Journal of Chromatography A, 1218 (2011) 639–646

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Directly suspended droplet microextraction with in injection-port derivatization coupled to gas chromatography–mass spectrometry for the analysis of polyphenols in herbal infusions, fruits and functional foods

Pilar Viñas, Nelson Martínez-Castillo, Natalia Campillo, Manuel Hernández-Córdoba*

Department of Analytical Chemistry, Faculty of Chemistry, University of Murcia, E-30071 Murcia, Spain

ARTICLE INFO

Article history:
Received 26 October 2010
Received in revised form 2 December 2010
Accepted 6 December 2010
Available online 14 December 2010

Keywords:
Directly suspended droplet microextraction
Injection-port derivatization
Gas chromatography–mass spectrometry
Polyphenols
Tea infusions
Juices
Fruits

ABSTRACT

A miniaturized liquid-phase extraction procedure based on directly suspended droplet microextraction is proposed for determining different classes of polyphenols. A derivatization reaction by means of in injection-port reaction with bis(trimethylsilyl)trifluoroacetamide is carried out to convert the polar non-volatile polyphenols into volatile derivatives. The separation and detection is carried out by coupling gas chromatography with mass spectrometry in the selected ion monitoring mode. The procedure uses undecanone, a low density organic solvent, and several factors influencing the extraction, collection efficiency and derivatization reaction are optimized. Excellent linearity was obtained for the range studied (0.05–500 ng mL^{-1}). The limits of detection are between 0.011 and 0.13 ng mL^{-1}, depending on the compound, and the limits of quantification between 0.037 and 0.43 ng mL^{-1}. The sensitivity and detection limits for polyphenols using the DSDME sample pretreatment method were very low. Enrichment factors are between 413 and 578. The recoveries obtained for spiked samples are satisfactory for all the compounds. The coupled miniaturized method is applied to the sensitive determination of both *cis*- and *trans*-resveratrol isomers, piceatannol, catechin, epicatechin, quercetin and fisetin in herbal infusions, fruits, juices and functional foods.

1. Introduction

An emerging field in mass spectrometry (MS) is the on-line coupling of several techniques to MS [1]. Besides the obvious combinations of gas (GC) or liquid chromatography (LC) with MS, others have been developed. Since the traditional ways for sample preparation are time-consuming and require large amounts of reagents [2], miniaturized procedures coupled to on-line GC–MS have several advantages, including [3] high analysis rates with good efficiency, low costs as solvent consumption is extremely low, high repeatability as the number of steps is lower and high sensitivity. Microextraction techniques [4,5] represent a challenge for the miniaturization of analytical laboratory [6], being the selective extraction of analytes based on differences in their physical–chemical characteristics (molecular weight, charge, solubility, polarity and volatility). Numerous microextraction approaches have been developed, depending on the analytes and samples involved [7]. Liquid-phase microextraction (LPME) includes several miniaturized techniques based on the extraction of analytes in a liquid phase using very low amounts of organic solvents [6]. Jeannot and Cantwell [8] developed single-drop microextraction (SDME), in which extraction was achieved into a small drop of a water-immiscible organic solvent. A wide range of solvents are suitable for the purpose. A different approach to LPME is known as directly suspended droplet microextraction (DSDME), in which a symmetrical rotated flow field is created by a stir bar placed on the bottom of a cylindrical sample cell to make a microdroplet of solvent suspended on the top centre of aqueous sample [9].

On the other hand, the relation between food intake and health is based on the nutritious aspects of food. In the field of nutrition, functional foods are defined as those which, while satisfying basic nutritional needs, provide health benefits or reduce the risk of illness [10–12]. The range of functional products containing bioactive ingredients has increased considerably in recent years in the framework of nutrigenome. Different studies have demonstrated the beneficial effects for health of products such as vegetables, fruits, oil, wine, cacao and tea. A typical tea drink is prepared by extracting 1 g of dry leaves in 100 mL of hot water. In the case of green tea, catechins correspond to 20–30% of the dry weight and flavanols 2–3% of the water soluble extract [13]. Functional drinks, such as those enriched with tea extracts, have contributed to the revolution of functional energetic drinks.

* Corresponding author. Tel.: +34 868887406; fax: +34 868884148.
E-mail address: hcordoba@um.es (M. Hernández-Córdoba).

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doi:10.1016/j.chroma.2010.12.026

The beneficial properties of functional products arise from their secondary metabolites, also named phytochemical or phytonutrient substances, which can be grouped as polyphenols, terpenes and sulphur compounds. In turn, polyphenols are a very numerous group of substances which can be classified into several classes according to their basic structure [14]; the most important in terms of their biological activity and contribution to the diet are flavonols, which include catechins, flavanols, such as quercetin and fisetin, stilbenoids, such as resveratrol and piceatannol, isoflavones, phenylpropanoids and benzoic acid derivatives [15].

Polyphenols are generally determined by LC using different detection systems [16]. Very few studies based on GC have been proposed [17–26], as these compounds are non-volatile, and a chemical derivatization step is needed [27,28]. However, the use of GC–MS, which is nowadays accesible to most laboratories provides important advantages because of the combination of the separation capabilities of GC and the power of MS as an identification and confirmation method. Furthermore, because of the interest in clean chemistry procedures, methods for food matrices should be, when possible, based on solvent-free methodologies. In this sense, polyphenols have been determined using miniaturized techniques such as solid-phase microextraction (SPME) [29–36] and liquid–liquid microextraction [37,38].

