Journal of Chromatography A, 1342 (2014) 44–53

Contents lists available at ScienceDirect

Journal of Chromatography A

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

High-throughput method based on quick, easy, cheap, effective, rugged and safe followed by liquid chromatography-multi-wavelength detection for the quantification of multiclass polyphenols in wines

Ariel R. Fontana*, Rubén Bottini

Laboratorio de Bioquímica Vegetal, Instituto de Biología Agrícola de Mendoza, Consejo Nacional de Investigaciones Científicas y Técnicas-Universidad Nacional de Cuyo, Almirante Brown 500, M5528AHB Chacras de Coria, Argentina

ARTICLE INFO

Article history:
Received 20 January 2014
Received in revised form 13 March 2014
Accepted 16 March 2014
Available online 25 March 2014

Keywords:
Polyphenols
Wine
QuEChERS
Dispersive-solid phase extraction
Sample preparation
Liquid chromatography-multi-wavelength detection

ABSTRACT

In this work, a reliable, simple, fast, inexpensive and robust sample preparation approach for the determination of multiclass polyphenols in wine samples is proposed. The polyphenols selected for this work were gallic acid, (+)-catechin, (−)-epicatechin, caffeic acid, syringic acid, coumaric acid, ferulic acid, *trans*-resveratrol, quercetin and cinnamic acid. The method is based on QuEChERS (quick, easy, cheap, effective, rugged and safe) extraction technique coupled with dispersive solid-phase extraction (d-SPE) clean-up. Under optimized conditions, the analytes were extracted from 5 mL wine samples (previously acidified with 1% formic acid) using 2.5 mL acetonitrile. For phase separation, 1.5 g NaCl and 4 g anhydrous $MgSO_4$ were added. Then, a 1 mL aliquot of the partitioned supernatant was cleaned-up using d-SPE with a combination of 150 mg $CaCl_2$, 50 mg primary-secondary amine (PSA) and 50 mg C_{18} as sorbents. A 250 μL aliquot of the obtained cleaned extract was concentrated to dryness and taken up with the initial mobile phase previous to liquid chromatography-multi-wavelength detection (LC-MWD). The proposed method provided limits of detection (LODs) ranging from 0.004 to 0.079 $\mu g\,mL^{-1}$ and an inter-day variability below 12% RSD for all analytes in red and white wine samples. Considering external calibration (red wines) and matrix-matched calibration (white wines) as quantification techniques, the overall recoveries (accuracy) of the method ranged between 75.0% and 119.6% for red and white wine samples, respectively. The developed method was applied for the determination of polyphenols in 10 wines produced in Argentina. Nine phenolic compounds were determined, at concentrations above detectable levels in the method. The maximum concentrations corresponded to (−)-epicatechin in white wines, while gallic acid and (+)-catechin were the most abundant in red wines.

1. Introduction

Because of the increase of consumers' interest that functional foods have acquired in recent years, there is need for the chemical identification of the characteristics and quality of food products. Phenolic composition is one of the most important quality parameters of wines, contributing to several organoleptic attributes such as bitterness, astringency, color, flavor, odor, and oxidative stability [1,2]. Polyphenols have also known health-promoting effects and other properties in different biological systems such as antioxidant, anti-inflammatory, anticarcinogenic, antimutagenic and antiproliferative activities [3]. In addition, a reduction in cardiovascular mortality in human populations has been associated to the regular consumption of red wine ("French paradox") [4]. These features are related to the antioxidant characteristics of polyphenols as reducing agents (i.e. by donating hydrogen they quench free radicals like singlet oxygen) that inhibit and/or delay oxidation of biomolecules (mainly lipids) in diverse cell-systems. Wine, especially red wine, may be an important source of polyphenols in the diet. In this sense, due to the reported health benefits of polyphenols and their impact on food quality, the development of analytical methodologies for characterization and determination of them in wine samples is a topic of increasing interest.

Because of the complexity of wines samples, which includes high quantity of target analytes of different chemical nature,

* Corresponding author. Tel.: +54 261 4135010x1228; fax: +54 2614960469.
E-mail addresses: afontana@mendoza-conicet.gob.ar, fontana_ariel@yahoo.com.ar (A.R. Fontana).

http://dx.doi.org/10.1016/j.chroma.2014.03.044
0021-9673/

sample preparation has a critical role for the highly selective determination of polyphenols. The most commonly used techniques for the determination of free phenolics are based on liquid chromatography (LC) coupled to different detectors such as UV–Vis, multi-wavelength (MWD), fluorescence, electrochemical and mass spectrometry (MS) [5–8]. Gas chromatography coupled to MS has been also reported, although low-volatile polar compounds, such as phenolics, exhibit low sensitivity and peak tailing. Therefore, the determination of these compounds by GC–MS requires a derivatization step, which sometimes is tedious and time consuming [5]. In the last decade, the development of ultra-high pressure pump systems and sub-2 μm packing materials has allowed significant improvement in the separation, speed and efficiency of modern LC. The innovative ultra-high pressure or UHPLC has made it possible to achieve 5- to 10-fold faster separations than with conventional LC systems, while maintaining or increasing resolution [7]. In the same way, diverse sample preparation strategies have been proposed for polyphenols, being liquid–liquid extraction (LLE) and solid-phase extraction (SPE) the most frequently used [5,9]. Although these techniques are effective for the extraction of polyphenols from wine samples, they comprise several steps that are time-consuming and require high volumes of organic solvents. In fact, LLE involve extensive additional clean-up procedures and solvent evaporation steps. In recent years, new sample preparation strategies such as SPE with several sorbents [10,11], micro-extraction by packed sorbents [12] and solid-phase micro-extraction [8] have been proposed for the isolation of low molecular weight polyphenols from wines and other food matrices. Recently, Anastassiades et al. [13] proposed the QuEChERS (quick, easy, cheap, effective, rugged and safe) extraction technique for the analysis of multi-residual pesticides in fruits and vegetables, which afterwards was applied to several analytes and matrices avoiding some drawbacks of the afore-mentioned techniques. This procedure involves an initial single phase extraction of compounds of interest with acetonitrile (MeCN) followed by salting-out extraction/partitioning step by adding a combination of salts. Subsequently, the clean-up is performed using dispersive-solid-phase extraction (d-SPE), which is based on the addition of the sorbent material into an aliquot of the extract to remove the matrix interferences. The d-SPE clean-up avoids passing the extracts through SPE cartridges, requiring much smaller quantities of sorbent and solvent. The principal advantages of QuEChERS method are its simplicity, repeatability, low cost, speed and wide applicability to different type of samples and analytes. QuEChERS has found interesting applications in different fields of analytical chemistry including the analysis of pesticides in foodstuffs [14–17]. QuEChERS was also successfully applied to determine analytes such as steroids and veterinary drugs [18], mycotoxins [19], pharmaceuticals [20], personal care products [21] and organic pollutants [22] in different samples with satisfactory results in terms of recovery of compounds. Silva et al. [23] reported an application of QuEChERS method to extract low molecular weight polyphenols from vegetables, providing a valuable and promising tool for quality evaluation of these foodstuffs.

