

## Analytical Methods

## Phenolic and antioxidant composition of by-products from the cider industry: Apple pomace

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## ARTICLE INFO

## Article history:

Received 17 November 2008

Received in revised form 25 February 2009

Accepted 12 April 2009

## Keywords:

Cider

By-products

Apple pomace

Polyphenols

Antioxidant capacity

DPPH

FRAP

## ABSTRACT

Eleven different cider apple pomaces (six single-cultivar and five from the cider-making industry) have been analysed for low molecular phenolic profiles and antioxidant capacity. The Folin index ranged between 2.3 and 15.1 g gallic acid per kg of dry matter. Major phenols were flavanols, dihydrochalcones (phloridzin and phloretin-2'-xyloglucoside), flavonols and cinnamic acids (chlorogenic and caffeic acids). The group of single-cultivar pomaces had higher contents of chlorogenic acid, (-)-epicatechin, procyanidin B2 and dihydrochalcones, whereas the industrial samples presented higher amounts of up to four unknown compounds, with absorption maxima between 256 and 284 nm. The antioxidant capacity of apple pomace, as determined by the DPPH and FRAP assays, was between 4.4 and 16.0 g ascorbic acid per kg of dry matter, thus confirming that apple pomace is a valuable source of antioxidants. PLSR analysis gave reliable mathematical models which allowed to predict the antioxidant activity of apple pomace as a function of the phenolic profile. The variables with the higher modelling power were phloridzin > procyanidin B2 > rutin + isoquercitrin > protocathechuic acid > hyperin.

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## 1. Introduction

Asturias (northern Spain) is one of the largest producers of cider of the world (AICV, 2000). More than 20,000 tons of apple pomace are produced per year and primarily used as cattle feed, although it still remains as a waste which causes economical and environmental problems. However, apple pomace, consisting of peel, seeds, core, stems and exhausted soft tissue, has long been recognised as a valuable material for nutritional, pharmacological or cosmetic purposes, as it is rich in dietary fibre and polyphenols (Četković et al., 2008; Guyot, Serrand, Le Queré, Sanoner, & Renard, 2007; Lu & Yeap Foo, 1997, 2000; Sudha, Baskaran, & Leelavathi, 2007; Yeap Foo & Lu, 1999). The antioxidant capacity of apple pomace is related to its phenolic profile. Procyanidins have long been recognised as the major contributors to antioxidant activity of apples (Chinnici, Bendini, Gaiani, & Riponi, 2004; Tsao, Yang, Xie, Sockovie, & Khanizadeh, 2005) and derivatives (Oszmianski, Wolniak, Wojdylo, & Wawer, 2008), which capacity depends on their polymerisation degree and substituents (Lotito et al., 2000). Also, the antioxidant activity of hydroxycinnamic and benzoic acids and flavonols has been ascertained (Kim, Lee, Lee, & Lee, 2002; Tsao et al., 2005).

Many methods to evaluate the antioxidant capacity of different materials have been reviewed (Huang, Ou, & Prior, 2005; Roginsky & Lissi, 2005). At the present time, *in vitro* assays based on the scavenging of free radicals such as DPPH and ABTS, and the mea-

sure of the ferric reducing power (FRAP) are some of the most employed techniques, however, there is not agreement about neither the standard methodology nor the reference compound to express the antioxidant capacity (Nenadis, Lazaridou, & Tsimidou, 2007), although Trolox or ascorbic acid are commonly recommended. The consensus is that different methods should be employed to assess the antioxidant capacity of foods (Roginsky & Lissi, 2005).

In the Asturian cider industry, a complex mixture of cider apples is used to obtain a must with mild acidic characteristics. The apples are washed, inspected to reject those rotten, milled and transferred to the press. At this step, and according to the technology available in the cellar, there are two possible alternatives of handling: traditional pressing, which takes 2–3 days, or pneumatic pressing, which is a fully automatic process lasting 2–5 h. This means that apple pomaces can experiment different degrees of oxidation during the cider making process, thus influencing the phenolic and antioxidant composition of this material. In this paper, a preliminary insight into the influence of technological processing factors (variety, pressing system, aeration) on the phenolic profiles and the antioxidant capacity of apple pomace is presented.

## 2. Materials and methods

## 2.1. Reagents and solvents

Folin–Ciocalteu reagent was supplied by Merck (Darmstadt, Germany). 2,2'-diphenyl-β-picrylhydrazyl (DPPH), 2,4,6-tris-(2-pyridyl)-s-triazine (TPTZ), ascorbic acid, ferric chloride and sodium

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acetate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Toluene- $\alpha$ -thiol was from Fluka (St. Louis, MO, USA). Polyphenol standards were supplied as follows: gallic acid, protocatechuic acid, (+)-catechin, (–)-epicatechin, phloridzin, and hydroxycinnamic acids (*p*-coumaric acid, caffeic acid and chlorogenic acid), by Sigma (St. Louis, MO, USA); procyanidin B2 and quercetin glycosides (hyperin, avicularin and quercitrin) by Extrasynthèse (Genay, France), and reynoutrin was from Apin Chemicals (Abingdon, UK). Phloretin-2'-xyloglucoside, trimer C1 and tetramer were kindly furnished by Dr. Lea (Reading, UK). Water was purified with a Milli-Q system from Millipore (Bedford, MA, USA). Solvents were purchased from Panreac (Barcelona, Spain) and were of analytical or HPLC grade.

