

## Liquid chromatographic method for quantifying polyphenols in ciders by direct injection

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### Abstract

An analytical method for the quantitative determination of the principal phenolic compounds (benzoic acids, hydroxycinnamic acids, 3-phenylpropionic acids, flavanols, procyanidins, dihydrochalcones, quercetin glycosides) in ciders, which successfully employs a RP-HPLC and photodiode-array detection system without prior treatment of the sample, is described. Parameters usually examined in the method validation were evaluated. Good linearity was obtained with correlation coefficients exceeding 0.999 and the detection limits ranged from 0.07 mg/L (*p*-hydroxybenzoic acid) to 2 mg/L (hydrocaffeic acid). Recoveries ranging between 90 and 104% and the reproducibility of the method was always <8% (RSD). The method was applied to a set of commercial samples and the results obtained may be helpful to establish a phenolic profile in Asturian cider.

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**Keywords:** Phenolic acids; Flavonoids; Cider; Direct injection; HPLC.

### 1. Introduction

Low molecular mass polyphenols have been exhaustively studied due to their contribution to the sensory quality (colour, taste, flavour) and their use for determining adulterations in apple derived products [1–5]. During the past decade the apple polyphenols have been subjected to a number of investigations due to their presence in human diet and their biological properties and benefit effects on health. Phenolic compounds seem to protect against cardiovascular disease and have certain potential anticarcinogenic properties due to their antioxidant activity and their function as free radical scavengers [6–9].

Polyphenols analysis, in cider and apple, is generally accomplished by reversed-phase high-performance liquid chromatography with UV–vis detection. However, the determination is usually preceded by several operations, such as extrac-

tion, purification and concentration, due to the complexity of analysis. Two methods for polyphenols fractionation have been used: liquid–liquid and solid–liquid extraction [10–13]. It should be noted that sample preparation is the time determining step of whole analytical procedure, which represents 2/3 of the total analysis time, and it is the primary source of error differences in the results obtained by different laboratories [14]. Polyphenols are compounds very reactive and substantial changes in sample composition may occur due to: isomerizations under exposure to UV radiation or daylight, the oxidative transformation and hydrolysis phenomena as result of the extraction procedure [15–19]. Therefore, a direct injection of the sample could be considered as an alternative to simplify the analysis of phenolic compounds, and to prevent many errors and any polyphenols degradation during the sample handling [19–22].

In this paper, a reversed-phase HPLC method with diode array detection for the separation and quantitation of phenolic compounds in ciders, by direct injection, without any prior purification of sample, is described and validated.

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## 2. Experimental

### 2.1. Reagents and standards

Polyphenol standards were supplied as follows: (+)catechin, (–)epicatechin, phloridzin, tyrosol, catechol, benzoic acids (gallic acid, protocatechuic acid and *p*-hydroxybenzoic acid), hydroxycinnamic acids (*p*-coumaric acid, caffeic acid, ferulic acid and chlorogenic acid), 3-phenylpropionic acids (hydrocoumaric acid, hydrocaffeic acid and hydroferulic acid,) by Sigma (St. Louis, MO, USA); and quercetin glycosides (hyperoside, isoquercitrin, avicularin, rutin and quercitrin) by Extrasynthèse (Genay, France). The phloretin-2'-xyloglucoside and procyanidins (B1, B2, B5, trimer C1, tetramer D and unknown trimer) were kindly furnished by Dr. A. Lea (Reading, UK). Water was purified on a Milli-Q system from Millipore (Bedford, MA, USA). Reagents and solvents were purchased from Panreac (Barcelona, Spain) and were of analytical or HPLC grade.

### 2.2. Samples

Eight Spanish ciders were purchased from local supermarkets of Asturias (Spain). Prior to HPLC analysis, the cider samples were degassed in an ultrasonic bath during 10 min in order to remove all carbon dioxide and filtered through a 0.45  $\mu\text{m}$  cellulose acetate membrane filter from Teknokroma (Barcelona, Spain).

### 2.3. HPLC analysis

Analyses were performed with a Waters system, equipped with a 717 automatic injector, provided with a column oven, two pumps (model 510), a diode array detector (model 996) and Millennium software v.2.1 data module. Separation of polyphenols was carried out on a reversed-phase Nucleosil 120 C<sub>18</sub> (250 mm  $\times$  4.6 mm I.D., 3  $\mu\text{m}$ ) column from Teknokroma (Barcelona, Spain). The column was thermostated at 25 °C and a flow rate of 0.8 mL/min was used. 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 and a final isocratic step of 20 min. Injection volume was 50  $\mu\text{L}$ .