The objective of this work was to develop and validate a simple, fast, inexpensive and robust method for the determination of 10 polyphenols representatives of different chemical classes (phenolic acids, flavanols, flavonols and stilbenes) in wines based on a QuEChERS method coupled to LC-MWD. Sample preparation conditions were optimized in order to maximize the yield and selectivity of extraction process. The analytical performance of the proposed QuEChERS-LC-MWD method was evaluated in terms of limits of detection (LODs), absolute recoveries, precision and linear range of work. Matrix effects (ME) and selectivity were also carefully evaluated to achieve unambiguous quantification of the studied compounds. The procedure was applied for the determination of target polyphenols in samples of commercial wine from Argentina, in order to establish the robustness of QuEChERS-LC-MWD method.

2. Experimental

2.1. Standards, solvents and sorbents

Standards of gallic acid (99%), (+)-catechin (≥99%), (−)-epicatechin (≥95%), caffeic acid (99%), syringic acid (≥95%), coumaric acid (99%), ferulic acid (≥99%), *trans*-resveratrol (≥99%), quercetin hydrate (95%) and cinnamic acid (99%) were purchased from Sigma-Aldrich (Steinheim, Germany). The selection of the mentioned polyphenols was based on their relative abundance and importance for wines quality. As well, the selected compounds cover the main polyphenols classes including phenolic acids, flavonols, flavanols and stilbenes. The chemical structures of these compounds and some properties of relevance to optimize extraction (QuEChERS) and LC separation processes are compiled in Table 1. Stock solutions of the above polyphenols were prepared in methanol (MeOH) at concentration levels of 1000 μg mL^{-1}. Further dilutions were prepared monthly in methanol and stored in brown bottles at −20 °C. Calibration standards used during optimization of LC-MWD conditions were dissolved in ultrapure water (0.1% formic acid; FA) water/methanol (80:20).

HPLC-grade MeCN, MeOH, FA and acetic acid were purchased from Mallinckrodt Baker (Inc. Pillispsburg, NJ, USA). Ultrapure water was obtained from a Milli-*Q* system (Millipore, Billerica, MA, USA).

Analytical grade sorbents (50 μm particle size) for d-SPE, including primary-secondary amine (PSA) and octadecylsilane (C_{18}) were both obtained from Waters (Milford, MA, USA). Reagent grade NaCl, anhydrous $MgSO_4$ and anhydrous $CaCl_2$ for QuEChERS development were purchased from Sigma-Aldrich.

2.2. Samples

Extraction conditions were optimized with aliquots of a pool of red wines (Malbec and Cabernet Sauvignon), considered as the most complex sample, spiked with target analytes at 5 μg mL^{-1}. Wine samples studied in this work were obtained from local supermarkets and wineries. The analyzed samples included different white and red wines produced in Argentina. The white wine samples corresponded to a Chardonnay varietal and a blend without varietal denomination. Red wines were the varietals Malbec, Cabernet Sauvignon, Tempranillo and two blends with varietal denomination as follows: Cabernet Sauvignon–Merlot–Malbec and Malbec–Cabernet Sauvignon. The selected samples included young wines as well as oak barrel aged wines, with the aim to test the proposed method with a wide range of matrices.

2.3. QuEChERS procedure

Wine (5 mL) was placed into a 15 mL PTFE centrifuge tube and acidified with FA (1%) by adding 57 μL of 88% w/v solution. Then, 2.5 mL MeCN were added and the tube was vigorously hand-shaken for 30 s to ensure adequate homogenization of sample and extraction solvent. For phase separation, 1.5 g of NaCl and 4 g of $MgSO_4$ were added; the tubes were shaken for 1 min and centrifuged for 10 min at 3000 rpm (900 rcf). Thereafter, 1 mL aliquot of the upper MeCN phase was transferred to a 2 mL d-SPE clean-up tube containing 150 mg $CaCl_2$, 50 mg PSA and 50 mg C_{18}. The mixture was then vortexed 30 s and centrifuged 2 min at 12,000 rpm (8400 rcf). Finally, an aliquot of 250 μL of extract was evaporated to dryness under gentle N_2 stream and the residue was reconstituted with

Table 1
Names, structures, chemical class and p*K*a of selected polyphenols.

