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Use of the ^{1}H Nuclear Magnetic Resonance Spectra Signals from Polyphenols and Acids for Chemometric Characterization of Cider Apple Juices

GLORIA DEL CAMPO,*,† J. IGNACIO SANTOS,† NURIA ITURRIZA,† IÑAKI BERREGI,† AND ARANTXA MUNDUATE‡

Applied Chemistry and Materials Physics Departments, Faculty of Chemistry, University of the Basque Country, P.O. Box 1072, E-20018 San Sebastián, Spain

The low field region (5.8−9.0 ppm) corresponding to aromatic protons and the region 1.8−3.0 ppm of the ^{1}H NMR spectra were used for characterization and chemometric differentiation of 52 apple juices obtained from six cider apple varieties. The data set consisted of 14 integrated areas corresponding to resonances from acids and phenolic compounds. Multivariate procedures based on hierarchical cluster and discriminant analysis were performed on selected signals of the spectra to determine whether it was possible to distinguish the different juices. Cluster analysis was able to satisfactorily classify the six apple varieties. Discriminant analysis, by means of stepwise procedure for variables selection and leave-one-out for cross-validation, was applied to 40 samples from the year 2001, obtaining recognition and prediction abilities of 100%. The most discriminant variables corresponded to poliphenols, (−)-epicatechin, phloridzin-phloretin, and *p*-coumaric, chlorogenic, and malic acids. The classification model was applied to 12 samples from apples harvested in the years 2002 and 2003, and the prediction ability was 91.7%.

KEYWORDS: ^{1}H NMR; cider apple juice; varietal characterization; chemometrics

INTRODUCTION

Cider apple has the same chemical features as other kinds of apple, but it has a higher acidity and a phenolic content of up to 10-fold that of dessert apples (*1*, *2*). Several studies have been dedicated to the quantification of sugars (*3*−*5*), organic acids (*3*, *6*), and poliphenolic compounds (*7*−*9*) in apples, and most of them involved separation of the liquid phase of the fruit, followed by chromatographic analysis.

Chromatographic methods only measure specimens corresponding to one specific chemical compound (sugars, organic acids, flavonoids, amino acids, etc.) and often require sample preparation before measurements. An alternative way to carry out this characterization is to use a very general analytical chemical screening tool that could detect a much wider range of different compounds in a single run without requiring separation or extraction steps. A possible screening tool is proton nuclear magnetic resonance spectroscopy (^{1}H NMR). NMR spectroscopy has played an increasingly important role in the quality assessment of horticultural products (*10*) and in the compositional study of food (*11*−*13*), particularly in fruit juices (*11*, *14*−*16*). Moreover, it is commonly recognized as a reliable technique for quantification of natural and synthetic samples. Recent reviews (*17*, *18*) show the increasing use that International Pharmacopeias make of quantitative NMR spectroscopy. The main advantages associated with this technique are easy sample preparation, rapidity, nondestructive character, and simultaneous detection of a great number of compounds. Among its disadvantages are the relatively high limit of detection and the dependence of the accuracy of quantification on a high number of parameters (*19*, *20*).

It has recently been shown that the combination of high-resolution NMR and chemometrics manages to accomplish the characterization or classification of products according to their origin, quality, or variety (*21*−*25*). Furthermore, the chemical origin of the discrimination between different groups of products can be interpreted by establishing the signals with the highest discriminant power and the subsequent assignment of these NMR signals.

Chemometric procedures have also been applied to discrimination between three apple varieties from English orchards (*26*) by using two regions of the ^{1}H NMR spectra: the central region, between 2.5 and 5.0 ppm, containing signals from the components of highest concentration, mainly sugars, and the high-field region between 0.8 and 2.5 ppm, containing signals from compounds of lower concentrations, mainly acids and amino acids. The low-field region contains resonance lines from aromatic protons that correspond to phenolic compounds, but this region has hardly been studied probably due to the fact that the intensities are the weakest of the spectrum. Moreover,

* To whom correspondence should be addressed. Tel: +34 943018213. Fax: +34 943015270. E-mail: qppcamag@sc.ehu.es.
† Applied Chemistry Department.
‡ Materials Physics Department.

