initial stage, the capillary was conditioned with 1 mL of nanopure water, then 8 mL of standard and water sample solutions were passed through the capillary with a precision glass syringe. For desorption of the analytes, 50 µL of methanol was passed and this solvent was collected to inject in hand-portable NanoLC. Figure 15 shows a schematic representation of this procedure. As mentioned in the previous section, for water samples, the previous procedure only involved filtration with 0.22 µm pore size PTFE.

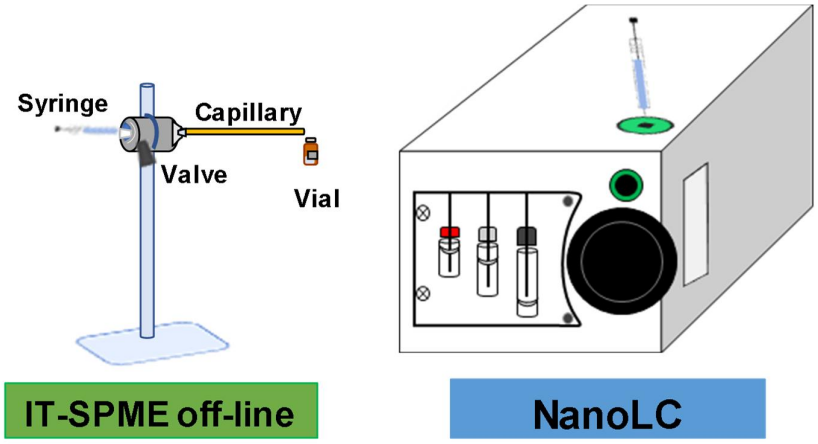


Figure 15. *Schematic representation of IT-SPME off-line.*

3.3.3 SPE

The solid-phase extraction procedure included five steps, which in this work were as follows: (1) activation with 1 ml of methanol; (2) conditioning with 1 mL of nanopure water; (3) loading of the standard and sample solutions; (4) cleaning-up with0.1 mL of nanopure water; and (5) elution of the analytes with two individual portions of 0.1 mL of methanol. Finally, the two fractions were separately filtered and injected into the hand-portable Nano LC system. Figure 16 shows the schematic SPE procedure for the analysis of water samples.

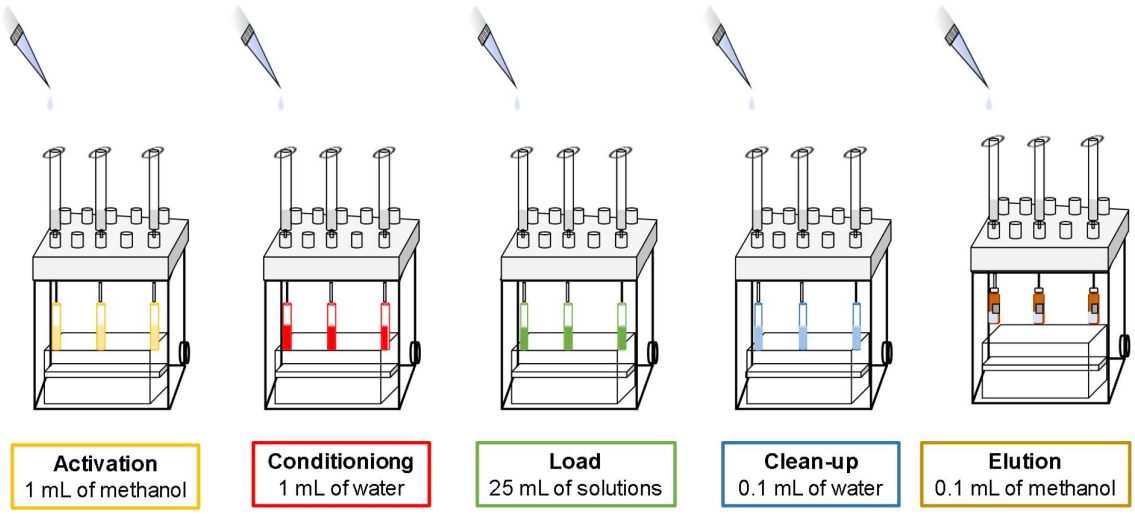


Figure 16. *Schematic* SPE *procedure for the analysis of water samples.*

3.4 SYNTHESIS OF NEW PHASES FOR EXTRACTION

As previously stated, the implementation of new phases to improve extraction efficiency in IT-SPME is one of the main topics of this technique. In this work, fused silica capillaries were coated with a silicon polymer using the sol-gel process. Also, this polymer was doped with NPs of SiO_2 and various metals and metal oxides to improve its extraction capacity. The experimental procedures carried out for the development of these extraction phases used in the studies in the framework of the development of this thesis are discussed in the following sections.

3.4.1 Capillary columns coated with a silicon polymer

Capillary columns of uncoated fused silica with an internal diameter of 320 µm were modified with silicon polymeric material using the procedure detailed by Silva et al. [250]. For this, in the first instance, the inner wall of the capillary was activated by passing an alkaline solution of 2M NaOH and placed for 2 hours at a temperature of 40°C. Next, the capillary was washed with a 0.1M HCl solution

and left to act for 30 minutes at room temperature. After this time, it was subjected to heat for 3 hours at 60°C.

Subsequently, the residues were washed with nanopure water, and the polymer dispersion was then passed. It was prepared with 65 mg of PEG, 50 µL of H_2O, 100 µL of TEOS, 100 µL of MTEOS and 2 mL of 0.1M NH_4OH. Once the volume filling was assured inside the capillary column, the ends of the cap were capped and warmed with a temperature program of 2 hours at 40°C and 12 hours at 120°C. Finally, the capillary was washed with water and acetonitrile to remove the excess of reagents present in the capillary. Likewise, capillary columns were modified following the previous procedure, incorporating nanoparticles of SiO_2, TiO_2, ZrO_2, CuO, ZnO or Al_2O_3 (0.05 mg mL^{-1}) to the monomer mixture [251].

3.5 PROCEDURES AND EXPERIMENTAL CONDITIONS

3.5.1 Chromatographic conditions

Experimental conditions employed for the chromatographic separation of all studies and procedures developed for CapLC and hand-portable NanoLC in the framework of this thesis are presented in Table 10. In all experiments, the mobile phase was filtered before use with 0.45 µm pore size nylon filters, and 0.22 µm for portable NanoLC.

Table 10. *Chromatographic conditions of the procedures used in this thesis.*

LC System	*Analytes*	*Sample treatment technique*	*Sorbent phase*	*Analytical column*	*Mobile phase*	*Flow rate (µL min^{-1})*	*Volume injected of sample solution*	*Detector*	*Section*
					Water - Methanol (for serum and oral fluid)				
			TRB-50						
			ZB 1701	Zorbax SB	1% aqueous acetic		Serum and saliva:		
	Caffeine		ZB WAX plus	C18 (150 x	acid- Methanol (for		25 µL	DAD	
	Paraxanthine	IT-SPME	ZB FFAP	0.5 mm, 5	urine)	15		(275 nm)	*4.1*
	Theobromine	online	TEOS-MTEOS-SiO2	µm)			Urine:		
	Theophylline		TEOS-MTEOS-TiO2		75:25 (0 min)		400 µL		
					70:30 (7 min)				
					50:50 (13 min)				
					50:50 (17 min)				
					100:0 (20 min)				
					Method A:				
					Water - Acetonitrile				
	Caffeine								
CapLC	Theobromine				85:15 (0 min)				
	Synephrine				80:20 (5 min)			DAD	
	Riboflavin				50:50 (9 min)				
	Caffeic acid				25:75 (11 min)	Method A:		Method A	
	Kaempferol				0:100 (15 min)				
	Limonene					10		200, 220,	
	α-Pinene	IT-SPME		Zorbax SB				275, 330,	
	Farnesene	online	Fused silica	C18 (150 x	Method B:		12 µL	370 nm	*4.3*
	Myrcene		untreated	0.5 mm, 5	0.1 % aqueous	Method B:			
	Linalool			µm)	phosphoric acid-				
	5-CQA				Methanol	12		Method B	
	3-CQA							330 nm	
	4-CQA				75:25 (0 min)				
	3,5-diCQA				70:30 (7 min)				
	3,4-diCQA				50:50 (15 min)				
	4,5-diCQA				25:75 (20 min)				
					0:100 (23 min)				

Table 10. *Continuation.*

LC System	*Analytes*	*Sample treatment technique*	*Sorbent phase*	*Analytical column*	*Mobile phase*	*Flow rate (µL min^{-1})*	*Volume injected of sample solution*	*Detector*	*Section*
CapLC	Limonene Lupenone β-amyrin α-amyrin Lupeol	IT-SPME online	Fused silica untreated	Zorbax SB C18 (150 x 0.5 mm, 5 µm)	Water: Acetonitrile 85:15 (Isocratic)	10	5 µL	DAD (200 nm)	*4.4*
	Caffeine Theobromine Theophylline	IT-SPME online	VP-HayeSep P HP-PLOT/Q	Phenomenex Synergi Fusion C18 (150mm x 0.5mm, 4 µm)	Water - Acetonitrile 80:20 (0 min) 50:50 (5 min) 25:75 (9 min) 0:100 (13 min) 0:100 (16 min)	15	4000 µL	DAD (275 nm)	*4.2*
Hand-portable NanoLC	Caffeine Theobromine Theophylline	IT-SPME off-line SPE	VP-HayeSep P C-18	Phenomenex Synergi Fusion C18 (150mm x 0.5mm, 4 µm)	Water - Acetonitrile 95:5 (0 min) 80:20 (4 min) 5:95 (4.5 min) 5:95 (4.7min)	2	10 µL	UV (255 nm)	*4.2*

3.6 ANALYZED SAMPLES

Four types of samples were analyzed for the development of this work, including biological, waters, food supplements, and natural resins samples. Figure 17 shows a summary schema for analyzed samples and analytes studied. Analysis of all samples was executed in triplicate at room temperature.

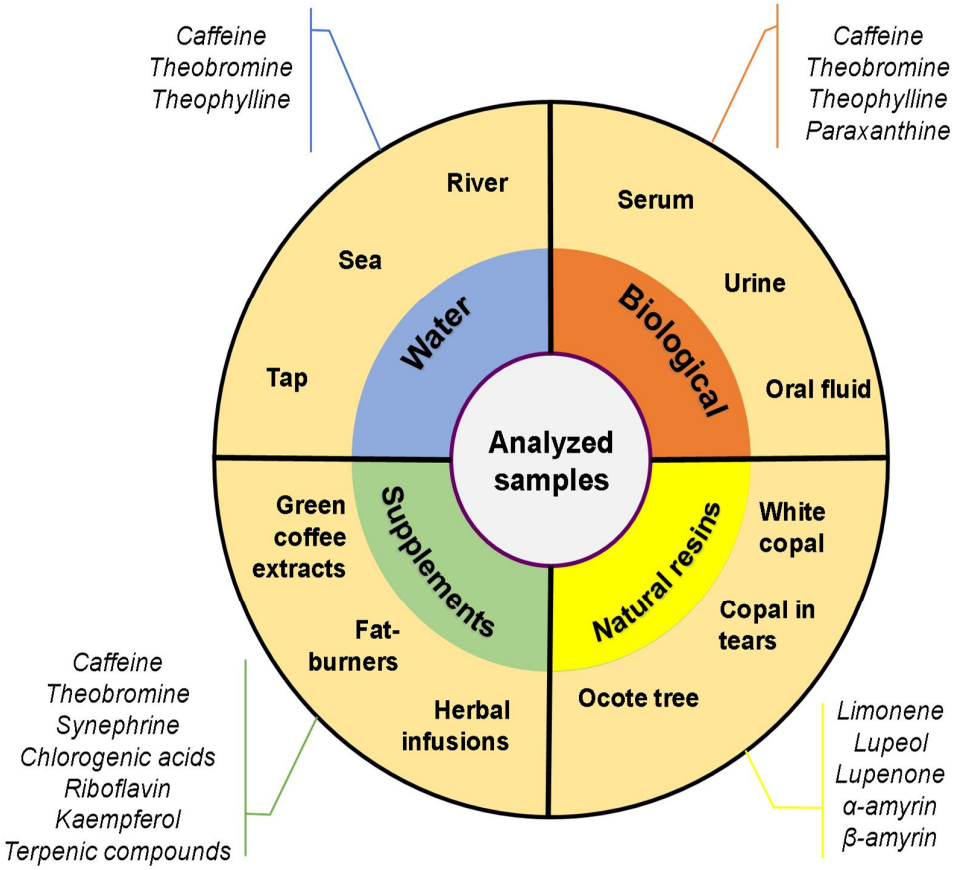


Figure 17. *Summary diagram of the matrices and analytes studied in this thesis.*

3.6.1 Biological samples

Biological samples were collected from two kinds of patients. For optimization of the analysis method, serum, urine, and oral fluid from healthy adult volunteers after 3 days on a trimethylxanthines-free diet, were stored refrigerated until analysis. Serum and oral fluid samples from preterm newborns, under treatment with a dosage of caffeine at the pediatric intensive care unit (La Fe University and Polytechnic Hospital - Valencia, Spain), were obtained after prior informed

consent from their parents. The collection and storage procedures for all specimens are detailed below.

3.6.1.1 Serum

Blood samples, obtained in plastic tubes containing gel for a total volume of 0.8 mL of sample (Mini Collect, Greiner Bio-One, Kremsmünster, Austria), were filled. Then, the tube was inverted until the blood completely mixed with the gel and centrifuged. Finally, the separated serum was stored frozen at −20 ◦C pending analysis.

3.6.1.2 Urine

Urine samples were obtained in sterile flasks of 120 mL of capacity, centrifuged and stored at −20 ◦C until analysis.

3.6.1.3 Oral fluid

Oral fluid samples were collected using cellulose spears devices (EYETEC, North Yorkshire, UK). The devices were placed in the mouth of the patient for 1–10 minutes until the spears were saturated with the fluid. Then, the saturated end of the spear was placed in the collection cone tube insert and cut off. Subsequently, the tube was frozen at −20 ◦C, and just before analysis, a centrifugation step was applied, and the supernatant was collected. A second procedure for the preservation of oral fluid samples was performed, differentiating itself from the first, in that the centrifugation step was carried out immediately after obtaining the sample. Although equivalent results were obtained for the same sample processed by both procedures, the first procedure shows slightly better repeatability, additionally fewer impurities were detected. With the idea of reducing the sample processing time, the first procedure will only require one centrifugation step.

3.6.2 Waters

For the development of this thesis, different types of water have been collected and analyzed, all of these taken at different points in the Comunidad Valenciana, Spain. All samples were collected in amber glass bottles, of 250 mL of capacity, previously cleaned in the laboratory with acetonitrile, methanol, and water, and rinsed with the sample water on-site and filled completely up to the rim to eliminate the headspace. The samples were transported from the sampling sites to the laboratory and were kept cold (±4 °C) until the analysis, which was done one week later. In all these samples, the presence of trimethylxanthines, including caffeine, theobromine, and theophylline, has been analyzed as biomarkers of human activity.

3.6.2.1 Superficial waters (river and sea water)

One-time water samples were obtained from the Turia river, near Pinedo wastewater treatment plant. Two sampling sites were established, the first one in front of WWTP and the second one, downstream (500 m) of the WWTP. Three samples from the Magro river, in the southwest of Valencia, were collected, following the same procedure above explained. Three sampling sites were established, one just before the WWPT, one upstream (1 km), and a third one downstream (100 m) of the wastewater treatment plant (WWTP). Finally, regarding the coastal water samples, these were taken at different points on the Valencian coast.

3.6.2.2 Tap water

Tap water samples were collected in a house located at Burjassot town, in amber glass bottles, following the same procedure for superficial samples. Then the bottles were transported to the laboratory and stored in a refrigerator at ±4°C until the analysis.

3.6.3 Dietary supplements

Different types of dietary supplements acquired in local supermarkets were analyzed, three brands of green coffee extract based products (GC), two fat burning formulations (FB), and finally, an orange herbal preparation (HP). All these products with differences in labeling, some of them specify the concentrations of the components present, and others only indicate the presence of some substance.

3.6.3.1 Green coffee extracts

Green coffee extract (GC-1), marketed in the form of bags, was prepared from extracts of different plants, including green coffee, and reported the presence of limonene, theobromine, and chlorogenic acids; the mean average mass of product per bag was 1.1 g. The sample GC-2 only reported 175 mg of decaffeinated green coffee extract per soft gelatin capsule (0.40 g). The label of sample GC-3 reported the presence of many compounds, including 200 mg of green coffee extract, 50 mg of green tea extract, 50 mg of *Citrus aurantium* (with 6 % synephrine), minor amounts of other vegetal species, and 0.7 mg of riboflavin per soft gelatin capsule (mean average mass, 0.50 g).

3.6.3.2 Fat burning capsules

Sample FB-1 reported the presence of a mixed extract of different plants including 125 mg of *Citrus aurantium* (6 % synephrine) per soft gelatin capsule (0.50 g); kaempferol was claimed to be present, although the amount was not reported. Sample FB-2 reported among its ingredients 125 mg of *Citrus aurantium* (also 6 % synephrine) and 55 mg of green tea per capsule (0.25 g), and some of the vitamins of the B complex (pantothenic acid and pyridoxine).

3.6.3.3 Herbal preparation

Sample HP (1.40 g per bag) was composed of a mixture of lime and leaves of orange trees intended for the preparation of infusions; the presence of synephrine, chlorogenic acids, linalool, α-pinene, and limonene was declared to be present, among other compounds like flavonoids and essential oils.

3.6.4 Natural resins

Different commercial samples of natural resins, identified as white copal and copal in tears, as well as a resin obtained from ocote trees, were purchased in Sonora market (Ciudad de México, México) in the year 2010. Figure 18 shows a photograph of the natural resins studied in this thesis.

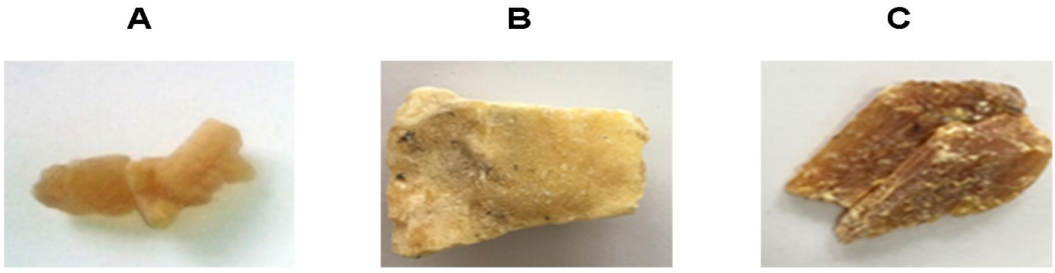


Figure 18. *Photograph of the natural resins studied in this thesis. A: Copal in tears; B: White copal; C: Ocote tree copal.*

CHAPTER 4. RESULTS AND DISCUSSION

4.1 CAFFEINE AND ITS METABOLITES IN BIOLOGICAL SAMPLES

As mentioned in the introductory section of this thesis, caffeine has been used as the drug of the first choice in the treatment of apnea of prematurity. The results obtained in the development of a procedure by IT-SPME-Cap LC for the determination of caffeine and its three main metabolites in biological samples are described and discussed below, within the framework of the collaborative study "CAFE-CINETIC. Non-invasive pharmacokinetic study of caffeine administered in newborns diagnosed with apnea in prematurity", between our MINTOTA research group and Neonatal research unit.

The main objective of the study was to develop a methodology capable of quantifying the serum and salivary concentrations of the target analytes to be used as a routine method in a hospital laboratory for preterm infants, and which met some important requirements, such as the use of a simple and green chemistry sample treatment technique, simple analytical procedure and a minimum amount of sample.

4.1.1. Optimization of the chromatographic conditions

Chromatographic separation of these compounds in previous studies points to the use of mobile phases containing aqueous dilutions of acids in conjunction with organic solvents such as methanol and acetonitrile [252-254]. In our study, better results, regarding efficiency and chromatographic resolution, were obtained with a mixture of water and methanol and was used as the mobile phase. Besides, different columns were tested to reach the total separation of the compounds, and satisfactory results were found with the Zorbax SB C18 column.

Once the mobile phase and column were studied, several gradient elution programs were analyzed to obtain a fast separation, as well as a good resolution of the four compounds, at the lower pressure possible. High polarity of these compounds (pKa values between -0.8 and -0.02) requires the use of a mobile phase mixture with low eluotropic power at the beginning of the chromatographic separation (percentage of methanol less than 25%), as well as a gradient with a slow increase of the organic phase.

The final conditions for the gradient were 25% of methanol at 0 min, after up to 30% in 7 min. Next, the increase of organic solvent up to 50% was applied in 6 min. Afterward, this percentage was held for 4 min to decrease to 0% at min 20. This last change was applied as a cleaning step, necessary to clean impurities retained on the column, especially in saliva samples, otherwise, system pressure increase with the number of samples processed. Finally, the initial conditions were obtained at 1 min and hold for a stabilization time of 2 min. Suitable separation of the analytes before 14 min at a flow

of 15 µL min^{-1} was reached, as Figure 19 shows. In optimized conditions, a library of UV spectra of the pure standard solutions was prepared to confirm the identity in biological real samples.

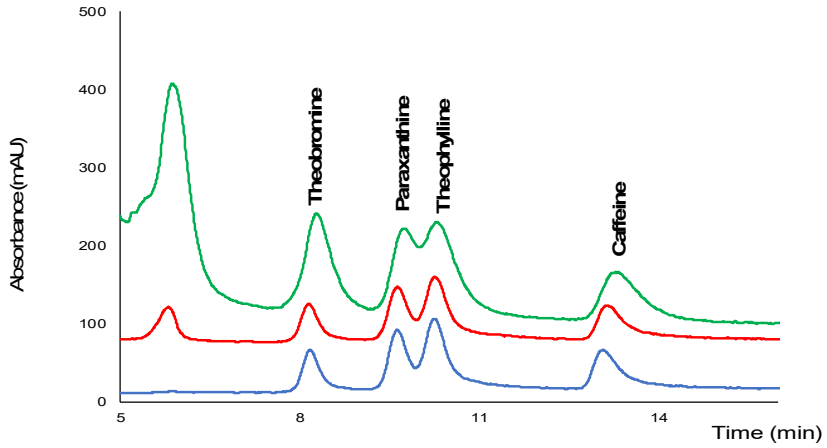


Figure 19. *Chromatogram obtained in optimized conditions. Blue: Standard solution 20 µg mL^{-1}; Red: Oral fluid spiked at 20 µg mL^{-1}; Green: Serum spiked at 50 µg mL^{-1}*

Subsequently, an evaluation of the separation of the analytes in fortified real samples was carried out. Good results were achieved for serum and saliva samples (Figure 19). On the other hand, urine analysis showed a high presence of impurities and endogenous substances of the matrix, in the chromatogram. Therefore, an alternative mobile phase, with acidification of the aqueous phase with acetic acid was required to minimize the matrix effect in urine samples. Figure 20 shows the chromatograms of a fortified urine sample analyzed under conditions with the mobile phase at neutral pH (black) and acidified (brown).

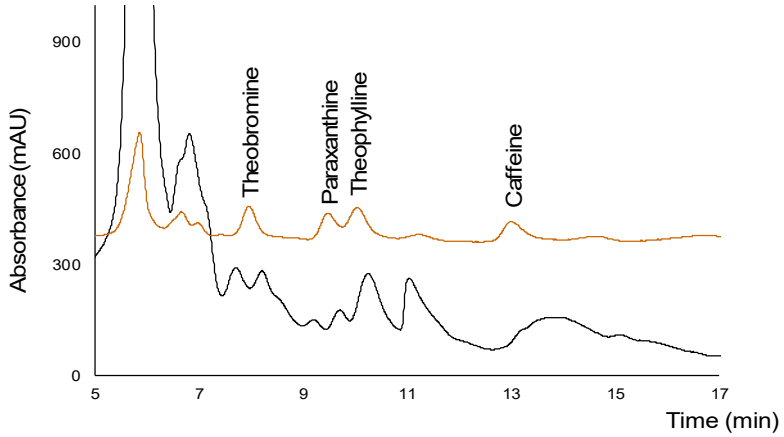


Figure 20. *Chromatograms obtained in urine analysis optimization. Black: urine sample spiked at 20 µg mL^{-1} in mobile phase with neutral pH; Brown: urine sample spiked at 20 µg mL^{-1} in mobile phase with acidification.*

4.1.2. Optimization of IT-SPME

Once the chromatographic separation was optimized, the sample treatment technique was optimized, for which the flow-through mode of IT-SPME was used. Here, a capillary column was used instead of the conventional injection loop connected to a six-port injection valve (Rheodyne®). The different parameters of the technique were optimized to achieve high recovery values.

4.1.2.1. Capillary sorbent selection

The selection of the sorbent phase is the most important step in the optimization of IT-SPME since its nature defines the interaction of the analytes, and hence, the retention and preconcentration of these in the sorption stage. In this work, conventional and modified phases, in decreasing order of polarity, were tested: SiO_2, TiO_2, ZB-FFAP (100% terephthalic modified PEG), ZB-WAX plus (100% polyethylene glycol (PEG)), TRB-50 (polydimethylsiloxane (PDMS) with 50% of diphenyl groups) and ZB1701 (polydimethylsiloxane with 14% of cyanopropylphenyl groups). To obtain the absolute recoveries of the IT-SPME procedure for each capillary column a 1 μg mL^{-1} solution of the analytes was prepared and 100 μL of this solution was processed manually in all experiments.

The absolute recoveries were calculated by comparing the amount of analyte extracted (the amount of the analyte transferred to the analytical column) to the total amount of analyte passed through the extractive capillary column. The amount of analyte extracted was established from a direct comparison of the peak areas in the resulting chromatographic analysis. The different recoveries values obtained for all analytes with the six different capillaries tested are shown in Figure 21. The results obtained concluded that caffeine presents a higher recovery value with the FFAP capillary, which can be attributed to the fact that this sorbent phase has the highest polarity, which generates greater retention for these compounds. For the rest of the compounds, the obtained percentages of recuperation were similar for FFAP and TRB-50 capillaries. This behavior may be due to the π-π interactions of the TRB-50 capillary, which increases the retention of these compounds in IT-SPME [255]. Finally, for all analytes, the lower recoveries were obtained with the capillary containing cyanopropylphenyl groups, the less polar tested phase.

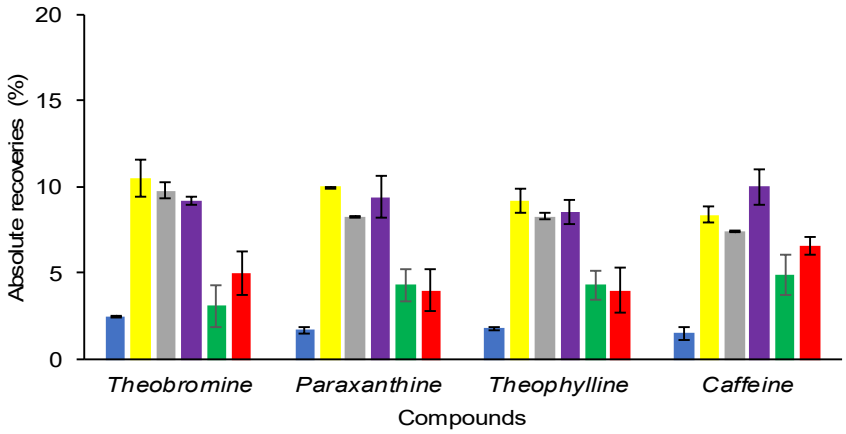


Figure 21. *Absolute recoveries (%) obtained with the IT-SPME with the capillaries tested: ZB 1701 (blue), TRB-50 (yellow), ZB-WAX plus (gray), ZB-FFAP (purple), TiO_2 (green) and SiO_2 (red). The error bars represent the standard deviation for n = 3.*

4.1.2.2. Sample preparation, pH and ionic strength

Following the requirements initially established for the development of the analytical procedure, concerning the use of the minimum sample volume of serum, saliva or urine, which is conditioned by the amount of total sample available in preterm newborns patients, the sample preparation optimization was carried out with the smallest volume that allows reaching an adequate sensitivity. Furthermore, simplifying the preparation procedure, with minimum steps will also facilitate its implementation in routine work in a clinical laboratory setting.

As mentioned in the introductory part of this thesis, the preparation of biological samples requires the application of some pre-treatment procedures to facilitate instrumental analysis. The acidification commonly is used to precipitate proteins, as well as provide similar pH and ionic strength for all the samples. Additionally, a sample pretreatment procedure commonly used before instrumental analysis is the dilution, with special application in biological fluids with high viscosity, as is the oral fluid. This high viscosity can cause increments in the pressure of the chromatographic system, so the dilution of the sample is necessary, but it is important to take an account that this procedure does not affect the sensitivity of analysis, as well as the analyte adsorption in the sorbent phase of the capillary column. Normally, urine sample preparation includes dilution and precipitation processes due to the high quantity of impurities present in this kind of sample.

