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Fast separation of (poly)phenolic compounds from apples and pears by high-performance liquid chromatography with diode-array detection

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Abstract

Polyphenolic compounds in apples and pears were analysed by HPLC on C_{18}-modified silica. Gradient elution with phosphoric acid–methanol mixtures and phosphoric acid–acetonitrile mixtures gave complete separation of all polyphenolics of interest. The use of methanol as modifier was preferred because it provides a more rapid separation (20 min). Diode-array detection was used for the provisional identification of polyphenolic compounds not available as standards.

Keywords: Apples; Pears; Phenols; Polyphenolic compounds

1. Introduction

Polyphenolic composition from apples and pears has been studied using HPLC reversed-phase chromatography employing gradient elution methods with phosphoric acid–methanol [1–5] and acetic acid–acetonitrile [6,7] mixtures as mobile phase and conventional C_{18} columns (25–30×0.46 cm I.D.) with 3–5 μm packing have been reported in most cases [1–4,7]. However, the run times often are excessive and the optimization of the gradient is required. The main difficulty in the optimization of the gradient methods for analysis of polyphenolic compounds from apples and pears is that some polyphenolic compounds involved in these fruits are not available as standards and, so, it is necessary to carry out their isolation and subsequently the final optimization of the method must be made with the real samples before their establishment. In this way, diode-array detection appears a good technique for the provisional identification of polyphenolic compounds not available as standards. Also, the co-elution between different polyphenolic classes involved in apples and pears, such as hydroxycinnamic acids, chalcones, flavan-3-ol and flavonol glycosides, can be seen by checking the purity spectra.

Apple and pear composition appears to be constituted by hydroxycinnamic acids, flavan-3-ol (catechins and procyanidins) and flavonol glycosides [4, 7–10]. The main difference between both fruits is the presence of arbutin and the lack of phloretin derivatives in pears [11]. The information found in the literature about this aspect is limited. However,

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the differences between apples and pears could always be found if separation is good.

Therefore, the aim of this work has been to optimize a rapid and reproducible gradient elution method for the analysis of polyphenolic compounds from apples and pears.

2. Experimental

2.1. Reagents and standards

The standards (+)-catechin and (−)-epicatechin as well as chlorogenic, caffeic and coumaric acids, arbutin, phlorizdin, rutin and quercitrin were acquired from Sigma (St. Louis, MO, USA). Methanol of HPLC grade was acquired from Sharlau and all other chemicals of analytical-reagent grade were purchased from Merck.

In all cases, the water used was of HPLC quality, purified in a Milli-Q system (Millipore, Bedford, MA, USA). All the prepared (solutions and extracts) were filtered through 0.45-μm membranes (Millipore) and degassed in an ultrasonic bath before being used.

2.2. Materials

Golden variety apples and Decana variety pears were acquired in a local supermarket in Alcalá de Henares (Alcalá de Henares, Madrid, Spain). Apples and pears were purchased from the same packets (October and November, 1997; January and March, 1998) under the same maturity state. The apples and pears were peeled and the peel was separated of the pulp. Peel fraction was carefully homogenized and the pulp was cut into little pieces. Immediately following the sampling, tissues were extracted and the extracts were stored at −20°C before analysis. Procyanidin fraction was isolated from Granny Smith apples and Decana pears.

2.3. Apparatus for HPLC

A HPLC Varian model system consisting of ternary solvent delivery system (9012), an autosampler (9100), and a photodiode array detector (9065) coupled with an analytical workstation was used. The column used was a Nucleosil 120 C_{18} (25×0.46 cm I.D.) with 5 μm packing.

2.4. Extraction procedure

Apple and pear samples, 5 and 10 g, of peel and fresh pulp, respectively, were extracted with pure methanol in presence of 2,6-di-*tert*-butyl-4-methylphenol (BHT) at 1% and in absence of light using an ultrasonic bath at room temperature. The extraction procedure was carried out according to the method previously optimized, in order to obtain a quantitative extraction. The sample was extracted twice with 10 ml of solvent for 1 h and 30 min, respectively. The extract was removed each time and a third extraction with 5 ml of methanol was performed for 30 min. The extracts were combined to a final volume of 25 ml. Solutions to be analysed by HPLC were filtered through membrane filters (0.5-μm pore size) prior to injection.