Identification of compounds was achieved by comparing their spectra and retention times with those of standards when available. Detection was performed at 313 nm for the hydroxycinnamic acids, at 355 nm for the flavonol glycosides and at 280 nm for the rest of phenolic compounds (benzoic and 3-phenylpropionic acids, flavanols, procyanidins and dihydrochalcones). The spectra were acquired from 200 to 400 nm with a sampling rate of 1.0 and the highest scanning resolution (1 nm).

Quantitation was performed according to an external standard method. For the compounds lacking of standards, or those which the amount at our disposal was too small, the

quantification was achieved from similar compounds: thus, the procyanidins were quantified as procyanidin B1, the phloretin-2'-xyloglucoside as phloridzin, the flavonol glycosides as quercitrin and the *p*-coumaroylquinic acid as *p*-coumaric acid.

## 3. Results and discussion

### 3.1. Separation and identification

Five families of phenols were taken as references for the analytical optimisation process in ciders: flavanols, hydroxycinnamic and 3-phenylpropionic acids, dihydrochalcones and flavonols.

Initial HPLC working conditions were selected on the basis of previously published works [12,23]. Two mobile phases

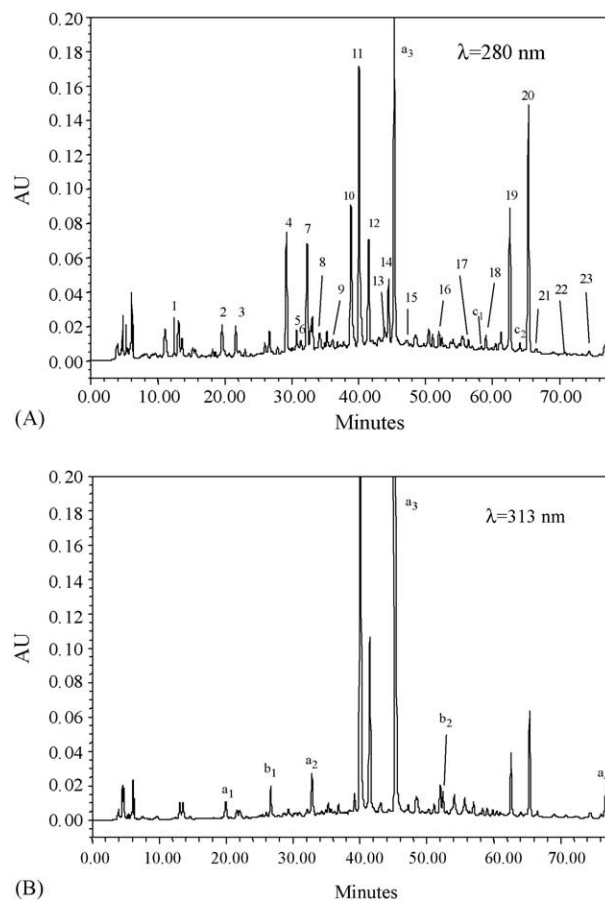


Fig. 1. Separation of phenolic compounds in cider at (A) 280 nm and (B) 313 nm. Peaks: 1, gallic acid; 2, catechol; 3, protocatechuic acid; 4, tyrosol; 5, procyanidin B1; 6, *p*-hydroxybenzoic acid; 7, hydrocaffeic acid; 8, (+)catechin; 9, unknown trimer; 10, procyanidin B2; 11, chlorogenic acid; 12, caffeic acid; 13, tetramer D; 14, trimer C1; 15, hydroferulic acid; 16, *p*-coumaric acid; 17, ferulic acid; 18, procyanidin B5; 19, phloretin-2'-xyloglucoside; 20, phloridzin; 21, hyperin; 22, avicularin; 23, quercitrin; a<sub>1</sub>–a<sub>4</sub>, *p*-coumaric derivatives ( $\lambda_{\text{max}} = 312.0 \text{ nm}$ ); b<sub>1</sub> and b<sub>2</sub>, hydroxycinnamic derivatives ( $\lambda_{\text{max}} = 326.3 \text{ nm}$ ); c<sub>1</sub> and c<sub>2</sub>, phloretin derivatives.

were tested: 2 mM phosphoric acid (solvent A)/methanol (solvent B) and 2% acetic acid (solvent A)/methanol (solvent B) elution gradient shapes, temperatures and flow rates in order to separate the maximum number of phenolic compounds.