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Table 1. Selected Resonances Used in the Chemometric Analysis

peak	compound	chemical shift (ppm)	integration interval (ppm)
1	quinic acid	1.90	1.86–2.00
2	quinic acid	2.06, 2.10, 2.14	2.04–2.36
	chlorogenic acid	2.09, 2.20, 2.24, 2.32	
3	succinic acid	2.56	2.53–2.59
4	malic acid	2.84, 2.92	2.80–2.96
5	citric acid	2.83, 2.98	2.96–3.04
	asparagine	2.92, 2.96	
6	phloretin	6.02	6.00–6.04
7	(–)-epicatechin	6.10, 6.11	6.08–6.16
	phloridzin	6.14	
8	*p*-coumaric acid	6.49	6.45–6.53
9	phloridzin	6.82	6.76–6.86
	phloretin		
10	polyphenol	6.92 broad	6.86–7.00
11	(–)-epicatechin	7.05	7.02–7.08
12	chlorogenic acid	7.13	7.08–7.16
13	chlorogenic acid	7.20	7.16–7.24
14	*p*-coumaric acid	7.75	7.70–7.80

to study this region, it is necessary to prevent the enzymatic oxidation of the phenolic compounds during the preparation of the juices.

The aim of this work was to evaluate the possible contribution of ^{1}H NMR spectroscopy in the characterization and differentiation of six cider apple varieties using multivariate statistical analysis [hierarchical cluster analysis (CA) and linear discriminant analysis (LDA)], on the basis of their phenolic and acid composition. Acids and polyphenols have a considerable effect in browning processes and contribute to the taste and stability of juices and ciders; hence, both have been used in the past for technological classification of these fruits (*1*).

MATERIALS AND METHODS

Sample Collection. Fifty-two representative samples of cider apples belonging to Gezamina (GE), Goikoetxea (GO), Moko (MO), Txalaka (TX), Urtebi Haundi (UH), and Urtebi Txiki (UT) cultivars were collected during the month of October from eight different orchards, located in six different areas of the Basque Country (Spain). Forty samples were collected in 2001, and 12 samples (one for each variety and year) were collected in 2002 and 2003. About 2 kg of each apple cultivar was crushed and pressed separately by using a small manual press. Ascorbic acid (5 g/100 mL) was immediately added to a portion of each juice in order to prevent enzymatic oxidation of the phenolic compounds and also to adjust the pH to the same value (2.74 ± 0.02) in all of the juices, since the chemical shifts of some compounds on the ^{1}H MNR spectra can vary from sample to sample because of the natural variation in the pH of the juices (*15*). The juices were then clarified by centrifugation (12000*g*, 15 min), microfiltered through a 0.45 μm pore size filter, and stored at −20 °C until the ^{1}H NMR spectra recording was performed.

^{1}H NMR Measurements. A 600 μL amount of the thawed sample was placed into a 5 mm outer diameter NMR tube, and 100 μL of a solution containing D_2O, 3-(trimethylsilyl)-1-propane-sulfonic acid (TSS), and 1,3,5-benzenetricarboxylic acid (BTC) was added according to the previously described conditions (*27*). One-dimensional spectra were recorded on a Bruker Avance-500 spectrometer (Karlsruhe, Germany). For each sample, 128 scans of 64 K data points were acquired by using a spectral width of 8012 Hz (16 ppm), an acquisition time of 4.09 s, a recycle delay of 1.00 s, and a flip angle of 90°. Solvent suppression was achieved using the Watergate pulse sequence (*28*). Free induction decay signals were processed before Fourier transforma-

