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Journal of Chromatography A, 897 (2000) 161–170

JOURNAL OF CHROMATOGRAPHY A

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Optimization strategy and validation of one chromatographic method as approach to determine the phenolic compounds from different sources

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Received 8 May 2000; received in revised form 14 July 2000; accepted 31 July 2000

Abstract

We have designed a novel working strategy to optimize a unique chromatographic method consisting of diode array detection for the analysis of the most representative phenolic compounds from different food sources. The simultaneous inclusion of standard phenolic compounds, phenolic compounds isolated from food sources and representative real extracts as an ultimate test in analysis has allowed to establish, for the first time, a unique liquid gradient to serve as an excellent medium for the investigation of phenolics in samples from different food sources. Under the optimized conditions, 21 commercially available phenolic compounds and 25 commercially unavailable phenolic structures were analyzed in less than 30 min. The chromatographic method was designed as an alternative for the provisional identification of these compounds before their full characterization. The optimized chromatographic method was carefully validated for precision and accuracy. A high reproducibility in the retention time (<2%), peak area and calibration slope (<5%) as well as recoveries higher than 95% were obtained in all cases. Consequently, the currently described method was successfully employed to study the phenolic compounds in the most representative food samples.

Keywords: Optimization; Validation; Gradient elution; Phenolic compounds

1. Introduction

Phenolic compounds are chemically complex substances widely distributed in plants and food plants and as such are common components of the human diet [1]. They have been thoroughly scrutinized by different researchers because of their physiological and physical–chemical properties as well as their anticarcinogenic and high antioxidant capacity [2–6].

High-performance liquid chromatography in the reversed-phase (RP-HPLC) with conventional columns (C_{18}) and, especially, HPLC with diode array detection (DAD) constitute a crucial, utterly reliable technique, which is routinely employed in the analysis of phenolics [1]. As is well known, the exact identification of phenolic compounds requires mass spectrometry (MS); however, the number of published studies dealing with liquid chromatography–mass spectrometry (LC–MS) techniques is so far limited [7,8]. According to the most relevant bibliography, DAD is an indispensable tool for the provisional identification of the main phenolic structures

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00817-7

present in foods [9,10]. In fact, the spectra from different phenolic classes (hydroxybenzoic and hydroxycinnamic acids as well as flavonoids) allow speedy identification of phenolic structures present in the samples upon careful analysis of the obtained results. Likewise, spectral differences can serve to distinguish among flavonoid structures (flavan-3-ol, flavonols, isoflavonoids and flavones) as well as glycosides and non-glycoside structures.

As far as we know there is no bibliographical reference concerning the application of only one chromatographic method (HPLC–DAD) to the study of the prominent phenolics from the most important phenolic sources (fruits, vegetables and legumes). The phenolic separations involve a large number of conjugated structures (many of them commercially unavailable) and subsequently, the design of a working strategy must take into account different sets such as phenolic standards, isolated phenolic and real extracts during chromatographic optimization. According to this working strategy and other conventional and well-known extraction techniques [11] the employment of only one HPLC method could be very useful because high variability of extraction and chromatographic conditions make it difficult to establish comparisons between the published results and qualitative and quantitative composition of phenolics in a given study.

On the other hand, the chromatographic methods are considered the most important analytical tools for the quantitative determination since the spectrophotometric methods offer a very low selectivity and generally overestimate the phenolic content [1].

Well aware of the above-mentioned drawbacks, we have concentrated on the optimization and evaluation of only one HPLC–DAD method, which could be used to separate, identify and quantify simultaneously, the most representative phenolic compounds present in foods such as hydroxybenzoic and hydroxycinnamic acids, but above all, flavonoids (both glycosides and non-glycoside structures). In order to fulfill this objective three phenolic groups were chosen in our strategy of optimization: standard phenolic compounds, isolated phenolic compounds and real extracts. Both the isolation of phenolic compounds not available as standards and the hydrolysis of glycosides used in the provisional identification have also been the aim of this work. Provided that the chromatographic method employed in all food samples was the same, the phenolic compounds, previously isolated from a particular food matrix could be used as pseudostandards and subsequently identified in other samples before carrying out more extensive characterization studies. Consequently, our approach could constitute an excellent investigation tool for carrying out a satisfactory comparison among a variety of phenolic sources of great interest in current analytical chemistry practice.