2.5. Isolation of procyanidins

Procyanidins were isolated from apples and pears using Sephadex LH-20. Two g were swelled in water and introduced into the column (30×0.7 cm). The bed was washed with methanol (20%) and 3 ml of apple extracts were carefully applied into the column. Phenolic acids were eluted with methanol (20%) and separated from the procyanidin fraction. These compounds were eluted from the column with methanol.

2.6. Hydrolysis of glycosides from pear extracts

A sample of 5 g of pear peel was extracted with ethyl acetate, after 3 ml of the extract were hydrolyzed in 2 N HCl at 100°C for 30 min, and the mixture was passed through a C_{18} Sep-Pak. Aglycons retained on the Sep-Pak were eluted with methanol.

2.7. HPLC conditions

The analysis was monitored at 280 nm and the absorption spectra of compounds were recorded between 210 and 350 nm. The gradient conditions are given in the figure captions. The column operated

at room temperature. The sample injection volume was 20 μl. Identification of compounds were made by comparing their t_R values and UV spectra with those of standards stored in a data bank.

3. Results and discussion

3.1. HPLC optimization of the mixture of phenolic compounds

To optimize the HPLC conditions for the analysis of all phenolic compounds from peel and pulp from apples and pears, an artificial mixture was prepared containing the polyphenolic compounds which were commercially available. Table 1 lists the common and systematic name of the polyphenolic compounds employed in the optimization studies.

Working according to conventional and well-known optimization strategies [12], two gradients emerge (see Fig. 1A and B). Under acetonitrile conditions the gradient range optimized was 2% for the initial composition and 35% for the final composition in acetonitrile during 35 min of elution. This gradient composition allowed to elute the first band (arbutin) near $2t_0$ and kaempferol at the end of the gradient without empty spaces at the beginning and at the end of the chromatogram, respectively (Fig. 1A). Under optimized conditions, the resolution obtained in all cases was good including the resolution obtained between (+)-catechin and chlorogenic acid (R_s=1.01). The resolution between these peaks was very important since both compounds are typical of apples and pears.

In a second attempt to optimize the conditions, water–phosphoric acid (0.01 *M*)–methanol mixtures were used as mobile phase. After trying different gradients, the best resolution with the lowest analysis time was obtained under the conditions shown in the caption to Fig. 1B. The gradient range optimized was 5% in methanol at the initial step and 100% in methanol at the end of the method for a gradient time of 25 min. These values for gradient range allowed the elution of arbutin (first band) and kaempferol (last band) at the beginning ($2t_0$) and at the end of the chromatogram, without empty spaces in their elution areas (Fig. 1B). Under these conditions, the resolution was acceptable in all cases, including rutin and phlorizdin (R_s=0.80). The separation between these peaks was also very important since both phenolic compounds are typical of peel apples.

Phosphoric acid–water–methanol mixtures allowed to eluate all phenolic compounds in less time than those obtained with phosphoric acid–water–acetonitrile mixtures. On the contrary, the best resolutions were obtained under acetonitrile conditions. Apart of the different elution order of caffeic acid and (−)-epicatechin, the most important difference found between both solvents was the elution order between phlorizdin and rutin. Phosphoric acid–methanol mixtures eluted phlorizdin first, followed by rutin, with similar retention times and an acceptable resolution, whereas phosphoric acid–acetonitrile mixtures first eluted rutin and then phlorizdin.

The precision of the optimized methods was calculated as the inter-assay reproducibility carried out on different days. The first attempt to evaluate precision was made by studying the retention be-

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	3-Cyanidol
4	Chlorogenic acid	Hydroxycinnamic acid	1,3,4,5-Tetrahydroxycyclohexane carboxylic acid
5	Caffeic acid	Hydroxycinnamic acid	3,4-Dihydroxycinnamic acid
6	(−)-Epicatechin	Flavan-3-ol	*cis*-2-[3,4-Dihydroxyphenol]-3,4-dihydro-1-benzopyrano-3,5,7-triol
7	*p*-Coumaric acid	Hydroxycinnamic acid	4-Hydroxycinnamic acid
8	Rutin	Flavonol glycoside	Quercetin-3-β-rutinosido
9	Phlorizdin	Dihydrochalcone	Phloretin-2′-β-D-glucoside
10	Quercetin	Flavonol aglycone	3,3′,4′,5,7-Pentahydroxyflavone
11	Kaempferol	Flavonol aglycone	3,4′,5,7-Tetrahydroxyflavone

