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A validated solid–liquid extraction method for the HPLC determination of polyphenols in apple tissues Comparison with pressurised liquid extraction

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Abstract

A solid–liquid extraction procedure followed by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with a photodiode array detector (DAD) for the determination of polyphenols in freeze-dried apple peel and pulp is reported. The extraction step consists in sonicating 0.5 g of freeze-dried apple tissue with 30 mL of methanol–water–acetic acid (30:69:1, v/v/v) containing 2 g of ascorbic acid/L, for 10 min in an ultrasonic bath. The whole method was validated, concluding that it is a robust method that presents high extraction efficiencies (peel: >91%, pulp: >95%) and appropriate precisions (within day: R.S.D. (n = 5) <5%, and between days: R.S.D. (n = 5) <7%) at the different concentration levels of polyphenols that can be found in apple samples. The method was compared with one previously published, consisting in a pressurized liquid extraction (PLE) followed by RP-HPLC-DAD determination. The advantages and disadvantages of both methods are discussed.

Keywords: Polyphenols; Apple; Solid–liquid extraction; Pressurised liquid extraction; HPLC; Validation

1. Introduction

Apples present a wide diversity of polyphenols classified into several major classes. Flavan-3-ols are preponderant, being present in monomeric forms named catechins, and in oligometric and polymeric forms known as procyanidins. Hydroxycinnamic acids are the second class in concentration, and together with catechins, they are involved in the browning phenomena that takes place during apple fruit processing, being responsible for the yellow or orange coloration of apple products [1]. Dihydrochalcones, flavonols and anthocyanins are minor components that contribute to the pigmentation of apples and to the potential antioxidant activity of apples and their derived foodstuffs [2].

In cider apple cultivars, polyphenol interest is due to the fact that they are responsible for the colour and the balance of bitterness to astringency, which defines the ‘overall mouth-

Abbreviations: ACY, anthocyanins; AVI, avicularin; CAT, (+)-catechin; CAT-2, unknown flavan-3-ol; CG-1, unknown anthocyanin; CQA, 5-caffeoylquinic acid; DHC, dihydrochalcones; EC, (−)-epicatechin; FA, flavan-3-ols; FO, flavonols; HCA, hydroxycinnamic acids; HYP, hyperoside; IDE, ideain; IQC, isoquercitrin; PB2, procyanidin B2; PCM, p-coumaric acid; PCQ, 4-*p*-coumaroylquinic acid; PLD-1, hydroxyphloretin diglycoside; PLD-2, hydroxyphloretin monoglycoside; PLG, phloridzin; PLXG, phloretin-2′-O-xyloglucoside; QCI, quercitrin; QG-1, unknown quercetin glycoside; RUT, rutin; GM, Geza miña; MK, Moko; MN111, Manttoni 111; MNEM7, Manttoni EM7; TX, Txalaka; UR, Urdin; DAD, diode array detector; DW, dried weight; HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantitation; nd, not detected; PLE, pressurized liquid extraction; PPO, polyphenoloxidase; R.S.D., relative standard deviation; S.D., standard deviation; SLE, solid–liquid extraction

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feel' of ciders [3]. Furthermore, they are implicated in the alcoholic and malolactic fermentations as metabolites, providing cider aroma, and as inhibitors of the microbiological growth, controlling fermentation rates and cider spoilage [4]. Phenolic compounds are also involved in the colloidal stability of cider [5]. In addition, polyphenols as natural antioxidant constituents of human diet are receiving increasing attention due to their health-protective properties [6].

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 [7,8]; cleanup and further fractionation by liquid–liquid extraction (LLE), usually with ethyl acetate [9,10]; column chromatography (CC) [11,12] or solid phase extraction (SPE) [13]. Finally, after the extract is concentrated, polyphenols are separated by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with UV–vis detection or mass spectrometry [14]. In other works, samples are extracted by sonication with solvent, and the crude extract is directly injected into the HPLC system [15]. Generally, in publications about natural products in plants (such as those about the determination of polyphenols in apples), little information is given about the optimisation procedures of the analytical methods used, and frequently, these methods have not been validated.

Sample extraction procedures are often regarded as bottlenecks in analytical methods. Moreover, classical sample preparation techniques are both time and solvent consuming, and sample handling can decrease the quality of the analytical results. In this sense, the sample preparation step accounts for at least one-third of the error generated by the analytical method [16]. Therefore, the importance of sample preparation in analytical methods should not be undervalued.

In this work, a method for the determination of polyphenols in apples was developed using a solid–liquid extraction (SLE) assisted by sonication and followed by RP-HPLC coupled with a photodiode array detector (DAD). This method was optimised and exhaustively validated by evaluating the selectivity, the linear range, the limits of detection and quantitation, the accuracy, the repeatabilities within day and between days, the robustness of the method and the polyphenol stabilities. In addition, the method was compared with a published validated method, which consists in a pressurised liquid extraction (PLE) followed by RP-HPLC analysis [17]. The advantages and drawbacks of both methods are commented.

2. Experimental

2.1. Reagents and standards

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 formic acid provided by Merck (Darmstdt, Germany) and ascorbic acid by Panreac (Barcelona, Spain) were of analytical quality. All solvents used were previously filtered through 0.45 μm nylon membranes (Lida, Kenosha, WI, USA).

