Detection of Apple Juice Concentrate in the Manufacture of Natural and Sparkling Cider by Means of HPLC Chemometric Sugar Analyses

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An HPLC method for sugar analyses in cider was used in order to detect the presence of apple juice concentrate. Sugars, previously derivatized with *p*-aminobenzoic ethyl ester, were analyzed by reversed-phase liquid chromatography using a C_8 column and a mobile phase of citrate buffer pH 5.5/tetrahydrofuran/acetonitrile, operated in gradient mode. The use of this analytical procedure together with chemometric techniques, such as principal component analysis and Bayesean analysis, allowed the authors to establish the minimum concentration of apple juice concentrate obtained by liquefaction or press technology that can be detected in natural cider.

KEYWORDS: Cider; monosaccharides; HPLC; chemometrics

INTRODUCTION

Natural and sparkling cider are two popular beverages in northern Spain. Natural cider is sold, basically, inside the producer regions, while sparkling cider is sold outside them. Natural cider is elaborated from a mixture of cider apple varieties belonging to different technological groups, namely, sharp, mild sharp, sweet, bittersweet, and bitter, by milling and slow pressing, resulting in an apple juice with mild sharp characteristics. Apple juice is spontaneously fermented by using microflora, yeast, and bacteria (alcoholic and malolactic fermentations). Sparkling cider is elaborated from practices with a high technological level. This cider can be made from rediluted apple juice concentrate and then carbonated.

Presently, Spanish regulation of natural cider production does not allow the use of apple juice concentrate. Therefore, it is very important to optimize analytical methods that can detect this fraudulent practice. The analysis of minor sugars such as xylose, arabinose, ribose, fucose, rhamnose, glucuronic acid, and galacturonic acid can be an interesting analytical strategy. As has been described by Knee (*1*), parenchymatous cells of ripening apples contain a rhamnogalacturonan consisting of chains of α-(1−4)-linked galacturonic acid units, interrupted by rhamnose molecules. This heteropolysaccharide contains a branched-chain arabinan attached to the rhamnose residues. Liquefaction is an enzymatic digestion process conducted by hydrolases, such as pectolytic enzymes, hemicellulases, and cellulases. This process is commonly used to make apple juice concentrate. The enzyme action on apple cell wall and apple middle lamella promotes the liberation of monosaccharide units such as galacturonic acid, rhamnose, galactose, xylose, and arabinose (*2*, *3*). As a consequence, we can expect a higher concentration of monosaccharides in the ciders elaborated from apple juice concentrate obtained by liquefaction than ciders made from fresh apple juice obtained by slow pressing. In the same way, but in minor extension, cider elaborated by using apple juice concentrate extracted by fast pressing and stabilized through a clarification process can present a different sugar profile when it is compared to natural cider.

Pattern recognition analysis is usually employed for food authentication purposes (*4*, *5*). Several multivariate statistical techniques can be used: principal component analysis (PCA) to visualize the data structure, and Bayesean and SIMCA analysis to model and classify the objects. Modeling methods are used to typify the boundaries for each class or category of samples (*6−10*).

The objective of this study was to detect the addition of apple juice concentrate in the manufacture of Spanish cider by analyzing the HPLC sugars profile of ciders with chemometric techniques.

MATERIALS AND METHODS

Chemicals. Aldose (xylose, arabinose, ribose, fucose, and rhamnose) and uronic acid (glucuronic and galacturonic acids) standards, *p*-aminobenzoic ethyl ester (ABEE), sodium cyanoborohydride, sodium citrate, and citric acid monohydrate were obtained from Sigma Chemical Co. (St. Louis, MO), acetic acid was obtained from Probus (Badalona, Spain), and HPLC-grade acetonitrile, methanol, chloroform, and tetrahydrofuran (THF) were purchased from Romil (Barcelona, Spain). Milli-Q water (Millipore, Milford, MA) was used throughout. All other chemicals and solvents were of analytical reagent or HPLC grade.

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Samples. Cider samples (total of 84)—natural cider (59) and sparkling cider (25)—were collected from cider-maker cellars. Natural ciders were made from cider apple varieties by milling and slow pressing. Sparkling ciders were made from fermentation of apple juice single strength obtained by appropriate redilution of two apple juice concentrate types: the apple juice concentrate made from apple juice extracted by pressing (press sparkling cider), and the apple juice concentrate obtained from apple juice extracted by liquefaction (liquefaction sparkling cider). The composition of 41 ciders belonging to the natural ciders group was modified by addition of different amounts of press and liquefaction apple juice concentrate (0.5, 1, 5, 10, 20, and 50%). Samples were frozen (−20 °C) or refrigerated (2−4 °C) prior to instrumental analyses.

