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Application of ^{1}H NMR and Multivariate Statistics for Screening Complex Mixtures: Quality Control and Authenticity of Instant Coffee

ADRIAN J. CHARLTON,* WILLIAM H. H. FARRINGTON, AND PAUL BRERETON

Department for Environment, Food and Rural Affairs, Central Science Laboratory, Sand Hutton, York YO41 1LZ, United Kingdom

Principal components analysis (PCA) followed by linear discriminant analysis (LDA) of the nuclear magnetic resonance (NMR) spectra from 98 instant spray-dried coffees, obtained from 3 different producers, correctly attributed 99% of the samples to their manufacturer. Blind testing of the PCA model with a further 36 samples of instant coffee resulted in a 100% success rate in identifying the samples from the 3 manufacturers. Coffees from one manufacturer were also assigned into 2 groups using these techniques, and the compound 5-(hydroxymethyl)-2-furaldehyde was identified as the primary marker of differentiation.

KEYWORDS: NMR; PCA; LDA; coffee; chemometrics; authenticity; quality assurance

INTRODUCTION

Instant coffee is produced by blending coffee beans, prior to manufacture of the retail product, until the desired organoleptic properties are obtained. Producing coffee with a reproducible flavor is primarily the responsibility of skilled coffee tasters, and, therefore, the chemical composition of the final product may vary from producer to producer. Variations in the chemical composition of complex mixtures can often be detected by nuclear magnetic resonance (NMR) spectroscopy (*1*, *2*), and this concept can be used to determine the reproducibility of production and identify the manufacturer of a particular product. The application of NMR and statistical methods for determining variance between sample sets is particularly useful as a quality assurance measure or for authentication of food products (*3*, *4*). For example, these methods may be used to determine whether a fraudulent retailer is selling inferior quality products marked as those from reputable manufacturers.

Coffee tasters blend green coffee beans from different geographical and botanical sources prior to manufacture of the retail product. Using this blending process they hope to obtain organoleptic properties that are characteristic of their product; however, flavor variation between jars of instant coffee of the same brand have been reported (*5*).

Because of the empirical nature of the blending process and the many variables that need to be controlled during roasting and extraction of the beans, the chemical composition of the final product will vary from manufacturer to manufacturer (*6*). Previous studies have identified many of the constituents of coffee (*7*, *8*), with caffeine, chlorogenic acid, and carbohydrates being among the most prevalent. Liquid and gas chromatographic methods have been extensively used to determine the chemical composition of coffee, but these methods are often time-consuming, and only a limited number of compounds can be investigated. An extensive study of espresso coffee using NMR spectroscopy (*9*) has identified many of its major constituents.

The NMR spectrum contains quantitative information about all of the ^{1}H containing compounds in a sample and can therefore be used to quickly characterize complex mixtures. NMR is particularly useful for identifying variation between the chemical compositions of solutions, and can be used as a fingerprinting method or to identify marker compounds. NMR has been used to detect unintended changes due to genetic modification (*10*) and for the detection of environmental effects in tomatoes (*11*). In both of these studies statistical methods were used to identify regions of the NMR spectrum that varied between the control and test sample sets. Similar methodology has been applied to studies of the speciation of both liverworts (*12*) and marine sponges (*13*) in which the NMR spectrum was used to identify chemical markers that could be used to assign organisms to the correct species. More recently, the use of NMR to identify characteristic marker compounds for the adulteration of food has been reported. Le Gall et al (*14*) have used NMR and multivariate statistics (chemometrics) to identify compounds that are indicative of the addition of pulp wash to pure orange juice. Multivariate statistics have also been used in conjunction with Fourier transform infrared spectroscopy (FTIR) to determine the botanical origin of instant coffee (*15*).

Chemometrics has been extensively used to analyze chemical data for the presence of patterns and trends from which sample classifications can be made (*16*, *17*). Classification of samples into groups by using patterns identified in analytical data can be performed in many ways, but essentially all of the methods

* Corresponding author. Tel: +44 1904 462513. Fax: +44 1904 462133. E-mail: adrian.charlton@csl.gov.uk.

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described use the same principle of model building (training) followed by model validation (testing) before predictions are made about the identity of unknowns.

Here we describe the application of discriminant analysis and class modeling methods to the ^{1}H NMR spectra of instant coffees from different manufacturing sources and to two instant coffees produced by the same manufacturer.