2. Experimental

2.1. Chemicals and samples

Methanol (MeOH) (HPLC grade; Scharlau, Barcelona, Spain), phosphoric acid (H_3PO_4) (Merck) and HPLC-grade water (Milli-Q system, Millipore, Bedford, MA, USA) were used during the preparation of mobile phases. The standards, arbutin, gallic, chlorogenic, caffeic and coumaric acids, (+)-catechin and (−)-epicatechin, phlorizdin, rutin, quercetin, myricetin, daidzein, genistein, apigenin and kaempferol were acquired from Sigma (St. Louis, MO, USA); procyanidins B1, B2 and B3 were isolated from apples and purified by using Sephadex LH-20.

Fresh red wine pomace (*Vitis vinifera*) was acquired from Valdepeñas (Spain). The different varieties of apples (*Malus domestica*) such as Golden, Reineta, Starky and Granny Smith and pears (*Pyrus comunis*) such as Agua, Blanquilla, Conference, Pasagrana and Decana were used; fresh and processed green beans (*Phaseolus vulgaris*) including two varieties (1 and 2) plus dried and processed lentils (*Lens culinaris*) were acquired from local supermarkets. All samples were studied as representative phenolic sources during the optimization process.

2.2. High-performance liquid chromatography

A HPLC Varian Model system consisting of ternary solvent delivery system (9012), an autosampler (9100), and a photodiode array detector (9065) coupled to an analytical workstation were used. A Waters system consisting of two pumps,

gradient controlled and a UV–Vis detector was also employed. Phenolics were detected at 280 nm. The injection volume was 20 μl. The separation was carried out with a Nucleosil 120 C_{18} column (25 cm×0.46 cm I.D.) with 5 μm packing. The elution solvents were A (aqueous 0.01 *M* phosphoric acid) and B (100% methanol). The samples were eluted according to the following binary gradient: the gradient was started with 5% B in order to reach 50% B at 10 min, 70% B at 15 min, 80% at 20 min and 100% at 25 min. The flow-rate was 1 ml/min.

2.3. Provisional identification of phenolic compounds

The compounds identification was carried out through comparison of their t_R values and UV spectra against standards stored in a data bank whenever the compounds were available commercially. In the cases of commercially unavailable phenolic compounds, the provisional identification was carried out by taking spectra characteristics or based on hydrolysis and isolation studies, as well as by comparison to the most relevant bibliographical data. Procyanidins were isolated from apples by using Sephadex LH-20 according the methods found in the literature [12,13]. Briefly, 2 g of Sephadex LH-20 was swelled in water and introduced into the column (30 cm×0.7 cm). A 3-ml volume of apple extracts was carefully applied to the column, and the bed was washed with aqueous methanol (20%, v/v). This fraction contained phenolic acids which were separated from the procyanidin fraction. The elution of phenolic acids was carefully controlled by spectroscopy and chromatographic methods, and the removal of the phenolic acids was confirmed by chromatographic run at 325 nm. Procyanidins were then eluted from the column with methanol and the obtained eluates were also carefully controlled by spectroscopy and chromatographic methods. Again, the chromatographic run at 325 nm showed the absence of phenolic acids in the procyanidin fraction. The isolated procyanidins were injected into the chromatographic system and checked by DAD. The application of the software indicated spectra characteristics typical of flavan-3-ols (only one band near to 277 nm) and no impurities in the five isolated peaks [(+)-catechin, (−)-epicatechin and three procyanidins]. The chromatographic run of the isolate was carried out under optimized conditions and the elution order guide found in the bibliography from different relevant articles allowed the provisional assignment to procyanidins B3, B1 and B2. With respect to hydrolysis studies, a 5-g amount of given samples was extracted with ethyl acetate, after that 3 ml of the extract were hydrolyzed in 2 *M* HCl at 100°C for 30 min under constant shaking conditions and the mixture was passed through a C_{18} Sep-Pak. Aglycons retained on the Sep-Pak were eluted with methanol, and then analyzed at 350 nm.

2.4. Procedures

Samples of apples and pears were purchased in different local supermarkets of Alcalá de Henares (Madrid, Spain). Both apples and pears were peeled and the peel was separated from the pulp. Whole green beans and peel fractions were carefully homogenized and the pulp was cut into little pieces. Lentils were ground to a particle size of 1 mm.