HPLC Analysis. Sugars were analyzed by HPLC using the methodologies previously described by the authors (*11*). A Shimadzu HPLC system equipped with two LC-10AD pumps, a UV−vis SPD-M10AD photodiode array detector, a Sil-10AD automatic injector, and a Gastor 150 LCD on-line degasser were used. A Kromasil C8 column (Teknokroma, 200 × 2.1 mm i.d., 3.5 μm) was used at 45 °C. Other conditions were are follow: mobile phase, solvent A (100 mM sodium citrate buffer pH 5.5/tetrahydrofuran, 88/12) and solvent B (acetonitrile); gradient program, 1−20 min at 1% B, 20−28 min at 20% B, 28−36 min at 1% B; flow rate, 0.15 mL/min. The analysis was monitored at 307 nm, and the absorption spectra of the compounds were recorded between 250 and 350 nm. The sample injection volume was 5 μL. Identification of compounds was carried out by comparing their retention time values and UV spectra with those of standards stored in a data bank. Quantitative determination was performed using the external standard method. All mobile-phase solutions were filtered through a 0.45-μm membrane filter.

Derivatization Procedure of the Monosaccharides. The procedure employed for the derivatization of sugars at their reducing end with ABEE was carried out according to the method of Wang et al. (*12*), modified by us (*11*). To 2.5 mL of cider were added 400 μL of 1.4 M NaBH$_3$CN in distilled water and 400 μL of glacial acetic acid, 2 mL of 0.6 M ABEE in methanol were added to this solution, and the mixture was heated at 80 °C for 10 min. After the mixture cooled to ambient temperature, the aqueous phase was extracted with 4 mL of chloroform to remove excess ABEE, and the aqueous layer was subjected to HPLC analysis.

Statistical Analysis. Multivariate analysis was performed with the PARVUS statistical package (*13*). A data matrix was structured with 84 rows representing ciders and 7 columns corresponding to the chemical variables (glucuronic acid, galacturonic acid, xylose, arabinose, ribose, fucose, and rhamnose). Forty-three samples were included in the training set; this set was used for constructing the multivariate models. The remainder 41 ciders with the composition modified by apple juice concentrate addition were included in the test set; this set was used to establish the detection limit of the quantity of the apple juice concentrate added to the natural cider samples. Ciders included in the training set were initially categorized as natural cider (N, 18 samples), liquefaction sparkling cider (L, 16 samples), and press sparkling cider (P, 9 samples). The chemical variables were autoscaled before statistical treatment. PCA was employed to visualize the data structure, and Bayesean analysis was used for classification.

RESULTS AND DISCUSSION

Table 1 lists the mean, minimum, and maximum values together with range and standard deviation for each chemical variable studied.

Factor Analysis of the Internal Structure. Two significant factors, validated according to full double-cross-validation (five groups for cancellation), which accounted for 76.7% of the variance, were chosen. **Figure 1** displays the projections of original variables and ciders belonging to the training set on the factorial plane formed by the two significant factors. As can be seen, the first principal component allows to one visualize three groups of samples, natural ciders (N), press sparkling ciders (P), and liquefaction sparkling ciders (L). Galacturonic

**Table 1.** Descriptive Statistics of Variables for Each Cider Category[a]

		variables		
category	mean (mg/L)	SD	min (mg/L)	max (mg/L)
		D-Glucuronic Acid		
NC	11.8	11.4	0.08	33.7
PSC	195.6	152.4	8.9	447.8
LSC	215.8	153.7	19.4	465.4
		D-Galacturonic Acid		
NC	132.1	104.0	21.8	302.2
PSC	615.9	389.0	126.0	1108
LSC	2850	328.8	2346	3379
		Xylose		
NC	17.7	27.8	0.13	92.2
PSC	125.2	48.4	89.4	212.0
LSC	179.1	119.4	60.0	459.0
		Arabinose		
NC	78.1	122.0	0.16	362.4
PSC	201.9	222.0	0.16	482.0
LSC	825.0	569.2	109	1779
		Ribose		
NC	7.9	9.9	0.18	28.2
PSC	6.7	24.8	1.51	15.0
LSC	12.1	16.1	0.18	44.9
		Fucose		
NC	16.8	10.8	5.7	35.1
PSC	14.3	8.6	4.9	27.9
LSC	51.5	6.4	39.5	63.3
		Rhamnose		
NC	25.5	50.4	14.0	34.0
PSC	29.5	22.1	8.7	68.8
LSC	87.0	11.4	73.8	118.7

[a] $n = 84$. SD, standard deviation; min, minimum values; max, maximum values; NC, natural ciders; PSC, press sparkling ciders; LSC, liquefaction sparkling ciders.
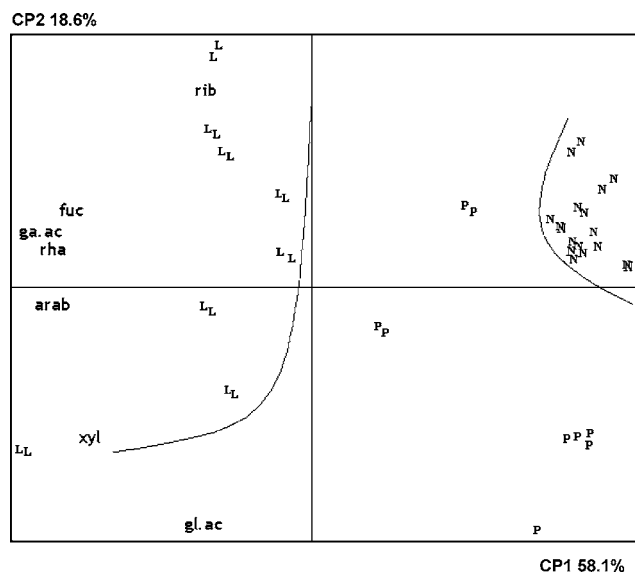
Figure 1. Projection of the variables and ciders onto the plane formed by the two first principal components: N, natural ciders; L, liquefaction sparkling ciders; P, press sparkling ciders; gl.ac, D-glucuronic acid; ga.ac, D-galacturonic acid; xyl, xylose; arab, arabinose; rib, ribose; fuc, fucose; rha, rhamnose.