Methanol, aqueous dilutions of phosphoric acid 1%, and formic acid 1% were tested as modifiers for pH and dilution solvent. Better results were obtained with the formic acid dilution because it improves the sensitivity of the compounds and also decreases the pressure in the

chromatographic system, as results of precipitation of protein and endogenous interferences. Also, an effect in buffer capacities both of ionic strength and pH regardless of the original sample nature was obtained.

Subsequently, the quantity of diluted acid and dilution factors were optimized. For oral fluid, ten microliters of the sample were mixed with the same volume of diluted formic acid, and for urine samples, 300 microliters of the diluted acid were added to 100 microliters of the sample. In both cases, a dilution factor of ten was employed using nano-pure water. Serum samples only needed a dilution factor of four with formic acid 1% solution. For this, 25 microliters of sample were mixed with 75 microliters of acid dilution.

4.1.2.3. Sample volume

As some papers of literature have reported, the extraction efficiency of IT-SPME decrease when the inner surface is saturated [17, 256], the extraction efficiency of IT-SPME decrease when the inner surface is saturated, so different sample volumes were prepared and processed to optimize this parameter. No significant differences in the analytical signals from 100 µL of saliva, 500 µL for serum and 1000 µL of urine to the values obtained with 10 µL, 25 µL, and 100 µL respectively.

Once the saturation of the sorbent phase is reached, even if the sample volume passed through the capillary column is increased, no more retention of the analytes is obtained. Instead, only possible interferences could be retained, but in our case, no effect of the impurities and endogenous compounds of the sample in the retention of the analytes in the capillary was found. Based on these results, and according to the initial objective of the work, reducing the amount of sample and considering the therapeutic concentration range of real samples, the minimum possible volume was selected.

4.1.2.4. Diluted sample volume processed

Since the diluted samples must be processed through IT-SPME, the optimal volume was obtained by comparison of the analytical signal of different volumes. This comparison showed that 25 µL of the diluted sample of oral fluid and serum generated a similar analytical signal to those obtained when 100 µL was used. The same behavior was obtained for urine samples since 400 and 1000 µL of the diluted sample produced equivalent signals.

It is possible to affirm that the absence of a preconcentration effect in the sorption phase for these compounds is due to its high polarity that not allows great interactions with the terephthalic modified PEG sorbent phase of the FFAP capillary. Therefore, for oral fluid and serum samples, 25 µL of the diluted sample was selected as the optimal volume and 400 µL for urine samples. Despite no

preconcentration effect was observed, the signal values obtained in the optimized conditions were satisfactory for the expected concentrations of the compounds in real samples, likewise, the absolute recoveries obtained are agreed with IT-SPME reported usually values [257].

4.1.2.5. Flushing of capillary

In IT-SPME a capillary washing step is required to minimize the presence of impurities of the sample in the chromatographic system due to endogenous substances and impurities of the matrix that can be retained in the capillary together with the analytes [258]. However, the high polarity of the trimethylxanthines studied limits the possibilities of using solvents with strong elution power such as acetonitrile. Two solvents were tested: water and a mixture of equal parts of methanol and water. In both cases the volume used was the internal volume of the capillary (25 µL). When the methanol/water mixture was used, the chromatographic signal of the analytes decreased. On the other hand, flushing with water before injection allowed the reduction of impurities without decreasing the signal of the analytes. Figure 22 shows the optimized procedure of IT-SPME for the analysis of serum, oral fluid, and urine samples.

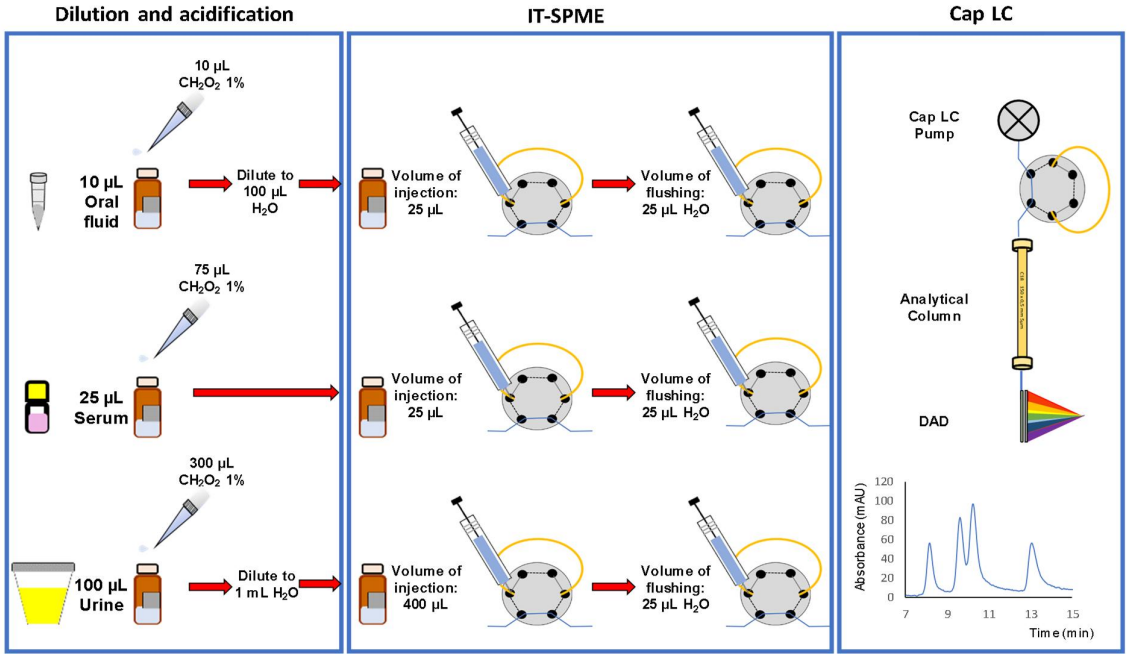


Figure 22. *Schematic procedure of analysis.*

4.1.2.6 Effect of proteins of oral fluid on the system pressure

Because the composition of saliva includes at least more than 400 types of protein, including albumin, these solid components may differ from person to person, and can even vary in the same individual at distinct times during a day [134]. For some oral fluid samples, a high presence of

precipitated matter (endogenous compounds) was observed, and in the analysis of these samples, the chromatographic system pressure increased. The quantification of albumin by the Biuret method [259] was applied to evaluate its effect on the overpressure.

10 µL of a bovine albumin solution prepared in nanopure water at 8 mg mL^{-1}, was mixed with 30 µL of water, 10 µL of sodium hydroxide (0.625 M) and 50 µL of biuret reactive. The biuret reactive was prepared by mixing 0.9 g copper(II) sulfate pentahydrate, 8.6 g sodium citrate tribasic dihydrate and 5.0 g sodium carbonate in 50 mL of nanopure water. The mixture was analyzed in a Carry-60 microcell spectrophotometer in a wavelength range between 500 and 700 nanometers, and the absorbance value of the complex formed was recorded at 545 nm. This procedure was repeated for different oral fluid real samples.

High amounts of protein were found in the samples that increased the pressure of the chromatographic system. Therefore, to avoid this effect, these samples were centrifuged twice. In any case, this additional treatment did not cause changes in the found concentrations of caffeine.

4.1.3. Analytical performance

Different bioanalytical parameters were evaluated to establish that the optimized method is suited to the analysis of the study samples with adequate performance, following the recommendations of some official guides [260,261]. The results of this evaluation are discussed next.

4.1.3.1. Calibration curve and matrix effect

An important parameter for the evaluation of bioanalytical methods is the matrix effect study since biological specimens contain varying levels of impurities and endogenous compounds, which probably may interfere in the precision and recovery of the analytes. For this, fortified blank serum, oral fluid, and urine samples were prepared and analyzed under the optimized procedure. For this purpose calibration standard solutions of the four trimethylxanthines at concentration levels of 5, 10, 20, 30, and 50 µg mL^{-1} in oral fluid and urine, and 1, 5, 10, 20, and 30 µg mL^{-1} in serum, were prepared. Then, the corresponding solutions were prepared in nano-pure water at the same concentration, and all the solutions were analyzed with the proposed method, following the procedure described in Figure 22.

Calibration curves (peak area vs concentration) with five points, by duplicate, were prepared. Correlation coefficients greater than 0.99 were observed for all compounds in all the matrix tested (Table 11). Likewise, the comparison of slope values obtained for the same analyte in the different matrices assayed was different and related to the dilution performed. Finally, the amount of diluted sample processed was different in each case.

Table 11. *Evaluation of linearity, limit of detection and quantification.*

Analytes	Parameter	Serum		Oral fluid		Urine	
		Matrix	Water	Matrix	Water	Matrix	Water
Theobromine	Slope	99 ±6	91 ±5	18.0 ±0.8	21 ±1	45 ±2	43 ±2
	Intercept	890 ±100	100 ±80	50 ±30	-80 ±30	-130 ±80	-20 ±70
	r	0.9949	0.9962	0.9971	0.9969	0.9956	0.9966
	r^2	0.989	0.9924	0.9942	0.9938	0.9912	0.9932
	LOD	0.1		0.5		0.5	
	MQL	0.4		1.5		1.5	
Paraxanthine	Slope	94 ±2	75±3	31.3 ±0.9	35.8 ±1.2	76.4 ±4	75 ±4
	Intercept	70 ±40	110 ±60	20 ±20	-60 ±40	-70 ±110	-90 ±100
	r	0.9991	0.9968	0.9987	0.9982	0.9962	0.9965
	r^2	0.998	0.9936	0.9974	0.9964	0.9924	0.9930
	LOD	0.1		0.5		0.5	
	MQL	0.4		1.5		1.5	
Theophylline	Slope	144 ±2	136 ±6	43.6 ±1.7	54.2 ±1.8	102 ±6	106 ±7
	Intercept	-50 ±40	70 ±100	100 ±50	-190 ±50	40 ±170	-160 ±190
	r	0.9996	0.9971	0.9976	0.9983	0.9946	0.9937
	r^2	0.9992	0.9942	0.9952	0.9966	0.9892	0.9874
	LOD	0.1		0.5		0.5	
	MQL	0.4		1.5		1.5	
Caffeine	Slope	66 ±4	69 ±5	28.4 ±1.3	30.8 ±1.4	72.7 ±1.5	77 ±3
	Intercept	150 ±70	98 ±80	-60 ±40	-80 ±40	-40 ±40	-180 ±80
	r	0.9936	0.9930	0.9970	0.9967	0.9993	0.9979
	r^2	0.987	0.986	0.9940	0.9934	0.9986	0.9958
	LOD	0.1		0.5		0.5	
	MQL	0.4		1.5		1.5	

Concentrations in µg mL^{-1} (Linear range for saliva and urine 5–50 µg mL^{-1}, for serum 1–30 µg mL^{-1}) (n=10). Equations expressed as y = ax +b. The values added after the ± sign represent the standard deviation of the slope and the intercept respectively. r: correlation coefficient; r^2: squared correlation coefficient. LOD: Limit of detection; MQL: Method quantification limit.

According to Andrade-Garda et al. [262], a statistical tool that circumvents the conflict about which variance must be used in the Student's test, represented as P value, was applied for comparison of the slopes of regression lines obtained in the analysis of solutions prepared in water, against those obtained from the solutions prepared in the biological matrix (serum, oral fluid, and urine). The results obtained are shown in Table 12, where is possible to affirm that for a 99% significance level, only theophylline in oral fluid showed statistical differences. Thus, the matrix effect is absent, and the quantification of the analytes can be done with water calibrations without further corrections (except for theophylline, as is explained above). Finally, the percentage of matrix effect (%ME) was also calculated according to Kwon et al.[263], using the following equation:

$$\%ME = [(S_m - S_r) / S_r)] \cdot 100$$

where S_m is the slope of matrix-matched calibration and S_r is the slope of the reagent-only calibration. For all analytes and all matrices, values lower than 25 %ME were obtained (Table 12).

Table 12. *Evaluation of matrix effect for all matrices and analytes.*

Analytes	Parameter	Serum	Oral fluid	Urine
Theobromine	% ME	9 ± 9	-15 ±10	5 ±6
	P value	0.327	0.587	0.584
Paraxanthine	% ME	25 ± 11	-13 ±7	1 ±3
	P value	0.035	0.025	0.958
Theophylline	% ME	8 ± 6	-20 ±10	-4 ±6
	P value	0.125	0.005*	0.672
Caffeine	% ME	-4 ± 6	-8 ±7	-6 ±5
	P value	0.702	0.268	0.269

%ME: Percentage of matrix effect. P value: F test significance level for slope equality (significant difference for 99% probability level).*

4.1.3.2. Limit of detection and quantification

For the calculation of the limits of detection (LOD) a signal-to-noise ratio (S/N) of 3 was used, and the method quantitation limits (MQLs) were estimated via S/N of 10. LOD for all analytes in oral fluid and urine was 0.5 μg mL^{-1}, instead, for serum samples was 0.1 μg mL^{-1}, which can be explained due to the lower dilution performed (dilution factor of 4). MQLs were 0.4 μg mL^{-1} for serum samples, and 1.5 μg mL^{-1} for oral fluid and urine samples (Table 11).

4.1.3.3. Precision

For the study of the precision, evaluated in terms of repeatability, values of HorRat(r) were calculated according to AOAC for intra laboratory studies [264], from the following equation:

$$HorRat(r) = (RSDr / PRSDR)$$

where the term *RSDr* is the Repeatability Relative Standard Deviation, it means the relative standard deviation calculated from within-laboratory data, and the term *PRSDR* is the Predicted Relative Standard Deviation, that is the reproducibility relative standard deviation calculated from the Horwitz formula. In that line, PRSDR is calculated according to Horwitz formula:

$$PRSDR = 2C^{-0.15}$$

Where the term *C* is expressed as a mass fraction, Horwitz calculation is applicable only for analyses that report the concentration in mass ratio units and was based on the statistics calculated for several hundred collaborative studies, including several analyte/matrix/level combinations from studies dating back to the early 20th century [265]. For repeatability, commonly acceptable HorRat(r) values are in the range of 0.3–1.3 [260].

The intra-day precisions using three determinations at two concentration levels for serum samples, three concentration levels in oral fluid samples and one concentration level for urine samples are shown in Table 13. According to the acceptance criteria pointed above, HorRat (r) values were inside the acceptable limits, except for theophylline at a high concentration level in saliva (50 μg mL^{-1}) which a higher value was found, and for caffeine, at a low concentration level in serum (0.4 μg mL^{-1}) a slightly lower value was obtained. Finally, the mean HorRat(r) values for serum, oral fluid, and urine were 0.65, 0.97 and 0.69 respectively, without significant differences among them.

For the inter-day precisions, using three determinations for serum and oral fluid samples at two concentration levels, and one concentration level of urine samples, the HorRat(r) values obtained were inside the acceptable limits. In this case, the means HorRat(r) values for serum, oral fluid, and urine were 0.63, 0.75 and 0.47, and just like for intra-day precision no significant differences among them were found.

Table 13. *Precision parameters for methylxanthines in biological samples by IT-SPME*

Analytes	Parameter	Serum				Oral fluid						Urine	
		0.4 µg mL^{-1}		**20 µg mL^{-1}**		**1.5 µg mL^{-1}**		**5 µg mL^{-1}**		**50 µg mL^{-1}**		**20 µg mL^{-1}**	
		RSD%	HorRat (r)	RSD%	HorRat (r)	RSD%	HorRat (r)	RSD%	HorRat (r)	RSD%	HorRat (r)	RSD%	HorRat (r)
Theobromine	Intra-day assay	14.3	0.78	12.4	1.22	13.2	0.88	13.9	1.11	8.4	0.95	5.4	0.53
	Inter-day assay	12.2	0.66	6.6	0.65	10.9	0.73	9.8	0.79	n.e		6.3	0.62
Paraxanthine	Intra-day assay	4.5	0.25	10.1	1.00	5.6	0.37	11.6	0.93	8.3	0.94	7.0	0.69
	Inter-day assay	17.2	0.94	8.3	0.82	6.5	0.43	8.2	0.66	n.e		4.9	0.48
Theophylline	Intra-day assay	11.2	0.61	5.9	0.58	8.0	0.54	15.2	1.22	15.7	***1.78***	5.4	0.53
	Inter-day assay	8.7	0.48	4.3	0.42	8.5	0.57	11.8	0.95	n.e		6.0	0.59
Caffeine	Intra-day assay	3.2	***0.18***	5.7	0.56	14.2	0.95	16.6	1.33	5.3	0.60	10.1	1.00
	Inter-day assay	12.0	0.66	4.6	0.45	14.3	0.96	11.7	0.94	n.e		3.0	0.30

n.e: not evaluated. In all cases n = 3. Values in bold outside the allowed range.

4.1.3.4. Recovery

Recovery values were calculated as the relative recovery [266] (RR%), comparing the peak area ratios of the analytes from the spiked serum, oral fluid, and urine blank samples to those obtained from the working standard solutions (prepared with water) at the same concentration. Recovery was tested at four different levels of concentrations, according to the FDA Guidance [261]. At the lower limit of quantification was evaluated for serum (0.4 µg mL^{-1}) and oral fluid (1.5 µg mL^{-1}) samples. Low-concentration for all samples was estimated at 5 µg mL^{-1}, instead, mid-concentration assayed were 30 µg mL^{-1} for oral fluid and urine, while for serum was 10 µg mL^{-1}. Finally, the high-concentration in serum was 30 µg mL^{-1} and 50 µg mL^{-1} for oral fluid and urine.

The relative recovery values obtained showed acceptable values in the range of 84-114%, for all the compounds at all levels of concentration evaluated, as it is shown in Table 14. For all compounds, the mean recovery value in the oral fluid was 96.7%, in serum 102.9% and for urine samples of 100.9%. Finally, regarding the compounds, the mean recoveries for all matrices were 104.3% for theobromine, 101.8% for paraxanthine, 95.1% for theophylline, and 99.3% for caffeine.

Table 14. *Results of relative recovery for methylxanthines in biological samples by IT-SPME.*

	Serum	***Oral fluid***	***Urine***
	Recovery (RR%)		
Lower concentration	***0.4 µg mL^{-1}***	***1.5 µg mL^{-1}***	
Theobromine	111.2	105.0	
Paraxanthine	97.9	97.0	not evaluated
Theophylline	95.6	93.0	
Caffeine	95.4	90.7	
Low concentration	***5 µg mL^{-1}***	***5 µg mL^{-1}***	***5 µ g mL^{-1}***
Theobromine	113.0	104.3	111.8
Paraxanthine	103.7	99.9	106.8
Theophylline	86.5	98.8	97.3
Caffeine	112.2	97.5	108.4
Mid concentration	***10 µg mL^{-1}***	***30 µg mL^{-1}***	***30 µg mL^{-1}***
Theobromine	100.7	107.1	97.9
Paraxanthine	112.2	100.0	98.5
Theophylline	92.3	96.8	94.9
Caffeine	101.7	98.3	98.5
High concentration	***30 µg mL^{-1}***	***50 µg mL^{-1}***	***50 µg mL^{-1}***
Theobromine	104.6	92.6	99.0
Paraxanthine	113.8	89.8	99.9
Theophylline	106.6	84.1	99.7
Caffeine	99.3	91.9	98.4

4.1.3.5. Specificity and selectivity

The specificity and selectivity of the method were established by the analysis of blank and spiked samples from five individual sources, in which nonappearance of interferences at the retention times of the analytes was shown. Furthermore, the absorption spectra of the corresponding peak for

the analytes in the enriched samples were compared with the spectra of a standard solution. Figure 23 shows the UV spectra recorded for the analytes studied.

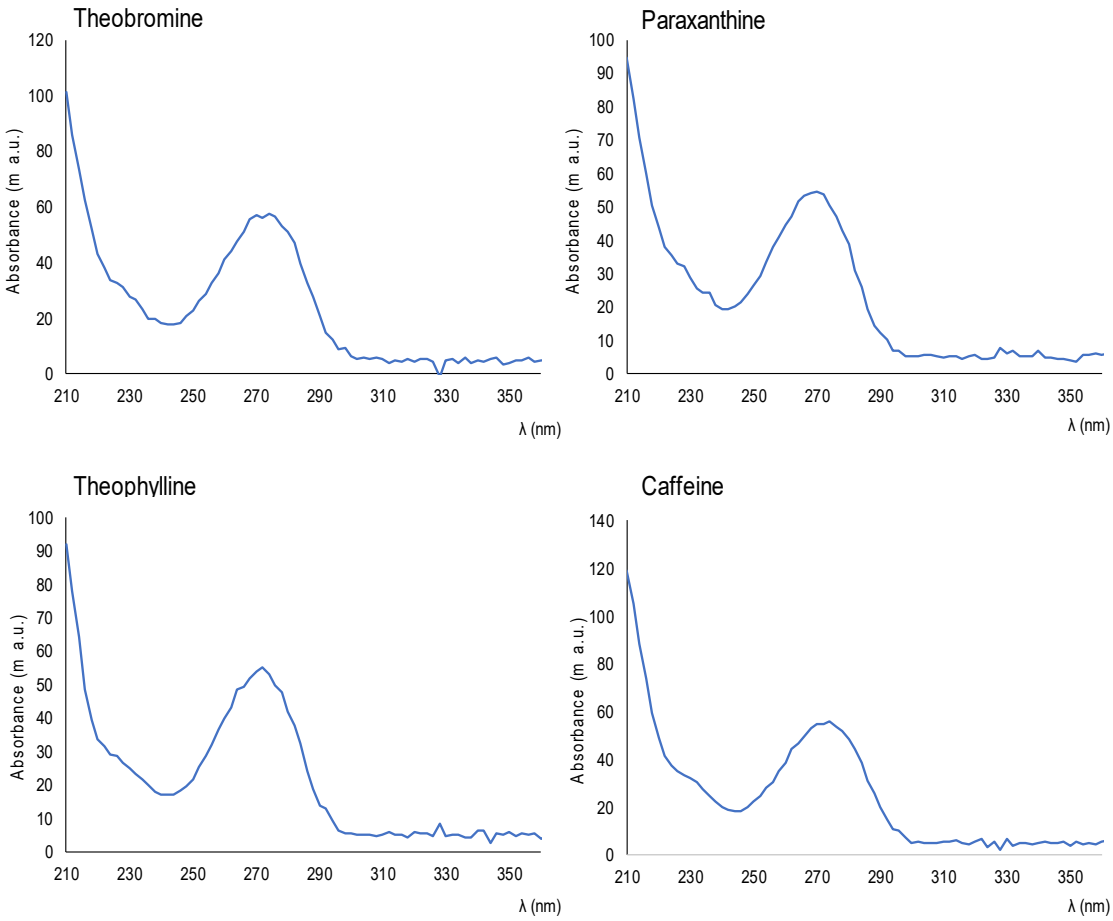


Figure 23. *UV spectra obtained for the trimethylxanthines studied.*

4.1.3.6. Stability

For bioanalytical assays, the stability parameter evaluation includes, at the bench-top, in extracts, freeze-thaw cycles, stock solution, and long-term [261]. In this work, the freeze-thaw and the long-term stability of the analytes were evaluated, being the only ones applicable to the IT-SPME procedure, due to the simplicity of this technique. For the freeze-thaw stability, two concentration levels, high and low, for serum (30 and 1 μg mL^{-1}) and oral fluid (50 and 5 μg mL^{-1}) with three cycles of frozen for at least 12 hours between cycles and with three determinations in each cycle, were tested. For all concentration levels, the recovery values were between 85.6-108.4% of the fortified concentration, being the acceptance criterion that the recovery at each level should be ± 15% of the nominal concentration.

In the same way, a real sample of oral fluid containing caffeine was subjected to freeze-thaw stability evaluations in the conditions explaining above (12 h, 3 cycles). Adequate precision has resulted (RSD %= 0.7). For the long-term stability of the analytes, a fortified oral fluid sample at 20 µg mL^{-1} concentration level, was analyzed by the proposed method and then stored in the freezer. After nine months the sample was re-analyzed, and the found concentrations of the analytes were within 15% of control values.

4.1.3.7. Robustness

For the evaluation of the robustness of the method a half fraction 2^{4-1} factorial design was applied [267], evaluating four variables according to the proposed methodology, which includes: a) columns from different suppliers (Agilent Zorbax SB C18 and Phenomenex Jupiter C18); b) formic acid concentration (0.95% or 1.05%); c) ionic strength (addition or not of sodium chloride solution) and diluted sample volume processed (24 or 26 µL). The two levels for each variable were combined in eight assays for each matrix (serum and oral fluid), using The Unscrambler software, as it is described in Table 15.

Table 15. *Half fraction 2^{4-1} factorial design for evaluation of robustness of the method.*

Factor	*Units*	*Evaluated values*		*Assays*							
		Lower values (-1)	*Upper values (+1)*	*A1*	*A2*	*A3*	*A4*	*A5*	*A6*	*A7*	*A8*
Analytical Column		Zorbax SB	Jupiter C18	-1	+1	-1	+1	-1	+1	-1	+1
Concentration of formic acid solution	Percentage (%)	0.95	1.05	-1	-1	+1	+1	-1	-1	+1	+1
Modification of ionic strength	Percentage (%)	0.0	0.2	-1	+1	+1	-1	+1	-1	-1	+1
Diluted sample volume	Microliters (µL)	24.0	26.0	-1	-1	-1	-1	+1	+1	+1	+1

To carry out the assays, in the first instance, serum and oral fluid blank samples were fortified at 20 µg mL^{-1} and 10 µg mL^{-1} respectively. Then for serum samples, 25 µL was mixed with 72 µL of aqueous formic acid solution (0.95% or 1.05%) and 3 µL of water or 3 µL of 0.2% NaCl solution. Then 24 µL or 26 µL were then processed by IT-SPME in the selected column. On the same way, for an oral fluid sample, 10 µL were mixed with the same volume of aqueous formic acid (0.95% or 1.05%), 3 µL of water or 3 µL of 0.2% NaCl solution and diluted to 100 µL with nanopure water. Finally, 24 µL or 26 µL were then processed by IT-SPME in the selected column.

According to Hund et al., the condition that interactions should be negligible is, in general, assumed to be fulfilled in robustness tests [268], therefore, the peak areas (signal responses) for all assays in each tested matrix, were statistically analyzed, and the studied variables were found not significant (for a 95% probability level) for all four compounds. Finally, through Pareto graphs, the visualization of the standardized effects of each variable studied is presented in Figures 24 and 25 for serum and oral fluid matrix respectively. For all compounds, the standardized effect was less than the established critical value (3.2).