The use of the phosphoric acid/methanol phase provided an overlapping of *p*-hydroxybenzoic and hydrocaffeic acids and worse resolution between procyanidin B2 and the unknown peak with hydroxycinnamic acid type spectra ( $\lambda_{\max} = 326$  nm). Best resolution and faster separation were observed by using methanol/acetic acid as eluents. The optimized chromatographic conditions (Section 2.3) showed a good separation with values of selectivity factors ( $\alpha$ ) above 1.00 for flavan-3-ol (procyanidin B1, catechin, unknown trimer, procyanidin B2, trimer C1, tetramer D, epicatechin and procyanidin B5), phenolic acids (benzoic and *p*-hydroxycinnamic acids) and between phloridzin and flavonols. Under these optimized conditions coelution was only detected between rutin and isoquercitrin, while the rest of quercetin glycosides were properly separated. In fact, the problem of coelution between these compounds was reported by other authors [24].

A typical RP-HPLC chromatogram of a cider is shown in Fig. 1. In this cider sample a total of 23 phenolic compounds were identified by comparison of their retention times and

UV–vis spectra (catechol, tyrosol, nine phenolic acids, seven monomeric and oligomeric flavan-3-ols, two dihydrochalcones and three quercetin glycosides). The identified peaks were then confirmed by spiking samples with standard mixtures. Furthermore, the following compounds were tentatively identified via their spectral features and the review of literature data: peaks a1–a4 exhibited spectral characteristics similar to *p*-coumaric acid ( $\lambda_{\max} = 312.0$  nm), the peaks b1 and b2 showed hydroxycinnamic acid type spectra ( $\lambda_{\max} = 326.0$  nm, shoulder at 297.7 nm) and peaks c1 and c2 had a phloridzin-like spectra. Probably, the peak a3 is *p*-coumaroylquinic the major phenolic acid quantified in cider together with chlorogenic acid [25,26], and the peak a4 could be *p*-coumaric ethyl ester [27]. Other quinic esters of hydroxycinnamic acids have been characterized in apple derivatives, such as chlorogenic acid and *p*-coumaroylquinic isomers and many derivatives of hydroxycinnamic acid and glucose can also be found in small quantities as *p*-coumaroylglucose and feruloylglucose [24,28]. With regard to the phloretin derivatives (peaks c1 and c2), the 3-hydroxyphloridzin has been identified in apple pomace and its chemical structure was established by NMR [29] and also tentatively identified by HPLC-MS in Basque apple cider [30]. Another dihydrochalcone was recently described in Golden Delicious apples [31].

Table 1  
Analytical characteristics of the calibration graphs and recoveries of phenolic compounds from cider

Compound	Calibration curve ( $n = 15$ )				LOD (mg/L)	Mean recovery (%) ( $n = 6$ )	RSD <sup>a</sup> (%)
	Linear range (mg/L)	Slope	Intercept	Correlation coefficient			
Catechol	3.0–30.3	86379.9	−11850.0	0.9999	0.3	92	3.0
Tyrosol	2.5–50.0	35207.0	−8444.6	0.9999	0.5	91	2.9
Benzoic acids							
Gallic	0.3–5.5	170572.6	−10366.7	0.9999	0.09	97	6.3
Protocatechuic	0.8–15.9	97787.7	−12904.1	0.9999	0.2	93	7.6
<i>p</i> -Hydroxybenzoic	0.6–6.4	189852.0	−6629.5	0.9999	0.07	92	5.0
Hydroxycinnamic acids							
Hydrocaffeic	10.7–214.0	54308.1	−59043.7	0.9999	2	94	2.6
Chlorogenic	3.9–39.0	158849.1	−40110.0	0.9996	0.6	104	3.7
Caffeic	4.2–21.0	325901.0	−75401.0	0.9999	0.5	92	1.0
Hydrocoumaric	2.6–51.5	38436.2	1763.9	0.9997	0.5	93	2.6
Hydroferulic	0.5–8.6	55459.3	−5985.6	0.9998	0.2	93	2.5
<i>p</i> -Coumaric	3.8–38.0	474518.0	−139986.0	0.9997	0.5	90	2.6
Ferulic	0.5–10.2	359849.5	−18505.1	0.9999	0.2	95	3.2
Procyanidin and flavanols							
Catechin	1.1–22.1	44382.2	−5025.5	0.9996	0.4	93	7.7
Epicatechin	1.6–32.1	45832.9	−15415.6	0.9994	0.8	99	7.4
Procyanidin B1	1.2–74.1	37013.6	−380.6	0.9997	0.3	94	3.8
Dihydrochalcone							
Phloridzin	3.8–61.7	141371.1	−37745.3	0.9999	0.6	94	3.2
Flavonol							
Quercitrin	0.6–9.9	101814.7	−4771.8	0.9994	0.2	93	3.7

LOD: Limit of detection.

