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Pressurized liquid extraction for the determination of polyphenols in apple

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Abstract

Pressurized liquid extraction (PLE) has been optimized for the determination of polyphenols in Golden Delicious apple peel and pulp. The effects of experimental variables, such as solvent composition, temperature, static extraction time and pressure, on PLE efficiency have been studied. Once the optimum conditions were established the recovery and the precision of the method for each analyte was tested by means of repeated analysis.

Keywords: Apples; Pressurized liquid extraction; Polyphenols; Flavonoids

1. Introduction

Phenolic compounds are one of the most diverse and widespread groups of natural constituents universally distributed among vascular plants. Thus, plant polyphenols are presented in human diet, being of great interest as they seem to present potential anticarcinogenic properties and reduce the incidence of cardiovascular diseases due to their antioxidant activity and their function as free radical scavengers [1]. Moreover, phenolic compounds have an important role in the nutritional, organoleptic and commercial properties of agricultural foodstuffs, since they contribute to their sensory properties such as color, astringency, bitterness and flavor [2,3].

The main groups of phenolic compounds present in apple fruit are catechins (monomeric flavan-3-ols and procyanidins (polymeric flavan-3-ols)), some dihydrochalcones (phloretin glycosides), some flavonols (quercetin glycosides) and hydroxycinnamic acid derivatives, and in the skin of red varieties, some anthocyanins (cyanidin glycosides) [4]. The methodology used to analyse these phenolic compounds in apples generally includes extractions with solvents, such as methanol, ethanol, acetone or mixtures of these with water [4,5]; cleanup and further fractionation by liquid–liquid extraction (LLE), usually with ethyl acetate [6], column chromatography (CC) [7] or solid-phase extraction (SPE) [8]. Finally, after the extract is concentrated, polyphenols are separated by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with UV–visible detection.

Sample extraction procedures are often regarded as bottlenecks in analytical methods. Moreover, classical sample preparation techniques are both time and solvent consuming. In addition, this step accounts for at least one-third of the error generated by

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the analytical method [9]. Therefore, the importance of sample preparation in analytical chemistry cannot be overemphasized.

Pressurized liquid extraction (PLE) is a new extraction technique that uses organic solvents at high pressures and temperatures above their normal boiling point. With PLE, a solid sample is packed into a stainless steel extraction cell and extracted with a suitable solvent under elevated temperature (40–200°C) and pressure (500–3000 p.s.i.) conditions for short periods of time (5–15 min). The sample extract is purged into a collection vial with the aid of a compressed gas.

Having made an exhaustive revision on PLE publications (Current Contents 1995–2000), we realized that most of them are dealing with environmental samples [10], whereas there are only five in which this technique is used to extract natural products from plants [11–15], but in any case, polyphenols.

In this paper, the optimization of polyphenol extraction from apple peel and pulp using PLE is reported. The effects of various experimental parameters, such as solvent composition, temperature, pressure and static extraction time are investigated.

2. Experimental

2.1. Reagents and standards

Polyphenol standards were supplied as follows: (+)-catechin, (−)-epicatechin, rutin, phloridzin, chlorogenic acid and *p*-coumaric acid by Sigma–Aldrich Chemie (Steinheim, Germany); hyperoside, isoquercitrin, avicularin and quercitrin by Extrasynthèse (Genay, France). Phloretin-2′-xyloglucoside and procyanidin B2 were kindly provided by Dr. F.A. Tomás-Barberán and Dr. C. Santos-Buelga, respectively. Methanol (Romil Chemical Ltd, Heidelberg, Germany) was of HPLC grade. Water was purified on a Milli-Q system from Millipore (Bedford, MA, USA). Glacial acetic acid and fuming hydrochloric acid 30% from Merck (Darmstdt, Germany) and ascorbic acid from Panreac (Barcelona, Spain) were of analytical quality. Diatomaceous earth was from Sigma–Aldrich Chemie (Steinheim, Germany). Nitrogen with 99.995% of purity was from Carburos Metálicos (Barcelona, Spain).