Samples of 5 g of fresh red wine pomace, 5 and 10 g of peel and pulp (apples and pears), respectively, 5 g of fresh green beans and 2 g of dried lentils were extracted at room temperature and in the absence of light with methanol (pure methanol for fruits and 80%, v/v, aqueous methanol for red wine pomace, fresh green beans and lentils) containing 1% 2,6-di-*tert*.-butyl-4-methylphenol (BHT) as an antioxidant agent using an ultrasonic bath. The conventional extraction procedure was optimized in order to obtain a quantitative extraction (higher than 95% in phenolic of interest). The samples were extracted with 10 ml of solvent for 1 h, 10 ml for 30 min, and then 5 ml for 30 min. The extracts were then combined to a final volume of 25 ml. The solutions chosen for HPLC analysis were filtered through membrane filters (0.5-μm pore size) prior to injection. Immediately after the sampling, tissues were extracted under no oxidation conditions and the extracts were stored at −20°C until chromatographic and spectrophotometric analysis (n=5). All the prepared samples (solutions and extracts) were filtered through 0.45-μm membranes (Millipore) and degassed in an ultrasonic bath before use.

All food samples were tested for their chromatographic profile after the final optimization of the

chromatographic method in order to carry out a quality analytical control.

2.5. Recovery studies

The recovery efficiency was determined by adding measured amounts of seven representative standards [arbutin, gallic acid, (+)-catechin, (−)-epicatechin, rutin and quercetin] to the samples prior to extraction of tissues samples. The samples were prepared as described above and 20 μl of the filtrate was injected into the HPLC column. Controls from all studied samples were prepared and subjected to the above specified extraction procedure. The recoveries were determined by subtracting the values obtained for the control matrix preparation from those samples that were prepared with the added standards, divided by the amount added and then multiplied by 100%. The recovery experiment was performed at three concentration levels, taking into account variety and matrix in a large number of samples. Mean values with standard deviations were calculated and included in the report.

2.6. Extraction efficiencies

The extraction was followed by using HPLC for the most representative phenolics in the samples studied. Percentages for each extraction were calculated as the amounts extracted relative to the total quantity extracted for each compound and from each sample.

3. Results and discussion

3.1. Liquid gradient optimization strategy

The development of only one liquid gradient for analysis of phenolics in food samples could constitute an interesting and quick solution approach for the study of the phenolic composition from different food sources prior to their complete characterization. The mentioned gradient might be used for the separation of both commercially available and unavailable phenolics as well as those present in real samples. Consequently, a suitable standard mixture and real samples might be chosen to carry out the liquid gradient optimization. For this reason, a standard mixture of representative 15 commercially available phenolic compounds and representative of all food groups (Table 1), a pseudostandard of procyanidins and real extracts as the ultimate test in the analysis were simultaneously employed. Real extracts were chosen from different sources: red wine pomace as a representative sample of the phenolic classes, fruits (apples and pears), vegetables (green beans) and legumes (lentils). The obtained

Table 1
Phenolic compounds employed in the optimization of the gradient method

Compound no.	Phenolic compound	Phenolic structure	Systematic name
1	Arbutin	Hydroquinone	Hydroquinone β-D-glucopyranoside
2	Gallic acid	Hydroxybenzoic acid	3,4,5-Trihydroxybenzoic acid
3	(+)-Catechin	Flavan-3-ol	[2*R*,3*S*]-2-[3,4-Dihydroxyphenyl]-3,4-dihydro-1[2H]-benzopyran-3,5,7-triol
4	Chlorogenic acid	Hydroxycinnamic acid	1,3,4,5-Tetrahydroxycyclohexane carboxylic acid
5	(−)-Epicatechin	Flavan-3-ol	[2*R*,3*R*]-2-[3,4-Dihydroxyphenyl]-3,4-dihydro-1[2H]-benzopyran-3,5,7-triol
6	Caffeic acid	Hydroxycinnamic acid	3,4-Dihydroxycinnamic acid
7	Coumaric acid	Hydroxycinnamic acid	4-Hydroxycinnamic acid
8	Phloridzin	Dihydrochalcone	Phloretin-2-β-D-glucoside
9	Rutin	Flavonol glycoside	Quercetin-3-β-rutinosido
10	Myricetin	Flavonol aglycone	3,3′,4′,5,5′,7-Hexahydroxyflavone
11	Daidzein	Isoflavonoid aglycone	4′,7-Dihydroxyisoflavone
12	Quercetin	Flavonol aglycone	3,3′,4′,5,7-Pentahydroxyflavone
13	Genistein	Isoflavonoid aglycone	4′,5,7-Trihydroxyisoflavone
14	Kaempferol	Flavonol aglycone	3,4′,5,7-Tetrahydroxyflavone
15	Apigenin	Flavone aglycone	4′,5,7-Trihydroxyflavone