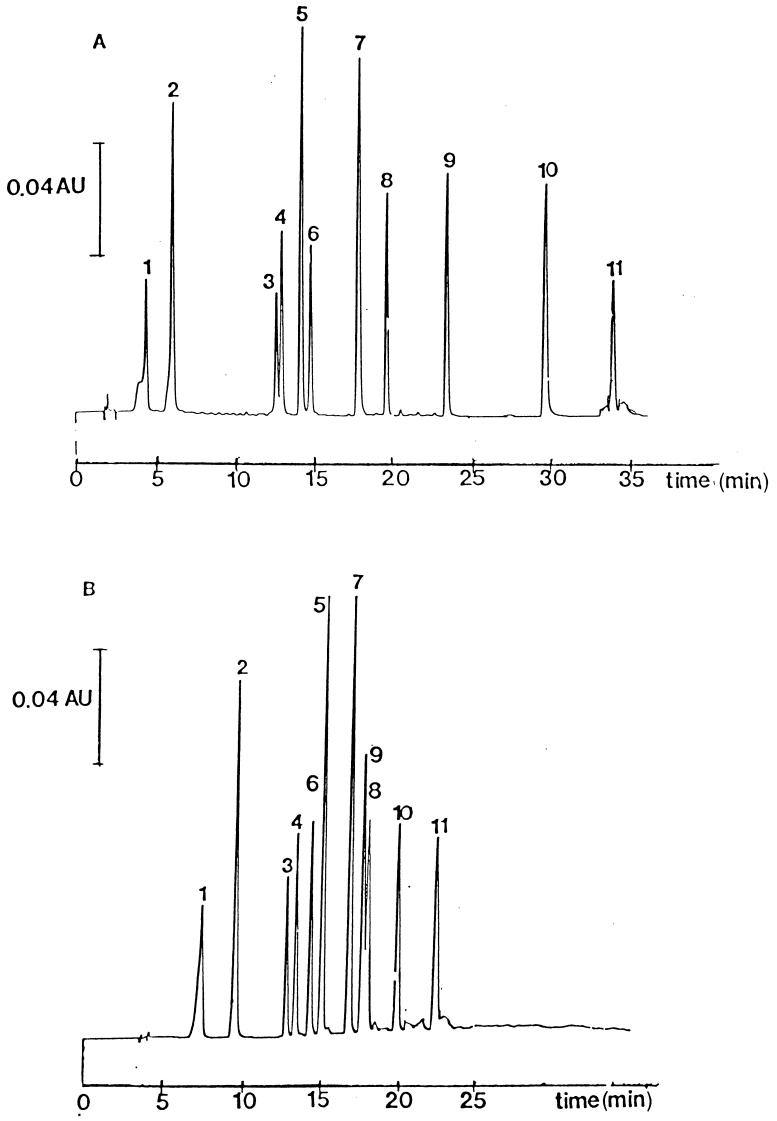

Fig. 1. HPLC of the mixture of phenolic compound standards. Detection at 280 nm. (A) Solvent system: solvent A, phosphoric acid 0.01 *M*; solvent B, acetonitrile. Elution gradient used: the gradient started with 2% B to reach 15% at 10 min and 35% B at 35 min. Solvent flow rate, 2 ml/min. (B) Solvent system: solvent A, phosphoric acid 0.01 *M*; solvent B, methanol. Elution gradient used: the gradient started with 5% in B to reach 50% at 10 min and 100% at 25 min. Solvent flow rate, 1 ml/min. Peaks: (1) arbutin, (2) gallic acid, (3) (+)-catechin, (4) chlorogenic acid, (5) caffeic acid, (6) (−)-epicatechin, (7) coumaric acid (8) rutin, (9) phlorizdin, (10) quercetin and (11) kaempferol.

haviour for the standard mixture using acetonitrile and methanol mixtures. High reproducibility was obtained with R.S.D. values ranging between 0.50 and 0.90% and 0.40 and 1.00%, under acetonitrile and methanol conditions, respectively.

