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Analysis of polyphenols in apple products

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Abstract Polyphenols of low molecular mass in apple samples were extracted by a C₁₈ solid-phase column, and analysed by HPLC. This extraction method had, in general, a greater extraction ability compared with liquid–liquid extraction using the ethyl acetate method.

Key words Phenolic compounds · Apples · Ethyl acetate · Solid-phase extraction · HPLC

Introduction

Consumer trends show an increasing demand for natural products, mainly fruit juices, of a high sensory quality. Polyphenols are important components in fruit product technology, because of their contribution to flavour [1, 2] and their involvement in colour changes that occur during processing, such as browning [3–5], or in the formation of hazes and sediments [3]. Knowledge of the phenolic compounds in fruits and the factors that affect the quantities in which they are found are relevant from an industrial point of view. Data on the quantity and type of phenolic compounds present can also be useful when determining the authenticity of fruit products [6–8].

Most of the studies of the phenolic compounds present in apples and apple derivatives have been carried out by liquid–liquid extraction using ethyl acetate [9–11]. However, this technique yields poor recovery of procyanidins, and low extraction efficiencies of different phenolic acids [12, 13]. The optimization of a solid-phase extraction method for the analysis of polyphenol compounds in apple musts and ciders has been recently described [14]. This method provides, as

compared with the liquid–liquid extraction method, higher recovery levels of flavonols and some phenolic acids present in apples.

In this paper, the suitability of the solid-phase extraction procedure in the analysis of different varieties of cider apples and apple juices is evaluated. A comparison of the solid-phase fractionation method with the liquid–liquid extraction method is also presented.

Materials and methods

Standards and reagents

(+)-Catechin, (–)-epicatechin, phloridzin, chlorogenic acid and *p*-coumaric acid were purchased from Sigma (St. Louis, Mo., USA), and glycosides of quercetin were from Extrasynthèse (Genay, France). Phloretin xyloglucoside and procyanidins were kindly provided by Dr. Lea (Reading, UK). Solvents employed were from Panreac, HPLC quality (Barcelona, Spain), and degassed with helium prior to use.

Samples

Single-variety musts were extracted by pressing from six cider apple varieties, at maturity, grown in the experimental orchard of the Research Centre in Villaviciosa. Other apple products included in this study were: experimental apple juices made from a mild-sharp mixture, stabilized by microfiltration and ultrafiltration [0.14-µm and 5000 nm (50,000 Å) pore membranes], and two commercial apple juices, one made from concentrate (concentrate juice) and the other made by pressing apples (pressed juice). Global parameters of the samples analysed are summarized in Table 1.

Sample preparation

Solid-phase extraction method. Polyphenols were extracted and fractionated into neutral and acidic compounds by means of a C₁₈ solid-phase cartridge, according to the method described elsewhere [14]. Extra-Sep C₁₈ columns (Lida, Kenosha, Wis., USA 1000 mg) were preconditioned for neutral phenols by sequentially passing 25 ml of methanol and 10 ml of water adjusted to pH 7.0.

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Table 1 Global parameters of laboratory-prepared apple juice and commercial apple juices

Samples	pH ^a	°Brix ^a	Total ^a acidity (g/l sulphuric acid)	Total ^b phenols (g/l tannic acid)
Raxao (sharp)	3.24	11.0	5.02	0.99
Clara (bitter-sweet)	3.90	12.8	1.34	1.99
Perezosa (mild-sharp)	3.46	13.0	3.92	0.90
Arbeya (bitter-sharp)	3.25	15.0	5.75	2.39
Marie (bitter)	3.70	14.4	1.50	3.10
Pepa (sweet)	3.97	15.1	1.84	1.25
Microfiltered juice (0.14 µm)	3.45	15.1	3.92	0.45
Ultrafiltered juice (50,000 Å; 5000 nm)	3.39	11.5	2.94	0.35
Commercial pressed juice	3.80	9.6	1.96	0.35
Concentrate juice	3.48	12.2	3.70	0.49