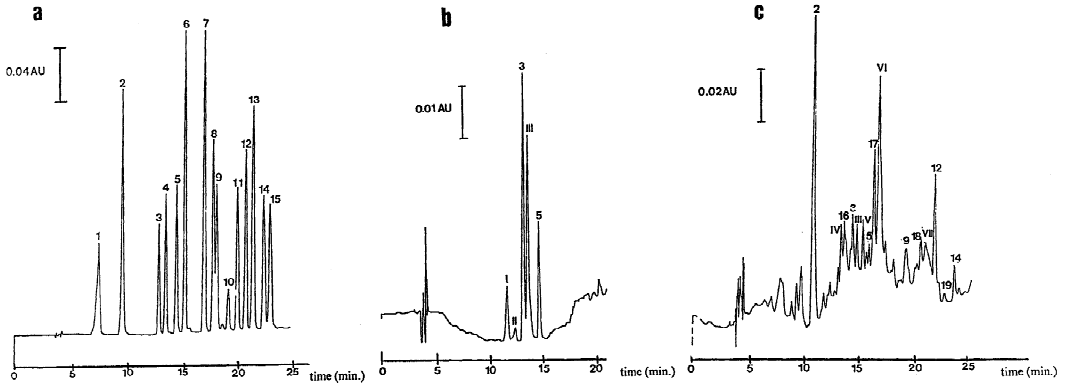

Fig. 1. Chromatograms at 280 nm obtained for phenolic compounds employed in the working strategy: (a) polyphenolic compounds standards, (b) isolated procyanidins and (c) red wine pomace. Peaks: available commercially: (1) arbutin, (2) gallic acid, (3) (+)-catechin, (4) chlorogenic acid, (5) (−)-epicatechin, (6) caffeic acid, (7) *p*-coumaric acid, (8) phloridzin, (9) rutin, (10) myricetin, (11) daidzein, (12) quercetin, (13) genistein, (14) kaempferol, (15) apigenin, (16) protocatechuic acid, (17) *p*-hydroxybenzoic acid, (18) ellagic acid and (19) cinnamic acid. Not available commercially: (I) procyanidin B3, (II) procyanidin B1, (III) procyanidin B2, (IV) hydroxybenzoic derivative, (VI) procyanidin structure and (VII) flavonoid structure.

chromatograms are shown in two figures: Fig. 1 shows the chromatograms obtained from the mixture of commercially available phenolic compounds (a), isolated procyanidins (b) and red wine pomace (c) as an initial analytical application of the currently developed method. Fig. 2 shows the chromatograms obtained from apple (a) and pear (b) peels, green pods (c) and lentils (d). After trying different gradients and solvents, methanol was chosen as an organic modifier because it showed the highest selectivity and it allowed the elution of currently studied phenolic compounds, (results with other modifiers are not shown). Taking into account the most important bibliographic data [13–19] that deal with phenolic composition in foods and well known optimization strategies [11], successive approaches were carried out to analyze as many as possible phenolics in a short chromatographic run. Gradient slope (range composition, time and flow) and gradient steps were optimized after injection of all compound groups (a, b and c) into the chromatographic system. After all optimization process, 21 commercially available phenolic compounds (Arabic numerals) and 25 commercially unavailable phenolic compounds (Roman numerals) were analyzed. Figs. 1 and 2 show chromatograms of the examined compounds. Table 2 lists retention times, including standard deviations (SDs) for six injections carried out on different days as well as UV absorption maximums of each peak, obtained by DAD from the studied phenolics. These parameters were employed toward the provisional identification of the commercially unavailable phenolic compounds. The optimized gradient consisted of four elution areas. The first gradient step (5–50% in methanol) allowed the elution of arbutin and gallic acid in both, standard mixture and real samples (red wine pomace and pears). Other structures such as single phenolics and hydroxybenzoic structures were also eluted (i.e., lentils). The main problem in the optimization of the second gradient step (50–70% in methanol) was the elution of (+)-catechin and (−)-epicatechin (commercially available) and their related procyanidins B1, B2 and B3 (commercially unavailable). Both types of compounds are of great interest in foods. Chlorogenic and caffeic acids were also eluted in this part of the liquid gradient. To achieve the complete optimization of this gradient step, procyanidins were isolated from apple and then they were used as a mixture of pseudostandards. Since the chromatographic analysis was carried out under same conditions and upon reaching final optimization, the procyanidins could be provisionally identified in other samples by comparing known retention time and UV spectra with those found for this fraction.

