Analytica Chimica Acta 739 (2012) 89–98

View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Repositório Digital da Universidade da Madeira

Analytica Chimica Acta

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

A sensitive microextraction by packed sorbent-based methodology combined with ultra-high pressure liquid chromatography as a powerful technique for analysis of biologically active flavonols in wines

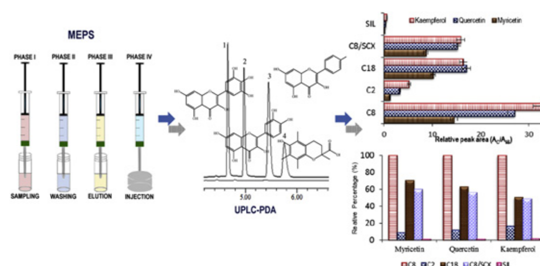
Catarina L. Silva, João L. Gonçalves, José S. Câmara*

CQM/UMa, Centro de Química da Madeira, Centro de Ciências Exactas e da Engenharia da Universidade da Madeira, Campus Universitário da Penteada, 9000-390 Funchal, Portugal

HIGHLIGHTS

- ► An innovative methodology to pre-concentrate bioactive flavonols, MEPS.
- ► Rapid screening for analysis of biological active flavonols in wines.
- ► MEPS reduced the sample volume and the time necessary for the analysis.
- ► Limits the consumption of organic solvents thus also reducing the testing cost.
- ► Good results were obtained in terms of selectivity, precision, sensitivity and accuracy.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 31 March 2012
Received in revised form 8 June 2012
Accepted 11 June 2012
Available online 19 June 2012

Keywords:
Wines
Flavonols
Microextraction by packed sorbent
Solid phase extraction
Ultra high pressure liquid chromatography

ABSTRACT

A new approach based on microextraction by packed sorbent (MEPS) and reversed-phase high-throughput ultra high pressure liquid chromatography (UHPLC) method that uses a gradient elution and diode array detection to quantitate three biologically active flavonols in wines, myricetin, quercetin, and kaempferol, is described. In addition to performing routine experiments to establish the validity of the assay to internationally accepted criteria (selectivity, linearity, sensitivity, precision, accuracy), experiments are included to assess the effect of the important experimental parameters such as the type of sorbent material (C2, C8, C18, SIL, and C8/SCX), number of extraction cycles (extract-discard), elution volume, sample volume, and ethanol content, on the MEPS performance. The optimal conditions of MEPS extraction were obtained using C8 sorbent and small sample volumes (250 μL) in five extraction cycle and in a short time period (about 5 min for the entire sample preparation step). Under optimized conditions, excellent linearity ($R^2_{values} > 0.9963$), limits of detection of 0.006 μg mL^{-1} (quercetin) to 0.013 μg mL^{-1} (myricetin) and precision within 0.5–3.1% were observed for the target flavonols. The average recoveries of myricetin, quercetin and kaempferol for real samples were 83.0–97.7% with relative standard deviation (RSD, %) lower than 1.6%. The results obtained showed that the most abundant flavonol in the analyzed samples was myricetin (5.8 ± 3.7 μg mL^{-1}). Quercetin (0.97 ± 0.41 μg mL^{-1}) and kaempferol (0.66 ± 0.24 μg mL^{-1}) were found in a lower concentration.

* Corresponding author. Tel.: +351 291705112; fax: +351 291705149.
E-mail address: jsc@uma.pt (J.S. Câmara).

0003-2670/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aca.2012.06.020

The optimized $MEPS_{C8}$ method was compared with a reverse-phase solid-phase extraction (SPE) procedure using as sorbent a macroporous copolymer made from a balanced ratio of two monomers, the lipophilic divinylbenzene and the hydrophilic N-vinylpyrrolidone (Oasis HLB) were used as reference. $MEPS_{C8}$ approach offers an attractive alternative for analysis of flavonols in wines, providing a number of advantages including highest extraction efficiency (from 85.9 ± 0.9% to 92.1 ± 0.5%) in the shortest extraction time with low solvent consumption, fast sample throughput, more environmentally friendly and easy to perform.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Flavonols are recognized as a subclass of flavonoids, distributed ubiquitously in the plant kingdom, from which the most common are kaempferol, quercetin and myricetin. The formation of flavonols myricetin, quercetin and kaempferol (Fig. 1) in grapes has been reported to occur through the phenylpropanoid metabolism [1]. Specifically, naringenin is transformed into quercetin and kaempferol by means of the enzyme flavanone 3β-hydroxylase (FHT), which leads to the production of dihydroflavonols [1–3]. Regarding myricetin, although it is also produced through the phenylpropanoid metabolism, its synthesis occurs in general terms further in the pathway [4–6].

These molecules are present in the grape mainly in the monoglycoside form, with the sugar residue linked to the hydroxyl group in position C-3 of the O-containing ring, but substitution can also occur at the 5′,7′,4′,3′, and 5′-positions [7]. The glycoside flavonols kaempferol, quercetin, and myricetin (Fig. 1) form co-pigments with anthocyanins (in red wines); together with oxidation products of tannins, are in the main responsible for the color of white grapes and wines.

Flavonoids have long been recognized to exhibit anti-inflammatory, antioxidant, anti-allergic, hepatoprotective, anti-thrombotic, anti-viral, anti-carcinogenic activities and immune modulating functions, among others [8–11]. The flavonoids also act as potent metal chelators and free radical scavengers and are powerful chain-breaking antioxidants [12], and control protein oxidation and advanced glycation end products (AGEs) formation [13]. By acting as free radical scavengers, flavonoids inhibit lipid peroxidation that can initiate LDL oxidation, a contributing factor to the development of atherosclerosis [14–19]. Epidemiological studies have shown that there is an inverse association between the intake of flavonols and flavones and the risk of coronary heart disease [20–22], stroke [23] and lung cancer [24]. The position and the degree of hydroxylation have been demonstrated to be the most important for their biochemical and pharmacological actions [25,26]. Wang et al. [11] examined the antioxidant and anti-inflammatory activities of selected flavonols, including kaempferol, quercetin and myricetin. Their results showed that flavonols exhibit distinctive antioxidant and anti-inflammatory potentials and that the structures, required to strengthen these activities, vary with sorts of free radicals and mechanisms. Quercetin blocked the aggregation of human platelets by ADP and thrombin, and this compound has gained considerable prominence as an inhibitor of carcinogens and of cancer cell growth in many experimental and human tumors [27,28].

The essential part of flavonol structure for exerting such activity is the *o*-dihydroxyl structure at the 3′- and 4′-position of the B-ring [29,30]. Hydroxyl groups at the 3′ and 4′-position scavenge free radicals by donation of hydrogen or an electron. Quercetin and myricetin can exert strong antioxidant activity because they possess an *o*-dihydroxyl structure, so-called catechol and pyrogallol, respectively [31]. Kaempferol is a weak antioxidant because of its monophenol structure in the B-ring [29].

Due to their proposed health-promoting effects, it is important to develop efficient and high throughput analytical methods able to assess the amount of flavonols in food commodities. In recent years, increasing knowledge of the positive health effects of food polyphenols has prompted the need to develop new separation techniques for their extraction, fractionation and analysis [29,32–34]. Many analytical methods have been described for determination of flavonols in wines. The most selective methods involve often liquid or gas chromatography coupled to detection by mass or tandem mass spectrometry [35–48].

Conventional techniques, based mainly on liquid–liquid extraction (LLE) and solid-phase extraction (SPE), are reported for analysis of wine flavonols. However, these procedures are quite time-consuming and need relatively high volume of solvent/sample, which is not appropriate for the routine analyses of many food commodities. Over the last years the-increasing demand for control analysis has contributed markedly to the renewal of interest in miniaturized analytical techniques. The usage of less amounts or no solvent, increasing sensitivity of analysis and user-friendly systems, should be pointed out. In this context, the microextraction by packed sorbent (MEPS) has emerged as an attractive alternative for sample preparation due to its simplicity, little solvent consumption (as small as 10 μL), the need of very small sample volumes, and can be easily interfaced to LC–MS [49] and GC–MS to provide a completely automated MEPS/LC–MS or MEPS/GC–MS system [50–52]. The commercially available MEPS uses the same sorbents as conventional SPE columns and so is suitable for use with most existing methods by scaling the reagent and sample volumes. Unlike conventional SPE columns, the MEPS sorbent bed is integrated into a liquid handling syringe that allows for low void volume sample manipulations either manually or in combination with laboratory robotics. When the sample has passed through the solid support, the analytes are adsorbed to the solid phase packed in a barrel insert and needle (BIN) [46,49]. The cartridge bed can be packed or coated to provide selective and suitable sampling conditions. Silica-based reversed phase (C2, C8 and C18), strong cation exchanger (SCX) using sulfonic acid bonded silica, restricted access material (RAM), HILIC, carbon, polystyrene-divinylbenzene copolymer (PS-DVB) or molecular imprinted polymers (MIPs), can be used as adsorbent materials. This extraction technique (MEPS) could be of interest in clinical, forensic toxicology and environmental analysis areas [49,53,54].The ultra high pressure liquid chromatography (UHPLC) has recently become a fast separation technique. The development of analytical columns of very small particle size and specially designed instruments allow for the use of much lower flows of mobile phase at very high pressures, which results in increased speed of analysis, higher separation efficiency and resolution, higher sensitivity and much lower sample and solvent consumption as compared to other analytical approaches.

