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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Phenolic Profile of Asturian (Spain) Natural Cider

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The polyphenolic composition of natural ciders from the Asturian community (Spain), during 2 consecutive years, was analyzed by RP-HPLC and the photodiode-array detection system, without previous extraction (direct injection). A total of 16 phenolic compounds (catechol, tyrosol, protocatechuic acid, hydrocaffeic acid, chlorogenic acid, hydrocoumaric acid, ferulic acid, (−)-epicatechin, (+)-catechin, procyanidins B2 and B5, phloretin-2′-xyloglucoside, phloridzin, hyperin, avicularin, and quercitrin) were identified and quantified. A fourth quercetin derivative, one dihydrochalcone-related compound, two unknown procyanidins, three hydroxycinnamic derivatives, and two unknown compounds were also found. Among the low-molecular-mass polyphenols analyzed, hydrocaffeic acid was the most abundant compound, representing more than 80% of the total polyphenolic acids. Procyanidins were the most important family among the flavonoid compounds. Discriminant analysis was allowed to correctly classify more than 93% of the ciders, according to the harvest year; the most discriminant variables were an unknown procyanidin and quercitrin.

KEYWORDS: Polyphenols; cider; HPLC; multivariate techniques

INTRODUCTION

Phenolic compounds are important constituents of apple derivatives because they greatly contribute to their sensory properties and other attributes. In particular, polyphenolic compounds have antioxidant activity, free-radical scavenging capacity, coronary heart disease prevention, and anticarcinogenic properties (*1*−*4*). Furthermore, phenolics are associated with bitterness, astringency, and color stability, and some of them have been used for detecting adulterations in apple products and could be inhibitors for microbiological growth-avoiding process spoilages (*5*−*8*).

From the quantitative point of view, there are five major groups of polyphenols found in apple derivatives: flavan-3-ols, procyanidins, flavonols, dihydrochalcones, and hydroxycinnamic acids and derivatives (*9*−*13*).

Asturias, a region located on the Atlantic coast of northern Spain, is the leading natural cider producer of the country with an average production of close to 40 million liters (*14*). Traditional natural cider is usually made from an acidic mixture of cider apple varieties, by milling and pressing. The must is racked to the tanks, where spontaneous fermentation (alcoholic and malolactic) takes place. The cider is matured during several months in the tanks and bottled without a stabilization process.

It is well-known that several factors such as fruit varieties, soil, climate, cultural conditions, and cider-making processes strongly influence the phenolic profiles of apples and ciders (*15*−*19*).

The analysis of these compounds is usually carried out by reverse-phase high-performance liquid chromatography (RP-HPLC) and diode array detection (DAD). Because of the complexity of the analysis, this is often carried out by previous extraction (*20*−*23*) and in some cases followed by thiolysis to quantify phenolic compounds according to their classes (*24*). However, the analysis of major polyphenols of low molecular mass in fermented beverages can be done by direct injection (*25*, *26*), avoiding an important error source in chemical analysis such as the extraction step with solvents and later concentration.

The aim of this work is to contribute to the characterization of Asturian natural ciders according to their phenolic profile. To do so, 92 commercial ciders from two consecutive harvests were analyzed using HPLC and DAD detection by direct injection.

MATERIALS AND METHODS

Samples. A total of 92 Asturian natural ciders available in the market, belonging to 51 different cidermakers and two harvests (years 1999 and 2000), were analyzed. Prior to HPLC analysis, the cider samples were degassed in an ultrasonic bath during 10 min to remove all carbon dioxide and filtered through a 0.45 μm cellulose acetate membrane filter from Teknokroma (Barcelona, Spain).

Reagents and Standards. Polyphenol standards were supplied as follows: (+)-catechin, (−)-epicatechin, phloridzin, tyrosol, catechol, 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), and quercetin glycosides (hyperin, avicularin, and quercitrin) by Extrasyntèse (Genay, France). Procyanidins B2 and B5 and phloretin-2′-

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xyloglucoside were kindly furnished by Dr. A. Lea (Reading, U.K.). Water was purified with a Milli-Q system from Millipore (Bedford, MA). Reagents and solvents were purchased from Panreac (Barcelona, Spain) and were of analytical or HPLC grade.

