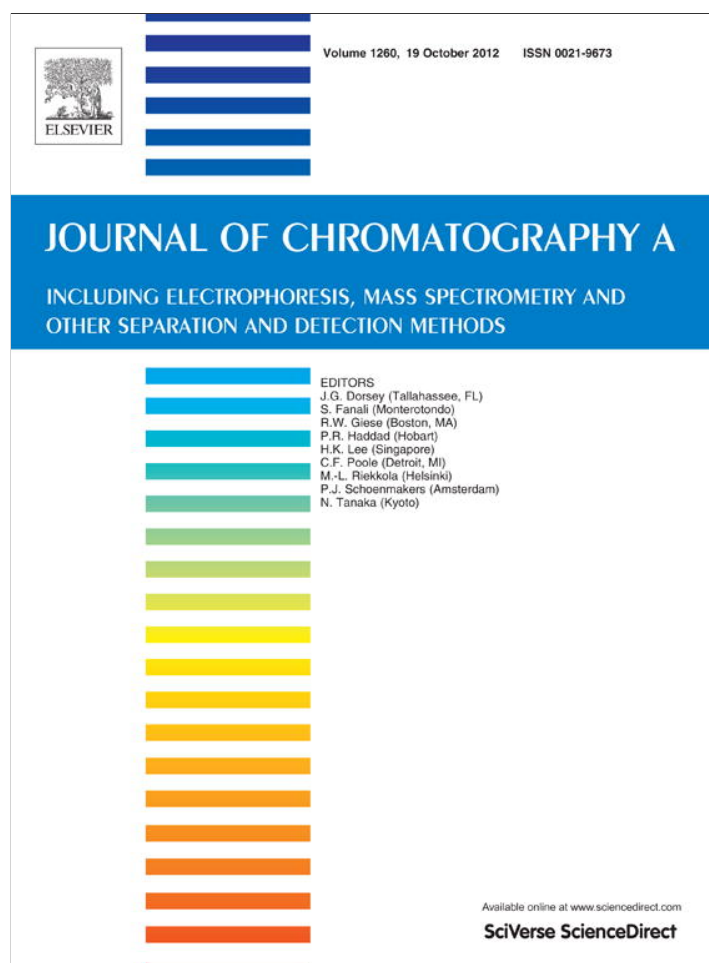


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# A new and improved strategy combining a dispersive-solid phase extraction-based multiclass method with ultra high pressure liquid chromatography for analysis of low molecular weight polyphenols in vegetables

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## ABSTRACT

This paper reports on the development and optimization of a modified Quick, Easy, Cheap Effective, Rugged and Safe (QuEChERS) based extraction technique coupled with a clean-up dispersive-solid phase extraction (dSPE) as a new, reliable and powerful strategy to enhance the extraction efficiency of free low molecular-weight polyphenols in selected species of dietary vegetables. The process involves two simple steps. First, the homogenized samples are extracted and partitioned using an organic solvent and salt solution. Then, the supernatant is further extracted and cleaned using a dSPE technique. Final clear extracts of vegetables were concentrated under vacuum to near dryness and taken up into initial mobile phase (0.1% formic acid and 20% methanol). The separation and quantification of free low molecular weight polyphenols from the vegetable extracts was achieved by ultrahigh pressure liquid chromatography (UHPLC) equipped with a photodiode array (PDA) detection system and a Trifunctional High Strength Silica capillary analytical column (HSS T3), specially designed for polar compounds.

The performance of the method was assessed by studying the selectivity, linear dynamic range, the limit of detection (LOD) and limit of quantification (LOQ), precision, trueness, and matrix effects. The validation parameters of the method showed satisfactory figures of merit. Good linearity ( $R^2_{\text{values}} > 0.954$ ; (+)-catechin in carrot samples) was achieved at the studied concentration range. Reproducibility was better than 3%. Consistent recoveries of polyphenols ranging from 78.4 to 99.9% were observed when all target vegetable samples were spiked at two concentration levels, with relative standard deviations (RSDs,  $n = 5$ ) lower than 2.9%. The LODs and the LOQs ranged from 0.005  $\mu\text{g mL}^{-1}$  (*trans*-resveratrol, carrot) to 0.62  $\mu\text{g mL}^{-1}$  (syringic acid, garlic) and from 0.016  $\mu\text{g mL}^{-1}$  (*trans*-resveratrol, carrot) to 0.87  $\mu\text{g mL}^{-1}$  ((+)-catechin, carrot) depending on the compound. The method was applied for studying the occurrence of free low molecular weight polyphenols in eight selected dietary vegetables (broccoli, tomato, carrot, garlic, onion, red pepper, green pepper and beetroot), providing a valuable and promising tool for food quality evaluation.

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## 1. Introduction

Fruits and vegetables contain several thousand of structurally diverse phytochemicals, such as polyphenols, carotenoids and tocopherols. Its consumption is encouraged due to the reported multiple biological effects namely antioxidant, anti-inflammatory, anticarcinogenic, antimutagenic and antiproliferative activities [1,2]. This physiological benefits have been attributed to their potential role on the prevention of 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 by acting as free radical scavengers [3–6], on inhibition of platelet aggregation [7–10], cell proliferation, migration, and angiogenesis [11]. They also can act as transient metal ion chelators [9], control protein oxidation and advanced glycation end products (AGEs) formation [12], and as potent pancreatic lipase inhibitors being potential candidates for obesity prevention, namely epigallocatechin-3-gallate, kaempferol and quercetin [13].