Stock standard solutions of (+)-catechin, (−)-epicatechin, rutin, phloridzin, chlorogenic acid and *p*-coumaric acid at a concentration of 1 mg ml^{-1} and hyperoside, isoquercitrin, and quercitrin at 0.6 mg ml^{-1} were prepared in methanol and stored at 4°C in darkness.

2.2. Samples

Apples from the Golden Delicious variety were carefully peeled and both peel and pulp were separately frozen, chopped and homogenized with an aqueous solution of ascorbic acid 0.1 g ml^{-1} (20 ml of ascorbic acid solution for each 100 g of peel or pulp) in a conventional food processor, frozen again and lyophilized. The freeze-dried material was stored at room temperature in a desiccator until analysis.

2.3. Pressurized liquid extraction

Pressurized liquid extractions were performed on a Dionex ASE 200 (Dionex Corp., Sunnyvale, CA, USA) system. Aliquots of freeze-dried apple peel (1 g) or pulp (3 g) were mixed with diatomaceous earth in a proportion (1:1) and placed into a 11- or 22-ml stainless steel extraction cell, respectively. The use of a dispersion agent, such as diatomaceous earth, is recommended in order to reduce the solvent volume used for the extraction [16]. The extraction cell was extracted under the extraction conditions.

Percentages of methanol in the solvent, temperature, pressure and static extraction time were the parameters under study. The pre-set default conditions were as follows: pre-heating period, 5 min; solvent flush volume, 60% of the extraction cell volume; number of extraction cycles, 1; purge, 90 s using pressurized nitrogen (150 p.s.i.); and collection, in 60 ml glass vials with teflon coated rubber caps (I-CHEM, New Castle, DE, USA). The solvent used was previously degassed in order to avoid the oxidation of the analytes under the operating conditions [16].

The extracts were stored in glass vials at −20°C in darkness until chromatographic analysis. Then, each one was filtered through a 0.45 μm nylon membrane (Lida, Kenosha, WI, USA) and transferred to a 50 ml

volumetric flask which was brought up to its volume with methanol. An aliquot (4–12 ml) was evaporated to dryness in a Turbovap LV Evaporator (Zymark, Hopkinton, MA, USA) provided with a nitrogen stream and a water bath at 35°C. After solvent evaporation, it was reconstituted in (0.5–2 ml) methanol–aqueous hydrochloric acid 0.1% (30:70, v/v) and filtered through a 0.45 μm PTFE filter (Waters, Milford, CA, USA) prior to injection into the HPLC system.

2.4. HPLC analysis

Analysis were performed on a Hewlett–Packard Series 1100 liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler and a DAD detector, connected to a HP ChemStation software. A reversed-phase Nova-Pak C_{18} (300×3.9 mm I.D., 4 μm) column and a Nova-Pak C_{18} (10× 3.9 mm I.D., 4 μm) guard column (Waters, Barcelona, Spain) were used. Solvents that constituted the mobile phase were A (acetic acid–water, 10:90, v/v) and B (methanol). The elution conditions applied were: 0–10 min, 0% B isocratic; 10–40 min, linear gradient 0–15% B; 40–60 min, 15% B isocratic; and finally, washing and reconditioning steps of the column were included (60–70 min, linear gradient 15–100% B; 70–75 min, 100% B isocratic; 75–85 min, linear gradient 100–0% B; and 5 min, 0% B isocratic. The flow-rate was 0.8 ml min^{-1} and the injection volume was 50 μl. The system operated at room temperature. Catechins and phloretin glycosides were monitored and quantified at 280 nm, quercetin glycosides at 370 nm and hydroxycinnamic acids at 320 nm.