^a See [16]^b See [17]

Solid-phase supports for phenolic acids were preconditioned by the sequential passing of 25 ml of methanol and 10 ml of 0.01 M HCl. A suitable volume of sample (to load 8 mg of total polyphenols per gram of solid phase), containing 1% ascorbic acid to avoid oxidation of polyphenols, was adjusted to pH 7.0 with NaOH, loaded onto the C₁₈ neutral fractionating column and washed with 10 ml of water (pH 7.0). The effluent portion was adjusted to pH 2.0 with 2 M HCl, passed through the preconditioned acidic column, and washed with 5 ml of 0.01 M HCl. The adsorbed fractions were eluted with 12 ml of methanol, and evaporated until dryness at 35°C. The residue was redissolved in 0.5 ml of methanol and filtered through a 0.22-µm polyvinylidene difluoride filter (PVDF, Lida, Kenosha).

Liquid-liquid extraction with ethyl acetate. Of the apple juice, 5 ml was adjusted to pH 7.0 with NaOH, and extracted three times with 5 ml of ethyl acetate. The organic phases were added together, and 9 ml of this mixture was evaporated until dryness at 35°C. The residue was redissolved in 0.5 ml of methanol and filtered through a 0.22-µm PVDF filter (Lida, Kenosha). The remaining aqueous layer, containing phenolic acids, was adjusted to pH 2.0 with 2 M HCl, and extracted under the same conditions as before.

HPLC analysis

Phenolic extracts (10 µl) were analysed, in duplicate, with a Waters Associates system, equipped with a 712 Automatic Injector, M510 pumps, a Millennium v.2.1 software data module and a 996 Diode Array Detector. Separation of polyphenols was carried out on a reversed-phase Spherisorb C₆ cartridge (250×4.6 mm, 5 µm; Teknokroma, Barcelona, Spain), using as solvents 2 mM phosphoric acid (solvent A) and methanol (solvent B). For neutral phenolics, the gradient is a sequence of linear increments: starting with 2% B to reach 11% B in 15 min, from 11% to 18.5% B in 5 min, from 18.5% to 39.5% B in 35 min. An additional flow rate gradient was included

to achieve the resolution of dihydrochalcones and flavonols: from 23 to 25 min, flow rate was lowered from 1.00 ml/min to 0.80 ml/min, and maintained until the end of the analysis. Phenolic acids (chlorogenic acid and a coumaric acid derivative) were analysed in isocratic mode, at 1.20 ml/min, using 6% MeOH in 2 mM phosphoric acid as the mobile phase. All the analyses were performed at 30°C. The phenolic compounds were identified by comparing spectra and retention times against the corresponding standards, when available. Quantification of neutral phenolics was done at 280 nm; phenolic acids were quantified at 320 nm.

Results and discussion

Comparison of the two extraction methods for the analysis of phenolic compounds

The suitability of two fractionation and extraction methods for the analysis of polyphenols has been tested using one single-apple must from an Asturian cider variety. The effect of these methods on the HPLC quantification of different kinds of phenolics is shown in Table 2.

The results obtained for catechin, epicatechin, phloridzin and some flavonols are quite similar (differences in mg/l less than 10%); however, procyanidins, phenolic acids and flavonols were poorly extracted by ethyl acetate, in agreement with previously reported results [10, 12, 13, 15]. Likewise the concentration of phloretin xyloglucoside was 71.6% less when the ethyl acetate extraction method was used, when compared to the solid-phase extraction method. Therefore, as this method was more appropriate for the extraction of low molecular mass phenolic compounds, it has been applied to the analysis of polyphenols in several apple samples.