In food, phenolics may contribute to the bitterness, astringency, color, flavor, odor, and oxidative stability of products. However, there is a limited amount of information on the content of phenolic compounds in common foodstuffs of plant origin and their antioxidant activities. In this respect, screening of various food products with beneficial health properties is very important.

Evidence on the health benefits of polyphenols and their impact on food quality have stimulated the development of analytical

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methods for their identification and quantification [14–18]. In recent years, the ability of several extraction techniques, such as solid–liquid extraction (SLE) [19], enzyme-assisted extraction [20], heat extraction [21], solid-phase extraction (SPE) [22], and solid-phase microextraction (SPME) [23], for the isolation free low molecular-weight polyphenols (LMW-PPs) from vegetables and other food matrices, has been proposed and evaluated as reliable alternatives to classic liquid–liquid extraction (LLE) technique. More recently, a novel analytical approach, based on miniaturized microextraction by packed sorbent (MEPS), followed by ultra-high pressure liquid chromatography (UHPLC) separation, has been proposed by Gonçalves et al. [24] for quantitative determination of wine biologically active phenolic constituents and *trans*-resveratrol [25]. In the last few years, a quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction technique for the multiclass, multiresidue analysis of pesticides in fruits and vegetables, was described by Anastassiades et al. [26]. This procedure involves initial single phase extraction with acetonitrile followed by salting-out extraction/partitioning by addition of  $MgSO_4$  plus NaCl, and finally using dispersive-solid-phase extraction (*d*SPE) for clean-up. In addition to pesticide multiresidue analysis in food-stuffs [27–34], QuEChERS concepts have been used for acrylamide [35,36], clinical [37], veterinary drug residue [38,39], food quality [40], supplement testing [41], perfluorinated compounds [42,43], alkaloids [44], environmental and mycotoxins [45–47] analytical applications. However, up to date no studies using the QuEChERS technique coupled to *d*SPE for the analysis of free LMW-PPs in vegetables or another kind of matrices have been published.

Therefore, in this paper, we report for the first time to the best of our knowledge, a new and reliable QuEChERS-based extraction technique combined with a *d*SPE clean-up procedure in order to investigate if this methodology is suitable for extracting the LMW-PPs from selected species of commonly consumed vegetables. Important parameters that may affect extraction efficiency, namely the partitioning solvents and the salts used to enhance the salting-out effect on the portioning process, were investigated and optimized. An effective extraction/clean-up procedure, using primary-secondary amine (PSA) and  $C_{18}$  sorbents, allows achieving clear extracts, no or low matrix effect and low LOD and LOQ, also reducing as much as organic solvents, fulfilling the purposes to establish a wider acceptability of the methodology. The QuEChERS procedure was optimized using carrot samples and evaluated in other seven selected vegetables. The LMW-PPs analysis were performed on an ultrahigh pressure liquid chromatography (UHPLC) equipped with a PDA detection system and a new analytical column specially designed for polar compounds. Chromatographic conditions were optimized in order to achieve increased sensitivity and high resolution on the free low molecular weight polyphenols analysis in addition to reduced analysis time (within 11 min). Novel aspects of the present study in comparison with similar works, both in the environmental or biological fields, are constituted by extraction, for the first time, of several LMW-PPs in a food matrix by QuEChERS-*d*SPE technique using a set of salts in the portioning process highly selective relatively to the generally used.

## 2. Experimental

### 2.1. Reagents, materials and standards

All chemicals and reagents were of analytical quality grade. HPLC grade acetonitrile (MeCN) and ethyl acetate (EtAc) were obtained from LabScan (Dublin, Ireland), formic acid (FA) from Fischer Scientific (Loughborough, UK), whereas sodium hydroxide, methanol (MeOH) and ethanol (EtOH) were supplied by Panreac

(Barcelona, Spain) and glacial acetic acid by Fluka Biochemica AG (Buchs, Switzerland).

LMW-PP standards, gallic acid monohydrate (98%, purity), ferulic acid (98%), gentisic acid (98%), cinnamic acid (99%), (–)-epicatechin ( $\geq 95\%$ ), *m*-coumaric acid (99%), *p*-coumaric acid (99%), *o*-coumaric acid (99%) and rutin ( $>95\%$ ) and kaempferol ( $\geq 97\%$ ) and protocatechuic acid (98%), (+)-catechin ( $\geq 95\%$ ), syringic acid (98%), and *trans*-resveratrol (99%) were obtained from Sigma–Aldrich (St. Louis, MO, USA), whereas quercetin (98%) was purchased from Riedel-de Haën (Seelze, Germany) and myricetin ( $\geq 97\%$ ) from Acros Organics (Geel, Belgium). Solvents were filtered with  $0.22\ \mu m$  membrane filters using a Solvent Filtration Apparatus 58061 from Supelco (Bellefonte, PA, USA). Ultrapure water from a Milli-Q ultrapure water purification system (Millipore, Bedford, USA) was used for preparing the LC mobile phase and other aqueous solutions. Filters of 13 mm with  $0.22\ \mu m$  PTFE membrane were used for filtration of the final extracts before analysis.

Bulk sorbents ( $50\ \mu m$  particle size) for *d*SPE, including primary–secondary amine (PSA), trifunctionally-bonded  $C_{18}$  silica and the QuEChERS extraction/partitioning tubes containing the buffered salts and the clean-up tubes, were obtained from Waters (Milford, MA, USA).