The current research study reports the development and validation of an ultra-fast, efficient, sensitive, reliable and high throughput MEPS-based methodology combined with UHPLC equipped with a PDA detection system, for the simultaneous determination of biologically active flavonols in wines. As stationary phase a new T3 bonding process (HSS T3), which utilizes a trifunctional C_{18} alkyl phases bonded at ligand density that promotes polar compound retention and aqueous mobile phase compatibility, in order to analyze a large batch of samples in a short period

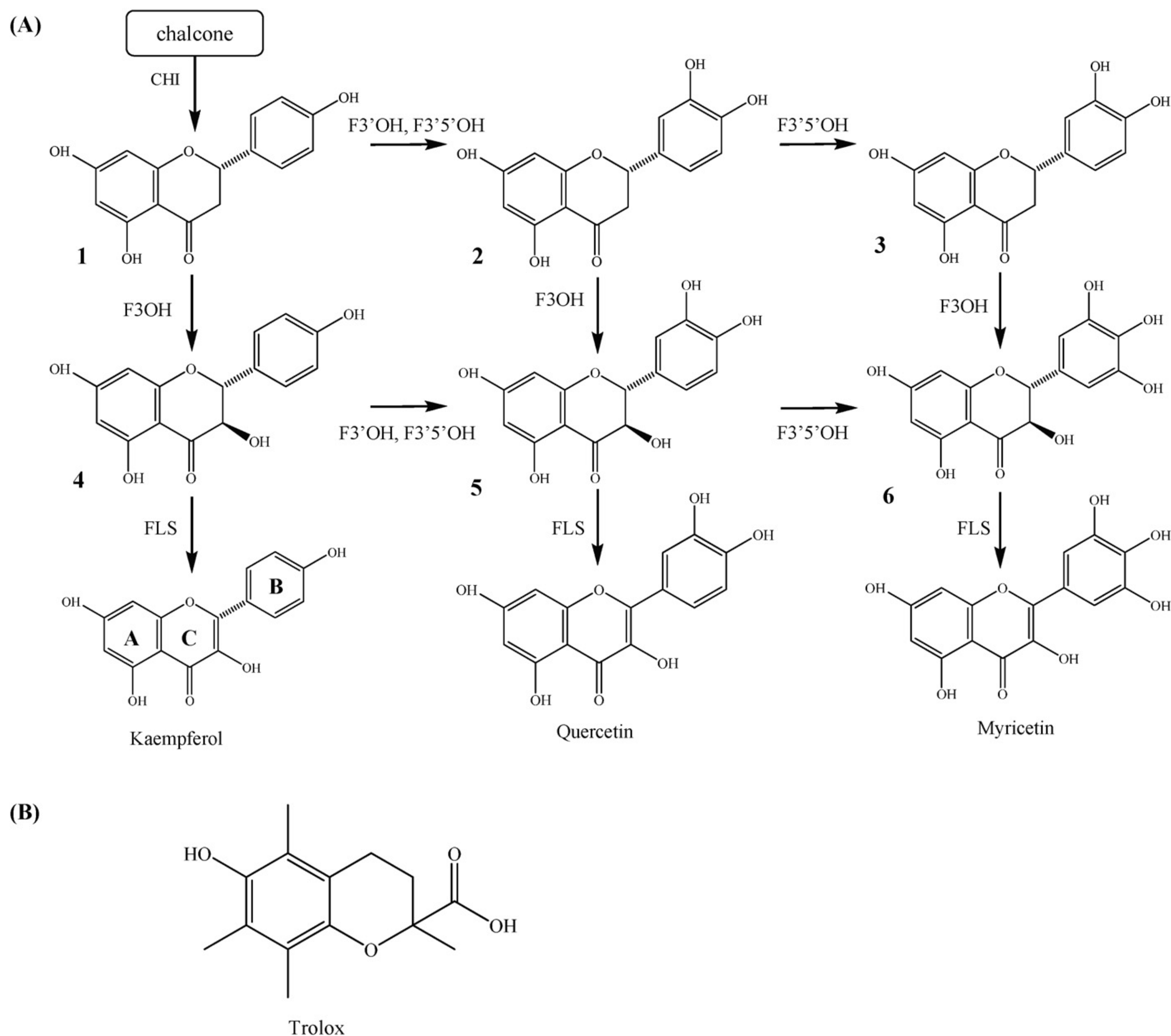


Fig. 1. General pattern for flavonol biosynthesis. (A) Precursors: 1: naringenin; 2: eriodictyol; 3: 3′,4′,5,5′,7-pentahydroxyflavanone; 4: dihydrokaempferol; 5: dihydroquercetin; and 6: dihydromyricetin. Enzymes: CHI, chalcone isomerase; F3OH, flavanone 3-hydroxylase; FLS, flavonol synthase; F3′OH, flavonoid 3′-hydroxylase; F3′5′OH, flavonoid 3′,5′-hydroxylase, and (B) Trolox chemical structure.

of time. Spiked synthetic wine solution, red wine (RW#3) and white wine (WW#26) were used to evaluate the performance of the developed method. Parameters affecting the MEPS extraction efficiency, such as type of sorbent material, number of extraction cycles (extract-discard), volume of eluent and sample volume, were carefully optimized. The method was compared to SPE technique commonly used for wine polyphenols analysis and applied to thirty wine samples from different varieties and vintage.

2. Experimental

2.1. Materials

LC-gradient grade methanol (99.9% purity) was obtained from Sigma–Aldrich (St. Louis, MO, USA), formic acid from Merck, (Darmstadt, Germany) and acetic acid from Riedel-de-Haën (Germany). Ethanol absolute (99.5% purity) and sodium hydroxide were obtained from Panreac (Barcelona, Spain). Kaempferol and Trolox (internal standard) were acquired from Fluka Biochemica AG (Buchs, Switzerland), and tartaric acid from Sigma–Aldrich. Myricetin was acquired from Acros Organics (Geel, Belgium). Ultrapure water (18 MΩ cm at 23 °C) was obtained by means of a Milli-Q water purification system (Millipore, Milford, MA, USA). All the extracts were filtered through 0.22 μm PTFE membrane filters. The MEPS gas-tight syringe (250 μL) and the BIN containing the sorbent material from SGE Analytical Science (Melbourne, VIC, Australia). The Waters Acquity UPLC HSS T3 analytical column (100 mm × 2.1 mm, 1.8 μm particle size) was purchased from Waters (Waters, Milford, MA, USA). A HANNA instruments pH209 pH meter (Woonsocket, USA) was used to pH adjustments.

2.2. Instrumentation

The analysis of flavonols was carried out on a Waters UPLC, Acquity H-Class system combined with a Waters Acquity quaternary solvent manager (QSM), an Acquity sample manager (SM), a

column heater, a 2996 PDA detector, and an in-line degasser system. The whole configuration was driven by Empower software v2.0 from Waters Corporation. Optimum separation was achieved with a binary mobile phase which consisted of (A) water containing 0.1% formic acid, and (B) methanol, with a constant flow rate of 250 μL min^{-1}, giving a maximum back pressure of 6000 psi, which is within the capabilities of the UHPLC. 2 μL of extracts were injected into the Waters Acquity UPLC system, equipped with an Acquity UPLCTM strength silica HSS T3 analytical column (1.8 μm particle size, 2.1 mm × 100 mm) and protected with an Acquity UPLCTM HSS T3 Van GuardTM Pre-column (Waters); column temperature was thermostated at 40 °C and the samples were kept at 4 °C in the sample manager. All solvents and samples were filtered through 0.22 μm pore size membrane filters, degassed ultrasonically and pumped in gradient mode through the chromatographic system as follows: 80% A (0 min); 80–70% A (0.50 min); 68% A (1 min); 20% A (7 min); and 80% A (7 min), followed by a re-equilibration time of 2 min, for bringing the column to the initial conditions after gradient analysis, resulted in a total analysis time of 9 min.

2.3. Procedures

2.3.1. Standard solutions

Analytical standard stock solutions of each flavonol (1000 μg mL^{-1}) were prepared in pure ethanol, aliquoted in 4 mL vials, coded and stored at −20 °C, in the dark; under these conditions they were stable for at least 4 month (as assessed by UHPLC). A working standard multicomponent solution was prepared daily from the individual stock solutions by diluting them in synthetic wine (5 g L^{-1} tartaric acid, 12% (v/v) ethanol and 8 g L^{-1} of sugar (glucose: fructose, 1:1). This standard was used both, to spike the wines to perform the assays for optimization of extraction conditions and for calibration studies [55,56]. The concentration ranges (Table 1) were selected in function of sensitivity of the UHPLC-PDA towards each flavonol compound. Trolox was used as internal standard (IS).