Quantification of polyphenols in musts

The results of the quantification of polyphenols are shown in Table 3. Clara cv. was the variety which contained the greatest amount of flavan-3-ols (556.5 mg/l), while Pepa cv. had the least (1.0 mg/l). (–)-Epicatechin and procyanidin B2 were the main flavan-3-ols found in the varieties studied, except for Raxao cv., in which the procyanidin tetramer and trimer C1 contents were slightly higher, and Clara cv., in which the amount of procyanidin C1 was similar to those of the aforementioned compounds (Table 3). Varieties included in the technological groups with bitter properties [namely, bitter (Marie cv.), bitter-sweet (Clara cv.) and bitter-sharp (Arbeya cv.)] had the highest level of B2 procyanidin and (–)-epicatechin, as can be seen in Table 3. The levels of (–)-epicatechin and procyanidin B2 found in these varieties were lower than those reported for some English cider apple varieties [3], even though the extraction method was different to that used here.

Table 2 Quantities of phenolic compounds (mg/l ± RSD) of an Asturian cider apple variety, as determined by solid-phase (SPE) and liquid–liquid (LLE) extraction

Phenolic compound	SPE (mg/l)	LLE (mg/l)
Catechin	96.2 ± 4.5	104.7 ± 7.5
B1	80.1 ± 6.5	39.5 ± 9.7
Epicatechin	157.4 ± 3.9	158.9 ± 6.3
<i>p</i> -Coumaryl derivative	17.7 ± 3.0	11.8 ± 20.5
Chlorogenic acid	103.4 ± 4.1	58.4 ± 6.5
B2	114.1 ± 4.7	79.8 ± 9.2
C1	113.2 ± 6.9	38.6 ± 18.1
Procyanidin tetramer	75.1 ± 8.1	16.1 ± 28.4
B5	11.5 ± 7.1	8.6 ± 9.0
Phloretin	10.9 ± 3.5	3.1 ± 21.2
xyloglucoside		
Phloridzin	41.3 ± 4.5	37.8 ± 6.2
Hyperin	2.8 ± 6.6	2.2 ± 7.6
Isoquercitrin + rutin	5.4 ± 4.0	2.6 ± 5.9
Unknown flavonol + avicularin	11.2 ± 3.3	11.0 ± 9.2
Quercitrin	3.6 ± 4.7	3.1 ± 14.4

Dihydrochalcone glycosides, phloretin xyloglucoside and phloridzin are characteristic of apples, since their presence has not been detected in any other fruit [8]. The quantity of phloretin xyloglucoside was higher than that of phloridzin in all the varieties studied; this behaviour is not in agreement with other reported results using ethyl acetate [10] – a phenomenon that can be related to the extraction procedure, since the SPE method used here is more efficient at extracting this compound. Raxao cv. contained the most phloretin xyloglucoside, while Clara cv. contained the most phloridzin.

Chlorogenic acid was the most important phenolic compound of low molecular mass found in the cider apple varieties studied. Its content decreased with the flavour of the apple variety, such that: bitter > bitter-sharp > sharp > mild-sharp > bitter-sweet > sweet, while similar levels of the coumaric acid derivative were found for all the varieties considered except for the bitter variety, which had the highest concentration of this phenolic compound.

Quantification of polyphenols in apple juices

As shown in Table 3, the amounts of phenolic compounds in commercial juices were lower than those of the single-variety and the experimental juices. The use of dessert apple varieties as a raw material for juice making, and the treatments used to stabilize and concentrate apple juices could explain why commercial juices contain a smaller amount of phenols. The commercial pressed juice contained only phloridzin, chlorogenic and the coumaric acid derivative. However, the main peaks observed had flavanone-type spectra, which are typical of citrus juice compounds.