### 2.2. Preparation of standard solutions

Stock solutions of each individual standard ( $1000\ \mu g\ mL^{-1}$ ) were prepared in methanol. These solutions were aliquoted in 2 mL vials, stored at  $-20\ ^\circ C$  and protected from light; under these conditions they are stable for at least 4 months (as assessed by UHPLC). A standard multicomponent solution was prepared by diluting each primary standard solution with the chromatographic mobile phase and was used for spiking the target vegetable samples, for preparing calibration standards and for linear dynamic range assessment of the UHPLC–PDA system. The choice of the LMW-PPs was mainly based on their importance and/or relevance for food quality, covering the major classes (flavonoids and non-flavonoids).

### 2.3. Vegetable samples

Samples of eight vegetables: carrot (*Daucus carota* L.), tomato (*Lycopersicon esculentum* L.), broccoli (*Brassica oleracea* L.), onion (*Allium cepa* L.), garlic (*Allium sativum* L.), green and red pepper (*Capsicum annuum* L.), and beetroot (*Beta vulgaris*), were purchased from a local market in Funchal, Portugal. For each vegetable sample, approximately 1 kg was randomly sampled from the market shelves, simulating consumer shopping behavior. Vegetables were washed in tap water and all inedible parts were removed manually or using a steel knife. Bruised or wounded vegetables were discarded. Carrot, beetroot, garlic and onions were peeled and analyzed only as pulp. For each independent analysis, at least 500 g of vegetable, added with 100 mL Milli-Q water, were put in a commercial juice extractor (Instant pulp, 200 W, Worten, Portugal), obtaining a fluid vegetable extract (FVE) which was used in all analyses. The FVE were then divided into fractions and stored at  $-20\ ^\circ C$  for later QuEChERS procedure. All determinations were performed using three independent aliquots of the same FVE each one analyzed in triplicate.

### 2.4. QuEChERS procedure for extraction of LMW-PPs

#### 2.4.1. Selection of extraction solvent and buffered salts

In order to get the highest extraction efficiency of LMW-PPs, different partitioning solvents namely methanol (MeOH; 100%), water ( $H_2O$ ; 100%), ethyl acetate (EtAc; 100%), acetonitrile (MeCN; 100%) and MeCN:EtAc (50:50, v/v), an two different sets of buffered salts, set S1 composed by sodium acetate (1.5 g) and anhydrous

magnesium sulfate (6g); and set S2 composed by trisodium citrate dihydrate (1g), disodium hydrogencitrate sesquihydrate (0.5g), sodium chloride (1g), and anhydrous magnesium sulfate (4g) were tested and compared. For clean-up procedure anhydrous  $\text{MgSO}_4$  (150 mg), PSA (25 mg) and  $\text{C}_{18}$  (25 mg) were used in all assays. Carrot (*Daucus carota* L.) samples were selected as matrix for optimization QuEChERS extraction procedure purposes.

#### 2.4.2. Extraction and clean-up procedure

A thoroughly homogenized sub sample (10 g) of the selected FVE was weighted into a 50 mL PTFE centrifuge tube and added 10 mL MeCN:EtAc (50:50, v/v) containing 1% FA; the tube was shaken vigorously for 2 min with vortex mixer ensuring that the solvent interacted well with the entire sample. Buffered salts, trisodium citrate dihydrate (1 g), disodium hydrogencitrate sesquihydrate (0.5 g), sodium chloride (1 g) and anhydrous  $\text{MgSO}_4$  (4 g) were added into the homogenized mixture and the shaking step was repeated for 1 min followed by a centrifugation at 5000 rpm for 3 min. An aliquot (1 mL) from the upper part of the extract (acetonitrile phase) was transferred into a 2 mL PTFE dSPE clean-up tubes containing 25 mg of PSA (removes various polar organic acids, polar pigments, some sugars and fatty acids), 25 mg of  $\text{C}_{18}$  sorbent (removes non-polar interfering substances like lipids) and 150 mg  $\text{MgSO}_4$  and subjected to clean-up by dSPE. The mixture was shaken in a vortex and centrifuged for 2 min at 3000 rpm. Then, 700  $\mu\text{L}$  aliquot of the extract were evaporated under nitrogen flow to near dryness and the residue was taken up with 100  $\mu\text{L}$  of initial mobile phase (0.1% FA in Milli-Q water and 20% of MeOH). All the sample and standard extracts were filtered through a 0.22  $\mu\text{m}$  Millipore PTFE filter membrane prior to UPLC analysis.

#### 2.5. UPLC–PDA conditions

The separation and quantification of LMW-PPs [48,49] was performed on a Waters Ultra Pressure 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. A high strength silica Acquity HSS T3 analytical column (2.1 mm  $\times$  100 mm, 1.8  $\mu\text{m}$  particle size) packed with a trifunctional  $\text{C}_{18}$  alkyl phase, kept at 40 °C, was used for the separation of LMW-PPs. A binary mobile phase with a gradient program was used, combining solvent A (0.1% FA) and solvent B (MeOH) as follows: 80% A (0 min); 80–70% A (0.50 min); 68% A (1 min); 20% A (8 min); and 80% A (11 min). The flow rate was 250  $\mu\text{L min}^{-1}$ , gave a maximum back pressure of 6000 psi, which is within the capabilities of the UPLC. The injection volume, of both the standard solutions and sample extracts, was 2  $\mu\text{L}$ . After each injection the needle was rinsed initially with 400  $\mu\text{L}$  of wash water:methanol solution at 90:10 and after with 200  $\mu\text{L}$  of water:methanol solution at 10:90. The samples were kept at 6 °C during the analysis. The system was re-equilibrated with the initial composition for 3 min, prior to next injection. The target compounds eluted within 11 min, while the additional equilibration at the initial mobile phase composition resulted in a total analysis time of 14 min. The UV detection wavelength was set to the maximum of absorbance for the compounds of interest. The identification of the LMW-PPs in investigated vegetables was based on the comparison of the retention times ( $t_R$ ) and PDA spectra of their peaks in samples with those previously obtained by the injection of pure standards.

