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Development of a novel microextraction by packed sorbent-based approach followed by ultrahigh pressure liquid chromatography as a powerful technique for quantification phenolic constituents of biological interest in wines

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ABSTRACT

A novel analytical approach, based on a miniaturized extraction technique, the microextraction by packed sorbent (MEPS), followed by ultrahigh pressure liquid chromatography (UHPLC) separation combined with a photodiode array (PDA) detection, has been developed and validated for the quantitative determination of sixteen biologically active phenolic constituents of wine. In addition to performing routine experiments to establish the validity of the assay to internationally accepted criteria (linearity, sensitivity, selectivity, precision, accuracy), experiments are included to assess the effect of the important experimental parameters on the MEPS performance such as the type of sorbent material (C2, C8, C18, SIL, and M1), number of extraction cycles (extract-discard), elution volume, sample volume, and ethanol content, were studied. 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). The wine bioactive phenolics were eluted by 250 μL of the mixture containing 95% methanol and 5% water, and the separation was carried out on a HSS T3 analytical column (100 mm × 2.1 mm, 1.8 μm particle size) using a binary mobile phase composed of aqueous 0.1% formic acid (eluent A) and methanol (eluent B) in the gradient elution mode (10 min of total analysis). The method gave satisfactory results in terms of linearity with $r^2_{\text{-values}} > 0.9986$ within the established concentration range. The LOD varied from 85 ng mL^{-1} (ferulic acid) to 0.32 μg mL^{-1} ((+)-catechin), whereas the LOQ values from 0.028 μg mL^{-1} (ferulic acid) to 1.08 μg mL^{-1} ((+)-catechin). Typical recoveries ranged between 81.1 and 99.6% for red wines and between 77.1 and 99.3% for white wines, with relative standard deviations (RSD) no larger than 10%. The extraction yields of the MEPS$_{C8}$/UHPLC–PDA methodology were found between 78.1 (syringic acid) and 99.6% (*o*-coumaric acid) for red wines and between 76.2 and 99.1% for white wines. The inter-day precision, expressed as the relative standard deviation (RSD%), varied between 0.2% (*p*-coumaric and *o*-coumaric acids) and 7.5% (gentisic acid) while the intra-day precision between 0.2% (*o*-coumaric and cinnamic acids) and 4.7% (gallic acid and (−)-epicatechin). On the basis of analytical validation, it is shown that the MEPS$_{C8}$/UHPLC–PDA methodology proves to be an improved, reliable, and ultra-fast approach for wine bioactive phenolics analysis, because of its capability for determining simultaneously in a single chromatographic run several bioactive metabolites with high sensitivity, selectivity and resolving power within only 10 min. Preliminary studies have been carried out on 34 real whole wine samples, in order to assess the performance of the described procedure. The new approach offers decreased sample preparation and analysis time, and moreover is cheaper, more environmentally friendly and easier to perform as compared to traditional methodologies.

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1. Introduction

The chemical composition present in a wine, cover a large number of metabolites including primary (e.g. sugars, organic acids, amino acids) and secondary metabolites (e.g. polyphenols such as flavonoids, anthocyanins and other pigments). All of these compounds have a strong influence on the quality and character of the wine, and are therefore important not only for the wine characterization, but also, reflects the history of the wine producing process, including the grape variety, the yeast strain, the containers used for fermentation and storage, and the enologic practices. As well as being a good source of vitamin C, dietary fiber, and minerals, berries contain high levels of natural polyphenol components that act as potent antioxidants. Grape and wine extracts, rich in polyphenols,

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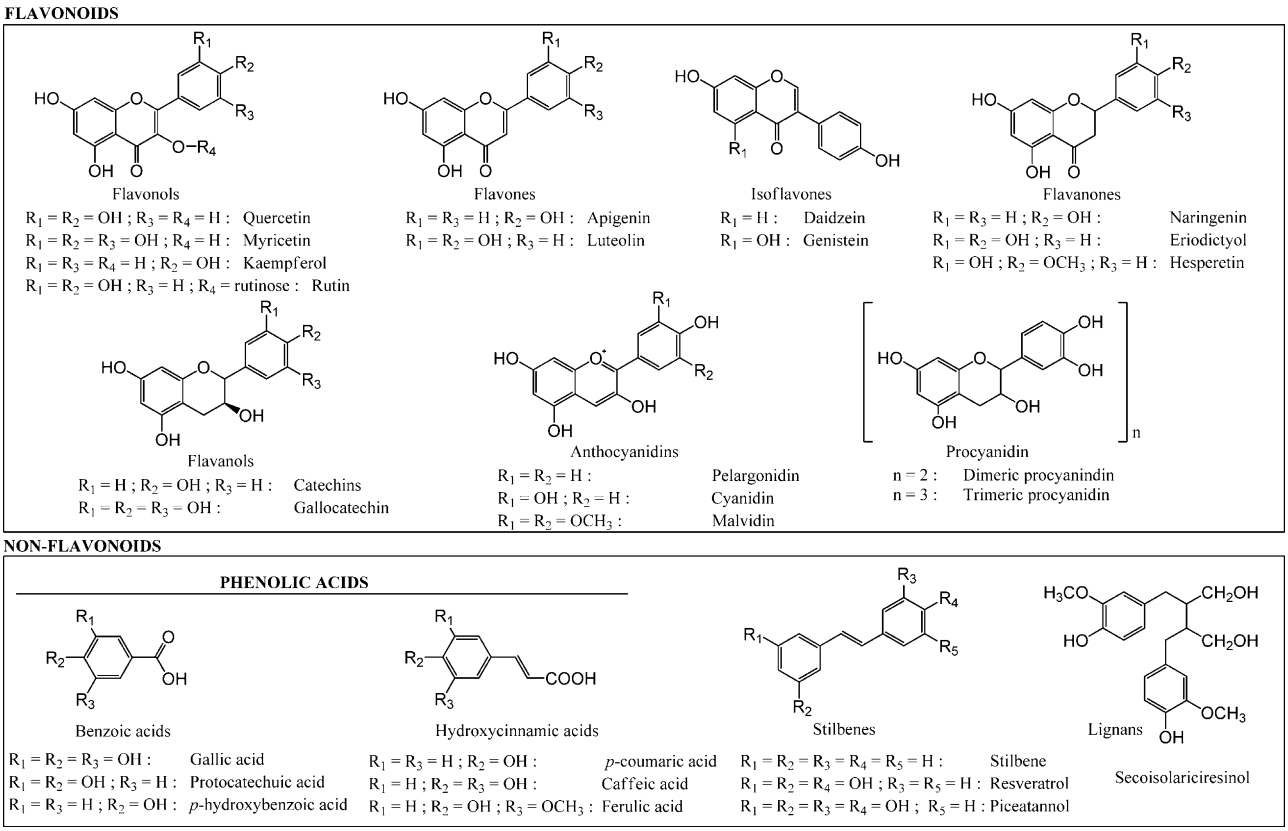

Fig. 1. Chemical structures of the biological active phenolic constituents found in wines.

have a range of biological effects that can have beneficial outcomes on human health [1,2].

Polyphenols arise biogenetically from either the shikimate/phenylpropanoid pathway or 'polyketide' acetate/malonate pathway, or both, producing monomeric and polymeric phenols and polyphenols, which fulfil a very broad range of physiological roles in plants [3]. Apparently act as defense (against herbivores, microbes, viruses or competing plants) and signal compounds (to attract pollinating or seed dispersing animals), as well as protecting the plant from ultraviolet radiation and oxidants [3]. They can be categorized as (i) flavonoids and (ii) nonflavonoid phenolic compounds [4]. To date, several hundreds of different flavonoids have been described and the number continues to increase [5]. This group comprises of 15 carbons, with 2 aromatic rings connected by a 3-carbon bridge (Fig. 1). According to the modifications of the central C-ring, they can be divided into different structural classes including flavonols (represented mainly by quercetin, kaempferol, and myricetin), flavones (represented by apigenin and luteolin), flavan-3-ols (ranging from the simple monomers (+)-catechin and its isomer (−)-epicatechin to the oligomeric and polymeric proanthocyanidins), flavanones, isoflavones, and anthocyanidins.