As far as the gradient range of 70–80% in methanol (step 3) was concerned, as can be expected, we encountered tremendous difficulties because at

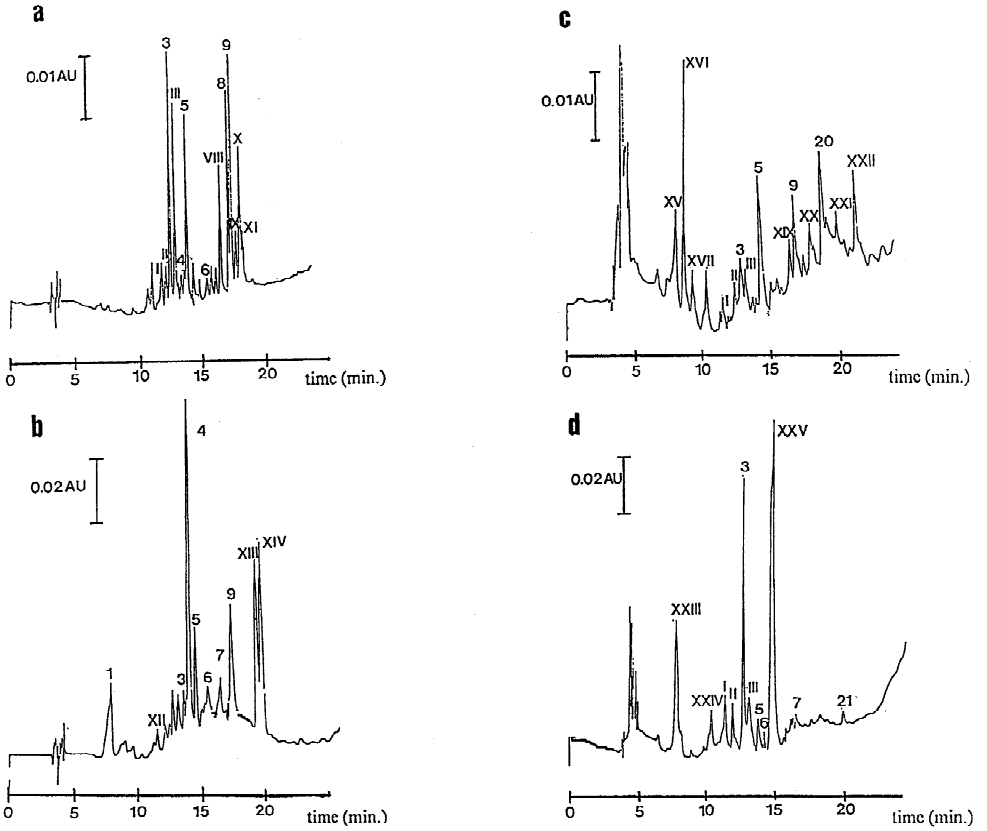

Fig. 2. Chromatograms at 280 nm obtained for different real extracts from phenolics employed in the working strategy: (a) apple and (b) pear peels, (c) green bean pod and (d) lentil. Peaks: available commercially: (1) arbutin, (3) (+)-catechin, (4) chlorogenic acid, (5) (−)-epicatechin, (6) caffeic acid, (7) *p*-coumaric acid, (8) phloridzin, (9) rutin and (21) luteolin. Not available commercially: (I) procyanidin B3, (II) procyanidin B1, (III) procyanidin B2, (VIII) phloretin xyloglucoside, (IX–XI) quercetin glycosides, (XII) hydroxybenzoic derivative, (XIII and XIV) isorhamnetin glycosides, (XV–XVIII) single phenols, (XIX) quercetin glycoside, (XX) kaempferol glycoside, (XXI) daidzein glycoside, (XXII) unknown structure, (XXIII) hydroxybenzoic derivative, (XXIV) unknown flavonoid and (XXV) kaempferol glycoside.