#### 2.6. Analytical method validation

Validation of the QuEChERS–dSPE/UHPLC–PDA procedure for the quantification of free LMW-PPs in dietary vegetables involved the assessment of the selectivity, linear dynamic range (LRD), instrument LODs and method LOQs, intra-day ( $\text{RSD}_I$ ) and inter-day ( $\text{RSD}_R$ ) precision, trueness (expressed as recovery percentage) and matrix effects.

The selectivity of the method was assessed by the absence of interfering peaks at the retention time of target LMW-PPs. To test the linear dynamic range of the method FVE samples of each vegetable, were spiked at six concentration levels, obtained by successive dilutions of the stock standard solution. Calibrations curves were constructed by plotting the LMW-PPs signal obtained against the concentration of LMW-PPs. Solvent-based standard solutions were also analyzed to assess the matrix effects. Least squares linear regression analysis was used to interpolate the data pairs obtained from each calibration solution. The slope ratios (slope matrix/slope solvent) were used as a mean to evaluate the matrix effects on the extraction efficiency. A value about 1 (100% similarity) indicates that matrix does not significantly influence the extraction efficiency. The LOD (the lowest analyte concentration that produces a response detectable above the noise level of the system) and the LOQ (the lowest level of analyte that can be accurately and precisely measured) were calculated for each analyte in each vegetable on the basis of the concentration that produced a signal-to-noise (S/N) ratio equal or higher than 3 and 10, respectively. The intra- and inter-day precision of the assay was evaluated by preparing and analyzing FVE samples of each vegetable spiked with known amounts of LMW-PPs at two different levels of concentrations (low level – lowest level of calibration curve; high level – highest level of calibration curve), in the range of expected concentrations, respectively, six times in the same day and five times in a week, and expressed as repeatability ( $\text{RSD}_I$  %) and reproducibility ( $\text{RSD}_R$  %), respectively. Trueness, expressed as recovery, was calculated as percent ratio between the concentration estimated from the calibration curve and the spiked concentration. All the experiments were done in triplicate.

#### 2.7. Statistics

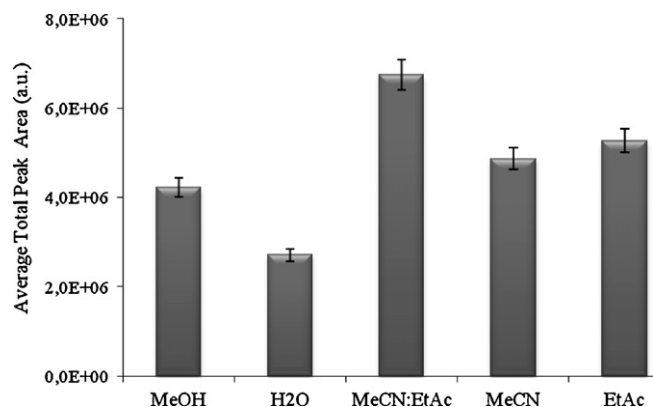
Statistical analysis of the results was carried out using SPSS for Windows, version 19.0 (SPSS, Inc., Chicago, IL) to apply one-way ANOVA followed by Bonferroni test. Trends were considered statistically significant when means of compared sets differed at  $p$  values  $< 0.05$ .

### 3. Results and discussion

#### 3.1. Selection of extraction/partitioning solvents and buffered salts

To get the highest extraction efficiency towards the target LMW-PPs, different solvents and mixtures,  $\text{H}_2\text{O}$  (100%), MeOH (100%), EtAc (100%), MeCN (100%), MeCN:EtAc (50:50, v/v), were assayed and evaluated based on the intensity of the response observed. A carrot FVE was selected as matrix for the optimization of QuEChERS procedure according to described in Section 2.4.2.

From the comparison of the graphic presented in Fig. 1, and on basis of the average total target LMW-PPs, it was found that the MeCN:EtAc (50:50, v/v) extraction/partitioning mixture was the most efficient solvent for the extraction of target LMW-PPs from carrot samples. Conversely, water was found the solvent with the lowest extraction efficiency for the target compounds. Thus, MeCN:AcEt was selected in all experiments for the extraction of



**Fig. 1.** Comparison of average peak area response obtained with different extractive solvents: MeOH, methanol; H<sub>2</sub>O, water; MeCN:EtAc, acetonitrile and ethyl acetate solution (50:50, v/v); MeCN, acetonitrile; EtAc, ethyl acetate.