## 2.2. Samples

Five apple pomaces (1 kg each one) were obtained from local cider-making industries and six single-cultivar pomaces (0.5–0.6 kg each one) from experimental pressings, which characteristics are summarised in Table 1. To obtain the experimental pomaces, between 1.5 and 2.0 kg of healthy and ripe apples were milled (SPEC RC2000) and quickly pressed by means of a small hydraulic press (Hafico). The resulting pomaces were immediately packed in plastic bags and frozen at  $-20^{\circ}\text{C}$ . The whole process (milling, pressing and packing) took about 15 min. Mean moisture content of apple pomaces were between 70% and 76%. The samples were dried (oven-dried at  $60^{\circ}\text{C}$  for 48 h) finely milled (sieve 0.5 mm) with a Cyclotek 1093 (Foss-Tekator) and kept protected from light and humidity until analysis.

## 2.3. Polyphenol extraction

Between 2 and 2.5 g of apple pomace powder were extracted with acetone:water (70:30) ( $3 \times 15$  mL) for 5 min in an ultrasonic bath at  $20^{\circ}\text{C}$ . The extracts were pooled, centrifuged (10 min at  $10^{\circ}\text{C}$  and 17,000g) and evaporated to dryness at  $30^{\circ}\text{C}$  with a rotary evaporator. The residue was reconstituted in 10 ml of methanol, filtered through 0.22  $\mu\text{m}$  PVDF membrane (Teknokroma, Barcelona, Spain) and analysed.

## 2.4. Analytical procedures

HPLC analysis was performed according to a previously validated method (Suárez, Palacios, Fraga, & Rodríguez, 2005), by means of a Waters system, equipped with a 717 automatic injector, a column oven ( $30^{\circ}\text{C}$ ), two pumps (model 510), a diode array detector (model 2996), and Empower v.2.0 software data module. The interval for absorbance measurements was between 190 and 450 nm. Separation of polyphenols was carried out on a

reversed-phase Nucleosil 120 C<sub>18</sub> ( $250 \times 4.6$  mm I.D, 3  $\mu\text{m}$ ) column from Teknokroma (Barcelona, Spain). The elution solvents were aqueous 2% acetic acid (solvent A) and 100% methanol (solvent B). The samples were eluted according to the following gradient: a linear step from 0% to 45% of solvent B in 55 min, an isocratic step for 15 min and a final linear increase of solvent B to 55% in 10 min. Flow rate was 0.8 ml/min and the injection volume was 10  $\mu\text{l}$ .

Quantification was done by the external standard method, using as standards (–)-epicatechin for flavanols, phloridzin for dihydrochalcones ( $\lambda = 280.0$  nm), quercitrin for flavonol glycosides ( $\lambda = 350.0$  nm), and *p*-coumaric acid ( $\lambda = 313.0$  nm) and chlorogenic acid ( $\lambda_{\text{max}} = 326.3$ ; sh. at 297.7 nm) for hydroxycinnamic acid derivatives.

Total phenol analyses were done by spectrophotometry by the Folin–Ciocalteu method, according to that described by the European Union Official Methods of Analysis (1998), by means of a Perkin–Elmer Lambda 35 equipped with an autosampler and a 1 cm optical quartz flow cell. The reaction is developed in 100 ml volumetric flasks, where the different reactants are added in this order: 1 ml of sample, 50 ml of water, 5 ml of Folin–Ciocalteu reagent, 20 ml of 20% sodium carbonate and water, to reach the final volume. The absorbance was measured at 750 nm, after 30 min at room temperature. The results are expressed as g gallic acid/kg of dry matter (DM).

## 2.5. Antioxidant capacity

### 2.5.1. DPPH assay

The antiradical activity was determined by spectrophotometry in 1 cm disposable plastic cells. 40  $\mu\text{l}$  of sample or standard (diluted 1/10 with methanol, reaching a concentration range of 100–300 mg gallic acid/L) were added to 1.460 mL of DPPH<sup>•</sup> solution (40 mg/L) in methanol, and left to stand in the dark (Brand-Williams, Cuvelier, & Berset, 1995). The absorbance at 515 nm was measured at the beginning ( $A_{t=0}$ ) and after 240 min ( $A_{t=240 \text{ min}}$ ), when the reaction reached the steady state. The inhibition percentage was calculated as follows:

$$\%IP = \frac{(A_{\text{control}} - A_{t=240 \text{ min}})}{A_{\text{control}}} \times 100$$

The antioxidant capacity was referred to as ascorbic acid (AA) equivalents (g AA/kg DM).

### 2.5.2. FRAP assay

The working FRAP reagent was prepared freshly every day by mixing 2.5 mL of TPTZ (10 mM in 40 mM hydrochloric acid), 2.5 mL of ferric chloride (20 mM) and 25 mL of sodium acetate buffer (300 mM, pH 3.6). The FRAP assay was carried out at  $37^{\circ}\text{C}$ , in 1-cm disposable plastic cells. 900  $\mu\text{l}$  of the FRAP reagent were mixed with 90  $\mu\text{l}$  of water and 30  $\mu\text{l}$  of apple pomace extract (diluted 1:25 with methanol). After 120 min, the absorbance at 595 nm was measured (Pulido, Bravo, & Saura-Calixto, 2000).

The antioxidant capacity of the apple pomaces were expressed as AA equivalents.