The main nonflavonoid phenolic compounds (Fig. 1) of dietary significance are the C6–C1 phenolic acids (gallic, p-hydroxybenzoic, protocatechuic, vanillic, and syringic acids), the C6–C3 hydroxycinammates (p-coumaric, caffeic, and ferulic acids, frequently accumulate as their respective tartrate esters, coutaric, caftaric, and fertaric acids) and their conjugated derivatives, and the polyphenolic C6–C2–C6 stilbenes (phytoallexins produced by plants in response to disease, injury, and stress).

In recent years the role of bioactive phenolic compounds and flavonoids as protective dietary constituents has become an increasingly important area of human nutrition research. Unlike the traditional vitamins, they are not essential for short-term well-being, but there is increasing evidence that modest long-term intakes may exhibit a potential for modulating human metabolism in a manner favorable contributing to the beneficial effects of fruit- and vegetable-rich diets. Indeed, there is reasonable evidence from epidemiological studies to support the notion that diets rich in phenolics (derived from fruits and vegetables) are associated with lower risks of cancer, osteoporosis, cardiovascular diseases, cataracts, and diseases associated with brain and immune dysfunction [6–13]. Furthermore, polyphenols from red wine have been reported to exert potent antioxidant effects that prevent low- and very-low density lipoprotein (LDL and vLDL) oxidation (crucial steps in atherosclerotic lesion formation) and DNA bases (relevant to the induction of cancer) by free radicals [14–17], inhibition of platelet aggregation [18–20], inhibition of cell proliferation, migration, and angiogenesis, and despite some reports of the absence of an association [21,22], they are serious candidates to explain the protective effects of vegetable and fruit consumption against several diseases. One possible reason for this protective effect may be the powerful antioxidant and free radical scavenging properties of various classes of phenolic compounds [23].

Developed methods regarding the analysis of biologically active phenolics in wines generally includes extractions with solvents (methanol, ethanol, acetone or mixtures of these with water), cleanup and further fractionation by liquid–liquid extraction (LLE) [24], usually with ethyl acetate [25]; column chromatography (CC) [26] or solid phase extraction (SPE) [27,28]. However, these procedures are quite time-consuming and need relatively high volume of solvent/sample, which is impractical for the routine analyses of many food commodities. The ever-increasing demand for control analysis has contributed markedly to the renewal of interest in miniaturized analytical techniques (MAT) which has taken place over the last years. The MAT had gained attention due to its many special features over classical approaches. Usage of little or no

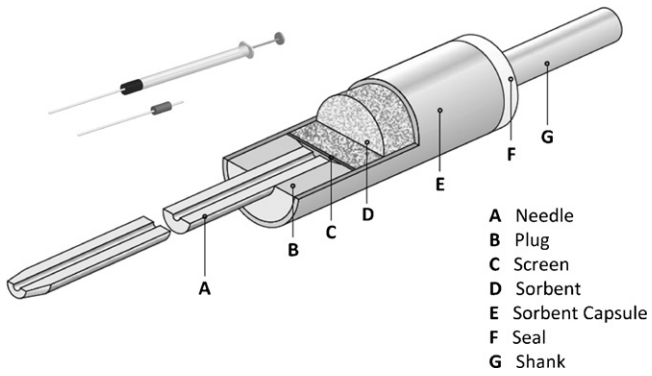

Fig. 2. Schematic diagram of the MEPS syringe and MEPS-BIN from SGE, Analytical Science.
Adapted from Wirth et al. [32].

solvent (reducing exposure of analyst to solvents), increasing sensitivity of analysis and user-friendly system, should be pointed out. In this context the microextraction by packed sorbent (MEPS) appears as a new format for solid-phase extraction (SPE) that has been miniaturized to work with sample volumes as small as 10 μL [29,30]. The commercially available presentation of 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) [30–32] (Fig. 2).

The cartridge bed can be packed or coated to provide selective and suitable sampling conditions. Silica based (C2, C8, 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 sorbent materials. This new technique is very promising because it is fast, simple and it requires very small volume of samples to produce comparable results to conventional SPE technique. Furthermore, this technique can be easily interfaced to LC–MS and GC–MS to provide a completely automated MEPS/LC–MS or MEPS/GC–MS system. This extraction technique (MEPS) could be of interest in clinical, forensic toxicology and environmental analysis areas [29,33–39].

The current available HPLC methods for quantification of bioactive phenolics in several matrices, including wines, have run times in the range of 10–50 min [24,28,40,41]. Recently, ultrahigh pressure liquid chromatography (UHPLC) has become widely spread technique and new trend in separation sciences [33]. Higher separation efficiency of sub-2-μm particle sorbents allows faster chromatographic separation keeping the same resolution compared to HPLC sorbents with conventional particle size [33,42,43]. This is expressed by Knox curves, where lower height equivalent of theoretical plate indicates improved separation efficiency and higher optimum linear velocity, which means decrease in analysis time [33,44].

The current research study describe the development and validation of an ultra-fast, efficient, sensitive, reliable and high throughput MEPS-based methodology in combination with UHPLC–PDA for the simultaneous determination of 16 bioactive phenolic constituents in wines. The UHPLC system used includes a binary solvent manager which delivers up to 15,000 psi pressure, a photodiode array (PDA) detector with a spectra in a range of between 200 and 400 nm, a 1.8 μm particle size column and a sample manager with small injection volume used (2 μL). Compared to LLE and SPE, MEPS saves preparation time and reduces solvent waste significantly. Spiked synthetic wine solution, red wine (Real Lavrador, coded as RL) and white wine (Grão Vasco, coded as GV) were used to evaluate the performance of the developed method. Some factors influencing the MEPS extraction efficiency, such as type of sorbent material, number of extraction cycles (extract-discard), volume of eluent and sample volume, were evaluated and optimized. The method was then applied to 34 wine samples from different producers and geographic origin. To the best of our knowledge no papers has been published for the analysis of bioactive phenolic constituents in wines by MEPS.

2. Experimental

2.1. Reagents, standards and materials

All chemicals were of analytical grade. Methanol (99.9% purity, Sigma–Aldrich), formic acid (Merck), and acetic acid (Riedel-de-Haën) of HPLC gradient were purchased through Labodidáctica-Equipamentos de Laboratório Didácticos, Lda. (Funchal, Portugal). Ethanol absolute (99.5% purity), and sodium hydroxide were obtained from Panreac (Barcelona, Spain). Bioactive phenolic standards, gallic acid, gentisic acid, (−)-epicatechin, *m*-coumaric acid, *o*-coumaric acid, cinnamic acid, cinnamic acid, vanillic acid, *p*-coumaric acid, rutin, ferrulic acid and kaempferol, all from Fluka Biochemica AG (Buchs, Switzerland), and protocatechuic acid, tartaric acid and syringic acid, from Sigma–Aldrich (St. Louis, MO, USA), were supplied through Labodidáctica-Equipamentos de Laboratório Didácticos, Lda. (Funchal, Portugal). Myricetin and syringaldehyde were acquired from Acros Organics (Geel, Belgium) and (+)-catechin, Cayman Chemical Company (Montigny-le-Bretonneux, France). Internal standard, Trolox, was acquired from Fluka Biochemica AG (Buchs, Switzerland). The purity of all polyphenolic standards and internal standard was greater than 95%. 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 eluates were filtered through 0.22 μm PTFE membrane filters (Millipore, Milford, MA, USA) supplied by Via Athena-Gestão de Laboratórios, Lda. (Sacavém, Lisbon, Portugal). The MEPS gas-tight syringe (250-μL) and the BIN (Barrel Insert and Needle) containing the sorbent material from SGE Analytical Science (Melbourne, VIC, Australia) were provided by I.L.C.-Instrumentos de Laboratório e Científicos, Lda. (Lisbon, Portugal). The Waters Acquity UPLC HSS T3 analytical column (100 mm × 2.1 mm, 1.8 μm particle size) was purchased from Via Athena-Gestão de Laboratórios, Lda. (Sacavém, Lisbon, Portugal). A HANNA instruments pH209 pH meter (Woonsocket, USA) was supplied by Labodidáctica-Equipamentos de Laboratório Didácticos, Lda. (Funchal, Portugal).