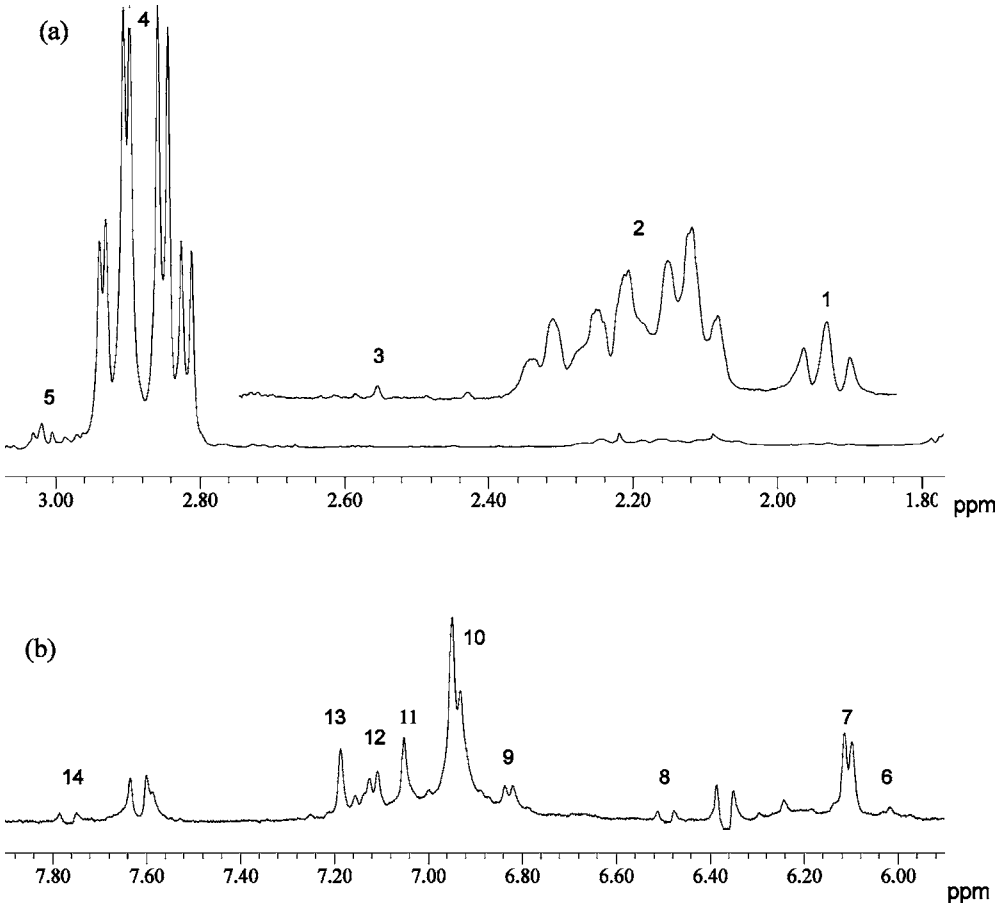

Figure 1. Expansions of the ^{1}H NMR spectrum of a cider apple juice showing the 14 signals used in chemometric analysis: (**a**) 1.80–3.08 and (**b**) 5.90–7.90 ppm (aromatic region).

tion by an exponential filter applying a line broadening factor of 0.4 Hz, using XWIN NMR (version 3.1, Bruker Gmbh, Germany). To attain reliable results, the phasing and the baseline correction of the spectra were critical. These processes were manually performed. The spectra were referenced to the TSS singlet peak at 0 ppm.

The definition of the baseline level affects areas of the selected signals and the proper integration of the selected signals were assessed considering the areas of the peaks corresponding to internal standards (TSS and BTC in the high and low regions, respectively). As they were added in equal amounts in all of the samples, their areas should also be equal in the different spectra. BTC has been used in our laboratory to develop new quantitative methods in the low field region (*27*, *29*). It gives only a single peak at about 8.75 ppm, which was never overlapped by any other signal from the apple juices. The mean values for BTC and TSS areas were 960.1 ± 68.9 and 11194.1 ± 96.6 ($n = 40$). For less than 5% of the spectra, the areas for BTC differed significantly from these values, and the baseline was additionally corrected over the integrated region. The 14 resonances chosen for the integration (in **Table 1**) were the clearly identified ones, which corresponded to phenolic and acid compounds.

Chemometrics. A matrix was constructed with rows representing apple juice samples and columns corresponding to the 14 selected peak areas. To avoid differences in measurement units, the variables were self-scaled, obtaining variables with zero mean and a unit standard deviation. All chemometric analyses were performed by means of the SPSS version 12.0 for Windows.