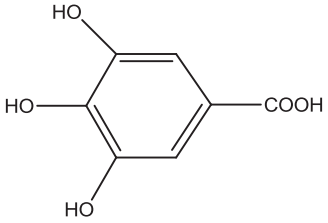
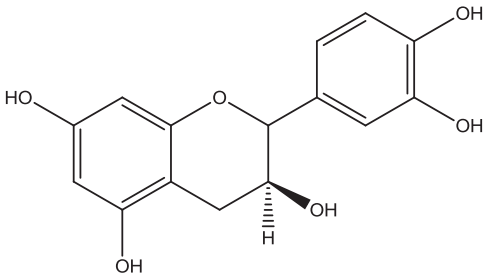
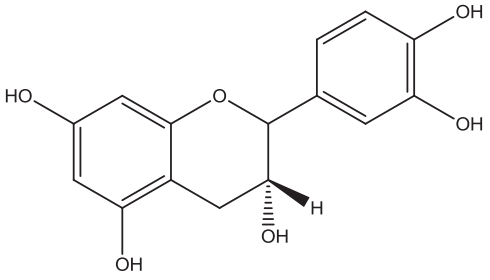
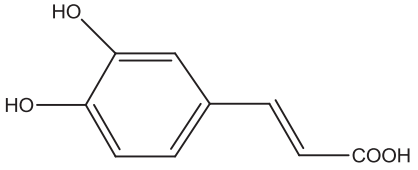
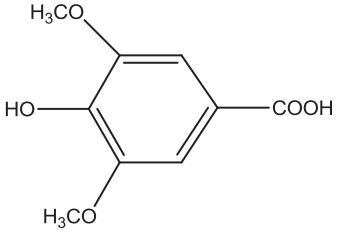
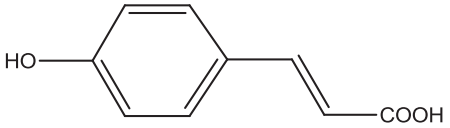
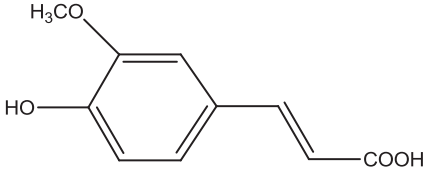
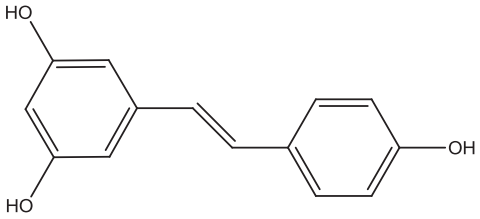
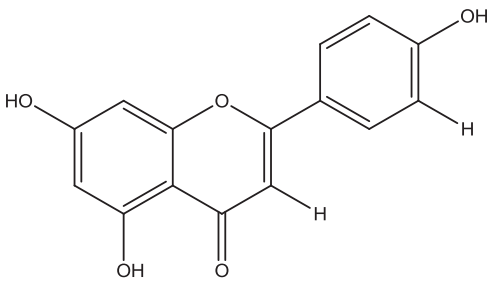
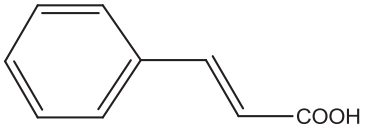
Analyte	Chemical structure	Chemical class	pKa_1	pKa_2
Gallic acid		Hydroxybenzoic acid	4.5	10.0
(+)-Catechin		Flavanol	8.7	9.7
(−)-Epicatechin		Flavanol	8.9	9.9
Caffeic acid		Hydroxycinnamic acid	4.4	–
Syringic acid		Hydroxycinnamic acid	4.3	–
p-Coumaric acid		Hydroxycinnamic acid	4.1	10.2
Ferulic acid		Hydroxycinnamic acid	4.0	10.2

Table 1 (*Continued*)

Analyte	Chemical structure	Chemical class	pKa_1	pKa_2
trans-Resveratrol		Stilbene	9.2	–
Quercetin		Flavonol	7.1	9.1
Cinnamic acid		Hydroxycinnamic acid	4.4	–

400 μL of LC initial mobile phase (0.1% FA in Milli-*Q* water and 20% of MeOH) and analyzed by LC-MWD.

2.4. LC-MWD analysis

Target polyphenols were determined using a LC-MWD system (Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany). The LC instrument was a Dionex Ultimate 3000 consisting of vacuum degasser unit, autosampler, quaternary pump and chromatographic oven. The detector was a Dionex MWD-3000 (RS) model with an analytical flow cell. The Chromeleon 7.1 software was used to control all the acquisition parameters of the LC-MWD system and also to process the obtained data.

LC separations were carried out in a reversed-phase Symmetry C_{18} column (4.6 mm × 250 mm, 5 μm particle size (Waters, Milford, MA, USA). Ultrapure water with 0.1% FA (A) and MeOH (B) were used as mobile phases. Analytes were separated using the following gradient: 0 min, 20% B; 0–2 min, 30% B; 2–8 min, 32% B; 8–14 min, 80% B; 14–22 min, 20% B; 22–32 min, 20% B. The mobile phase flow was 1 mL min^{-1} and the column temperature 35 °C. The injection volume for standards and sample extracts was 10 μL. The identification and quantification of the target polyphenols in the wine samples studied was based on the comparison of the retention times (t_R) and maximum absorbance value of detected peaks in samples of interest with those obtained by the injection of pure standards. The t_R and wavelength used for quantification of each compound are overviewed in Table 2.

2.5. Matrix effects, absolute recoveries and sample quantification

Potential ME in % for each compound caused by interferences occurring during LC-MWD analysis were calculated as follows:

$$ME\% = \left(1 - \frac{\text{slope MM curve}}{\text{slope solvent curve}}\right) \times 100$$

being MM the matrix-matched calibration standards. If we assume that the slopes of the solvent calibration curve and the matrix-matched curve are equal, then no ME is present and the slope ratio is 1.

Sample quantification was performed and compared by using calibration standards involving both matrix-matching (standards added to blank extracts) and non-matrix-matching (solvent-based standards in solutions of initial mobile phase). For matrix-matching, the sample extract was fortified with standards after d-SPE clean-up (we compared adding the standard either before or after d-SPE without observing significant differences in results).

The absolute recoveries (*R%*) of the proposed method were calculated as the difference between the concentrations measured for extracts from spiked (C_s) and non-spiked aliquots (C_b) of wine divided by the theoretical concentration (C_t) added to the sample, and multiplied by 100,

$$R\% = \left[\frac{C_s - C_b}{C_t}\right] \times 100$$

where C_s and C_b were established against calibration curves obtained for matrix-matched standards (white wine) and external calibration with solvent-based standards (red wine).

3. Results and discussion

The proposed technique includes a two-step sample preparation approach viz. an extraction based on LLE followed by a salting-out process and a clean-up using d-SPE. Sample preparation strategies should be designed and tested to minimize ME. This can be beneficial for determining the zones of the chromatograms that are severely affected by wine co-extractives helping to identify the best sample clean-up approach to eliminate interferences. In this sense, the critical variables affecting the extraction efficiency and selectivity of the technique were optimized.

Table 2
Linearity, limits of detection (LODs), recoveries, intra- and inter-day precision of the QuEChERS-LC-MWD method for the determination of multiclass polyphenols in white and red wines.

Analyte	t_R (min)	λ (nm)	Linear range (μg mL^{-1})	R^2	LOD (μg mL^{-1})	Precision (RSD, %)				Recovery (%)[c]			
						Intra-day[a]		Inter-day[b]		White wine		Red wine	
						White wine	Red wine	White wine	Red wine	1 μg mL^{-1}	10 μg mL^{-1}	5 μg mL^{-1}	25 μg mL^{-1}
Gallic acid	3.59	280	0.1–50	0.9992	0.010	3.7	3.5	7.4	2.3	75.7	75.9	87.6	75.5
(+)-Catechin	5.28	280	0.5–50	0.9961	0.055	2.0	0.9	8.9	8.9	113.6	115.6	108.4	94.8
(−)-Epicatechin	7.11	280	0.5–50	0.9989	0.079	7.2	1.1	8.3	6.8	77.4	119.6	87.5	75.3
Caffeic acid	8.03	320	0.1–50	0.9994	0.010	1.8	3.0	8.9	4.4	77.5	92.0	96	111.4
Syringic acid	8.49	280	0.1–50	0.9992	0.008	3.5	2.5	2.9	5.9	103.7	122.7	82.8	115.9
p-Coumaric acid	12.01	320	0.1–50	0.9994	0.008	3.8	2.5	2.6	3.8	114.4	106.3	89.6	119.3
Ferulic acid	12.57	320	0.2–50	0.9995	0.017	2.9	2.4	11.6	6.9	116.0	98.9	103.1	115.1
trans-Resveratrol	13.89	320	0.1–50	0.9991	0.005	2.4	2.0	2.4	4.4	114.5	115.4	116.6	112.3
Quercetin	15.48	370	0.5–50	0.9995	0.031	4.2	4.5	8.3	5.5	75.6	117.4	75.0	110.1
Cinnamic acid	16.07	280	0.1–50	0.9997	0.004	3.0	2.8	3.2	8.7	119.5	95.2	105.4	105.8