3. Results and discussion

3.1. Optimization of PLE procedure

The optimization of the PLE procedure was performed using freeze-dried apple peel (without any previous spiking) and the optimal conditions found for apple peel were applied to apple pulp. Next, analytical performance data were determined. It is worthwhile to add that only one apple variety was used because all compounds considered in this study are known to be present in all apple varieties. In addition, optimizing the PLE procedure for peel is justified because all components present in apple pulp are also present in apple peel, though in higher amounts in peel.

3.2. Solvent composition

Solvents generally used in PLE are the same ones that are employed in classical extraction procedures [17]. Therefore, since the most used solvents to extract phenolic compounds from apple are methanol and mixtures of this with water, only hydromethanolic mixtures were studied as solvent. Fig. 1 shows that the extraction yield increased with the percentage of methanol in the extraction solvent, following a similar tendency for all the four classes of phenolic compounds (catechins, phloretin glycosides, quercetin glycosides and hydroxycinnamic

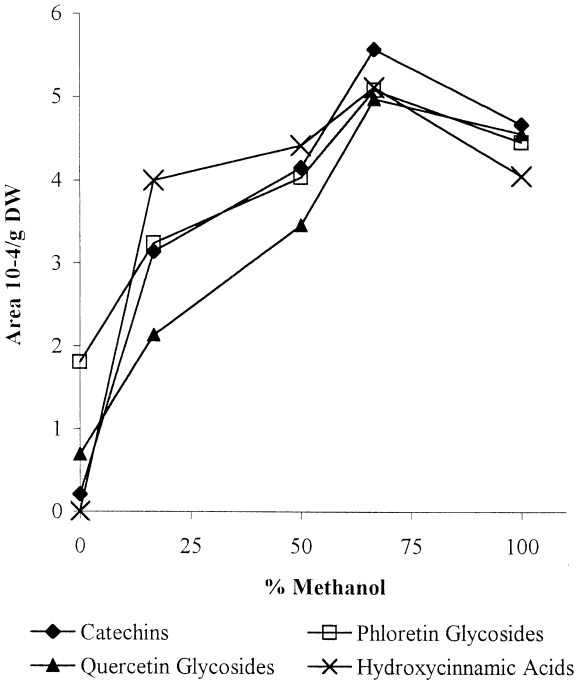

Fig. 1. Sum of the areas of chromatographic peaks of polyphenols belonging to the same class per gram of dried weight (DW) of apple peel as a function of the composition of hydromethanolic mixtures used as the extraction solvent for PLE. PLE operating conditions: temperature, 40°C; pressure, 1000 p.s.i.; and static extraction time, 10 min.

acids), being the behaviour at methanol 100% an exception. With pure methanol, the amount of analyte extracted slightly decreased, in accordance with the fact that polyphenols with several hydroxyl groups, such as glycosides, are hydrophilic, and generally present higher solubilities in hydroalcoholic mixtures than in a pure alcohol solvent [18]. This behaviour is also observed for catechins and hydroxycinnamic acids since, these compounds are even more hydrophilic than the glycosides considered, as the order of elution in reversed-phase HPLC shows. Therefore, it seemed that the most powerful extraction solvent would be an hydroalcoholic mixture that would contain about two-thirds of methanol. However, preliminary studies with this new technique showed that the presence of water in the extraction solvent or in the sample (fresh apple peel or pulp were assayed, mixing crude material even with three times of drying agent) decreased the precision of the method, resulting higher RSDs (about 10% for fresh peel and about 20% for fresh pulp). Moreover, taking into account the slight difference in the amount of extracted analytes between using pure methanol or the optimum hydroalcoholic mixture, pure methanol was chosen as the extraction solvent. This selection also favoured the later evaporation of the extract.