The present study describes a new miniaturized method for the sensitive determination of both *cis*- and *trans*-resveratrol isomers, piceatannol, catechin, epicatechin, quercetin and fisetin in fruits, herbal infusions and functional foods by coupling DSDME–GC–MS and derivatization in the injection-port with bis(trimethylsilyl)trifluoroacetamide (BSTFA). A comparison with classical methods show advantages as simplicity, speed, low cost, high recovery and use of minimal amounts of toxic organic solvents. As a new small fraction of the solvent is used for each extraction, no memory effects are produced and enrichment factors are high. Compared with other LPME methods, DSDME did not require special equipment, the organic drop is more stable and the equilibrium is reached quickly. In comparison with SPME methods for polyphenols determination, the cost is lower, as the fibres are expensive and have a limited lifetime. Other advantages are the higher sensitivity, the absence of memory effects and the higher analysis rate.

2. Experimental

2.1. Reagents

Analytical reagent grade methanol was purchased from Lab-Scan (Dublin, Ireland). Isooctane, toluene, octanone, undecanone, undecanol and decanol were provided by Aldrich (Steinheim, Germany), and sodium chloride by Fluka (Buchs, Switzerland). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Commercially available polyphenols (>98%) were obtained from Sigma (St. Louis, MO, USA): *trans*-resveratrol, piceatannol, catechin, epicatechin, quercetin and fisetin. Stock solutions (100 mg L^{-1}) were prepared by dissolving the commercial products, without previous purification, in methanol. They were kept at −18 °C in dark bottles sealed with PTFE/silicone caps. Working standard solutions were prepared daily by diluting with methanol. The solution of *cis*-resveratrol was obtained by UV irradiation of the *trans*-isomer using a cracker with UV light for 1 min. The continuous flow system cracker was so effective that total isomerization was achieved after only 1 min of irradiation. The amount of *trans*-resveratrol remaining was not detectable, at least, by UV detection. Consequently, the concentration of *cis*-resveratrol considered for calibration was the same as that for *trans*-resveratrol. This solution was irradiated on the same day of use. The derivatizing reagent was BSTFA, N,O-bis(trimethylsilyl)triflouroacetamide, obtained from Supelco (Bellefonte, PA, USA) and maintained at room temperature in the dark.

Table 1
Retention time and target and qualifier ions for the polyphenols.

Compound	t_R (min)	T	Q_1 (Q_1/T%)	Q_2 (Q_2/T%)	Q_3 (Q_3/T%)
cis-Resveratrol	7.83	444	445 (68)	446 (40)	369 (15)
trans-Resveratrol	8.97	444	445 (60)	446 (50)	369 (10)
Epicatechin	9.13	369	368 (86)	388 (40)	446 (30)
Catechin	9.46	368	369 (78)	446 (60)	388 (50)
Piceatannol	9.56	532	516 (41)	575 (30)	446 (20)
Fisetin	10.67	471	487 (70)	446 (40)	388 (20)
Quercetin	11.40	647	575 (70)	662 (44)	446 (40)

2.2. Instrumentation

GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source and provided with a split–splitless injection port and a liner of single bevel deactivated with an internal volume of 900 μL and 4 mm ID. The mass spectrometer was operated using electron-impact (EI) mode (70 eV). The carrier gas, helium, was maintained at a constant flow of 0.5 mL min^{-1}. A HP-U1MS (100% dimethylpolysiloxane, 30 m × 0.25 mm ID, 0.25 μm thickness) capillary column (Agilent) was used. Injection was carried out in the splitless mode at 240 °C. The GC temperature was programmed as follows: start temperature of 100 °C, increasing to 320 °C at 30 °C min^{-1} and holding for 5 min. The temperatures of the ion source and the transfer line were 230 and 320 °C, respectively. The compounds were quantified in the SIM mode in order to improve the detection limits. Identification was confirmed by the retention time of the target ion and the qualifier-to-target ion ratios for each compound (Table 1).

For extraction and collection procedures, 4 mL amber glass vials sealed with hole-caps and PTFE/silicone septa were used and the solutions were stirred with a magnetic stirrer (IKA RH KT/C, Supelco) using PTFE-coated magnetic stir bars (10 mm × 6 mm OD). To control the extraction temperature, a home made heating system consisting of a drilled block provided with an electronic temperature control system was used. A 10 μL Agilent manual syringe with fitted plunger, fixed needle (needle gauge 26, length 50 mm, tip bevel) was used to collect the floating organic solvent.

A PSA 10570 UV cracker from a PSA Millenium Excalibur continuous flow system (PS Analytical, Orping, UK) was used to obtain the *cis*-resveratrol. An IKA-A11 grinder and an UP 200H ultrasonic probe processor (Dr. Hielscher, Teltow, Germany) were also used to homogenize the samples.

2.3. Samples

Different samples of tea (green, black, red, breakfast, Ceylan and green tea with mallow, anise and elder), camomile and lime blossom were purchased in a supermarket in tea bag format. Isotonic tea-based drinks (with black tea and peach flavor tea), juices (peach, peach and grapes, apple) and fruits (black grape, white grape, apple and pear) were also obtained commercially and stored in glass containers at 4 °C before analyzing. Fresh grapes were analyzed unwashed and with the peel intact. A representative portion of sample (50 g whole fruit) was chopped into small pieces and triturated in an IKA-A11 grinder. The herbal infusions were prepared by introducing the weighed bag (2 g approximately) for 5 min in 80 mL of water (approximately a cup) which had just boiled. After cooling, the solution was made up to 250 mL using a calibrated flask. Recovery experiments were carried out using different type of samples

which were spiked with a standard mixture of polyphenols at two different concentration levels. The samples were treated as indicated above and were allowed to stand at 4 °C for at least half an hour before starting the extraction procedure.