## 2.6. Thiolytic of the crude apple pomace and extracts

In order to confirm the structure of the unknown flavanols, the thiolytic reaction was applied both to the acetone extract and directly to the dried apple pomace, according to the procedure described elsewhere (Guyot, Marnet, Sanoner, & Drilleau, 2001). Briefly, between 50 and 100 mg of apple pomace or 500  $\mu\text{l}$  of the pomace acetone:water extract were treated with acidic methanol (400  $\mu\text{l}$ , HCl 3.3% v/v) and toluene- $\alpha$ -thiol (800  $\mu\text{l}$  5% in methanol), incubated at  $40^{\circ}\text{C}$  for 30 min, cooled, filtered through a PVDF

**Table 1**  
Technological descriptions of the apple pomaces.

Origin	Characteristics	References
Industrial pomaces	Mixture of Asturian apples, hydraulic press, and different degrees of exposure to air during the pressing process	M1, M3: 48 h
		M2: 10 h
		M4: 36 h
		G: 1.5 h
Single-cultivar pomaces	Limón Montés (acidic)	LM
	Meana (bittersharp)	M
	Durona de tresali (acidic, mild bitter)	DT
	De la Riega (mild sharp)	DR
	Perezosa (Sweet)	P
	Carrió (Mild sharp)	C

membrane and injected. The mean polymerisation degree (DP<sub>n</sub>) was calculated by the molar ratio between all the flavan-3-ols (benzylthioether adducts + terminal units) to the sum of terminal units ((+)-catechin and (–)-epicatechin).

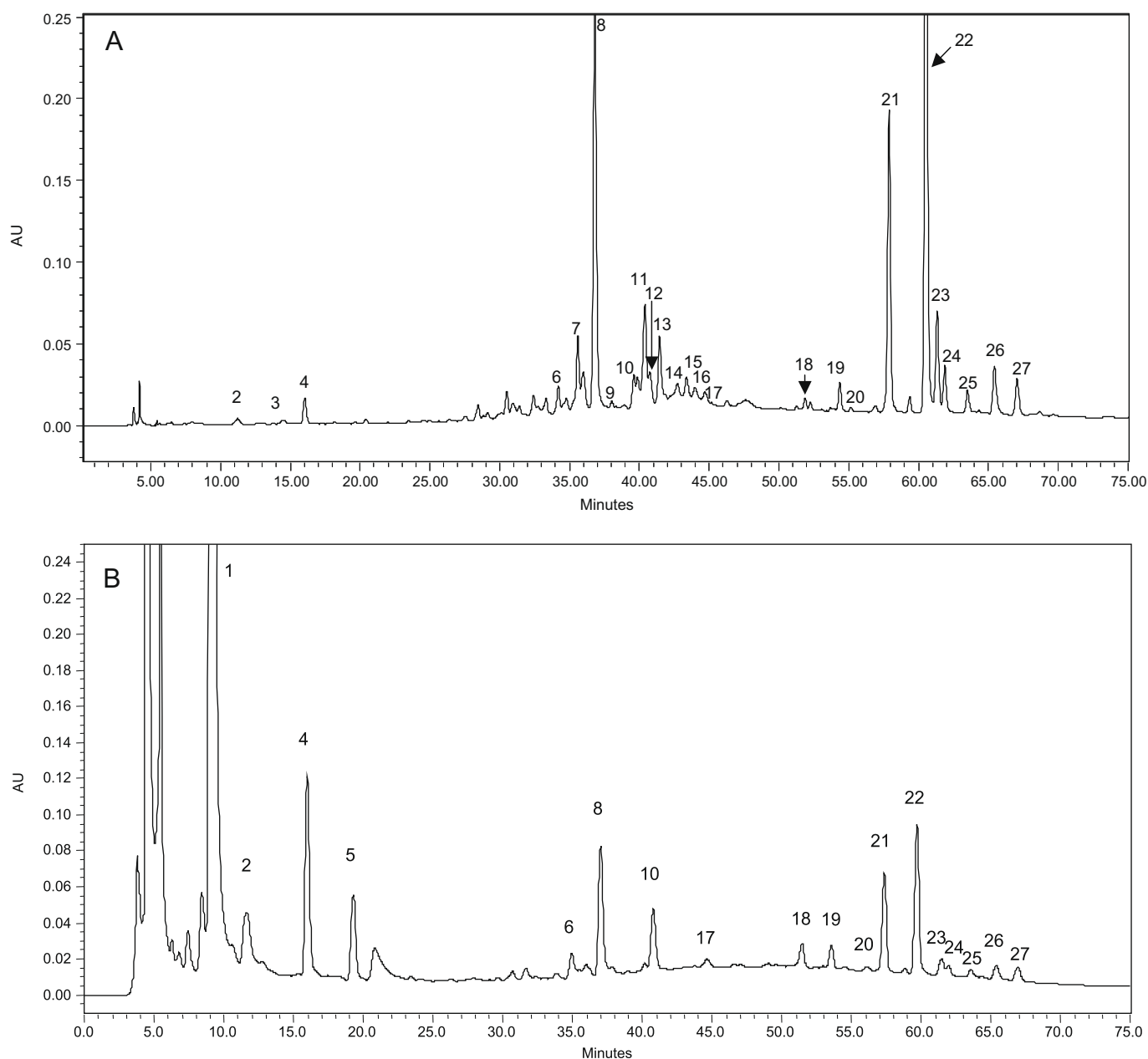
### 2.7. Statistical analyses

ANOVA, non-linear regression and Principal Component Analysis, taking a 95% confidence interval were performed by means of the SPSS v.11.0 statistical package for Windows. Multivariate partial least squares regression (PLSR) analyses were done with the PARVUS v.1.0 statistical package (Forina, Leardi, Armanino, & Lanteri, 1988).