3.2. HPLC analysis of phenolic compounds in apples and pears

Fig. 2 shows the HPLC of the phenolic compounds from Golden apples (A) and Decana pears

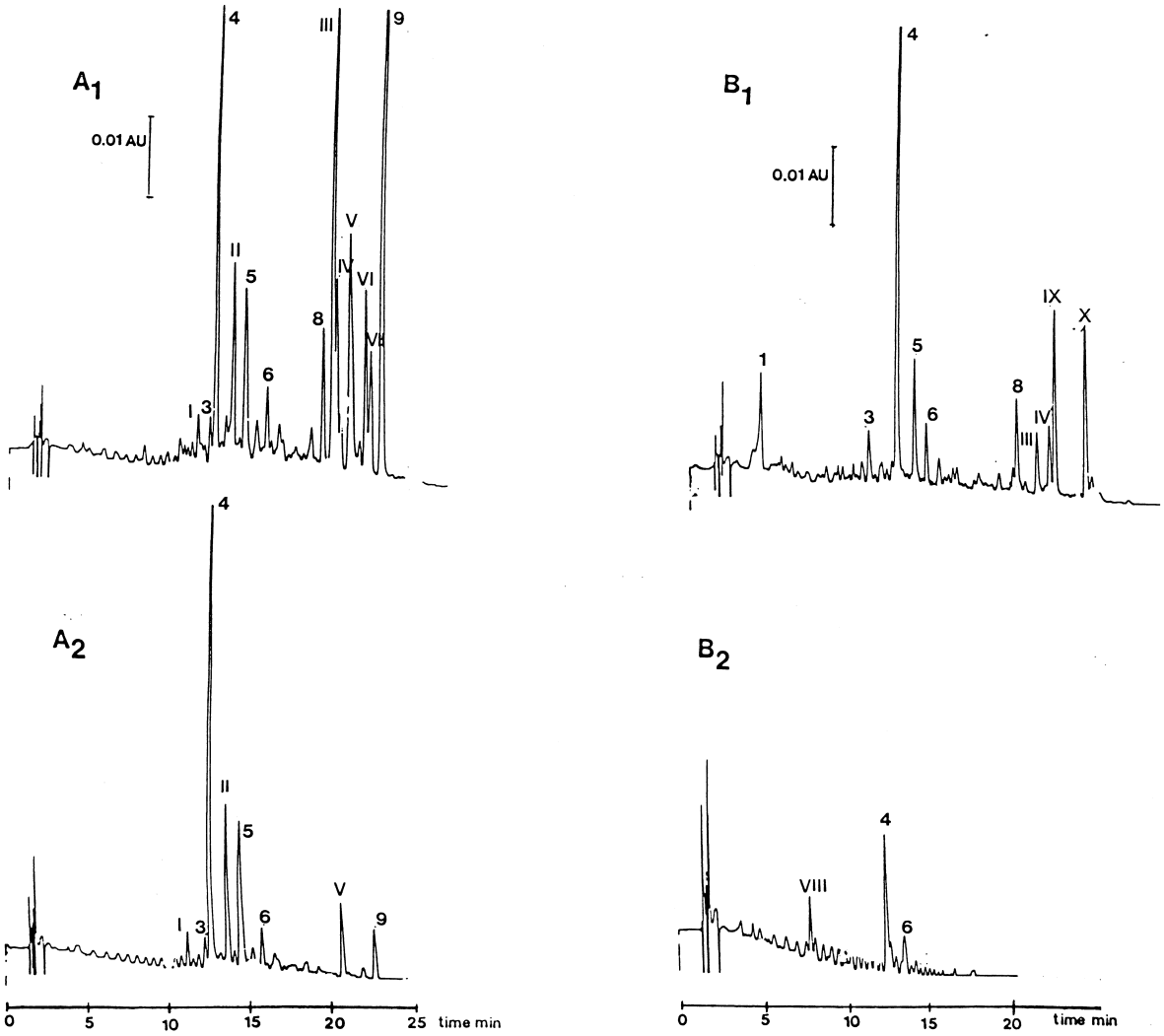

Fig. 2. HPLC of phenolic compounds in apple (A) and pear (B) peel (1) and pulp (2). Detection at 280 nm. Solvent system phosphoric acid–acetonitrile. (A) Apple peaks: (I) procyanidin B1, (3) (+)-catechin, (4) chlorogenic acid, (ii) procyanidin B2, (5) caffeic acid, (6) (−)-epicatechin, (8) rutin, (III, IV) quercetin glycosides, (V) phloretin xyloglucoside, (VI, VII) quercetin glycosides and (9) phlorizdin. (B) Pear peaks: (1) arbutin, (VIII) benzoic derivative, (3) (+)-catechin, (4) chlorogenic acid, (5) caffeic acid, (6) (−)-epicatechin, (8) rutin, (XI, XII) quercetin glycosides and (IX, X) isorhamnetin glycosides.

(B) in both peel (1) and pulp (2) extracts chromatographed using phosphoric acid (0.01 *M*)–acetonitrile mixtures. In respect to apple peel (Fig. 2A), chlorogenic and caffeic acid, (+)-catechin, (−)-epicatechin, rutin and phlorizdin were identified from the retention time and spectra of those standards. The elution of other peaks not included in the standard mixture were noticed. Procyanidin compounds were isolated from apple peels by using a Sephadex column, and they were characterized by chromatographic and spectroscopic methods. The results obtained allowed the provisional identification of peaks 1 and 4 as procyanidins B3 and B1, respectively. Their elution order was established by the

reported values under similar chromatographic conditions [1,3,4,10]. Quercetin glycosides were tentatively identified on the basis of their spectra. In respect to chalcone composition, apart of phlorizdin other phloretin derivative (peak V) was provisionally identified because of their spectra characteristics. Their lower retention time indicated a more polar structure which was identified as phloretin xyloglucoside [6]. In respect to the pear peel, arbutin, (+)-catechin, (−)-epicatechin, chlorogenic, caffeic and coumaric acids, as well as rutin, were identified. Other bands not included in the initial standard mixture were detected at the end of the chromatogram (peaks IX and X). These compounds were tentatively identified as isorhamnetin glycosides since their hydrolysis released isorhamnetin aglycone. Likewise, the optimized method allowed the elution of arbutin and another new peak at the beginning of the chromatogram (chromatogram of pear pulp, peak VIII). This compound was tentatively identified as a benzoic derivative since the spectrum was identical to ones found for gallic acid.