[a] $n = 3$ extractions in the same day (1 μg mL^{-1}).
[b] $n = 9$ extractions in 3 consecutive days (2 μg mL^{-1}).
[c] Recoveries were calculated as described in Section 2.5. $n = 3$ replicates.

3.1. Optimization of QuEChERS extraction conditions

The original QuEChERS technique is characterized by a single phase solvent extraction using polar organic solvents and phase separation after salting out and centrifuging the mixture [13]. Among the most commonly extraction solvents used for sample preparation, MeCN is the solvent of choice for a flexible QuEChERS method due to its distinct properties which were deeply explained in previous works [13,24].

The sample to solvent ratio was studied with the objective to achieve the highest recoveries with the minimum sample and solvent consumption, as well as to get the required sensitivity for polyphenols in wines. To determine the influence of extraction solvent volume, a series of separate sets of extractions were performed using 5 mL of red wine with different MeCN volumes (1.5, 2.5, 3.5 and 5 mL). The results are summarized in Fig. 1. As it can be observed, the best results for the 10 studied polyphenols were achieved when 2.5 mL of MeCN were used. Lower volumes rendered lower recoveries of analytes, probably because the reduced volumetric recovery of extraction phase when 1.5 mL were used, being insufficient to quantitatively extract the target analytes. The volumetric recovery of extraction phase for volumes between 2.5 and 5 mL was 73%, about twice the obtained for 1.5 mL MeCN (33%). For MeCN volumes of 3.5 and 5 mL, lower recoveries due to dilution

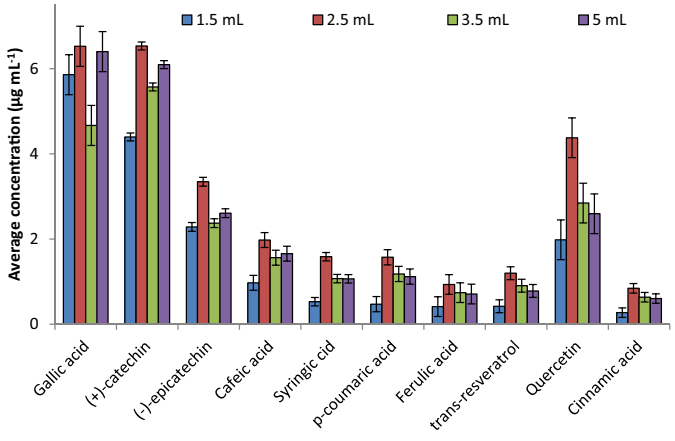


Fig. 1. Effect of sample to solvent ratio on the average concentration of polyphenols. Extraction conditions as described in text. d-SPE: 1 mL extract, 150 mg anhydrous $CaCl_2$, 100 mg PSA and 100 mg C_{18}.

effect of polyphenols were observed. Thus, taking into account the achieved results a sample to solvent ratio of 2:1 was selected to perform further assays.

The acidification of samples prior to extraction is a commonly used strategy in QuEChERS methods to increase the extraction of acidic analytes. In general, low molecular weight polyphenols are more stable at lower pH. The acidification helps to prevent oxidation of polyphenols and increase the efficiency of phenolics extraction since, under these conditions, the phenol-phenolate equilibrium shifts toward the less polar phenol form and phenolic acids are mostly neutral molecules, facilitating the extraction with MeCN [25]. The possibility of acidification of wine samples was evaluated by studying the effect of FA and acetic acid addition. A set of extractions was performed by adding 2.5 mL MeCN to 5 mL acidified wine (1% FA, 1% acetic acid or without acid addition). The obtained results showed that by using any of both acids a remarkable increase in recoveries was observed. These results could be justified according to the p*K*a's of studied polyphenols (see Table 1). For phenolic acids (p*K*a's between 4.1 and 4.5), the observed increases in recoveries where the highest (between 75% and 96%, as compared to non-acidified samples), followed by quercetin, (+)-catechin and (−)-epicatechin which have higher p*K*a's and so they were less affected by lowering the pH. For *trans*-resveratrol no significant differences in recoveries were observed with and without acidification. This could be explained due to a higher p*K*a, so the acidification does not exert strong effect on the compound ionization and therefore the extraction efficiency remained unaffected. Besides the recoveries for some target polyphenols, such as (+)-catechin, (−)-epicatechin, *p*-coumaric acid, ferulic acid, *trans*-resveratrol, quercetin and cinnamic acid were not affected by the type of acid. On the contrary, gallic, caffeic and syringic acids showed an appreciable higher recovery when 1% FA was used. Thus, FA was selected as acidification agent.

The percentage of acidification with FA was studied by extracting 5 mL of wine sample acidified with different amounts of FA (1 and 2.5%). The results are shown in Fig. 2. The pH was measured in the extract after the salting-out step (1) and after d-SPE clean-up (2). The achieved pH values were: pH without FA, (1) pH 3, (2) pH 5; acidification with 1% FA, (1) pH 2, (2) pH 2; acidification with 2.5% FA, (1) pH 2, (2) pH 1.5. As can be seen, when samples were not acidified the phenolic acids presented a significant reduction in their recoveries. Observing the pH value obtained after d-SPE in samples without FA (pH 5), this value is above the p*K*a's of phenolic acids (see Table 1). Thus, the equilibrium of these compounds