Analytical Procedures. HPLC analysis was performed according to the method validated by our research group (*26*). A Waters system, equipped with a 717 automatic injector, a column oven, two pumps (model 510), a diode array detector (model 996), and Millennium software version 2.1 data module, was used. Separation of polyphenols was carried out on a reversed-phase Nucleosil 120 C_{18} (250 × 4.6 mm I.D., 3 μm) 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. The injection volume was 50 μL. All analyses were carried out in duplicate.

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 the phenolic compounds (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 the external standard method. For the compounds lacking standards or those in which the amount at our disposal was too small, the quantification was achieved from similar compounds; thus, procyanidins were quantified as (−)-epicatechin, phloretin-2′-xyloglucoside and the unknown dihydrochalcone as phloridzin, flavonol glycosides as quercitrin, *p*-coumaric acid derivatives (λ_{max} = 313.0 nm) as *p*-coumaric acid, and hydroxycinnamic acid derivatives (λ_{max} = 326.3 nm, and shoulder at 297.7 nm) as chlorogenic acid. The levels of peak unknowns 1 and 2 were estimated as area × 10^{-4}.

Statistical Analysis. Analysis of variance (ANOVA) was carried out for detecting significant differences in the analyte concentrations depending upon the harvest year, and linear discriminant analysis (LDA) and step LDA were used for classification and characterization purposes. The program used was SPSS (*27*).

RESULTS AND DISCUSSION

Chromatographic Analysis. A typical chromatogram from Asturian natural cider is shown in **Figure 1**. In this cider, a total of 14 phenolic compounds (catechol, protocatechuic acid, tyrosol, hydrocaffeic acid, (+)-catechin, procyanidins B2 and B5, hydrocoumaric acid, (−)-epicatechin, phloretin-2′-xylogluside, phloridzin, hyperin, avicularin, and quercitrin) were identified by comparison of their retention times and UV−vis spectra with those of the standards. Furthermore, other unknown chromatographic peaks were assigned to a polyphenol family according to their spectral features and the review of the literature data (*8*, *20*). In this sense, peak 8 shows a hydroxycinammic-acid-type spectrum (λ_{max} = 326.3 nm, and shoulder at 297.7 nm); peaks 13 and 22 exhibited spectral characteristics similar to *p*-coumaric acid (λ_{max} = 313.0 nm); peaks 10 and 15 showed a procyanidin spectrum; and peak 17 showed a phloridzin-like spectrum. Probably, the peak 13 is *p*-coumaroylquinic, the major *p*-coumaric acid derivative quantified in cider (*28*). Peaks 1 and 2 were not assigned to any group of phenolic compounds because their UV−vis spectra did not correspond to any of the available standards. Their spectra are displayed in **Figure 2**.

The range of concentrations of phenolic compounds determined from the analysis of Asturian natural ciders elaborated in years 1999 (consumed in 2000) and 2000 (consumed in 2001) are presented in **Table 1**, together with the results of ANOVA ($p < 0.05$).