Multiple Box−Whiskers Plot. In these plots, the three horizontal lines of each box were set at (from bottom to top) the 25th, 50th (median), and 75th percentile values. Vertical lines from each end of the box came to the lowest and the highest value in the data set that was not an outlier.

CA. CA is an unsupervised classification method, i.e., does not know to which variety a sample belongs (*30*). A dissimilitary matrix $S_{40\times14}$ was constructed, and a hierarchical method was used. This was a linkage method that used the square of the Euclidean distance of each object from the rest (rescaled distance cluster combine, RDCC) as a similarity measure.

LDA. One of the best studied and widely used pattern recognition methods is LDA (*30*). Its starting point is to find a discriminant function, which is a linear combination of the original variables. The LDA procedure implemented here used the Mahalanobis distance as the measurement of distance between samples; each sample was assigned to the group from which it had the shortest squared Mahalanobis distance. The stepwise LDA procedure was applied as follows: from the n variables, the most discriminating subset was selected. The criterion for the selection was the Wilks' λ, which is a measure of the quality of the separation.

RESULTS AND DISCUSSION

^{1}H NMR Spectra. In **Figure 1**, two expansions of the ^{1}H NMR spectrum of an apple juice are shown, and the 14 integrated signals used for the statistical analysis are numbered, corresponding to the following: 1, quinic acid; 2, quinic and chlorogenic acids; 3, succinic acid; 4, malic acid (although protons of the CH_2 groups, corresponding to citric acid, and β-CH_2, corresponding to asparagine, also show partially overlapped signals with those malic acid, their intensities are negligible in comparison to malic acid); 5, citric acid and asparagine; 6, phloretin; 7, (−)-epicatechin and phloridzin; 8, *p*-coumaric acid; 9, phloridzin and phloretin (dihydrochalcones); 10, polyphenols; 11, (−)-epicatechin; 12, chlorogenic acid; 13, chlorogenic acid; and 14, *p*-coumaric acid. The chemical components that contribute to the major peaks shown were assigned to the spectra on the basis of previously published data (*15*, *31*, *32*). Moreover, to assist in the assignment, ^{1}H NMR spectra of standards of the 11 compounds included in our analysis, known to be present in apple juice (*3*, *6*−*9*), were run under the same conditions as the juice samples. Peak areas within spectra were used as a guide to estimate relative concentrations of species within the samples.

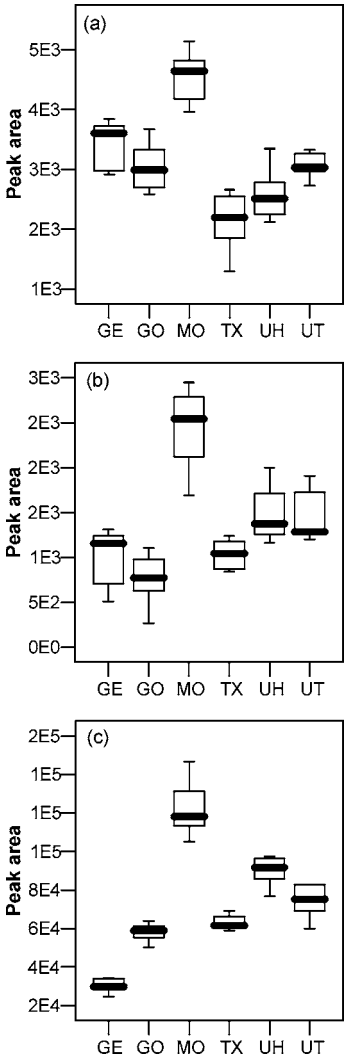

Figure 2. Multiple Box–Whiskers plot, according to apple variety, for areas from (**a**) peak 1, quinic acid; (**b**) peak 3, succinic acid; and (**c**) peak 4, malic acid. Codification: GE, Gezamina; GO, Goikoetxea; MO, Moko; TX, Txalaka; UH, Urtebi Haundi; and UT, Urtebi Txiki.