2.3.2. Wine samples

Thirty representative wine samples from different vintages, grape varieties, and from different Portuguese regions (mainland, Madeira and Azores Islands), produced according to standard procedures were kindly donated by the winemakers.

Synthetic wine was prepared by adding 5 g L^{-1} of tartaric acid and 8 g L^{-1} of sugar (glucose: fructose, 1:1) to ethanol solution at 12% (v/v). Synthetic wine pH 3.4 was adjusted with sodium hydroxide 1 M.

To minimize the influence of ethanol on extraction efficiency, wine samples and synthetic wines were dealcoholized under vacuum at 40 °C, up to 1/4 of initial volume. The volume of dealcoholized extracts was adjusted to initial sample volume with ultrapure water. The aqueous extracts were used for both techniques (MEPS and SPE). All samples were analyzed in triplicate.

2.3.3. Flavonols extraction

2.3.3.1. Optimization of the factors affecting the MEPS performance. The MEPS procedure was carried out by means of a SGE Analytical Science apparatus (I.L.C., Lisbon, Portugal), consisting of a 250 μL gas-tight syringe with a removable needle. The syringe was fitted with a BIN containing 4 mg of the sorbent material and was used to draw and discharge samples and solutions through the BIN. A synthetic wine sample spiked with known amounts of flavonols was used to optimize the MEPS procedure. Several important MEPS-influencing extraction factors, such as the nature of adsorbent material, number of extraction cycles, elution volume, sample volume, and ethanol content, were evaluated.

At a first step, the performance of the five MEPS sorbent materials, C2 (ethyl-silica), C8 (octyl-silica), C18 (octadecyl-silica), SIL (unmodified silica) and C8/SCX (a mixed mode sorbent containing 80% C8 and 20% strong cationic exchange (SCX)) was tested and compared. To select the number of extraction cycles (extract-discard) and sample volume, an aliquot of 50 μL, 100 μL and 250 μL of synthetic wine was pumped up and down 1, 5 and 10 times, in order to obtain the best extraction efficiency. Different elution volumes (50, 100, 250, 350 and 500 μL) were also tested. The flow rate during aspiration is limited to about 20 ± 2.3 μL s^{-1} (average values of n = 12 assays) to prevent cavitation. This will increase analyte/sorbent contact time and extraction efficiency.

To overcome the co-solvent effect of ethanol towards flavonols and to improve the extraction efficiency, synthetic wine, red wine and white wine samples were dealcoholized under roto-evaporation to 1/4 of initial volume. The volume of the dealcoholized extract was adjusted to initial volume with different solutions to select that gives best results: (a) 100% water; (b) 90% aqueous solution of formic acid at 0.1% and 10% methanol; and (c) 80% aqueous solution of formic acid at 0.1% and 20% methanol (mobile phase used at initial step of gradient elution). Since the influence of methanol on MEPS polyphenols extraction efficiency was similar to ethanol, and taking into account their use as mobile phase in the UHPLC system, methanol was chosen to evaluate the effect of alcoholic content on extraction efficiency. All optimization procedures were carried out in triplicate.

2.3.3.2. MEPS procedure for flavonols analysis. MEPS experiments were conducted using 4 mg of C8 sorbent, selected, in the optimization step, as the best sorbent to isolate the target flavonols. Before being used for the first time, the sorbent was manually conditioned first with 100 μL methanol and then with 100 μL water (containing 0.1% formic acid). This step activates the sorbent and ensures reproducible retention of the analytes [57,58]. 250 μL of sample were passed through the C8 sorbent five times at a flow rate of about 20 ± 2.3 μL s^{-1} on average. The solid phase was then washed with 100 μL of water containing 0.1% formic acid to remove interferences, at a speed of about 50 μL s^{-1}. The analytes were eluted with 250 μL of 95% methanol and 5% water directly into a vial. Between every extraction, the sorbent was rinsed with 250 μL methanol followed by 250 μL of the washing solution (0.1% formic acid aqueous solution). This step decreased memory effects (carry-over), but also functioned as conditioning step before the next extraction. The extracts were filtered through Millipore membrane PTFE filters (0.22 μm particle size). An aliquot of 2 μL of this solution was injected in triplicate into the UHPLC-PDA system. The same packing bed was used for about 100 extractions; then it was discarded due to both the low analyte extraction yields and clogging of the sorbent. All MEPS steps including activation, loading, washing, and elution were carried out manually. In all measurements (standards and samples), Trolox was added as internal standard (IS).

2.3.3.3. SPE. The MEPS procedure was compared to SPE reverse-phase using Oasis HLB extraction cartridges, developed by Silva et al. [43]. Briefly, 1 cc (30 mg) Oasis HLB extraction cartridges were conditioned with 1 mL of ethyl acetate and methanol and equilibrated with 1 mL of water. An aliquot of sample (900 μL), previously prepared (see Section 2.3.2.) was passed through the SPE cartridges, at around 1.5 mL min^{-1}, using an Alltech extraction unit (SGE). The cartridges were washed with a solution of methanol 5% and subsequently the flavonols were eluted, into the same vial, with 500 μL methanol, 1000 μL ethyl acetate and finally 500 μL methanol. The extracts were evaporated to dryness under a nitrogen stream. The dried residue was dissolved in 1000 μL of initial mobile phase (0.1% formic acid aqueous solution and 20% of methanol solution), homogenized in a vortex agitator and filtered

Table 1
Comparison of parameters for calibration: linearity, LOD, LOQ and matrix effect in red and white wines between $MEPS_{C8}$/UHPLC-PDA and $SPE_{Oasis\ HLB}$/UHPLC-PDA.

Peak number	RT (min)	λ_{max}[a] (nm)	Flavonols	Method	Conc. Range ($\mu g\ mL^{-1}$)	Regression equation ($y = ax^b + b$)	R^2[c]	LOD[d] ($\mu g\ mL^{-1}$)	LOQ[e] ($\mu g\ mL^{-1}$)	Matrix effect[f]	
										Red wine (±SD%)	White wine (±SD%)
1	4.420	372	Myricetin	$MEPS_{C8}$	0.1–5	$0.0420x - 0.0156$	0.9963	0.013	0.042	1.01 ± 7.5	1.07 ± 5.9
				$MEPS_{C8}$(RW[g])	0.1–5	$0.0415x + 0.1202$	0.9797				
				$MEPS_{C8}$(WW[h])	0.1–5	$0.0393x + 0.0233$	0.9923				
				$SPE_{Oasis\ HLB}$	0.1–5	$0.0136x - 0.0009$	0.9946	0.038	0.128	1.15 ± 8.0	1.20 ± 8.1
2	4.731	360	Quercetin	$MEPS_{C8}$	0.05–5	$0.0810x + 0.0362$	0.9966	0.006	0.012	0.85 ± 9.7	0.88 ± 8.3
				$MEPS_{C8}$(RW)	0.05–5	$0.0692x + 0.1889$	0.9603				
				$MEPS_{C8}$(WW)	0.05–5	$0.0718x + 0.0222$	0.9400				
				$SPE_{Oasis\ HLB}$	0.05–5	$0.0411x + 0.0066$	0.9815	0.010	0.032	1.03 ± 9.5	0.92 ± 7.3
3	5.252	366	Kaempferol	$MEPS_{C8}$	0.05–3	$0.0968x + 0.0215$	0.9989	0.011	0.037	0.96 ± 11.2	0.90 ± 6.4
				$MEPS_{C8}$(RW)	0.05–3	$0.0932x + 0.0317$	0.9925				
				$MEPS_{C8}$(WW)	0.05–3	$0.0879x + 0.0233$	0.9837				
				$SPE_{Oasis\ HLB}$	0.05–3	$0.0344x + 0.0036$	0.9886	0.059	0.198	0.95 ± 10.3	0.98 ± 11.5
4	5.524		Trolox (IS[i])								

[a] Maximum absorbance values obtained in PDA system detection.
[b] x = flavonol concentration.
[c] Correlation coefficient, give an estimating how well the experimental points fit a straight line.
[d] Limit of detection.
[e] Limit of quantification. Values obtained from ordinary least-squares regression data.
[f] Matrix effect was calculated by the quotient between the slopes of the standards in synthetic wine and spiked red and white wines.
[g] RW: calibration curve in red wine.
[h] WW: calibration curve in white wine.
[i] IS: internal standard.

through a 0.22 μm membrane filters before injection into UHPLC-PDA system. All extractions were carried out in triplicate, and each extract was injected three times.

2.4. Validation of $MEPS_{C8}$/UHPLC method

The newly developed $MEPS_{C8}$/UHPLC-DAD approach was fully validated based on selectivity, linearity, limits of detection (LOD) and quantification (LOQ), inter- and intra-day precisions and accuracy. The assays were carried out using the C8 sorbent and a Waters Acquity H-Class equipped with a 100 mm × 1.8 μm UPLC analytical column.