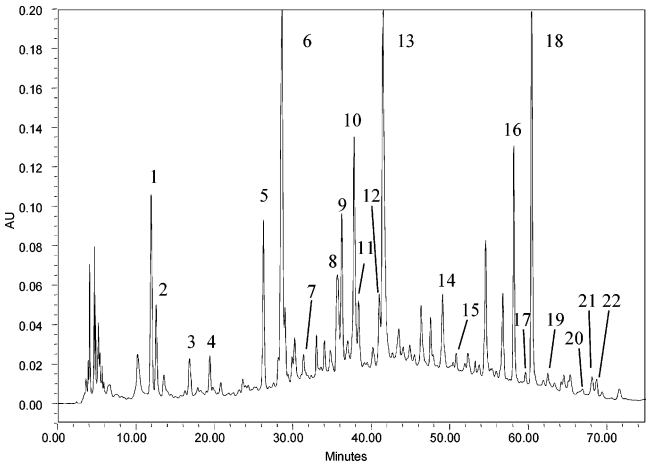

Figure 1. HPLC chromatogram at 280 nm of phenolic compounds in Asturian natural cider. Peaks: (1) unknown 1 (λ_{max} = 284.6 nm), (2) unknown 2 (λ_{max} = 292.9 nm), (3) catechol, (4) protocatechuic acid, (5) tyrosol, (6) hydrocaffeic acid, (7) (+)-catechin, (8) unknown hydroxycinnamic acid derivative (HCA-1, λ_{max} = 326.3 nm, and shoulder at 297.7 nm), (9) procyanidin B2, (10) unknown procyanidin PC-1, (11) hydrocoumaric acid, (12) (−)-epicatechin, (13) *p*-coumaroylquinnic acid, (14) procyanidin B5, (15) unknown procyanidin PC-2, (16) phloretin-2′-xyloglucoside, (17) unknown chalcone, (18) phloridzin, (19) hyperin, (20) avicularin, (21) quercitrin, (22) unknown hydroxycinnamic acid derivative (HCA-2, λ_{max} = 313.0 nm).

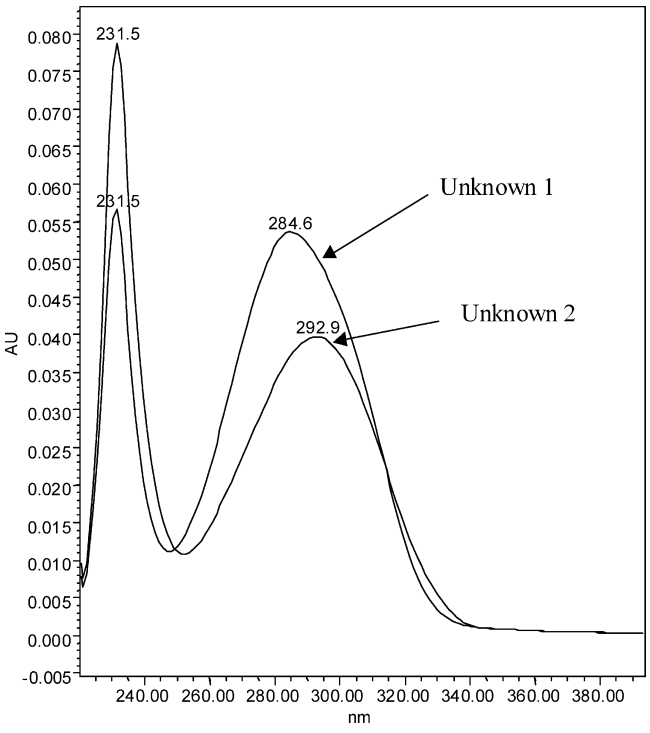

Figure 2. UV−vis spectra of peaks 1 and 2 in **Figure 1**.

In our case, phenolic acids were the most important family of nonvolatile phenols analyzed, with levels representing around 60% (**Table 1**). In both years, phenylpropionic acids (hydrocaffeic acid and hydrocoumaric acid), representing more than 80% of the total polyphenolic acids, were more abundant in accordance with previous works reported by our group (*22*, *26*). This could be explained on the basis of the metabolism of hydroxycinnamic acids by microorganisms during fermentation and postfermentation steps (*29*). In this sense, chlorogenic acid and *p*-coumaroylquinic acid are hydrolyzed into caffeic acid/

Table 1. Phenolic Composition of Asturian Natural Ciders (mg/L)