Polyphenol standards were supplied as follows: (+)-catechin, (−)-epicatechin, rutin, phloridzin, 5-caffeoylquinic acid and *p*-coumaric acid by Sigma–Aldrich Chemie (Steinheim, Germany); hyperoside, isoquercitrin, avicularin, quercitrin and ideain chloride by Extrasynthèse (Genay, France). 4-*p*-Coumaroylquinic acid, phloretin-2′-*O*-xyloglucoside and procyanidin B2 were kindly provided by Dr. Guyot, Dr. F.A. Tomás–Barberán and Dr. C. Santos–Buelga, respectively. Stock standard solutions of (+)-catechin, (−)-epicatechin, rutin, phloridzin, 5-caffeoylquinic acid and *p*-coumaric acid at a concentration of 1 mg mL^{-1} and hyperoside, isoquercitrin, quercitrin and ideain at 0.6 mg mL^{-1} were prepared in methanol and stored at 4 °C in dark. The other standards were prepared in approximately concentrations and only used for chromatographic peak identification.

2.2. Plant materials

Apple cultivars used for the optimization and validation of the solid–liquid extraction of polyphenols from apple peel and pulp were some local varieties used for cidermaking: Urdin (UR), Txalaka (TX), Mantonni 111 (MN111), Manttoni EM7 (MNEM7), Geza Miña (GM) and Moko (MK). Apples were harvested in the Experimental Orchards of the Diputación Foral de Gipuzkoa in Hondarribia (Guipúzcoa, Spain) and the Diputación Foral de Bizkaia in Zalla (Vizcaya, Spain) during the 2000 season.

2.3. Apple powder preparation

Fruits were harvested at maturity, which was tested by the lugol index [18]. For each variety, fruits were mechanically peeled and cored, and sprayed with an aqueous solution of 3% formic acid in order to avoid polyphenol oxidation. Peels and pulps were immediately frozen in liquid nitrogen and then they were freeze-dried. An aliquot for each variety was used to determine the fresh/dry matter ratio. The dried tissues were crushed in closed vessels to avoid hydratation, obtaining an homogenous powder that was stored at room temperature in a desiccator until analysis. Aliquots of 0.5 g of freeze-dried apple peel or pulp were used for each analysis.

2.4. Analytical procedures

2.4.1. Solid–liquid extraction of freeze-dried samples

In broad outline, the procedure proposed for the solid–liquid extraction (SLE) of polyphenols from apple peel and pulp consisted in a direct extraction of the freeze-dried plant material with an appropriate solvent in an ultrasonic bath (Selecta, Barcelona, Spain) during a certain period of time.

The most influent experimental variables on the extraction procedure, which were the composition and the volume

Table 1
Design matrix and normalised values in the central composite design for SLE experiments

Run no.	Data matrix			Normalised values													
	MeOH (%)	V (mL)	t_{ext} (min)	EC	PB2	CAT-2	CQA	PLD-1	PLD-2	PLXG	PLG	HYP	IQC	QG-1	AVI	QCI	IDE	CG-1
1	30	10	10	0.76	0.76	0.77	0.71	0.89	0.81	0.65	0.64	0.67	0.73	0.69	0.70	0.72	0.68	0.69
2	70	10	10	0.86	0.89	0.84	0.90	0.82	0.87	1.00	0.97	0.88	0.89	0.87	0.87	0.86	0.95	0.99
3	30	20	10	1.16	1.01	0.87	1.15	1.26	1.26	0.94	0.87	0.91	0.93	1.05	0.88	0.90	0.98	0.96
4	70	20	10	1.07	1.10	1.13	1.11	1.01	0.92	1.24	1.23	1.16	1.16	1.15	1.15	1.14	1.16	1.14
5	30	10	30	0.97	0.84	0.69	0.96	1.00	1.00	0.92	0.90	0.84	0.89	0.85	0.76	0.83	0.88	0.87
6	70	10	30	1.06	1.12	1.16	1.11	1.01	0.90	1.23	1.21	1.12	1.14	1.13	1.14	1.14	1.15	1.13
7	30	20	30	0.90	0.97	0.94	0.97	1.07	1.10	0.86	0.84	0.96	0.96	0.93	0.90	0.93	0.98	1.00
8	70	20	30	1.13	1.09	1.05	1.17	1.04	0.91	1.16	1.13	1.14	1.05	1.08	1.12	1.14	1.16	1.22
9	10	15	15	0.99	0.96	0.76	0.99	1.19	1.14	0.79	0.77	0.79	0.83	0.81	0.72	0.80	0.73	0.75
10	90	15	15	1.23	1.16	1.25	1.12	1.01	0.93	1.26	1.31	1.24	1.21	1.25	1.33	1.25	1.16	1.22
11	50	5	15	0.78	0.78	0.79	0.75	0.81	1.19	0.92	0.91	0.88	0.83	0.79	0.79	0.81	0.86	0.77
12	50	25	15	1.16	1.20	1.30	1.15	1.10	1.19	1.17	1.21	1.20	1.20	1.18	1.32	1.22	1.11	1.17
13	50	15	5	0.89	0.96	1.07	0.89	0.85	0.84	0.94	0.92	0.93	0.93	0.96	0.98	0.94	0.88	0.88
14	50	15	40	1.15	1.18	1.25	1.13	1.13	1.09	0.99	1.09	1.19	1.19	1.21	1.23	1.23	1.11	1.12
15	50	15	15	0.98	0.97	1.06	0.89	0.88	0.92	0.93	0.95	1.00	0.99	1.01	1.04	1.00	1.02	0.91
16	50	15	15	0.99	1.00	1.04	0.98	0.95	0.95	0.98	1.01	1.02	1.01	1.02	1.05	1.03	1.09	1.07
17	50	15	15	0.93	1.02	1.05	1.01	0.98	0.97	1.03	1.02	1.06	1.05	1.01	1.05	1.05	1.10	1.11