Detection of outliers was checked by means of the Box−Whiskers graphs, and because none of the juice samples showed outliers for more than one variable, all of the samples were included in the subsequent analysis. The apple acid fraction affects the fruit-processing quality. Moreover, there is considerable variation in acidity among cultivars but only small differences within cultivars (*33*); therefore, the acid fraction study is important to cider apple characterization. The major acid in apples is malic acid and its acid salt. Chlorogenic, quinic, and succinic acids are also present, and they can be measured in ^{1}H NMR spectra. The results obtained for malic, quinic, and succinic acids are shown in the form of Box−Whiskers graphs in **Figure 2**. As can be seen, malic acid was the most discriminant and completely differentiated MO from the GE variety, which had the highest and the lowest malic acid contents, respectively (**Figure 2c**). MO variety also showed the highest content in quinic and succinic acids, but only a few differences were observed among other apple varieties (**Figure 2a**,**b**).

The aromatic region showed large differences between cultivars, which are evidenced in the Box−Whiskers graphs corresponding to the main phenolic compounds (**Figure 3**). An

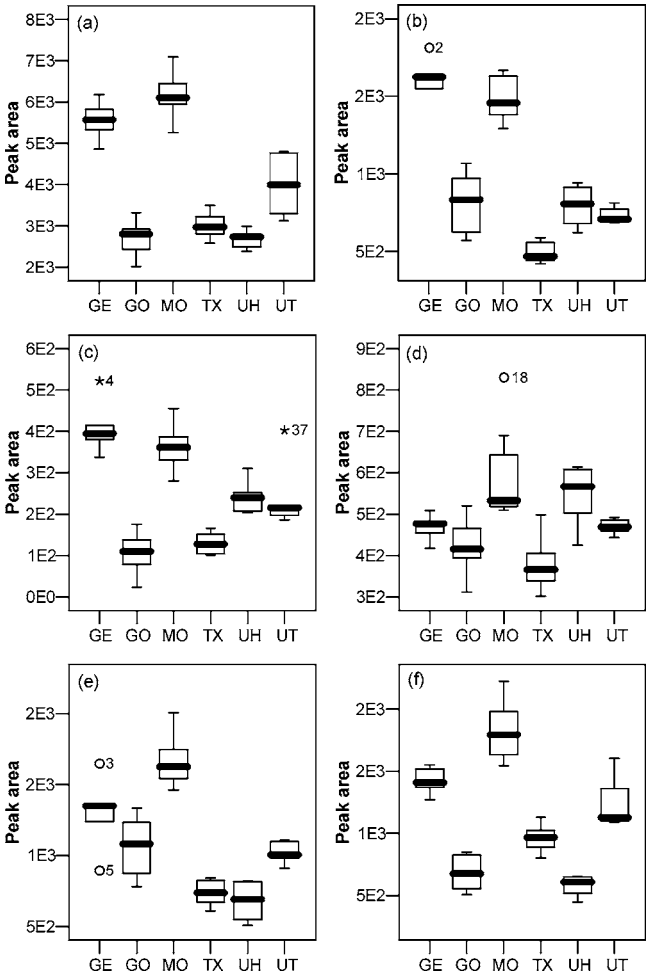

Figure 3. Multiple Box–Whiskers plot, according to apple variety, for areas from (**a**) peak 10, polyphenols; (**b**) peak 13, chlorogenic acid; (**c**) peak 14, *p*-coumaric acid; (**d**) peak 6, phloretin; (**e**) peak 9, phloridzin-phloretin; and (**f**) peak 11, (–)-epicatechin. Codification is given in **Figure 2**.