2.4. DSDME–GC–MS procedure

All analyses were performed with 4 mL amber vials containing 200 μL of the infusion or isotonic drink or, alternatively, 500 mg of the juice or homogeneized fruit sample, 0.24 g (6%, m/v) sodium chloride, 1 mL of 0.1 M phosphate buffer (pH 5) and water up to 4 mL. A 10 μL volume of undecanone and a magnetic stir bar (10 mm × 6 mm OD) were introduced in the vial. This was then placed in the home-made heating module previously programmed at 30 °C and maintained under magnetic stirring (1000 rpm) for 20 min. To avoid evaporation of the solvent, glass vials were sealed with hole-caps and PTFE/silicone septa. After this extraction step, the supernatant organic solvent was collected, while stirring to maintain the vortex, with a micropipette and transferred to a 100 μL eppendorf tube. Derivatization of the polyphenols was carried out in the injection port of the GC in the splitless mode at 240 °C. A volume of 2 μL of the derivatizing reagent BSTFA was initially injected and immediately an aliquot of 3 μL of the sample extract was injected to carry out the derivatization reaction. After 30 s the furnace program was started and the derivatized analytes injected into the column. The syringe was methanol-washed several times between injections. Each sampling was performed in duplicate. To avoid contamination between samples, the magnetic bars were washed with methanol after each extraction.

3. Results and discussion

3.1. GC–MS separation

The polyphenols separation conditions were optimized by comparing two different capillary columns coated with non-polar stationary phases, HP-U1MS and HP-U5MS. Satisfactory resolution was achieved for all the compounds using the HP-U1MS column. Several temperature programs were tested using undecanone as the solvent in order to achieve the best separation of the polyphenols in the lowest analysis time. The program selected is summarized in Section 2. The flow of helium gas was varied from 0.5 to 4 mL min^{-1} and an optimal value of 0.5 mL min^{-1} was selected. Table 1 shows the retention times as well as the target and the qualifier ions selected for the seven polyphenols studied under the chromatographic conditions finally used in the SIM mode.

3.2. DSDME optimization

Organic solvents for use in DSDME must have certain chemical characteristics, such as poor solubility in water to prevent dissolution into the aqueous phase, high affinity for the analytes and low volatility to avoid solvent evaporation during extraction. A derivatization step is required to analyze polyphenols, the initial conditions for which were the following: temperature of the injection port 250 °C, BSTFA volume 2 μL, sample volume 2 μL and derivatization time 1 min. The optimal solvent was selected from among several low density organic solvents differing in polarity, including toluene, isooctane, decanol, undecanol, octanone and undecanone. The volume of the extraction solvent was 20 μL and the sample was stirred while the drop was being collected. The results showed (Fig. 1) that best extraction efficiencies were obtained using undecanone for most analytes, which was selected.

The volume of the acceptor phase was optimized as the sensitivity of the method can be increased by decreasing the volume ratio of the acceptor/donor phase. The volume of the organic solvent was varied in the 5–30 μL range and extraction increased up to 10 μL and then continuously decreased for higher volumes for all the analytes due to the dilution effect. Volumes lower than 5 μL were not useful as the floating drop could not be collected. A volume of 10 μL was selected for DSDME.

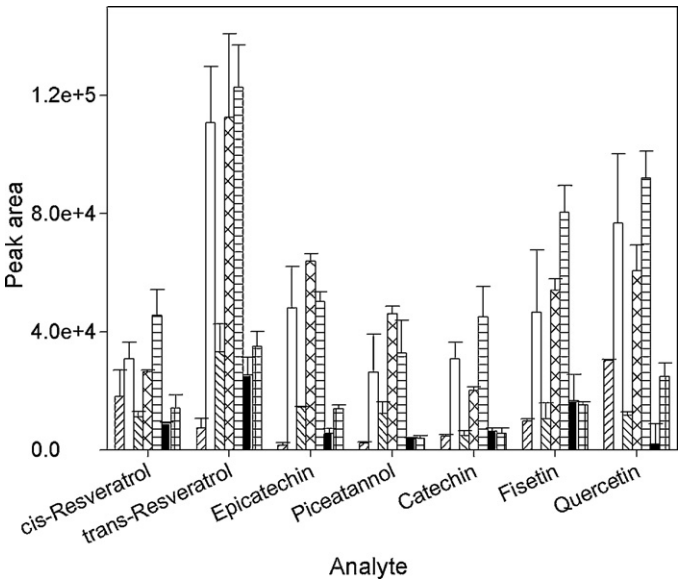

Fig. 1. Selection of extraction solvent for DSDME. Sample volume, 4 mL; organic solvent volume, 20 μL; extraction at 30 °C for 20 min at 1500 rpm. Analyte concentration: *cis*-resveratrol, *trans*-resveratrol, epicatechin, catechin, piceatannol (70 ng mL^{-1}), fisetin and quercetin (150 ng mL^{-1}). Bars correspond to: decanol (▨), undecanone/octanone 1:1 (□), undecanol (▧), octanone (▩), undecanone (▤), isooctane (■) and toluene (◫). Vertical segments correspond to standard deviation (n = 3).