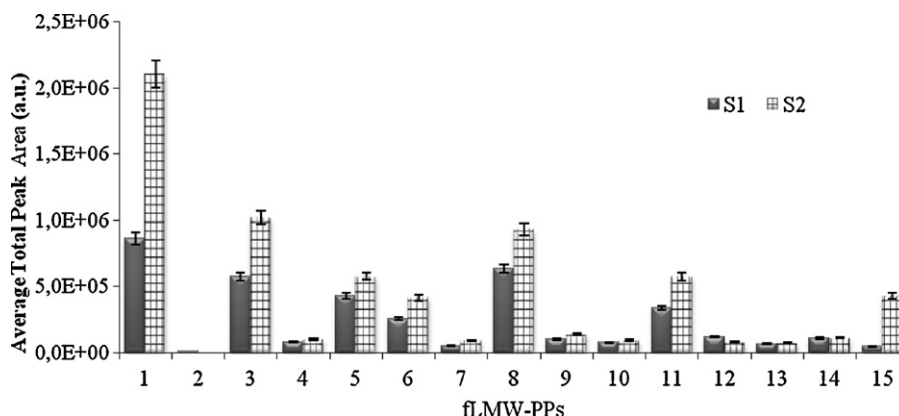
LMW-PPs. MeCN is easily and effectively separated from FVE by adding polar substances including buffered salts, namely NaCl and MgSO<sub>4</sub>.

Furthermore, in order to improve the LMW-PPs extraction efficiency, two different sets of buffered salts were compared. Besides the concentration of some target LMW-PPs, such as (+)-catechin, (–)-epicatechin, seringaldehyde, ferulic acid, *m*-coumaric acid, *trans*-resveratrol, *o*-coumaric acid, cinnamic acid, was not significantly affected by the nature of buffered salts, as showed in Fig. 2. The average content of the target LMW-PPs was significantly high using the S2 set, composed by trisodium citrate dehydrate, disodium hydrogencitrate sesquihydrate, sodium chloride and anhydrous MgSO<sub>4</sub>. Thus, S2 set was used in all further experiments.

In all assays, 150 mg MgSO<sub>4</sub>, 25 mg PSA and 25 mg C<sub>18</sub> were used in the clean-up step. The use of C<sub>18</sub> associated to PSA and MgSO<sub>4</sub> to remove lipids is of crucial importance to maximize the sensitivity of LMW-PPs and to minimize the presence of interfering compounds in the extract.

### 3.2. Method validation

To demonstrate the feasibility of the present approach for the determination of LMW-PPs and to test its practicability, the performance of the method was fully evaluated in terms of selectivity, linearity, LODs, LOQs, intra/inter-day precision, trueness, and matrix effects. Table 1 describes the LMW-PPs identification (coded



**Fig. 2.** Comparison of average peak area response obtained by using two different sets of buffered salts on the extraction/partitioning mechanism: S1 – CH<sub>3</sub>COONa (1.5 g), MgSO<sub>4</sub> (6 g); S2 – Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub> (1 g), C<sub>6</sub>H<sub>8</sub>Na<sub>2</sub>O<sub>8</sub> (0.5 g), NaCl (1 g) and MgSO<sub>4</sub> (4 g). The numbers correspond to LMW-PPs described in Table 1. a.u. – arbitrary units.

**Table 1**

Peak number, retention time ( $t_R$ ) and maximum wavelength for the 15 investigated LMW-PPs.

No.	$t_R^a$ (min)	$\lambda_{max}^b$ (nm)	LMW-PPs <sup>c</sup>
1	3.027	259	Protocatechuic acid
2	3.345	278	(+)-Catechin
3	3.961	327	Genitistic acid
4	4.292	278	(–)-Epicatechin
5	4.518	260	Vanillic acid
6	4.718	274	Syringic acid
7	5.208	308	Seringaldehyde
8	5.581	309	<i>p</i> -Coumaric acid
9	5.807	322	Ferulic acid
10	6.148	277	<i>m</i> -Coumaric acid
11	6.406	356	Rutin
12	6.629	304	<i>trans</i> -Resveratrol
13	6.813	275	<i>o</i> -Coumaric acid
14	8.126	317	Cinnamic acid
15	8.997	366	Kaempferol

<sup>a</sup>  $t_R$ , average retention times (RSD < 2%).

<sup>b</sup>  $\lambda_{max}$ , maximum absorbance values obtained in PDA system detection.

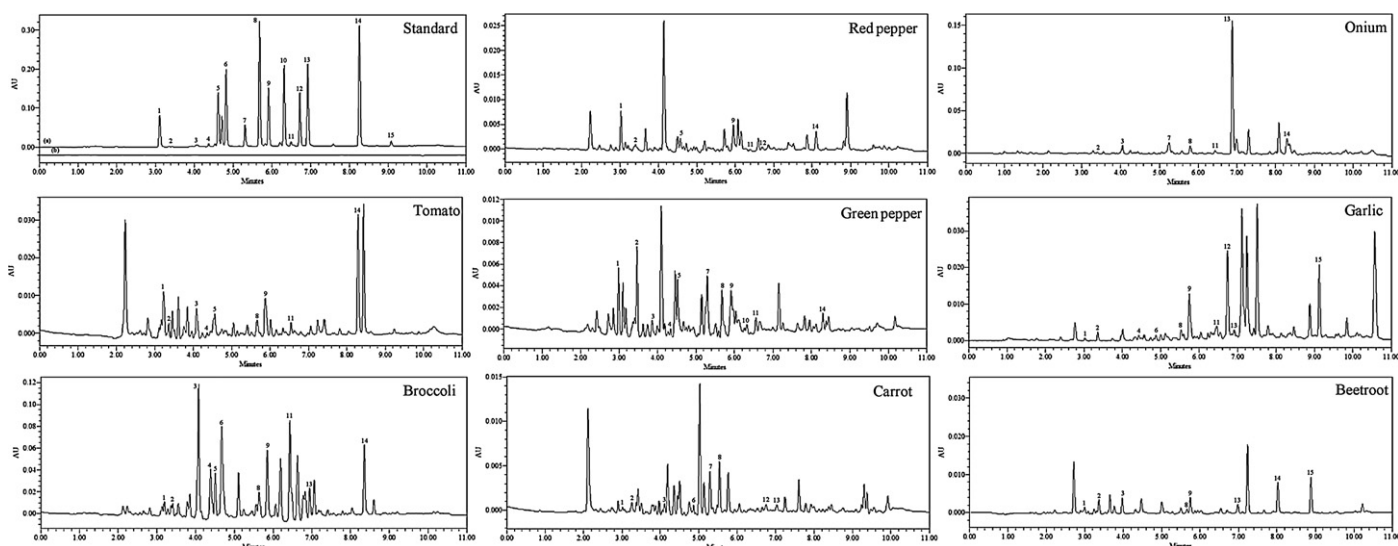
<sup>c</sup> LMW-PPs, low molecular weight polyphenols.