Table 3 Quantities of phenolic compounds (mg/l ± RSD) in apple samples

Phenolics	Raxao	Clara	Perezosa	Arbeya	Marie	Pepa	Filtered juice (5000 nm)	Filtered juice (0.14 µm)	Commercial pressed juice	Commercial concentrate
Catechin	6.3 ± 2.9	5.8 ± 2.2	2.0 ± 5.6	17.0 ± 4.2	3.1 ± 8.5	ND	7.2 ± 8.5	10.9 ± 1.8	ND	ND
B1	14.0 ± 1.2	10.0 ± 5.4	3.6 ± 5.4	39.6 ± 3.2	10.1 ± 0.7	ND	12.2 ± 4.9	14.9 ± 4.9	ND	ND
Epicatechin	31.6 ± 2.8	171.5 ± 3.4	13.4 ± 3.0	111.1 ± 3.8	57.6 ± 0.2	1.0 ± 6.2	31.8 ± 8.2	42.8 ± 4.1	ND	6.2 ± 7.8
B2	28.9 ± 3.5	117.5 ± 3.2	6.0 ± 3.2	147.5 ± 4.7	63.7 ± 2.2	ND	22.2 ± 9.0	23.5 ± 7.5	ND	4.9 ± 0.7
Procyanidin trimer	14.6 ± 2.3	25.6 ± 4.1	5.1 ± 0.9	35.8 ± 8.7	13.8 ± 1.5	ND	ND	ND	ND	ND
C1	38.6 ± 4.0	124.9 ± 5.8	5.5 ± 4.6	63.3 ± 8.1	36.3 ± 0.2	ND	8.4 ± 5.5	7.7 ± 5.3	ND	2.3 ± 12.4
Procyanidin tetramer	41.5 ± 6.2	73.4 ± 4.3	ND	35.7 ± 6.9	27.9 ± 2.8	ND	2.9 ± 11.2	4.2 ± 3.7	ND	6.2 ± 6.9
B5	6.4 ± 5.2	27.8 ± 3.6	ND	9.5 ± 1.0	5.4 ± 3.2	ND	2.3 ± 11.1	3.9 ± 6.3	ND	ND
Σ flavan3-ols	181.9	556.5	35.6	459.5	217.9	1.0	87.0	107.9	0.0	19.6
Phloretin	95.3 ± 2.6	78.1 ± 3.6	28.5 ± 2.8	54.4 ± 3.3	40.4 ± 2.0	13.9 ± 0.3	27.1 ± 3.3	31.5 ± 7.7	ND	12.8 ± 0.5
xyloglucoside										
Phloridzin	11.7 ± 2.3	31.7 ± 3.5	22.6 ± 2.4	22.2 ± 2.9	18.3 ± 2.5	9.8 ± 4.8	18.5 ± 2.8	19.3 ± 6.9	3.4 ± 15.8	16.1 ± 0.3
Quercitrin	0.9 ± 3.5	1.3 ± 4.0	0.5 ± 4.5	ND	1.0 ± 0.8	1.8 ± 3.0	ND	ND	ND	ND
Coumaric derivative	7.1 ± 3.6	7.4 ± 2.4	7.5 ± 7.9	15.8 ± 2.9	76.1 ± 2.3	10.0 ± 3.2	13.0 ± 5.6	12.4 ± 5.0	1.1 ± 16.8	8.1 ± 10.2
Chlorogenic acid	252.7 ± 2.4	182.9 ± 3.5	230.2 ± 6.8	402.3 ± 3.6	557.4 ± 6.5	171.8 ± 2.7	195.7 ± 6.8	197.1 ± 5.1	10.8 ± 9.3	138.5 ± 9.9

The profiles of the phenolic compounds in experimental apple juices were all quite similar; however, the quantity of monomeric flavan-3-ols was less in the ultrafiltered juice. This fact could be related to the greater degree of oxidation and the adsorption ability of the cake layer developed on the membrane ultrafiltration surface.

The method using solid-phase extraction is a powerful tool for the fractionation and analysis of polyphenol compounds in different types of apple samples. Quantitative profiles of phenolic compounds are useful for the detection of mixtures and adulterations and, probably, for the classification of cider apples into technological groups.

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