AVI, avicularin; CQA, 5-caffeoylquinic acid; CAT-2, unknown flavan-3-ol; CG-1, unknown anthocyanin; EC, (−)-epicatechin; HYP, hyperoside; IDE, ideain; IQC, isoquercitrin; PB2, procyanidin B2; PLD-1, hydroxyphloretin diglycoside; PLD-2, hydroxyphloretin monoglycoside; PLG, phloridzin; PLXG, phloretin-2′-*O*-xyloglucoside; QCI, quercitrin; QG-1, unknown quercetin glycoside; MeOH, percentage of methanol in the extraction solvent; *V*, extraction solvent volume; t_{ext}, extraction time in the ultrasonic bath.

of the extraction solvent and the extraction time in the ultrasonic bath, were preliminary studied by a central composite experimental design. A two-level factorial design plus star orthogonal composite design involving 14 runs plus three central points was chosen. The 17 experiments of the design matrix (Table 1) were performed on 0.5 g of Urdin apple peel. Then, crude extracts were centrifuged at 3600 r.p.m. for 20 min. Aliquots of the supernatant were extracted three times with the same volume of hexane in order to eliminate interferences (chlorophylls, carotenoids, lipids) that can be extracted jointly with polyphenols during the solid–liquid extraction of the plant material [19]. Aliquots of the aqueous phase were 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, they were reconstituted in an adequate volume of methanol–aqueous acetic acid 10% (10:90, v/v) and filtered through a 0.45 μm PTFE filter (Waters, Milford, CA, USA) prior to injection into the HPLC system.

Once the results of the central composite design were evaluated, further experiments were carried out in order to optimise the experimental conditions of the extraction procedure. In this sense, changes were consecutively made in the experimental procedure. These changes are described and justified in the results (Section 3.2).

2.4.2. Reversed-phase HPLC analysis

Chromatographic analysis were performed on a Hewlett-Packard series 1100 system, equipped with a vacuum degasser, a quaternary pump, a thermostatted autosampler, a thermostatted column compartment and a DAD detector, connected to a HP ChemStation software. A reversed-phase Nova-Pak C18 (300 mm × 3.9 mm i.d., 4 μm) column and a Nova-Pak C18 (10 mm × 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 the column. The flow rate was 0.8 mL min^{-1} and the injection volume was 50 μL. The chromatographic separation was carried out at 25 °C. Flavan-3-ols and dihydrochalcones were monitored and quantified at 280 nm, hydroxycinnamic acids at 320 nm, flavonols at 370 nm and anthocyanins at 530 nm. Polyphenol identification was achieved by comparison of their retention times and their UV–vis spectra with those of the standards that were available. Some other chromatographic peaks were assigned to a particular polyphenol class according to their UV–vis spectra and bibliographic sources. In this sense, an unknown chromatographic peak that exhibits flavan-3-ol spectra was appointed as CAT-2; those with a spectrum of dihydrochalcone as PLD-1 and PLD-2, of flavonol as QG-1, and of anthocyanin as CG-1. Quantification was performed by reporting the measured integration areas in the calibration equation of the corresponding standards. Thus, procyanidin B2 and CAT-2 were quantified as (+)-catechin; phloretin-2′-*O*-xyloglucoside, PLD-1 and PLD-2 as phloridzin; avicularin and QG-1 as rutin; 4-*p*-coumaroylquinic acid as *p*-coumaric acid; and CG-1 as ideain.

2.5. Method validation procedure

The method was validated in terms of selectivity, accuracy, precisions within day and between days, recoveries achieved,

robustness and polyphenol stabilities. The selectivity of the method was assessed by comparing the chromatograms of the extracts of representative samples, which presented low polyphenol concentrations (peel: TX and ER; pulp: MN111 and MNEM7), to those of the method blank (extraction solvent), and to a solution of standards. Peak shapes, retention times and spectral purity of the chromatographic peaks were considered in order to detect possible interferences.

Calibration curves were obtained by injecting in the chromatographic system standard solutions of the polyphenols. The mathematical expressions of the calibration curves, the repeatabilities and the limits of detection and quantitation were determined. In order to establish the linear ranges, the correlation coefficients (r^2) and the distribution of residues were considered. The residues were statistically analysed to confirm that they were randomly distributed.

The limit of detection for each polyphenol was estimated as three times the signal (peak height) of the background noise, and the limit of quantitation, as 10 times the signal (peak height) of the background noise or as the lower limit of the linear range of the calibration curve.

The accuracy of the method was determined by successive extractions of representative samples of each matrix at two concentration levels: low (peel: TX; pulp: MN111) and high (peel: GM; pulp: MK). The recoveries and the standard deviation (three replicates) for the first extraction step in relation to the total analyte recovered in all the successive extractions steps were determined for each polyphenol studied.

The precision of the method was evaluated using representative samples of each matrix at two levels of concentrations: low (peel: TX; pulp: MN111) and high (peel: GM; pulp: MK).