The mass transfer between the aqueous and the organic phases is strongly affected by the temperature and the duration of the extraction step. With this technique, the equilibrium of the compounds between the organic and the aqueous phases is not instantaneous and a compromise time had to be selected to ensure high sample throughput. The diffusion coefficients of the analytes normally increase with temperature, although very high temperatures might decrease extraction due to the solvent evaporation. The effect of the temperature was studied between 25 and 50 °C using a time of 20 min. As shown in Fig. 2A, the extraction efficiency increased up to 35 °C for some polyphenols and decreased for others, while higher temperatures produced a decrease in the signals. A temperature of 30 °C was selected. When the extraction time was varied in the 5–35 min range (Fig. 2B), optimal signals for most analytes were achieved at around 20 min, which was selected to decrease the total analysis time. After this time the signal decreased because at higher extraction times, a portion of the organic solvent drop will be evaporated and/or dissolved in the sample matrix.

The samples were stirred to accelerate the extraction kinetic. An increase in the stirring rate usually increases the extraction efficiency because this facilitates the diffusion of analytes through the liquid interfaces, thus improving the repeatability of the extraction method. Stirring rates in the 0–1700 rpm range were studied. Fig. 2C shows that extraction efficiency increased for most compounds up to a 1000 rpm value, which was selected. However, in DSDME the excessive agitation can produce the break of the drop and its dispersion in the aqueous phase.

The effect of ionic strength on the extraction efficiency has been extensively studied in LPME because the addition of salts can increase the partition coefficient of the compounds to the organic phase, resulting in higher preconcentration. Consequently, the salting-out effect was studied by adding 0–36% (m/v) sodium

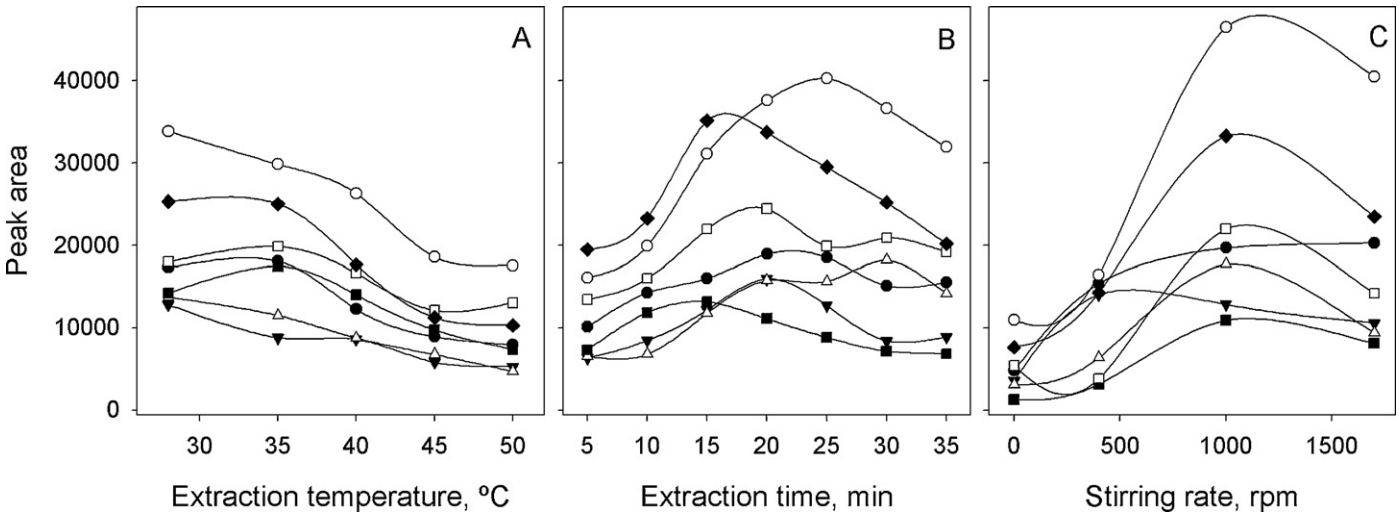

Fig. 2. Influence of the extraction temperature (A), extraction time (B) and stirring rate (C) for DSDME–GC–MS. Sample volume, 4 mL; organic solvent volume, 10 μL. Analyte concentration: *cis*-resveratrol, *trans*-resveratrol, epicatechin, catechin, piceatannol (10 ng mL^{-1}), fisetin and quercetin (50 ng mL^{-1}). Symbols correspond to: *cis*-resveratrol (●), *trans*-resveratrol (○), epicatechin (▼), catechin (■), piceatannol (△), fisetin (□) and quercetin (♦).

chloride. As shown in Fig. 3A, extraction improved for most compounds up to 5–10% (m/v) and then decreased, as the amount of collected solvent decreased. This effect was produced because the high ionic strength can also increase the solubility of the extractant solvent in water. A 6% (m/v) concentration was selected. The pH of the donor aqueous solution can also be adjusted to decrease the solubility of target analytes in the sample solution and to provide efficient transfer into the organic phase. The pH effect was studied in the 2–8 range, using 0.01 M phosphate buffer solutions. Maximal extraction for most polyphenols was obtained for pH 6, except for *cis*- and *trans*-resveratrol which were best extracted at pH 4 (Fig. 3B). Finally, a value of pH 5 was selected.

3.3. Derivatization reaction for polyphenols

Polyphenols are polar compounds and cannot be directly analyzed by GC–MS. Derivatization converts them into non-polar compounds, which are more easily extractable, thermically stable and more volatile [27]. A recent review revised the derivatization reactions in miniaturized techniques [39], which can be carried out in different modes, pre-extraction or simultaneously with microextraction (in-sample derivatization) and post-extraction. Post-extraction derivatization refers to quasi-simultaneous mass transfer and reaction in the same phase but also the injection-port transformation of analytes. The injection-port derivatization of polar analytes at the high temperatures associated with the GC injection port permitted volatile derivatives of the target compounds to be obtained [39]. In-sample derivatization was not possible for this procedure, as the reagent was not compatible with the aqueous solution, for which reason post-extraction injection-port derivatization was used, leading to a simple technique which improves separation, recovery and sensitivity of the analytical method [40].