## 3. Results and discussion

### 3.1. Phenolic profiles of cider apple pomaces

In Fig. 1 are shown two characteristic profiles of apple pomaces. As seen in Fig. 1A, major compounds correspond to dihydrochalcones (phloridzin and phloretin-2'-xyloglucoside, and others, probably 3-hydroxyphloretin), acids (protocatechuic, chlorogenic, caffeic and others), flavanols ((–)-epicatechin, procyanidin B2, trimer, tetramer, and others), and flavonols (hyperin, isoquercitrin, rutin, reynoutrin, avicularin, and quercitrin). At the beginning of both chromatograms (Fig. 1A and B), a series of four unknown compounds were observed. These components were not assigned



**Fig. 1.** Typical chromatogram at 280 nm of apple pomace (A: single-cultivar; B: industrial). Analytical conditions, see Section 2. Peaks: 1, Unk-1 ( $\lambda_{\max}$  268 nm); 2, Unk-2 ( $\lambda_{\max}$  270 nm); 3, Unk-3 (protocatechuic acid-type;  $\lambda_{\max}$  256 nm); 4, Unk-4 ( $\lambda_{\max}$  284 nm); 5, protocatechuic acid; 6, cinnamic acid-1 ( $\lambda_{\max}$  315 nm; sh. 283 nm); 7, procyanidin B2; 8, chlorogenic acid; 9, caffeic acid; 10, cinnamic acid-2 ( $\lambda_{\max}$  312 nm); 11, trimer + tetramer; 12, (–)-epicatechin; 13, cinnamic acid-3 ( $\lambda_{\max}$  312); 14–16, unknown flavanols; 17, cinnamic acid-4 ( $\lambda_{\max}$  312 nm); 18–20, unknown dihydrochalcones; 21, phloretin-2'-xyloglucoside; 22, phloridzin; 23, hyperin; 24, rutin + isoquercitrin; 25, reynoutrin; 26, avicularin; 27, quercitrin.

to any group because their UV-spectra did not correspond to any of the phenolic families analysed. The absorption maxima ranged between 256 and 284 nm, and their amounts were expressed in  $\text{area} \times 10^{-6}/\text{kg DM}$ .

The compounds tentatively identified as flavanols by spectra and by co-elution with the corresponding standards (procyanidin B2, trimer and tetramer, (–)-epicatechin) were confirmed as flavanols. Thus, one single-cultivar apple pomace, both the direct powder and the extract, were submitted to thiolysis, according to the method reported elsewhere (Guyot et al., 2001). As expected, the reaction with the thiol reactant gave rise to the disappearance of the peaks assigned to procyanidin B2, trimer + tetramer and other flavanols, by cleavage of the interflavanil linkages, leading to the formation of the benzylthioether adducts at the end of the chromatogram, and the release of the terminal monomeric units ((+)-catechin and (–)-epicatechin), thus confirming these components as flavanols. No differences were observed between the treatments with toluene thiol of the dried apple pomace powder and the corresponding extract. The resulting mean polymerisation degree was close to 4 in both samples. The (–)-epicatechin monomer content was 4.5-fold higher than that of (+)-catechin.

### 3.1.1. Single-cultivar samples

Six single-cultivar pomaces from different technological groups were chosen among the cider apple varieties included in the Protected Designation of Origin “Cider from Asturias”.

Significant differences were observed among varieties for all of the parameters analysed ( $p < 0.05$ ). Folin index varied in this order:  $C > DR = M > DT = P > LM$ . The flavanol contents ranged between 1.7 and 2.5 g/kg. As shown in Table 2, Meana was the cultivar with the lowest amount of trimer + tetramer, and the highest in other flavanols, followed by De la Riega and Carrió. Phloridzin was al-

ways the main dihydrochalcone present in the apple pomaces, with contents ranging between 0.6 and 1.5 g/kg. Phloretin-2'-xyloglucoside was the second one, with contents in the range of 0.08 and 1.0 g/kg. Considering phenolic acids, their contents were between 0.5 and 1.6 g/kg, being chlorogenic acid the major one in all the cases. Chlorogenic acid contents varied in the order  $DR > C > DT > P = LM > M$ . Maximum content of caffeic acid was 25 mg/kg, and corresponded to DT cultivar. Protocatechuic acid was always a minor component, excepting in LM cultivar, with 144.7 mg/kg. Six quercetin glucosides were identified in the apple pomaces: hyperin, isoquercitrin, rutin, reynoutrin, avicularin and quercitrin, as described by others (Lu & Yeap Foo, 1997; Schieber, Hill, Conrad, Beifuss, & Carie, 2002; Sánchez Rabaneda et al., 2004). These components were present in high levels in apple pomaces, ranging between 0.4 and 1.4 g/kg, hyperin being the major one.

Comparing with previous results on single-cultivar apple pomaces (Ćetković et al., 2008), the samples reported here exhibited higher levels of polyphenols. Phenolic profiles are closely related to cultivar (Alonso-Salces et al., 2004; Mangas, Rodríguez, Suárez, Picinelli, & Dapena, 1999; Marks, Mullen, & Crozier, 2007), the cultural conditions of the apple trees (Chinnici et al., 2004; Lea & Beech, 1978; Veberic et al., 2005) and season (Lata, Przeradzka, & Bińkowska, 2005).