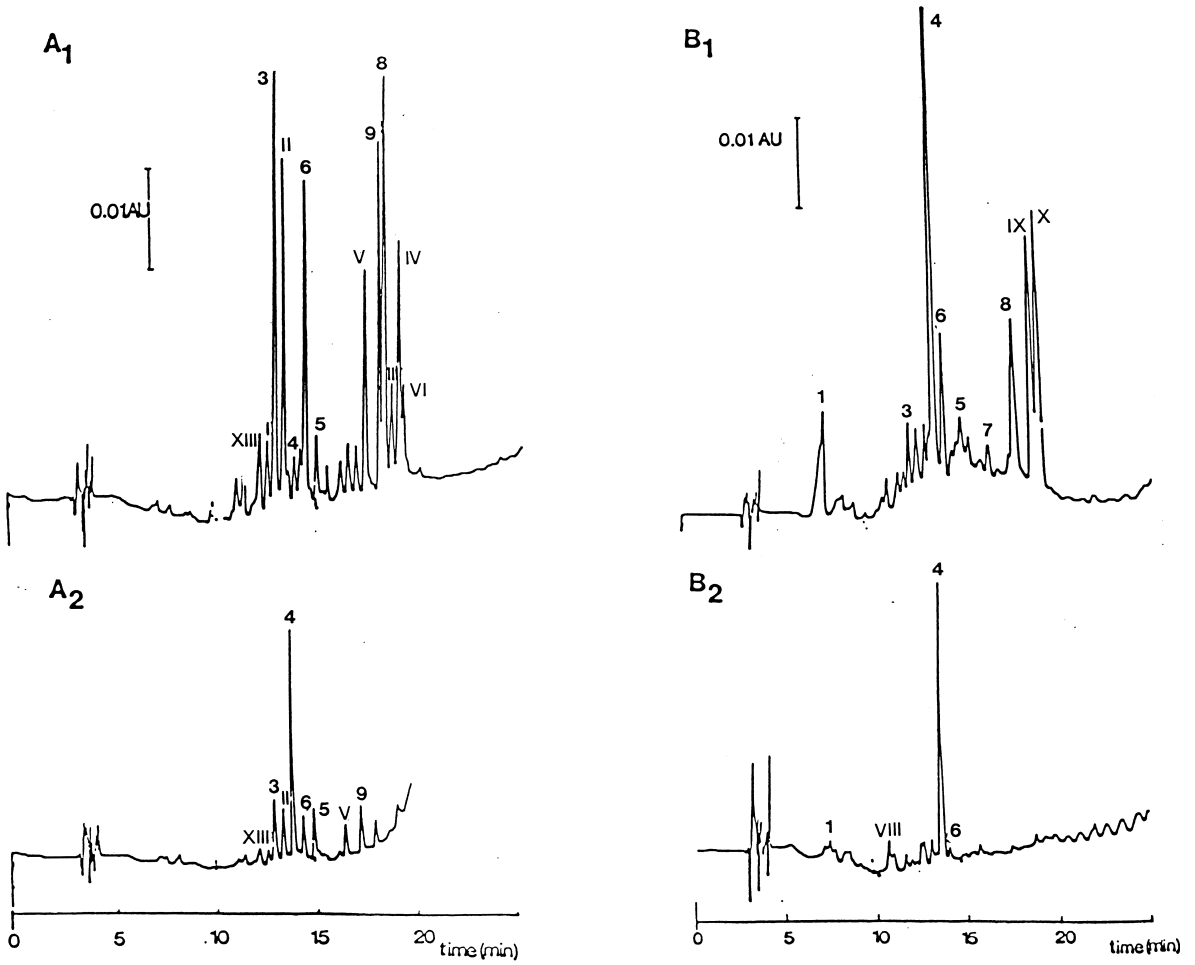

Fig. 3. HPLC of phenolic compounds in apples (A) and pears (B) in peels (1) and pulps (2). Detection at 280 nm. Solvent system phosphoric acid (A)–methanol (B): the gradient started with 5% in B to reach 50% at 10 min, 70% in B at 15 min, 80% in B at 20 min and 100% at 25 min). (A) Apple peaks: (XIII) procyanidin B3, (I) procyanidin B1 (3) (+)-catechin, (II) procyanidin B2, (4) chlorogenic acid, (6) (−)-epicatechin, (5) caffeic acid, (V) phloretin xyloglucoside, (9) phlorizdin, (8) rutin and (III, IV, VI) quercetin glycosides. (B) Pear peaks: (1) arbutin, (VIII) benzoic derivative, (3) (+)-catechin, (4) chlorogenic acid, (6) (−)-epicatechin, (5) caffeic acid, (7) *p*-coumaric acid, (8) rutin and (IX, X) isorhamnetin glycosides.

Table 2
Characteristics of the gradient methods

Phosphoric acid (0.01 *M*)–acetonitrile (B)							Phosphoric acid (0.01 *M*)–methanol (B)						
Step no.	%B	*F* (ml/min)	t_G (min)	Slope (%B/min)	Shape	Structure eluted	Step no.	%B	*F* (ml/min)	t_G (min)	Slope (%B/min)	Shape	Structure eluted
0	2	2.0	5	0	Isocratic		0	5	1.5	5	0	Isocratic	
1	2	2.0	Initial conditions. Starting				1	5	1.0	Initial conditions. Starting			
2	15	2.0	10	1.3	Linear	ArbutinGallic acid	2	50	1.0	10	4.5	Linear	Arbutin
3	35	2.0	35	1.0	Linear	Procyanidin B1	3	70	1.0	5	4.0	Linear	Gallic acid Procyanidins B1 B3 (+)-Catechin Procyanidin B2 Chlorogenic acid
						(+)-Catechin Chlorogenic acid Procyanidin B2 Caffeic acid (−)-Epicatechin Quercetin glycosides Ph. Xyloglucoside Isorhamnetin glycosides Phlorizdin	4	80	1.0	5	2.0	Linear	(−)-Epicatechin Caffeic and coumaric acids Ph. derivatives Quercetin and Isorhametin glycosides
							5	100	1.0	5	4.0	Linear	QuercetinKaempferol
4	2	2.0	5	6.6	Linear	Reversed gradient	6	5	1.0	5	19.0	Linear	Reversed gradient
Separation time: 35 min							Separation time: 20 min						