2.2. Standard preparation and wine samples

Individual stock solutions of each polyphenol tested were prepared in pure methanol at concentration of 1000 μg mL^{-1}, aliquoted in 4 mL vials, and stored at −20 °C, in the dark. Under these conditions they were stable for at least 4 month (as assessed by UHPLC). Working standard solutions containing the 16 polyphenols was prepared daily from the individual stock solutions by diluting them in the synthetic wine (5 g L^{-1} tartaric acid, 12% (v/v) ethanol). This standard was used both to spike the wines in order to perform the assays for optimization of extraction conditions and for calibration study at different concentrations. The chemical structures of the investigated bioactive metabolites are reported in Fig. 1. Calibration standards (1–50 μg L^{-1}) were prepared fresh on the day of the analysis by diluting the appropriate working solutions in synthetic wine solution. The ranges of concentrations were selected

Table 1
Varietal composition, ethanol content and production year of investigated wines.

Wine	Region	Year	Alcohol (%, v/v)	Varietal composition
Red wine				
Esteva	Douro	2009	13	Tinta Roriz, Touriga Franca, Tinta Barroca and Touriga Nacional
JP Azeitão	Setúbal		13	Castelão, Aragonez and Syrah
Terras d'el Rei	Alentejo	2010	13	Trincadeira, Castelão, Moreto and Aragonez
Reguengos	Alentejo	2010	13	Aragonez, Trincadeira and Castelão
Monte Velho	Alentejo	2009	14	Aragonez, Trincadeira and Castelão
Casa de Santar	Dão	2007	13.5	Touriga Nacional, Alfrocheiro and Tinta Roriz
Periquita	Azeitão	2008	13	Castelão, Aragonez and Trincadeira
Contemporal	Dão	2008	12	Touriga Nacional, Touriga Francesa, Tinta Roriz and Tinta Barroca
Frei Bernardo	Beiras	2009	12.5	Rufete, Marufo and Tinta Roriz
Real Lavrador	Alentejo	2010	13	Castelão
Porca da Murça	Douro	2009	13	Touriga Nacional, Tinta Roriz, Toutiga Francesa and Tinto Cão
Adega de Borba	Alentejo	2009	14	Aragonez, Tincadeira and Alicante Bouschet
Fonte da Serrana	Alentejo	2009	14	Aragonez, Trincadeira, Cabernet Sauvignon and Alicante Bouschet
Pelão	Douro	2009	12	Baga and Touriga Nacional
Terras Altas	Dão	2007	12	Bastardo, Jean, Touriga National and Alfrocheiro
Grão Vasco (GV)	Dão	2008	13	Jaen, Tinta Roriz and Touriga Nacional
Torcaz	Madeira	2004	12	Tinta Negra Mole
TMR1	Madeira	2009		
Terras de Lava	Azores	2009	13	Merlot, Cabernet Sauvignon, Saborinho and Syrah
Terras de Lava	Azores	2010	13	Merlot, Cabernet Sauvignon, Saborinho and Syrah
Basalto	Azores	2009	12	Periquita, Agronómica and Saborinho
Basalto	Azores	2010	12	Periquita, Agronómica and Saborinho
White wine				
Real Lavrador (RL)	Alentejo	2010	12.5	Roupeiro and Rabo-de-ovelha
Vidigueira	Alentejo	2009	13.5	Antão Vaz, Manteúdo, Perrum and Roupeiro
Navegante	Alentejo	2009	13	Antão Vaz, Manteúdo, Perrum and Roupeiro
Planalto	Douro	2009	12.5	Malvasia Fina, Viosinho, Gouveio and Códega
Periquita	Azeitão	2008	12	Moscatel and Arinto
Grão Vasco	Dão	2009	13	Encruzado, Bical and Malvasia Fina
Viñatigo Gual	Canary	2005	13	Albillo Real
TMW1	Madeira	2010		
TMW2	Madeira	2010		
TMW3	Madeira	2010		
Lajido	Açores	1999	16.5	Arinto, Verdelho and Terrantez
VG-Verdelho	Madeira	2004		

in function of sensitivity of the UHPLC–PDA towards each bioactive phenolic compound. Physical-chemical characteristics affect the analytical signal and for some compounds higher concentrations had to be used in order to detected the compound. Trolox was used as internal standard (IS).

Thirty-four representative commercial table wines available from different regions of Portugal, and Macaronesia Islands (Madeira, Azores and Canary), which were produced according to standard procedures and defined varietal composition, were analyzed. The list of the studied wines, production year and their varietal composition are summarized in Table 1.

All samples were taken from bottled wines ready for sale and stored at −20 °C.

Synthetic wine samples were prepared by an ethanol solution at 12% (v/v) to which 5 g L^{-1} tartaric acid were added. Solution pH was adjusted to 3.4 with 1 M sodium hydroxide.

Wine samples and synthetic wines were dealcoholized under vacuum at 40 °C, up to ¼ of initial volume, in order to avoid interferences of ethanol. The volume of dealcoholized extracts was adjusted to initial sample volume with water and the pH adjusted to 3.4 with 30% (v/v) acetic acid. All samples were analyzed in triplicate.

2.3. Optimization of the factors affecting the performance of MEPS

The MEPS procedure was carried out by means of a SGE Analytical Science (I.L.C., Lisbon, Portugal) apparatus, consisting of a 250-μL gas-tight syringe with a removable needle. The syringe was fitted with a BIN (Barrel Insert and Needle) 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 bioactive phenolics was used to optimize the MEPS procedure. Several important MEPS-influencing extraction factors such as the type of sorbent material, number of extraction cycles, elution volume, sample volume, and ethanol content, were evaluated.

Selection of sorbent is an important factor to achieve acceptable clean-up and get high extraction yield [30]. Therefore the performance of the five MEPS sorbent materials: C2 (ethyl-silica), C8 (octyl-silica), C18 (octadecyl-silica), SIL (unmodified silica) and M1 (a mixed mode sorbent containing 80% C8 and 20% strong cationic exchange (SCX)) was tested and compared, in order to select the best sorbent for the target analytes. C2–C8 phases are suitable for lipophilic analytes (non-polar) and polymeric phases such as polystyrene–divinylbenzene or mixed mode phases (anion–cation exchange mode) are suitable for polar analytes such as acidic and basic compounds. 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 once, five and ten 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 μL s^{-1} to prevent cavitation. This will increase analyte/sorbent contact time and extraction efficiency. In order to check the influence of ethanol content, a synthetic wine with 12% (v/v) of ethanol content was compared to dealcoholized synthetic wine extract. The dealcoholized extract was adjusted to initial volume using solutions with different alcoholic content: (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 it was used as eluent in the UHPLC system, we select the methanol to evaluate the effect of alcoholic content on extraction efficiency. All optimization procedures were carried out in triplicate.

2.3.1. MEPS procedure for bioactive phenolic constituents

The precision engineering used in the design and manufacture of MEPS allows the same functions as SPE, such as the removal of interfering matrix components and the selective isolation and concentration of analytes. MEPS experiments were conducted using 4 mg of solid-phase material (C8 sorbent selected, in the optimization step, as the best sorbent to isolate the target analytes). Before being used for the first time, the sorbent was manually conditioned first with 100 μL methanol and then with 100 μL water (0.1% formic acid). This step activates the sorbent and ensures reproducible retention of the analytes [30]. 250 μL samples were passed through the C8 sorbent five times at a flow rate of about 20 $\mu L\,s^{-1}$. 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 $\mu L\,s^{-1}$. The analytes were then 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. 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 the internal standard (IS).