On a dry weight basis, it is possible to compare the phenolic contents of the cider apple varieties analysed in this paper with others. Thus, these Asturian cultivars present higher mean contents of (–)-epicatechin (232.4 mg/kg), chlorogenic acid (824.6 mg/kg) and phloridzin (913.1 mg/kg) than those from the Basque region, (respectively, 162.1, 409.0 and 21.0 mg/kg, as calculated from Alonso-Salces et al. (2004)). The concentrations of (–)-epicatechin in the Spanish cider varieties were remarkably lower

**Table 2**  
Mean polyphenolic contents (mg/kg) and antioxidant capacity of single cultivar cider apple pomaces.

	P	M	DR	LM	C	DT
Folin (g gallic acid/kg)	6.2b	7.2c	7.7c	5.5a	10.9d	6.3b
TP <sub>HPLC</sub> (mg/l)	4818.3	5910.0	5230.4	4672.5	5862.6	5198.5
DPPH (g AA/kg)	11.4a	11.1a	13.5c	12.4b	15.9d	11.1a
FRAP (g AA/kg)	9.5a	10.8a	10.7a	9.8a	13.8b	9.7a
<i>Phenolic acids</i>						
Protocatechuic	20.8c	nd	nd	144.7d	4.1b	1.7a
Chlorogenic	693.2b	393.2a	1415.5e	681.5b	927.2d	836.9c
Caffeic	nd	20.7 cd	16.8c	10.5b	17.3c	25.1 cd
Other acids	230.6c	109.7a	110.6ab	118.1b	278.3d	409.5e
Sum of phenolic acids	944.7	523.7	1542.9	954.7	1226.9	1273.3
<i>Flavanols</i>						
(–)-Epicatechin	394.9d	222.8c	314.6d	161.1b	163.0b	136.0a
Procyanidin B2	477.0b	590.2c	437.3b	329.1a	348.7a	553.9c
Trimer C1 + tetramer	525.3b	372.0a	576.0b	572.9b	585.0b	569.6b
Other flavanols	664.1b	1228.6e	944.3d	652.1b	772.0c	619.6a
Sum of flavanols	2061.2	2413.7	2272.1	1715.8	1868.7	1881.2
<i>Dihydrochalcones</i>						
Phloretin-2'-xyloglucoside	332.8d	996.2f	136.9b	82.9a	457.2e	228.2c
Phloridzin	797.3c	1435.4f	730.2b	587.2a	1053.0e	875.7d
Other dihydrochalcones	38.7c	104.0f	22.7b	18.0a	61.7e	51.0d
Sum of dihydrochalcones	1168.8	2535.7	889.7	688.2	1571.9	1154.9
<i>Flavonols</i>						
Hyperin	223.4b	186.7a	175.3a	377.1d	462.9e	252.5c
Isoquercitrin + rutin	136.0c	44.6a	63.3b	159.6d	208.9e	218.7f
Reynoutrin	61.7c	37.5b	16.8a	144.3f	104.4e	84.2d
Avicularin	129.5b	99.2a	168.1c	364.0f	242.9e	223.5d
Quercitrin	93.0b	69.0a	96.0b	252.0e	168.7d	103.8c
Sum of flavonols	643.6	436.9	525.6	1313.7	1195.0	889.2

Different letters in the same line indicate significant differences at  $p < 0.05$ .

TP<sub>HPLC</sub>: Sum of HPLC polyphenols.

nd: Not detected.

For cultivar references, see Table 1.

**Table 3**  
Mean polyphenolic contents (mg/kg) and antioxidant capacity of industrial cider apple pomaces.

	G	M1	M2	M3	M4
Folin (g gallic acid/Kg)	4.7a	4.0a	7.4b	3.9a	13.9c
TP <sub>HPLC</sub> (mg/l)	2467.5	3652.4	4418.9	1045.9	2059.5
DPPH (g AA/kg)	8.2c	7.6b	12.5e	4.5a	10.3d
FRAP (g AA/kg)	8.2b	8.1b	11.9c	4.3a	12.1c
<i>Phenolic acids</i>					
Protocatechuic	108.9b	36.8a	93.6b	29.6a	263.9c
Chlorogenic	259.8b	586.7d	602.4d	96.1a	375.3c
Caffeic	10.6b	18.3d	21.4e	8.2a	17.2c
Other acids	84.3a	229.4b	609.0d	63.3a	328.8c
Sum of phenolic acids	463.5	871.2	1326.4	197.2	985.2
<i>Flavanols</i>					
(–)-Epicatechin	167.5b	nd	nd	nd	nd
Procyanidin B2	144.6c	92.1b	287.1d	nd	nd
Trimer C1 + tetramer	96.0c	1316.2e	748.7d	nd	49.7b
Other flavanols	153.1d	105.4c	305.5e	43.5a	64.5b
Sum of flavanols	561.3	1513.6	1341.3	43.5	114.2
<i>Dihydrochalcones</i>					
Phloretin-2'-xyloglucoside	172.8c	150.4b	351.4d	19.7a	174.3c
Phloridzin	451.6c	302.5b	594.7d	159.4a	292.5b
Other dihydrochalcones	58.5b	59.5b	115.8d	11.4a	101.0c
Sum of dihydrochalcones	682.8	512.3	1061.8	190.5	567.8
<i>Flavonols</i>					
Hyperin	189.2b	232.0d	207.5c	191.6b	112.6a
Isoquercitrin + rutin	102.8c	119.6d	137.3c	71.1b	57.3a
Reynoutrin	17.6a	76.3d	70.4c	67.6c	41.7b
Avicularin	255.2d	180.6c	162.8b	160.8b	90.5a
Quercitrin	186.3e	144.2d	109.9b	118.3c	87.1a
Sum of flavonols	751.1	752.7	687.9	609.4	389.2
<i>Unknown compounds<sup>1</sup></i>					
Unk-1 ( $\lambda_{\max}$ 268 nm)	250.5a	548.2a	505.6a	397.7a	19777.0b
Unk-2 ( $\lambda_{\max}$ 260 nm)	nd	25.9b	53.4c	46.9bc	397.4d
Unk-3 ( $\lambda_{\max}$ 256 nm)	8.4c	6.8b	10.3d	6.5b	nd
Unk-4 ( $\lambda_{\max}$ 284 nm)	160.5c	130.1b	72.6a	189.1d	1061.0e

Different letters in the same line indicate significant differences at  $p < 0.05$ .