as a number), the maximum wavelength for each analyte, and the retention time obtained with the instrumental conditions used.

The selectivity was assessed by the analysis of three blank solutions (elution solution) extracted by the optimized QuEChERS method. No interference was detected at the analyte retention time (Fig. 3a). The linear dynamic range (LDR) of the method was established on standard solutions and spiked FVE samples of each vegetable, prepared and analyzed using the described extraction procedure (QuEChERS-dSPE/UHPLC-PDA) in the range of 0.1–25  $\mu\text{g mL}^{-1}$  (six calibrators evenly distributed, five replicates). Along with each calibration curve, a zero sample (FVE of each vegetable without spiking) was also analyzed.

Calibration curves were obtained by plotting the average peak-area of each analyte against analyte concentration. The UHPLC-PDA system gave a linear response all throughout the respective investigated range of concentrations. Least-squares linear regression analysis of the data provided excellent correlation coefficient values for all LMW-PPs investigated ( $R^2 > 0.954$ ), and the calibrators' residuals were considered adequate, being within  $\pm 10\%$  of the nominal concentration for all levels.

To evaluate the impact of the matrix on the analytes, the slopes obtained in the calibration with matrix-matched standards were compared with those obtained with solvent-based standards, calculating matrix/solvent slope ratios for each of the 15 studied LMW-PPs in all FVE matrices. Table 2 summarizes the results.



**Fig. 3.** Representative UHPLC–PDA chromatograms of MeCN:EtAc extracts of the (a) standard mixture of 15 LMW-PPs and (b) blank solution for selectivity evaluation, and from the investigated vegetables. For peak assignment (see Table 1).

We consider that, if the value was in the range of 0.85–1.1, the matrix effect could be ignored; if the value was lower than 0.85, it could show matrix suppression effect; if the value was higher than 1.1, it could show matrix enhancement. For some of the investigated analytes the matrix effect values are into this range. As it can be seen in Table 2, the signal is affected for the matrix in most cases: soft matrix effect was observed for 75% of LMW-PPs while 25% of LMW-PPs showed strong matrix effect (equal or up to 0.15).

Based on the obtained results, it can be observed that the matrix effect was negligible for some target LMW-PPs in some matrices, and in this case, solvent based standards could be used for accurate quantification of the target analytes. However for other LMW-PPs a strong matrix effect of the matrix was observed, and in this case, matrix-matched calibration solutions should be used for LMW-PPs quantification purposes. Therefore, in this study matrix-matched standards were used as calibration mode, to quantify the 15 LMW-PPs in all target dietary vegetables, in order to compensate the errors associated with matrix induced suppression or enhancement effects. Thus, we can deduce that the matrix effect depends strongly on the nature of matrix, on some specific compounds, and also on the chemical nature of the analytes.

Based on calibration curves, method LODs and LOQs were estimated from the theoretical calculations of the lowest concentration level, obtaining a S/N ratio equal or higher than 3 and 10, respectively, the theoretical concentrations of which was then experimentally tested by the injection of the corresponding concentration, followed by confirmation of the expected/required response. The QuEChERS–dSPE/UHPLC–PDA methodology gave in general very low LODs (Table 3), ranging between 0.005 for *trans*-resveratrol in carrot FVE and  $0.62 \mu\text{g mL}^{-1}$  for syringic acid in garlic FVE, while LOQs ranged from  $0.016 \mu\text{g mL}^{-1}$  for *trans*-resveratrol to  $0.87 \mu\text{g mL}^{-1}$  for (+)-catechin, both obtained with carrot FVE, showing that the method is enough sensitive for the determination of very low levels of LMW-PPs in vegetables.

The precision was measured through inter-day (reproducibility,  $\text{RSD}_R$ ) and intra-day (repeatability,  $\text{RSD}_r$ ) studies, calculated using the measurement of relative peak area of each LMW-PP in the matrix (Table 4). Intra-day precision and trueness (expressed as the relative error (RE));  $\text{RE} = ((\text{spiked concentration} - \text{nominal concentration}) / \text{nominal concentration} \times 100)$  were evaluated by analyzing in the same day 6 replicates of carrot samples spiked with polyphenols at two concentration levels (lowest and

highest levels of calibration curve). The obtained RSDs were in general lower than 2.9% for all compounds at both tested concentrations, presenting a mean relative error within a  $\pm 5.1\%$  interval. Inter-day precision and trueness were evaluated at two concentrations within a 5-day period. The calculated RSDs were lower than 3.0% for all compounds at all concentration levels, while trueness was within a  $\pm 16\%$  interval. Table 4 summarizes the intra- and inter-day precision and trueness data.