2.4. UPLC–PDA analysis and operating conditions

Besides maximum enrichment performance by MEPS, the determination of the target analytes, in small sample volumes requires a sensitive detection method. The analysis of bioactive secondary metabolites was carried out on a Waters Ultra Performance Liquid Chromatographic Acquity system (UPLC, Acquity H-Class) (Milford, MA, USA) combined with a Waters Acquity quaternary solvent manager (QSM), an Acquity sample manager (SM), a column heater, a 2996 PDA detector, and a degassing 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 at 0.1% formic acid, and (B) methanol, with a constant flow rate of 250 $\mu L\,min^{-1}$, giving a maximum back pressure of 6000 psi, which is within the capabilities of the UPLC. 2 μL of extracts were injected into the Waters Acquity UPLC system, equipped with an Acquity UPLC™ strength silica HSS T3 analytical column (1.8 μm particle size, 2.1 mm × 100 mm) and protected with an Acquity UPLC™ HSS T3 Van Guard™ Pre-column (Waters, Milford, MA, USA); column temperature was thermostated at 40 °C and the samples were kept at 4 °C in the sample manager. The 5 min gradient was as follows: 80% A (0 min); 80–70% A (0.50 min); 68% A (1 min); 20% A (8 min); and 80% A (10 min), followed by a re-equilibration time of 3 min, for bringing the column to the initial conditions after gradient analysis, given a total run time of 13 min. All solvents and samples were filtered through 0.22 μm membrane filters from Millipore (Millipore, Milford, MA, USA), before use. For quantification purposes the PDA detection was conducted by using four distinct channels that were set to the maximum absorbance wavelength of each polyphenol, as indicated in Table 2. They were identified by comparing the retention time and spectral characteristics of their peaks with those of

Table 2
Peak number, retention time (RT), UV–vis data, and calibration parameters for the validation of sixteen bioactive phenolic compounds on synthetic wine, by the newly developed methodology ($MEPS_{C8}$/UHPLC–PDA).

Peak number	RT (min)	Bioactive phenolics	λ_{max}[a] (nm)	Analytical performance						
				Conc. Range ($\mu g\,mL^{-1}$)	Regression equation	r^2[b]	LOD[c] ($\mu g\,L^{-1}$)	LOQ[d] ($\mu g\,L^{-1}$)	% Matrix effect	
									Red wine	White wine
1	1.894	Gallic acid	270	1–50	0.0015x[e] − 0.0005	0.9989	0.22	0.73	87.3	93.3
2	3.045	Protocatechuic acid	260	1–50	0.057x + 0.0034	0.9986	0.036	0.12	84.4	87.7
3	3.333	(+)-Catechin	278	2–50	0.0014x + 0.0017	0.9993	0.32	1.08	95.7	81.4
4	4.030	Gentisic acid	325	1–30	0.0028x − 0.0012	0.9992	0.085	0.28	90.7	90.7
5	4.256	(−)-Epicatechin	280	1–25	0.002x + 0.0017	0.9980	0.18	0.59	95.0	90.0
6	4.507	Vanillic acid	260	1–50	0.0193x + 0.0027	0.9994	0.044	0.15	84.8	88.8
7	4.695	Syringic acid	277	1–25	0.0226x − 0.0029	0.9998	0.050	0.17	86.6	95.7
8	5.167	Syringaldehyde	308	1–50	0.0334x + 0.0043	0.9997	0.070	0.23	93.1	87.7
9	5.555	*p*-Coumaric acid	308	1–40	0.0686x − 0.0432	0.9990	0.013	0.043	83.5	79.2
10	5.775	Ferulic acid	323	1–25	0.0643x − 0.009	0.9998	0.0085	0.028	90.0	91.8
11	6.181	*m*-Coumaric acid	278	1–40	0.0653x − 0.0427	0.9991	0.028	0.093	89.1	88.4
12	6.360	Rutin	256	1–25	0.0542x + 0.0464	0.9958	0.15	0.50	87.2	102.6
13	6.781	*o*-Coumaric acid	276	1–40	0.0659x − 0.0359	0.9993	0.026	0.088	86.0	90.6
14	7.029	Myricetin	372	1–30	0.0699x − 0.0084	0.9995	0.078	0.26	112.6	110.4
15	8.112	Cinnamic acid	277	1–40	0.1617x − 0.0468	0.9995	0.026	0.088	99.6	102.0
16	8.965	Kaempferol	366	1–25	0.1659x + 0.0049	0.9993	0.022	0.074	97.6	87.6
17	9.178	Trolox	289	50	–	–	–	–	–	–

[a] Maximum absorbance values obtained in PDA system detection.
[b] Correlation coefficient, give an estimating how well the experimental points fit a straight line.
[c] Limit of detection.
[d] Limit of quantification. Values obtained from ordinary least-squares regression data.
[e] x = analyte concentration.

Table 3
Accuracy, extraction yield, intra- and inter-day performance, obtained for bioactive phenolic compounds by using $MEPS_{C8}$/UHPLC–PDA methodology.

Bioactive phenolics	Fortification level ($\mu g\,mL^{-1}$)		Accuracy (%)		Extraction yield (%)		RSD (%)	
			Red wine	White wine	Red wine	White wine	Intra-day ($n=7$)	Inter-day ($n=25$)
Gallic acid	LL[a]	1	85.7	82.8	84.3	86.4	4.7	5.9
	ML[a]	30	89.9	94.2	89.4	94.5	3.2	4.0
	HL[a]	50	95.1	96.7	80.1	96.8	2.6	1.4
Protocatechuic acid	LL	1	83.1	86.6	88.8	82.3	1.1	1.9
	ML	20	95.0	87.0	95.2	88.5	1.2	1.6
	HL	50	98.9	95.6	98.9	95.8	1.6	0.7
(+)-Catechin	LL	2	89.2	80.2	82.4	87.7	3.5	7.5
	ML	20	91.3	83.6	79.3	79.1	2.2	2.5
	HL	50	88.9	88.7	80.9	89.8	1.9	1.5
Gentisic acid	LL	1	87.9	82.0	81.9	85.2	3.2	4.0
	ML	10	90.1	89.6	91.0	83.0	1.7	1.0
	HL	30	97.1	93.7	97.2	94.1	1.5	1.2
(−)-Epicatechin	LL	1	91.2	94.9	83.0	95.2	4.7	5.6
	ML	10	87.4	98,0	85.4	98.0	1.3	1.8
	HL	25	89.1	99.1	82.7	99.1	1.1	1.6
Vanillic acid	LL	1	89.0	82.4	86.3	85.0	0.7	1.4
	ML	20	80.7	94.9	83.8	95.1	0.7	0.8
	HL	50	93.7	98.1	94.0	98.1	2.0	0.4
Syringic acid	LL	1	80.3	78.9	85.8	76.2	1.6	1.8
	ML	10	86.9	84.1	78.1	86.3	0.6	0.7
	HL	25	91.6	91.7	84.4	92.3	0.6	0.6
Syringaldehyde	LL	1	95.8	89.6	80.5	90.6	2.0	1.3
	ML	20	95.9	97.8	96.1	97.9	1.6	0.9
	HL	50	98.6	99.3	98.6	99.3	2.4	0.6
p-Coumaric acid	LL	1	85.8	86.0	90.9	91.0	0.5	0.9
	ML	20	88.6	93.7	89.7	94.0	0.4	0.2
	HL	40	94.7	97.1	95.0	97.2	0.4	0.3
Ferulic acid	LL	1	94.0	85.3	94.4	89.1	0.3	1.3
	ML	10	90.3	93.3	91.1	93.7	0.5	0.7
	HL	25	95.3	96.6	95.5	96.7	0.3	0.4
m-Coumaric acid	LL	1	81.3	92.2	83.0	92.8	1.0	1.0
	ML	20	87.8	97.9	81.8	98.0	0.4	0.4
	HL	40	89.6	99.0	90.6	99.0	0.2	0.3
Rutin	LL	1	82.6	81.4	83.1	89.3	0.5	3.8
	ML	10	84.7	93.6	93.4	94.0	0.4	2.9
	HL	25	90.1	96.6	88.8	96.7	0.6	0.5
o-Coumaric acid	LL	1	93.4	77.1	93.8	81.4	0.8	0.9
	ML	20	99.0	94.7	99.0	95.0	0.7	0.2
	HL	40	99.6	97.6	99.8	97.7	0.8	0.3
Myricetin	LL	1	87.0	91.8	81.4	82.4	0.6	2.6
	ML	10	81.1	81.5	84.1	84.4	0.5	1.4
	HL	30	92.4	94.8	92.9	95.1	0.6	0.18
Cinnamic acid	LL	1	85.1	86.9	84.1	81.2	0.5	0.9
	ML	20	90.6	94.7	91.4	95.0	0.2	0.5
	HL	40	94.9	97.4	95.2	97.5	0.4	5.7
Kaempferol	LL	1	94.2	82.0	94.6	87.5	0.4	0.7
	ML	10	99.0	80.4	99.0	83.6	0.4	0.6
	HL	25	99.4	91.6	99.4	92.2	0.3	0.5

[a] Concentration levels used in $MEPS_{C8}$/UHPLC–PDA validation studies: LL-low level; ML-medium level; HL-high level.

standards and they were quantified using the standards of each one.