this composition the most complex structures (flavonoids) were eluted. In fact, flavonoid fraction is a very complex class that included flavan-3-ols (eluted before), flavonols (aglycones and their related glycosides), chalcones, flavones and isoflavones. Besides the separation of chalcones (phlorizdin and phloretin xyloglucoside) and quercetin glycosides from apples and the elution of the isorhamnetin from pears, other difficulties were also observed. Thus, quercetin and kaempferol glycosides were a complex fraction noticed in vegetable samples. In all the cases, the hydrolysis of the glycosides was carried out to improve their provisional identification. However, kaempferol glycosides could be easily identified on the basis of their spectroscopic characteristics and subsequently it could be easily distinguished from other glycosides [20]. Since the spectral behavior of quercetin glycosides was identical, different gradient slopes were plotted in this step, in order to achieve the complete separation. Likewise, in this step of the gradient it was possible to separate *p*-hydroxybenzoic and ellagic acids from red wine pomace (available standards), chalcones (available and unavailable phenolics) from apples and coumaric and caffeic acids (available standards) from green beans and lentils.

At the last optimized gradient step (80–100% in methanol) the only distinguishable species were the

Table 2
Chromatographic and spectroscopic parameters employed in the provisional identification of phenolics in the samples

Phenolic compounds commercially available				Phenolic compounds not commercially available			
Peak no.	Phenolic compound	$t_R \pm$SD (min)	UV bands (nm)	Peak no.	Phenolic compound	$t_R \pm$SD (min)	UV bands (nm)
1	Arbutin	7.65±0.08	281	I	Procyanidin B3	12.11±0.02	277
2	Gallic acid	9.85±0.10	270	II	Procyanidin B1	12.84±0.02	277
3	(+)-Catechin	13.02±0.07	277	III	Procyanidin B2	13.48±0.03	278
4	Chlorogenic acid	14.00±0.09	240; 326 (max)	IV	Hydroxybenzoic derivative	12.33±0.33	270
5	(−)-Epicatechin	14.48±0.08	277	V	Procyanidin structure	13.50±0.03	279
6	Caffeic acid	15.27±0.11	238; 324 (max)	VI	Procyanidin structure	13.82±0.04	279
7	Coumaric acid	17.03±0.10	224; 309 (max)	VII	Flavonoid	20.32±0.04	286
8	Phlorizdin	17.89±0.10	283	VIII	Phloretin xyloglucoside xyloglucoside	17.41±0.02	282
9	Rutin	18.48±0.11	256 (max); 356	IX	Quercetin glycoside	18.75±0.03	255 (max); 357
10	Myricetin	19.24±0.11	251 (max); 367	X	Quercetin glycoside	19.08±0.03	255 (max); 357
11	Daidzein	20.07±0.10	246 (max); 299	XI	Quercetin glycoside	19.45±0.03	255 (max); 357
12	Quercetin	20.78±0.11	253 (max); 367	XII	Hydroxybenzoic derivative	10.97±0.09	270
13	Genistein	21.48±0.12	258 (max); 341	XIII	Isorhametin glycoside	19.33±0.10	255 (max); 357
14	Kaempferol	22.43±0.13	262 (max); 367	XIV	Isorhametin glycoside	19.77±0.12	255 (max); 357
15	Apigenin	22.96±0.14	265; 335 (max)	XV	Phenol derivative	7.86±0.04	254
16	Protocatechuic acid	12.57±0.02	252 (max); 285	XVI	Phenol derivative	8.48±0.04	254
17	*p*-Hydroxybenzoic	15.45±0.04	271	XVII	Phenol derivative	9.27±0.04	254
18	Ellagic acid	19.42±0.04	251	XVIII	Phenol derivative	10.50±0.04	254
19	Cinnamic acid	21.58±0.05	308	XIX	Quercetin glycoside	17.01±0.03	255 (max); 355
20	Phloretin	20.05±0.02	282	XX	Kaempferol glycoside	19.45±0.03	262 (max); 348
21	Luteolin	21.96±0.07	265; 345 (max)	XXI	Daidzein glycoside	20.60±0.03	258 (max); 299
				XXII	Unknown flavonoid	22.03±0.03	292
				XXIII	Hydroxybenzoic derivative	7.66±0.06	270
				XXIV	Procyanidin structure	10.65±0.05	277
				XXV	Kaempferol glycoside	15.67±0.05	263 (max); 344

aglycones (except in fruits). However, this gradient step was needed for controlling the hydrolysis because isorhametin, quercetin and kaempferol aglycones were eluted in this area.

The results obtained by applying the optimized gradient elution and their characteristics are summarized in Table 3. As can be observed, the following elution order could be established: hydroxybenzoics>hydroxycinnamics>flavonoids. As far as the flavonoids were concerned, the elution order became difficult to establish; however, the following could be proposed: flavan-3-ols> chalcones>flavonol glycosides>flavonol. The optimized gradient could be very suitable to study the phenolics of interest, as well as to trace the hydrolysis of the all the conjugates.