In order to evaluate the trueness of the analytical method, a recovery study of the MeCN:AcEt extracts from FVE samples of each vegetable was carried out by adding LMW-PPs standards with known amounts of each LMW-PP (Table 4). Satisfactory results were found for most of LMW-PPs with recovery values ranging from 78.4% (*trans*-resveratrol; tomato) to 99.9% (cinnamic acid; carrot), respectively. The % RSD of the average recovery is less than 2.9%. Approximately 71% (7 LMW-PPs) of the analyzed LMW-PPs yielded recoveries of 90–99.9%, 28% yielded recoveries of 80–89%, and 0.4% yielded recoveries of lower than 80%.

In comparison with other methods to quantify the LMW-PPs, the QuEChERS method offers better selectivity than the SPE<sub>Oasis</sub> HLB technique [22] and better sensitivity to some analytes than obtained by MEPC<sub>8</sub> [24]. However, regarding the figures of merit of the different techniques, similar results were obtained for LODs, LOQs, trueness and precision.

### 3.3. Application of QuEChERS–dSPE/UHPLC–PDA for the analysis of LMW-PPs on vegetable samples

After validation, the herein described procedure was applied to a set of selected commonly consumed vegetables. In particular, carrot, tomato, broccoli, green and red pepper, onion, garlic and beetroot, purchased from a local market and simulating consumer shopping behavior, were included in this study. The resulting chromatograms of investigated vegetables, obtained by UHPLC–PDA analysis, are shown in Fig. 3. Good peak shape and resolution were achieved for all the compounds with low interference from the vegetable matrix. The chromatograms for the different tested vegetables showed quite different profiles (Fig. 3) and their complexity increases or decreases according to the wavelength. The maximum absorbance value of each LMW-PP listed in Table 1 was used for quantification purposes.

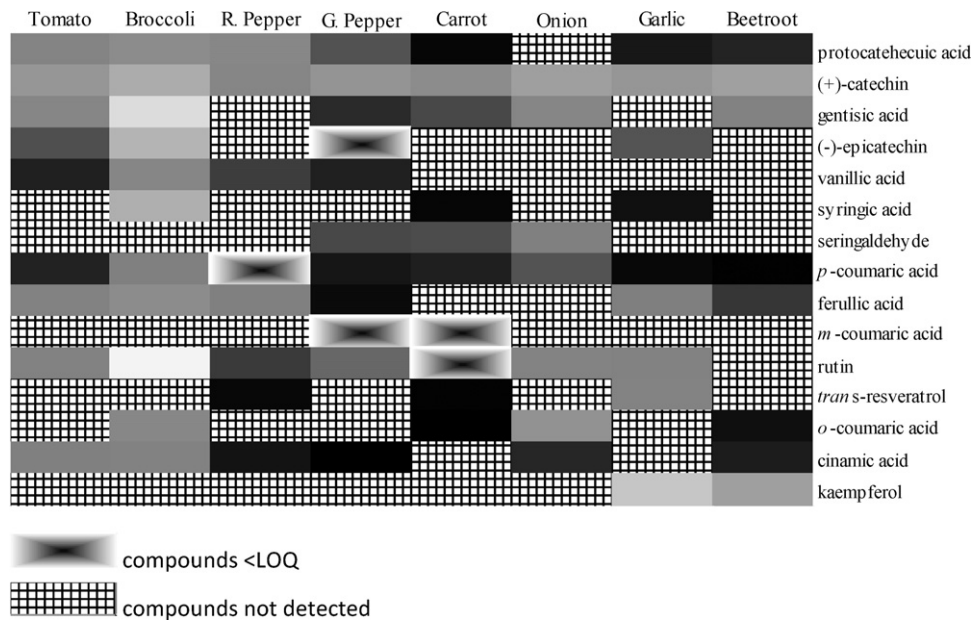


Fig. 4. Concentration of the LMW-PPs in investigated vegetables as heat map representation.

Only the areas of the compounds that were clearly recognized by their PDA spectrum were extracted to eliminate false positives. As can be seen, the separation of the target LMW-PPs is very fast, being achieved within only 11 min.

The number of LMW-PPs detected at concentration above the LOQ varied in the different matrices: it was possible to quantify 11

LMW-PPs in broccoli, 10 in green pepper, 9 in garlic and tomato, 8 in carrot and beetroot, 7 in red pepper and onion. Furthermore some LMW-PPs were detected in these samples at concentrations lower than the LOQ and could not be quantified (Table 1S). The results of the analysis are shown in the form of heat map in which the concentration values are within a gray scale (Fig. 4).