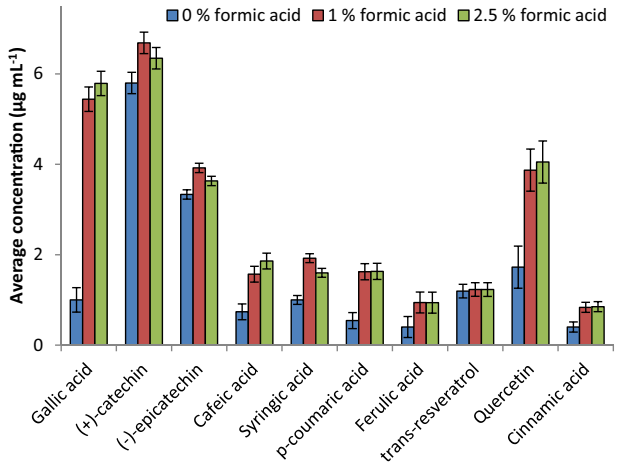


Fig. 2. Effect of acidification with FA on the average concentration of polyphenols. Extraction conditions as described in Fig. 1.

is not completely shifted to its neutral form, which could be easily extracted with MeCN, justifying the lower recoveries without acidification. There is also an effect related to the PSA properties because PSA strongly retains the acidic analytes, unless there is sufficient acid to saturate the sorbent, blocking the –OH interactions of the analytes with the PSA. On the other hand, when samples where acidified with FA no significant differences were observed between both levels. The acidification performed in wine samples allows the achievement of a pH well below the p*K*a's of studied analytes (in the extracts and after d-SPE), increasing the neutral form of phenolics as well as saturating the PSA (actually PSA cannot retain phenolics) which augment the recovery of target analytes. In this sense, wine samples were acidified with 1% FA prior to its extraction with 2.5 mL MeCN.

3.2. Optimization of d-SPE clean-up

After the salting-out extraction, an additional efficient clean-up of sample extract using d-SPE is often a crucial step of the traditional QuEChERS approach. This is essential, particularly for complex MeCN extracts coming from wine samples, in order to reduce the content of sugars and pigments (anthocyanin) that negatively affect the identification of polyphenols. For the clean-up optimization, the effects of sorbent type and composition of sorbent mixture on the purification efficiency and extraction recovery were evaluated, and the highest sorbent amounts for maximizing clean-up effectiveness without affecting the recoveries were selected for this purpose.

For the development of d-SPE, PSA and C_{18} were evaluated alone and in different combinations. The achieved results showed that the use of a combination of $CaCl_2$, PSA and C_{18} give the best results in terms of the recoveries of analytes. As well, a reduction in the chromatograms background, particularly near the *trans*-resveratrol peak, was observed by using this combination of sorbents. The utilization of $CaCl_2$ without PSA and C_{18} showed the lowest recoveries for the polyphenols. In the same way, the application of a combination of $CaCl_2$ + PSA or C_{18} separately, did not show improvement in the recoveries of analytes. This could be explained as follows: PSA sorbent, as a weak anion exchanger, is commonly used to remove various co-extractive interferences due to its remarkable trapping activity for fatty acids, some sugars, acidic analytes and anthocyanin pigments (the last were abundant in the MeCN extract before d-SPE) [13,24]. Considering the pH value of d-SPE extract under optimized conditions (pH 2), the amount of acid was sufficient to saturate the PSA sorbent, allowing the partition of neutral analytes to MeCN. Anhydrous $CaCl_2$ retains the remaining water after the salting-out step and increase the ionic strength of the medium, so these facets changes the adsorption capacity of PSA and C_{18} to the analytes and matrix components. In fact, the lower amounts of water and the high ionic strength favor the partition of neutral analytes (phenolic acids are well below their p*K*a's) to MeCN phase. In this sense, a combination of sorbents containing PSA and C_{18}, plus $CaCl_2$ were used instead of individual sorbents for the removal of various types of interfering matrix components in the MeCN extracts. The amounts of sorbents in d-SPE were studied within the range of 35–200 mg of each, maintaining the $CaCl_2$ amount in a fixed mass of 150 mg. This study is relevant to achieve an adequate clean-up without affecting the recovery of target polyphenols. The results are presented in Fig. 3. The procedure was the same as described above. It was observed that by increasing the sorbent amount of each sorbent from 35 mg to 50 mg, the relative responses for most of the analytes increased. This may be because the high amount of anthocyanins present in the extract, which may affect negatively the PSA sorbent efficiency. In this sense, Anastassiades et al. [13] reported that for strawberry extracts, which were rich in anthocyanins, higher amounts of PSA were needed to avoid saturation of the PSA. When 100 mg d-SPE sorbents were used, (+)-catechin, (−)-epicatechin and caffeic acid showed an increase (about 10%) of their relative responses, although the majority of the analytes responses decreased with higher amounts of d-SPE sorbents. It could be that for a higher amount of PSA, the remaining acid was insufficient to saturate PSA, so acidic analytes were able to interact with the sorbent, being strongly retained and reducing the recovery. Higher amounts of PSA also increase the pH of the medium, thus acids are in their ionic forms at pHs well above their p*K*a's and the ionic strength from the $CaCl_2$ was not enough to force the ions into MeCN. In view of the mentioned results, we selected a compromised situation to achieve a satisfactory recovery of analytes and avoid using excessive amount of sorbents. Thus, a combination of 150 mg anhydrous $CaCl_2$, 50 mg PSA and 50 mg C_{18} was selected for further studies.

3.3. Method validation

In order to evaluate the ME on the analytical signals of polyphenols, the slopes of the calibration graph obtained with matrix-matched standards were compared with those obtained with solvent-based standards, calculating the matrix to solvent slope ratios as described in Section 2.5 for each of the analytes studied in white and red wine matrices. According to established parameters, previously developed and accepted for pesticide analysis, ME values from −20 to +20% are considered suitable indicating minor ME [26]. Often, this ±20% range is used as a cutoff value to justify using solvent calibration in place of matrix-matched standards. To test the developed method, a comparison of the percent of compounds that fell within ±20% of the solvent curve values was performed. The obtained results for red wine showed that 100% of evaluated analytes showed a minimum ME (−12% for *trans*-resveratrol–+3% for (−)-epicatechin), showing the efficiency of the proposed QuEChERS approach and supporting the use of external calibration as quantification technique. For white wines, about 30% of the investigated analytes (viz. (−)-epicatechin, ferulic acid and quercetin) showed a significant ME (ME%: +22 for (−)-epicatechin–+29% for quercetin). In order to finally test the possibility of quantification of polyphenols in red wines by using external calibration, a comparison between the concentrations determined by using calibration standards involving both matrix-matching (standards added to sample extracts) and non-matrix-matching (solvent-based standards) was performed. The attained results showed that there were not significant differences between the quantification with both methods,