3.2. Validation of the chromatographic method

The chromatographic method was carefully evaluated according to their analytical characteristics. As far as qualitative and quantitative analysis were concerned, two types of characteristics were verified. With respect to qualitative analysis; the method was evaluated by taking into account the precision in the retention time, elution purity, analysis time and phenolic compounds eluted in each type of food samples. A high reproducibility in the retention time was obtained with relative standard deviations (RSDs) less than 5% in all cases studied. The elution purity was studied in the prominent peaks. The spectra were taken from the upslope, the apex and the downslope of the peak for each compound of interest. In all cases examined non-impurities were observed. The phenolic structures eluted in each part of the gradient as well as the analysis and separation time employed for each sample are also shown in Table 3. Upon comparison (whenever possible) of apples, pears and red wine pomace, the results obtained in this work coincided with those reported

Table 3
Elution of the phenolic compounds in the gradient elution method proposed in representative food samples

Gradient characteristics		t_G (min)	Fruit samples			Vegetable samples, Green beans, *Phaseolus vulgaris*	Legume samples, Lentils, *Lens culinaris*
Step no.	MeOH (%)		Red wine pomace, *Vitis vinifera*	Apples, *Malus domestica*	Pears, *Pyrus comunis*		
1	5	0	Gallic acid	Empty elution area in their chromatogram	Arbutin	Single phenols	Hydroxybenzoic derivative
2	50	10	Protocatechuic acid (+)-Catechin (−)-Epicatechin Procyanidins	(+)-Catechin (−)-Epicatechin Procyanidins B1, B2, B3 Chlorogenic acid	Hydroxybenzoic derivative (+)-Catechin (−)-Epicatechin Chlorogenic acid	(+)-Catechin (−)-Epicatechin Procyanidins B1, B2, B3	(+)-Catechin (−)-Epicatechin Procyanidins B1, B2, B3
3	70	15	*p*-Hydroxybenzoic acid Rutin Ellagic acid	Caffeic acid Phloretin derivative Phlorizdin Rutin and other quercetin glycosides	Caffeic acid Coumaric acid Rutin Isorhamnetin glycosides	Rutin and other quercetin and kaempferol glycosides	Caffeic acid Coumaric acid Kaempferol glycoside
4	80	20	Quercetin aglycone Cinnamic acid	Empty elution area in their chromatogram	Empty elution area in their chromatogram	Daidzein glycoside	Luteolin
5	100	25	Kaempferol aglycone				
Analysis time (min)			35	30	30	30–35	35

in the bibliography that deals with qualitative composition [13,15–19,21–23]. The analysis time ranged between 30 and 35 min, including the re-equilibration time in order to secure reproducible results. These times were excellent when compared to those found in the bibliography. Therefore, analysis time, elution purity and number of eluted structures could be used as a measurement of method's quality.

On the other hand, and purely for the quantitative purposes, the method was also carefully evaluated for precision and accuracy. The precision was evaluated from both, the peak area of the phenolics of interest and the calibration slopes at different times intervals and operators. The accuracy was evaluated from extraction efficiencies and the recovery studies of the commercially available phenolic compounds and taking into account all the investigated samples. Table 4 shows both, the extraction efficiencies and optimized extraction conditions for the most representative phenolic structures (commercially available and unavailable) from the studied samples. An ultrasonic bath at room temperature was used as a suitable conventional extraction system because it allowed relatively rapid extraction of the phenolic contained in the samples. Pure and aqueous methanol were used as extraction solvents because they permitted phenolic extraction characterized by a high yield. The extraction time depended on the studied food matrix and it ranged between 45 and 60 min. Multiple extractions were performed under optimized extraction conditions (ultrasonic bath, solvent and equilibrium time) to obtain quantitative yields. Relative yields in the ranges of 52–70%, 21–41% and 3–14% for the first, second and third extraction were obtained for the studied phenolics. A fourth extraction was carried out and in all studied cases. The observed chromatographic signal was lower than the detection limit of the employed analytical technique. Finally, as can be deduced from Table 4, three extraction are necessary to secure extraction efficiencies close to 100%. Table 5 shows the obtained precision and accuracy as well as the values of the analytical characteristics used to evaluate the optimized chromatographic method optimized. The obtained low variation coefficient (<5%) in the peak areas as well as the calibration slopes, and the high