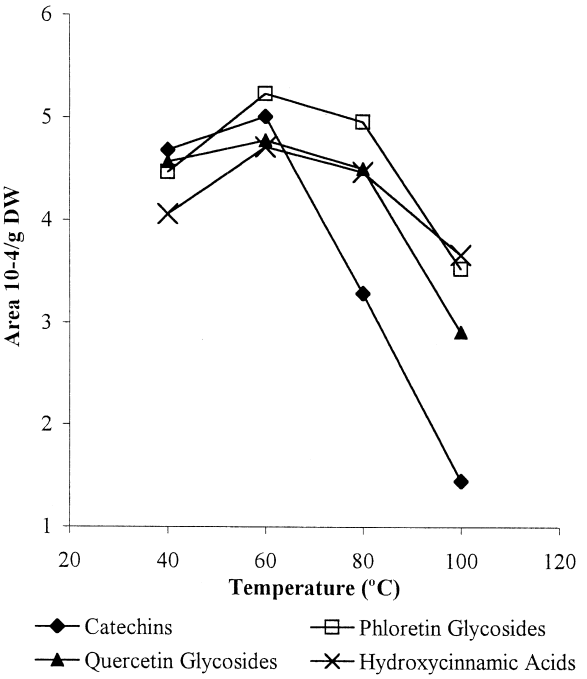

Fig. 2. Sum of the areas of chromatographic peaks of polyphenols belonging to the same class per gram of dried weight (DW) of apple peel as a function of the extraction temperature for PLE. PLE operating conditions: pressure, 1000 p.s.i.; static extraction time, 10 min; and extraction solvent, pure methanol.

3.3. Temperature, static extraction time and pressure

Fig. 2 shows the extraction efficiencies for each polyphenolic class as the operating temperature was increased. All of them experimented a slightly increase in their extraction yield at 60°C and a decrease at higher temperatures, that is especially sharp for catechins. This decrease in the extraction yield could be due to a possible degradation of polyphenols at temperatures above 40°C, caused by hydrolysis, internal redox reactions and polymerizations [19]. Moreover, an increasing temperature led to obtain cloudy extracts; only at 40°C, the extract was a transparent solution. Taking into account these facts, 40°C was the temperature selected as the optimum.

Studying the static extraction time (5, 10 and 15 min and PLE operating conditions: pressure, 1000 p.s.i.; temperature, 40°C; and extraction solvent, methanol), it was observed that the amounts of extracted analytes were practically constant during these periods of time, so a 5 min static extraction time was selected for further experiments.

When the extraction pressure was examined (1000, 1250 and 1500 p.s.i. and PLE operating conditions: static extraction time, 5 min; temperature, 40°C; and extraction solvent, methanol), any significant influence on the efficiency of the process was detected, what had been earlier reported, for instance when extracting phathalates in polymeric samples [17]. The purpose of pressurizing the extraction cell is to prevent the solvent from boiling at the extraction temperature and to ensure that the solvent remains in intimate contact with the sample. Thus, the lighter operating pressure, 1000 p.s.i., was chosen.

3.4. PLE method: recovery and repeatability

The recovery efficiency for the PLE procedure

was determined by performing consecutive pressurized liquid extractions on the same sample under the optimized PLE conditions (solvent, temperature, static extraction time and pressure), until no phenolic compound was detected by HPLC analysis. The limits of detection for each analyte are indicated in Table 2; in all cases their values represent less than 1% of the total amount of compound found in samples, except for hyperoside and isoquercitrin in pulp (9 and 4%, respectively). The recovery experiment was performed with three replicates. Taking into account the results obtained with this experiment, the optimal number of extraction cycles was selected. We concluded that two successive extraction cycles would be acceptable, since the recoveries obtained for every analyte were higher than 80% and with good precisions (Table 1). Better recoveries could be obtained with more extraction cycles, but it would suppose an unnecessary increase of the amount of solvent used and the extraction time. Thus, the experimental conditions of the PLE method proposed for the extraction of polyphenols from freeze-dried apple peel and pulp were: solvent, methanol; temperature, 40°C; static extraction time, 5 min; pressure, 1000 p.s.i. and two extraction cycles. The total volumes of solvent required for these conditions were 25 ml or 40 ml approximately, when using an 11- or 22-ml extraction cell, respectively. With these conditions the repeatability, expressed by means of RSDs, was better for peel than for pulp. This fact is due to the evidence that the amounts of analytes in peel are much higher than in pulp (Table 2).