TP<sub>HPLC</sub>: Sum of HPLC polyphenols.

nd: Not detected.

For sample references and characteristics, see Table 1.

<sup>1</sup> Expressed in Area  $\times 10^{-6}$ /kg DM.

than those found in the English cider cultivars (632.2 mg/kg, calculated from Marks et al. (2007)).

Regarding the not identified compounds, that referred to as Unk-4 was the major one, followed by Unk-3 and Unk-1 (Table 4). The order observed for compound Unk-4 was  $M \approx P > DR > DT > C > LM$ . Likewise, cultivars M and P exhibited the highest amounts of compounds Unk-3 and Unk-1, whereas the compound referred to as Unk-2 was present in DT cultivar only (data not shown).

### 3.1.2. Industrial samples

In Table 3 are presented the results for the industrial apple pomaces. As seen, the sample referred to as M4 had the highest Folin index, followed by M2. There were significant differences among the samples for all the parameters analysed. Unlike the single-cultivar samples, in the industrial pomaces protocatechuic acid was present in all the cases; among the flavanols, (–)-epicatechin was absent in all the samples, except that referred to as G. Chlorogenic acid was predominant among the phenolic acids, and phloridzin was the main dihydrochalcone, as observed for single-cultivar samples. It is worth to note the unknown compound 1, which proportion in the sample referred to as M4 is remarkable (Table 3). This component was found to be the major one among the non identified phenols, followed by Unk-4 and Unk-2.

Comparing with the single-cultivar pomaces, significant differences were found for many of the phenolic compounds analysed.

No differences were observed in the cases of Folin index, caffeic and other acids, trimer + tetramer, other dihydrochalcones, most of the flavonols and the compound referred to as Unk-4 (Table 4).

Among the phenolic acids, chlorogenic acid content was significantly higher in the group of the single cultivars, whereas the industrial pomaces had higher concentrations of protocatechuic acid. Another difference between the two groups of pomaces were the contents in (–)-epicatechin procyanidin B2 and other flavanols, significantly lower in the industrial ones, or absent. Although not significantly different, the maximum concentration of trimer + tetramer observed in the industrial residues was nearly twice that of the single-cultivar pomaces (Table 4). Finally, the contents of dihydrochalcones were also lower in the industrial samples than in the experimental single cultivar ones.

The areas of the unknown compounds referred to as 1, 2 and 4 were significantly higher in the industrial pomaces than in the single cultivar samples, whereas the last ones had higher contents of compound referred to as 3 (Table 4). Even though chlorogenic acid, and in a lesser extent, flavanols and phloridzin are good substrates for enzymatic oxidation catalysed by polyphenol oxidases (Bernillon, Guyot, & Renard, 2004), and that the apple pomaces were exposed to different degrees of oxidation, we did not observed any trace of those compounds associated with phloridzin oxidation ( $\lambda_{\max} = 430$  nm), reported elsewhere (Le Guernevé, Sanoner, Drilleau, & Guyot, 2004). Instead, the unknown compounds analysed in the apple pomaces, which identification is still in progress, could be related to the colourless compounds resulting from the enzymatic oxidation of phloridzin by apple polyphenoloxidase at low pH values (Le Guernevé et al., 2004).

A data matrix was constructed with all the samples analysed and nine variables (protocatechuic acid, chlorogenic acid, (–)-epicatechin, procyanidin B2, other flavanols, phloretin-2'-xyloglucoside, phloridzin, hyperin and rutin + isoquercitrin), and a PCA was performed. Three significant components explaining the 84.17% of the variance were obtained. In Fig. 2 is shown the projection of samples and variables onto the plane formed by the first two principal components. From this figure, a data structuration can be visualised, since the single-cultivar pomaces (referred to as 1) were placed on the right side of the first axis and the industrial ones (referred to as 2) were on the left side. Procyanidin B2, other flavanols and phloridzin had the highest score on the positive side of this axis, whereas hyperin and rutin + isoquercitrin had the highest score on the second axis.

### 3.2. Phenolic profiles and antioxidant activity of apple pomace

DPPH and FRAP assays are two of the most employed for antioxidant quantitation, due to their operating simplicity. The first one is usually performed by monitoring at 515 nm the changes of a mixture of the reactant and the antioxidant, until the absorbance is stable. The percentage of the DPPH remaining is proportional to the antioxidant concentration. The second one is typically run at 595 nm, taking 4 min as reaction time (Benzie & Strain, 1996).

One extract of apple pomace, properly diluted with methanol to reach a final range of total polyphenols between 30 and 300 mg gallic acid/L, was used to optimise the analytical methods for antioxidant activity.