On the other hand, Fig. 3 shows the HPLC of the phenolic compounds from Golden apples (A) and Decana pears (B) in both peel (1) and pulp (2) extracts chromatographed using phosphoric acid (0.01 *M*)–methanol mixtures as mobile phase. Under these conditions, one minor modification in the gradient slope in order to adjust the band spacing to get acceptable resolution ($R_S > 0.80$) was needed (new gradient conditions are given in figure caption). Apple peel chromatograms showed the presence of chlorogenic and caffeic acids, (+)-catechin, (−)-epicatechin, phlorizdin and rutin. These compounds were identified by comparison of the retention time and UV spectra with those standards. Peaks numbered as XIII, I and II were provisional identified as procyanidins B3, B1 and B2, respectively, after their isolation from apple peel. Likewise, the absence of coumaric acid allowed the elution of phloretin xyloglucoside in this part of the chromatogram. Pear peel chromatograms (Fig. 3B), showed the presence of arbutin, chlorogenic, coumaric and caffeic acids, (+)-catechin, (−)-epicatechin and rutin, which were identified by comparison of their retention times and UV spectra with those of standards. Peaks IX and X, seen at the end of the chromatogram, were tentative identified as isorhamnetin glycosides since their hydrolysis released isorhamnetin aglycone.

In respect to pulp extracts (Figs. 2 and 3) the optimized method allowed the separation of the polyphenolic compounds involved in these type of matrices from both fruits.

From the results obtained with both modifiers (acetonitrile and methanol) we can establish a comparison between the optimized elution gradients. Acetonitrile mixtures were more efficient for glycosides structures, whereas methanol conditions were more efficient for procyanidin fraction. Acetonitrile mixtures showed the best resolutions, whereas methanol mixtures allowed the separation of polyphenolic compounds with lower analysis time and with an acceptable resolution. Also, both gradient elution methods allowed the inclusion of an internal standard (quercetin and kaempferol) at the end of their chromatograms for controlling the reliability of the analysis, and to show the main polyphenolic differences between both fruits. The main difference between both modifiers was the elution order between phloretin derivatives and quercetin glycosides.

On the other hand, in both elution gradient methods the analysis time employed was lower than those offered in the literature for the separation of the same compounds under similar chromatographic conditions. In fact, the chromatographic column and solvents employed in the literature were very similar, but the workers did not pay attention at the empty spaces observed in their chromatograms. Programs shown in the literature eluted the first band (procyanidin B1) at 37 min using methanol and phosphoric acid as solvents [4]. Other programs have not eliminated the time between the elution of gallic acid and (+)-catechin; also, the elution of chlorogenic acid was not near (+)-catechin [3]. Other authors employed 70 min in the elution of very similar phenolic compounds in apples of the same varieties under acetonitrile conditions [7]. In the last work, only procyanidin B2 was identified, probably due to using acetonitrile as mobile phase.

Finally, the chromatographic characteristics of both gradient methods are summarized in Table 2.

4. Conclusion

Methanol mixtures are proposed for the HPLC analysis of polyphenolic compounds in apples and pears, since methanol mixtures are less expensive and toxic than acetonitrile mixtures, and they also allowed the most rapid separation of the prominent and less prominent polyphenolic compounds from apples and pears with acceptable resolution. Under optimized chromatographic conditions, only 20 min were needed for the separation of polyphenolic compounds in both fruits with a high elution purity and a good reproducibility. In regard to polyphenolic composition, the method proposed allowed to establish the main differences between the fruits.

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