The selectivity of the method was assessed by the absence of interfering peaks at the retention time of target flavonols. The linearity was evaluated building three calibration curves (peak $area_{flavonol}$/peak $area_{IS}$ ratio plotted against the corresponding standard concentration) for each flavonol using standards prepared in synthetic wine, at 7 different concentrations levels, including the zero point (Table 1). The zero point (not spiked synthetic wine) enables to verify that none of the compounds showed residual level or background signal. Each level of concentration was prepared in triplicate and injected three times, so there were a total of nine replicates.

The sensitivity was assessed by determining the LOD (the lowest analyte concentration that produces a response detectable above the noise level of the system) and LOQ (the lowest level of analyte that can be accurately and precisely measured) for each compound. LOD and LOQ were calculated with the data generated in the linearity studies, being LOD defined as ($a + 3S_{a/b}$) and LOQ as ($a + 10S_{a/b}$), where "a" represents origin ordinate, "S_a" the origin ordinate variance and "b" the slope [59]. These parameters were calculated for each analyte from the standard solutions used to obtain the corresponding calibration curves, using the UHPLC developed method.

To check the accuracy of the proposed method a recovery study was carried out by spiking a red wine (RW#3) and a white wine (WW#26), in triplicate at two concentration levels corresponding to the LL (low level, lowest concentration of calibration curve) and HL (high level, highest concentration of calibration curve), and subjected to the $MEPS_{C8}$ procedure described above. The recovery values were calculated according to the Eq. (1).

$$\text{Accuracy} = 100 \times \left\{ \frac{([\text{flavonol}]_{\text{after spiking}} - [\text{flavonol}]_{\text{before spiking}})}{[\text{flavonol}]_{\text{added}}} \right\} \quad (1)$$

where $[\text{flavonol}]_{\text{after spiking}}$ is the flavonol concentration measured in spiked wine; $[\text{flavonol}]_{\text{before spiking}}$ is the flavonol concentration measured in unspiked wine, and $[\text{flavonol}]_{\text{added}}$ is the nominal concentration of the flavonol added to wine.

Method precision (expressed as relative standard deviation, RSD %) was tested by spiking a synthetic wine at three different concentration levels, corresponding to the low level (LL), medium level (ML) and highest point (HL) of calibration curve of each target flavonol and treated by $MEPS_{C8}$. Seven replicates ($n = 7$) were performed in the same day to obtain repeatability (intra-day precision, RSD_r%). For inter-day precision (reproducibility, RSD_R,%) evaluation, the same protocol was followed but six replicates of each level were analyzed daily through three different days ($n = 18$).

The matrix effect was evaluated by the quotient between the slopes of the standards in "blank" matrix (synthetic wine) and those obtained by spiking RW#3 and WW#26 wines (standard addition method).

3. Results and discussion

To enable a high throughput, robust and accurate method for quantification of flavonols in wines, a MEPS approach combined with UHPLC-PDA system was developed. Their performance was compared with the SPE technique using Oasis HLB as sorbent.

The extracts were monitored at four different wavelengths, 375, 371, 366 and 289 nm, corresponding to maximum absorbance wavelength of myricetin, quercetin, kaempferol and Trolox (IS), respectively. They were identified by comparing the retention time and spectral characteristics of their peaks with those of standards (Fig. 1S; Supplementary figure) and quantified using the standards

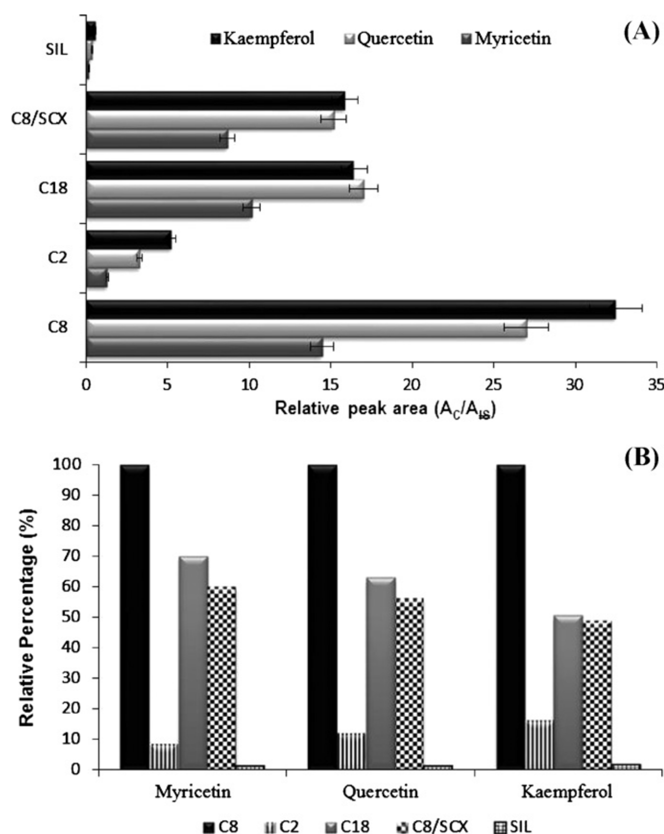


Fig. 2. (A) Comparison the performance characteristics (relative peak areas obtained for each adsorbent) of different MEPS adsorbents for isolation of target flavonols from wines; and (B) normalized extraction efficiencies measured for C2, C8, C18, SIL and C8/SCX.

of each one.Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.aca.2012.06.020.

3.1. MEPS optimization

The development of the MEPS procedure for the analysis of flavonols involved consideration and optimization of the following factors: nature of the adsorbent, number of extraction cycles, elution volume, sample volume, and ethanol content. Experiments to evaluate these factors were conducted using synthetic wine mixtures of flavonols.

3.1.1. Nature of sorbent

The selection of an appropriate sorbent is of major importance to achieve acceptable clean-up and extraction yield [60]. Five different MEPS sorbents namely the silica-based C2, C8 and C18 phases (suitable for lipophilic analytes), as well as the mixed bonded silica C8/SCX (suitable for polar analytes such as acidic and basic compounds) containing both reversed phase and cationic exchange groups, and the polar silica phase (SIL), were tested. Fig. 2A shows the UHPLC-PDA response for the target analytes using different sorbents.

The performance of each commercially available MEPS adsorbent was evaluated in terms of intensity of the response observed (relative peak area) and reproducibility. It was observed that the C8 sorbent exhibits better extraction efficiency for all target flavonols, as demonstrated by the highest UHPLC-PDA responses (based on peak area) (Fig. 2A). On the other hand SIL sorbent showed the lowest performance. The use of C8/SCX phase did not induce high response, indicating that the sulfonyl groups of the phase did not play a role in the retention of flavonols. Therefore, the C8 sorbent was used in subsequent method optimization stages. The sum of the target flavonols peak areas obtained for each sorbent was normalized in relation to the sum of the flavonols peak areas obtained for the C8 sorbent. The extraction efficiency was calculated for each sorbent, and the results are presented in Fig. 2B. In the case of the C8 sorbent, this study illustrated excellent extraction efficiencies for all compounds studied (Fig. 2B), thus supporting the conclusion that the C8 approach provides the best extraction efficiency for the purposes of this research.

The effect of pH on the C8 sorbent partition mechanism was evaluated by adjust the sample pH, prior to extraction, to pH 3.4, 5, 7 and 9 (using diluted hydrochloric acid solution or dilute sodium hydroxide solution). The stability of flavonols is influenced by pH being stable in strongly acidic conditions. The optimum pH for the analysis of the target flavonols was found to be pH 3.4 (Supplementary Fig. 2S).Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.aca.2012.06.020.

3.1.2. Number of extraction cycles and volume of sample

In MEPS the retention on the sorbent phase is affected by the number of extraction cycles performed and by the elution flow rate (about $20 \pm 2.3\,\mu L\,s^{-1}$, on average). The sample can be drawn through the needle into the syringe up and down, once or several times (cycles) without discarding it. Fig. 3A shows the effect of the number of extraction cycles (1, 5 and 10, extract-discard) and the impact of sample volume (50, 100 and 250 μL) on the extraction efficiency of flavonols. Assays showed that the competition for active adsorption sites of the C8 sorbent increased slightly as the applied extraction number and sample volume increased (Fig. 3A and B), thereby increasing extraction efficiency of $MEPS_{C8}$. Secondly, the volume of sample was optimized. 250 μL of wine sample was the optimal volume (Fig. 3A) required to recover the target flavonols without any further dilution of the extract. Small sample volumes given rise to increasing extraction cycles (extract-discard cycles) and furthermore increase the extraction amount in unit volume of sample of organic solvent. Statistically, no significant differences were observed when 250 μL of synthetic wine sample are passed through the C8 sorbent once, five and ten times. For this reason $5 \times 250\,\mu L$ was selected, since the results showed that five extraction cycles gave a good recovery and can extend the lifetime of the MEPS cartridge. Similar outcomes were concluded by Zhang et al. [61] in their studies associated with the optimization of condition for analysis of five estrogens in urine and milk.