MATERIALS AND METHODS

Materials. A total of 98 instant spray-dried coffee samples were provided by a manufacturer of instant coffee. Of these, 39 of the coffees were from population A_1, 26 were from population A_2, 18 were from population B, and 15 were from population C. Population A coffees were produced by manufacturer A, population B coffees were produced by manufacturer B and population C coffees were produced by manufacturer C. A further 36 blind samples were provided, and these were instant coffees from a variety of sources, with some being coffees produced by manufacturers A, B, and C.

Deuterated solvents and sodium 3-(trimethylsilyl) propionate-d_4 (TSP) were purchased from Goss Scientific (Cambridge, UK), and 5-(hydroxymethyl)-2-furaldehyde was a gift from one of the coffee manufacturers.

Extraction Protocol. A 140-mg aliquot of each instant coffee was dissolved in 2.4 mL of D_2O (containing 1 mM TSP). The sample was mixed until homogeneous and heated for 30 min at 100 °C. The coffees were centrifuged for 5 min, and the supernatant was removed and filtered sequentially through a 0.45- and a 0.2-μm PTFE syringe filter. Each sample (540 μL) was placed in a 5-mm NMR tube, and 60 μL of 10 mM sodium azide was added to prevent bacterial growth. The pH of all the samples was determined to be 4.85 $\pm$ 0.05.

NMR Spectroscopy. One-dimensional NMR spectra were acquired on a Bruker ARX 500 spectrometer using a 5-mm broad band probe tuned to detect ^{1}H resonances at 500.13 MHz. Data were collected at 300 K, without sample rotation, as 32-K complex points using a 30° pulse length. Using an inversion recovery sequence, TSP was found to have the longest spin−lattice relaxation time (2.6 s), and subsequently a 7.5 s relaxation delay was found to be sufficient for the acquisition of quantitative data. 300 scans were acquired with a spectral width of 6250 Hz. The data were processed using FELIX software (MSI). A sinebell-shaped window function phase shifted by 90° was applied to the free induction decay (FID) over the first 1024 points. The FID was reduced to 1024 points prior to Fourier transformation. Phase and baseline corrections were applied to the data, and the spectrum was referenced to the TSP peak at 0 ppm. The spectral data were saved as ASCII formatted text to facilitate statistical analysis.

The spinlock for two-dimensional total correlation spectroscopy (TOCSY) was achieved using a MLEV17 pulse sequence of 150 ms duration. Data were acquired into 400 complex files, each containing 4 K points with a spectral width of 6250 Hz in both F_2 and F_1. A sine-squared bell window function was applied to both t_1 and t_2 data. The data were transformed into a 4 K $\times$ 4 K real matrix. Real-time phase corrections and a baseline correction were applied in both dimensions, and the spectrum was referenced to the TSP peak at 0 ppm.

Repeatability. The reproducibilities of the extraction protocol and the NMR data acquisition and processing parameters were assessed. One-dimensional ^{1}H NMR spectra of six extractions of the same sample and six repeat measurements on the same extract, were used to determine the mean peak area and the standard deviation, for uncorrelated resonances at 9.12 and 7.50 ppm.

Chemometrics. After removing the water peak from the NMR spectrum, all of the remaining data points were compressed using covariance principal components analysis (PCA) to calculate the first 10 principal components (PCs). All of the chemometrics discussed were performed using WinDAS software (*18*).

A total of 30 samples from population A_1 and 16 from population A_2 were used to calculate the first 10 principal components from their ^{1}H NMR spectra. Linear discriminant analysis (LDA) was applied to the principal components, and the squared Mahalanobis distance was used to determine the proximity of each sample to the group centers for populations A_1 and A_2. Each sample was assigned to the group from which it had the shortest squared Mahalanobis distance. The identities of the remaining 9 samples from population A_1 and 10 samples from population A_2 were predicted using the squared Mahalanobis distance from the group centers defined at the training stage.

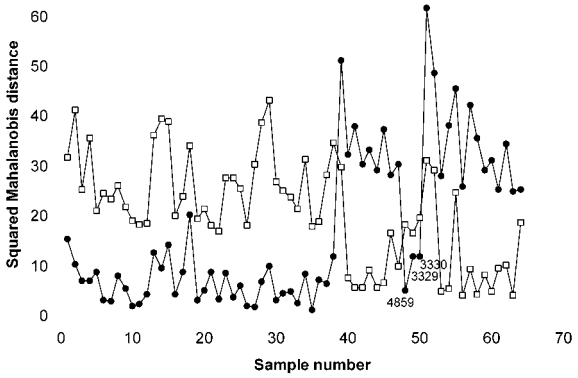

Figure 1. Squared Mahalanobis distance from the group center for population A_1 (●) and for population A_2 (□) calculated using LDA for each of the population A coffees.