2.5. Method validation design

The newly developed $MEPS_{C8}$/UHPLC-DAD approach was fully validated in terms of selectivity, linearity, limits of detection (LOD) and quantification (LOQ), inter- and intra-day precisions, accuracy and extraction efficiency (Table 2). The assays were carried out at Laboratory of Analytical Chemistry and Enology (Centro de Química da Madeira) using the C8 sorbent and a Waters Acquity H-Class equipped with a HSS T3 analytical column (100 mm × 2.1 mm, 1.8 μm particle size).

The selectivity of the method was assessed by the absence of interfering peaks at the elution times of the bioactive metabolites. The linearity of the analytical method was evaluated building three calibration curves (the peak $area_{analyte}$/peak $area_{IS}$ ratio obtained were plotted against the corresponding standard concentration) for each bioactive phenolic using standards, prepared in synthetic wine from individual stock solutions, at 7 different concentrations levels, including the zero point (Table 3). The zero point (unspiked 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 of the method 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 [45]. These parameters were calculated for each analyte from the standard solutions used to obtain the corresponding calibration curves, using the UHPLC developed method.

Precision is a function of concentration and it was calculated by dividing the standard deviation (SD) by the means of concentration to obtain the coefficient of variation (C.V.), which when expressed on a percentage basis gives the relative standard deviation (RSD). The use of the RSD values, which describes the closeness of agreement between series of measurements, facilitates comparisons of variabilities at different concentration. Method precision was evaluated 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 (Table 3) of each phenolic compound and treated by $MEPS_{C8}$. Seven replicates ($n = 7$) was performed in the same day to obtain repeatability (intra-day precision). For inter-day precision (intermediate precision—IP) evaluation, the same protocol was followed but six replicates of each level were analyzed daily through three different days ($n = 25$; 7 + 18).

In order to check the accuracy of the proposed method a recovery study was carried out by spiking a red wine (RL) and a white wine (GV), in triplicate at three concentration levels corresponding to the LL, ML and HL (Table 3), and subjected to the $MEPS_{C8}$ procedure described in Section 2.3. The RL and GV wines phenolics concentration was previously determined by $MEPS_{C8}$, and the recovery values were calculated according to the following formula: $\text{Accuracy} = 100 \times ([\text{analyte}]_{\text{after spiking}} - [\text{analyte}]_{\text{before spiking}})/[\text{analyte}]_{\text{added}}$; where $[\text{analyte}]_{\text{after spiking}}$ is the analyte concentration measured in spiked wine; $[\text{analyte}]_{\text{before spiking}}$ is the analyte concentration measured in unspiked wine, and $[\text{analyte}]_{\text{added}}$ is the nominal concentration of the analyte added to wine. Extraction efficiency (EE) was determined by replicate analysis ($n = 3$) of synthetic wine spiked with bioactive phenolics at three concentration levels (LL, ML, and HL; see Table 3) and subjected to $MEPS_{C8}$ procedure (C_{SW_MEPS}); a second set of different aliquots of synthetic wine was subjected to $MEPS_{C8}$ and the extracts spiked with bioactive phenolics at LL, ML, and HL concentration levels (C_{SW}). The peak area ratio obtained for spiked synthetic wine matrix before and after $MEPS_{C8}$ was used to calculate the corresponding concentration through regression analysis (interpolation of signals in calibration graphs). The EE was then calculated as follow: $\%EE = (C_{SW\_MEPS}/C_{SW}) \times 100$, where C_{SW_MEPS} and C_{SW} represents the concentration of the bioactive phenolics before and after $MEPS_{C8}$ procedure.

The matrix effect was evaluated by the percentage of the quotient between the slopes of the standards in synthetic wine and those obtained by spiking RL and GV wines (standard addition method).

3. Results and discussion

3.1. Optimization of the MEPS procedure

To optimize microextraction by packed sorbent, factors affecting the recovery such as nature of sorbent, number of extraction cycles, elution volume, sample volume, and ethanol content, were carefully investigated. Selection of sorbent is important to achieve acceptable clean-up and extraction yield, therefore the performance of different kinds of sorbents such as C2, C8, C18, SIL and M1 (mixed mode C8 + SCX) was evaluated. Fig. 3a shows the UHPLC–PDA response for the target analytes using different sorbents.

Each MEPS sorbent was evaluated in terms of extraction efficiency and reproducibility. M1, C2 and C8 sorbents (Fig. 3a) provided the best extraction yields, however C8 sorbent was chosen for the MEPS procedure because provided better results for extraction yield and precision compared to both M1 and C2 sorbents. In addition the C8 sorbent showed good reproducibility and stability for repeated use. It was used for more than 100 extractions without loss of extraction power. On the other hand, the SIL sorbent (silica) gave low extraction yields of analytes. The influence of alcoholic content on the MEPS extraction efficiency was evaluated by testing different solutions to adjust the dealcoholized wine extract to initial volume and comparing them with the efficiency obtained by using synthetic wine with 12% (v/v) of ethanol. The results showed that the best efficiency was obtained when ultra-pure water was used to adjust the dealcoholized wine extract volume to initial sample volume.

In MEPS the retention of the analytes to the sorbent phase is affected by the number of extraction cycles performed and the speed applied. Practically, an aliquot of the volume of the sample can be drawn up and down through the syringe, once or several times (cycles) without discarding it. The multiply extraction cycles can be carried out from the same aliquot (draw–eject in the same vial) or by draw up from aliquot and discard in waste (extract–discard). The last option was selected in this study. The influence of the number of extraction cycles and sample volume on extraction efficiency of bioactive phenolic compound studied is illustrated in Fig. 3b. 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. 3b.

Statistically, no significant differences were observed between once, five and ten times of 250 μL sample wine are passed though the C8 sorbent. For this reason 5× 250 μL was selected, since the results showed that five extraction cycles gave a good recovery and can extend the lifetime of the MEPS cartridge. Fig. 3c shows the UHPLC–PDA response for different elution volumes, namely 50 μL, 100 μL, 250 μL, 350 μL, and 500 μL. The results showed that a volume of 50 μL is enough to obtain the highest extraction efficiency.

3.2. Method validation

To demonstrate the feasibility of the present approach for determination of wine bioactive phenolics and to test its practicability, the method was fully validated considering the linearity, limit of detection (LOD), limit of quantification (LOQ), extraction yield, accuracy and intra/inter-day precision. These parameters were calculated for each bioactive phenolic using concentrations usually found in wines. The validation parameters are shown in Tables 2 and 3.

For each compound a linear regression of the peak $\text{area}_{\text{analyte}}/\text{paek area}_{\text{IS}}$ vs concentration, was calculated to determine the linearity of the method using three replicates at seven levels of concentration (Table 2).