	harvest 1999 ($n = 50$)				harvest 2000 ($n = 42$)			
compound	mean	max	min	SD	mean	max	min	SD
volatile phenols								
catechol[a]	3.16	9.06	1.22	1.46	4.58	30.17	1.51	4.70
tyrosol[a]	21.66	37.93	10.09	5.42	24.61	38.16	11.28	5.11
benzoic acids								
protocatechuic acid	0.80	2.43	nd[b]	0.53	0.54	10.23	nd[b]	1.64
hydroxycinnamic acids and derivatives								
hydrocaffeic acid	90.10	147.19	26.05	21.91	84.09	128.90	41.13	18.34
chlorogenic acid[a]	0.12	6.25	nd[b]	0.88	1.85	32.24	nd[b]	6.00
hydrocoumaric acid	10.34	19.83	5.72	2.85	9.83	52.35	nd[b]	7.37
p-coumaroylquinic acid	12.80	21.78	4.81	3.72	12.39	35.40	5.66	5.30
ferulic acid	0.55	1.06	0.17	0.17	0.56	0.96	nd[b]	0.18
HCA-1[c]	8.99	14.02	5.81	1.81	8.38	17.70	5.14	2.51
HCA-2[a,d]	0.85	1.12	0.44	0.15	0.74	1.31	0.14	0.24
procyanidins and flavan-3-ols								
catechin[a]	0.11	2.41	nd[b]	0.46	0.45	3.03	nd[b]	0.87
epicatechin	2.81	20.34	nd[b]	3.45	3.29	19.67	nd[b]	4.56
B2	12.73	81.92	nd[b]	17.19	12.62	40.90	2.22	8.33
B5	18.01	65.41	nd[b]	13.16	17.21	38.67	nd[b]	9.89
PC-1[e]	20.61	66.11	nd[b]	10.74	25.15	58.42	8.57	12.02
PC-2[a,e]	1.63	4.61	nd[b]	0.93	2.46	4.01	0.64	0.68
dihydrochalcones								
phloretin-2′-xyloglucoside	5.21	36.99	1.42	6.79	5.97	32.98	1.20	7.58
unknown dihydrochalcone	0.22	2.31	nd[b]	0.48	0.29	2.12	nd[b]	0.56
phoridzin[a]	20.29	53.64	2.94	12.35	14.85	33.26	0.65	10.21
flavonols								
hyperin	1.50	4.94	nd[b]	1.26	1.44	4.91	nd[b]	1.20
unknown flavonol[a]	0.49	1.52	nd[b]	0.35	0.29	1.32	nd[b]	0.43
avicularin	0.65	2.17	nd[b]	0.46	0.66	1.94	nd[b]	0.47
quercitrin[a]	2.36	5.65	nd[b]	1.30	1.76	3.76	nd[b]	1.02
others								
unknown 1[a,f,g]	168.90	269.91	90.31	41.80	201.43	409.22	96.86	61.96
unknown 2[f,h]	35.55	69.83	1.67	13.53	39.27	70.42	15.46	10.19

[a] Significant differences between harvests ($p < 0.05$). [b] nd = not detected. [c] HCA 1 = unknown hydroxycinnamic acid derivative ($\lambda_{max} = 326.3$ nm, and shoulder at 297.7 nm). [d] HCA 2 = unknown hydroxycinnamic acid derivative ($\lambda_{max} = 313.0$ nm). [e] PC-1 and PC-2 = unknown procyanidins. [f] Expressed in area $\times 10^{-4}$. [g] Unknown 1 ($\lambda_{max} = 284.6$ nm). [h] Unknown 2 ($\lambda_{max} = 292.9$ nm).

quinic acid and *p*-coumaric acid/quinic acid, respectively, and later, the hydroxicinnamic acids (caffeic acid and *p*-coumaric acid) are reduced into hydrocaffeic and hydrocoumaric acids. Two facts support this point: the slight presence of chlorogenic acid in ciders (detected only in six samples) and the absence of caffeic and *p*-coumaric acids, although the hydrolysis of *p*-coumaroylquinic acid is not complete, according to the levels found in ciders, around 12 mg/L. Furthermore, another hydroxycinnamic acid, HCA-1 (**Table 1**), was found in moderate levels, with mean concentrations around 8 mg/L in all of the ciders. The other phenolic acids (HCA-2 and ferulic acid) were found in very low levels, and their contribution to total polyphenolic acids was never higher than 2% (**Table 1**).

On the other side, the levels of flavan-3-ols showed wide ranges as revealed by their standard deviations, and they were generally low. Procyanidins PC-1, B5, and B2 were the most abundant among the flavonoid compounds analyzed, and they were detected in 99, 96, and 78% of the ciders studied, respectively. Likewise, a minor procyanidin (PC-2) was found in 95% of the Asturian natural ciders.

Phloridzin and phloretin-2′-xyloglucoside were the major dihydrochalcones quantified in this work, with mean values that varied from 7 to 10% for phloridzin and from 2 to 3% for phloretin-2′-xyloglucoside of the total of polyphenols analyzed. The quantity of phloretin-2′-xyloglucoside was higher than phloridzin in only 4 of the 92 ciders assayed. These dihydrochalcones are characteristic of apple products (*30*), and they are useful for food authenticity studies.