acid, arabinose, fucose, and rhamnose were the sugars most correlated to the first factor. At the same time, glucuronic acid was the variable most correlated to the second factor. These results agree with the structure model of cortical parenchyma cell walls in apple fruit proposed by Knee (*1*). In fact, when apple fruit is digested by a liquefaction process, the liberation of certain monosaccharides such as galacturonic acid, rhamnose,

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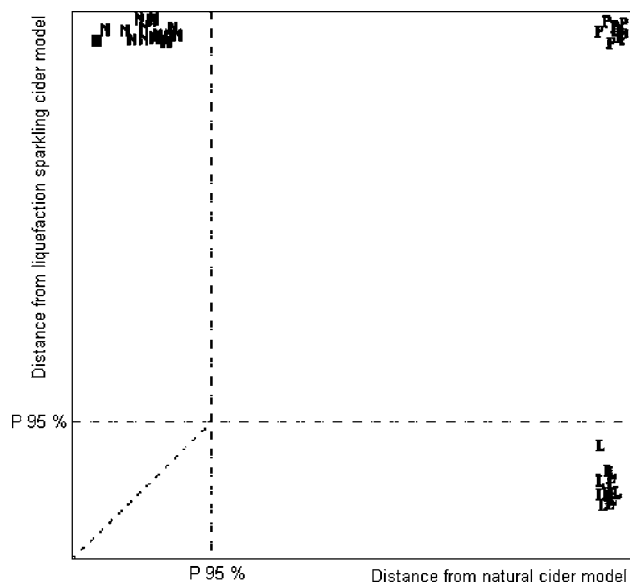
Figure 2. Coomans' diagrams obtained from Bayesean analysis: N, natural ciders; L, liquefaction sparkling ciders; P, press sparkling ciders.

and arabinose is expected, which is in accordance with the correlation observed in the factorial plane between these variables and ciders made from liquefaction apple juice concentrate (L category).

Stepwise Linear Discriminant Analysis. To ascertain the most relevant variables for classification purposes, a stepwise LDA technique was employed using the criterion of minimization of Wilks' lambda. We thus employed an *F* test, taking into account an *F*-to-enter of 3.84 and an *F*-to-remove of 2.71 (confidence level above 90%). The most relevant variables were galacturonic and glucuronic acids, the estimated value of Wilks' lambda being 3.1×10^{-2}. These results agree with the PCA analysis, since these acids were the variables most correlated to the two first factors.

Bayesean Analysis. The use of modeling techniques allows one to compute classification rules in order to classify samples of unknown kind. The Bayesean technique constructs for each category a hyperellipsoid characterized by the points of space placed to a critical Mahalanobis distance, where a 95% probability of the multivariate normal distribution is accumulated. The construction of robust models from Bayesean analysis requires, for each category, a number of observations that should be, at minimum, twice the number of variables. In our case, it is necessary to carry out a reduction of original variables. Using a correlation analysis, we have rejected arabinose and rhamnose from the data matrix; arabinose was highly correlated to xylose ($r = 0.8141$), and rhamnose was highly correlated to galacturonic acid ($r = 0.9341$). From the new data matrix (43 observations × 5 variables), Bayesean models (,atural, N; liquefaction, L; and press, P) were computed for each category. All models presented high sensitivity (100%) and were highly specifics (100%); classification hits were 100% for all categories. These results can be seen in **Figure 2**, where Coomans' diagrams are displayed.

At the same time, ciders included in the test set were classified according to the mathematical rules computed by the Bayesean models. The natural cider model accepted all natural ciders whose composition was modified up to 5% with press apple juice concentrate, while natural ciders with percentages added of press apple juice concentrate higher than 10% were rejected. Natural ciders with percentages of liquefaction apple juice concentrate added smaller than 1% were accepted by the natural

cider model, while all natural ciders with percentages of liquefaction apple juice concentrate greater than 5% were rejected.

Conclusions. The PCA method provided an adequate data structurization using only two dimensions: two predictive components were chosen that accounted for 76.7% of the variance. The Bayesean analysis allows one to classify ciders according to the origin of the apple juice used in their manufacture (fresh, liquefaction concentrate, and press concentrate). Likewise, it allows one to detect the addition of apple juice concentrate to the natural cider in amounts higher than 5% for liquefaction concentrate and higher than 10% for press concentrate.

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