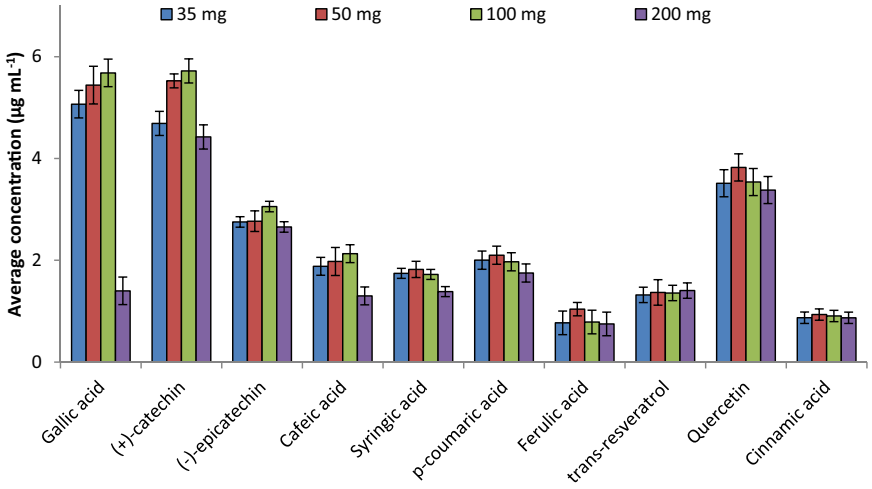


Fig. 3. Effect of d-SPE sorbents amount on the average concentration of polyphenols. Extraction conditions as described in Fig. 2. d-SPE: 150 mg $CaCl_2$ + PSA and C_{18} in amounts showed in the figure.

justifying the application of external calibration as quantification technique for red wines. For white wines, the differences were statistically significant, and matrix-matched calibration should be used to achieve accurate quantification of the target analytes. For both calibration curves, linear ranges between 0.1 and 50 μg mL^{-1} were obtained, with coefficient of determination (R^2) higher than 0.9961 for all the studied polyphenols (see Table 2).

Based on the obtained results, in this study external calibration using standards prepared in solvent (initial mobile phase: 0.1% FA in Milli-Q water and 20% of MeOH) was used to quantify the 10 polyphenols in red wines. On the other hand, in order to compensate the errors associated with the observed interferences in white wines, matrix-matched standards were used as calibration technique to quantify the analytes in these samples.

The analytical figures of merit of the optimized method are summarized in Table 2. The LODs of the analytes for extraction of 5 mL wine sample, calculated as three times the signal-to-noise ratio ($S/N = 3$), were ranged between 0.004 for cinnamic acid to 0.08 μg mL^{-1} for (−)-epicatechin. The achieved LODs showed that the proposed QuEChERS-LC-MWD method shows a suitable sensitivity according to the polyphenols levels commonly found in wines. The limits of quantification (LOQs), calculated as ten times the signal-to-noise ratio ($S/N = 10$) were 0.03, 0.18, 0.26, 0.03, 0.03, 0.03, 0.06, 0.02, 0.10, 0.02 μg mL^{-1} for gallic acid, (+)-catechin, (−)-epicatechin, caffeic acid, syringic acid, coumaric acid, ferulic acid, *trans*-resveratrol, quercetin and cinnamic acid, respectively.

The selectivity of the QuEChERS method for the determination of polyphenols was evaluated by the comparison of t_R and spectral behavior achieved by analyzing a standard solution of polyphenols and a QuEChERS extract of wine after applying the optimized method (see Section 3.1). As can be observed from Fig. 4, the t_R obtained after analyzing a wine sample did not show significant differences with the obtained for the standard as well as any interference was detected at the polyphenols t_R.

The precision was evaluated through inter-day (reproducibility) and intra-day (repeatability) studies, calculated using the measurement of relative peak area of each polyphenol in 5 mL aliquots of the pooled matrices of red and white samples. Intra-day precision was evaluated by analyzing in the same day 3 replicates of wine samples spiked with polyphenols at 1 μg mL^{-1} level. The obtained RSDs were ranged between 0.9% ((+)-catechin, red wine) and 7.2% ((−)-epicatechin, white wine). The inter-day precision was assessed with 5 mL aliquots of the pooled matrices, spiked at the 2 μg mL^{-1} level and processed in triplicate during 3 consecutive days. The calculated RSDs were lower than 11.6% for all compounds. Table 2 overviews the intra- and inter-day precision data.

The absolute recoveries (*R*%) of the overall procedure, considered as an estimation of the accuracy, were assessed using red and white wines spiked at two different concentration levels. The obtained results are summarized in Table 2. In all cases, spiked and non-spiked aliquots were processed in triplicate and the concentrations of polyphenols in the corresponding extracts determined by external calibration (red wine) and matrix-matched calibration (white wine). The obtained *R*% ranged between 75.0% and 119.6% with associated standard deviations between 1.5% and 8.1%.

3.4. Samples analysis

The developed and validated QuEChERS-LC-MWD method was applied for the determination of polyphenols to a total of 10 samples of white (2 specimens) and red (8 specimens) wines from different grape varieties cultivated in Argentina. The levels of polyphenols in the analyzed samples are summarized in Table 3. Fig. 4(b and c) shows the chromatograms obtained for the same red wine sample with and without addition of the studied polyphenols. As can be observed, good peak shape and resolution were achieved for all the compounds with low interference from wine matrix. According to the wavelength, the complexity of chromatograms increase or decrease, showing the importance of adequate selection of maximum absorbance value of each polyphenols when quantification is performed (see Table 2). The number of polyphenols detected and its concentration varied according to the type of matrix: white wines reported a considerable lower quantity and concentration of studied analytes. One polyphenol (cinnamic acid) remained below the LOD of the method in all the processed samples. In white wines was possible to quantify 8 compounds, being (−)-epicatechin the most abundant with a maximum concentration of 44.5 μg mL^{-1}. On the other hand, red wines contained measurable levels of 9 polyphenols. The total concentration of target polyphenols in red wines was approximately two-fold higher than for white wines (102.8 vs. 51.2 μg mL^{-1}, considering the maximum levels for each one).