Flavonols are a minor source of polyphenols found in apple derivatives. Four quercetin glycosides were detected (quercitrin, hyperin, avicularin, and an unknown flavonol). Only two flavonols, quercitrin and hyperin, accounted for at least 78% of total flavonols in all of the ciders analyzed. In general, quercitrin, present in 97% of the samples, was the major flavonol; however, it is worth noting that hyperin was the major one in 22% of the ciders analyzed. On the other hand, the unknown flavonol could correspond to reynoutrin (*31*).

The chromatographic method also allowed us to determinate two volatile phenolics: catechol and tyrosol. Tyrosol, formed during fermentation from tyrosine, was the major volatile phenol detected, with mean values of 21.54 and 24.05 mg/L; this fact is in accordance with other fermented beverages such as beer or wine (*32*). However, the presence of catechol could be explained by the metabolism of lactic bacteria. In this sense, some cider lactic bacteria (*Lactobacillus plantarum*) have shown the ability to metabolize quinic and shikimic acids under anaerobic conditions to give catechol as an end product (*33*).

Moreover, the other two major compounds were detected (unknowns 1 and 2). Although they could not be identified, both analytes must be taken into account because they were present in all of the analyzed ciders. On the other hand, it must be pointed out that these compounds are formed during the fermentative process.

The polyphenolic profiles of Asturian natural cider present differences when they are compared to data reported in ciders from other places (*5*, *19*, *28*). This fact is not surprising because polyphenol concentrations show a great variability according to raw materials: apple varieties, fruit maturities, cultivation conditions (*5*, *10*, *11*, *13*, *17*, *31*, *34*), and the cidermaking procedures (*6*, *18*, *19*, *29*). Furthermore, differences in the

concentrations of polyphenols could be due to distinct methodologies used for the extraction and quantitative determination of phenolics. In this study, the direct injection avoids the purification step and the possible generation of artifacts or the degradation of the sample during analytical determination.

In this way, it must be pointed out that the cidermaking process in Asturias is very similar to that used in the Basque Country (North of Spain). Natural cider is produced by milling, followed by traditional pressing (3 days) or pneumatic pressing (6 h), spontaneous clarification, natural fermentation to dryness, and long maturation times (4−10 months); moreover, the addition of sugars and CO_2 in this kind of cider is prohibited by the Spanish legislation (*35*). Spanish natural cider is dry and acidic, with volatile acidity < 2 g of acetic acid/L and a balanced aroma described by freshness.

Indeed, the profiles of phenolic acids (chlorogenic and *p*-coumaroylquinic acids) and flavanols [(+)-catechin, (−)-epicatechin] are in agreement to these observed in Basque ciders. Likewise, the not identified peaks (unknowns 1 and 2) could correspond with compounds reported by Alonso-Salces et al. (*19*) present in ciders by the action of polyphenoloxidase (PPO), which catalyses the oxidation of phenolic compounds containing two *o*-dihydroxy groups to the corresponding *o*-quinone (*36*). In comparison to the Basque ciders, Asturian ones analyzed in this work showed higher levels of flavonols and no presence of the aglycone form of flavonols (quercetin) and dyhidrochalcones (phloretin). As commented above, the analytical method employed by these authors could explain the presence of aglycone forms only, derived from the degradation of their respective glycosides (*10*, *18*, *24*). Likewise, it is very surprising that the phenylpropionic acids (hydrocaffeic and hydrocoumaric acids) are absent in Basque ciders. At present, in an attempt to determine whether compositional variations in phenolics were related to the analytical method, ciders from different countries are being studied by direct injection. The partial results indicated that the main phenolic acid found in Basque cider is hydrocaffeic acid (data not shown).

Chemometric Analysis. *ANOVA.* For determining the presence of significant differences for the analytes found in ciders, on the basis of the year of harvest, an analysis of variance was carried out.

In general, significant differences were detected for at least one compound of each group of phenolics, although in some cases, this fact should not be taken into account. In this sense, the significant differences detected for chlorogenic acid and (+)-catechin, detected only in <15% of samples analyzed, are not outstanding.