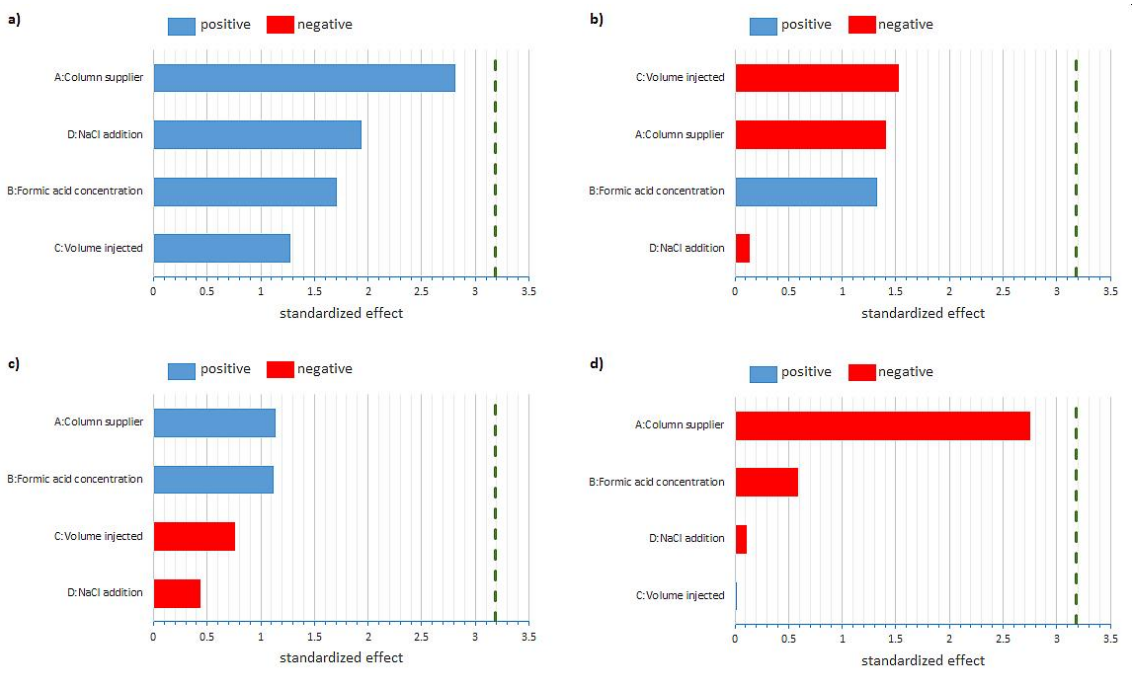


Figure 24. *Standardized effects determined using a half-fraction 2^{4-1} factorial design in serum matrix: a) Theobromine; b) Paraxanthine; c) Theophylline; and d) caffeine.*

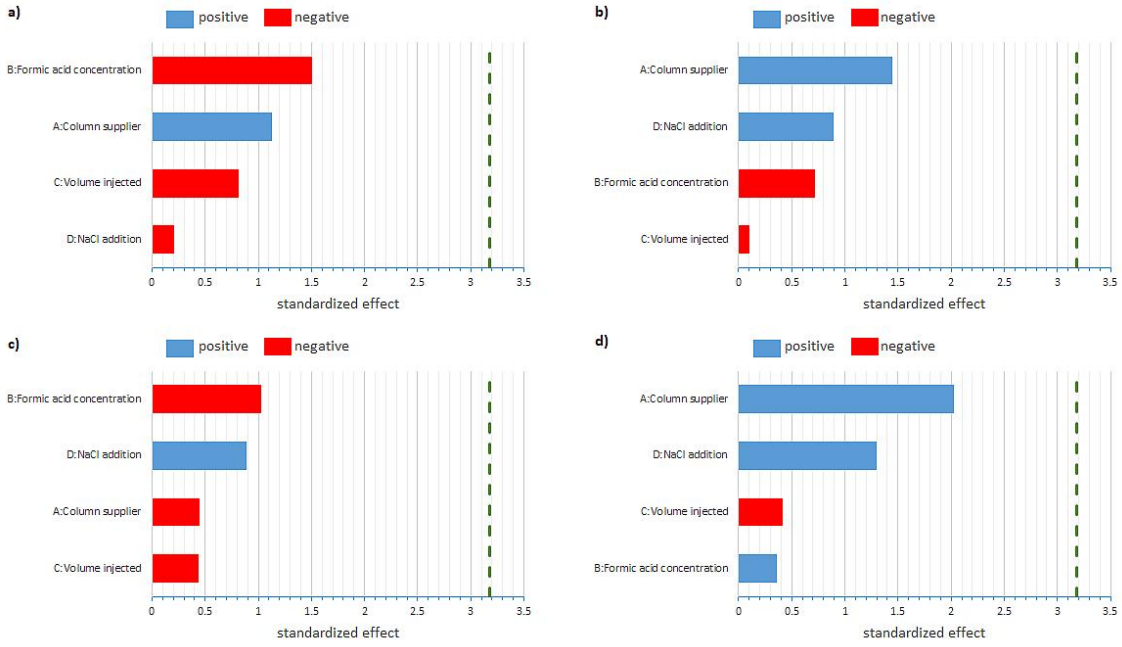


Figure 25. *Standardized effects determined using a half-fraction 2^{4-1} factorial design in oral fluid matrix: a) Theobromine; b) Paraxanthine; c) Theophylline; and d) caffeine.*

4.1.4. Analysis of real samples

Serum and oral fluid paired samples from preterm newborns were obtained in the framework of the non-invasive pharmacokinetic study of caffeine administered in preterm infants diagnosed with apnea at the pediatric intensive care unit of La Fe University and Polytechnic Hospital - Valencia, Spain, to determinate the concentrations of caffeine, theobromine, paraxanthine, and theophylline, because they are related as it has been reported in some papers of the literature [269-271].

4.1.4.1. Concentration of caffeine in serum and saliva samples

The developed method was applied for the determination of 35 paired samples by duplicate. Representative chromatograms of serum and oral fluid samples, as well as a standard solution, are shown in Figure 26. In all samples, the presence of caffeine was determined and none of the metabolites were found. The absence of metabolites in the samples can be explained, on the one hand, due to the high doses of caffeine that are administered, as Nobile et al. reported [272], and on the other hand, some papers have reported that the caffeine plasma half-life can vary between 65 and 100 h, and the unmodified caffeine fraction eliminated in the urine is 86% in the first six days [269]. In some serum samples, a peak with a retention time near to theobromine was observed. However, by comparing the absorption spectrum, it was ruled out that it was one of the analytes studied.

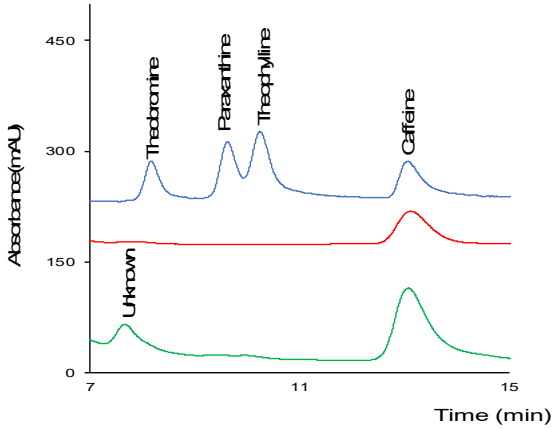


Figure 26. *Representative chromatograms from preterm newborns samples: Blue: Standard solution 20 µg mL^{-1}; Red: Oral fluid sample; Green: Serum sample.*

Table 16 shows the caffeine average concentration found in the analyzed samples, with concentration range values between 7.0 and 54.0 µg mL^{-1} in serum samples and between 5.5 and 56.2 µg mL^{-1} in oral fluid samples.

Table 16. *Caffeine average concentration found in the analyzed paired samples.*

Sample identification	Caffeine concentration (µg mL^{-1})		Sample identification	Caffeine concentration (µg mL^{-1})	
	Serum	Oral fluid		Serum	Oral fluid
P1/PK-C/M1	26.99	27.93	*P20/PK-C/M1*	29.84	20.81
P3/PK-C/M1	15.88	10.16	*P20/PK-C/M2*	12.90	8.47
P3/PK-C/M2	6.96	7.57	*P21/PK-C/M1*	23.97	12.23
P5/PK-C/M1	14.23	14.25	*P21/PK-C/M2*	31.63	26.17
P7/PK-C/M1	9.36	15.90	*P22/PK-C/M1*	14.05	11.20
P7/PK-C/M2	19.86	14.93	*P23/PK-C/M1*	24.23	18.32
P8/PK-C/M1	17.34	16.02	*P23/PK-C/M2*	17.68	15.41
P8/PK-C/M2	13.96	14.68	*P25/PK-C/M1*	23.07	15.17
P9/PK-C/M1	14.74	10.92	*P25/PK-C/M2*	15.83	13.52
P9/PK-C/M2	11.10	15.11	*P26/PK-C/M1*	22.81	17.08
P11/PK-C/M1	13.04	5.54	*P26/PK-C/M2*	26.06	16.59
P12/PK-C/M1	17.51	15.94	*P27/PK-C/M1*	35.00	20.81
P13/PK-C/M2	40.67	56.18	*P27/PK-C/M2*	24.63	20.32
P16/PK-C/M1	54.01	50.22	*P31/PK-C/M2*	27.17	17.35
P18/PK-C/M1	31.67	46.65	*P31/PK-C/M3*	20.82	13.98
P15/PK-C/M1	15.95	10.32	*P32/PK-C/M1*	17.67	14.57
P19/PK-C/M1	24.51	12.49	*P32/PK-C/M2*	19.08	10.16
P19/PK-C/M2	21.36	13.56			

4.1.4.2. Correlation between serum and saliva concentrations

The study of serum caffeine concentrations and those found in oral fluid showed a close correlation, as it is shown in Figure 27, with intercept (-2 ± 3) which is statistically equal to 0; a slope equal to 0.99 ± 0.14 (statistically equal to 1), and finally, a correlation coefficient close to 0.8. Likewise, when the paired-samples means were compared by a significance test, no evidence for a significant difference in the methods at =0.05 (t_{exp} = 1.43; t_{tab} = 2.03) was obtained [273].

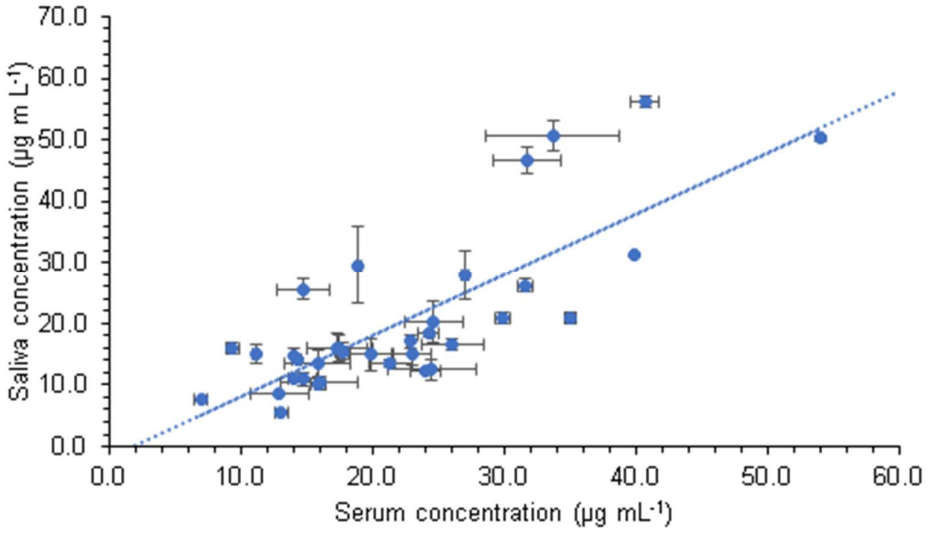


Figure 27. *Correlation plot of concentration values of caffeine in serum and saliva (n = 35). Regression equation: C saliva = (-2 ± 3) + (0,99 ± 0,14)·C serum. r = 0.786.*

Since some authors have pointed, the application of simple linear regression and the coefficient of determination is not recommended as a method for assessing the comparability between analytical methods, due to the correlation studies the relationship between one variable and another, not its differences [274]. An alternative way to address the shortcomings of simple linear regression and the coefficient of determination for evaluation of laboratory tests is the Bland-Altman method, which yields a plot useful in qualitatively assessing differences in measurements between two assays [275,276].

The Bland-Altman graphic for the concentrations of caffeine in serum (method A) and caffeine in oral fluid (method B) of the paired samples is shown in Figure 28. No significant differences in the results for both methods were obtained, with mean difference value being statistically equal to 0 at a confidence interval of [-0.80, 4.60].

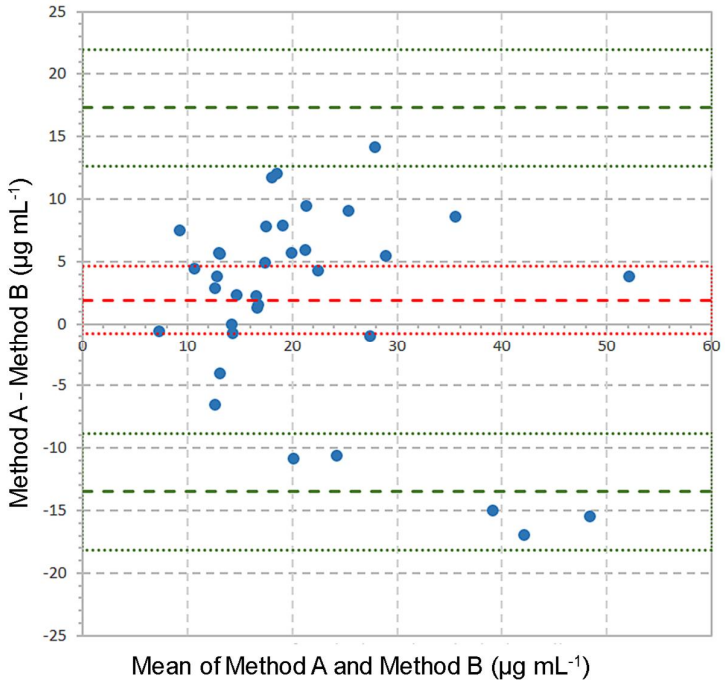


Figure 28. *Bland-Altman graphic (plot of differences between method A (caffeine in serum) and method B (caffeine in saliva) vs. the mean of the two measurements). It presents confidence interval limits for mean and agreement limits.*

4.1.4.3. Evaluation of precision from duplicate results

According to Thompson and Howarth [277], duplicate analytical results can be used to give a rapid and realistic estimate of precision in analytical systems. In this thesis, the evaluation of the standard deviation from duplicate results of the analysis of serum and oral fluid samples were performed according to Synek [278]. The relative standard deviation (% RSD) obtained from all analysis was calculated with the following equation:

$$\% \text{ RSD} = (S / X)\ 100$$

Where *S* is the term for standard deviation and *X* represents the average concentration. For serum samples, the result was 7.6%, and for oral fluid samples, it was estimated at 8.4%, with caffeine mean of about 20 µg mL^{-1} in both cases. These values are consistent with those previously obtained at the validation step. Figure 29 shows the graphic representation of the relationship between the mean concentration of caffeine and %RSD for duplicate analytical results.

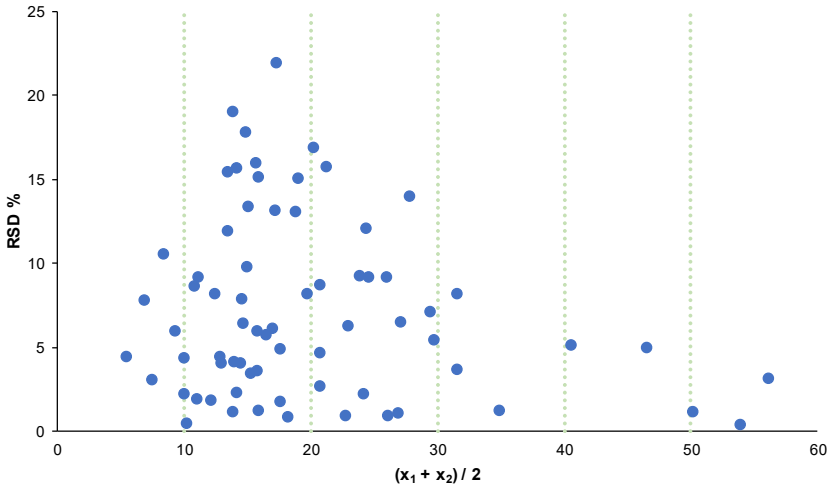


Figure 29. *Plot of the relationship between the mean concentration of caffeine and % RSD for duplicate analytical results.*

4.1.5. Conclusions

In the present work, the application of IT-SPME, using a conventional capillary column with terephthalic modified PEG as the sorbent phase, in line with Capillary LC-DAD, demonstrated a remarkable performance for the simultaneous quantitation of caffeine and its three primary metabolites, theobromine, paraxanthine, and theophylline in serum, oral fluid, and urine samples. The developed method offers a complete analysis in 20 min, with a low sample volume consumption, less than 10 µL of serum, oral fluid, and urine samples for a single run. Likewise, no more preparation than a dilution of the samples and injection in the IT-SPME loop is necessary.

In terms of analytical parameters, high sensitivity was obtained, with limits of detection of 0.5 µg mL^{-1} in urine and oral fluid, and 0.1 µg mL^{-1} for serum, recovery values of RR% between 84–114%, and adequate repeatability with a coefficient of variation lower than 15%. The characteristics of the developed method make it useful for its implementation in a pediatric hospital laboratory in the case of special patients, as it is the case of preterm newborns, where the sample availability and the urgency of the results, are important analytical requirements.

The applicability of the newly developed method was demonstrated for the determination of caffeine concentrations in paired samples of serum (invasive method) and oral fluid (non-invasive method) from preterm newborns who were given doses of caffeine for the treatment of apnea of prematurity, providing similar results between them.

4.2 TRIMETHYLXANTHINES IN WATERS

As described earlier, the high consumption of coffee, tea, cola, energy drinks, processed cocoa products, as well as medications, with trimethylxanthines as components, make possible the presence of these substances in environmental matrices, such as water, so they are considered as emerging pollutants, indicators of anthropogenic activity and lifestyle.

The application of miniaturized and portable liquid chromatography systems has a particular interest in water analysis to obtain instant results in the site of sampling. In the development of this thesis, two procedures for the analysis of caffeine, theobromine and theophylline in water samples have been optimized, using two approaches. On one hand, IT-SPME-CapLC-DAD, and on the other hand, SPE with portable NanoLC. Both procedures were applied to the analysis of sea and river water samples. The results and the comparison of the two approaches, in terms of advantages and limitations, are presented in detail below.

4.2.1. Optimization of the chromatographic separation

4.2.1.1. Portable NanoLC

At the beginning of the optimization of chromatographic separation with the portable nano-LC system, initial mobile phase initial conditions were taken from manufacturer recommendations, a mixture of water and acetonitrile, adding a 3% of each solvent in the reservoir of the other, which for the water reservoir, can be explained as prevention for bacterial growth, and its corresponding compensation in the proportions for acetonitrile reservoir.

A mixed standard solution of the analytes at 50 µg mL^{-1} was prepared in acetonitrile as a solvent and analyzed in the chromatograph. It was observed that the peak profiles obtained for the analytes, especially for caffeine, in these conditions show a bad efficiency (split and double peaks). From these results, the effect of the solvent in which the analyte solution is prepared was studied. For this, caffeine solutions with a concentration of 50 µg mL^{-1} were prepared in acetonitrile, water, methanol and acetonitrile-water mixtures with different proportions. The solutions were injected into the chromatograph and the analyte chromatographic peak was observed, as shown in Figure 30.

All solutions prepared with acetonitrile showed a split and double peak, on the other hand, those prepared in methanol and water showed improved results in terms of efficiency and signal detection, however, the last was ruled out due to incompatibility with sample treatment techniques applied. Consequently, methanol was the solvent used in further experiments.

Subsequently, the gradient elution program was optimized, for this, a mix standard solution of the analytes at 10 µg mL^{-1}, prepared in methanol, was injected into the chromatograph. The high polarity of the compounds requires a mobile phase composition with low elution strength at the beginning of the program. Different percentages of acetonitrile and gradient ranges were assayed to obtain the conditions that favor the detection of individual peaks of the compounds, in the shortest possible run time.

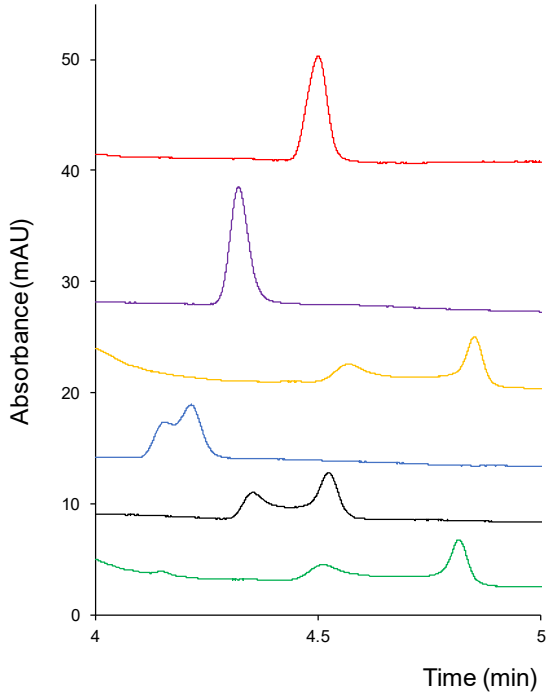


Figure 30. *Chromatograms obtained in the study of the effect of the solvent on the separation efficiency. Solution of caffeine standard at 50 µg mL^{-1} prepared in different solvents. Green: in acetonitrile; Black: in a mixture of acetonitrile and water (8:2); Blue: in a mixture of acetonitrile and water (5:5); Orange: in a mixture of acetonitrile and water (2:8); Purple: in water; Red: in methanol.*

The final gradient conditions started with a low percentage of acetonitrile (5%) increased to 20% at min 4, and to 95% at 4.5 min. This composition was kept constant for 0.2 min, and finally, initial conditions were set in 0.3 min and hold until the end of the run (5.2 min). With these conditions, satisfactory separation of the analytes in less than 4.5 minutes was obtained (Figure 31.Blue).

4.2.1.2. Capillary LC

For the separation of the three analytes in the CapLC system, the initial conditions were taken from the results obtained for the analysis of trimethylxanthines in biological samples discussed in section 4.1 of this thesis and the optimized conditions for the portable NanoLC system, explained

above. A C18-column (150 x 0.5 mm, 4 µm) and a mixture of water and acetonitrile in gradient elution mode was used for the separation of studied compounds. The percentage of acetonitrile was increased from 20 % at 0 min, to 50 % at 5 min, to 75% at 9 min, and to 100 % at 13 min; the mobile-phase flow rate was 15 µL min^{-1} and the signal was monitored at 275 nm.

For manual injection, a 20 cm length and 0.32 mm i.d. segment of a fused-silica capillary was used as an injection loop, so that the sample volume injected was 16 µL (internal volume of the capillary) of a standard solution prepared in nanopure water. In these conditions, the resolution was also suitable, and the three analytes were separated in less than 8.5 min, as depicted in Figure 31 (Red). The chromatograms of Figure 31 show that the resolution between the analytes was much better with the portable NanoLC as the peaks were much narrower.

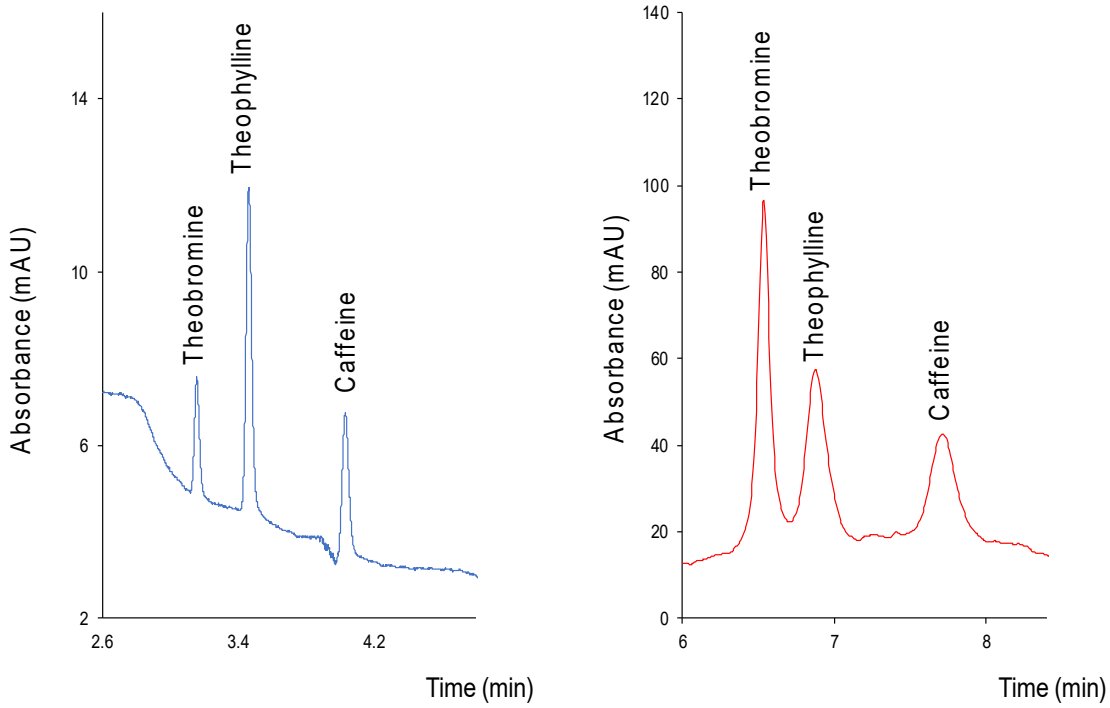


Figure 31. *Chromatograms obtained under the selected conditions with: (Blue) the portable nano LC; (Red) CapLC systems. Concentration of each analyte: 10 µg mL^{-1} for Blue signal, and 10 ng mL^{-1} for Red signal.*

4.2.1.3. Comparison of analytical parameters achieved by Capillary LC and portable NanoLC

The comparison of the relevant features of the two tested chromatographic systems is summarized in Table 17. From the experimental results is possible to affirm that the IT-SPME-Cap LC could be applied to detect and measure lower concentrations of the analytes because instrumental limits of detection (LODs) for this system were 20 times lower, which can be explained by the fact that

the sample volume injected into the CapLC system was just under 20 µL, whereas in NanoLC chromatograph only 40 nL of the working solutions were sent to the analytical column.

Moreover, is important to note that with the NanoLC system, the chromatograms were registered at 255 nm, whereas in the CapLC the signals were registered at 275 nm, which is the maximum wavelength of the UV spectra for caffeine; likewise, the maxima of the UV spectra of theophylline and theobromine was close to 275, as Figure 23 showns, and thus, the absorbances of the three analytes at 275 nm were higher than those measured at 255 nm. Additionally, with Cap LC is possible to register the spectrum of each analyte at any position of the corresponding chromatographic peak, which improved selectivity about the measurement at 255 nm with portable NanoLC system.

The instrumental linearity of each system was evaluated by processing standard solutions, prepared in nanopure water, in the concentration ranges of 1-50 µg mL^{-1} and 50-400 ng mL^{-1} for the portable NanoLC and CapLC systems, respectively. The determination coefficients for all compounds in the two systems were higher than 0.99. By processing consecutively three replicates of the working solutions, at 10 µg mL^{-1} and 5 ng mL^{-1} for the portable NanoLC and CapLC systems, respectively, the instrumental precision, expressed as relative standard deviation (% RSD), was calculated for both systems, and the values were slightly lower with the portable chromatograph.

On the other hand, the comparison of the analysis times showed that the chromatographic separation was faster with the portable NanoLC system. This fact, accompanied by the lower mobile phase flow rates, resulted in much lower mobile phase consumption with the portable system, being of interest from a point of view of green analytical chemistry. Using a rough estimation of the mobile phase consumption by injection carried out for both systems, it was possible to establish that the portable system only needs 20 µL of the mobile phase per analyzed injection, conversely, the CapLC chromatograph, require 10 mL for each injection. That means that the portable system reduces the production of waste about 500 times less than the benchtop chromatograph.

Table 17. *Comparison of the features of the two chromatographic systems used in this study.*

Feature		Portable NanoLC			CapLC		
		Calibration equation [a] (n = 10)	**RSD [b], (n = 3) (%)**	**LOD (ng mL^{-1})**	**Calibration equation [a], (n = 10)**	**RSD [b], (n = 3) (%)**	**LOD (ng mL^{-1})**
Analytical performance	Theobromine	0.36 x +0.11 (R^2 = 0.998)	1	300	1.7 x + 10.4 (R^2 = 0.9990)	7	15
	Theophylline	0.72 x + 0.07 (R^2 = 0.9990)	1	300	2.8 x - 35.9 (R^2 = 0.9990)	6	15
	Caffeine	0.55 x + 0.02 (R^2 = 0.9990)	2	300	1.6 x -21.2 (R^2 = 0.997)	6	15
Volume of sample		40 nL			20 µL		
Time of the chromatographic run		5 min			16 min		
Solvent consumption per sample		20 µL			10 mL		

(a) In the 1-50 µg mL^{-1} and 50 - 400 ng mL^{-1} concentration ranges for the portable NanoLC and CapLC systems, respectively; Equations expressed as y = ax +b

(b) Measured at a concentration of 10 µg mL^{-1} and 5 ng mL^{-1} for the portable NanoLC and CapLC systems, respectively.

4.2.2. Sample treatment techniques

Once the chromatographic parameters were characterized, sample treatment was optimized for the two chromatographic systems. Here, two possible strategies for sample conditioning suitable for on-site analysis with the portable NanoLC system were explored, namely off-line IT-SPME and solid-phase extraction (SPE) with cartridge format. On the other hand, for the CapLC system, IT-SPME on-line was optimized.

4.2.2.1. On-line IT-SPME

As it is mentioned in the introductory part of this work, the selection of the extractive phase for IT-SPME is essential to obtain suitable extraction efficiency, and it is a key parameter especially for highly polar compounds like trimethylxanthines studied. According to the explanation of section 4.1.2.1, various extractive capillaries coated with different sorbents were tested, namely polydimethylsiloxane (PDMS) with diphenyl and with cyanopropylphenyl groups, polyethylene glycol (PEG), terephthalic acid modified PEG, all of them conventional capillaries, and polymers obtained from tetraethyl orthosilicate (TEOS) and triethoxymethylsilane (MTEOS) functionalized with SiO_2 and TiO_2 nanoparticles. It was demonstrated that the best results, in terms of extraction efficiency, were obtained with the terephthalic acid modified PEG phase.