Gallic acid and (+)-catechin were the most abundant compounds in the analyzed red wine samples with concentrations between 14.0 and 30.5 μg mL^{-1}, followed by (−)-epicatechin, caffeic acid

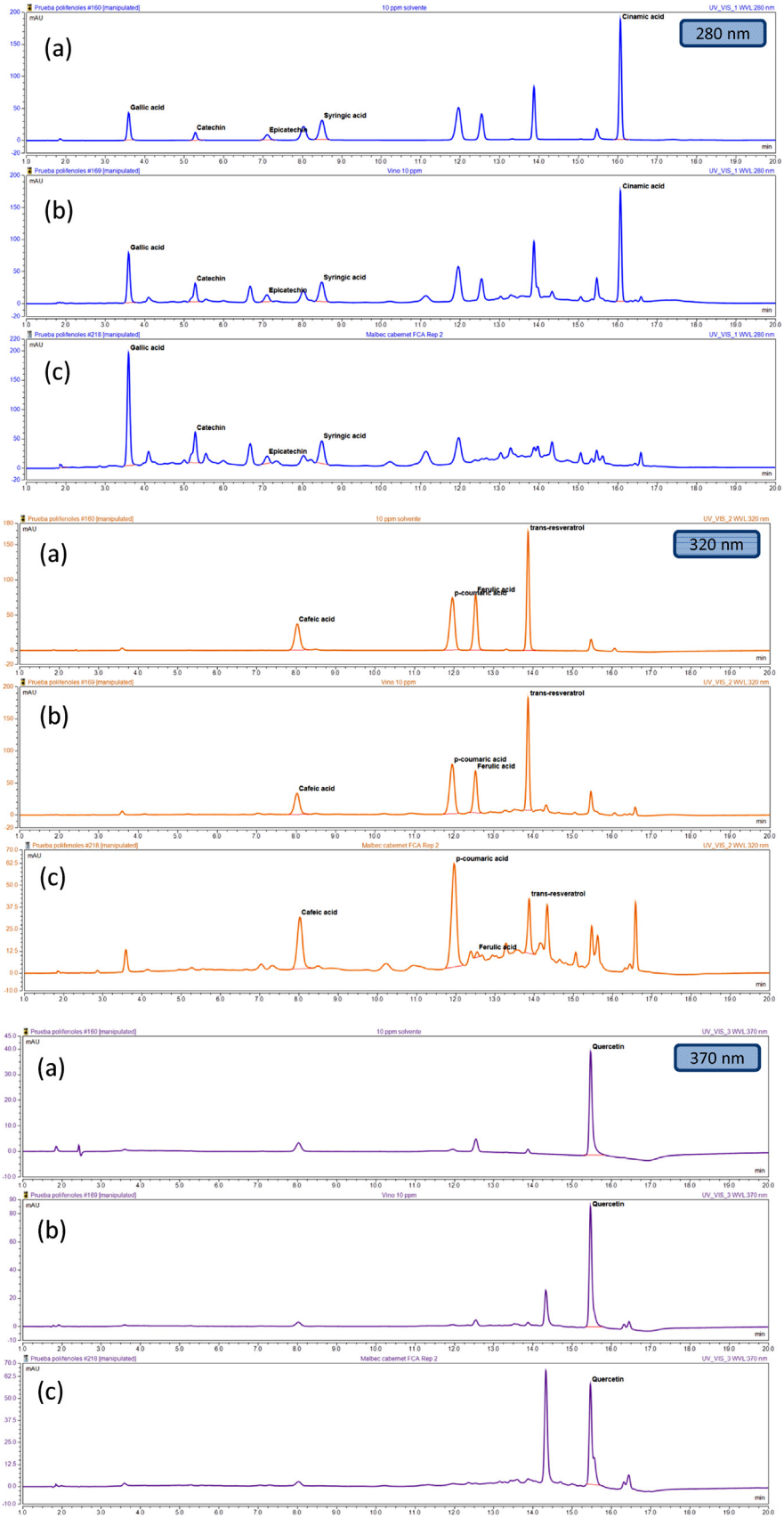


Fig. 4. Extracted chromatograms of each detection wavelength. (a) Solvent standard at 10 μg mL^{-1}; (b) QuEChERS extract of a red wine sample (Code R2) spiked with 10 μg mL^{-1} of standards of each polyphenol; (c) QuEChERS extract of a red wine sample (Code R2) without addition of standards.

Table 3
Levels of target polyphenols in non-spiked wine samples. Average concentrations ($\mu g\,mL^{-1}$) with their standard deviations, $n = 3$ replicates.

Code	W1	W2	R1	R2	R3	R4	R5	R6	R7	R8
Grape varieties	n.i.	Chardonnay	Cabernet Sauvignon	Malbec–Cabernet Sauvignon	Malbec	Malbec	Malbec–Cabernet Sauvignon	Malbec	Tempranillo	Cabernet Sauvignon–Merlot–Malbec
Gallic acid	0.71 ± 0.03	0.72 ± 0.01	17.4 ± 1.8	24.3 ± 2.3	21.2 ± 0.4	29.1 ± 0.3	22.0 ± 3.4	30.5 ± 1.9	27.7 ± 4.2	21.6 ± 1.6
(+)-Catechin	3.2 ± 0.2	6.82 ± 0.01	24.5 ± 3.8	24.5 ± 1.2	26.7 ± 0.9	21.2 ± 0.9	29.00 ± 0.06	23.8 ± 0.6	14.0 ± 1.9	27.4 ± 0.6
(−)-Epicatechin	44.5 ± 2.5	33.0 ± 0.6	12.2 ± 1.4	10.0 ± 0.3	14.5 ± 0.4	9.9 ± 0.4	19.7 ± 0.6	14.9 ± 1.9	7.9 ± 1.2	16.1 ± 0.8
Caffeic acid	1.9 ± 0.1	0.58 ± 0.02	4.4 ± 0.4	5.6 ± 0.4	6.1 ± 0.2	7.5 ± 1.3	6.1 ± 0.4	4.74 ± 0.02	8.0 ± 1.0	6.8 ± 0.4
Syringic acid	0.16 ± 0.01	0.45 ± 0.02	0.56 ± 0.01	1.37 ± 0.03	0.94 ± 0.01	10.7 ± 0.4	7.3 ± 0.2	5.77 ± 0.03	6.6 ± 0.7	6.05 ± 0.07
p-Coumaric acid	0.46 ± 0.04	2.76 ± 0.01	4.13 ± 0.05	7.5 ± 0.1	9.4 ± 0.2	5.6 ± 0.4	9.12 ± 0.04	3.5 ± 0.9	6.8 ± 0.6	8.69 ± 0.08
Ferulic acid	0.14 ± 0.01	0.20 ± 0.02	<LOQ	0.54 ± 0.01	0.37 ± 0.05	0.6 ± 0.2	0.65 ± 0.01	2.4 ± 0.2	0.49 ± 0.06	0.65 ± 0.01
trans-Resveratrol	0.16 ± 0.03	0.41 ± 0.15	1.74 ± 0.02	2.10 ± 0.01	3.37 ± 0.03	2.6 ± 0.2	2.5 ± 0.1	1.2 ± 0.2	2.03 ± 0.08	2.20 ± 0.03
Quercetin	<LOD	<LOD	7.3 ± 0.2	4.2 ± 0.2	5.5 ± 0.2	5.4 ± 0.22	6.40 ± 0.06	6.22 ± 1.08	5.8 ± 0.5	4.52 ± 0.06
Total conc. ($\mu g\,mL^{-1}$)	51.2	44.7	72.2	80.3	88.1	92.7	102.8	93.18	79.4	94.0

n.i., no information.
Codes W and R correspond to white and red wines, respectively.
<LOD, below the limit of detection of the method.
<LOQ, below the limit of quantification of the method (minimum level of calibration curve).
Cinnamic acid was not included in the table because it was not detected in the analyzed samples.