3.5. Identification and quantitation of phenolic compounds

Chromatogram of a PLE extract from freeze-dried apple peel is shown in Fig. 3. The identification of those compounds for which we had standards was

Table 1
PLE recoveries (%) of phenolic compounds from freeze-dried apple peel and pulp (mean and percentage of relative standard deviation for three replicates)

	Peel			Pulp	
	Peak	Mean	RSD (%)	Mean	RSD (%)
Catechins					
(+)-Catechin	*1*	100	1	83	6
Procyanidin B2[a]	*2*	86	2	83	6
(−)-Epicatechin	*3*	98	1	82	7
Unknown procyanidin[a]	*4*	84	3	84	6
Phloretin glycosides					
Phloretin-2′-xyloglucoside[b]	*5*	96	1	83	6
Phloridzin	*6*	93	2	84	7
Quercetin glycosides					
Hyperoside	*7*	82	5	81	8
Isoquercitrin	*8*	86	4	84	7
Unknown quercetin glycoside+Rutin[c]	*9*	85	3	84	7
Avicularin[c]	*10*	92	3	81	8
Quercitrin	*11*	90	3	84	6
Hydroxycinnamic acids					
Chlorogenic acid	*12*	95	1	86	4
p-Coumaric acid derivative[d]	*13*	95	1	84	6

[a] Quantified as (+)-catechin.
[b] Quantified as phloridzin.
[c] Quantified as rutin.
[d] Quantified as *p*-coumaric acid.

Table 2
Amounts of phenolic compounds in freeze-dried Golden Delicious apple peel and pulp (μg of compound per gram of freeze-dried weight (DW)) extracted under the optimized PLE conditions and analysed by HPLC–DAD, their repeatabilities (mean and percentage of relative standard deviation for three replicates) and limits of detection (LOD)

	Peel				Pulp		
	Peak	Mean ($\mu g\ g^{-1}$ DW)	RSD (%)	LOD ($\mu g\ g^{-1}$ DW)	Mean ($\mu g\ g^{-1}$ DW)	RSD (%)	LOD ($\mu g\ g^{-1}$ DW)
Catechins							
(+)-Catechin	*1*	43	7	0.2	18	8	0.08
Procyanidin B2[a]	*2*	1190	2	0.2	340	5	0.08
(−)-Epicatechin	*3*	1080	7	0.08	220	6	0.03
Unknown procyanidin[a]	*4*	97	1	0.2	22	7	0.08
Phloretin Glycosides							
Phloretin-2′-xyloglucoside[b]	*5*	230	5	0.7	48	6	0.2
Phloridzin	*6*	429	1	0.7	59	8	0.2
Quercetin Glycosides							
Hyperoside	*7*	1680	1	0.3	0.8	5	0.09
Isoquercitrin	*8*	310	2	0.2	2	9	0.07
Unknown quercetin glycoside+Rutin[c]	*9*	530	2	0.09	6	5	0.03
Avicularin[c]	*10*	920	2	0.09	5	4	0.03
Quercitrin	*11*	630	2	0.3	31	5	0.1
Hydroxycinnamic Acids							
Chlorogenic acid	*12*	410	9	0.3	540	7	0.09
p-Coumaric acid derivative[d]	*13*	17	7	0.2	46	5	0.06

[a] Quantified as (+)-catechin.
[b] Quantified as phloridzin.
[c] Quantified as rutin.
[d] Quantified as *p*-coumaric acid.

carried out by comparison of their retention time and their UV–visible spectra with those obtained injecting standards in the same conditions. Some other chromatographic peaks were assigned to a particular polyphenol class according to their UV–visible spectra and bibliographic sources. Peaks 4 and 13 exhibited spectral characteristics identical to those of catechins and *p*-coumaric acid, respectively, so they were identified as a procyanidin and a *p*-coumaric derivative and were quantified as (+)-catechin and *p*-coumaric acid, respectively [20]. Peak 9 was constituted by two peaks partially overlapped of two quercetin glycosides, being the former, unknown, and the latter, rutin. These overlapped peaks were quantified together as rutin. Avicularin, phloretin-2′-xyloglucoside and procyanidin B2 were identified by comparison with standards, but were quantified as rutin, phloridzin and (+)-catechin, respectively, since the available amounts of solid standards were not enough to perform an accurate quantitation.