Table 4
Optimized ultrasonic extraction conditions and efficiencies

Sample	Phenolic compound[a]	Solvent	Extraction time[b] (min)	Extraction efficiencies[c] (%)		
				Extraction I	Extraction II	Extraction III
Red pomace	Gallic acid	80% (v/v) MeOH	45	70	24	7
	Quercetin			78	17	5
Apple peel	(+)-Catechin	MeOH	60	67	24	9
	Chlorogenic acid			68	21	12
	(−)-Epicatechin			67	21	10
	Phlorizdin			66	25	9
Pear peel	Arbutin	MeOH	60	52	33	14
	Chlorogenic acid			60	31	9
	Rutin			56	41	4
Green beans	Phenol structure	80% (v/v) MeOH	45	60	26	14
	(−)-Epicatechin			64	25	12
	Rutin			52	36	12
Lentils	Hydroxybenzoic structure	80% (v/v) MeOH	60	70	29	1
	(+)-Catechin			67	30	3
	Kaempferol glycoside			70	23	4

[a] Phenolics most representative from all structures in the samples: available and not available commercially.
[b] Equilibrium time corresponding to first extraction.
[c] Percentages are amounts extracted relative to the total quantity extracted from each sample.

Table 5
Analytical characteristics and evaluation of the gradient method proposed

Phenolic structure	Phenolic compound	Sensitivity ($\cdot 10^{-3}$)[a]	Detection limit[b] (μg/ml)	Recovery[c]				
				Fruits			Vegetables, Lentils, *Phaseolus vulgaris*	Legumes *Lens culinaris*
				Red wine pomace, *Vitis vinifera*	Apples, *Malus domestica*	Green beans, Pears, *Pyrus comunis*		
Hydroquinone	Arbutin	8±0.5	1.10	–	–	97.5±2.4	–	–
Hydroxybenzoic acid	Gallic acid	74±2.9	0.12	101.3±5.4	–	–	94.1±1.3	104.5±2.0
Hydroxycinnamic acids	Chlorogenic acid	27±0.2	0.33	–	95.9±1.9	98.0±2.6	98.3±0.8	96.7±1.9
	Caffeic acid	58±2.0	0.15	–	–	–	–	–
	Coumaric acid	95±1.9	0.10	–	–	–	–	–
Flavan-3-ols	(+)-Catechin	15±0.3	0.61	–	101.6±4.5	–	–	102.8±0.5
	(−)-Epicatechin	14±0.8	0.61	–	95.3±4.3	102.3±7.6	98.9±3.7	–
Chalcones	Phlorizdin	42±1.6	0.21	–	–	–	–	–
Flavonol glycoside	Rutin	22±1.0	0.40	–	103.0±3.7	100.9±6.1	100.0±4.3	104.4±2.5
Flavonols	Quercetin	40±0.5	0.22	97.9±4.3	–	–	–	–
	Kaempferol	50±0.5	0.18	–	–	–	–	–

[a] Mean value±standard deviation obtained in the calibration slopes between different days ($n=6$). Regression equation: $y=a+bx$ where y is peak area and x the concentration in μg/ml.
[b] Detection limit obtained by using the calibration slope shown in the table.
[c] Expressed as recovery studies.

recoveries values allowed to establish a high reproducibility and a satisfactory accuracy in the proposed method when applied to all studied phenolic structures from involved samples. Likewise, the accuracy was independent of phenolic structure, sample and the investigated matrix.

The limits of detection were calculated as signal-to-noise ratio of 3:1. The obtained values are also summarized in Table 5. The values ranged between 0.10 and 1.10 μg/ml, which suggested full capacity of the method for the quantification of each phenolic compound investigated in this study can be observed.

4. Conclusions

The present study discussed one liquid gradient where methanol was used as modifier on C_{18}-modified silica which allowed for the first time the separation of the prominent phenolic compounds from very different sources. The chromatographic method yielded a very satisfactory separation of the standards, which involved all representative phenolic classes present in foods and it also allowed the elution of the aglycones released from the hydrolysis for the provisional identification of the glycosides. Under the same chromatographic conditions the separation of the prominent phenolics of interest was successfully achieved in the studied real samples. The optimized chromatographic method could constitute an excellent research tool for the study of phenolic from different sources, and it offers an alternative for the provisional identification of these compounds prior to their full analytical characterization. The recently validated method could be used as a quality control screening in very different food sources.

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