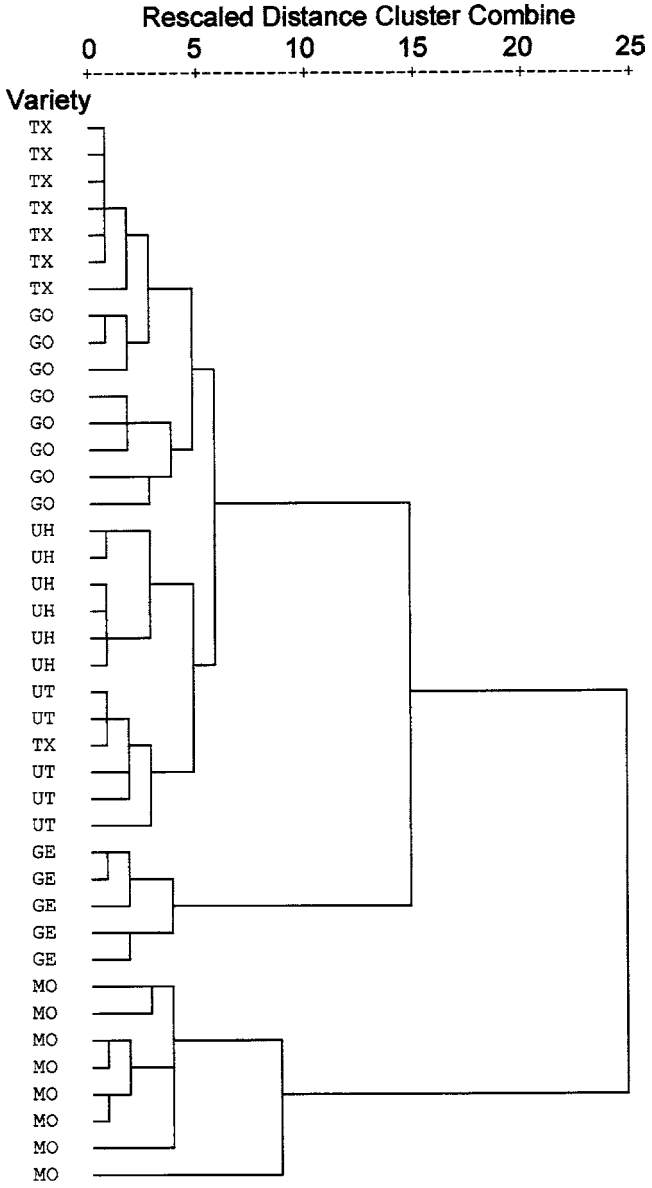

Figure 4. Dendrogram built with all of the variables using average linkage between groups method and Euclidean squared distance. Codification is given in **Figure 2**.

intense broad peak between 6.86 and 7.00 ppm (peak 10) is assigned to condensed polyphenol species; the signals from (−)-epicatechin and chlorogenic acid appear overlapped in this peak, an area that can be considered as an estimation of the total phenolic content. The GE and MO varieties had the highest contents of these compounds, and GO, TX, and UH had similar contents between them, being the lowest of the six varieties studied (**Figure 3a**). GE and MO varieties also showed a high content of chlorogenic (**Figure 3b**) and *p*-coumaric acids (**Figure 3c**); however, the MO variety had higher contents in phloretin (**Figure 3d**) phlorizin-phloretin (**Figure 3e**), and (−)-epicatechin (**Figure 3f**) than the GE variety. The TX variety exhibited the lowest mean contents in chlorogenic acid and phloretin. Both the UH and the UT varieties had similar contents in *p*-coumaric and chlorogenic acids, but the UH variety had lower contents in (−)-epicatechin and phloridzin-phloretin than the UT variety. The GO variety had a moderate content in each phenolic compound, although some juices of this variety presented the lowest content in *p*-coumaric acid. The ^{1}H NMR data inspection shows some of the differences existing between the acidic and the phenolic profiles of the apple varieties, which are consistent with results obtained in previous studies, using the high-performance liquid chromatography technique (*9*, *34*).

Hierarchical CA. This analysis was performed in order to determine whether the selected variables had sufficient explanatory capacity in finding clusters between juices of different apple varieties. The dendrogram in **Figure 4** presents the clustering of cider apple juices. RDCC is a relative scale with zero value for each individual sample and a maximum value of 25 for the most distant samples. At a RDCC = 4.5, seven clusters were found, corresponding to the six cider apple juice varieties, except for a TX sample, which was included within UT samples. The most similar cultivars were GO−TX (joined at RDCC = 5.0) and UH−UT (joined at RDCC = 5.0); these clusters were put together with GE samples at RDCC = 15. A sample of the MO cultivar formed a single cluster, which was joined with the remainder MO samples at RDCC = 8.8.