and *p*-coumaric acid. The stilbene *trans*-resveratrol reported levels between 1.2 and 3.4 $\mu g\,mL^{-1}$. For any of the studied samples, a characteristic profile for polyphenols was observed. However a large number of samples should be analyzed to assess varietal characterization of each wine. Besides, other factors involved in winemaking processes, as well as genetic, vineyard conduction and environmental conditions, may affect the phenolic composition of the final product [27].

4. Conclusions

The QuEChERS-LC-MWD developed method allows the selective determination of studied polyphenols in red and white wine samples, showing a good enough sensitivity to guarantee reliable determination at levels commonly found in wines, suitable precision and linear response ranges. As well, satisfactory robustness of the method was observed when the recovery study was performed over different white and red wine samples.

The extraction and clean-up procedures are very simple and require little sample preparation, providing adequate clean-up to wine extracts and allowing increasing the sample throughput of the method.

Data obtained in this research indicate that ME in red wines were avoided, allowing the use of external calibration as quantification technique and simplifying the routine determination of compounds. For white wines, the results showed the possibility of using matrix-matched standards as quantification technique.

All these results disclose that QuEChERS-LC-MWD is a simple, robust and reliable method for the sensitive quantification of multiclass polyphenols in wines and could be successfully applied for the determination of these bioactive compounds in wine samples with different matrices in a routine work scale.

Acknowledgments

The authors are grateful for funds provided by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) (PICT 2008-1666, PAE-PID 2007-0149) and SECTyP-UNCuyo to R.B. A.F. and R.B. are fellows of CONICET.

References

[1] M. Atanacković, A. Petrović, S. Jović, L.G. Bukarica, M. Bursać, J. Cvejić, Food Chem. 131 (2012) 513.
[2] A.S. Arribas, M. Martínez-Fernández, M. Chicharro, Trends Anal. Chem. 34 (2012) 78.
[3] M. Šeruga, I. Novak, L. Jakobek, Food Chem. 124 (2011) 1208.
[4] S. Renaud, M. De Lorgeril, Lancet 339 (1992) 1523.
[5] E. de Rijke, P. Out, W.M.A. Niessen, F. Ariese, C. Gooijer, U.A.T. Brinkman, J. Chromatogr. A 1112 (2006) 31.
[6] K.M. Kalili, A. De Villiers, J. Sep. Sci. 34 (2011) 854.
[7] M.-J. Motilva, A. Serra, A. Macià, J. Chromatogr. A 1292 (2013) 66.
[8] P. Viñas, N. Campillo, N. Martínez-Castillo, M. Hernández-Córdoba, J. Chromatogr. A 1216 (2009) 1279.
[9] K. Pyrzynska, M. Biesaga, Trends Anal. Chem. 28 (2009) 893.
[10] S.N.N.S. Hashim, L.J. Schwarz, R.I. Boysen, Y. Yang, B. Danylec, M.T.W. Hearn, J. Chromatogr. A 1313 (2013) 284.
[11] C.L. Silva, J. Pereira, V.G. Wouter, C. Giró, J.S. Câmara, Talanta 86 (2011) 82.
[12] J. Gonçalves, B. Mendes, C.L. Silva, J.S. Câmara, J. Chromatogr. A 1229 (2012) 13.
[13] M. Anastassiades, S.J. Lehotay, D. Stajnbaher, F.J. Schenck, J. AOAC Int. 86 (2003) 412.
[14] P. Payá, M. Anastassiades, D. Mack, I. Sigalova, B. Tasdelen, J. Oliva, A. Barba, Anal. Bioanal. Chem. 389 (2007) 1697.
[15] A. Lozano, Ł. Rajski, N. Belmonte-Valles, A. Uclés, S. Uclés, M. Mezcua, A.R. Fernández-Alba, J. Chromatogr. A 1268 (2012) 109.
[16] F. Dong, X. Chen, X. Liu, J. Xu, Y. Li, W. Shan, Y. Zheng, J. Chromatogr. A 1262 (2012) 98.
[17] I.R. Pizzutti, A. de Kok, C. Dickow Cardoso, B. Reichert, M. de Kroon, W. Wind, L. Weber Righi, R. Caiel da Silva, J. Chromatogr. A 1251 (2012) 16.
[18] M.V. Salvia, E. Vulliet, L. Wiest, R. Baudot, C. Cren-Olivé, J. Chromatogr. A 1245 (2012) 122.
[19] N. Arroyo-Manzanares, A.M. García-Campaña, L. Gámiz-Gracia, J. Chromatogr. A 1282 (2013) 11.
[20] W. Peysson, E. Vulliet, J. Chromatogr. A 1290 (2013) 46.

[21] V. Homem, J. Avelino Silva, C. Cunha, A. Alves, L. Santos, J. Sep. Sci. 36 (2013) 2176.
[22] H.R. Norli, A. Christiansen, E. Deribe, J. Chromatogr. A 1218 (2011) 7234.
[23] C.L. Silva, N. Haesen, J.S. Câmara, J. Chromatogr. A 1260 (2012) 154.
[24] B. Guo, Z. Huang, M. Wang, X. Wang, Y. Zhang, B. Chen, Y. Li, H. Yan, S. Yao, J. Chromatogr. A 1217 (2010) 4796.
[25] V. Ivanova, M. Stefova, B. Vojnoski, A. Dörnyei, L. Márk, V. Dimovska, T. Stafilov, F. Kilár, Food Res. Int. 44 (2011) 2851.
[26] C. Ferrer, A. Lozano, A. Agüera, A.J. Girón, A.R. Fernández-Alba, J. Chromatogr. A 1218 (2011) 7634.
[27] M. Anastasiadi, N.G. Chorianopoulos, G.J.E. Nychas, S.A. Karoutounian, J. Agric. Food Chem. 57 (2009) 457.