3.1.3. Elution volume

The extraction performances by different elution volumes namely 50 μL, 100 μL, 250 μL, 350 μL, and 500 μL of methanol/water 0.1% formic acid (80:20) solution, were compared. The results in Fig. 3B indicated that the extraction efficiencies were enhanced by decreasing volume of elution solution. On the other hand a complex matrix such as wines can saturate the adsorbent phase and the partitioning may be hindered by interferences. For the wine flavonols extraction the optimal recovery (Fig. 3B) was obtained when 50 μL of elution solution containing 80% of methanol and 20% aqueous solution of formic acid was used.

3.2. MEPS performance. Comparison with $SPE_{Oais\ HLB}$

To demonstrate the feasibility of the present approach for determination of wine flavonols and to evaluate their practical applicability, the method was fully validated considering the selectivity, linearity, LOD, LOQ, intra/inter-day precision, accuracy and matrix effects. Their performance was compared with a previously $SPE_{Oais\ HLB}$ validated method in our laboratory by Silva et al. [43]. The validation parameters are shown in Table 1.

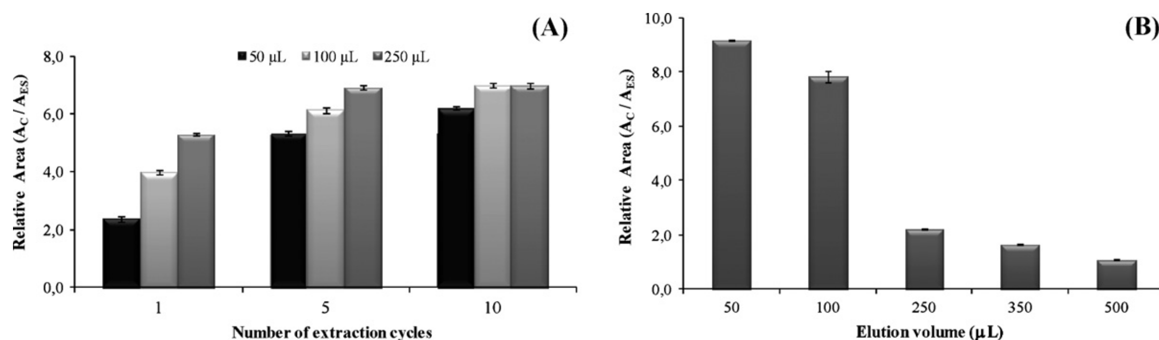


Fig. 3. Effect of the experimental conditions on the extraction performance of the MEPS procedure, including: (A) influence of number of extraction cycles (extraction-discard) as a function of applied sample volume on the extraction efficiency of flavonols; (B) effect of elution volume on UHPLC-PDA response. Errors bars show the standard deviation of the mean ($n = 3$).

Table 2
Recovery (%) of flavonols added to each of two wines at high concentration (HL) and low concentration (LL) level.[a]

Flavonol	$MEPS_{C8}$				Overall mean ± SD	$SPE_{Oasis\ HLB}$				Overall mean ± SD
	RW_{LL}[b]	RW_{HL}[c]	WW_{LL}[d]	WW_{HL}[e]		RW_{LL}	RW_{HL}	WW_{LL}	WW_{HL}	
Myricetin[f]	90.6 ± 1.6	93.2 ± 0.8	94.1 ± 0.4	88.6 ± 0.2	91.6 ± 0.7	85.0 ± 8.4	111.0 ± 5.3	89.0 ± 5.6	99.0 ± 1.1	96.0 ± 4.7
Quercetin[g]	83.0 ± 0.8	87.2 ± 0.9	89.2 ± 0.8	84.5 ± 1.2	85.9 ± 0.9	104.0 ± 9.2	102 ± 6.9	78.0 ± 5.0	89.2 ± 4.9	93.2 ± 6.0
Kaempferol[h]	92.5 ± 1.3	97.0 ± 0.5	88.9 ± 0.3	90.1 ± 0.1	92.1 ± 0.5	82.0 ± 7.5	76 ± 8.0	89 ± 4.6	102 ± 3.1	87.2 ± 5.5

[a] Each wine was assayed three times before and after each addition, and average of results is presented.
[b] RW_{LL} red wine fortified with flavonols at low concentration level.
[c] RW_{HL} red wine fortified with flavonols at high concentration level.
[d] WW_{LL} white wine fortified with flavonols at low concentration level.
[e] WW_{HL} white wine fortified with flavonols at high concentration level.
[f] Myricetin: LL = 0.2 $\mu g\ mL^{-1}$; HL = 4.0 $\mu g\ mL^{-1}$.
[g] Quercetin: LL = 0.1 $\mu g\ mL^{-1}$; HL = 4.0 $\mu g\ mL^{-1}$.
[h] Kaempferol: LL = 0.1 $\mu g\ mL^{-1}$; HL = 3.0 $\mu g\ mL^{-1}$.

For each flavonol a linear regression of the peak $area_{flavonol}$/peak $area_{IS}$ vs concentration, was calculated to determine the linearity of the method using three replicates at seven levels of concentration (Table 1).

The UHPLC-PDA system gave linear response over the studied range of concentrations and the least-squares linear regression analysis of the data provided excellent correlation coefficient (R^2) values for all compounds tested with $R^2 > 0.9963$ for MEPS and $R^2 > 0.9815$ for $SPE_{Oasis\ HLB}$ method, over synthetic wine, and residuals not exceeding ±6.3%.

The LOD and LOQ were calculated from ordinary least-squares regression data [62]. The standard deviation chosen to calculate the LOD and LOQ values is the residual standard deviation of the regression line for all flavonols in the analyzed matrix. The $MEPS_{C8}$/UHLPC-PDA methodology gave in general very low detection limits, ranging between 0.006 (quercetin) and 0.013 $\mu g\ mL^{-1}$ (myricetin), while limits of quantification were in the range 0.012–0.042 $\mu g\ mL^{-1}$, for quercetin and myricetin, respectively (Table 1). The $SPE_{Oasis\ HLB}$ technique gives LOD and LOQ values slightly higher than the developed ($MEPS_{C8}$) technique.

The matrix effect was evaluated through the coefficient between the calibration curve obtained with standards in synthetic wine and those obtained from spiked RW#3 and WW#26 wines (standard addition method). Matrix effect values ranged from 0.85 ± 9.7 (quercetin) to 0.11 ± 5.9% (myricetin) with an average value of 0.99% for $MEPS_{C8}$ technique. Regarding $SPE_{Oasis\ HLB}$ procedure, the average values of matrix effect was found to be similar, 0.99% on average, with the individual values ranging between 0.92 ± 7.3 and 1.2 ± 8.1%. Therefore no significant matrix effect was observed (Table 1).

The recovery studies were carried out by spiking RW#3 and WW#26 wine samples at two concentration levels (LL and HL), with a known amount of each flavonol (Table 2). The concentration of phenolics added to the wines was chosen to cover the expected

Table 3
Precision[a] of $MEPS_{C8}$/UHPLC-PDA and $SPE_{Oasis\ HLB}$/UHPLC-PDA assays for the target flavonols.

Flavonol	$MEPS_{C8}$ Intra-day precision ($n = 7$)				Inter-day precision ($n = 18$)				$SPE_{Oasis\ HLB}$ Intra-day precision ($n = 7$)				Inter-day precision ($n = 18$)			
	LL	ML	HL	Overall mean (±SD%)	LL	ML	HL	Overall mean (±SD%)	LL	ML	HL	Overall mean (±SD%)	LL	ML	HL	Overall mean (±SD%)
Myricetin[b]	3.1	1.5	0.9	1.8 ± 1.1	1.3	1.8	1.4	1.5 ± 0.3	7.8	3.6	2.9	4.8 ± 2.7	6.3	5.9	4.2	5.5 ± 1.1
Quercetin[c]	2.2	0.8	0.7	1.2 ± 0.8	2.8	0.7	0.9	1.5 ± 1.2	4.6	1.9	1.1	2.5 ± 1.8	7.1	8.9	2.6	6.2 ± 3.2
Kaempferol[d]	1.4	0.5	0.9	0.9 ± 0.5	1.6	1.2	0.6	1.1 ± 0.5	3.7	1.7	0.3	1.9 ± 1.7	4.7	3.2	4.1	4.0 ± 0.8

[a] Synthetic wine spiked with flavonols; at high concentration level.
[b] Myricetin: LL = 0.2 $\mu g\ mL^{-1}$; ML = 1.4 $\mu g\ mL^{-1}$; HL = 5.0 $\mu g\ mL^{-1}$.
[c] Quercetin: LL = 0.1 $\mu g\ mL^{-1}$; ML = 2.0 $\mu g\ mL^{-1}$; HL = 5.0 $\mu g\ mL^{-1}$.
[d] Kaempferol: LL = 0.1 $\mu g\ mL^{-1}$; ML = 0.8 $\mu g\ mL^{-1}$; HL = 3.0 $\mu g\ mL^{-1}$.