As an attempt to enhance the analyte detectability by improving the limits of detection of the analytes, a polystyrene-divinylbenzene (PS-DVB) phase was tested. According to Serra-Mora et al. this sorbent provided excellent results for the IT-SPME of compounds with aromatic rings [279], and considering that because of their structure, the analyzed compounds exhibit aromatic-like properties [280]. Additionally, Villamena et al. demonstrated the nonspecific yet selective adsorption of caffeine by some polymers as the divinylbenzene (DVB) [281].

Two different PS-DVB capillaries columns were tested, HP-PLOT/Q and VP-HayeSep P, with 20 µm and 10 µm thickness, respectively. In the first experiments, the effect of preconcentration on the HP-PLOT/Q capillary was tested. For this, a standard solution mixture of the analytes at 50 ng mL^{-1} was prepared in nanopure water and variable volumes of this solution were processed through the capillary of 25 cm length. The signal intensity of the three analytes versus processed volume is represented in Figure 32, and the results showed an increment of the signal when the volume of the solution is increased. This behavior of preconcentration was especially marked for caffeine when 4 mL of the solution was passed. Therefore, this volume was selected as the optimum.

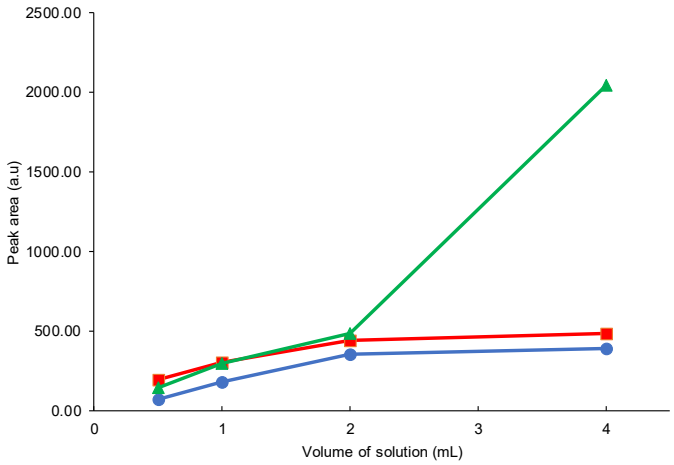


Figure 32. *Study of the effect of volume on preconcentration in the HP-PLOT/Q capillary. Experimental conditions: standard solution at 50 ng mL^{-1}; capillary length of 25cm; Volume of flushing 100 µL of water. Blue: Theobromine; Red: Theophylline; Green: Caffeine.*

Another strategy to improve the analyte detectability in IT-SPME is to increase in the length of the capillary column. Therefore, two lengths were compared, 25-cm-long and 50-cm-long columns, passing 4 mL of a standard aqueous solution with a concentration of 5 ng mL^{-1} in each capillary. Higher signal intensity values for the three analytes were obtained when the length was increased. This increase in capillary length did not cause changes in system pressures. Figure 33 shows the obtained results of the evaluation of capillary length.

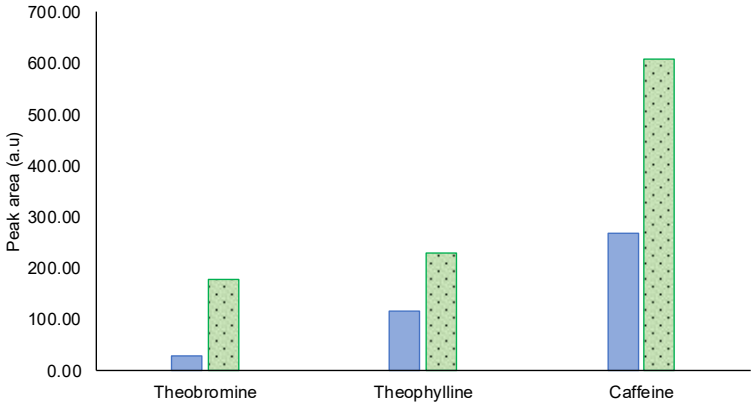


Figure 33. *Study of the effect on capillary length with the HP-PLOT/Q capillary. Experimental conditions: standard solution at 5 ng mL^{-1}; processed volume of 4 mL; Volume of flushing 100 µL of water. Blue: 25 cm; Green: 50cm.*

Finally, when the parameters of IT-SPME were optimized for HP-PLOT/Q capillary, the extraction efficiency was compared with the performance of VP-HayeSep P capillary, in equal conditions. For this, a standard solution mix of the analytes in nanopure water at 10 ng mL^{-1} was prepared and 4 mL of the solution were passed through both capillaries, with a length of 50 cm, while the manual injection valve was in the loading position. After a flushing step with 100 µL of water, the injection valve was changed to the injection position. The peak area intensity obtained for all compounds in the two polymeric capillaries was compared with those achieved with the terephthalic acid-modified PEG-coated capillary (FFAP). For the latter capillary, the IT-SPME conditions included a standard solution concentration of 1 µg mL^{-1}, 100 µL of the solution were processed, and 25 µL of water for flushing.

Figure 34 compares the responses obtained with the three capillaries tested. As noted, the PS-DVB phase provided higher peak areas than those obtained with the PEG-based phase, even processing half the mass of the analytes. The comparison of the two polymeric capillaries shows that no significant differences between PS-DVB with coatings of 10 µm and 20 µm were observed. However, due to the greater sorbent phase thickness, the 20 µm capillary column retained more impurities from the matrix, therefore the background chromatograms were lower with the 10 µm thickness capillaries, which were then selected for further work.

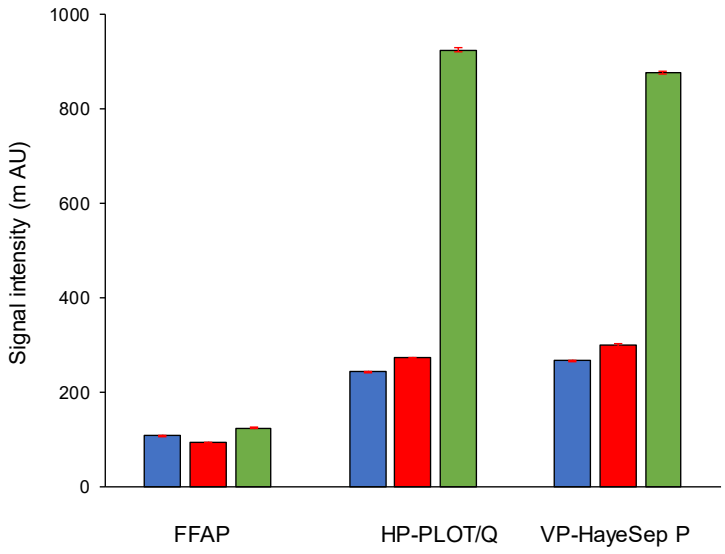


Figure 34. *Analyte responses obtained SPME with the different capillaries tested coupled on-line to the CapLC system. Concentration of each analyte: 1 µgmL^{-1} in the modified PEG (FFAP) and 10 ng mL^{-1} for the polymeric phases (HP-PLOT/Q and VP-HayeSep P); Processed volume 0.1 mL for FFAP and 4 mL for polymeric phases. Blue: Theobromine; Red: Theophylline; Green: Caffeine. Error bars represent standard deviation for n = 3.*

4.2.2.2. Off-line IT-SPME

There are two important aspects to consider in the election of the sample treatment technique previous the analysis in the portable NanoLC system. On one hand, the design of the chromatography instrument constrains the sample treatment to off-line procedures and, bearing in mind that this equipment is intended to field tests, sample treatments should involve a minimum of sample handling and, ideally, no additional instrumentation.

As is refers in the introduction part of this thesis, IT-SPME off-line does not required additional instrumentation, as only a manual injection valve and syringes to load the samples and desorb the trapped analytes are necessary. Additionally, as previously the IT-SPME online procedure was previously optimized for the CapLC system, off-line IT- SPME was selected as the first choice as a sample treatment technique for the portable NanoLC analysis.

Consequently, this approach was tested by using the capillary column selected in the previous section, the PS-DBV 10 μm thickness coated capillary. Initially, aliquots of the standard mix solution, containing 500 μg mL^{-1} of each analyte, prepared in nanopure water, were processed through the capillary of 50 cm length (internal volume of 40 μL), followed by 40 μL of water, as a cleanup step. Then, the capillary was flushed with a volume of methanol to desorb the retained analytes, and the collected extracts were then filtered (0.22 μm nylon membranes) and, finally, injected into the portable NanoLC system. The selection of methanol as a desorption solvent was due to the positive results observed in the optimization of the chromatographic separation. The minimum volume of methanol that led to reproducible volumes of the extracts was 100 μL; for this volume, the volume of the collected extracts was about 50 μL.

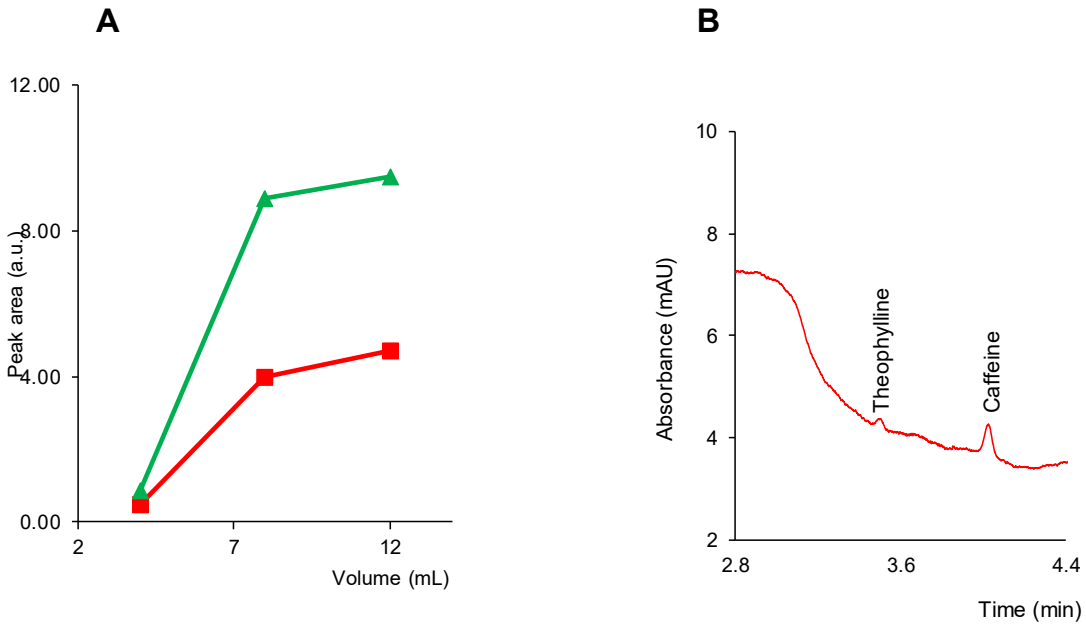


Figure 35. *Results obtained by off-line SPME/portable NanoLC: (A) effect of the sample volume on analyte responses; (B) chromatogram obtained for a sample volume of 12 mL. Concentration of each analyte, 500 µg mL^{-1}. Capillary: VP-HayeSep P (50 cm).* Volume of flushing: *40 µL of water. Desorption volume: 100 µL methanol.*

In those conditions, and to increase analytes detectability as much as possible, aliquots of the standard solution of 4, 8, and 12 mL were processed though the IT-SPME device. Increasing the volume from 4 mL to 8 mL resulted in an increment in peak areas of the analytes, but a further increment to 12 mL had a modest effect (Figure 35.A). Even for the higher amount of processed volume of the working solutions (12 mL), the peak of theobromine could not be detected, as observed in Figure 35B. This could be most probably explained by the breakthrough of the analyte, as this was the most polar of the three tested compounds (log Kow value equal to -0.8).

In the last attempt to improve the detection of the analytes, the capillary length was increased from 50 to 100 cm was tested. However, this modifies the internal volume of the capillary, which in turn increases the volume necessary to desorb the analytes. As expected, no improvement was seen with the longest capillary, since in IT-SPME the nature of the sorbent and the interactions of the analytes with it is more important for the extraction efficiency than the quantity of sorbent phase contained in the capillary [257].

Despite all the efforts made to reduce the detection limits, at this point the reached results for the off-line IT-SPME procedure show that this sample treatment technique could detect levels higher than 0.1 µg mL^{-1} for caffeine, but it is expected that the trimethylxanthines could be at lower

concentration levels in some environmental waters. In this sense, only for raw wastewater samples, the reported concentrations of caffeine have been higher than 0.1 μg mL^{-1}, for example, Bo et al. reported the presence of caffeine in influent waters of WWTPs between 0.1 and 0.6 μg mL^{-1} [282]. In contrast, analysis of samples of treated wastewater and river water has reported maximum concentrations of caffeine equal to 0.03 μg mL^{-1} [283] and 0.01 μg mL^{-1} [284], respectively.

4.2.2.3. SPE

In SPE the aqueous sample passes through a cartridge containing the solvent phase, with higher amounts than in IT-SPME. It allows reaching a better detection limit, due to the greater sample loading capacity, as well as a higher preconcentration [285]. Like IT-SPME, in SPE different parameters of the extraction process must be optimized to raise the efficiency of the technique.

In this study, different sorbents were tested, including two commercially available cartridges with PS-DVB and C18 phases, and two homemade silica-amino sorbents with two particle sizes, < 100 μm and >100 μm, prepared according to the procedure proposed by the LIFE LIBERNITRATE project from rice straw.

The extraction efficiency of the four tested cartridges was compared. For this, aliquots of 8 mL of a standard mix solution of the analytes at 500 ng mL^{-1} were processed. Then, the analytes were desorbed from the cartridges with 100 μL of methanol. Subsequently, the extracts were filtered and injected into the portable NanoLC system. Unsuitable results, with an absence of signals for the three compounds in the analysis with the silica amino sorbent cartridges, were observed, whereas with the PS-DVB and C18 sorbents satisfactory peak areas were obtained. However, the peak areas measured with the last packed cartridge were significantly higher, as seen in Figure 36. This behavior can be explained because the C18 sorbent has an end-capped technology, which improves the retention of polar compounds, as is the case of the studied compounds.

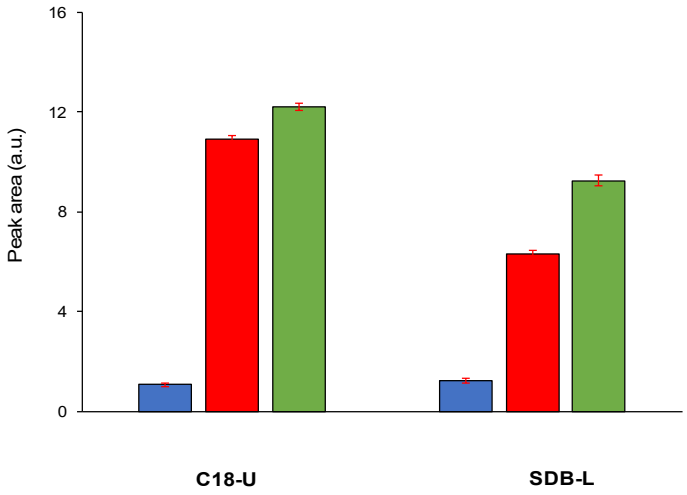


Figure 36. *Analyte responses obtained with two of the four cartridges tested and the portable nanoLC system. Concentration of each analyte: 500 ng mL^{-1}; Processed volume: 8 mL. Blue: Theobromine; Red: Theophylline; Green: Caffeine. Error bars represent standard deviation for n = 3.*

Next, the load volume was optimized and different volumes, between 4 and 35 mL, of the same solution of the analytes (500 ng mL^{-1}) were processed through the C18 packed cartridges. The chromatograms of Figure 37 shows that increasing the sample volume up to 25 mL had a linear positive behavior on the peak areas concerning the load volume for all compounds. However, no improvement was observed when using higher volumes were used (35 mL).

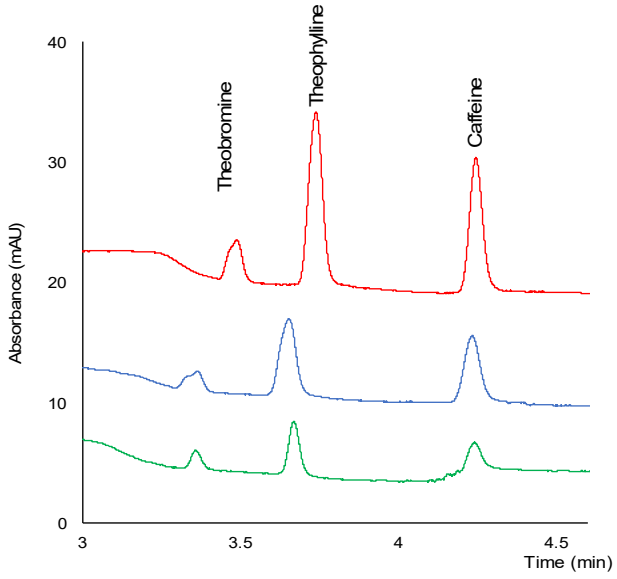


Figure 37. *Chromatograms of the analysis for different load volumes: Green: 8 mL; Blue: 16 mL; Red: 25 mL. Concentration of each analyte: 500 ng mL^{-1}; cartridge: C18-U.*

The volume of elution is an important parameter in SPE, since a high volume of the desorption solvent will dilute the concentration of the analytes, affecting the detectability in the instrumental analysis, and on the other hand, with lower volumes, the analytes could be only partially eluted, decreasing the extraction efficiency [286]. To optimize this volume, 25 mL of the standard mix solution of 500 ng mL^{-1}, were processed and, then, three aliquots of 100 µL of methanol were successively flushed through the cartridges and the extracts were then collected and processed individually.

It was observed that most of the theobromine eluted in the firsts 100 µL, whereas theophylline and caffeine were predominant in the second part of the extract. Moreover, the signal of the analytes found in the third part of the extract was negligible. Based on these results, another extraction, with the same conditions, but using one only fraction of 200 µL of methanol to desorb the analytes was carried out. However, the peak area found for caffeine was significantly lower than that observed when processing the second eluting extract. The results of these experiments are shown in Figure 38.

In brief, processing separately the first eluting extract for theobromine and the second eluting extract for theophylline and caffeine was selected as the best option, considering that each chromatographic assay with the portable nanoLC chromatograph takes less than four and half minutes. Under such conditions, not only the three analytes could be satisfactorily detected and quantified, but also the peak areas measured for theophylline and caffeine were much higher than those measured by the off-line IT-SPME approach.

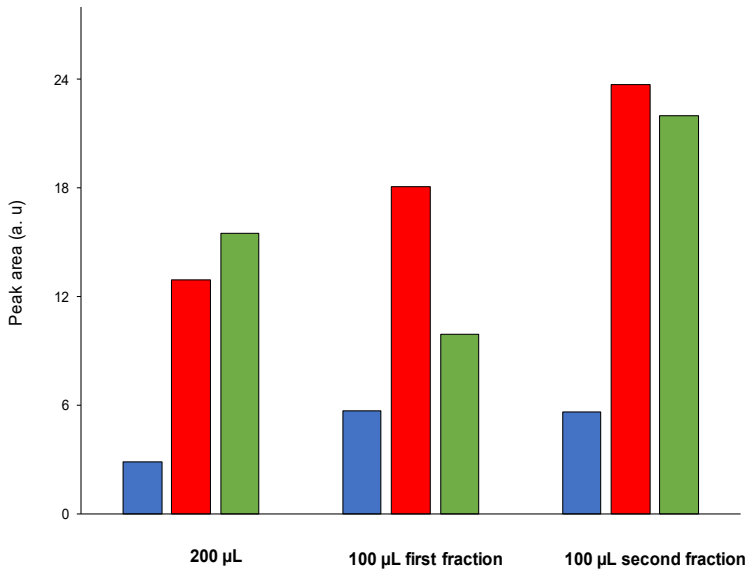


Figure 38. *Analyte responses obtained for the study of the volume of elution in SPE. Concentration of each analyte: 500 ng mL^{-1}; Load volume: 25 mL; cartridge: C18-U. Blue: Theobromine; Red: Theophylline; Green: Caffeine.*

One strategy to increase analyte detectability commonly used in SPE is the evaporation of the extract to dryness with subsequent reconstitution. However, in the present study, this option was discarded, since it moves away from the portable NanoLC approach, intended to be used for on-site measurements.

Due to the high polarity of the analytes studied, the use of organic solvents for cleaning the possible impurities of real samples retained in the sorbent was ruled out, so the effect of using water at this stage was studied. For it, a seawater sample was fortified with a standard solution of the analytes to obtain a final concentration of 500 ng mL^{-1}. Three extraction procedures, in optimized conditions, were carried out as follows: first a sample without fortification or cleaning process; then a fortified sample without cleaning process, and finally, a fortified sample and cleaning with 100 µL of nanopure water (this volume has previously been shown to be sufficient for the amount of sorbent in the cartridge).

Figure 39 shows the chromatograms for the analysis of the two fortified samples, with and without the cleaning process. No difference for theophylline signal was obtained by the cleaning process selected, and a minimal diminution effect was observed for theobromine and caffeine signals. Moreover, no signals of the analytes were shown in the chromatogram of the seawater sample without fortification. According to these results, a clean-up stage with 100 µL of water was included in the final procedure of SPE.

Consequently, SPE with C18 was the option selected for treating the samples when using the portable nanoLC system. The conditions finally selected were as follows: C18 cartridges were conditioned with 1 mL of methanol and 1 mL of water; next, 25 mL of the samples followed by 100 µL of water were flushed through them. Finally, two portions of 100 µL of methanol were passed successively through the cartridges, and the extracts were collected separately, filtered, and injected into the portable NanoLC equipment.

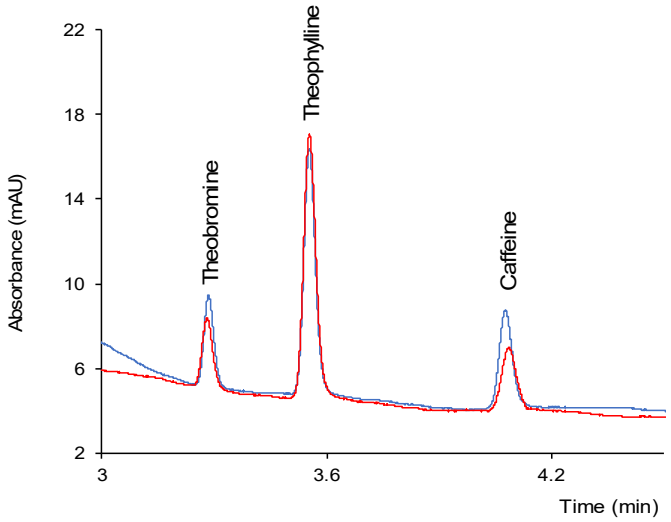


Figure 39. *Chromatograms of the optimization of the cleanup process. Blue: Fortified sample without cleaning process; Red: Fortified sample and cleaning with 100 µL of nanopure water. The fortified concentration of each analyte: 500 ng mL^{-1}; cartridge: C18-U; load volume: 25 mL; desorption with two fractions of 100 µL of methanol.*

4.2.3. Analytical performance IT-SPME-CapLC and SPE/portable NanoLC

Once the analytical procedures were optimized, the analytical performance of the SPE/portable NanoLC and IT-SPME-CapLC approaches were evaluated and compared using the conditions optimized in the above sections. The results found are discussed below.

The concentration intervals tested were 1-50 µg mL^{-1} and 0.2-15 ng mL^{-1} for the SPE/portable NanoLC and IT-SPME-CapLC approaches, respectively. Suitable linearity for all compounds with the two approaches tested was obtained, with determination coefficient values higher than 0.99 in the tested range. The LODs for both approaches were calculated as the ratio signal-to-noise higher than 3, which for the IT-SPME-CapLC system were 3-20 times lower than those reached with the SPE/portable NanoLC. Nevertheless, it has to be noted that when considering the registers obtained by the two methods at 255 nm, the LODs provided by IT-SPME- CapLC were only 2-10 times lower.

The results that some literature papers have reported in the analysis of caffeine in environmental water samples indicate the presence in raw wastewater up to above 0.1 µg mL^{-1} [287]; instead, for surface waters concentrations between 0.7 and 1.6 ng mL^{-1} have been found [170]. Thus, the LOD for caffeine with the SPE/portable NanoLC method is suitable for the analysis of caffeine in these kinds of samples. Finally, LOQs were calculated, with an S/N ratio higher than 10, and adequate values were reported. Table 18 summarizes the obtained results for linearity, LODs, and LOQs with the two approaches applied.

Table 18. *Analytical parameters obtained with the SPE/portable NanoLC and IT-SPME-CapLC approaches.*

Analyte	**SPE/portable NanoLC**			**IT-SPME-CapLC**		
	Calibration equation[a], (n =9)	**LOD**	**LOQ**	**Calibration equation[b], n=10**	**LOD[c]**	**LOQ**
	y = ax + b	**(ng mL^{-1})**	**(ng mL^{-1})**	**y = ax + b**	**(ng mL^{-1})**	**(ng mL^{-1})**
Theobromine	0.05 x + 0.02	10	30	20.0 x - 25.2	0.5 (**1**)	2
	(R^2 = 0.994)			(R^2 = 0.999)		
Theophylline	0.11 x + 0.22	1.5	10	25.9 x + 1.1	0.5 (**1**)	2
	(R^2 = 0.996)			(R^2 = 0.999)		
Caffeine	0.10 x + 0.08	1.5	10	78.1 x +7.3	0.1 (**0.5**)	0.5
	(R^2 = 0.995)			(R^2 = 0.995)		

(a) In the 10-100 ng mL^{-1} concentration ranges for theophylline and caffeine, and 30-100 ng mL^{-1} concentration ranges for theobromine.
(b) In the 2-15 ng mL^{-1} concentration ranges for theophylline and theobromine; and 0.5-15 ng mL^{-1} concentration ranges for caffeine.
(c) The values in brackets are the LODs at 255 nm

To evaluate the intraday-precision (expressed as % RSD), three aliquots of the samples at three concentrations within the respective tested concentration ranges were processed consecutively. The RSDs values obtained are listed in Table 19. The values obtained by the two approaches were rather similar. Given the results of the features of the two chromatographic systems listed in Table 17, it can be deduced that the variability associated with the SPE procedure is higher. This can be explained by the fact that the manipulation of the sample with the IT-SPME is reduced only to loading the sample. Finally, the inter-day precision values were obtained by the two methods and are also comparable, as Table 19 shows.

Table 19. *Precision of the two tested approaches, RDS (%); n=3.*

Compound /	**SPE/portable NanoLC**						**IT-SPME - CapLC**					
	Intraday			**Interday**			**Intraday**			**Interday**		
Concentration (ng mL^{-1})	**10**	**50**	**100**	**10**	**50**	**100**	**2**	**5**	**10**	**2**	**5**	**10**
Theobromine	-	2	6	-	7	6	5	5	2	4	5	6
Theophylline	8	4	4	6	5	5	2	4	4	2	6	4
Caffeine	3	4	7	5	5	4	4	4	4	5	3	6

The last parameter evaluated was the extraction recovery. For this, the seawater sample was spiked to contain the analytes at three different concentration levels and then processed in order to obtain the extraction recoveries. This sample was selected due to, as mentioned in the optimization of cleanup solvent selection for SPE procedure, none of the analytes were found. The recovery percentages were calculated by comparing the peak areas obtained from the spiked samples with those measured for standard solutions with the analytes at the same concentration.

Figure 40 shows representative chromatograms obtained for the analysis of seawater, blank, and fortified samples by the SPE/portable NanoLC and IT-SPME – CapLC approaches. The obtained values ranged within the 65-89 % and 83-103 % with the SPE/portable NanoLC and IT-SPME-CapLC methods, respectively. Recovery values for the three analytes in all concentration levels, for the two procedures, with n = 3, are listed in Table 20.