For DPPH assay, as reaction time is a critical point of control of antioxidant activity assessment, the percentage of inhibition was monitored against time until not significant changes were observed. The steady stage of the inhibition reaction was obtained at 220 min, thus, a final time of 4 h was selected for further analyses. Subsequently, the antioxidant activity of three apple pomaces were determined five times within 1 day for repeatability, while reproducibility was examined in triplicate for three different days.

**Table 4**  
Mean polyphenolic contents (mg/kg) and antioxidant capacity of the cider apple pomaces.

		Industrial				Single-cultivar			
		Mean	sd	Max	Min	Mean	sd	Max	Min
Folin (g gallic acid/kg)	ns	6.8	4.0	14.1	2.3	7.3	1.8	11.3	5.0
DPPH (g AA/kg)	**	6.6	2.8	12.8	4.4	12.6	1.8	16.0	10.7
FRAP (g AA/kg)	*	8.9	3.0	12.5	4.1	10.7	1.6	14.5	8.0
<i>Phenolic acids</i>									
Protocatechuic	**	106.6	88.2	268.5	28.2	28.6	54.0	147.4	nd
Chlorogenic	**	384.1	200.5	607.2	94.5	824.6	322.2	1463.2	383.4
Caffeic	ns	15.1	5.1	22.0	7.6	15.1	8.6	25.3	nd
Other acids	ns	262.9	205.8	633.3	62.2	209.5	113.8	412.8	106.5
<i>Flavanols</i>									
(–)-Epicatechin	**	33.5	69.4	169.6	nd	232.4	104.6	410.6	127.3
Procyanidin B2	**	104.8	110.6	295.6	nd	456.0	102.3	619.3	311.9
Trimer C1 + tetramer	ns	442.1	533.5	1352.3	nd	533.5	83.3	677.8	367.8
Other flavanols	**	134.4	96.8	310.0	42.2	813.6	221.6	1255.2	589.5
<i>Dihydrochalcones</i>									
Phloretin-2'-xyloglucoside	*	173.7	109.4	357.9	19.4	372.4	313.9	1020.7	79.4
Phloridzin	**	360.1	155.5	641.8	149.8	913.1	281.6	1476.6	560.2
Other dihydrochalcones	ns	69.2	38.0	117.2	10.9	49.4	29.6	108.4	16.8
<i>Flavonols</i>									
Hyperin	**	186.6	41.5	233.9	110.9	280.5	108.3	466.9	175.3
Isoquercitrin + rutin	*	97.6	30.8	139.7	57.0	139.5	67.1	227.6	43.6
Reynoutrin	ns	54.7	22.9	77.3	14.9	74.8	43.6	147.3	15.9
Avicularin	ns	169.9	54.6	258.0	80.2	204.5	89.6	375.7	97.5
Quercitrin	ns	129.2	35.2	189.7	86.3	130.4	64.2	256.4	67.6
<i>Unknown compounds<sup>1</sup></i>									
Unk-1 ( $\lambda_{max}$ 268 nm)	*	4295.8	8014.1	20008.0	246.1	10.0	10.5	31.9	nd
Unk-2 ( $\lambda_{max}$ 260 nm)	*	104.7	153.2	404.7	nd	6.5	15.0	40.4	nd
Unk-3 ( $\lambda_{max}$ 256 nm)	**	6.4	3.6	11.0	nd	23.8	8.9	42.3	15.3
Unk-4 ( $\lambda_{max}$ 284 nm)	ns	322.7	384.2	1068.0	69.9	194.3	90.8	419.9	108.8

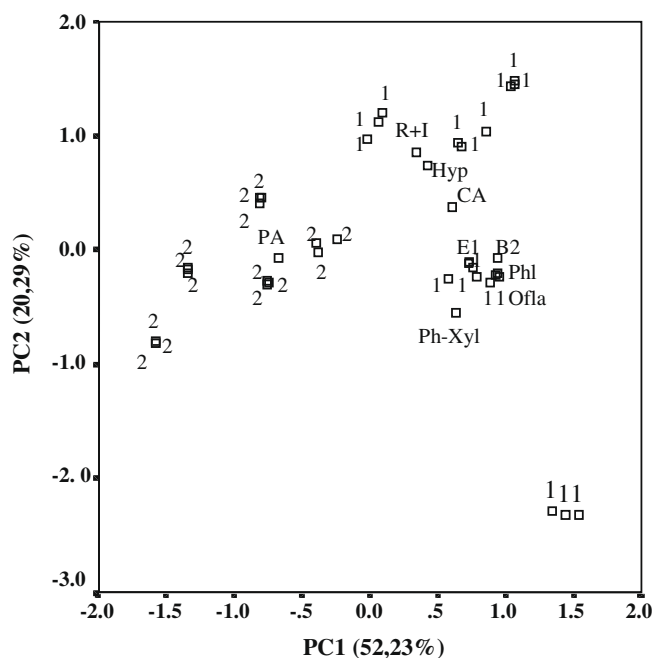
ns: Not significant.

nd: Not detected.

<sup>1</sup> Expressed in Area  $\times 10^{-6}$ /kg DM.

\* Significant at the 5% level.

\*\* Significant at the 1% level.



**Fig. 2.** Projection of samples and variables on the factorial plane formed by the first two principal components. Sample references: 1, single cultivars; 2, industrial apple pomaces. Abbreviations: CA, chlorogenic acid; B2, procyanidin B2; E, (–)-epicatechin; R+I, rutin + isoquercitrin; Ofla, other flavanols; Hyp, hyperin; PA, protocatechuic acid; Phl, phloridzin; Ph-Xyl, phloretin-2'-xyloglucoside.