Silylation is a common procedure for the GC analysis of non-volatile and thermolabile polyphenols [41], trimethylsilyl (TMS) derivatives being more volatile, less polar and more thermostable. During silylation, an active hydrogen is replaced by a trimethylsilyl group [29], although the technique is not without problems because

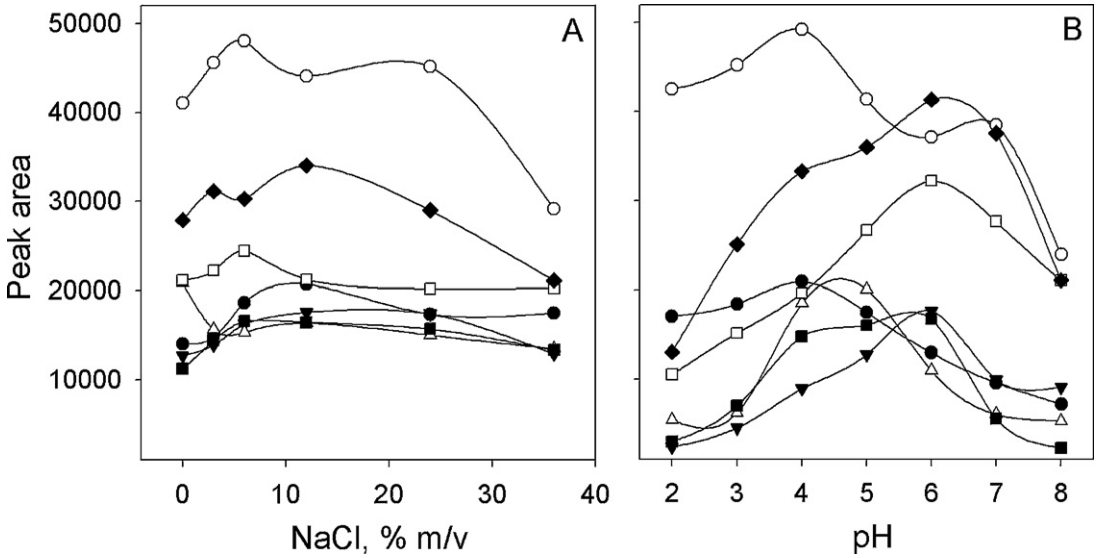

Fig. 3. Influence of the salting-out effect (A) and pH (B) for DSDME–GC–MS. Sample volume, 4 mL; organic solvent volume, 10 μL. Analyte concentration: *cis*-resveratrol, *trans*-resveratrol, epicatechin, catechin, piceatannol (10 ng mL^{-1}), fisetin and quercetin (50 ng mL^{-1}). Symbols correspond to: *cis*-resveratrol (●), *trans*-resveratrol (○), epicatechin (▼), catechin (■), piceatannol (△), fisetin (□) and quercetin (♦).

Table 2
Analytical data for polyphenol standards using the DSDME–GC–MS method/.

Compound	Linearity ($ng\,mL^{-1}$)	r^2	DL ($ng\,mL^{-1}$)	QL ($ng\,mL^{-1}$)	EF
cis-Resveratrol	0.5–200	0.9991	0.11	0.36	541
trans-Resveratrol	0.05–200	0.9987	0.011	0.037	413
Epicatechin	0.1–300	0.9998	0.040	0.13	430
Catechin	0.5–300	0.9900	0.059	0.20	550
Piceatannol	0.1–200	0.9932	0.027	0.09	578
Fisetin	0.5–500	0.9978	0.13	0.43	503
Quercetin	0.5–500	0.9980	0.061	0.20	552

Table 3
Slopes ($mL\,ng^{-1}$) of standard addition calibration graphs using DSDME–GC–MS.

Compound	Aqueous	Tea infusion	Red grape	Apple
cis-Resveratrol	920 ± 15	940 ± 30	903 ± 26	884 ± 42
trans-Resveratrol	5650 ± 120	5605 ± 176	5638 ± 153	5330 ± 261
Epicatechin	1927 ± 17	1915 ± 95	1746 ± 16	1799 ± 88
Catechin	1148 ± 53	1130 ± 67	1074 ± 61	1100 ± 76
Piceatannol	2183 ± 104	2172 ± 133	2120 ± 80	2081 ± 103
Fisetin	883 ± 29	849 ± 29	861 ± 17	832 ± 44
Quercetin	1290 ± 33	1177 ± 84	1253 ± 28	1239 ± 39

the reagents and the silylmethylated derivatives are hydrolyzed in aqueous solutions. To avoid this, derivatization was carried out in the injection port of the GC, after extraction. The analytes were just derivatized by exposure to the reagent gaseous phase, thus minimizing contact between the TMS derivatives and the aqueous sample matrix. The reagent was BSTFA. Several variables were examined to determine their influence on the silylation process. Thus, different injection modes (split, splitless and pulsed splitless) were tested and best recoveries for most analytes were obtained in the splitless mode, which was selected. The order of injection between the reagent and analyte in the port was also tested, and sensitivity was seen to be greater when the BSTFA was injected first, followed by the analytes.