On the other hand, the ANOVA showed significant differences for phloridzin, which could reflect the employment of non-Asturian cider apples, if we take into account that ciders harvested in 2000 were made from blends of apples from other places (*37*). In relation with this fact, it is important to observe that cultural practices for cider apple orchards influence the levels of this dihydrochalcone in cider apples; when nitrogenous fertilization is moderate or poor, as in the Asturian traditional growing of apple trees, higher accumulation of phloridzin in fruits can be expected (*17*), as it was observed for samples harvested in 1999.

Three phenolics formed during the fermentation process, catechol, tyrosol, and the unknown compound 1 (unknown 1, λ_{max} = 284.6 nm), showed significant differences because of the harvest year. However, catechol, present in all of the samples analyzed, exhibits anomalous high contents in two ciders (30.2 and 17.5 mg/L) in the year 2000, as evidenced by its standard deviation (**Table 1**). Thus, the ANOVA result should be taken with care.

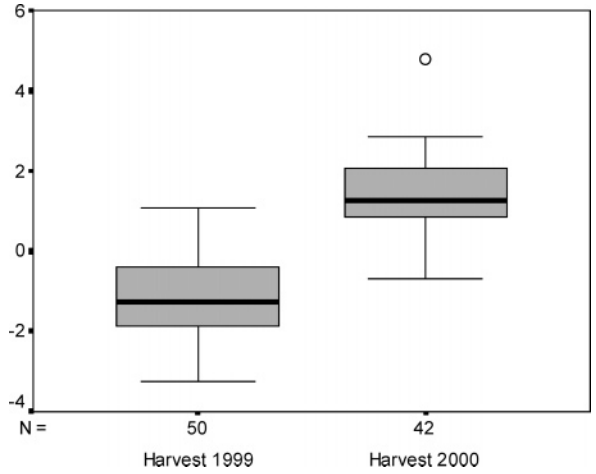

Figure 3. Box plot for discriminant scores of samples categorizes according to the year of the harvest.

Multivariate Analysis. With the aim of classifying the ciders according to the year of harvest, an univariate analysis using the Fisher's test was carried out in the first place. The most discriminant variable was the procyanidin PC-2 (Fisher's weight = 0.539 79, p < 1%). However, the use of this variable only did not allow the correct classification of ciders; therefore, multivariate treatment of the data was needed.

Previously, to detect the most correlated variables, an analysis of correlation was carried out using a confidence level of 99%. Only two variables (unknown dihydrochalcone and phloretin-2′-xyloglucoside) showed a correlation higher than 0.9; therefore, the unknown dihydrochalcone was removed. Thus, the new data matrix dimension was 92 objects (ciders) × 24 variables (phenolics).

Linear discriminant analysis (LDA) was carried out to establish a decision rule that could allow us to classify ciders according to the year of harvest. With the 24 selected variables, the classification capacity was 90.2% for both groups, with a similar capacity for each one (92.0% for 1999 samples and 88.1% for 2000 samples). Nevertheless, when the discriminant scores of samples were displayed in a box-whisker plot (see **Figure 3**), it was detected that one object of the 2000 harvest fell outside of the box. This outlier, characterized for its high content in chlorogenic acid (usually not present in the analyzed ciders), was removed from the database, and the model was recalculated. The new model improved the number of samples correctly classified in the 2000 harvest (95.1%) and maintained the hits obtained in 1999 (92.0%), resulting in 93.4% of overall hits. Furthermore, the prediction capacity, estimated by cross-validation using the U method (each case in the analysis is classified by the functions derived from all cases other than that case), was 83.5%.

To establish which phenolic compounds were more valuable to differentiate the samples according to the harvest, an stepwise discriminant analysis was employed. Values of 3.84 and 2.71 were used for F statistics to enter and remove variables, respectively. From the 24 phenolics previously selected, 2 were picked: the unknown procyannidin PC-2 and quercitrin. When these variables were used to classify the ciders, 72.5% of the hits was obtained.

In summary, the polyphenolic profile of Asturian natural ciders had shown similar levels in two consecutive harvests. In

this way, the presence of high concentrations of phenylpropionic derivatives could be considered as characteristic for natural ciders on the basis of technological criteria. Moreover, the use of multivariate technique analyses enables the ciders to be differentiated on the basis of the year of harvest used in the cider making.

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