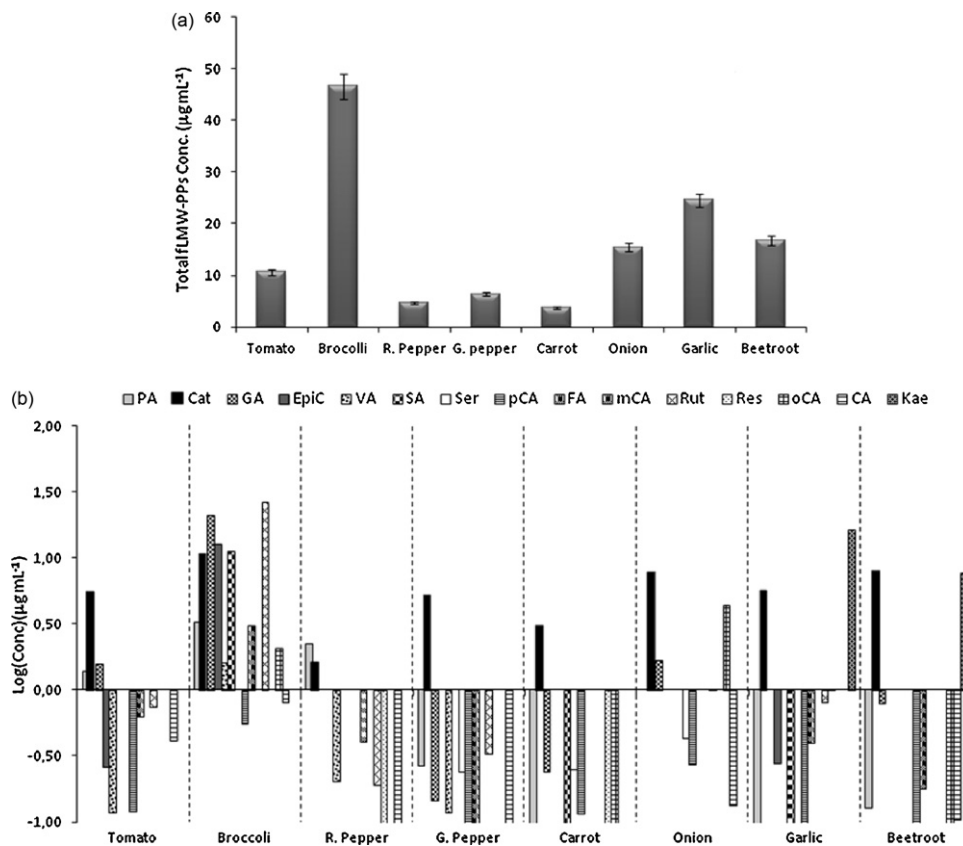


Fig. 5. (a) Total content of LMW-PPs in the investigated vegetables obtained using the optimized conditions (extraction solvent: MeCN:EtAc (50:50) in the presence of buffered salts:  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ ,  $\text{C}_6\text{H}_8\text{Na}_2\text{O}_8$ , NaCl and  $\text{MgSO}_4$ ; clean-up with: 150 mg  $\text{MgSO}_4$ , 25 mg PSA and 25 mg C18); (b) distribution of the concentration profile (Conc.) of the assayed LMW-PPs among the studied vegetables. Note that the y axis sets on a log scale.







As shown in Fig. 4 not all LMW-PPs were detected in each vegetable or the LOQ was too low to ensure that the LMW-PP was quantified. The results concerning the quantitative determination of the LMW-PPs found in the selected vegetables are summarized in Table 1S. The total concentration of the target LMW-PPs (Fig. 5a) significantly differed between the investigated vegetables being higher in the broccoli samples than the other investigated vegetables (Table 1S). The total content of the target LMW-PPs was approximately 38-fold higher for broccoli than for carrot, the vegetable with the lowest LMW-PPs content.

Kaempferol and (+)-catechin followed by protocatechuic acid were the major constituents of all identified LMW-PPs in the target vegetables. (+)-Catechin was detected and quantified in all the vegetables unlike *m*-coumaric acid which was not found in any of the investigated sample. Syringic acid was only found in broccoli. Kaempferol was detected and quantified in broccoli, garlic and beetroot, however, was found the major target LMW-PPs in these vegetables. The content of rutin and (+)-catechin determined in broccoli is significantly higher than that found in the other samples. In red pepper the major LMW-PP is protocatechuic acid whereas (+)-catechin was the most abundant in tomato, green pepper, carrot, onion and beetroot. The main LMW-PP determined in garlic was kaempferol followed by (+)-catechin. The highest content of *trans*-resveratrol was found in garlic, whereas carrot and red pepper exhibited minor amounts.

#### 4. Conclusions

This paper describes for the first time a quick, simple and sensitive analytical method based on QuEChERS-*d*SPE/UHPLC-PDA for the simultaneous determination of 15 LMW-PPs in common dietary vegetables.

The extraction and clean-up procedures of the described method are very simple and required little sample preparation or pre-treatment, providing adequate clean-up to the FVE. Moreover, gradient elution by the mobile phase acetonitrile–water yields good separation and resolution and the analysis time required for the chromatographic determination of the 15 LMW-PPs is very short (around 11 min for a chromatographic run).

Satisfactory validation parameters, such as linearity, recovery, precision and LODs and LOQs, were obtained. The effectiveness of different extraction/partitioning conditions was systematically investigated, and the joint use of MeCN:EtAc in presence of trisodium citrate dihydrate, disodium hydrogen citrate sesquihydrate, NaCl and MgSO<sub>4</sub> as buffered salts on the extraction/partitioning, and MgSO<sub>4</sub>, PSA and C18 as clean-up reagents, was recommended in our final method. For all target LMW-PPs the sensitivity of the method was good enough to ensure reliable determination at levels commonly found in dietary vegetables. These data suggests that the analytical method represents an attractive, reliable and promising high-throughput approach for the quantification of LMW-PPs in a wide range of dietary vegetable samples, and their application could be successfully applied to the analysis of a range of these health-related secondary metabolites in fruits and other food commodities.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chroma.2012.08.082>.

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