<sup>a</sup> RSD of mean recovery.

### 3.2. Validation procedure

To check the linearity of the response of detector, a linear regression analysis of absolute areas versus concentration of the phenolic compounds was used. The linearity was determined by the square correlation coefficients of the calibration curves generated by three repeated injections of standard solutions at five concentrations levels, with concentrations included the range expected in real samples. The limit of detection was estimated from the residuals of calibration graph [32]. Calibration parameters are shown in Table 1. All the compounds showed a good linearity with regression coefficients >0.9990 the limit of detection (LOD) ranging from 0.07 mg/L for *p*-hydroxybenzoic to 2 mg/L for hydrocaffeic acid. These results suggest that the proposed HPLC method is sufficiently sensitive for the determination of phenolic compounds in apple derivatives, in accordance with previous reports [31,33,34].

Recovery experiments were performed in order to study the accuracy of the method. Known amounts of pure standards were added to a sample cider, at three different concentrations levels, in duplicate (Table 1). Recoveries ranged between 90 and 104% and these values testify the accuracy of the proposed method.

Precision was studied in a real sample in two ways: retention times and peak areas. The repeatability of peak areas and retention times were calculated by the RSD of five injections carried out on the same day. The RSD for the retention time of all peaks was <0.7% and the coefficient of variation for the peak areas was <2%. The reproducibility of the method was evaluated during recovery experiments and the RSD were always <8% (Table 1).

To improve the selectivity, different wavelengths were used for quantify the phenolic compounds: 313 nm for the hydroxycinnamic acids, 355 nm for quercetin glycosides and 280 for the rest of compounds. Additionally, the peak pu-

Table 2  
Contents of phenolic compounds in commercial ciders

Compound	Content <sup>a</sup> (mg/L)								
	<i>t<sub>R</sub></i> (min)	Cider 1	Cider 2	Cider 3	Cider 4	Cider 5	Cider 6	Cider 7	Cider 8
Gallic acid	12.56	nd	nd	1.2	0.4	0.1	0.6	nd	nd
Catechol	19.58	4.3	2.7	3.9	1.8	3.0	7.2	nd	3.2
Protocatechuic acid	21.62	0.5	0.6	1.5	1.3	1.8	nd	3.0	3.6
Tyrosol	29.22	15.0	31.6	28.8	35.1	41.5	22.0	23.1	30.5
Procyanidin B1	30.74	5.8	5.6	15.5	11.4	4.0	13.7	7.9	16.6
<i>p</i> -Hydroxybenzoic	31.40	1.2	1.1	0.8	1.6	0.9	1.6	1.4	1.3
Hydrocaffeic acid	32.29	68.4	74.7	93.5	97.3	94.9	110.5	55.8	110.5
Catechin	34.14	1.6	1.1	1.3	2.0	0.8	2.1	2.7	7.9
Unknown trimer <sup>b</sup>	36.12	5.0	2.9	6.8	6.3	2.4	7.1	3.6	5.1
Procyanidin B2 <sup>b</sup>	38.84	36.0	33.9	61.4	43.9	23.6	65.1	31.6	66.8
Chlorogenic acid	40.07	nd	nd	nd	nd	nd	nd	21.5	nd
Caffeic acid	41.48	nd	nd	nd	nd	nd	nd	10.3	12.3
Hydrocoumaric acid	42.18	42.6	19.5	48.7	44.3	23.3	32.9	15.9	8.8
Tetramer D <sup>b</sup>	43.00	5.5	4.3	11.4	6.9	2.0	10.9	4.7	nd
Trimer C1 <sup>b</sup>	43.82	12.4	12.0	14.6	11.7	12.3	17.2	12.7	nd
Epicatechin	44.49	nd	nd	6.6	nd	4.1	3.2	9.9	30.3
<i>p</i> -Coumaroylquinic <sup>c</sup>	45.31	13.6	14.6	20.9	15.8	10.6	22.0	12.5	21.3
Hydroferulic acid	47.82	4.0	nd	1.3	nd	3.9	1.7	2.9	2.9
<i>p</i> -Coumaric acid	52.43	nd	nd	nd	nd	0.8	nd	0.7	nd
Unknown procyanidin <sup>b</sup>	55.00	4.0	4.5	4.6	6.7	3.2	nd	9.4	nd
Ferulic acid	56.41	0.8	0.3	0.4	0.4	nd	nd	1.6	1.1
Unknown phloretin derivative (1) <sup>d</sup>	58.36	1.6	1.2	2.2	1.1	1.5	2.6	0.6	2.0
Procyanidin B5 <sup>b</sup>	59.01	3.1	5.7	7.0	2.6	10.4	9.5	6.1	2.6
Phloretin 2'-xyloglucoside <sup>d</sup>	62.59	7.4	2.0	9.3	25.4	4.6	5.8	36.5	28.5
Unknown phloretin derivative (2) <sup>d</sup>	64.10	nd	nd	nd	0.8	0.8	nd	1.8	1.4
Phloridzin	65.36	24.8	19.6	38.0	27.5	21.6	53.7	8.9	34.1
Hyperin <sup>e</sup>	66.52	2.0	1.3	3.8	1.6	0.5	6.8	nd	1.5
Rutin + isoquercitrin <sup>e</sup>	67.58	0.3	0.2	0.6	nd	nd	nd	0.6	nd
Unknown flavanol <sup>e</sup>	68.75	0.5	0.3	0.9	0.4	nd	1.8	nd	0.4
Avicularin <sup>e</sup>	70.50	1.0	0.5	1.1	0.6	0.1	2.4	0.2	0.9
Quercitrin	74.42	2.2	2.5	4.3	2.7	2.7	6.3	2.4	3.1
Unknown <i>p</i> -coumaric acid derivative <sup>c</sup>	76.26	0.6	0.6	1.0	0.7	1.0	1.0	0.7	0.1