When the temperature in the injection port was varied between 160 and 280 °C, extraction efficiency increased up to 240 °C for most analytes (Fig. 4A). The derivatization time was varied between 0 and 60 s and optimal efficiencies were obtained at 30 s (Fig. 4B). The optimal ratio BSTFA volume/extracting volume was also considered by testing the values 1/1, 1/2, 2/2 and 2/3 μL and maximal efficiency was obtained when 2 μL of BSTFA and 3 μL of the extractant undecanone containing the analytes were used.

3.4. Method performance

The method was validated for its linearity, detection and quantification limits, selectivity, accuracy and precision. Calibration curves using DSDME–GC–MS were obtained by least-squares linear regression analysis of the peak area versus analyte concentration using six concentration levels in duplicate. Quantification was performed by the external standard procedure. The validation parameters, range of linearity and the correlation coefficients for the polyphenols are shown in Table 2. Excellent linearity ($r^2 > 0.99$) was obtained for the range studied. The limits of detection (DL, calculated as three times the signal-to-noise ratio) and the limits of quantification (QL, calculated as ten times the signal-to-noise ratio) are included in Table 2. It can be seen from the data that the sensitivity and detection limits for polyphenols using the DSDME sample pretreatment method were very low. The enrichment factor (EF) was calculated as the ratio between the analyte concentration in the floating organic phase after extraction and the initial concentration of analyte in the aqueous solution; values between 413 and 578 were obtained.

The selectivity of the method was judged from the absence of interfering peaks at the elution times of the polyphenols for blank chromatograms of different unspiked samples. No matrix compounds existed that might give a false positive signal in the blank samples.

3.5. Matrix effect and recovery study

The response of the detector system to target analytes may be affected by the presence of co-extractives from the sample. The matrix effect for the polyphenols was evaluated by comparing the slopes of aqueous standards and standard additions calibration graphs for different samples, obtained by plotting concentration (at six levels) against peak area and following linear regression analysis. Table 3 shows that the slopes were very similar for all the samples. A statistical study was carried out to compare the slope values of the different samples and the aqueous standards and an

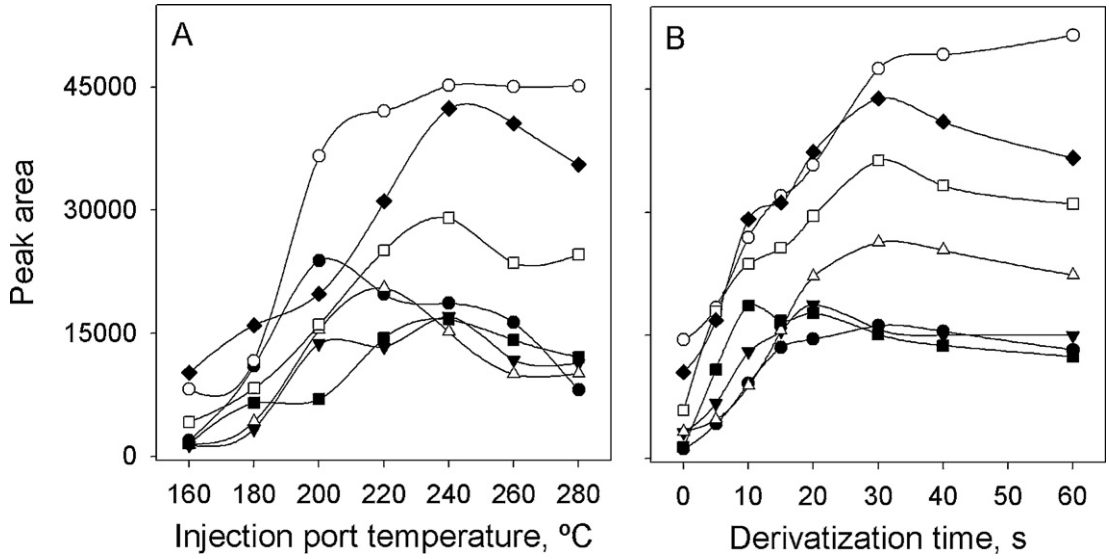

Fig. 4. Influence of the in injection port-derivatization temperature (A) and the derivatization time (B) in DSDME–GC–MS. Sample volume, 4 mL; organic solvent volume, 10 μL. Analyte concentration: *cis*-resveratrol, *trans*-resveratrol, epicatechin, catechin, piceatannol (10 $ng\,mL^{-1}$), fisetin and quercetin (50 $ng\,mL^{-1}$). Symbols correspond to: *cis*-resveratrol (●), *trans*-resveratrol (○), epicatechin (▼), catechin (■), piceatannol (△), fisetin (□) and quercetin (♦).

Table 4
Polyphenol contents in infusions, functional drinks, juices and fruits.