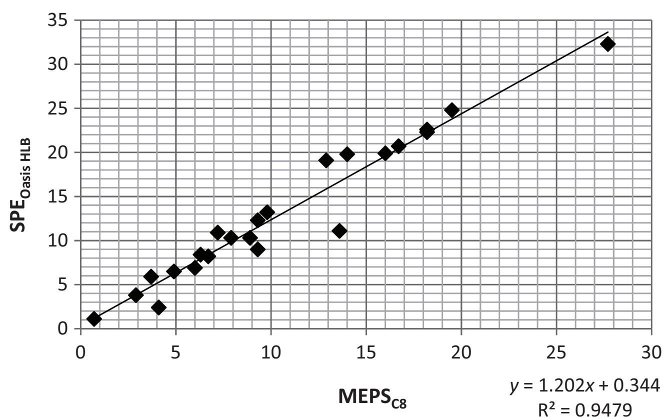


Fig. 4. Correlation between the $MEPS_{C8}$ and $SPE_{Oasis\ HLB}$ extraction techniques for isolation the target wine flavonols.

values in the wine samples. The accuracy was determined according to the equation presented in Section 2.4. The obtained values for each fortification level are listed in Table 2.

At all concentration levels, satisfactory results were found with recovery values ranging from 83.0 ± 0.8 (RW_{LL}) to $97.0 \pm 0.5\%$ (RW_{HL}), and between 82.0 ± 7.5 (RW_{LL}) and $111.0 \pm 5.3\%$ (RW_{HL}), for $MEPS_{C8}$ and $SPE_{Oasis\ HLB}$, respectively. At low flavonols concentration (LL), the extraction yields were slightly lower than that obtained at high concentration level (HL). The fact that the sorbent's chemistry surface can be changed by interfering compounds from wine, which therefore can change the sorption properties may explain this observation [52]. This effect is more pronounced at low concentrations of the analyte when analyte/matrix ratio is very low. The same behavior was observed for accuracy.

The precision was measured through inter-day reproducibility and intra-day repeatability studies, expressed by the RSD% and calculated using the measurement of relative peak area of each flavonol in the matrix. The repeatability was measured by comparing standard deviation of spiked synthetic wine (LL, ML, HL) run in the same day ($n = 7$). The reproducibility was determined by analyzing spiked synthetic wine samples for three alternate days. The results are satisfactory with RSD values lower than 3.1% for each measured analyte at all spiking levels (Table 3). The repeatability values at the three different levels ranged from 0.5 (kaempferol at ML concentration) to 2.2% (quercetin at LL concentration) ($n = 7$), whereas the reproducibility varied from 0.6 (kaempferol at HL concentration) to 1.3% (quercetin at LL concentration).

Combination of fast MEPS technique together with quick UHPLC-PDA system proves to be an improved strategy, with excellent recoveries, sensitivity, and repeatability, which make it possible to use as a quick approach to analyze the selected biological active constituents in wines.

3.3. *Determination of flavonols in wines by $MEPS_{C8}$ and $SPE_{Oasis\ HLB}$*

In order to test the applicability of the developed method, MEPS procedure was first applied to a mixture of flavonols standards and then to thirty wine samples (22 red and 8 white wines) (Table 4). In addition, the proposed method was compared with $SPE_{Oasis\ HLB}$ technique. The correlation between the two sampling methods is provided by the results given in Fig. 4. It shows the plot of the $MEPS_{C8}$/UHLPC-PDA data against $SPE_{Oasis\ HLB}$/UHLPC-PDA data for the 30 studied wines.

As can be seen, the values obtained with both techniques are well correlated ($R^2 = 0.9479$), which means that in terms of quantification, both methods can be used. However, several other experimental parameters should be taking into account, such as the extraction efficiency, the extraction time, solvent consumption, fast sample throughput, among others. In this regard MEPS technique offers advantages than SPE.

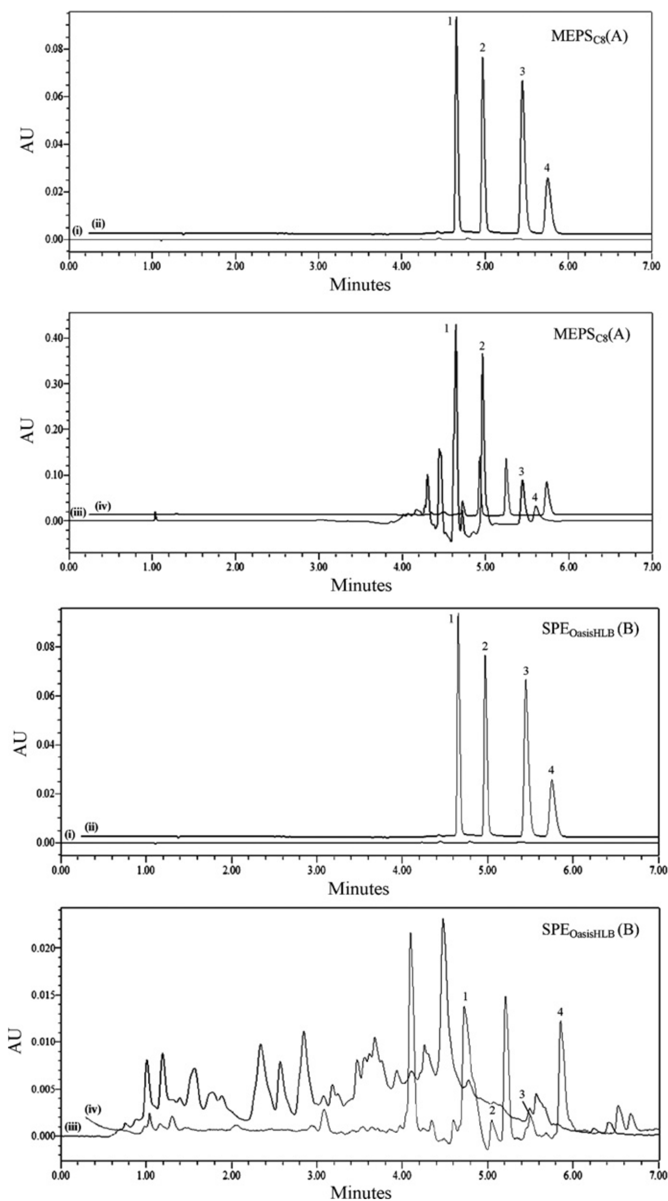


Fig. 5. Comparison of the wine flavonols UHPLC-PDA profile measured at maximum wavelength of each flavonol (See Table 1), after extracted with: (A) optimized $MEPS_{C8}$ – (i) synthetic wine (selectivity); (ii) synthetic wine spiked with flavonols; (iii) not spiked red wine; and (iv) not spiked white wine; (B) $SPE_{Oasis\ HLB}$ – (i) synthetic wine (selectivity); (ii) synthetic wine spiked with flavonols; (iii) not spiked red wine; and (iv) not spiked white wine; (for peak identification see Table 1).

The resulting chromatograms of both methods are shown in Fig. 5. The separation of the target flavonols is very fast, being achieved within only 7 min.

Accurate quantification and improved sensitivities for all three flavonols analysis in red and white wines were achieved by $MEPS_{C8}$ (Fig. 5A). In addition, the chromatographic profile resulting from $MEPS_{C8}$ extracts is much cleaner than that obtained by $SPE_{Oasis\ HLB}$ (Fig. 5B), meaning that the selectivity of Oasis HLB sorbent towards flavonols is poorer tha C8 sorbent. The chromatograms obtained for white and red wines samples showed quite different profiles and their complexity increases or decreases according to the

Table 4
Flavonol composition[a] of representative commercial wines ($\mu g\,mL^{-1}$) (flavonol data have been corrected for recoveries).