LDA. Two interesting questions arise. First, is it possible to obtain discriminant functions for discrimination among cultivars, and second, can they be applied in different years? In answer to the first question, a stepwise DA was applied and five discriminant functions (D1−D5) were obtained, which were linear combinations of the following variables: *p*-coumaric acid, polyphenols, (−)-epicatechin, chlorogenic acid, phloridzin-phloretin, and malic acid. Although two discriminant functions explained 82.5% of the total variance (D1: eigenvalue, 26.9;

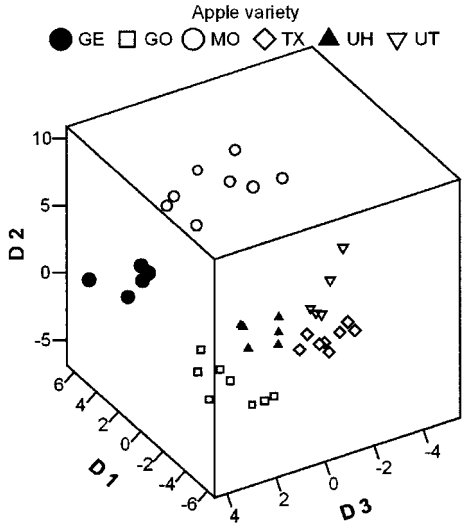

Figure 5. Scattered plot of the samples in the space defined by the three first discriminant functions. Codification is given in **Figure 2**.

% contribution, 50.0%; D2: eigenvalue, 17.4; % contribution 32.4%), a third axis allows for a supplementary discrimination of 11.8%, giving a total of 94.3% of the total variance explained by the analysis. The values of their canonical correlations, which show the correlation of a function with the discriminant scores, were higher than 0.929. The values of Wilk's λ were very small (<0.001) indicating that the selected variables had an effective discriminating power. The tolerance values, from 0.768 for *p*-coumaric acid to 0.262 for (−)-epicatechin, indicated low redundancy among the variables. The first function, D1, was dominated by phloridzin-phloretin (with a negative sign), (−)-epicatechin, and polyphenols; the second function, D2, was dominated by malic acid, and the third function, D3, was dominated by (−)-epicatechin and chlorogenic acid (with a negative sign). A three-dimensional plot of the discriminant space is shown in **Figure 5**. As can be seen, the samples are distributed in six differentiated groups, which coincide with the six cultivars. The high values of D1 and D2 for MO variety are due to a highly acidic character and phenolic of this cultivar, which can also be observed in **Figures 2** and **3**. The GE variety also has a high phenolic content, but the negative values of D2, explained by its low content in malic acid (**Figure 2a**), separated it from the remaining varieties. **Figure 5** also shows the proximity among the TX, GO, UH, and UT apple varieties, according to results from CA.

Considering the subsequent probabilities, a recognition ability of 100% was obtained for each cultivar. Validation of these results was performed using the leave-one-out procedure, obtaining a prediction ability of 100%. To prove the predictive ability of new samples from different years, the discriminant functions were applied to juices obtained from the six cider apple varieties (one for each cultivar) harvested in the years 2002 and 2003, 12 samples in total. The prediction ability on the two years together was 91.7%. Five samples from the year 2002 were correctly classified, but a sample of the GO variety was classified as TX variety. For the six apple juices from 2003, the prediction ability was 100%. Taking the three years into account, all juices from GE, MO, TX, UH, and UT apple varieties were correctly classified, so the results can be considered as acceptable.

The results obtained indicate that the low field region (5.8−9.0 ppm), corresponding to aromatic protons and the interval 1.8−3.0 ppm of the ^{1}H NMR, make a very useful tool for the characterization and differentiation of cider apples using chemometric techniques. The spectra can be obtained without any time-consuming purification and chemical derivatization while offering a reliable overview of many components present in the juices.

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