Sample	*trans*-Resveratrol	*cis*-Resveratrol	Piceatannol	Catechin	Epicatechin	Quercetin	Fisetin
Black tea[a] 1	56 ± 5	6 ± 0.5	14 ± 1	214 ± 19	238 ± 29	1075 ± 23	109 ± 10
Black tea[a] 2	51 ± 5	24 ± 4	53 ± 6	329 ± 21	174 ± 17	945 ± 38	124 ± 8
Red tea[a] 1	60 ± 6	26 ± 6	34 ± 2	141 ± 19	208 ± 4	920 ± 66	320 ± 29
Red tea[a] 2	44 ± 6	7 ± 0.5	40 ± 3	120 ± 11	228 ± 15	799 ± 40	78 ± 5
Green tea[a] 1	76 ± 9	25 ± 3	53 ± 3	324 ± 23	252 ± 22	1533 ± 37	539 ± 17
Green tea[a] 2	64 ± 5	10 ± 1	14 ± 2	98 ± 5	204 ± 12	904 ± 28	183 ± 11
Breakfast tea[a]	43 ± 4	10 ± 1	36 ± 3	93 ± 9	304 ± 10	1003 ± 13	160 ± 5
Ceylan tea[a]	36 ± 4	8 ± 1	49 ± 4	147 ± 6	153 ± 9	781 ± 29	174 ± 12
Lime blossom[a]	48 ± 9	18 ± 2	68 ± 2	111 ± 6	196 ± 12	581 ± 74	39 ± 4
Camomile[a]	45 ± 4	63 ± 3	11 ± 0.2	99 ± 5	125 ± 6	555 ± 10	28 ± 3
Black tea drink[b]	116 ± 11	39 ± 2	48 ± 1	146 ± 26	223 ± 9	568 ± 13	105 ± 7
Peach flavor tea drink[b]	167 ± 6	25 ± 2	23 ± 2	220 ± 17	146 ± 34	630 ± 24	139 ± 5
Apple juice[b]	62 ± 5	11 ± 2	15 ± 3	130 ± 18	69 ± 4	48 ± 5	ND
Peach/grape juice[b]	50 ± 6	13 ± 2	36 ± 5	149 ± 12	292 ± 19	167 ± 19	43 ± 3
Peach juice[b]	29 ± 3	11 ± 3	14 ± 4	55 ± 3	26 ± 4	43 ± 6	19 ± 2
Apple[c]	ND	ND	ND	10 ± 2	7 ± 1	61 ± 5	ND
Pear[c]	ND	ND	ND	16 ± 1	19 ± 1	51 ± 5	ND
Red grape[c]	1639 ± 71	405 ± 31	374 ± 16	260 ± 8	497 ± 38	392 ± 23	255 ± 15
White grape[c]	239 ± 24	82 ± 12	43 ± 5	132 ± 12	212 ± 21	191 ± 27	176 ± 8

Values are mean ± standard deviation ($n = 3$). ND means not detected.
[a] Concentrations given in $\mu g\,g^{-1}$.
[b] Concentrations given in $ng\,mL^{-1}$.
[c] Concentrations given in $ng\,g^{-1}$.

analysis of variance (ANOVA) showed that there were no statistically significant differences. Quantification can therefore be carried out using aqueous standards.

To check the repeatability of the method, ten replicate analyses of a polyphenol mixture were performed at two levels of 1 and 100 $ng\,mL^{-1}$ for each compound, with an RSD of <10% being obtained in all cases. These values indicate that the precision of the method was satisfactory for control analysis. In order to check the accuracy of the proposed method, a recovery study was carried out by fortifying two samples (a functional drink and a fruit) at two different concentration levels. The recoveries of the polyphenols from spiked samples varied from 81 to 116% with an average recovery ± SD ($n = 56$) of 99 ± 6. The similarity in recoveries obtained for each polyphenol in the different samples corroborates the absence of a matrix effect. The precision of the method was calculated by evaluating ten consecutive analyses of the above fortified samples, obtaining values for the relative standard deviations ranging between 1.2 and 10%.

3.6. Analysis of samples

Different infusions, fruits and functional foods were analyzed under the optimized conditions. Fig. 5A shows a typical chromatogram as well as the ions corresponding to each peak obtained by DSDME–GC–MS in SIM mode for a green tea infusion sample under the selected conditions. Similar chromatograms were obtained for the other samples. The profiles demonstrated the absence of interfering peaks at the retention times for the analytes. The polyphenols in the samples were identified by comparing the retention time, identifying the target (T) and qualifier ions (Q) and comparing the qualifier-to-target ratios (Q/T %) of the peaks in both the sample and the standards solution. The average values for the retention times of polyphenols pointed to very good agreement between the retention data obtained in the different samples. The T and Q abundances were determined by injecting individual standards under the same chromatographic conditions, except in full scan mode. The Q/T percentage was determined by dividing the abundance of the selected qualifier ion by the target ion (see Table 1). Fig. 5B shows the mass spectra, which confirmed the identity.

After identification of the peaks, different samples of infusions (green tea, black tea, red tea, green tea with mallow, anise and elder, breakfast tea, Ceylan tea, camomile and lime blossom), two isotonic tea-based functional drinks (with black tea and peach flavor tea), fruit juices (apple, peach, peach and grapes) and fresh fruits (pear, apple, red grape and white grape) were analyzed using the DSDME–GC–MS procedure. All samples were analyzed in triplicate. Table 4 shows the results obtained. As expected, higher levels were recorded for all the polyphenols in tea infusions and grapes than in other samples. The content of flavanols and flavonoids was higher than that of stilbenoids. Of the stilbenoids, the levels of piceatannol were lower than those of resveratrol, while the isomer *trans*-resveratrol was found in higher amounts than the *cis*-resveratrol. Among the flavonoids, the levels of catechin and epicatechin were similar and both were lower than the levels of quercetin, which was found in very high concentrations. Lower levels were found for the flavonol fisetin. The polyphenols levels found in the literature vary widely because they depend of many factors such as sample preparation form, sample origin, stage of ripeness, post-harvesting conservation and processing, and the climate or light conditions [42].