nd: not detected; LOD: Limit of detection; *t<sub>R</sub>*: retention time.

<sup>a</sup> Mean values for three injections.

<sup>b</sup> Quantified as procyanidin B1.

<sup>c</sup> Quantified as *p*-coumaric acid.

<sup>d</sup> Quantified as phloridzin.

<sup>e</sup> Quantified as quercitrin.

rity was checked by means of the PDA Millennium software spectral contrast facilities.

### 3.3. Cider sample analysis

The optimized and validated method was applied to the analysis of eight different commercial ciders from Asturias (Spain). Table 2 lists the range of concentrations of the phenolic compounds determined.

The phenolic compounds found in ciders varied quantitatively with the nature of raw materials (apple varieties, cultivation conditions) and the cidermaking procedures [1,26,35–38]. In all the ciders analysed the major phenolic compound was hydrocaffeic acid with levels that represent a 30% of the total polyphenols assayed. This compound is probably generated by hydrolysis of caffeic acid derivatives by microorganisms during fermentation and post-fermentation steps [25,40]. In contrast, caffeic acid was detected in two ciders only, while chlorogenic acid was found in one of the samples. The procyanidin B2 was always the major compound among the flavonoids, with values that varied from 23.6 mg/L (24%) to 66.8 mg/L (33%). Phloridzin and phloretin-2'-xyloglucoside were found in all samples analysed, confirming the usefulness of these compounds as chemical markers for apple derivatives [5]. Likewise, flavonols should be considered as a minor class, because they represent less than 5% of total phenolic compounds. An unknown flavonol ( $t_R = 68.75$  min) detected could correspond to reynoutrin [41,42] or a quercetin pentoside reported by Shieber et al. [42] and Lommen et al. [43]. Tyrosol, which is produced during fermentation by deamination of the amino acid tyrosine, was present in all samples analysed.

Spanish natural cider presents differences [26,38,44] when compared to French or English ones, which are reflected in the sensorial properties and chemical compositions of the product. These differences are attributable to multitude of variables such as apple variety used, cultivation conditions, climatology, processed and fermentation technology used and maturation and aging. Spanish natural cider is dry and acidic with a balanced aroma described by fresh, while French ciders are characterised by sweeter, tannic and apple-like flavours, whereas English ciders are carbonated products, with varying degrees of sweetness and an alcoholic proof, being and the flavour generally smoother compared with that described 30 years ago [25,26,45,46].

## 4. Conclusion

A method for accurately quantifying the phenolic compounds in cider using a direct injection has been successfully developed. The analytical method described is suitable for routine analysis of phenolic acids and flavonoids in a single chromatogram avoiding the extra time required in purification steps and the possible generation of artefacts or degradation of sample during the extraction step.

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