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 values for all compounds tested ($r^2 > 0.9986$). The calibration was performed by the use of synthetic wine-matched calibration standards prepared as described in Section 2.2. The quantitation of the samples was performed using the means of equation of

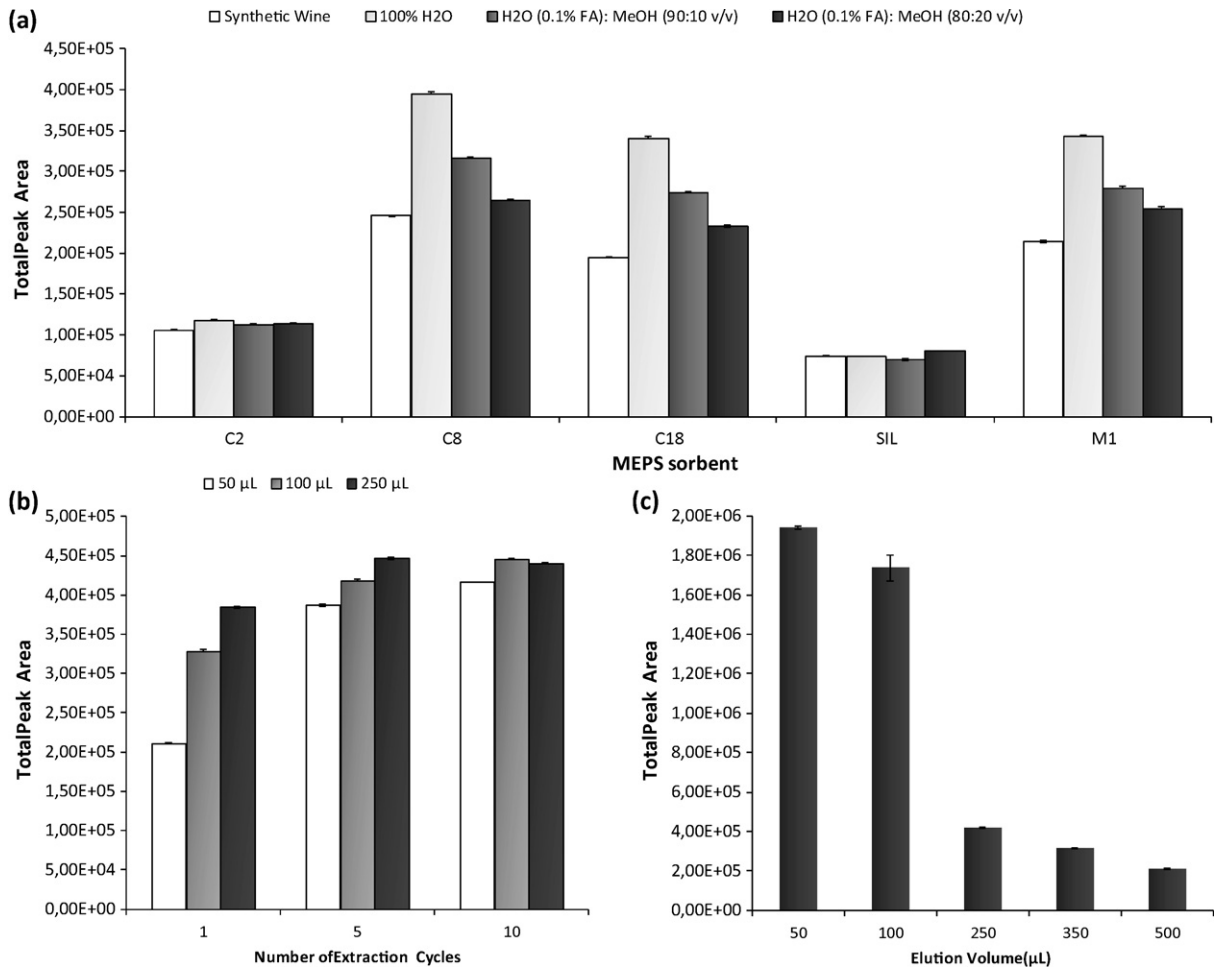

Fig. 3. (a) Comparison of the performance of five different MEPS sorbents and the influence of the alcoholic content; (b) effect of number of extraction cycles (extraction-discard) and sample volume; and (c) influence of different elution volumes; on the extraction efficiency of bioactive phenolic compounds in wines by MEPS. Values expressed as mean ± standard deviation ($n = 3$).

calibration curves, obtained from ordinary least-squares regression data (Table 2).

The limits of detection (LOD) and limits of quantification (LOQ) (corresponding to the bioactive phenolic amount for which the area is equal to 3 times and 10 times the chosen standard deviation, respectively) were calculated from ordinary least squares regression data [46]. The standard deviation chosen to calculate the LOD and LOQ values is the residual standard deviation of the regression line for all bioactive phenolic compounds in the analyzed matrix. As it can be seen in Table 2, the $MEPS_{C8}$/UHLPC–PDA methodology gave in general very low detection limits, ranging between 0.0085 and 0.32 $\mu g\,mL^{-1}$, while limits of quantification ranged from 0.028 to 1.08 $\mu g\,mL^{-1}$, for ferulic acid and (+)-catechin, respectively.

The percentage of matrix effect was evaluated through the percentage of the quotient between the slopes of the standards obtained in synthetic wine and those obtained from spiked RL (red) and GV (white) wines (standard addition method). Matrix effect values ranged from 79.2 to 110.4% for white wine and from 83.5 to 112.6% for red wine, therefore no significant matrix effect was observed (Table 2).

In order to evaluate the accuracy of the method, a recovery study was carried out by spiking RL red wine and GV white wine samples at three concentration levels, with a known amount of each bioactive phenolic (Table 3). The concentration of phenolics added to the wines was chosen to cover the expected values in the wine samples. The accuracy was determined according to the equation presented *in* Section 2.5.

The mean accuracy of the bioactive polypenols for each fortification level is listed in Table 3. At all concentration levels, satisfactory results were found with recovery values ranging from 81.1 (myricetin at 10 $\mu g\,mL^{-1}$) to 99.6% (*o*-coumaric acid at 40 $\mu g\,mL^{-1}$) for red wines, and between 77.1 (*o*-coumaric acid at 1 $\mu g\,mL^{-1}$) and 99.3% (syringaldehyde at 50 $\mu g\,mL^{-1}$). The extraction yields (absolute recovery) when using MEPS single pushing and pulling off 250 µL of sample through the C8 sorbent mode was examined. The results showed an average extraction yield of about 89.2% for red wine and 91.3% for white wine at all concentration levels investigated (Table 3). Furthermore, the absolute extraction yield increase slightly from low concentration level (86.1% for red wines, and 86.4% for white wines) to medium (89.2% red wines, and 91.3% for white wines) and high concentration level (92.1% for red wines, and 96.4% for white wines). Therefore, low concentration of bioactive phenolic compounds results in lower extraction yields than the obtained to the middle and high fortification level. The reason for this may be due to the fact that the surface chemistry of sorbent can be changed by interfering compounds from wine and therefore sorption properties can be changed and may mixed retention mechanism was involved [31]. 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.

Table 4
Concentration[a] of bioactive phenolic constituents in studied wines (values obtained were not corrected by the recoveries).