The NMR spectra of 30 samples from population A_1 were pooled with the NMR spectra of 16 samples from population A_2 and these were designated as group 1. Similarly 12 spectra from population B and 10 spectra from population C were assigned to group 2. The first 10 principal components were calculated using these 68 samples. The identity of the 19 remaining samples from population A and 11 from population B or C, was predicted using both LDA and CVA models with 3 to 10 principal components. All of the CVA and LDA models were applied to the 36 blind samples, and predictions about their identity were made.

Thirty-one population A_1 samples and 21 population A_2 samples were used to construct a SIMCA and a UNEQ model using 3 to 10 principal components. Group membership was assessed using Student's *t* test for SIMCA and the squared Mahalanobis distance from the group center for UNEQ. A further 8 population A_1 samples and 5 population A_2 samples, plus all of the population B and C samples (33 in total), were used to test each of the SIMCA and UNEQ models. The blind samples were then classified using the SIMCA and UNEQ models that included 3 to 10 PCs.

RESULTS AND DISCUSSION

The NMR spectra obtained from instant coffee, using the protocol described, suggests that the concentration of the extracted molecules is generally high enough to be clearly detected. The standard deviation from the mean peak area for two baseline resolved resonances at 9.12 and 7.50 ppm were found to be 1.7 and 1.6% respectively, using the ^{1}H NMR spectra of six extracts of the same sample. Acquisition of six spectra from a single coffee sample gave a standard deviation of 1.0% for the areas of both of the aforementioned peaks.

During the training stage, using PCA followed by LDA, all of the population A_1 and A_2 samples were correctly distinguished using 3 principal components. During the testing stage all of the population A_1 samples were correctly classified using a one principal component model. Of the 10 population A_2 samples, 7 were also correctly classified when a single principal component was used. However, all of the samples could be assigned to the correct group when a 3 principal component model was adopted.

Figure 1 shows the squared Mahalanobis distance from the group centers defined by the population A_1 and A_2 coffees, calculated using the first principal component. Each sample in population A_1 was found to have a squared Mahalanobis

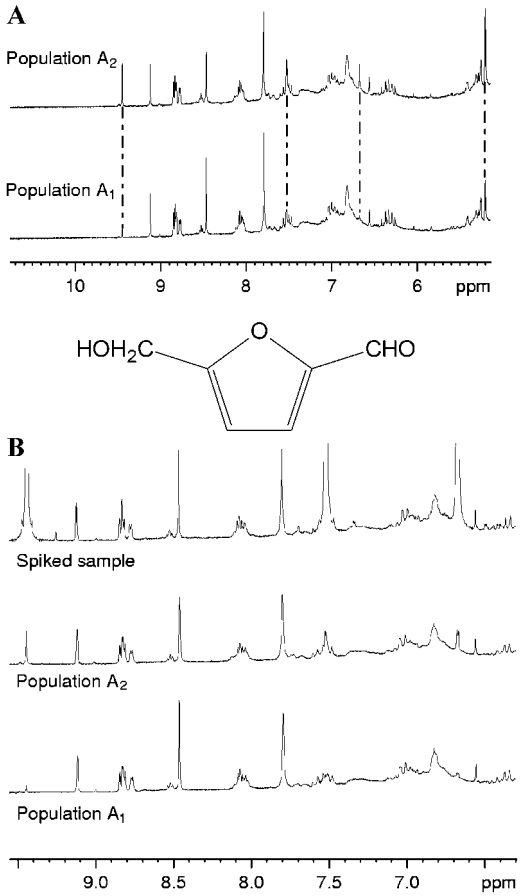

Figure 2. (A) Downfield region of a ^{1}H NMR spectrum of a representative coffee sample from populations A_1 and A_2. (B) Increase in intensity of the 4 peaks identified as markers for the population A_2 samples, on addition of 5-(hydroxymethyl)-2-furaldehyde (center).

distance to the group center of population A_1 that was shorter than that to the group center of population A_2. Based on the first PC score, all of the population A_2 samples were found to be more similar to each other than they were to the population A_1 samples, with the exception of samples 4859, 3329, and 3330.