Wine sample	Vintage	Varieties	Myricetin		Quercetin		Kaempferol		Total flavonols		$\Delta_{flavonols}$
			MEPS	SPE	MEPS	SPE	MEPS	SPE	MEPS	SPE	
Red wines (RW)											
#1	2008	Tinta Barroca, Tinta Roriz, Touriga Franca	7.2 ± 1.6	6.6 ± 1.5	n.d.[b]	4.3 ± 1.2	<LOQ[c]	n.d.	7.2	10.9	3.7
#2	NA[d]	Syrah, Castelão and Aragonez	8.9 ± 3.2	8.7 ± 3.5	n.d.	n.d.	0.4 ± 0.01	0.3 ± 0.1	9.3	9.0	−0.3
#3	2010	Aragonez, Trincadeira and Castelão	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–	–	–
#4	2010	Syrah, Trincadeira and Castelão	6.4 ± 1.2	8.0 ± 0.9	n.d.	n.d.	0.3 ± 0.4	0.2 ± 0.09	6.7	8.2	1.5
#5	2009	Touriga Nacional and Castelão	8.7 ± 0.7	10.3 ± 1.4	n.d.	n.d.	0.2 ± 0.1	n.d.	8.9	10.3	1.4
#6	2007	Touriga Nacional, Alfrocheiro and Tinta Roriz	13.4 ± 0.6	16.1 ± 1.0	n.d.	n.d.	4.8 ± 0.9	6.2 ± 1.0	18.2	22.3	4.1
#7	2008	Castelão, Aragonez and Trincadeira	2.9 ± 0.5	3.8 ± 1.3	n.d.	n.d.	n.d.	n.d.	2.9	3.8	0.9
#8	2008	Touriga Nacional, Touriga Francesa, Tinta Roriz and Tinta Barroca	5.6 ± 1.1	6.6 ± 2.6	n.d.	n.d.	0.4 ± 0.1	0.3 ± 0.07	6.0	6.9	0.9
#9	2009	Rufete, Marufo and Tinta Roriz	12.4 ± 1.7	14.2 ± 3.2	4.1 ± 1.2	6.4 ± 1.7	0.2 ± 0.9	0.1 ± 0.06	16.7	20.7	4.0
#10	2010	Castelão	5.8 ± 0.1	8.1 ± 0.8	n.d.	n.d.	0.5 ± 0.4	0.3 ± 0.1	6.3	8.4	2.1
#11	2009	Touriga Nacional and Tinta Roriz	10.7 ± 2.2	13.2 ± 2.9	3.3 ± 0.4	6.6 ± 0.8	<LOQ	n.d.	14.0	19.8	5.8
#12	2009	Aragonez, Tincadeira and Alicante	2.9 ± 0.2	3.4 ± 0.7	5.2 ± 2.2	7.4 ± 1.4	1.7 ± 0.41	2.4 ± 0.8	9.8	13.2	3.4
#13	2009	Aragonez, Trincadeira, Cabernet	16.8 ± 2.5	15.7 ± 2.7	7.5 ± 1.8	10.6 ± 1.5	3.4 ± 0.6	6.0 ± 2.6	27.7	32.3	4.6
#14	2009	Baga and Touriga Nacional	3.9 ± 1.4	6.3 ± 2.9	0.4 ± 0.1	0.2 ± 0.1.	0.6 ± 0.2	<LOQ	4.9	6.5	1.6
#15	2007	Tinta Negra Mole	4.1 ± 0.7	2.4 ± 0.9	n.d.	n.d.	<LOQ	n.d.	4.1	2.4	−1.7
#16	2009	Tinta Roriz and Touriga Nacional	4.3 ± 0.5	6.6 ± 1.6	2.2 ± 0.7	4.4 ± 0.8	2.8 ± 1.7	1.3 ± 0.8	9.3	12.3	3
#17	2006	Bastardo and Touriga National	5.7 ± 0.8	2.9 ± 1.3	9.3 ± 4.4	11.1 ± 1.8	3.2 ± 0.9	7.3 ± 1.0	18.2	22.6	4.4
#18	2008	NA	13.1 ± 4.5	15.0 ± 3.4	2.8 ± 1.3	4.9 ± 0.4	0.8 ± 4.5	n.d.	16.0	19.9	3.9
#19	2009	Merlot, Cabernet Sauvignon, Saborinho	10.4 ± 2.8	5.7 ± 2.8	3.2 ± 0.9	5.4 ± 2.6	< LOQ	n.d.	13.6	11.1	−2.5
#20	2010	Merlot, Cabernet and Syrah	7.8 ± 1.7	5.3 ± 1.7	11.0 ± 3.4	19.2 ± 3.5	0.7 ± 0.3	0.3 ± 0.2	19.5	24.8	5.3
#21	2009	Periquita and Saborinho	7.9 ± 0.8	10.3 ± 3.3	n.d.	n.d.	<LOQ	n.d.	7.9	10.3	2.4
#22	2010	Agronómica and Saborinho	4.1 ± 0.2	3.2 ± 0.6	7.9 ± 3.3	14.3 ± 0.2	0.9 ± 2.4	1.6 ± 1.9	12.9	19.1	6.2
White wines (WW)											
#23	2009	NA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#24	2010	Rabo-de-ovelha	n.d.	n.d.	3.4 ± 1.7	5.8 ± 1.6	0.3 ± 0.1	0.1 ± 0.06	3.7	5.9	2.2
#25	2009	Roupeiro, Antão Vaz and Perrum	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#26	2009	Antão Vaz and Roupeiro	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#27	2010	Malvasia Fina and Gouveio	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#28	2010	Moscatel and Arinto	n.d.	n.d.	<LOQ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#29	2009	Encruzado, Bical and Malvasia Fina	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
#30	2010	NA	n.d	n.d.	n.d.	n.d.	n.d.	1.10 ± 1.1	0.7	1.1	0.4

[a] The content of each of the flavonols analyzed in the wine samples tested is the mean of three replicates ± SD and indicated as $\mu g\,mL^{-1}$.
[b] n.d.: not detected.
[c] <LOQ: lower than quantification limit.
[d] NA: data not available.

wavelength. The maximum absorbance wavelength of each flavonol, listed in Table 1, was used for quantification purposes.

The content of the target flavonols found in the assayed wines is summarized in Table 4. As can be observed, the concentration of flavonols in investigated red wines is significantly higher than the concentrations determined in white wines. The fact that the polyphenols content in red wines is higher than white wines was widely described before in the literature [35,39].

The average values obtained by $SPE_{Oasis\ HLB}$ technique {(14.0 $\mu g\,mL^{-1}$, from 2.4 $\mu g\,mL^{-1}$ (RW#15) to 32.3 $\mu g\,mL^{-1}$ (RW#13)} are slightly higher than that found with $MEPS_{C8}$ technique {(11.4 $\mu g\,mL^{-1}$, from 2.9 $\mu g\,mL^{-1}$ (RW#7) to 27.4 $\mu g\,mL^{-1}$ (RW#13)}.

The highest flavonol content, considering the sum of the target flavonols assayed ($\sum_{flavonols/MEPSC8} = 27.7\ \mu g\,mL^{-1}$ and ($\sum_{flavonols/SPEOasis\ HLB} = 32.3\ \mu g\,mL^{-1}$) was determined in red wine #13, followed by red wines #20 and #17, with a flavonol composition around 19.5 $\mu g\,mL^{-1}$ and 18.2 $\mu g\,mL^{-1}$ obtained by $MEPS_{C8}$ and 24.8 $\mu g\,mL^{-1}$ and 22.6 $\mu g\,mL^{-1}$ by $SPE_{Oasis\ HLB}$, respectively.

Regarding to individual flavonols, myricetin was the most abundant in all investigated wines, followed by quercetin and, in lowest concentration, kaempferol. Myricetin was not found in any of the investigated white wines (#23–30), however, it was found in all investigated red wines with exception of red wine #3.

4. Conclusions

In conclusion a novel, ultra-fast, sensitive and reproducible $MEPS_{C8}$/UHPLC-PDA-based methodology, was developed, validated and successfully applied to the simultaneous determination of flavonols in wines. As stationary phase a T3 bonding process (HSS T3), which utilizes a trifunctional C_{18} alkyl phases bonded at ligand density that promotes polar compound retention and aqueous mobile phase compatibility, was used. The combination of the shorter running time with a smaller flow rate also reduced drastically the solvent consumption and thus is more environmental friendly. The validated method showed a good performance with regard to selectivity, LODs, LOQs, linearity, extraction yields,

accuracy and intra/inter-day precisions. The results obtained from the application of the methodology to wine samples suggested that this method can be potentially useful to quantitate each of these compounds and serve as promising alternative to existing methodology for flavonols determination. Moreover, the method can be applied, as an attractive and very promising approach, to the analysis of flavonols in other food matrices, such as vegetables and fruits, due to the possibility of automation, easy to use, fast and low cost analysis, when compared to conventional SPE.

Wine extractions using the $MEPS_{C8}$ method provided higher sample throughput, selectivity and sensitivity equal to or slightly greater than the $SPE_{Oasis\ HLB}$ method.

Acknowledgments

The authors thank the financial support of FEDER (Transnational Cooperation MAC 2007–2013 Program) through VinSaudeMAC project (MAC/1/M105) and Fundação para a Ciência e Tecnologia through the MS Portuguese Networks (REDE/1508/RNEM/2005) and Pluriannual base funding (QUI-Madeira-674).