	Concentration															
	Gal. ac.[b]	Prot. ac.[b]	Cat.[b]	Gent. ac.[b]	Epicat.[b]	Van. ac.[b]	Syr. ac.[b]	Syr.[b]	*p*-Coum. ac.[b]	Fer.[b]	*m*-Coum. ac.[b]	Rut.[b]	*o*-Coum. ac.[b]	Myr.[b]	Cin. ac.[b]	Kaemp.[b]
Red wines																
Real Lavrador	12.8 ± 0.1	–[c]	15.6 ± 0.3	–	1.2 ± 0.1	–	2.6 ± 0.02	–	2.4 ± 0.02	–	–	4.3 ± 0.1	–	3.2 ± 0.04	–	<LOQ[d]
Fonte da Serrana	13.5 ± 0.3	–	11.5 ± 0.8	–	1.4 ± 0.1	–	2.6 ± 0.02	–	2.4 ± 0.02	–	–	2.3 ± 0.1	–	5.8 ± 0.2	–	0.4 ± 0.001
Torcaz	10.2 ± 0.2	0.2 ± 0.008	–	–	–	1.6 ± 0.02	4.2 ± 0.02	–	1.6 ± 0.008	–	–	<LOQ	–	–	–	–
Pelão	10.5 ± 0.1	–	8.1 ± 0.1	–	1.3 ± 0.03	–	3.1 ± 0.04	–	1.6 ± 0.01	–	–	1.7 ± 0.1	–	3.0 ± 0.03	–	0.3 ± 0.004
JP Azeitão	12.5 ± 0.1	–	15.1 ± 0.2	–	0.9 ± 0.001	–	2.5 ± 0.001	–	1.9 ± 0.004	–	–	2.8 ± 0.01	–	4.4 ± 0.03	–	0.2 ± 0.001
Porca de Murça	9.7 ± 0.1	–	10.5 ± 0.6	–	0.7 ± 0.0001	–	1.9 ± 0.02	–	1.5 ± 0.04	–	–	4.4 ± 0.05	–	6.7 ± 0.04	–	0.8 ± 0.003
Periquita	10.5 ± 0.05	–	9.2 ± 0.2	–	2.4 ± 0.04	–	3.8 ± 0.02	–	2.0 ± 0.008	–	–	3.3 ± 0.05	–	2.2 ± 0.04	–	–
Contemporal	9.6 ± 0.2	–	8.4 ± 0.1	–	3.4 ± 0.01	–	1.8 ± 0.01	–	1.7 ± 0.04	–	–	4.0 ± 0.002	–	3.8 ± 0.04	–	0.4 ± 0.01
Reguengos	8.7 ± 0.02	–	10.0 ± 0.05	–	–	–	2.4 ± 0.01	–	1.8 ± 0.003	–	–	4.2 ± 0.02	–	1.8 ± 0.01	–	0.2 ± 0.001
Frei Bernardo	22.2 ± 0.2	–	19.5 ± 0.1	–	3.5 ± 0.04	–	1.5 ± 0.01	–	–	–	–	3.7 ± 0.06	–	3.8 ± 0.004	–	0.5 ± 0.002
Grão Vasco	6.8 ± 0.1	–	11.7 ± 0.1	–	<LOQ	–	2.1 ± 0.01	–	2.3 ± 0.002	–	–	1.4 ± 0.03	–	8.1 ± 0.01	–	<LOQ
Casa de Santar	9.7 ± 0.1	–	10.7 ± 0.02	–	2.2 ± 0.05	–	3.9 ± 0.01	–	1.9 ± 0.002	–	–	3.9 ± 0.03	–	2.2 ± 0.004	–	0.6 ± 0.002
Adega de Borba	10.0 ± 0.1	–	11.8 ± 0.02	–	0.7 ± 0.001	–	2.4 ± 0.01	–	2.5 ± 0.02	–	–	5.0 ± 0.04	–	4.5 ± 0.002	–	0.9 ± 0.005
Terras Altas	12.4 ± 0.4	–	7.2 ± 0.0001	–	2.4 ± 0.04	–	3.1 ± 0.002	–	2.2 ± 0.01	–	–	0.8 ± 0.009	–	2.2 ± 0.02	–	0.1 ± 0.001
TMR1	19.1 ± 0.04	–	10.0 ± 0.05	–	0.9 ± 0.0001	–	1.7 ± 0.003	–	–	–	–	1.6 ± 0.02	–	3.2 ± 0.004	–	0.1 ± 0.0002
Esteva	6.2 ± 0.2	–	8.6 ± 0.1	–	–	–	2.8 ± 0.01	–	1.8 ± 0.001	–	–	1.8 ± 0.001	–	3.0 ± 0.001	–	0.2 ± 0.001
Monte Velho	5.6 ± 0.1	–	9.0 ± 0.02	–	<LOQ	–	2.8 ± 0.001	–	1.7 ± 0.001	–	–	4.2 ± 0.001	–	3.8 ± 0.003	–	0.8 ± 0.001
Terras d'el Rei	8.5 ± 0.1	–	9.7 ± 0.01	–	0.8 ± 0.001	–	3.7 ± 0.01	–	1.9 ± 0.004	–	–	3.8 ± 0.005	–	1.2 ± 0.004	–	0.1 ± 0.001
TLT 2009	22.4 ± 0.2	0.2 ± 0.001	20.0 ± 0.07	–	8.5 ± 0.1	–	2.7 ± 0.01	–	2.8 ± 0.004	1.2 ± 0.02	–	3.4 ± 0.005	–	6.7 ± 0.03	–	<LOQ
TLT 2010	29.1 ± 0.4	–	21.3 ± 0.2	–	8.9 ± 0.2	1.1 ± 0.01	1.7 ± 0.01	–	–	–	–	3.1 ± 0.06	–	5.1 ± 0.04	–	0.5 ± 0.01
Basalto 2009	23.7 ± 0.5	0.2 ± 0.003	16.6 ± 0.1	–	6.6 ± 0.02	–	1.6 ± 0.005	–	2.6 ± 0.03	–	–	2.4 ± 0.02	–	5.2 ± 0.04	–	<LOQ
Basalto 2010	23.0 ± 0.1	–	31.4 ± 0.2	–	7.3 ± 0.2	2.1 ± 0.01	1.8 ± 0.01	–	2.2 ± 0.03	–	–	<LOQ	–	2.8 ± 0.006	–	0.6 ± 0.01
White wines																
Grão Vasco	1.9 ± 0.01	–	3.2 ± 0.2	–	<LOQ	–	–	–	1.7 ± 0.001	0.7 ± 0.0006	–	<LOQ	–	–	–	–
Vidigueiro	1.7 ± 0.03	–	1.6 ± 0.1	–	<LOQ	–	–	–	–	0.3 ± 0.001	–	3.4 ± 0.01	–	–	–	0.3 ± 0.002
Navegante	2.5 ± 0.01	–	3.8 ± 0.2	–	<LOQ	–	–	–	–	–	–	3.1 ± 0.02	–	–	–	–
Planalto	1.5 ± 0.03	–	<LOQ	–	<LOQ	–	–	–	2.1 ± 0.002	0.6 ± 0.002	–	<LOQ	–	–	–	–
Real Lavrador	17.0 ± 0.05	–	–	–	–	–	–	–	1.5 ± 0.002	0.5 ± 0.002	–	<LOQ	–	–	–	–
Periquita	1.0 ± 0.03	<LOQ	–	–	–	–	–	–	–	0.4 ± 0.001	–	–	–	–	–	–
TMW1	–	<LOQ	–	–	–	–	–	–	0.9 ± 0.002	0.5 ± 0.001	–	0.6 ± 0.03	–	–	–	–
TMW2	–	<LOQ	–	–	<LOQ	–	–	–	0.8 ± 0.01	0.3 ± 0.0003	–	<LOQ	–	–	–	–
Viñatigo Gual	–	<LOQ	–	–	–	–	–	–	1.5 ± 0.003	0.4 ± 0.0003	0.7 ± 0.001	–	–	–	–	–
TMW3	3.6 ± 0.03	–	5.9 ± 0.02	–	<LOQ	0.6 ± 0.01	0.9 ± 0.002	–	–	–	–	0.9 ± 0.02	–	0.4 ± 0.002	–	–
Lajido	4.3 ± 0.04	<LOQ	–	–	–	–	–	–	3.0 ± 0.003	0.5 ± 0.004	–	–	–	–	–	–
VG-Verdelho	–	<LOQ	–	–	–	0.3 ± 0.002	1.0 ± 0.004	0.8 ± 0.004	1.0 ± 0.001	–	–	<LOQ	–	0.4 ± 0.0004	–	–

[a] The content of each of the 16 polyphenols analysed in the wine samples tested is the mean of three replicates ± SD and indicated as $\mu g\,mL^{-1}$.