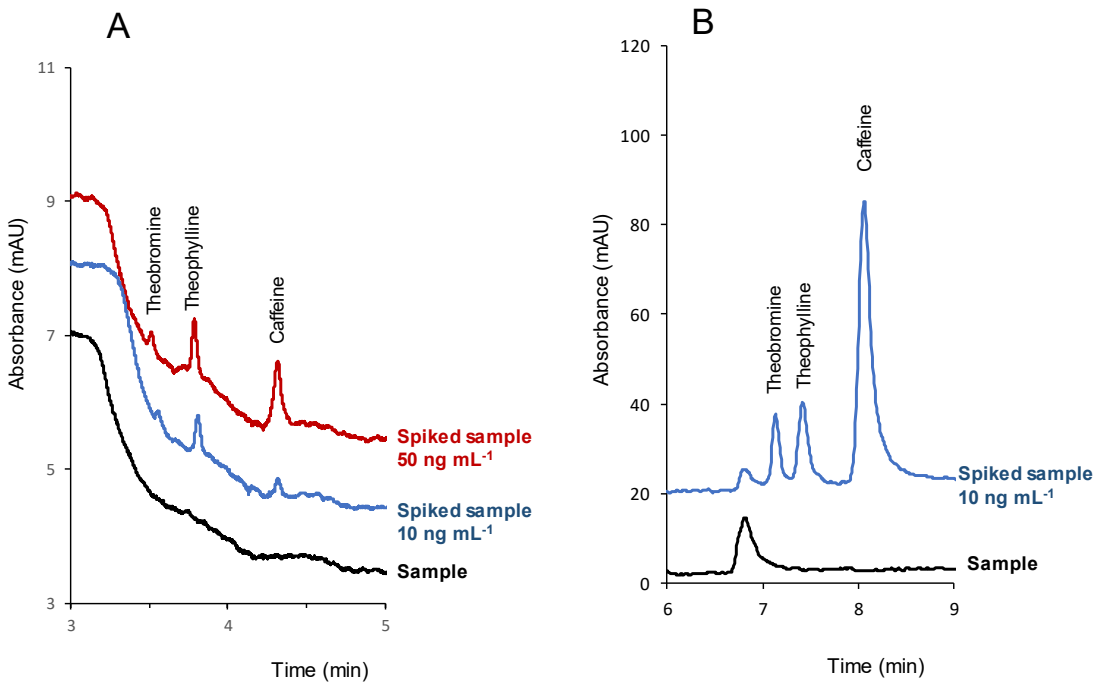


Figure 40. *Chromatograms obtained for sea water analysis. A: SPE/portable NanoLC; B: IT-SPME-CapLC .*

Table 20. *Recoveries obtained by the two tested approaches in spiked seawater, RDS (%); n=3.*

Compound	Extraction recovery (%) (n = 3)					
	SPE/portable NanoLC			IT-SPME - CapLC		
	10 ng mL^{-1}	50 ng mL^{-1}	100 ng mL^{-1}	2 ng mL^{-1}	5 ng mL^{-1}	10 ng mL^{-1}
Theobromine	-	65 ± 4	70 ± 2	94 ± 2	92 ± 5	83 ± 4
Theophylline	81 ± 7	78 ± 3	78 ± 3	103 ± 8	93 ± 2	87 ± 6
Caffeine	88 ± 3	89 ± 6	88 ± 4	88 ± 5	92 ± 4	96.9 ± 0.4

4.2.4. Analysis of real samples

The developed procedures were applied to the analysis of sea and tap water samples, five river water samples, two from the Turia river, and three from the Magro river. Caffeine was found in one of the Turia river samples assayed by SPE/portable NanoLC method at a concentration close to its LOD. The presence of this compound in such a sample was confirmed by spiking the sample with a standard solution of the analytes (Figure 41.A). This was consistent with the results obtained by the IT-SPME-CapLC method, which led to a concentration of caffeine of (1.94 ± 0.05) µg mL^{-1} (n = 3). None of the analytes was found in the second Turia river sample analyzed by the SPE/portable NanoLC

method; however, this sample was positive for caffeine when using the IT-SPME-CapLC method, being its concentration (1.79 ± 0.11) µg mL^{-1}, (n=3).

In the rest of the samples, none of the analytes were found by any of both methodologies. The application of the second approach allows confirming the presence of caffeine by the concordance between the spectra of the suspected peak and that recorded for a standard solution of caffeine, as Figure 41.B shows.

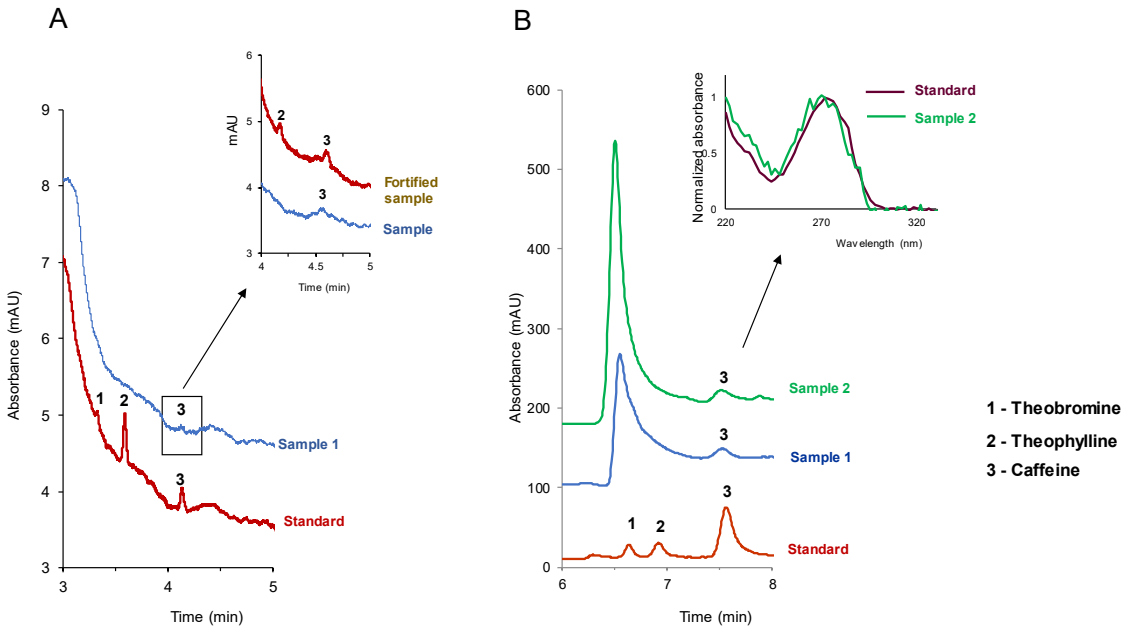


Figure 41. *Chromatograms obtained for the Turia river water samples: (A) chromatograms obtained by the SPE/portable NanoLC approach for a sample positive for caffeine, for an standard solution of the analytes (10 ng mL^{-1}, each compound), and detail of the registers obtained for the same sample fortified with the analytes (2 ng mL^{-1}, each); (B) registers obtained by the IT-SPME - CapLC approach for the samples and for an standard solution of the analytes (10 ng mL^{-1}), and normalized spectra of the peak assigned to caffeine in sample 2 and the spectra corresponding to a standard solution of caffeine.*

4.2.5. Conclusions

In this thesis, a method for the detection and quantification of the emerging contaminant caffeine and other trimethylxanthines (theophylline and theobromine) using a portable NanoLC chromatograph has been developed. The results obtained along the study indicate that this instrument can be applied for the analysis of the contaminants in water analysis, provided that the proper sample treatment is applied to the samples.

According to this, a protocol based on SPE has been developed which can be carried out on-site, as it involves neither extensive sample manipulation nor additional instrumentation (for solvent evaporation, for instance).The proposed method is adequate to quantify the tested methylxanthines at μg mL^{-1} levels, and provides satisfactory linearity and precession similar to that of the IT-SPME-Cap LC approach, although the sensitivity attainable is slightly lower. Additional advantages of the SPE/NanoLC approach are better resolution, lower times of analysis, and a significant reduction in solvents consumption. The work represents a pioneer study in the application of portable NanoLC systems for the analysis of organic pollutants in different kinds of water samples.

4.3 ANALYSIS OF DIETARY SUPPLEMENTS

In the framework of the development of this thesis, the application of capillary liquid chromatography with UV-diode array detection (DAD) for the chemical characterization of botanical dietary supplements was proposed. As is pointed in the introductory part of this work, the development of analytical methods for this type of products can be useful to estimate its effectiveness and safety through the consideration of the contents of major constituents such as caffeine, chlorogenic acids, and synephrine, whereas minor compounds as theobromine, riboflavin, limonene, α-pinene, farnesene, myrcene, linalool, and kaempferol, with potential physiological effects, can be used to evaluate the sensory characteristics of the samples.

The advantages that miniaturized chromatographic systems show in terms of high-resolution power and sensitivity attainable, make possible the determination of seventeen compounds that can be found in dietary supplements at different concentration levels, under the approach of analysis of both major and minor components in this kind of samples.

4.3.1. Optimization of the chromatographic separation

The structural and polarity variety of the compounds, with log Kow values with a wide range, from -0.8 for theobromine to 6.1 for farnesene, denotes the need to apply a gradient separation. Additionally, the reverse-phase mechanism is the most appropriate for this type of chromatographic separation, due to the variety of solvents and stationary phases available for this type of mechanism. Based on the UV cutoff wavelength, defined as the wavelength at which solvent absorbs 1.0 AU in a 10-mm cell [288], of some of the solvents commonly used in reverse-phase, and the maximum absorption wavelength of the analytes, some eluents such as alcohols, tetrahydrofuran or buffers were found unsuitable as they present significant absorbances at the low wavelengths ranges (<210 nm) required for the detection of the terpenic compounds.

According to the graph in Figure 42, in addition to the effect of methanol, isopropanol, and tetrahydrofuran, the use of aqueous solutions with acetic acid could affect the detection of synephrine. For the rest of the compounds, minimal effects can be produced due to its absorption at high wavelengths. Finally, the graph shows that water and acetonitrile turn out to be the best option to optimize the chromatographic separation.

For the initial experiments, diluted standard solutions of the analytes were prepared at concentrations of 1 µg mL^{-1} for synephrine, caffeine, theobromine, riboflavin, caffeic acid, kaempferol, and 5-CQA, and 5 µg mL^{-1} for the rest of analytes, all of them prepared in water. Different gradient elution programs were tested to separate the seventeen compounds. However, caffeic acid and the chlorogenic acids could not be satisfactorily resolved. It can be explained due to the similarity of their chemical structures, and also, because the chlorogenic acids studied are, as stated earlier, structural isomers, which increases the difficulty to separate all these analytes in a single run [289].

Consequently, two chromatographic separations were developed. The first one for the determination of caffeic acid and chlorogenic acids, called the "chlorogenic acids method", and the second one for the rest of compounds called the "multi-class method". Both are discussed below.

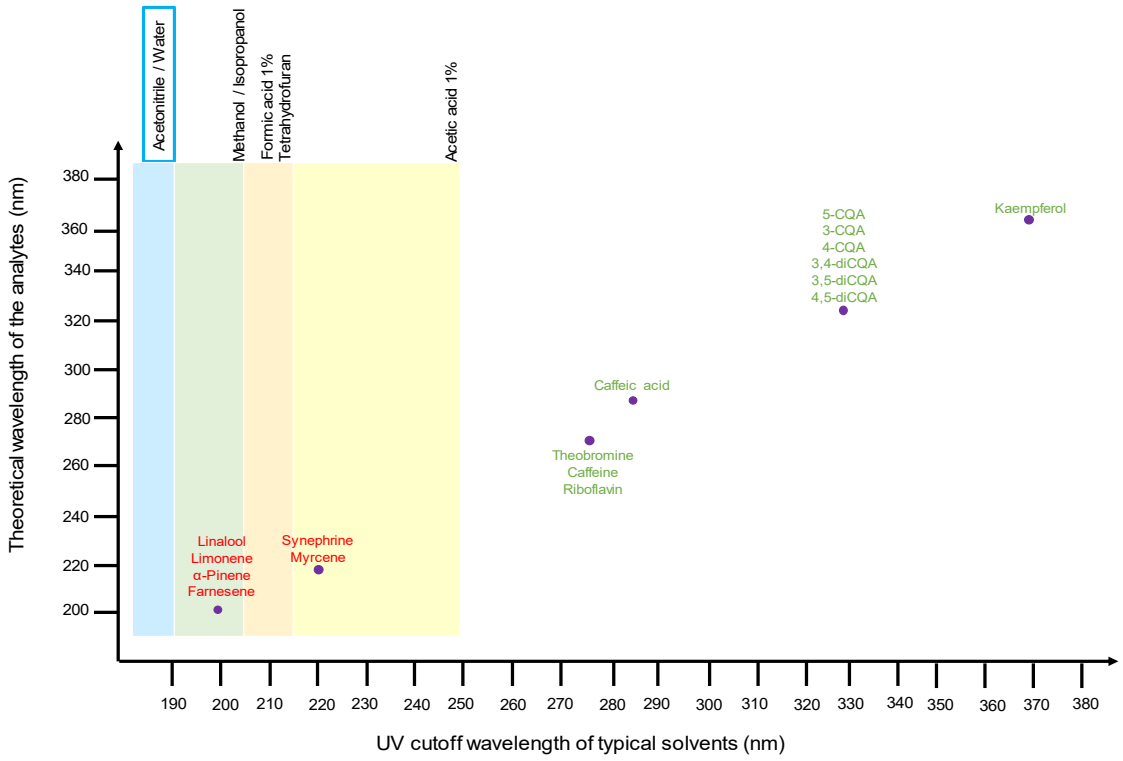


Figure 42. *Graphical evaluation of the effect of the UV cutoff wavelength of the solvents commonly used as a function of the maximum absorption wavelength of the studied compounds.*

4.3.1.1. Multi-class method

For the correct separation of the most polar compounds, such as synephrine and theobromine, it is foreseeable to start the gradient with a low percentage of acetonitrile. On the other hand, the terpenic compounds will require a higher percentage of the organic solvent (100%) at the end of the separation. In that way, applying 15% of acetonitrile at the beginning of the separation, and a slow increase in the first 5 minutes, allows the separation of the polar compounds.

However, poor peak profile for synephrine was found in all the conditions tested, which has also been reported by some literature papers, such as the case of Roman et al., who have mentioned that the high polarity of this biogenic amine causes poor retention in traditional reverse-phase systems, necessitating the use of an anionic ion-pairing agent to achieve retention [290].

In that sense, the use of a modifier in the working standard solution was tested. Improved peak profiles for synephrine were observed by acidifying. Different acids were assayed, included phosphoric, formic, and hydrochloric acid, all of them at 1% (v/v). Figure 43 shows the resulting chromatograms. Although all acidic solutions prevented peak split, better results, in terms of narrower peak, were observed with the use of hydrochloric acid.

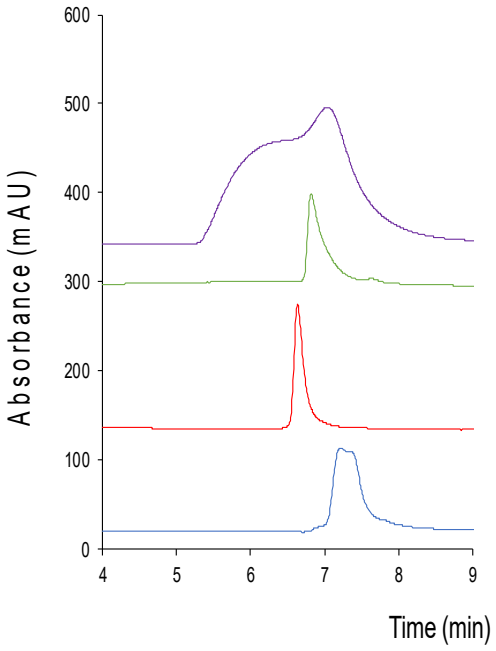


Figure 43. *Chromatograms resulting from the study of the effect of acid modifiers on the chromatographic efficiency of synephrine. Blue: without modifier; Red: with hydrochloric acid; Green: with ortho-phosphoric acid; Purple: with formic acid. Concentration of synephrine: 1 µg mL^{-1}.*

For hydrochloric acid, different proportions were assayed to find the adequate quantity that allows a good chromatographic efficiency of the peak, without causing a decrease in the pH of the solution below 2, to avoid negative effects on the stationary phase. Adequate results were observed when adding 0.1 % hydrochloric acid to the working solutions in a proportion of 1:10 (v/v). Finally, with the addition of the modifier, the retention times of the rest of the analytes were relatively unaffected.

The elution program finally selected was started with a 15% of acetonitrile at zero min, and linearly increased to 20% at 5 min, to 50% at 9 min, and to 75% at 11 min. Finally, the percentage of acetonitrile was increased to 100 % at 15 min and kept constant until the end of the run. Figure 44 shows the chromatograms obtained under the selected conditions for a standard solution of the analytes at 200, 220, 275, 300, and 370 nm. As observed most compounds were satisfactorily resolved, unfortunately, caffeine and riboflavin overlapped.

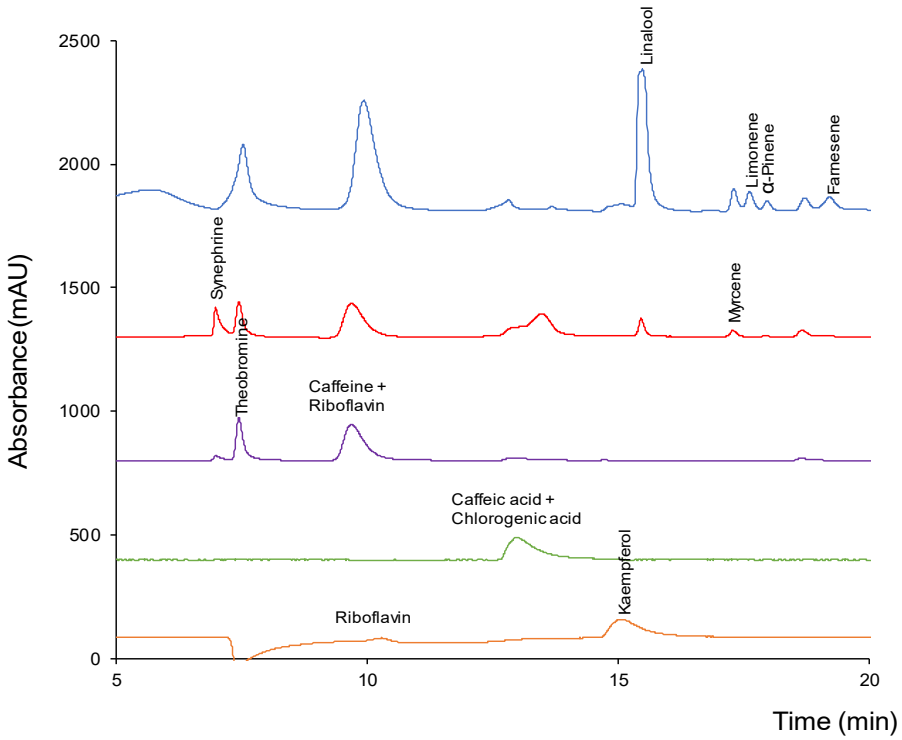


Figure 44. *Chromatograms obtained with the optimized gradient. Blue: at 200 nm; Red: at 220 nm; Purple: at 275nm; Green: at 330 nm; Orange: at 370 nm. For a solution containing synephrine (1 µg mL^{-1}), theobromine (1 µg mL^{-1}), caffeine (1 µg mL^{-1}), Riboflavin (1 µg mL^{-1}), 5-CQA (1 µg mL^{-1}), caffeic acid (1 µg mL^{-1}), linalool (5 µg mL^{-1}), myrcene (5 µg mL^{-1}), limonene, (5 µg mL^{-1}), α-pinene (5 µg mL^{-1}), farnesene (5 µg mL^{-1}) and kaempferol (1 µg mL^{-1}).*

Although both compounds have different absorption spectra (Figure 45), and riboflavin presents an absorption band at 370 nm, for samples with a caffeine content much higher than those of riboflavin, overestimated results could be obtained for the latter compound. On the other hand, the low signal intensity of riboflavin at 370 nm could affect the detectability of the analyte. Therefore, it was decided to develop an alternate gradient, if required, for complete separation of both compounds. For this gradient, the initial percentage of acetonitrile was at the minimum value possible, 5% at 0 min. Then it was increased to 10 % at 5 min, to 25 % at 12 min, to 75 % at 15 min, and finally to 100 % at 19 min. As Figure 45 shows, the proper separation of both compounds was achieved after 15 minutes.

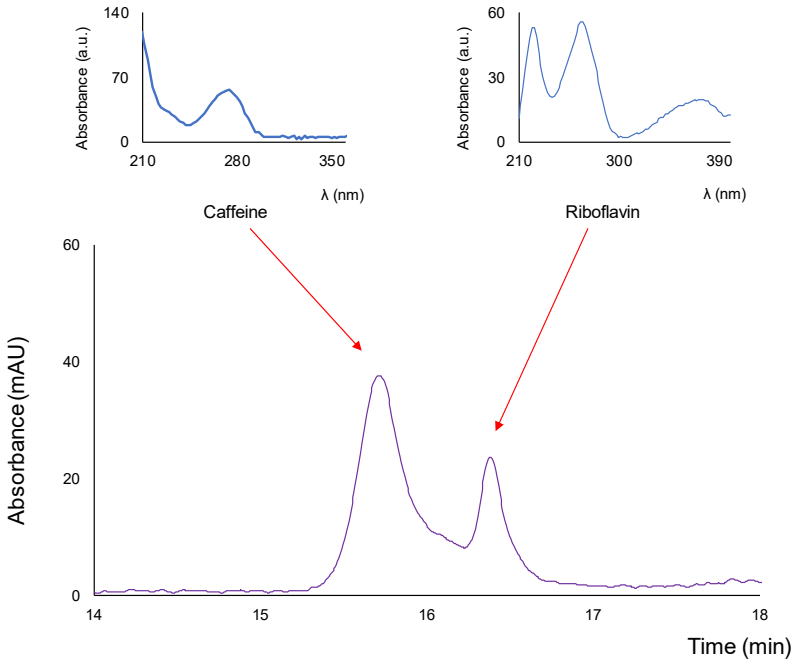


Figure 45. *Chromatogram obtained at 275 nm for a mixture of caffeine and riboflavin (1 µg mL^{-1} each) with the gradient modified for the analysis of these compounds. At the top the absorption spectra of each compound appear.*

4.3.1.2. Chlorogenic acids method

As stated earlier, the separation of caffeic acid and the chlorogenic acids with the multi-class method was not possible. In those conditions, these compounds eluted as a wide peak between 12.5 and 14.0 min (Figure 44). Consequently, different elution conditions were tested to achieve his separation. Unlike with terpenic compounds, caffeic acid and chlorogenic acids have maximum absorbances at high wavelengths, around 330 nm, which allows the use of solvents other than acetonitrile and water or even modifiers in the mobile phase.

On this line, initial tests were carried out using water acidified with 0.1% phosphoric acid and acetonitrile as the mobile phase, and a standard solution of the compounds at 1 µg mL^{-1} level was prepared in water and chromatographed. An unsuitable separation resulted in all of the conditions assayed, with a coelution of at least three compounds (Figure 46).

Therefore, it was decided to modify the selectivity of the mobile phase, replacing acetonitrile with methanol, and maintaining the 0.1% phosphoric acid solution. The optimized gradient started with a percentage of methanol from 25 % at zero min and increased to 30 % at 7 min, to 50 % at 15 min, kept constant until 16 min, and then increased to 75 % at 20 min and 100 % at 23 min. In these conditions, adequate separation of the analytes was achieved in less than 22 minutes, although the separation of 3,4-diCQA and 3,5-diCQA was not possible. Other papers have reported the separation of these analytes in more than 45 minutes [291, 292]. Figure 46 shows the chromatograms obtained at 330 nm for a standard mixture of caffeic acid and chlorogenic acids (1 µg mL^{-1}) with the mobile phases tested.

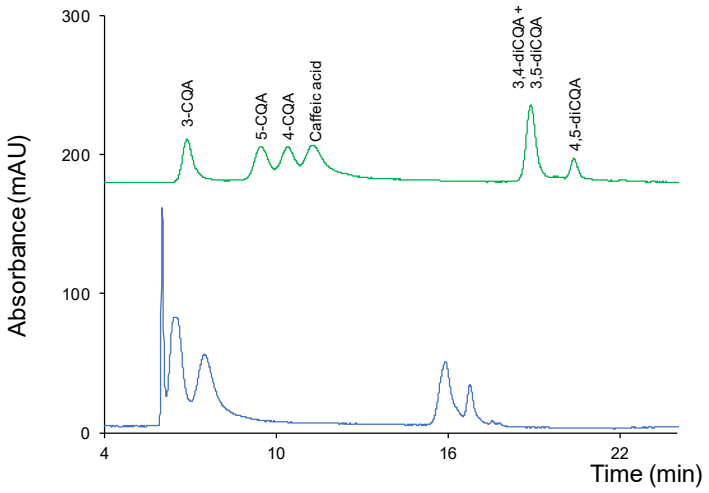


Figure 46. *Chromatograms obtained at 330 nm for a standard mixture of caffeic acid and chlorogenic acids (1 µg mL^{-1}) with different mobile phases. Blue: acetonitrile and 0.1% phosphoric acid; Green: methanol and 0.1% phosphoric acid.*

4.3.2. Extraction optimization

For the isolation of the compounds of interest in the dietary supplements, ultrasound-assisted extraction was the option selected, which has been widely used for the treatment of vegetal origin samples due to its advantages over traditional extractions as maceration and Soxhlet [293]. Despite that in ultrasound-assisted extraction, there are different parameters to optimize such as

temperature, frequency, power, solvent type, and solvent to the material ratio, the last two of these have a major significance in the extraction efficiency [294].

Due to the high polarity characteristics of some of the analytes, it was decided to start the extraction experiments using methanol as the solvent. Preliminary experiments were carried out with all the samples, placing 25 mg of each and adding 1 mL of the solvent, and then they were placed in the ultrasonic bath for 5 minutes. Supernatants were collected and filtered, and an aliquot of the extract was treated with hydrochloric acid as described in the above section and chromatographed. The results of these experiments allowed to estimate the present amounts of the analytes, as well as selecting the sample GC-3 to optimize the solvent to material ratio to extract the analytes because more intense background signals in the chromatogram were observed with it.

Three portions of 25 mg of the sample were placed in glass vials. Then, variable volumes of methanol (1, 2, and 5 mL) were added to the vials. The resulting suspensions were first vortexed and then placed in an ultrasonic bath for 5 min. The extracts were subjected to the procedure detailed above. Then, the sample residue was treated with another portion of methanol, and the extraction was repeated.

By comparing the chromatograms obtained after the first and the second extractions it was observed that, although most compounds were extracted with 1-2 mL of methanol, significant amounts of caffeine and chlorogenic acids remained in the solid residue, as Figure 47 shown.

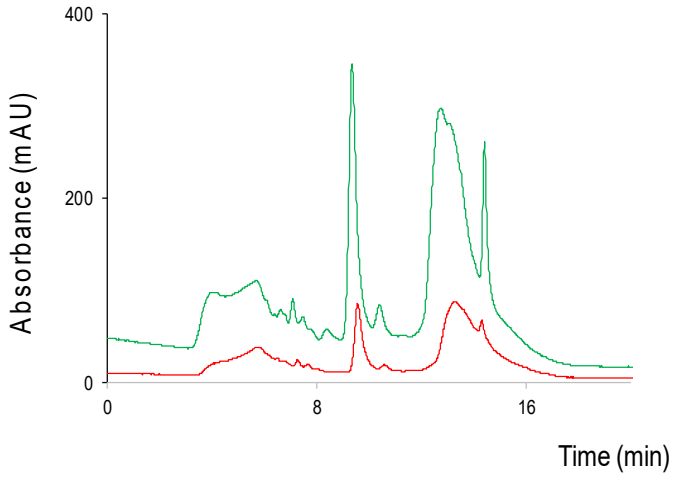


Figure 47. *Chromatograms obtained at 275 nm for sample GC-3, extracted with 2 mL of methanol, with a dilution factor of 50 and analyzed by multi-class method. Red: extraction with the first portion of methanol; Green: extraction with the second portion of methanol.*

In contrast, no significant peaks were observed in the chromatograms obtained for residues that had been treated with 5 mL of methanol (Figure 48). Therefore, extraction with 5 mL of methanol was the option applied in further assays.

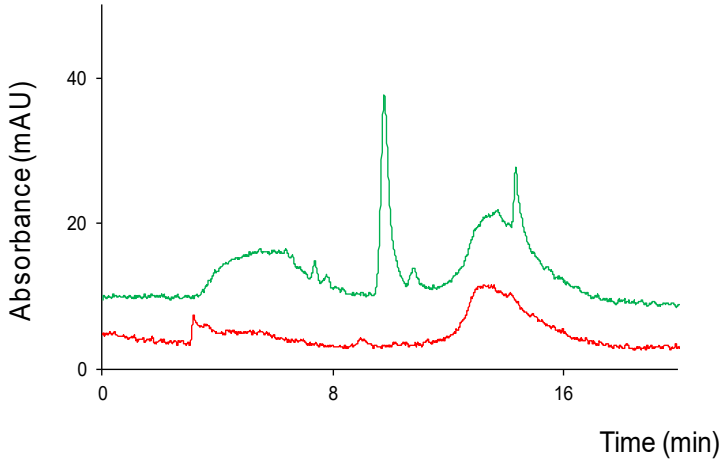


Figure 48. *Chromatograms obtained at 275 nm for sample GC-3, extracted with 5 mL of methanol, with a dilution factor of 100 and analyzed by multi-class method. Red: extraction with the first portion of methanol; Green: extraction with the second portion of methanol.*

4.3.3. Analytical performance

To evaluate the analytical performance of the two developed methods, different parameters were studied for the estimation of the linearity, limits of detection (LOD) and limits of quantification (LOQ), repeatability and reproducibility, and extraction recoveries, following the guide of Eurachem group [295]. For these experiments, standard solutions of the analytes, blank, and fortified samples were prepared.