Robustness of the method was assayed by performing the following changes in the experimental variables: (i) ± 1.7% of methanol in the extraction solvent; (ii) ± 2% of acetic acid in the extraction solvent; and (iii) ± 30 s in the extraction time in the ultrasonic bath. These experiments were carried out in duplicate.

Stability of polyphenols were studied: (i) in standard solutions under storage conditions; (ii) in the extracts of each kind of matrix (peel: GM; pulp; MN111) inside the thermostatted autosampler at 4 °C for 48 h; (iii) once the extracts and the vegetal materials had been in contact at room temperature for 3 h; and (iv) after leaving the extracts in a closed vial at room temperature for 3 h.

3. Results

3.1. Central composite experimental design for SLE

A central composite design was carried out using three variables: the percentage of methanol in the solvent (methanol–water–acetic acid, v/v/v, containing 1% of acetic acid) (M), the solvent volume (V) and the extraction time (t). Table 1 gives the design matrix and the normalised concentrations obtained in each run.

The analysis of these results was performed by the nonlinear regression analysis program NLREG [20]. Eq. (1) shows the most general function for central composite design:

$$Y = \beta_1 M + \beta_2 V + \beta_3 t + \beta_{12} MV + \beta_{13} Mt + \beta_{23} Vt + \beta_{11} M^2 + \beta_{22} V^2 + \beta_{33} t^2 + \beta_{123} MVt \quad (1)$$

where M, percentage of methanol in the solvent (methanol–water–acetic acid v/v/v, containing 1% of acetic acid); V, solvent volume; t, extraction time; β_1, β_2, β_3, β_{12}, β_{13}, β_{23}, β_{11}, β_{22}, β_{33}, β_{123}, parameters. The β_0 term was not included in Eq. (1) since the results had been previously normalised. The results obtained by NLREG are given in Table 2. The normalised equation followed by the studied analytes contained the terms due to β_1, β_2, β_3 and β_{23}, the other parameters were systematically eliminated from the models, since their probability of being zero was higher than 10% ($P > 0.1$). It could be concluded that the three variables, percentage of methanol in the extraction solvent (β_1), solvent volume (β_2) and extraction time (β_3), had an influence on the extraction efficiency. The four parameters (β_1, β_2, β_3 and β_{23}) had the same sign and magnitude for all the polyphenolic compounds studied, within each family of polyphenols and between families. Therefore, the same experimental extraction conditions can be used for all of them. Moreover, it was observed that the percentage of methanol influences positively on the extraction efficiency. Thus, the higher the percentage of methanol in the extraction solvent was, the larger the polyphenol extraction was. However, from the values of the parameters obtained by NLREG, it was difficult to conclude how the extraction solvent volume and the extraction time in the ultrasonic bath affected the extraction efficiency, due to the negative interaction between both variables ($\beta_{23} < 0$). The three-dimensional plots of the response surface,

Table 2
Values of the parameters obtained for the central composite design for the SLE experiments[a,b]

Class	Polyphenol	β_1	β_2	β_3	β_{23}	r^2
FA	PB2	0.004	0.049	0.029	−0.002	0.78
	EC	0.003	0.052	0.033	−0.002	0.74
	CAT-2	0.006	0.042	0.022	−0.001	0.70
HCA	CQA	0.003	0.051	0.032	−0.002	0.83
DHC	PLXG	0.007	0.040	0.027	−0.002	0.93
	PLG	0.007	0.038	0.026	−0.001	0.94
FO	HYP	0.006	0.040	0.025	−0.001	0.88
	IQC	0.005	0.044	0.029	−0.002	0.88
	QG-1	0.005	0.045	0.027	−0.002	0.89
	AVI	0.007	0.038	0.019	−0.001	0.84
	QCI	0.006	0.040	0.025	−0.001	0.87
ACY	IDE	0.006	0.040	0.026	−0.001	0.85
	CG-1	0.006	0.038	0.022	−0.001	0.90

[a] *Abbreviations*: See Table 1; FA, flavan-3-ols; HCA, hydroxycinnamic acids; DHC, dihydrochalcones; FO, flavonols; ACY, anthocyanins.
[b] β_1, β_2, β_3, β_{23}, parameters of Eq. (1); r^2, correlation coefficient.

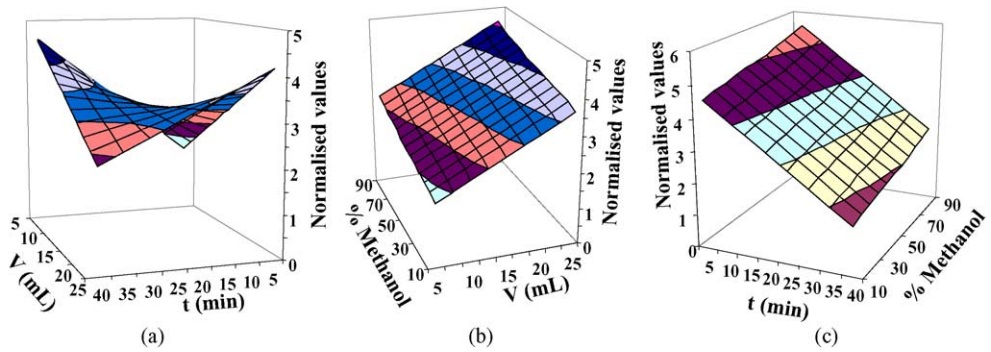

Fig. 1. Three-dimensional plots of the response surface, keeping one of the variables fixed: (a) 30% of methanol, (b) 10 min for the extraction time in the ultrasonic bath, and (c) 25 mL of extraction solvent.

keeping one of the variables fixed, provided a maximum of the extraction efficiency on the range limits but not inside the experimental ranges considered (Fig. 1).