References

[1] H. Halbwirth, I. Puhl, U. Haas, K. Jezik, D. Treutter, K. Stich, J. Agric. Food Chem. 54 (2006) 1479–1485.
[2] R. Lukacin, L. Britsch, Eur. J. Biochem. 249 (1997) 748–757.
[3] R. Lukacin, I. Groning, E. Schiltz, L. Britsch, U. Matern, Arch. Biochem. Biophys. 375 (2000) 364–370.
[4] A. Crozier, J. Burns, A.A. Aziz, A.J. Stewart, H.S. Rabiasz, G.I. Jenkins, C.A. Edwards, M.E. Lean, Biol. Res. 33 (2000) 79–88.
[5] F. de la Pena Moreno, G.P. Blanch, M.L. Ruiz del Castillo, J. Agric. Food Chem. 58 (2010) 11639–11644.
[6] R. Lukacin, F. Wellmann, L. Britsch, S. Martens, U. Matern, Phytochemistry 62 (2003) 287–292.
[7] A. Crozier, I.B. Jaganath, M.N. Clifford, Nat. Prod. Rep. 26 (2009) 1001–1043.
[8] R.B. Rucker, in: C.A. Rice-Evans, L. Packer (Eds.), Flavonoids in Health and Disease, Marcel Dekker, Inc, New York, 2004.
[9] I. Erlund, R. Koli, G. Alfthan, J. Marniemi, P. Puukka, P. Mustonen, P. Mattila, A. Jula, Am. J. Clin. Nutr. 87 (2008) 323–331.
[10] S.N. Nichenametla, T.G. Taruscio, D.L. Barney, J.H. Exon, Crit. Rev. Food Sci. Nutr. 46 (2006) 161–183.
[11] L. Wang, Y.-C. Tu, T.-W. Lian, J.-T. Hung, J.-H. Yen, M.-J. Wu, J. Agric. Food Chem. 54 (2006) 9798–9804.
[12] C. Kandaswami, E. Middleton, Free Radicals in Diagnostic Medicine, Headington Hill Hall, New York, 1994.
[13] M. Exner, M. Hermann, R. Hofbauer, S. Kapiotis, P. Quehenberger, W. Speiser, I. Held, B.M. Gmeiner, Cardiovasc. Res. 50 (2001) 583–588.
[14] N. Yamamoto, J.H. Moon, T. Tsushida, A. Nagao, J. Terao, Arch. Biochem. Biophys. 372 (1999) 347–354.
[15] E.N. Frankel, J. Kanner, J.B. German, E. Parks, J.E. Kinsella, Lancet 341 (1993) 454–457.
[16] E.N. Frankel, A.L. Waterhouse, J.E. Kinsella, Lancet 341 (1993) 1103–1104.
[17] Y. Hanasaki, S. Ogawa, S. Fukui, Free Radic. Biol. Med. 16 (1994) 845–850.
[18] S.V. Nigdikar, N.R. Williams, B.A. Griffin, A.N. Howard, Am. J. Clin. Nutr. 68 (1998) 258–265.
[19] R.L. Walzem, S. Watkins, E.N. Frankel, R.J. Hansen, J.B. German, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 7460–7464.
[20] M.G. Hertog, E.J. Feskens, P.C. Hollman, M.B. Katan, D. Kromhout, Lancet 342 (1993) 1007–1011.
[21] M.G. Hertog, D. Kromhout, C. Aravanis, H. Blackburn, R. Buzina, F. Fidanza, S. Giampaoli, A. Jansen, A. Menotti, S. Nedeljkovic, et al., Arch. Intern. Med. 155 (1995) 381–386.
[22] M.G. Hertog, E.J. Feskens, D. Kromhout, Lancet 349 (1997) 699.
[23] S.O. Keli, M.G. Hertog, E.J. Feskens, D. Kromhout, Arch. Intern. Med. 156 (1996) 637–642.
[24] P. Knekt, R. Jarvinen, R. Seppanen, M. Hellovaara, L. Teppo, E. Pukkala, A. Aromaa, Am. J. Epidemiol. 146 (1997) 223–230.
[25] M. Furusawa, T. Tanaka, T. Ito, A. Nishikawa, N. Yamazaki, K.-i. Nakaya, N. Matsuura, H. Tsuchiya, M. Nagayama, M. Iinuma, J. Health Sci. 51 (2005) 376–378.
[26] J.P. Hu, M. Calomme, A. Lasure, T. De Bruyne, L. Pieters, A. Vlietinck, D.A. Vanden Berghe, Biol. Trace Elem. Res. 47 (1995) 327–331.
[27] E.E. Deschner, J. Ruperto, G. Wong, H.L. Newmark, Carcinogenesis 12 (1991) 1193–1196.
[28] M. Yoshida, M. Yamamoto, T. Nikaido, Cancer Res. 52 (1992) 6676–6681.
[29] C.D. Stalikas, J. Sep. Sci. 30 (2007) 3268–3295.
[30] A. Sekher Pannala, T.S. Chan, P.J. O'Brien, C.A. Rice-Evans, Biochem. Biophys. Res. Commun. 282 (2001) 1161–1168.
[31] W. Bors, C. Michel, Ann N Y Acad Sci. 957 (2002) 57–69.
[32] C. Stalikas, Y. Fiamegos, V. Sakkas, T. Albanis, J. Chromatogr. A 1216 (2009) 175–189.
[33] T. Hyötyläinen, M.-L. Riekkola, Anal. Chim. Acta 614 (2008) 27–37.
[34] P. Alberts, M.A. Stander, A. de Villiers, J. Chromatogr. A 1235 (2012) 92–102.
[35] Z. Spacil, L. Novakova, P. Solich, Talanta 76 (2008) 189–199.
[36] B. Klejdus, J. Vacek, L. Lojkova, L. Benesova, V. Kuban, J. Chromatogr. A 1195 (2008) 52–59.
[37] D. Guillarme, C. Casetta, C. Bicchi, J.L. Veuthey, J. Chromatogr. A 1217 (2010) 6882–6890.
[38] J. Oszmianski, C.Y. Lee, Am. J. Enol. Vitic. 41 (1990) 204–206.
[39] B. Fuhrman, N. Volkova, A. Suraski, M. Aviram, J. Agric. Food Chem. 49 (2001) 3164–3168.
[40] M.P. Saenz-Navajas, V. Ferreira, M. Dizy, P. Fernandez-Zurbano, Anal. Chim. Acta 673 (2010) 151–159.
[41] M.N. Bravo, S. Silva, A.V. Coelho, L.V. Boas, M.R. Bronze, Anal. Chim. Acta 563 (2006) 84–92.
[42] A. Escarpa, M.D. Morales, M.C. González, Anal. Chim. Acta 460 (2002) 61–72.
[43] C.L. Silva, J. Pereira, V.G. Wouter, C. Giró, J.S. Câmara, Talanta 86 (2011) 82–90.
[44] V. Pereira, J.S. Camara, J. Cacho, J.C. Marques, J. Sep. Sci. 33 (2010) 1204–1215.
[45] V. Pereira, M. Pontes, J.S.C. Ã¢mara, J.C. Marques, J. Chromatogr. A 1189 (2008) 435–443.
[46] E. Boselli, M. Minardi, A. Giomo, N.G. Frega, Anal. Chim. Acta 563 (2006) 93–100.
[47] I. Novak, P. Janeiro, M. Seruga, A.M. Oliveira-Brett, Anal. Chim. Acta 630 (2008) 107–115.
[48] I.O. Vvedenskaya, R.T. Rosen, J.E. Guido, D.J. Russell, K.A. Mills, N. Vorsa, J. Agric. Food Chem. 52 (2004) 188–195.
[49] R. Mandrioli, L. Mercolini, D. Lateana, G. Boncompagni, M.A. Raggi, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 879 (2010) 167–173.
[50] D. Guillarme, D.T. Nguyen, S. Rudaz, J.L. Veuthey, J. Chromatogr. A 1149 (2007) 20–29.
[51] M. Abdel-Rehim, J. Chromatogr. A 1217 (2010) 2569–2580.
[52] Z. Altun, M. Abdel-Rehim, Anal. Chim. Acta 630 (2008) 116–123.
[53] L. Novakova, Z. Spacil, M. Seifrtova, L. Opletal, P. Solich, Talanta 80 (2010) 1970–1979.
[54] M.A. Saracino, G. Lazzara, B. Prugnoli, M.A. Raggi, J. Chromatogr. A 1218 (2011) 2153–2159.
[55] J.O. Fernandes, M.A. Ferreira, J. Chromatogr. A 886 (2000) 183–195.
[56] D. Di Majo, L. La Neve, M. La Guardia, A. Casuccio, M. Giammanco, J. Food Compos. Anal. 24 (2011) 265–269.
[57] L.G. Blomberg, Anal. Bioanal. Chem. 393 (2009) 797–807.
[58] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, 4th ed., Prentice Hall Ptr, Essex, UK, 2005.
[59] V.F. Samanidou, M.K. Nika, I.N. Papadoyannis, J. Sep. Sci. 30 (2007) 2391–2400.
[60] B. Fuhrman, A. Lavy, M. Aviram, Am. J. Clin. Nutr. 61 (1995) 549–554.
[61] Q. Zhang, G.S. Ma, H. Greenfield, X.Q. Du, K. Zhu, D.R. Fraser, Asia Pac. J. Clin. Nutr. 12 (Suppl.) (2003) S46.
[62] A.A. Bertelli, D.K. Das, J. Cardiovasc. Pharmacol. 54 (2009) 468–476.