4.3.3.1. Multi-class method

First, the concentration ranges were selected to obtain peak areas of approximately the same order for all analytes at their respective working wavelengths, which were selected according to their UV spectra. Figure 49 shows the UV spectra of compounds that have not been included so far, it is important to underline the absence of characteristic absorption bands for terpenic compounds.

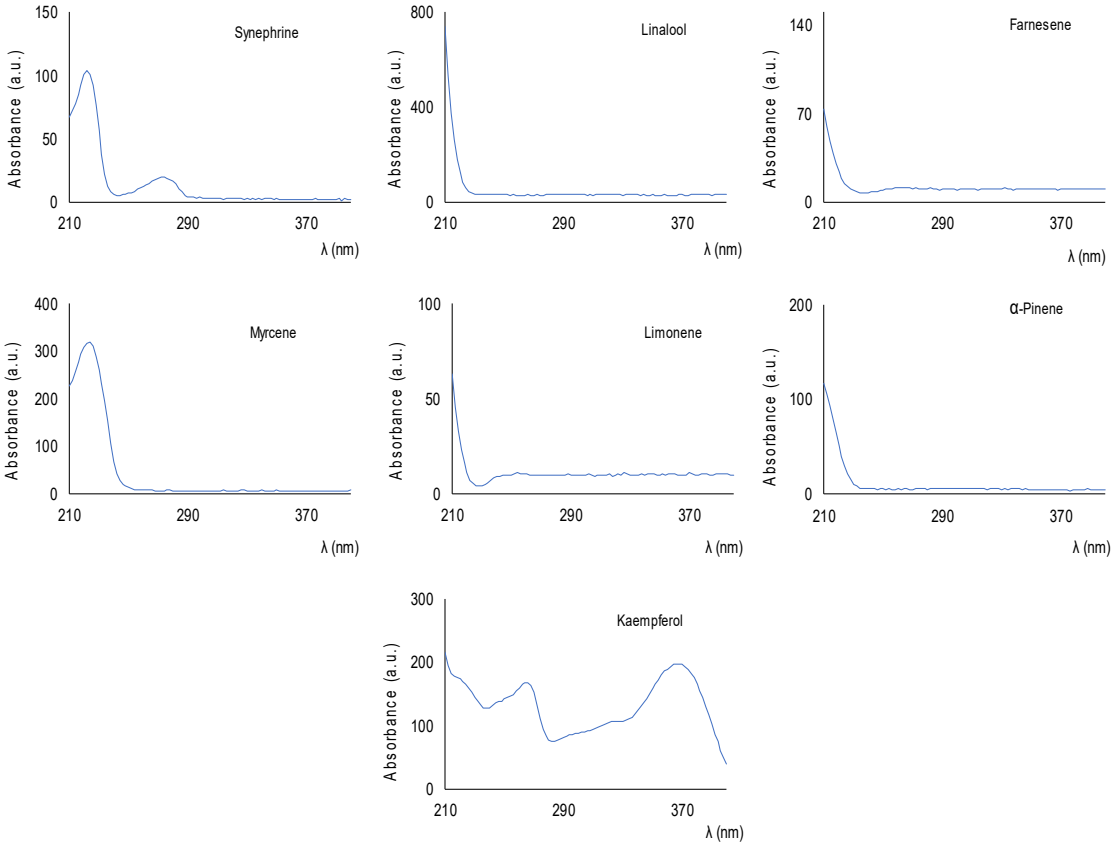


Figure 49. *UV spectra obtained for some of the compounds analyzed by the multi-class method.*

The linearity was evaluated by processing in duplicate five concentrations within the tested range. In all instances, satisfactory results were observed, with determination coefficients between 0.99 and 0.999. The limits of detection (LODs) and limits of quantification (LOQs) were established as the concentrations of analyte that resulted in signal-to-noise ratios of 3 and 10, respectively. These values were obtained by processing solutions with decreasing concentrations of the analytes; before analyzing each solution, water was processed to confirm the absence of contaminants and/or memory effects.

The LODs ranged from 0.005 to 0.25 μg mL^{-1}, with the lowest values being found for linalool, caffeine, theobromine, and myrcene. On the other hand, as expected, the highest values were those presented by terpenic compounds such as limonene, α-pinene, and farnesene. The LOQs were in the 0.02-1.0 μg mL^{-1} interval. Finally, to establish the instrumental precision, a successive injection of three replicates of standard solutions of the analytes at concentrations of 0.5-5.0 μg mL^{-1} was effected. Then the relative standard deviations (RSDs) were obtained, and the values were ranged from 2-11%. Table 21 shows the concentration ranges and wavelengths used, as well as the relevant analytical parameters obtained.

Table 21. *Analytical parameters of the multi-class method.*

Compound	Wavelength (nm)	Concentration range (µg mL^{-1})	Linearity (n =10) y = ax +b			LOD (µg mL^{-1})	LOQ (µg mL^{-1})	Precision [a], (n=3) RSD %
			a ± s_a	b ± s_b	R^2			
Synephrine	220	0.2 - 5.0	1840 ± 30	- 15 ± 35	0.997	0.05	0.2	8
Theobromine	275	0.05 - 1.0	2360 ± 40	- 21 ± 20	0.998	0.01	0.04	7
Caffeine	275	0.05 - 2.5	1840 ± 30	- 15 ± 35	0.997	0.01	0.04	4
Riboflavin	275	1.0 - 7.0	391 ± 6	- 18 ± 23	0.998	0.10	0.3	3
Kaempferol	370	1.0 - 5.0	270 ± 10	- 50 ± 30	0.990	0.25	1.0	10
Linalool	200	0.02 - 1.0	20200 ± 200	67 ± 90	0.999	0.005	0.02	11
Myrcene	220	0.05 - 2.5	530 ± 20	- 20 ± 20	0.992	0.01	0.04	2
Limonene	200	1.0 - 10.0	920 ± 40	- 600 ± 300	0.993	0.25	1.0	3
α-Pinene	200	1.0 - 10.0	166 ± 6	- 60 ± 30	0.993	0.25	1.0	11
Farnesene	200	1.0 - 5.0	900 ± 40	- 200 ± 100	0.991	0.25	1.0	11

([a]) Established at a concentration of 0.5 µg mL^{-1} for synephrine, 5 µg mL^{-1} for riboflavin and 1 µg mL^{-1} for the rest of compounds.

For the study of the recoveries, a green coffee sample was spiked with known amounts of the analytes. The amount of each compound added to the sample was 0.25 μg g^{-1}. For compounds expected at higher percentages, such as caffeine and synephrine, the samples were also spiked with 25 μg g^{-1} of the analytes. The recoveries were calculated by comparing the increments of the peak areas in the spiked samples with those obtained for standard solutions containing an equivalent concentration of each compound. The values obtained are listed in Table 22. As observed, the main recoveries showed adequate results ranged from 82 % to 109 %.

It has to be noted that with the recoveries values found and the LOQs presented in Table 21, the theoretical minimum amounts of the analytes that could be quantified by the present method (for a weight of 25 mg of the samples) ranged from 0.004 mg g^{-1} for linalool to 0.2 mg g^{-1} for limonene and α-pinene. If required, the minimum amounts could be further reduced by two possibilities. On one hand, increasing the amount of sample, and on the other hand, by evaporating the methanolic extract to dryness and subsequent redissolution in a lower volume of solvent.

Finally, to study the intra-day precision of the entire procedure, three portions of the samples were spiked with the analytes and processed consecutively. The inter-day precision was obtained in the same way, from six replicates of the spiked samples processed on different days. The results are shown in Table 22. The RSD values obtained were of about the same order than those found for standard solutions of the analytes (instrumental precision) listed in Table 21.

Table 22. *Precision and recovery obtained in spiked green coffee samples for* of the multi-class method.

Compound	Added amount (mg g^{-1})	Mean recovery, (n=6) (%)	Precision, RSD (%)	
			Intra-day (n=3)	Inter-day (*n*=6)
Synephrine	0.25	109	11	12
	25.0	82	7	3
Theobromine	0.25	105	15	12
Caffeine	0.25	94	11	10
	25.0	93	6	5
Riboflavin	0.25	92	11	9
Kaempferol	0.25	93	10	11
Linalool	0.25	96	5	4
Myrcene	0.25	106	3	10
Limonene	0.25	95	11	13
α-Pinene	0.25	98	10	13
Farnesene	0.25	105	7	6

4.3.3.2. Chlorogenic acids method

In a similar way that for the multiclass method, the same parameters were evaluated for the chlorogenic acids method, measuring the peak areas at 330 nm as analytical signals, as their UV spectra show (Figure 50). The values obtained are shown in Table 23. The analytical responses for all compounds were linear within the tested concentration range, being the precision suitable (RSDs ≤ 8 %) and the determination coefficients between 0.990 and 0.998. The concentration range tested was from 0.3 to 2.0 µg mL^{-1} for all compounds, except for 3-CQA and 5-CQA, where the range was for 0.2 to 2.0 µg mL^{-1}.

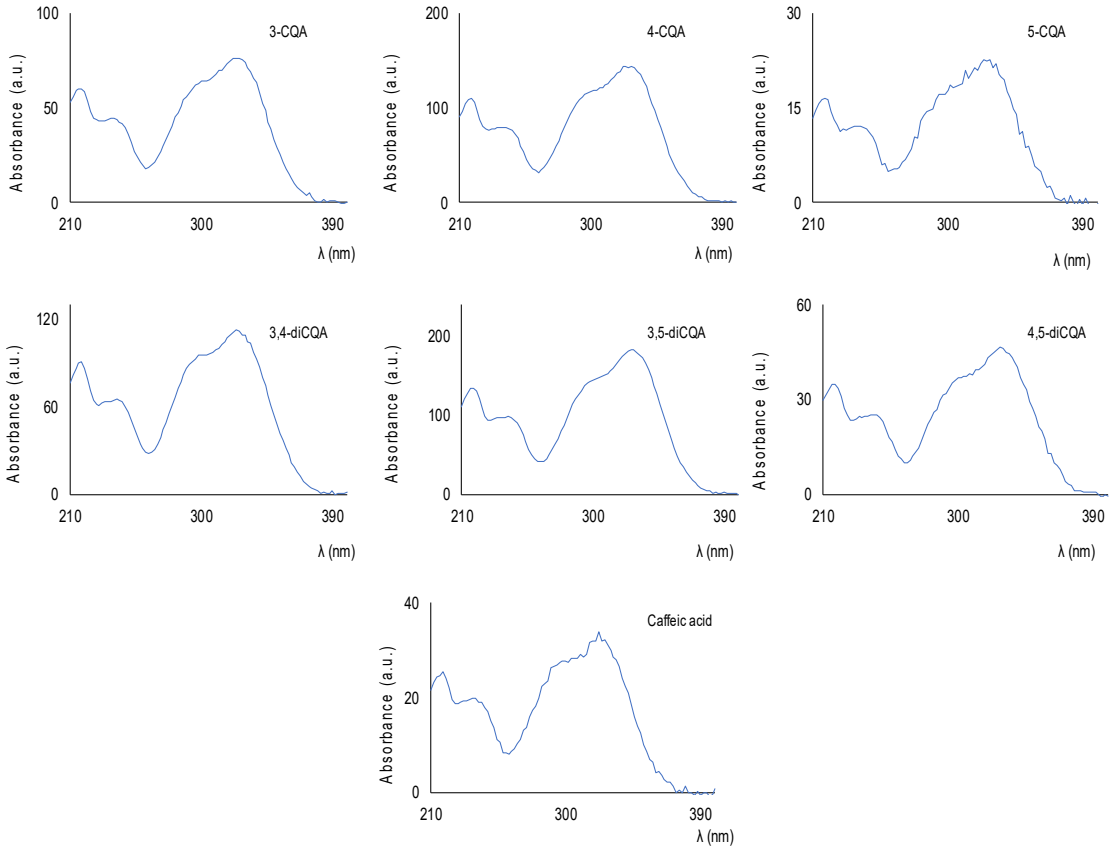


Figure 50. *UV spectra obtained for chlorogenic acid compounds analyzed under the chlorogenic acids method.*

The LODs and LOQs were of 0.05-0.1 µg mL^{-1} and 0.2-0.3 µg mL^{-1}, respectively. It has to be noted that the slopes of the calibration equations obtained for 3,4-diCQA and 3,5-diCQA (both eluted at 18.1 min) were equivalent. Therefore, as Figure 46 (green) shows, the area of the obtained for the peak observed at this retention time can be used as an estimation of the total amount of both compounds in the samples.

Table 23. *Analytical parameters of the chlorogenic acids method.*

Compound	Wavelength (nm)	Concentration range (µg mL^{-1})	Linearity (n =10) y = ax +b			LOD (µg mL^{-1})	LOQ (µg mL^{-1})	Precision [a], (n=3) RSD %
			a ± s_a	b ± s_b	R^2			
3-CQA	330	0.2 - 2.0	1180 ± 20	- 80 ± 20	0.998	0.05	0.2	6
5-CQA	330	0.2 - 2.0	1160 ± 30	- 110± 30	0.996	0.05	0.2	2
4-CQA	330	0.3 - 2.0	1200 ± 20	- 200 ± 30	0.998	0.10	0.3	5
3,4-diCQA	330	0.3 - 2.0	650 ± 40	- 90 ± 40	0.990	0.10	0.3	6
3,5-diCQA	330	0.3 - 2.0	670 ± 30	- 20 ± 30	0.990	0.10	0.3	4
4,5-diCQA	330	0.3 - 2.0	560 ± 20	- 60 ± 20	0.993	0.10	0.3	8
Caffeic acid	330	0.3 - 2.0	2300 ± 100	- 600 ± 100	0.993	0.10	0.3	8

([a]) Established at a concentration of 1 µg mL^{-1} for the all compounds.

To obtain the recoveries and the precision in two aspects, intra- and inter-day, the same green coffee sample used above was spiked with 5 µg g^{-1} of all compounds. Additionally, another sample was spiked with 25 µg g^{-1} of 5-CQA, whose concentration in the samples is expected at higher percentages, according to the preliminary experiments with the samples. The obtained results for these parameters are shown in Table 24.

Table 24. *Precision and* recovery obtained in spiked green coffee samples for chlorogenic acids method.

Compound	Added amount (mg g^{-1})	Mean recovery, (n=6) (%)	Precision, RSD (%)	
			Intra-day (n=3)	Inter-day (*n*=6)
3-CQA	5.0	87	3	6
5-CQA	5.0	85	5	8
	25.0	79	2	8
4-CQA	5.0	90	2	6
3,4-diCQA	5.0	87	1	1
3,5-diCQA	5.0	89	3	2
4,5-diCQA	5.0	89	3	2
Caffeic acid	5.0	85	3	7

4.3.4. Analysis of real samples

The proposed methods were applied to the analysis of different dietary supplements, namely three green coffee extracts claimed to enhance physical performance, two fat-burnings for losing weight, and one herbal preparation (relaxant). Portions of 25 mg of the samples were treated with 5 mL of methanol, then vortexed for 30 seconds, and then they were placed in the ultrasonic bath for 5 minutes. Finally, the supernatants were collected and filtered. One aliquot of the extract was treated with hydrochloric acid and diluted with water to adjust the peak areas of the analytes to their respective linear intervals, and the resulting dilution was chromatographed by the multi-class method. Another aliquot of the extract only was diluted and injected with the optimized conditions of the chlorogenic acids method. Figure 51 shows the schema of the procedure for the analysis of the samples.

4.3.4.1. Multi-class method

As mentioned before, the preliminary studies allowed to identify the compounds of the samples, what was established from the concordance between the retention times and spectra of the suspected peak, and that of a standard solution, and it was further confirmed by spiking the extract

with such compound. The concentrations of the analytes in the extracts were calculated from the calibration equations of Table 21 and transformed into amounts in the samples considering the dilution factors (if applicable) and the recovery values of Table 22.

In the analysis of the three green coffee samples, caffeine presence resulted positive in all samples, although at variable contents. The highest amount was found in sample GC-1, which contained a mean value of 89 mg per capsule, equivalent to a content of 81 mg g^{-1}, whereas the lowest content (1.30 mg g^{-1}) was found in sample GC-2, which was marketed as a decaffeinated product. According to the label, this product contained 350 mg of coffee extract. Thus, the sample contained about 3.7 mg of caffeine per g of extract, which corresponds to the result for decaffeinating coffee extracts reported by Meinhart et al [296].

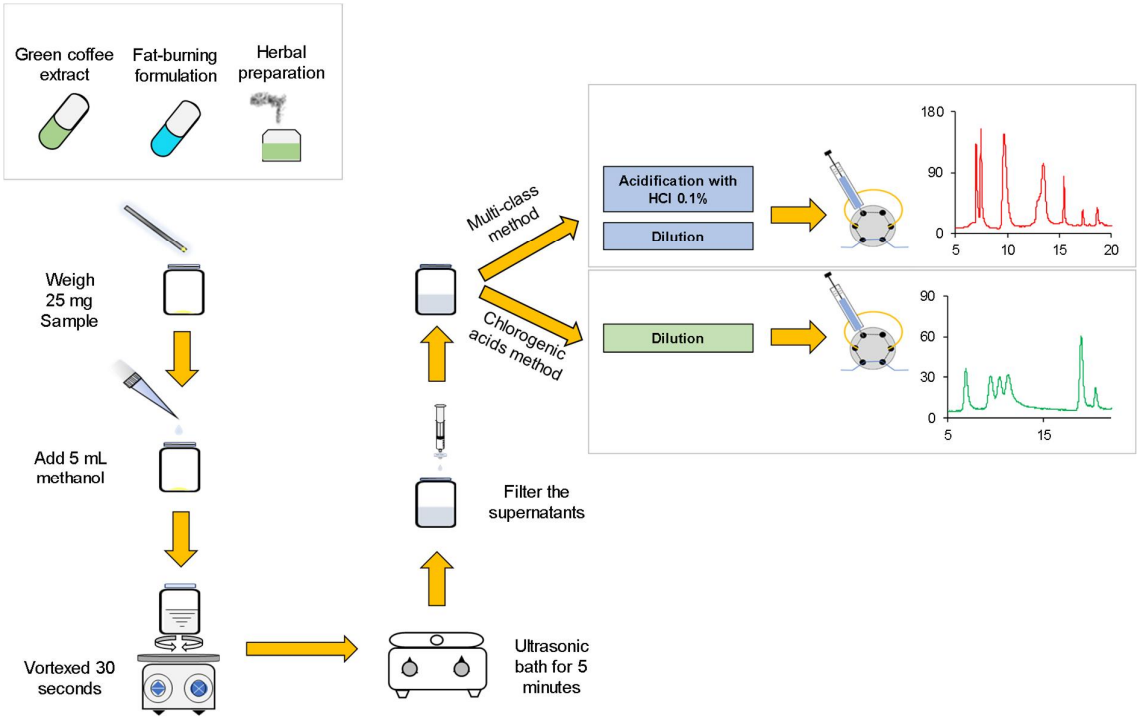


Figure 51. *Schema of the procedure for the analysis of the samples by the two methods.*

Figure 52 shows the chromatograms obtained for the three green coffee samples at 275nm. GC-1, which reported higher amounts of caffeine, is shown in Figure 52A, whereas Figure 52B represents the analysis for GC-2 and GC-3. A peak identified as theobromine was found in sample GC-3.

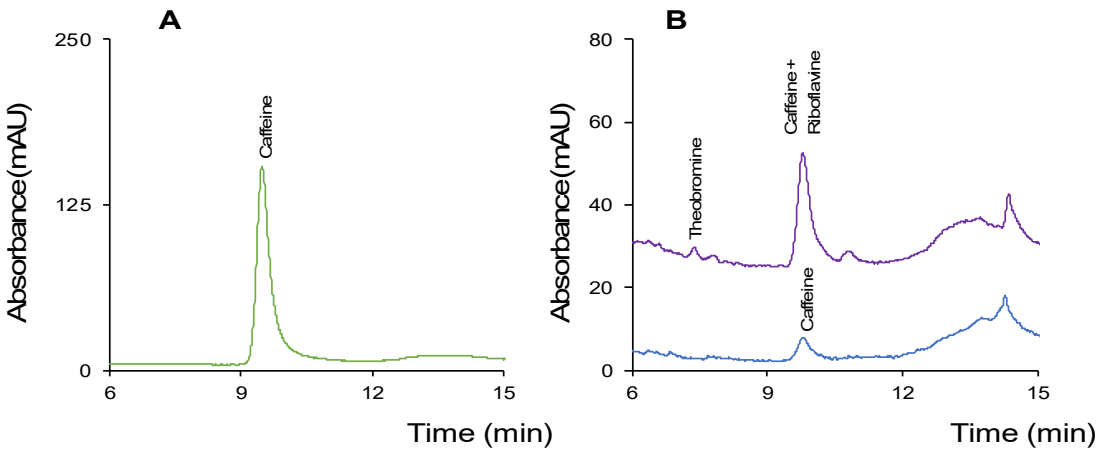


Figure 52. *Chromatograms obtained for the analysis of the green coffee samples at 275nm. A: GC-1 with dilution factor of 200; B: GC-2 (Blue) with dilution factor of 100; GC-3 (Purple) with dilution factor of 100.*

As observed in Figure 52 B of the GC-3 sample, despite a large amount of caffeine found, riboflavin could be identified, which matches with the declared value on its label. Thus, the alternate gradient for the separation of caffeine and riboflavin was applied for this sample, and the found concentration for riboflavin was 0.56 mg, whereas the declared amount was 0.7 mg. Figure 53 shows these results.

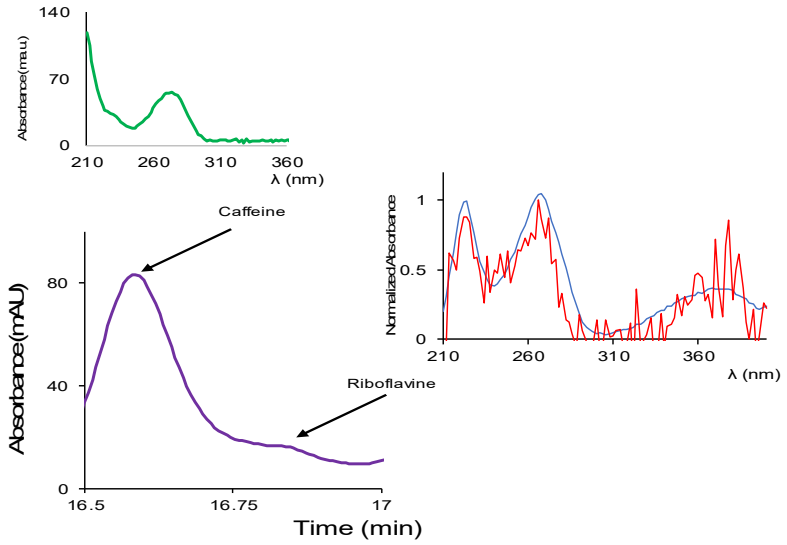


Figure 53. *Chromatogram obtained at 275 nm for sample GC-3 with the Multi-class method and the modified gradient for the analysis of caffeine and riboflavin (purple), and normalized spectra of the peak assigned to riboflavin in the sample (red) and the spectra corresponding to a standard solution of riboflavin (blue). In green appears the spectra corresponding to the peak assigned to caffeine.*

It has to be noted that different peaks at retention times of 12.5-14.0 min were observed for the three samples assayed, as Figure 52 is shown. The UV spectra registered at different positions of such peaks were indicative of the presence of caffeic and/or chlorogenic acids. Consequently, those samples were further processed using the chlorogenic acids method. Besides, in sample GC-3 was also found a significant amount of synephrine. This product was labeled to contain 50 mg of Citrus aurantium (dry extract) with 6 % of synephrine (w/w). Thus, the theoretical amount of synephrine was 3 mg/capsule, being the percentage found equal to 86 % (2.6 mg/capsule).

Finally, limonene and myrcene were found in samples GC-1 and GC-3. It is interesting to note that neither these compounds nor the other minor components were found in sample GC-2, which contained decaffeinated green coffee as the main ingredient. This suggests that minor volatile compounds were likely lost during the decaffeination process, as some researchers have attributed changes in the levels of important bioactive compounds during this process [297].

Synephrine was the most abundant compound in the fat-burning supplements. The highest amount of synephrine was found in sample FB-1 (13.2 mg per capsule), which also contained a significant amount of caffeine (0.9 mg per capsule), whereas the sample FB-2 contained 2.56 mg of synephrine per capsule. Figure 54 depicted the chromatogram registered at 220 nm for sample FB-1 and FB-2. Unlike the result found for sample GC-3, the amounts of synephrine found did not match with the declared values, which were 7.5 mg and 6.6 mg for FB-1 and FB-2, respectively. Interestingly, the three products that used Citrus aurantium as ingredient reported the same percentage of synephrine, 6 %. This suggests that this percentage is used as a mere reference or mean value for this kind of extract. The results of our study indicate that the real concentrations may be significantly different.

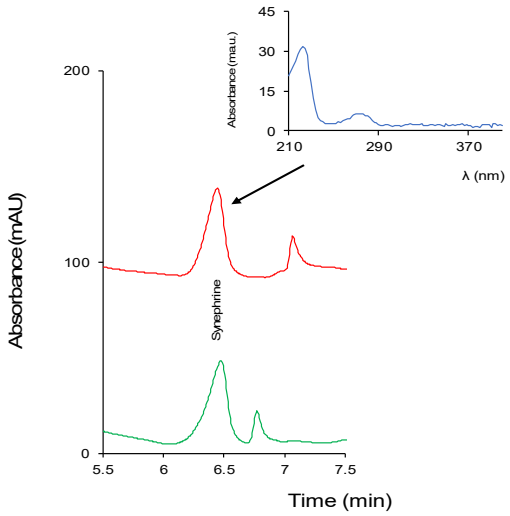


Figure 54. *Chromatogram obtained at 220 nm with the multi-class method for the analysis of Fat-burning supplements. Green: FB-1 with dilution factor of 1000; Red: FB-2 with dilution factor of 500. At the top appears the spectra corresponding to the peak assigned to synephrine (blue).*

Minor compounds of terpenic compounds, including limonene, myrcene, and linalool were present at much lower percentages (<0.04 %) in fat-burning supplements. As an example, the peak identified as linalool in the chromatogram obtained at 200 nm for sample FB-1 is shown in Figure 55A. This figure also shows the registers obtained from an extract of the same sample fortified with linalool and for a standard solution of this compound. Figure 55B shows the chromatogram obtained at 220 nm for sample FB-2 and normalized spectra of the peak assigned to myrcene in the sample. As regards the caffeic and chlorogenic acids, only a small peak was observed at 330 nm for sample FB-1, which result was confirmed by the chlorogenic acids method.

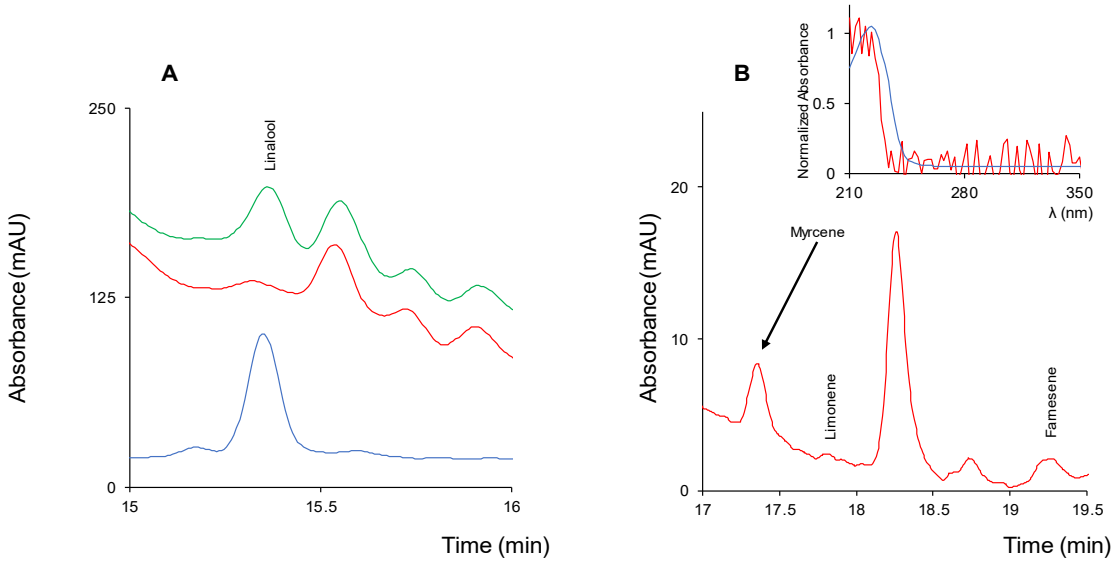


Figure 55. *A: Chromatogram obtained at 200 nm for the analysis of FB-1 sample. Blue: standard solution of Linalool (5 µg mL^{-1}); Red: Sample with dilution factor of 2; Green: Sample spiked with linalool (1µg mL^{-1}). B: Chromatogram obtained at 220 nm for the analysis of FB-2 sample. Red: Sample with dilution factor of 2; At the top appears the normalized spectra of the peak assigned to myrcene in the sample (red) and the spectra corresponding to a standard solution of myrcene (blue).*

Finally, limonene (1.90 mg g^{-1}) and synephrine (0.51 mg g^{-1}) were the most abundant compounds in the herbal preparation, which is consistent with the declared composition (lime and orange tree leaves). The other compounds found were linalool, myrcene, and farnesene, which are characteristics of orange essential oils, and responsible for the sensory characteristics of these products [298]. In Figure 56 is shown the register obtained for this sample at 200 nm. No peaks corresponding to caffeic/chlorogenic acids were observed for this sample.