3.2. SLE optimisation

Once the results of the central composite design were evaluated, the optimisation of the extraction conditions was continued in order to minimise the laboriousness of the procedure, the time needed for each experiment, and the consumption of organic solvents. In this sense, it was interesting to eliminate the liquid–liquid extraction (LLE) clean-up of the crude extract with hexane as well as the methanol evaporation step, and to consider short ultrasonic extraction times and small solvent volumes.

In order to eliminate the LLE step, the SLE had to be performed using a solvent containing less than 70% of methanol. If solvents containing 70% or higher percentages of methanol were used, hexane solutions from the LLE of the extracts obtained by SLE were coloured, indicating that chlorophylls and lipids were co-extracted from apple peel. When not eliminated, these compounds precipitated as soon as the extracts were reconstituted in an appropriate solvent for their injection into the HPLC system. Solvents with 50% of methanol or less did not extract such quantities of chlorophylls and lipids; therefore, hexane LLE extracts did not present any colour, and no precipitates appeared when the SLE extracts were reconstituted after the evaporation step. Hence, the LLE step was not further needed. Otherwise, in order to eliminate the solvent evaporation step and to inject the crude extract directly into the HPLC system, an extraction solvent containing a percentage of methanol of 40% or smaller was required, so that the chromatographic peaks were not distorted. On the other hand, extraction time not longer than 15 min are desirable when large sample sets have to be analysed, and solvent volume not greater than 30 mL are also preferred.

The recovery efficiency for the SLE procedure was determined by performing consecutive extractions on 0.5 g of freeze-dried apple peel (Urdin apple cultivar) with methanol–water–acetic acid (30:69:1, v/v/v), until no phenolic compound was detected by HPLC analysis. Solvent volumes and extraction times in the ultrasonic bath used in each successive extraction were: (i) 30 mL, 10 min; (ii) 20 mL, 10 min; (iii) 10 mL, 10 min; (iv) 5 mL, 10 min; (v) 2.5 mL, 10 min, (vi) 2.5 mL, 10 min. Once the vegetal material was extracted in each step, the extract was filtered with a vacuum system and the vegetal material was cleaned with water, filtered, and then re-extracted. This experiment was carried out by triplicate, achieving good repeatabilities (%R.S.D. (n = 3) <5% for all the polyphenols studied). The results showed that in the first extraction more than 96% of the total amount extracted of each polyphenol studied was obtained, and that with the first three successive extractions, recoveries of 99.9% were achieved for all analytes. Hence, an SLE procedure with one extraction step (30 mL, 10 min) was considered to be adequate, being not necessary to perform additional extractions.

The extraction efficiency of the procedure proposed was compared with a published extraction method, which uses methanol–acetic acid (99:1, v/v) as the extraction solvent [15]. When using this bibliographic extraction procedure, the largest extract volume that could be injected into our HPLC system was 10 μL, since greater injection volumes highly distorted the chromatographic peaks. This led to worse peak resolutions and higher quantitation errors. Moreover, some polyphenols were not detected (CG-1, PLD-1, PLD-2, CAT-2) due to the small injection volume (10 μL). With regard to extraction efficiencies for the analytes studied, the bibliographic method achieved recoveries between 10 and 40% lower depending on the family of polyphenols considered. Taking into account dihydrochalcones, it was observed that the total dihydrochalcones extracted with both procedures were not significantly different; however, the individual concentration of each glycoside was different. Thus, PLXG and PLG concentrations were higher in the acidified methanol extract than in the acidified hydroalcoholic medium, whereas if PLD-1 and PLD-2 were considered, the opposite occurred.

When both extraction procedures were applied to freeze-dried apple pulp, it was observed that acidified methanol attained better recoveries than the acidified hydroalcoholic mixture, the opposite occurring than in apple peel extractions. Otherwise, pulp browning was observed when pulp was extracted with methanol–water–acetic acid (30:69:1, v/v/v). This fact led us to conclude that the enzymatic oxidation of polyphenols by polyphenoloxidase (PPO) was responsible

for the low extraction efficiencies obtained with the hydroalcoholic mixture. It seems that methanol reduce PPO activity, while solvents containing low percentages of methanol do not inactivate the enzyme completely, resulting in low extraction efficiencies [21]. This fact also explains the enzymatic oxidation by PPO cresolase activity of monophenols, such as phloretin glycosides (PLXG and PLG), into their *ortho*-diphenols, PLD-1 and PLD-2 (hydroxyphloretin glycosides) [22]. PPO activity has been detected in both apple peel and pulp, even though, in most apple cultivars, this activity was higher in pulp than in peel [23]. In order to avoid polyphenol oxidation, an antioxidant, ascorbic acid (2 g L^{-1}), was added to the extraction solvent. This way, good recoveries were achieved in pulp, comparable to those obtained using acidified methanol or even better considering some polyphenols (CAT and PB2).