4. Conclusion

The efficiency of pressurized liquid extraction is comparable to conventional techniques to extract polyphenols from apple peel and pulp. The solvent volume required in PLE is similar or even smaller, but this extraction procedure is less time-consuming and the handling of the sample is reduced.

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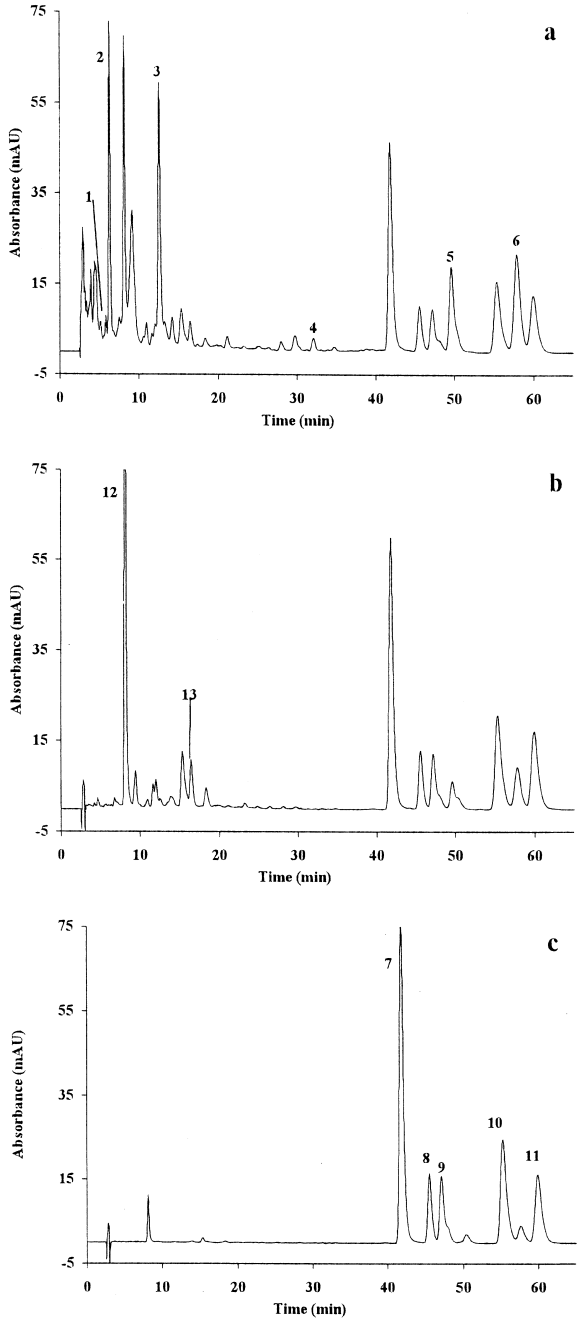

Fig. 3. Chromatogram of a PLE extract of freeze-dried apple peel recorded at: (a) 280, (b) 320, and (c) 370 nm. 1, (+)- catechin; 2, procyanidin B2; 3, (−)-epicatechin; 4, unknown procyanidin; 5, phloretin-2′-xyloglucoside; 6, phloridzin; 7, hyperoside; 8, isoquercitrin; 9, unknown quercetin glycosides+rutin; 10, avicularin; 11, quercitrin; 12, chlorogenic acid; 13, *p*-coumaric acid derivative.

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