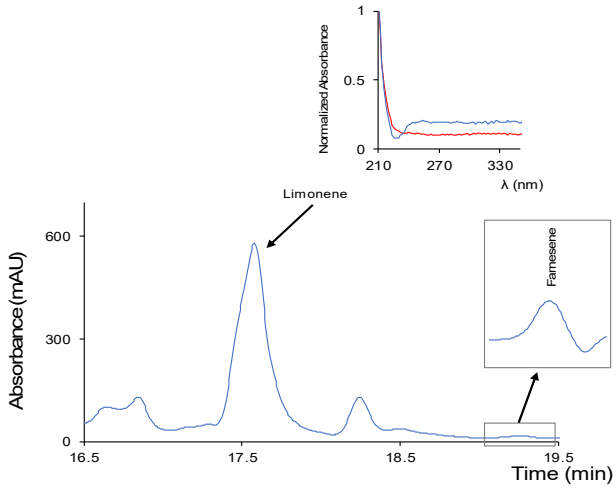


Figure 56. *Chromatogram obtained at 200 nm for the analysis of HP sample without dilution. Peaks of limonene and farnesene were identified. At the top appears the normalized spectra of the peak assigned to limonene in the sample (red) and the spectra corresponding to a standard solution of limonene (blue).*

The results obtained from the six samples tested with the multi-class method are listed in Table 25. In total nine compounds were identified, of which eight could be correctly quantified.

4.3.4.2. Chlorogenic acids method

Once the samples were analyzed by the multi-class method, those that resulted positive for chlorogenic acids were analyzed using the chlorogenic acids method. These samples were the three green coffee samples and sample FB-1. The peaks corresponding to all the isomers included in the study were detected in the three green coffee samples, whereas only 5-CQA was found in FB-1. On the other hand, caffeic acid was not observed in any of the samples. Figure 57 shows the record obtained from the four samples at 330 nm and the comparison between the spectra of the peak assigned to 5-CQA in sample FB-1 with the theoretical spectrum of the compound.

Table 25. *Results obtained for the real samples analyzed with the multi-class method (n=3).*

Sample	Found compounds	Dilution factor	Found amount	
			mg per unit [a]	mg g^{-1}
GC-1	Chlorogenic acids	200	detected	-
	Caffeine	200	89 ± 4	81 ± 4
	Limonene	2	0.350 ± 0.004	0.318 ± 0.003
	Myrcene	2	0.059 ± 0.003	0.054 ± 0.003
GC-2	Chlorogenic acids	100	detected	-
	Caffeine	100	0.51 ± 0.01	1.30 ± 0.03
GC-3	Chlorogenic acids	100	detected	-
	Caffeine	100	3.34 ± 0.03	6.7 ± 0.1
	Theobromine	100	0.24 ± 0.02	0.48 ± 0.05
	Synephrine	100	2.71 ± 0.02	5.4 ± 0.3
	Limonene	10	0.84 ± 0.01	1.68 ± 0.01
	Myrcene	10	1.3 ± 0.2	2.6 ± 0.3
	Riboflavin [b]	10	0.56 ± 0.06	1.1 ± 0.1
FB-1	Chlorogenic acids	2	detected	-
	Synephrine	100	13.2 ± 0.8	27 ± 2
	Caffeine	100	0.9 ±0.2	1.8 ± 0.4
	Limonene	2	0.129 ± 0.001	0.258 ± 0.002
	Linalool	2	0.0031 ± 0.0001	0.006 ± 0.001
	Myrcene	2	0.0200 ± 0.001	0.039 ± 0.003
FB-2	Synephrine	50	2.56 ± 0.08	10.4 ± 0.3
	Limonene	2	0.130 ± 0.002	0.520 ± 0.005
	Linalool	2	0.003 ± 0.001	0.012 ± 0.001
	Myrcene	2	0.003 ± 0.005	0.012 ± 0.001
HP	Synephrine	5	0.71 ± 0.04	0.51 ± 0.3
	Limonene	-	2.60 ± 0.05	1.90 ± 0.04
	Linalool	-	< LOQ	< LOQ
	Myrcene	-	0.150 ± 0.001	0.110± 0.001
	Farnesene	-	0.100 ± 0.003	0.070 ± 0.002

(a) capsule or bag; (b) measured with modified gradient.

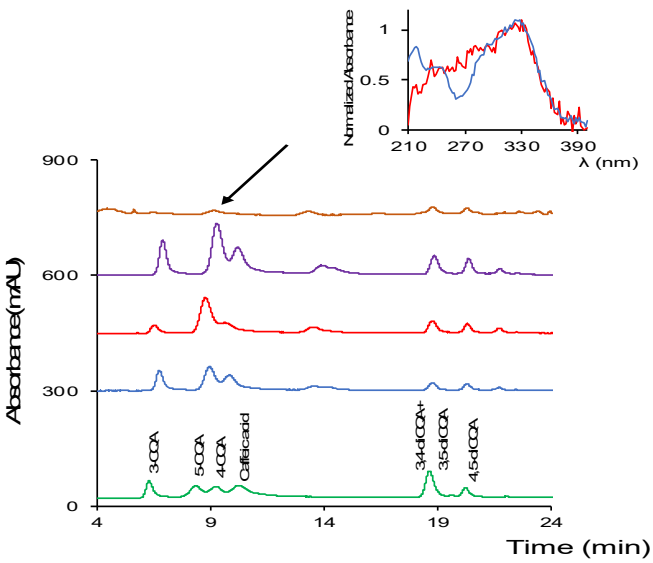


Figure 57. *Chromatograms obtained at 330 nm for the analysis of GC-1, GC-2, GC-3, and FB-1 samples. Green: standard solution (each compound at 1 µg mL^{-1}); Blue: GC-2 sample with dilution factor of 300; Red: GC-3 sample with dilution factor of 300; Purple: GC-1 sample with dilution factor of 100; Brown: FB-1 sample with dilution factor of 5; at the top appears the normalized spectra of the peak assigned to 5-CQA in the sample (red) and the spectra corresponding to a standard solution of 5-CQA (blue).*

It has to be noted that besides the peaks of the chlorogenic acids included in this study, minor peaks with spectra similar to chlorogenic acid were observed between 13 and 15 min (Figure 58). As is pointed out in the introduction part of this thesis, there are three main subgroups of chlorogenic acids, and each one of these groups has, at least, three isomers. Thus, these minor peaks can correspond at feruloylquinic acids (FQA) group, which include the 3-FQA, 4-FQA, and 5-FQA, and this may coincide with the findings reported by Takahashi et al [299].

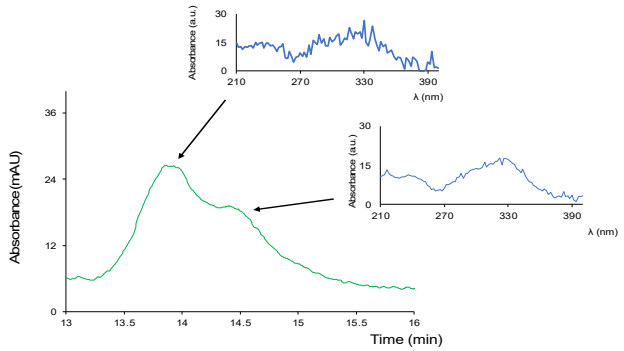


Figure 58. *Chromatogram obtained at 330 nm for the analysis of the GC-1 sample with a dilution factor of 100; at the top appears the UV spectra of the unknown peaks.*

The concentrations of the analytes obtained after the proper dilution of the extracts are summarized in Table 26. In this table, the total content of 3,4-diCQA and 3,5-diCQA is given expressed as 3,5- diCQA, as indicated in section 4.3.3.2. The total amounts of chlorogenic acids found were 37.1, 39.0, and 87.5 mg per capsule or bag in samples GC-1, GC-2, and GC-3, respectively. The amount of chlorogenic acids found in this study for sample GC-3 was slightly lower than the declared value (100 mg per capsule). The difference can be explained by the presence of other chlorogenic acids not included in the present study observed at retention times of 13-15 min.

Nevertheless, the value found for sample GC-2 was much lower than the declared value (78.75 mg per capsule), which suggests that other factors (inaccurate label or sample deterioration) may be also responsible for the discrepancy. Only a small amount of 5-CQA was found in sample FB-1. The contents of chlorogenic acids in samples GC-1 and FB-1 were not provided by the suppliers of the products.

Table 26. *Results obtained for the real samples analyzed with the chlorogenic acids method (n=3).*

Sample	Found compounds	Dilution factor	Found amount	
			mg per unit [a]	mg g^{-1}
GC-1	3-CQA	100	6.2 ± 0.7	5.6 ± 0.6
	5-CQA		12 ± 2	11 ± 1
	4-CQA		8.6 ± 0.2	7.8 ± 0.2
	3,4-diCQA + 3,5-diCQA		4.1 ± 0.1	3.7 ± 0.1
	4,5-diCQA		6.2 ± 0.1	5.6 ± 0.1
			Total: 37.1 ± 3.1	
GC-2	3-CQA	300	8.4 ± 0.1	21.0 ± 0.3
	5-CQA		13 ± 1	33 ± 3
	4-CQA		9.3 ± 0.8	23 ± 2
	3,4-diCQA + 3,5-diCQA		3.7 ± 0.2	9.3 ± 0.5
	4,5-diCQA		4.6 ± 0.1	12.0 ± 3.0
			Total: 39.0 ± 2.2	
GC-3	3-CQA	300	6.1 ± 0.4	12.2 ± 0.8
	5-CQA		39 ± 1	78 ± 2
	4-CQA		10 ± 1	20 ± 2
	3,4-diCQA + 3,5-diCQA		16.6 ± 0.9	33 ± 2
	4,5-diCQA		14 ± 1	28 ± 2
			Total: 85.7 ± 4.3	
FB-1	5-CQA	5	0.23 ± 0.01	0.46 ± 0.02

(a) capsule

4.3.5. Conclusions

Numerous methods for the analysis of dietary supplements have been described in the literature. However, they are focused on individual substances or on a family of compounds. On the other hand, the methods presented for multi-class analysis require expensive and specialized instrumentation. In this work, an analytical method has been developed that can be used to obtain information not only on major components, but also on minor constituents such as vitamins or those compounds present in essential oils (myrcene, limonene), which may have potential effects on health consumers.

As a treatment technique, ultrasound-assisted extraction was successfully applied, requiring only 5 mL of methanol for the complete isolation of the compounds present in the samples, with a minimum of experimental work. Two chromatographic separations were successfully developed and applied for the determination of the studied compounds, using a miniaturized CapLC system. On the one hand, a multi-class method capable of identifying and quantifying major components, such as caffeine, synephrine, and theobromine, as well as minor compounds, including riboflavin, kaempferol, limonene, myrcene, and farnesene. Also, this method is capable of identifying the presence of compounds related to chlorogenic acid. In those samples with a positive presence of these compounds, it is possible to use a second chromatographic separation for the adequate quantification of the isomers of chlorogenic acid and caffeic acid.

The analytical parameters obtained were satisfactory and comparable with those described in the literature for the analysis of these kinds of products. Therefore, the method can be used as a simple alternative for more extensive characterization of the samples and facilitate the detection of undeclared substances added to increase the effectiveness of the dietary supplements.

4.4 TERPENIC COMPOUNDS IN MICROSAMPLES OF NATURAL RESINS

Based on the results achieved in the previous section of this thesis, for the study of terpenic compounds by applying capillary liquid chromatography, a method for the quantification of representative components in natural resins was developed. The study included the determination of both volatile and non-volatile compounds typically found in these kinds of samples, due to the high sensitivity achieved with miniaturized LC systems, especially important for the analysis of micro samples, as in natural resins analysis.

Limonene, a volatile monoterpene, and lupeol, lupenone, α-amyrin, and β-amyrin, triterpenes with high molecular weight, all of them with high octanol-water partition coefficients, have been selected as model compounds. The analytical performance of the proposed method has been tested, and examples of application to real samples were made. In the following sections, the findings for the developed method are discussed in detail.

4.4.1. Optimization of the chromatographic separation

Initially, standard solutions of the analytes at 100 µg mL^{-1} prepared in methanol with a volume of injection of 5 µL was used in order to optimize the separation and detection of the target compounds. For this, different acetonitrile-water mixtures were tested, with percentages of acetonitrile ranged from 60% to 95%. As expected, mobile phases with high contents of acetonitrile (>70%) were necessary for the analytes to be eluted at reasonable run times (<40 min). For instance, see the chromatogram in Figure 59.

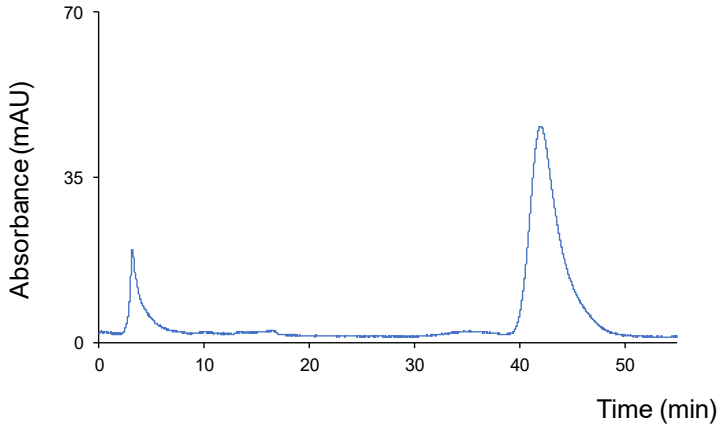


Figure 59. *Chromatogram obtained at 200 nm for the analysis of Lupenone standard solution at 100 µg mL^{-1}, employing a mixture of water and acetonitrile (10:90) as mobile phase.*

At this point, the UV spectra of all compounds were obtained, and according to Figure 60, all the analytes presented decreasing absorbances within the 190-210 nm range and nearly null absorbance at higher wavelengths. Thus, 200 nm was selected as the working wavelength.

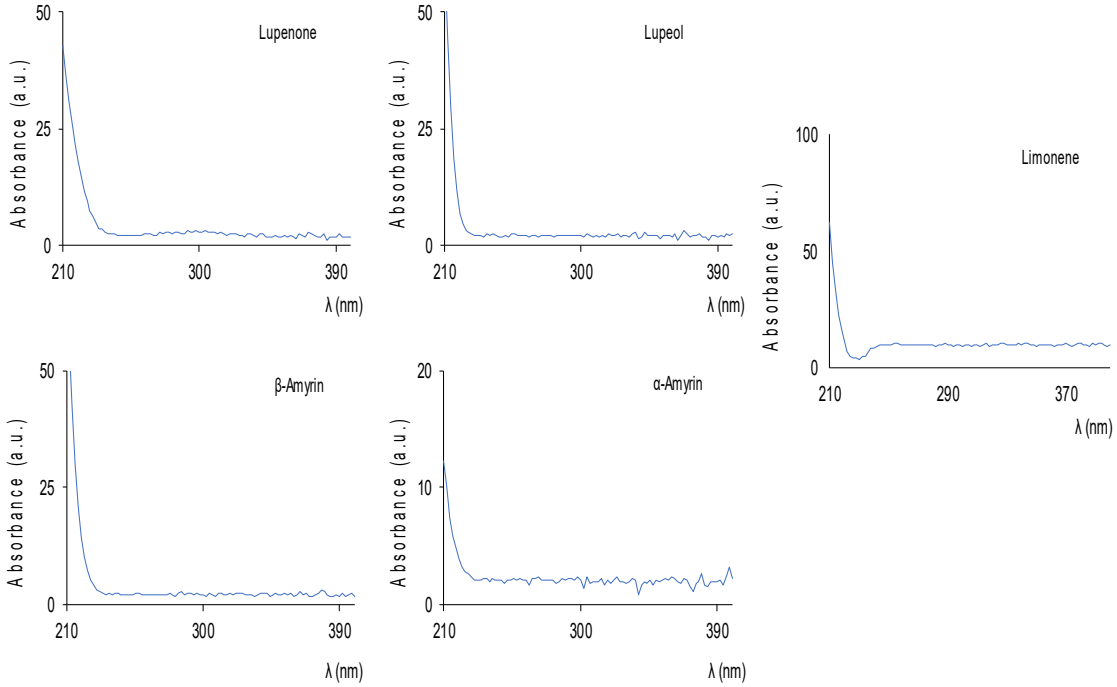


Figure 60. *UV spectra obtained for analyzed compounds.*

For the following experiments, a standard solution mix of the analytes at 10 µg mL^{-1} was prepared in methanol. For all the elution conditions assayed suitable separation of the analytes was obtained except for limonene since its isolation was particularly difficult due to the presence of an intense peak corresponding to the injection solvent (methanol). Due to its high intensity, such a peak partially overlapped with that of limonene. An option to improve the resolution between the two peaks could be to use a gradient elution program, but this will cause an increase in the total execution time. For the rest of the compounds, a good resolution was obtained even with a mobile phase of 100% acetonitrile. With this eluent, the chromatographic run time was <20 min, as Figure 61 shows. Besides the peaks of the solvent and analytes, two minor peaks were detected at 12.1 min and 15.3 min, which were identified as impurities of β-amyrin.

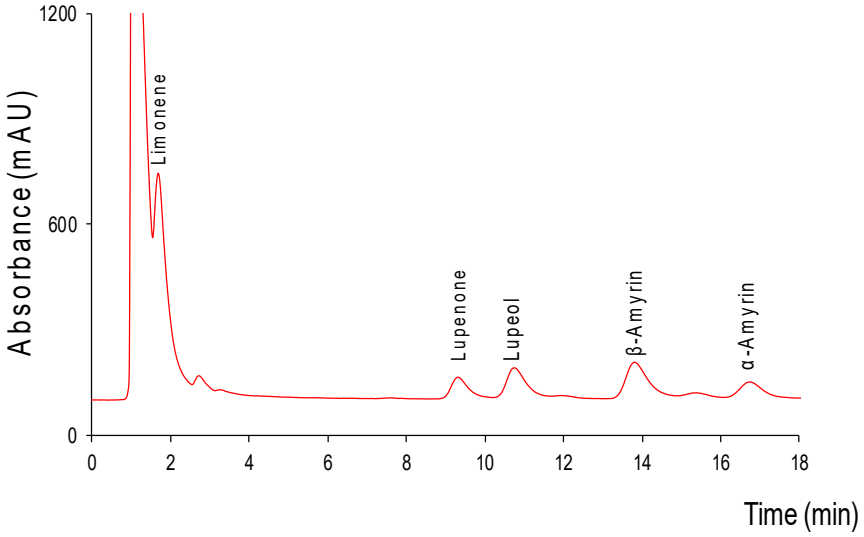


Figure 61. *Chromatogram obtained for standard solution mix of the analytes (10 µg mL^{-1}) in methanol injected directly; volume of injection 5 µL; eluent, 100% acetonitrile; detection wavelength 200nm.*

Since the elution strength of tetrahydrofuran is higher than that of acetonitrile, it was tested as an elution solvent. However, due to its significant absorbance at wavelengths <212 nm (as was pointed in Figure 42 of section 4.3), the background noise at the wavelength necessary to detect the analytes was unacceptable. Therefore, this solvent was no longer used. Then, standard solutions mixtures of the analytes at 100 µg mL^{-1} were prepared in different solvents (as injection solvents) to avoid the overlap of methanol with the peak of limonene. However, as Figure 62 shows, unsuitable results were obtained with chloroform, isopropanol, and ethyl acetate. When using acetonitrile, no greater variation was observed in the limonene peak, while for the rest of the compounds, wider and lower peaks resulted, that is, a lower sensitivity. Therefore, methanol was maintained as an injection solvent.

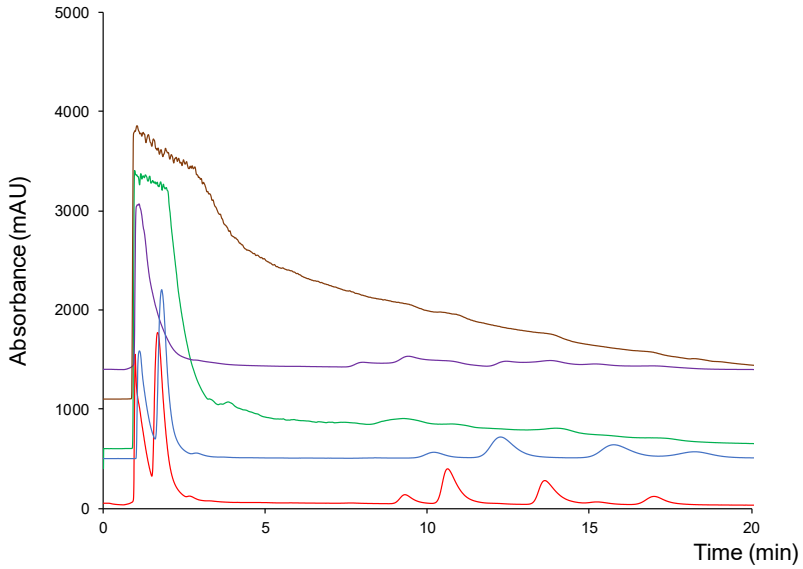


Figure 62. *Chromatograms obtained at 200 nm for standard solution mix of the analytes (100 µg mL^{-1}) in: Red: methanol; Blue: acetonitrile; Green: Ethyl acetate; Purple: isopropanol; Brown: Chloroform. Volume of injection 5 µL; eluent, 100% acetonitrile.*

Another strategy to try to reduce the solvent peak and to improve the resolution of limonene was to test different methanol-water mixtures as a solvent. For this, standard solutions of the analytes at 5 *µg mL^{-1}* prepared with mixtures of water-methanol in percentages of 10, 20 and 35 were injected (v/v). Ideally, in LC samples should be injected in an injection solvent with similar to or lower elution strength than that of the mobile phase used to avoid peak-shape problems [300]. However, the results showed that the presence of water in the injected solutions caused a diminution of the peak areas of some of the analytes, effect which was especially high for α-amyrin, as Figure 63 shows. This behavior suggested that at the working concentration the analytes were not completely dissolved in the mixture of methanol- water, which is consistent with their high Kow values.

As an alternative, the introduction of an aliquot of water in the injection capillary loop was tested before loading the sample, because it could prevent peak broadening at the entrance of the chromatographic column. Variable volumes of water in the 5-25 µL range were loaded in the injection loop before loading 5 µL of the standard solution prepared in methanol. The resulting chromatograms were compared with those observed for the same directly injected solution and are shown in Figure 61. As a result, the introduction of water into the injection capillary loop had a strong effect, on the one hand, on the retention times of the analytes, and on the other hand, on the shapes of the analytes peaks.

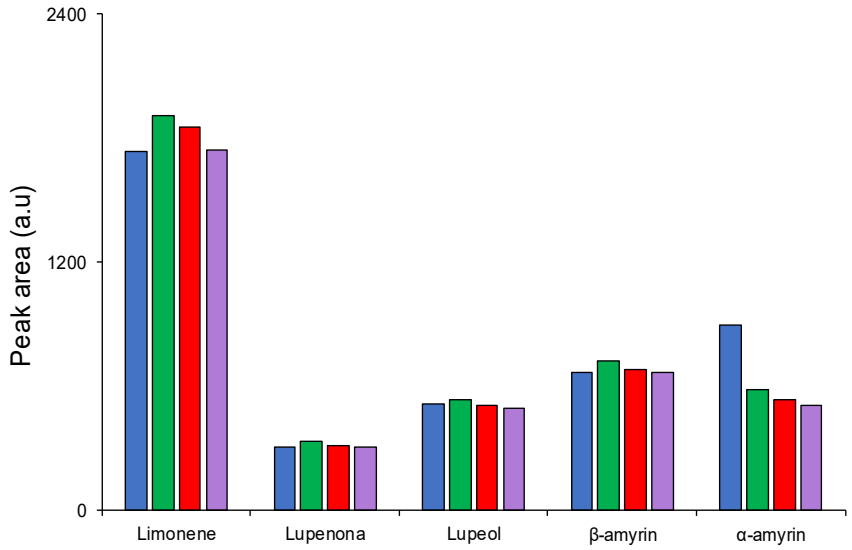


Figure 63. *Analyte responses obtained for the study of the percentage of water in injection solvent. Blue: 0% of water; Green: 10% of water; Red: 20% of water; Purple: 50% of water. Concentration of each analyte: 5 µg mL^{-1}; Volume of injection 5 µL; eluent, 100% acetonitrile.*

As observed in Figure 64, in the chromatogram obtained after the consecutive introduction of 5 µL of nanopure water and 5 µL of the working solution all the analytes eluted about 1.5 min later. This effect was particularly positive for the complete separation of the limonene peak from the solvent peak. Furthermore, the presence of water had also a positive effect on the peak shapes of the other compounds. Finally, no substantial differences in the chromatographic registers were observed when the volume of water was increased up to 25 µL.

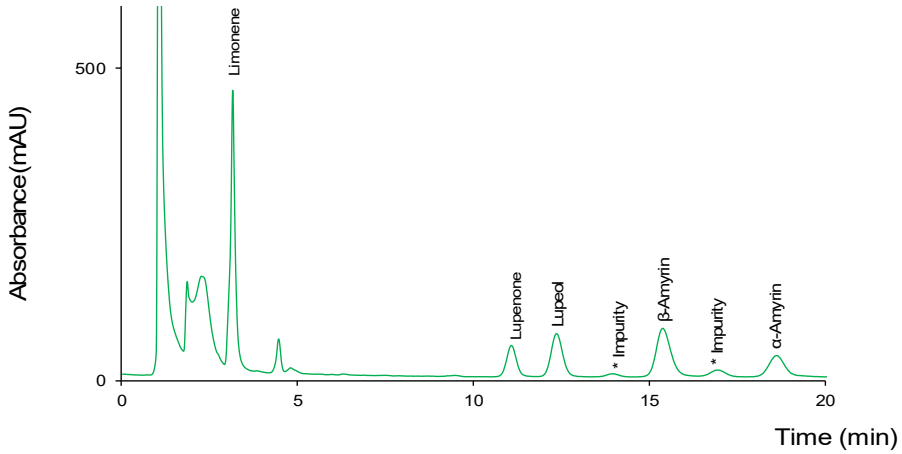


Figure 64. *Chromatogram obtained for standard solution mix of the analytes (10 µg mL^{-1}) in methanol after loading 5 µL of nanopure water in the injection loop. Volume of injection 5 µL; eluent, 100% acetonitrile; detection wavelength 200nm.*

Finally, the effect of the injection volume was evaluated in the range of 5-25 µL. Although the absolute peak areas increased as the injection volume increased as expected, this increment also resulted in wider peaks. Besides, the retention times of the compounds decreased, which may be due to the increased volume of methanol entering the system. As Figure 65 shows, the increase in the injection volume resulted in an unsuitable separation between lupeol and lupenone.