3.3. Validation of the method

The analytical method proposed for the determination of polyphenols in apple peel and pulp consisted in a solid–liquid extraction of 0.5 g of freeze-dried material with 30 mL of methanol–water–acetic acid (30:69:1, v/v/v) containing 2 g of ascorbic acid/L, for 10 min in an ultrasonic bath, and an analysis of the crude extract by HPLC-DAD.

The method was validated in terms of selectivity, accuracy, precisions within day and between days, recoveries achieved, robustness and polyphenol stabilities. Moreover, the linear ranges of polyphenol calibration curves, the repeatabilities for each range, the sensitivity, the analytical resolution and the limits of detection and quantitation were determined.

Regarding selectivity, no interfering peaks were observed in the blank chromatograms at the quantitation wavelengths (280, 320, 370 and 530 nm). In most cases, peak purity and the degree of match with the standard spectra were greater than 99%, except for those polyphenols whose concentrations were below the quantitation limit, and for some polyphenols whose standards were not available. In these particular cases (CMA-2, CAA-2, PCQ, QG-1, QG-3, CAA-1 and PLXG), their spectra were compared with the spectra of a standard of the same family.

The calibration curve made with standard solutions was divided in several linear ranges. Tables 3 and 4 show the origin ordinate, the slope and the repeatability for each range, and the limits of detection and quantitation for the polyphenols used for quantitation.

In Table 5, the recoveries and the standard deviation (three replicates) for the extraction procedure for each polyphenol studied are summarised. SLE presents satisfactory efficiencies at both high and low concentration levels of polyphenols, showing recoveries higher than 91% in peel, and than 95% in pulp.

For most polyphenols, the repeatabilities within day (five replicates), expressed by means of the percentage of relative standard deviation (%R.S.D. (n = 5)), were lower than 5%, except for some minor components such as ideain in peel and

Table 3
Linear ranges, slope (±S.D.) and origin ordinate (±S.D.) of the calibration curves for polyphenols in standard solutions[a]

Polyphenol[b]	Linear ranges (μg mL^{-1})	Slope	Origin ordinate	r^2
CAT	0.03–1	42.1 ± 0.7	−0.3 ± 0.3	0.9992
	1–10	42.4 ± 0.4	−1 ± 2	0.99997
	10–50	42 ± 1	(2 ± 2) 10^2	0.998
EC	0.03–1	38.5 ± 0.6	−0.1 ± 0.4	0.9992
	1–12	40.9 ± 0.8	−6 ± 6	0.9998
	12–300	37.8 ± 0.6	(11 ± 9) 10	0.9996
	300–2500	8.5 ± 0.1	(−1 ± 2) 10^2	0.9993
CQA	0.01–0.1	(16 ± 2) 10	0 ± 1	0.995
	0.1–1	181 ± 8	−5 ± 5	0.9991
	1–11	186 ± 7	(−3 ± 5) 10	0.9993
	11–550	174 ± 2	(7 ± 5) 10^2	0.9996
PCM	0.01–0.1	(33 ± 6) 10	(0 ± 3)	0.989
	0.1–1	419 ± 8	−9 ± 5	0.9998
	1–10	418 ± 8	(−3 ± 5) 10	0.9998
	10–250	369 ± 7	(16 ± 9) 10^2	0.9993
PLG	0.05–0.3	117 ± 8	−1 ± 1	0.99992
	0.3–1	138 ± 6	−5 ± 3	0.9993
	1–10	120 ± 7	(2 ± 4) 10	0.9986
	10–500	128 ± 2	(3 ± 5) 10^2	0.9993
HYP	0.06–1	80 ± 1	−0.5 ± 0.6	0.9998
	1–11	86 ± 2	(−1 ± 1) 10	0.9997
	11–550	78.2 ± 0.7	(3 ± 2) 10^2	0.9997
IQC	0.03–1	107 ± 3	0 ± 1	0.998
	1–10	116 ± 2	(−1 ± 1) 10	0.9998
	10–500	111 ± 1	(−2 ± 3) 10^2	0.9995
RUT	0.03–1	77.5 ± 0.8	0.1 ± 0.4	0.9997
	1–11	79 ± 4	(0.3 ± 2) 10	0.9990
	11–525	80 ± 1	(2 ± 3) 10^2	0.9991
QCI	0.03–0.3	70 ± 8	0 ± 1	0.995
	0.3–1	78 ± 3	−1 ± 2	0.9993
	1–10	83 ± 2	(−1 ± 1) 10	0.9998
	10–500	81 ± 1	(1 ± 2) 10^2	0.9995
IDE	0.01–1	209 ± 3	0 ± 2	0.9986
	1–12	227 ± 1	−22 ± 7	0.999992
	12–120	(14 ± 1) 10	(11 ± 7) 10^2	0.997

[a] S.D., standard deviation; r^2, correlation coefficient.
[b] *Abbreviations*: See Table 1; CAT, (+)-catechin; PCM, *p*-coumaric acid; RUT, rutin.

Table 4
Limits of detection (LOD) and limits of quantitation (LOQ) for polyphenols

Polyphenol[a]	LOD (μg mL^{-1})	LOQ (μg ML^{-1})	LOD (μg g^{-1} DW)[b]	LOQ (μg g^{-1} DW)[b]
CAT	0.009	0.02	0.5	1
EC	0.003	0.03	0.2	2
CQA	0.01	0.02	0.7	0.9
PCM	0.007	0.01	0.4	0.6
PLG	0.03	0.05	2	3
HYP	0.01	0.03	0.6	2
IQC	0.009	0.02	0.5	1
RUT	0.004	0.03	0.2	2
QCI	0.01	0.05	0.7	3
IDE	0.005	0.007	0.3	0.4

[a] *Abbreviations*: See Tables 1 and 3.
[b] μg g^{-1} of peel or pulp dried weight.