3.6.1. Comparison of the proposed DSDME procedure with other extraction techniques

The determination of polyphenols by DSDME offers a new sample pre-treatment method which combines sampling, extraction and preconcentration. Compared with classical extraction methods it has the advantages of simplicity of handling, speed, low cost, high recovery and the use of minimal amounts of toxic organic solvents. As a new solvent fraction of only a few microlitres is used for each extraction, no memory effect exists and high enrichment factors are achieved. However, the main disadvantage of the method is the limitation in the selection of the extracting solvent. Compared with other LPME methods, DSDME does not require special equipment or supporting material for the drop such as hollow fibre or syringe needle. It is more flexible as regards the solvent volume and stirring speed, there is no problems due to drop instability and the equilibrium is reached more quickly. Compared with previous methods for quantifying polyphenols using SPME the proposed method is cheaper, since the SPME fibres are expensive and have a limited life time. Additional advantages are the

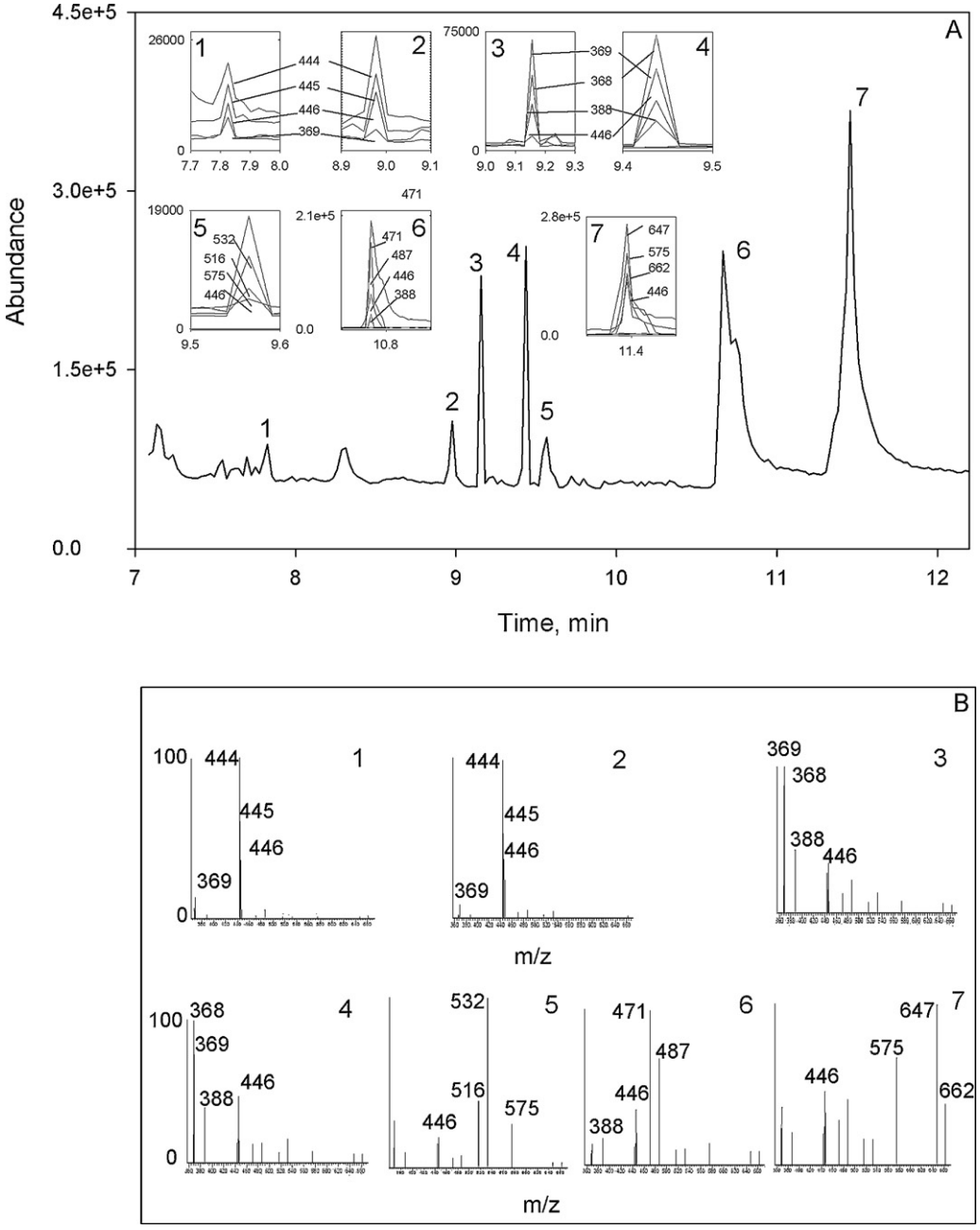

Fig. 5. (A) Elution profile obtained for a green tea infusion sample by DSDME–GC–MS showing the extracted ions for each peak. Peaks correspond to: 1, *cis*-resveratrol; 2, *trans*-resveratrol; 3, epicatechin; 4, catechin; 5, piceatannol; 6, fisetin and 7, quercetin. (B) Mass spectra of compounds.

absence of cross-memory effects and the fact the analyses take less time.

4. Conclusion

The miniaturized analytical method here described allows an excellent sensitivity to be obtained for the determination of some of the main bioactive ingredients of the vegetable and fruit products used for the elaboration of functional foods. Very low quantities of solvent are needed, the procedure resulting environmentally friendly with the advantage of an easy sample handling.

Acknowledgements

The authors are grateful to the Spanish MICINN (Project CTQ2009-08267) for financial support. N.M.-C. acknowledges a fellowship from Departamento de Formación del Personal Académico de la Universidad Centroccidental Lisandro Alvarado (Venezuela).

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