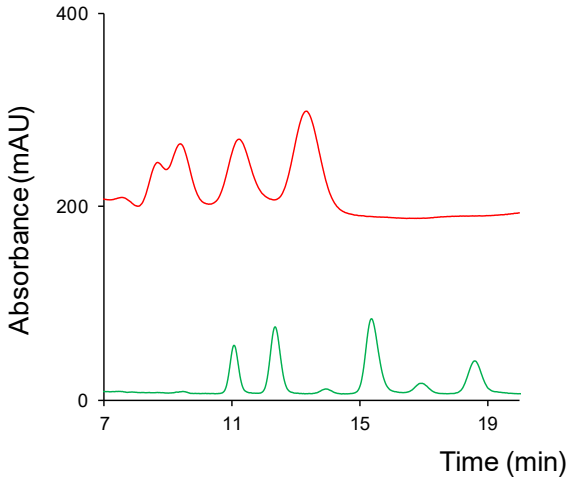


Figure 65. *Chromatograms obtained for standard solution mix of the analytes (10 µg mL^{-1}) in methanol after loading 5 µL of nanopure water in the injection loop. Red: volume of injection 15 µL; Green: volume of injection 5 µL. Eluent, 100% acetonitrile; detection wavelength 200nm.*

Based on the above results, the successive injection into the loop of 5 µL of water and 5 µL of the working and sample solutions was selected as the best option. For the mobile phase, as a compromise between resolution and chromatographic run time, a mixture of acetonitrile:water 85:15 (v/v) was selected as optimum conditions.

4.4.2. Extraction optimization

Optimized chromatographic conditions were applied to the analysis of the target compounds in three natural resins, namely white copal, copal in tears, and resin obtained from ocote trees. In the first instance, different solubility studies with the resin samples were carried out to optimize the solvent extraction. For this, homogenized portions between 1 and 15 mg of the three resins were treated with 1 mL of extracting solvent. Then, the resultant solutions were vortexed for 1 minute and finally filtered with 0.2 µm nylon membranes. Methanol, acetonitrile, ethyl acetate, isopropanol, and chloroform were tested as extraction solvents.

According to the results presented in Figure 62 of the above section, the employment of ethyl acetate, isopropanol, and chloroform was unsuitable. It was concluded that the use of such solvents

would require a step of evaporation of the extracts followed by their redissolution in methanol or acetonitrile prior to chromatographic analysis. Thus, to simplify the whole analytical process, as well as to avoid possible losses of the volatile analyte limonene, these solvents were not used in further experiments.

The registered chromatograms for the analysis of white copal resin extract with acetonitrile (red) and methanol (green) are presented in Figure 66. Slightly higher signals were observed for the extract with methanol, so methanol was finally preferred as an extraction solvent for the following experiments.

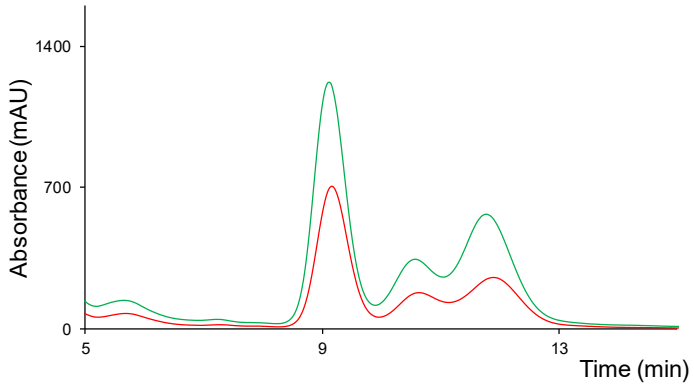


Figure 66. *Chromatograms obtained for the extract of white copal sample. Red: extracted with acetonitrile; Green: extracted with methanol. Eluent, 100% acetonitrile; detection wavelength 200nm.*

Figure 67 shows the photographs of the extracts obtained with methanol for the three resin samples. The white copal (67a) and ocote tree (67b) samples were satisfactorily dissolved in methanol, however, for 10–15 mg of the copal in tears resin sample was treated in the same way (67c) significant amounts of solid matter were observed, most probably due to the presence of highly polar gum compounds, characteristics of resins samples [301].

Thus, for copal in tears sample, a further study of the solid residue was carried out, applying a centrifugation stage after which the liquid phase was separated. Then, the insoluble solid residue was treated with 1 mL of water, and complete dissolution was observed (67d), which confirmed the presence of a high percentage of gum in this sample.

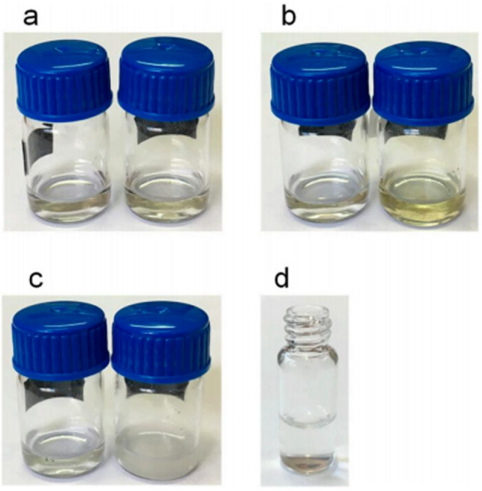


Figure 67. *Photographs of the extracts obtained after adding 1 mL of methanol. a: white copal; b: ocote; c: copal in tears; left vials in (a–c), 1 mg of homogenized samples; right vials in (a–c), 15 mg of the homogenized samples. d: solution obtained after treating the residue insoluble in methanol of copal in tears (10 mg) with 1 mL of water.*

4.4.3. Analytical performance

The analytical performance of the proposed method was evaluated to establish its suitability for the analysis of the natural resins [302]. Working solutions of the target compounds were prepared in methanol at concentrations in the range 0.25-10 µg mL^{-1} and then were analyzed. With the obtained results parameters for linearity, limits of detection (LODs), limits of quantification (LOQs), accuracy, and precision were studied.

For all tested compounds the peak areas showed a linear relationship with the concentration within the range 0.25-10.0 µg mL^{-1}, with determination coefficients ranging from 0.994 to 0.997 (n =15). For checking the accuracy, solutions of the target analytes were prepared and tested at low-low-intermediate (2.5 µg mL^{-1}) and high-intermediate (7.5 µg mL^{-1}) concentrations. The corresponding calibration equations were used to establish the concentration of each compound and the relative errors found ranged from −13% to +16%. These values were satisfactory according to the standards set for these kinds of samples [303], and it was therefore concluded that the accuracy was adequate. Table 27 shows the results obtained.

Table 27. *Evaluation of the linearity and accuracy of the proposed method.*

Compound	Linearity (*n* =15) y = ax +b				Mean found concentration (n=3)	
	Concentration range (µg mL^{-1})	a ± s_a	b ± s_b	R^2	2.5 µg mL $^{-1}$	7.5 µg mL $^{-1}$
Limonene	0.25 - 10.0	433 ± 6	- 77 ± 2	0.997	2.3 ±0.1	6.5 ±0.4
Lupenone	0.5 - 10.0	63.9 ± 1.6	- 22 ± 9	0.994	2.4 ±0.1	70. ±0.1
Lupeol	0.5 - 10.0	111 ± 2	- 42 ± 12	0.996	2.6 ±0.1	7.4. ±0.2
β-Amyrin	0.5 - 10.0	135 ± 3	- 20 ± 17	0.995	2.3 ±0.1	7.5 ±0.6
α-Amyrin	0.5 - 10.0	313 ± 8	72 ± 42	0.994	2.9 ±0.2	8.4 ±0.1

a: intercept; s_a: standard deviation of the intercept; b: slope; s_b: standard deviation of the slope; R^2: squared correlation coefficient.

The precision was evaluated by calculating the relative standard deviations (RSDs) of the areas measured for three consecutive injections (intra-day RSD) and in three different working sessions (inter-day RSDs) for the same solutions used to determinate the accuracy. Intra- and inter-day precision were determined at 2.5 µg mL^{-1} and 7.5 µg mL^{-1}, and although for α-amyrin the RSDs were slightly higher, values <8% were found for the rest of the compounds.

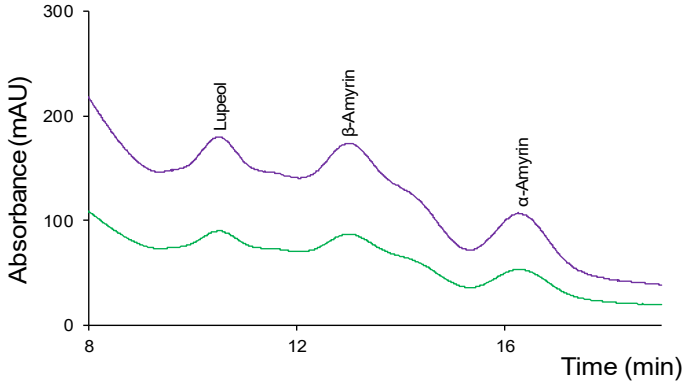
Although there are different options available to determinate the LODs and LOQs, in this study these parameters were calculated as the concentrations that resulted from signal-to-noise ratios of 3 and 10, respectively [304]. The LODs and LOQs were obtained by injecting solutions with decreasing concentrations of the analytes and to prevent errors coming from contaminants and/or memory effects, before analyzing each solution, water was processed. The LODs were 0.1 µg mL^{-1} for limonene, whereas for the rest of the analytes was 0.25 µg mL^{-1}. On the other hand, the LOQs were 0.4 µg mL^{-1} for limonene and 0.8 µg mL^{-1} for the other analytes. Table 28 summarized the results obtained.

Table 28. *Evaluation of the precision, LOD and LOQ of the proposed method.*

Compound	Precision [a], (*n*=3) RSD %				LOD (µg mL^{-1})	LOQ (µg mL^{-1})
	Intraday		Interday			
	2.5 µg mL $^{-1}$	7.5 µg mL $^{-1}$	2.5 µg mL $^{-1}$	7.5 µg mL $^{-1}$		
Limonene	2	0.6	3	4	0.1	0.4
Lupenone	4	0.8	7	7	0.25	0.8
Lupeol	1.4	2	7	8	0.25	0.8
β-Amyrin	3	8	8	8	0.25	0.8
α-Amyrin	9	17	16	17	0.25	0.8

rsd: residual standard deviation.

For the study of the effect of the sample matrix in the analytical response, sample extracts from copal in tears were fortified with known amounts of the analytes, and the recoveries were calculated. The tested concentration was 5 µg mL^{-1} of each analyte. The increment on the peak areas between the spiked and unspiked extracts was used to calculate the added concentration, using the calibration equations of Table 27. The concentration values obtained were then compared with the added concentrations to calculate the recoveries, and values ranging from 52% to 103% were found. Figure 68 shows the chromatograms of the copal in tears sample with and without fortification, besides the recovery values found for all analytes.



Compound	*Recovery %*
Limonene	103 ±4
Lupenone	101 ±1
Lupeol	79 ±9
β-Amyrin	52 ±5
α-Amyrin	75 ±3

Figure 68. *Chromatograms obtained in the matrix effect study for Copal in tears sample. Green: sample without fortification; Purple: fortified sample with 5 µg mL^{-1} of the analytes. On the right, the recoveries values found for all analytes (n=3).*

According to the LOQs obtained and the recoveries values listed in Figure 68, the minimum percentages of the analytes that could be measured were calculated for samples of 10 mg. These values were ranged from 0.004% for limonene to 0.02% for β-amyrin. These values were considered low enough for most applications, making unnecessary extra pre-concentration operations.

4.4.4. Analysis of real samples

Finally, the optimized method was applied for the quantitative analysis of the three resins tested. For this purpose, different portions of the homogenized samples ranging from 1 to 15 mg were analyzed under the conditions described in the previous sections. In all three natural resin samples, the presence of the analytes was evaluated from the concordance between the retention times and UV spectra of the suspected peaks and those observed for the standard solutions. Besides, the presence of a compound was confirmed by fortifying the extracts with standard solutions of such compounds.

α-amyrin was the only analyte found in the three resins samples analyzed, limonene was found in the white copal and ocote resins, whereas lupeol and β-amyrin were found only in the copal in tears sample. As expected in these kinds of samples, in addition to the peaks of some of the analytes, peaks of unknown compounds were observed in the samples, particularly at retention times close to that of limonene, which means the polar fraction of the resins. However, they could be easily differentiated from limonene through their respective UV spectra. On the other hand, the peaks of the impurities found in the standard solutions of β-amyrin were not identified in any sample.

To calculate the percentages of each of the analytes found in the samples, the peak areas of the analytes were used, as well as the calibration equations of Table 27 and considering the recoveries determined for each compound, listed in Figure 68. The final values are summarized in Table 29, and as can be deduced from this table, the percentages of the triterpenic compounds (C=30) were <1%. Therefore, for the quantification of these compounds, a higher amount of the sample was used (10-15 mg). Whit 1mg of white copal resin sample, the concentration of α-amyrin was below its LOD, and for ocote resin sample the concentration was between its LOD and LOQ. For this last resin, even when processing 10 mg of the sample, the concentration of α-amyrin in the extract was close to its LOQ.

Table 29. *Percentages of the analytes found in the analyzed natural resin samples (n = 3).*

Sample		Percentage [a] (%), (n = 3)				
		Limonene	Lupenone	Lupeol	β-Amyrin	α-Amyrin
White copal	1 mg	0.9 ± 0.2	<LOD	<LOD	<LOD	<LOD
	15 mg	1.2 ± 0.2	<LOD	<LOD	<LOD	0.020 ± 0.002
Copal in tears	10 mg	<LOD	<LOD	0.034 ± 0.001	0.069 ± 0.002	0.011 ± 0.001
Ocote	1 mg	9.3 ± 0.2	<LOD	<LOD	<LOD	<LOQ
	10 mg	9.3 ± 0.1	<LOD	<LOD	<LOD	0.093 ± 0.003

a: All values expressed with digits known plus the first uncertain digit.

Higher percentages of the monoterpene limonene were found in white copal and ocote resins. In fact, for the quantification of this analyte in the ocote tree resin, the extract of the sample had to be diluted with methanol whit a dilution factor equal to 20, to adjust the analyte concentration to the linear working interval evaluated. Due to its relative abundance in white copal and ocote samples (≥1%), the percentage of limonene could be established using both 1 mg and 10–15 mg of the samples. The values obtained by using different amounts of the samples were then compared statistically [305].

The t calculated values were 2.01 and 0.17 for white copal and ocote resins, respectively, whereas the t tabulated at 95% confidence level was 2.776. For this calculation, equivalent variances were assumed, as F calculated were 1.15 and 4.86 for the white copal and ocote resins, respectively, being the F tabulated at 95% confidence level = 19.00. Therefore, it was concluded that the percentages obtained were not dependent on the sample size. Representative chromatograms obtained for white copal and ocote tree resins are shown in Figure 69; some of the pictures have been zoomed for better visualization of the peaks of interest.

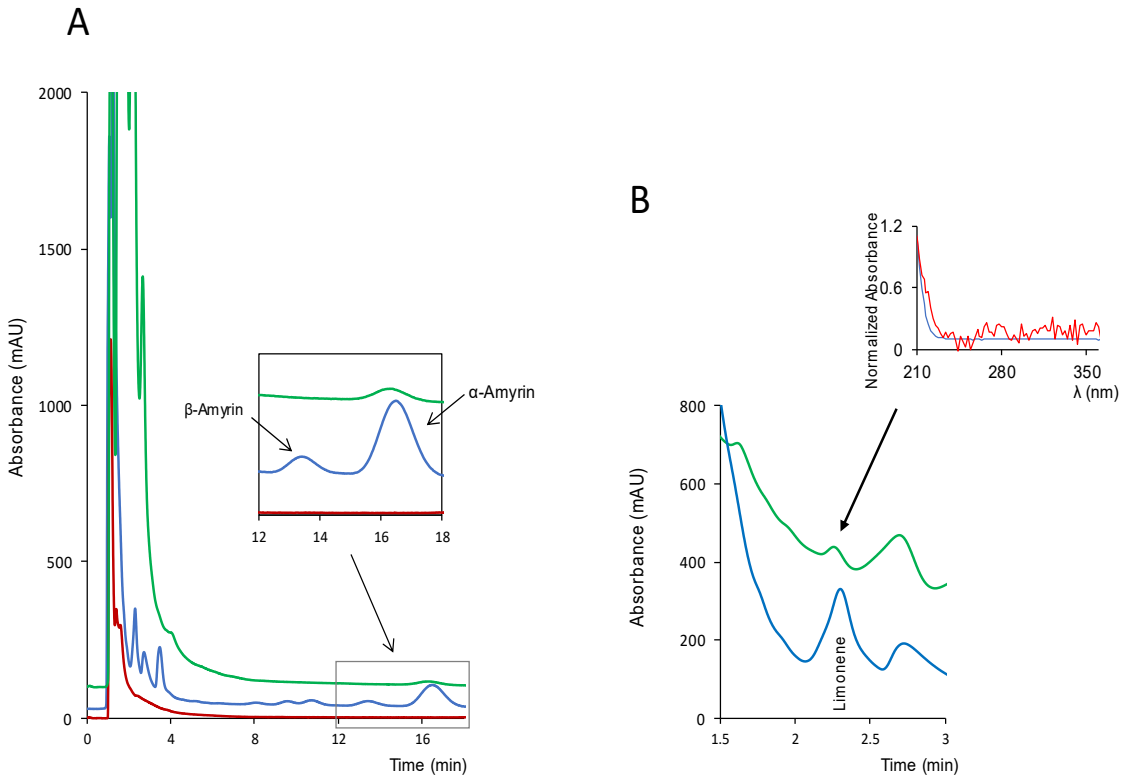


Figure 69. *Chromatograms obtained in the analysis of the resin samples. A: White copal; Red: blank of methanol; Blue: standard solution at 5 µg mL^{-1};Green: sample. B: Ocote resin with dilution factor of 20; Blue: standard solution at 5 µg mL^{-1}; Green: sample. At the top appears the normalized spectra of the peak assigned to limonene in the sample (red) and the spectra corresponding to a standard solution of limonene (blue).*

To evaluate the effect of the thermal stability on the sample composition, portions of copal in tears and ocote tree, samples with different composition profiles, were subjected to different treatments. For this purpose, homogenized portions of the samples were spread on the surface of glass vials. Then the vials were exposed to ambient conditions for five days before analysis. Additionally, portions of the same samples were dried at 40 °C in an oven until constant weight and

then processed. The results obtained in these experiments are shown in Table 30, and as observed, the composition of the copal in tears sample was not significantly modified by any of the treatments applied. In contrast, both treatments led to lower contents of limonene in the ocote resin, whereas the percentage of α-amyrin increased.

Besides, the results found for this sample indicate that limonene was partially volatilized both at ambient conditions and after drying at 40 °C. It is important to note that the loss of limonene, and possibly other volatile compounds, resulted in higher percentages of non-volatile compounds such α-amyrin. The absence of limonene in the copal in tears resin, suggests that the volatile compounds had been previously lost, which is consistent with the fact that the percentages of the triterpenes remained approximately constant after exposing the sample at ambient conditions or after the thermal treatment applied.

Table 30. *Percentages of the analytes found in the resin samples after the stability study (n = 3).*

Sample		Percentage [a] (%), (n = 3)				
		Limonene	Lupenone	Lupeol	β-Amyrin	α-Amyrin
Copal in tears	10 mg [b]	<LOD	<LOD	0.033 ± 0.001	0.074 ± 0.001	0.010 ± 0.003
	10 mg [c]	<LOD	<LOD	0.035 ± 0.002	0.082 ± 0.005	0.010 ± 0.004
Ocote	10 mg [b]	7.2 ± 0.1	<LOD	<LOD	<LOD	0.16 ± 0.01
	10 mg [c]	7.3 ± 0.3	<LOD	<LOD	<LOD	0.16 ± 0.02

a: All values expressed with digits known plus the first uncertain digit. b: Exposed at ambient conditions for 5 days; c: Dried at 40◦C until constant weight.

4.4.5. Conclusions

In the framework of this thesis, a quantitative study for some relevant terpenoids typically used to characterize natural resins has been developed based on a method by capillary LC with UV detection. The determination of volatile and non-volatile analytes within the same chromatographic run with the adequate sensitivity to be applied when only small size samples are available (a few mg), has been accomplished due to the optimization of the extraction and separation chromatographic conditions.

The results obtained throughout the study for the performance characteristics of the method have proved that quantitative performance is suitable. To the best of our knowledge, this is the first method validated for the quantification of limonene and representative triterpenes in microsamples of resins. Therefore, the developed procedure can be considered a useful tool to increase the

knowledge about the chemical composition of natural resins, as most existing methods so far are limited to obtaining their chemical fingerprints. Furthermore, it is important to note that, for classification purposes, the quantitative composition of these kinds of samples can be used to obtain information about the history (age and ambient conditions) of samples of similar origin.

CHAPTER 5. GENERAL CONCLUSIONS

New knowledge was attained about miniaturized and portable liquid chromatography, following the current trends of green analytical chemistry, and also its coupling to in-tube solid-phase microextraction, as a sustainable sample treatment technique, for the analysis of different kinds of matrices, including biological samples, environmental waters, dietary supplements, and natural resins.

The study of the ability and characterization of commercial and synthesized extractive phases for in-tube solid phase microextraction for the determination of highly polar compounds in serum, urine, and oral fluid samples were carried out. A pioneer study with a portable NanoLC for the determination on-site of trimethylxanthines in environmental waters evaluating different strategies for sample treatment was made. The high-resolution power, sensitivity, and the viability to use a small sample volume attainable with Capillary LC system were demonstrated from the analysis of natural resins and dietary supplements for quantification of a wide number of compounds of very different physic-chemical properties.

Good results were achieved by the use of FFAP (100% nitroterephthalic modified polyethylene glycol) as the extractive phase in In-tube solid-phase microextraction (IT-SPME) coupled on-line to capillary liquid chromatography with diode array detection for the analysis of caffeine and its three primary metabolites (theobromine, paraxanthine, and theophylline) in micro samples of serum, oral fluid, and urine samples. With the optimized procedure, the sample amount required for one analysis was only 2.5 µL of oral fluid, 6.25 µL of serum or 40 µL of urine, which is of special ability for its implementation in a hospital laboratory for preterm newborns. Analytical performance of the proposed method was similar to those proposed by other methodologies but using lower sample volume and a faster and simpler sample treatment and analysis. Finally, paired samples of serum and oral fluid from preterm newborns treated with caffeine were analyzed by the method, with statistically equivalent results for caffeine concentrations.

For the analysis of environmental waters, two methods were proposed, for one hand, a fast lab method based on IT-SPME coupled online with a capillary liquid chromatograph (CapLC) with diode array detection (DAD), and on the other hand, using a portable nano liquid chromatograph (NanoLC) with UV detection at 255 nm for in-place analysis. The analytical performance of both procedures for the determination of trimethylxanthines as target analytes was done. Different strategies for sample treatment technique was applied, included IT-SPME online and off-line, and, solid-phase extraction for improving instrumental parameters, related to detection capacity and selectivity. IT-SPME or SPE/portable NanoLC based methods were superior in terms of chromatographic resolution and organic solvent consumption per sample, around 200 µL vs 10 mL for IT-SPME-CapLC-DAD, instead,

the lab method provided better LOD. Finally, both systems were tested for the analysis of real water samples with suitable results.

The characterization of botanical dietary supplements by capillary liquid chromatography with UV-diode array detection (DAD) was achieved by the optimization of separation and quantification conditions in order to analyze not only the major constituents such as caffeine, chlorogenic acids, and synephrine, but also trace compounds as theobromine, riboflavin, limonene, α-pinene, farnesene, myrcene, linalool, and kaempferol. The proposed procedure was used for the analysis of different types of products, including green coffee extract-based supplements, fat burning formulations, and herbal preparations; managing to estimate the effectiveness and safety of these products with a stimulant, antioxidant, and slimming effects, through the consideration of the contents of major compounds, whereas minor compounds can be used to evaluate the sensory characteristics of the samples.

Despite the difficulties involved in the analysis of natural products such as vegetal resins, a method was developed for the separation and quantification of terpenic compounds typically used as markers in the chemical characterization of these kinds of samples, based on capillary liquid chromatography with UV detection. Notwithstanding the analyzed compounds have different polarities and volatilities, a simple sample treatment procedure, an adequate separation, and remarkable detectability were achieved in a single chromatographic run for micro samples of three resins namely, white copal, copal in tears, and ocote tree resin. According to the state of the art in the analysis of this kind of samples, the proposed method can be considered complementary to existing protocols aimed at establishing the chemical fingerprint, as a useful tool to increase the knowledge about the chemical composition of natural resins.

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APPENDIX

A.1 ABBREVIATIONS

AAS	Atomic absorption spectroscopy
AIBN	Azobisisobutironitrilo
AOP	Apnea of prematurity
APTS	3-(Aminopropil)trimetoxisilano
AU	Absorbance units
CapLC	Capillary liquid chromatography
CGA	Chlorogenic acid
CTAC	Cetrimonium chloride
d.i.	Internal diameter
DAD	Diode array detection
DBS	Dried blood spot
DHEP	Bis(2-ethylhexyl) phthalate
di-CQA	dicaffeoylquinic
DLLE	Dispersive liquid-liquid Microextraction
DPX	disposable pipette tip extraction
DSS	Dried saliva spot
DVB	Divinylbenzene
ESI	Electrospray ionization
FD	Fluorescence detector
FDA	Food and drugs administration of USA
FFAP	Polyethylene glycol modified with nitro terephthalic acid
FID	Flame ionization detector
FQA	Feruloylquinic

GC	Gas chromatography
HF-LPME	Hollow-fiber liquid-phase microextraction
HILIC	Hydrophilic interaction liquid chromatography
HLB	Hydrophile-lipophile balance
HS-SPME	Head-space solid-phase microextraction
IgA	Immunoglobulin A
ILs	Ionics liquids
IT-SPME	In-tube solid-phase microextraction
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LLSME	Liqui-liquid-solid microextraction
LOD	Limit of detection
LOQ	Limit of quantification
m/v	Mass/volume
MEPS	Microextraction by packed sorbent
MIP	Molecular imprint polymer
MS	Mass spectrometry
MS/MS	Mass spectrum in tandem
MTEOS	Triethoxymethylsilane
NanoLC	Nano Liquid chromatography
NPs	Nanoparticles
PAHs	Polycyclic aromatic hydrocarbons
PDMS	Polydimethylsiloxane
PEEK	Polyether ether ketone
PEG	Polyethylene glycol

PETE	Polyethylene terephthalate
PS-DVB	Divinylbenzene polystyrene
PTFE	Polytetrafluoroethylene
QToF-MS	Quadrupole time-of-flight mass spectrometry
RAM	Restricted access material
REC	Research ethics committees
RNA	Ribonucleic acid
RSD	Relative standard deviation
SBSE	Stir-bar extraction
SDME	Single-drop microextraction
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
TEOS	Tetraethyl orthosilicate
TQD-MS	Triple quadrupole mass spectrometer
tR	Retention time
TRB-5	Polydimethylsiloxane modified with 5% phenyl groups
TRB-20	Polydimethylsiloxane modified with 20% phenyl groups
TRB-35	Polydimethylsiloxane modified with 35% phenyl groups
TRB-50	Polydimethylsiloxane modified with 50% phenyl groups
ua	Arbitrary units
UE	European Union
UHPLC	Ultra-high pressure liquid chromatography
UPLC	Ultra pressure liquid chromatography
UV-vis	Ultraviolet-visible
v/v	Volume/volume

WWTP	Wastewater treatment plant

A.2 LIST OF FIGURES

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A.4 CONTRIBUTION IN PUBLICATIONS

1. Ponce-Rodríguez, H.D., García-Robles, A.A., Sáenz-González, P., Verdú-Andrés, J. and Campíns-Falcó, P. (2020). On-line in-tube solid phase microextraction coupled to capillary liquid chromatography-diode array detection for the analysis of caffeine and its metabolites in small amounts of biological samples. Journal of Pharmaceutical and Biomedical Analysis, 178, 112914. Impact Factor (JCR 2018): 2.983. Contribution 100 %.

2. Ponce-Rodríguez, H.D., Herráez-Hernández, R., Verdú-Andrés, J. and Campíns-Falcó, P. (2019). Quantitative Analysis of Terpenic Compounds in Microsamples of Resins by Capillary Liquid Chromatography. Molecules, 24, 4068. Impact Factor (JCR 2018): 3.060. Contribution 100 %.

3. Ponce-Rodríguez, H.D., Verdú-Andrés, J., Herráez-Hernández, R. and Campíns-Falcó, P. (2020). Innovations in extractive phases for in-tube solid-phase microextraction coupled to miniaturized liquid chromatography: A critical review. Molecules, 25, 2460. Impact Factor (JCR 2018): 3.060. Contribution 100%.

4. Ponce-Rodríguez, H.D., Verdú-Andrés, J., Herráez-Hernández, R. and Campíns-Falcó, P. (2020). Exploring hand-portable nano-liquid chromatography for in place water analysis: determination of trimethylxanthines as a use case. Science of the Total Environment (In revision). Contribution 100 %.

5. Ponce-Rodríguez, H.D., Verdú-Andrés, J., Herráez-Hernández, R. and Campíns-Falcó, P. (2020). Multi-class analysis of botanical dietary supplements by capillary liquid chromatography. Pending submit. Contribution 100 %.