Table 5
Polyphenol recoveries (% ± S.D. (n = 3)) at two concentration levels in apple peel (low: TX and high: GM) and apple pulp (low: MN111 and high: MK)[a,b]

Polyphenol	Recoveries (mean (%) ± S.D. (n = 3))			
	Apple peel		Apple pulp	
	Low	High	Low	High
CAT	94.5 ± 0.3	95.0 ± 0.1	97.6 ± 0.3	98.4 ± 0.2
EC	94.58 ± 0.07	94.36 ± 0.07	96.4 ± 0.3	98.0 ± 0.2
PB2	94.7 ± 0.1	94.5 ± 0.2	97.9 ± 0.2	97.9 ± 0.2
CAT-2	96.1 ± 0.2	93.6 ± 0.4	100	97.1 ± 0.5
CQA	95.5 ± 0.1	94.6 ± 0.1	97.6 ± 0.3	98.1 ± 0.1
PCQ	100	96.0 ± 0.3	100	100
PLD-1	95.5 ± 0.4	93.5 ± 0.5	100	97.8 ± 0.3
PLD-2	93.2 ± 0.1	92.2 ± 0.2	100	100
PLXG	100	94.4 ± 0.2	100	98.3 ± 0.4
PLG	100	94.3 ± 0.2	100	96.3 ± 0.5
HYP	92.6 ± 0.5	91.7 ± 0.2	–	100
IQC	94.6 ± 0.3	92.2 ± 0.2	100	100
QG-1	94.2 ± 0.3	91.6 ± 0.4	100	100
AVI	92.7 ± 0.2	91.2 ± 0.2	–	100
QCI	93.3 ± 0.3	91.1 ± 0.4	100	100
IDE	–	93.2 ± 0.4	–	–

[a] *Abbreviations*: See Tables 1 and 3; PCQ, 4-*p*-coumaroylquinic acid.
[b] *Apple cultivars*: GM, Geza Miña; MK, Moko; MN111, Manttoni 111; TX, Txalaka.

avicularin and hyperoside in pulp that presented %R.S.D. (n = 5) of 6%, 9% and 8%, respectively (Table 6). The repeatabilities between days (five replicates) expressed as %R.S.D. were lower than 7%, except for some minor components in pulp (Table 6).

Regarding robustness, the results obtained from the batch of experiments performed for evaluating this feature were not significantly different from those achieved by the validated method; therefore, it was concluded that the method was robust.

Regarding stability, standard stock solutions of 500 or 1000 μg mL^{-1} in methanol (or in methanol–hydrochloric acid (99.9: 0.1), for ideain) at 4 °C and in the dark were stable at least for 5 months, except for (+)-catechin and (−)-epicatechin, that were degraded about 15%, and ideain, about 5%. These three polyphenols were stable at least for 1 month under the mentioned conditions. With regard to the polyphenol stability in the extracts, no degradation of interesting chromatographic peaks was observed when the extract had been inside the thermostated autosampler at 4 °C for 48 h. As well, no degradation was observed once the extract and the vegetal material had been in contact at room temperature for 3 h, neither when the extract was left in a closed vial at room temperature for 3 h.

4. Discussion

4.1. SLE versus PLE

The method for the analysis of polyphenols in apple material validated above was compared with the method described by Alonso-Salces et al. [17], which consisted in a pressurised liquid extraction (PLE) of the freeze-dried apple material followed by an HPLC-DAD analysis of the crude extract. Both methods used the same experimental conditions for HPLC-DAD analysis. Therefore, the different performances of these methods were due to the different extraction techniques that they included.

SLE presents higher efficiencies at both high and low concentration levels of polyphenols, showing recoveries higher than 91% (in peel) and 95% (in pulp), whereas PLE recoveries in peel are higher than 90%, except for PB2, CAT-2, HYP, IQC y QG-1 (82–86%), and in pulp, are between 81 and 86% [17].

SLE repeatabilities within day in peel (R.S.D. (n = 5) ≤ 4%) are similar to those obtained by PLE, except for CAT, EC, CA, PCQ and PLXG (%R.S.D. (n = 3) = 5–9% by PLE) [17]. In pulp, repeatabilities obtained by SLE (%R.S.D. (n = 5) ≤ 4%) for flavan-3-ols, hydroxycinnamic acids and dihydrochalcones are better than by PLE (%R.S.D. (n = 3) = 5–8%). For flavonols, precisions of both methods are similar (%R.S.D. ≤ 9%).