The relative standard deviations in terms of ascorbic acid equivalents were, respectively, 2.6% and 5.4%.

Regarding the FRAP assay, most of the previous reports on the application of this assay for antioxidant measurements used a reaction time of 4 min, however, different time-dependent kinetic behaviours are observed when reaction times longer than 4 min are taken (Pulido et al., 2000). In the case of the apple pomace, the steady stage was not reached but the absorbance at 595 nm continuously increased. A final time of 120 min was chosen. Within and intra-day precision were calculated as described before. Mean values obtained for relative standard deviation were, respectively, 1.8% and 5.7%.

The antioxidant activities of cider apple pomaces ranged between 4.1 and 14.5 (FRAP assay), or 4.4–16.0 (DPPH assay) g ascorbic acid/kg DM (Table 4). Considering that this material is constituted by a 25% of dry matter, the presented values are equivalent to 1.0–4.0 g AA/kg of fresh weight (FW). Thus, comparing with previous studies, the apple pomace exhibited similar antioxidant activity than that of fresh apples (1.36 g AA/kg FW, reported by Kim et al. (2002), for Gala apples) or extracts of peels and pulps (1.25 g AA/kg FW for pulps, 3.3 g AA/kg FW for peels, as reported by Chinnici et al. (2004)), which confirms the suitability of apple pomace as a valuable material.

Among the single-cultivar pomaces, the antioxidant capacity determined by the DPPH assay showed this order: C > DR > LM > P = M = DT, whereas the results from the FRAP assay did not show significant differences among varieties (Table 2). Regarding the industrial samples, that referred to as M3 exhibited the lower antioxidant value. M2, exposed to air for 10 h, presented

the maximum value, followed by M4, aerated for 36 h (Table 3). The samples referred to as G (1.5 h pressing) and M1 (48 h pressing) had intermediate values for antioxidant capacity. Thus, no apparent relationship could be inferred from the present data between the pressing time, and therefore, the exposure of the pomaces to oxygen, and their antioxidant capacity.

Removing from the sample set those referred to as M, among the single-cultivars, and M4, among the industrial ones, highly significant correlations were observed between the antioxidant activity and several of the parameters analysed. Phloridzin (0.89;  $p < 0.01$ ) and total phenols (0.85;  $p < 0.01$ ) exhibited the highest values, followed by those of other flavanols (0.84;  $p < 0.01$ ), chlorogenic acid (0.78;  $p < 0.01$ ), procyanidin B2 (0.73;  $p < 0.01$ ) and phloretin 2'-xyloglucoside (0.69;  $p < 0.01$ ). Lower but still significant values for correlation were found for (–)-epicatechin, and for hyperin and rutin + isoquercitrin when using the DPPH assay. In general, the correlation values were higher when the antioxidant capacity was measured by the DPPH method, except for chlorogenic acid.

A multivariate partial least squares regression analysis was performed taking the antioxidant activity of the apple pomaces as dependent variables ( $A$ ) and their phenolic profiles ( $X_n$ ) as predictor ones. The linear models were constructed as:

$$A = b_0 + b_1X_1 + b_2X_2 + \dots + b_nX_n$$

A final data matrix containing fourteen phenolic compounds (protocatechuic acid, chlorogenic acid, procyanidin B2, other flavanols, phloretin 2'-xyloglucoside, phloridzin, other chalcones, and flavonols) and all the samples analysed gave two models with three latent variables and good prediction ability. Values obtained for explained and cross-validated variances for prediction of the antioxidant activity as measured by the DPPH were 91.4% and 80.3%, respectively, and 84.4% and 72.2% in the FRAP assay. The variables with the higher modelling power were phloridzin > procyanidin B2 > rutin + isoquercitrin > protocatechuic acid > hyperin. All these compounds have been previously reported for their antioxidant activity (Chinnici et al., 2004; Nakamura, Watanabe, Miyake, Kohno, & Osawa, 2003; Natella, Nardini, Di Felice, & Scaccini, 1999; Tsao et al., 2005). It is interesting to note that phloridzin was the most relevant variable (even though its antioxidant activity is much lower than that of flavanols and flavonols), whereas chlorogenic acid, another major compound in cider apple pomace, with recognised antioxidant capacity (Moreira, Monteiro, Ribeiro-Alves, Donangelo, & Trugo, 2005) was not included in these models. In this sense, as quite long reaction times have been used in these assays, the influence on the antioxidant activity of less active compounds, as phloridzin, or more complex structures, as highly polymerised procyanidins may become important.

#### 4. Conclusions

Apple pomace is a valuable source of polyphenols relevant for their antioxidant activity, as flavanols and flavonols. The antioxidant activity of apple pomace should be mainly related to apple cultivars, and it resulted to be equivalent to that previously reported for fresh apples. The apple pomaces coming from the cider-making industry exhibited lower, but still appreciable antioxidant activity than the single-cultivar samples considered in this study, which can be attributed to differences in their phenolic profiles. Both DPPH and FRAP assays, with optimised reaction times, allowed to perform reliable routine determinations of antioxidant capacity of apple pomace, and provided equivalent results. The antioxidant activity of apple pomace can be predicted by the contents of phloridzin, procyanidin B2, rutin + isoquercitrin, protocatechuic acid and hyperin.

#### Acknowledgements

Financial support for this work was provided by the National Institute of Agricultural of Research and Agro-Food Technology (INIA) and co-financed with ERDF and ESF funds (Project RTA2006-00-00069), the Regional Government of Asturias and the Town Hall of Villaviciosa for a grant (YDG).

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