[b] Gal. ac. (gallic acid), Prot. ac. (protocatechuic acid), Cat. ((+)-catechin), Gent. (gentisic acid), Epicat. ((−)-epicatechin), Van. ac. (vanillic acid), Syr. ac. (syringic acid), Syr. (syringicaldehyde), *p*-coum. ac. (*p*-coumaric acid), Fer. (ferulic acid), *m*-coum. ac. (*m*-coumaric acid), Rut. (rutin), *o*-coum. ac. (*o*-coumaric acid), Myr. (myricetin), Cin. ac. (cinnamic acid) and kaemp. (kaempferol).

[c] (–) not detected.

[d] <LOD: lower than limit of quantification.

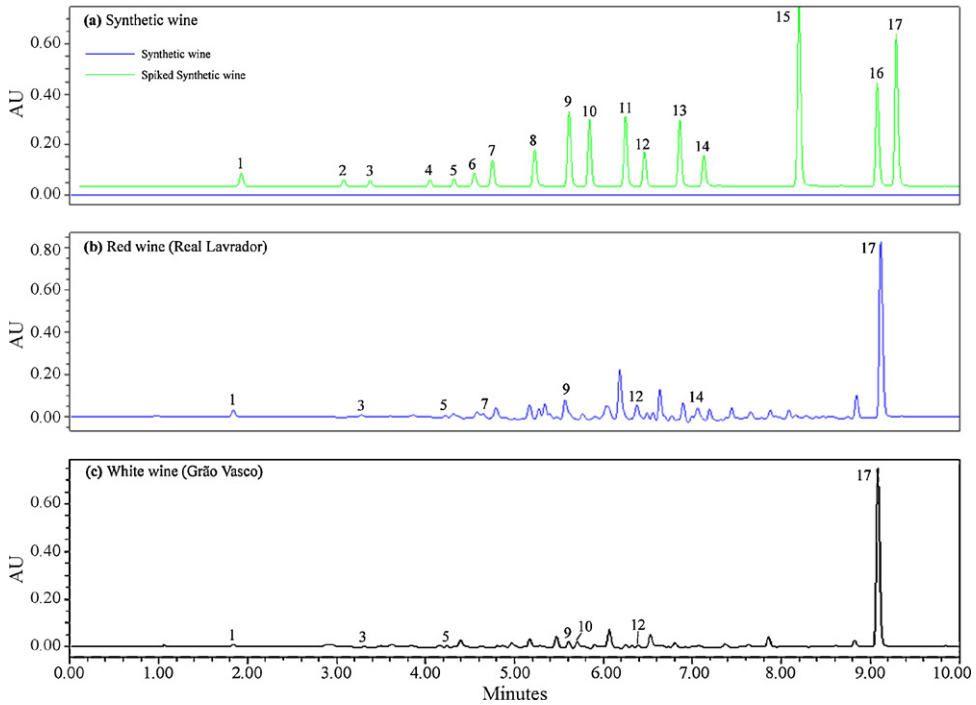

Fig. 4. Profile of $MEPS_{C8}$/UHPLC–PDA chromatogram of (a) synthetic wine and synthetic wine spiked with 16 bioactive polyphenols; (b) unspiked red wine (RL); and (c) unspiked white wine (GV) (for peak identification see Table 2).

Method precision was measured through repeatability inter-day and intra-day studies, expressed by the relative standard deviation (RSD) and calculated using the measurement of peak area of each bioactive phenolic compound in the matrix. The intra-day precision was measured by comparing standard deviation of spiked synthetic wine (LL, ML, HL) run in the same day ($n = 7$). The inter-day precision was determined by analyzing spiked synthetic wine samples for three alternate days. The results are satisfactory with RSD values lower than 8% for each measured analyte at all spiking levels (Table 3). The intra-day precision values at the three different levels ranged from 0.2 (*m*-coumaric acid at 40 $\mu g\,mL^{-1}$ and cinnamic acid at 25 $\mu g\,mL^{-1}$) to 4.7% (gallic acid and (−)-epicatechin at 1 $\mu g\,mL^{-1}$) ($n = 7$), whereas the inter-day precision varied from 0.2 (*p*-coumaric and *o*-coumaric acid at 25 $\mu g\,mL^{-1}$) to 7.5% ((+)-catechin at 1 $\mu g\,mL^{-1}$) ($n = 18 + 7$).

Combination of fast MEPS technique together with quick UHPLC–PDA system proves to be an improved, 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 bioactive phenolics in wines by $MEPS_{C8}$/UHPLC–PDA

UHPLC can be regarded as a new direction for liquid chromatography. Using sub-2 μm particles and mobile phases at high linear velocities, and instrumentation that operates at higher pressures than those used in HPLC, dramatic increases in resolution, sensitivity, and speed of analysis can be obtained.

In order to test the applicability of the developed method, the microextraction by packed sorbent procedure was first applied to a mixture of polyphenols standards and then to 34 wine samples (22 red and 12 white wines) (Table 4). As shown in Fig. 4a, the separation of the standard mixture of 16 phenolic metabolites is very fast, being achieved within only 10 min. All samples were analyzed in triplicate.

Good peak shape and resolution was achieved for all the compounds with no interference from the wine matrix (Fig. 4a). The chromatograms for the white and red wines samples showed quite different profiles (Fig. 4b and c) and their complexity increases or decreases according to the wavelength. The maximum absorbance values of each bioactive phenolic compound listed in Table 2 was used for quantification purposes.

The content of bioactive phenolic compounds found in the wine samples assayed is summarized in Table 4. As can be easily observed, the bioactive phenolics analyzed are about six times more abundant in red wines. The fact that polyphenols content is higher in red wines was widely described before in the literature [47]. The phenolic acids gentisic, *o*-coumaric and cinnamic along to syringaldehyde, were not found in any of the studied wines. On contrast, gallic acid, (+)-catechin, syringic acid, rutin and myricetin were found in all red wines analyzed.

Red wine from Azores, Basalto, was by far the one that showed higher polyphenolic content considering the sum of the 16 bioactive polyphenols tested (almost 71.24 $\mu g\,mL^{-1}$), followed by Terras de Lava, with polyphenolic composition around 70.68 $\mu g\,mL^{-1}$. In white wines, the polyphenolic content is significantly lower, varying from about 1.09 $\mu g\,mL^{-1}$ in the TMW1 wine up to 18.98 $\mu g\,mL^{-1}$ in RL wine. This fact makes white wines less prone to be effective in health protection against oxidative damage as the protective effects associated to moderate wine consumption have been attributed to their content in polyphenols. In fact, Fuhrman et al. [48] have shown that it was possible to have white wine with antioxidant characteristics similar to those of red wine just by increasing its polyphenols content, what could be easily obtaining by increasing the extraction of grape skin polyphenols during the maturation process [48].

Regarding to the individual phenolic constituents, gallic acid and rutin were the most abundant biologically active found in all wines investigated, followed by *p*-coumaric acid, (+)-catechin and (−)-epicatechin. These results confirmed that wine polyphenolic composition is very heterogenic, being dependent of diverse

factors, namely local climate and vinification conditions that are quite different among the wine samples used in this work.

4. Conclusions

A novel, ultrafast, sensitive and reproducible $MEPS_{C8}$/UHPLC–PDA-based methodology, using a 100 mm analytical column (Acquity HSS T3) packed with 1.8 μm particle size, was developed, validated and successfully applied to the simultaneous determination of 16 bioactive phenolics in wine matrices. The extraction procedure is simpler, more efficient and less time-consuming, and moreover can be used for small sample volumes (50 μL) as well as large volumes (>1000 μL). After a careful selection of the eluent systems, it was demonstrated that the chromatographic separation of the bioactive phenolic metabolites could be achieved in less than 10 min with high resolving power. 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 packed syringe can also be used several times (100 or more) depending from the matrix nature. 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 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 bioactive phenolic constituent determination. Moreover, the method could be applied to the analysis of bioactive phenolic in other food matrices (vegetables, fruits), as an attractive and very promising approach for the analysis of other groups of compounds due to the possibility of automation, easy to use, rapid and minimum cost of analysis when compared to conventional SPE.

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