The limits of detection attained by the PLE method (peel: 0.08–0.7 μg g^{-1} DW, pulp: 0.03–0.2 μg g^{-1} DW) [17] are lower than SLE method (0.2–2 μg g^{-1} DW), as a result of the concentration step that the former method includes. However, this concentration step (evaporation to dryness of an aliquot of the extract and reconstitution with an appropriate solvent for its injection into the HPLC system) increases considerably the total time of the analysis and the laboriousness of the procedure. Moreover, this handling of the samples is probably responsible for the slightly worse precisions that presented the PLE method (peel: 1–9% R.S.D. (n = 3), pulp: 4–9% R.S.D. (n = 3)) [17]. If the evaporation steps in the PLE method was substituted by a dilution of the extract aliquot

Table 6
Repeatabilities within day and between days (%R.S.D. (n = 5)) at two concentration levels of polyphenols in apple peel (low: TX and high: GM) and apple pulp (low: MN111 and high: MK)[a]

Polifenol	Apple peel						Apple pulp					
	Low			High			Low			High		
	Mean ± S.D. ($\mu g\,g^{-1}$ DW)[b]	% R.S.D. (n = 5)		Mean ± S.D. ($\mu g\,g^{-1}$ DW)	% R.S.D. (n = 5)		Mean ± S.D. ($\mu g\,g^{-1}$ DW)[b]	% R.S.D. (n = 5)		Mean ± S.D. ($\mu g\,g^{-1}$ DW)[b]	% R.S.D. (n = 5)	
		Within day	Between days		Within day	Between days		Within day	Between days		Within day	Between days
CAT	54 ± 2	2	1	516 ± 10	2	2	78.5 ± 0.9	1	1	66 ± 4	5	1
EC	772 ± 11	1	3	3971 ± 103	2	2	237 ± 4	1	2	1766 ± 13	0.6	2
PB2	1094 ± 11	0.8	3	3468 ± 88	2	4	514 ± 5	0.7	0.7	2525 ± 9	0.3	1
CAT-2	105 ± 2	2	4	342 ± 11	3	3	45 ± 2	4	1	211 ± 1	0.4	2
CQA	303 ± 2	0.6	1	3575 ± 77	2	0.7	1078 ± 7	0.5	0.7	2932 ± 20	0.5	0.7
PCQ	24.1 ± 0.3	1	3	219 ± 6	2	2	139.6 ± 0.8	0.5	3	104 ± 3	2	0.9
PLD-1	126 ± 3	2	3	520 ± 18	3	6	14.5 ± 0.6	3	5	137 ± 1	0.7	2
PLD-2	343 ± 9	2	3	1787 ± 61	3	2	11.4 ± 0.5	3	10	28.4 ± 0.9	3	4
PLXG	76 ± 3	3	4	488 ± 24	4	6	53.5 ± 0.9	1	4	396 ± 5	1	0.7
PLG	322 ± 8	2	3	2555 ± 140	4	7	55 ± 2	2	3	136 ± 2	1	4
HYP	821 ± 20	2	4	2153 ± 92	3	7	nd	–	–	1.2 ± 0.1[b]	8	12
IQC	219 ± 3	1	3	548 ± 23	3	5	nd	–	–	5.89 ± 0.06	0.8	6
QG-1	494 ± 9	2	3	959 ± 25	2	7	nd	–	–	9.7 ± 0.6	5	7
AVI	553 ± 6	0.9	3	2069 ± 78	3	4	nd	–	–	5.4 ± 0.6	9	9
QCI	319 ± 3	0.7	3	907 ± 39	3	6	nd	–	–	21.9 ± 0.8	3	2
IDE	nd	–	–	22 ± 2	6	6	nd	–	–	nd	–	–

[a] *Abbreviations*: See Tables 1, 3, 4 and 5; nd, not detected.
[b] Under LOQ.

to obtain a final extract containing 30% of methanol, so that it could be injected directly in the chromatographic system, the detection limits achieved in pulp (0.2–2 $\mu g\,g^{-1}$ DW) [17] were similar to those presented by the SLE method, whereas in peel, the PLE method showed not as good detection limits (0.5–5 $\mu g\,g^{-1}$ DW) [17], but also acceptable.

Regarding organic solvent consumption, in the SLE method (9.3 mL per sample), it is considerable lower than in the PLE method (more than 50 mL per sample). In relation to the time of analysis, although the chromatographic analysis (85 min) is the limiting step, the SLE method allows to extract a large number of samples simultaneously in 10 min (plus the time used for adding the extraction solvent to each sample). Instead, the ASE 200 (Dionex Corp., Sunnyvale, CA, USA) equipment used in the PLE method performs the sample extractions consecutively, the time used for each extraction being 20 min. Moreover, the time for preparing the extraction cells also has to be considered. This drawback is partially solved, since PLE is an automated technique.

However, having been demonstrated that PLE is an efficient technique for extracting polyphenols [17,24,25], the possibilities that it presents in other fields should not be despised. For instance in industry, which is nowadays very interested in the development of new extraction methods for phenolic compounds from waste materials, such as grape and apple pomace resulting from elaboration of juices, wine or ciders. Until now, these materials have been used as animal feed or compost [26]. Grape and apple pomaces contain polyphenols linked to the cellular walls, which are not extracted during pressing [27] and which are very interesting due to their biological properties [28]. Analytical extraction methods based on a solid–liquid extraction, usually, are not adequate from an industrial point of view, mainly because these methods imply long extraction time and the use of large volumes of solvents as a result of the great size of the sample to be extracted. Moreover, polyphenol degradation by light, oxygen and polyphenoloxidase enzymes present in the pomace is favoured with these kinds of methods. In these sense, PLE presents the advantage of performing extractions under inert atmosphere and protected from the light. Moreover, temperature and high pressures enhance extraction, decreasing solvent volume consumption and extraction time. And last but not least, it is an automated technique.

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