Closer examination of the NMR spectra of a representative member of populations A_1 and A_2 suggested that four peaks had an increased intensity in the population A_2 samples when compared to those from population A_1 (**Figure 2**A). The chemical shifts of these peaks suggested that they were probably derived from an aldehyde group (9.5 ppm), two peaks from methine protons (6.7 and 7.5 ppm) and a methylene proton (5.2 ppm).

5-(Hydroxymethyl)-2-furaldehyde is present in instant coffee and contains only the protons described above. When 5-(hydroxymethyl)-2-furaldehyde was added back to a population A_1 sample, the chemical shifts of its protons were found to be the same as those differentiating the population A samples (**Figure 2B**). A TOCSY experiment was performed on a population A_2 coffee and this confirmed that three of the resonances that were more pronounced in the population A_2 coffee were part of the same molecule. **Figure 3** shows the correlation between the peaks at 5.2 ppm (shifted to 4.8 ppm in the TOCSY spectrum) and the peaks at 7.5 and 6.7 ppm. A strong correlation between the peaks at 7.5 and 6.7 ppm can also be seen. This confirms that 5-(hydroxymethyl)-2-furaldehyde is the compound responsible for the major differences in peak intensity between the A_1 and A_2 samples. These variations in the concentration are

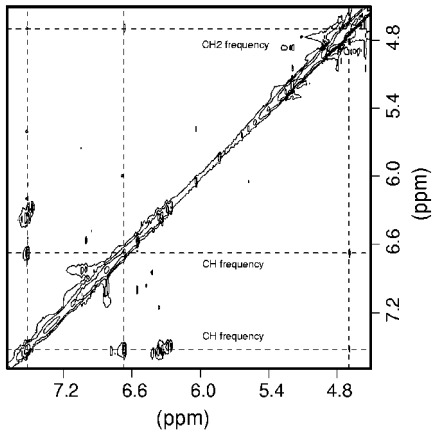

Figure 3. Part of a TOCSY spectrum acquired from a population A_2 coffee.

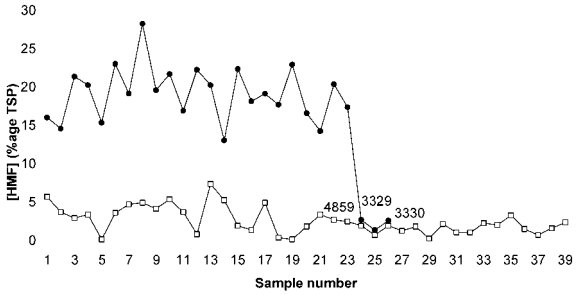

Figure 4. Concentration of 5-(hydroxymethyl)-2-furaldehyde expressed as a percentage of the internal reference compound TSP for population A_1 (□) and A_2 (●) coffees.

the likely basis for the discrimination of the two sample sets using the first principal component and the squared Mahalanobis distances. The concentration of 5-(hydroxymethyl)-2-furaldehyde was determined from the NMR spectrum (**Figure 4**). The three samples that were not correctly identified using LDA (**Figure 1**), have a level of 5-(hydroxymethyl)-2-furaldehyde that is indicative of a population A_1 sample, but their correct classification should have been population A_2. This confirms that 5-(hydroxymethyl)-2-furaldehyde is the major variable identified in the first principal component and that the first PC is sufficient to correctly classify 62 out of 65 samples. By introducing a further two principal components to the calculation all of the samples could be correctly classified, suggesting that more subtle differences were present in the NMR spectra of the population A samples.

Data clusters for manufacturer A (group 1), and manufacturers B and C (group 2) coffees are evident when then first two PC scores are plotted (**Figure 5**). By extending this plot into five dimensions (i.e., using 5 PC scores) and applying LDA, the manufacturer A coffees were differentiated from those of manufacturers B and C. The manufacturers of all of the remaining samples used to test the model were correctly identified with the exception of a single coffee produced by manufacturer B. When the blind coffee samples were subjected to linear discriminant analysis, it was possible to correctly assign all of the coffees from the three manufacturers, using a model containing 5 or more PCs (**Table 1**). The test set of data was known to contain only samples from manufacturers A, B, and C. However, the blind samples contained coffees which were not produced by any of the three manufacturers and these were therefore classified as group 1 or group 2 coffees.

CVA correctly identified the manufacturer of 95% of the samples during the training stage using a 7 PC model. The remaining 5% of the training samples were unclassified. During

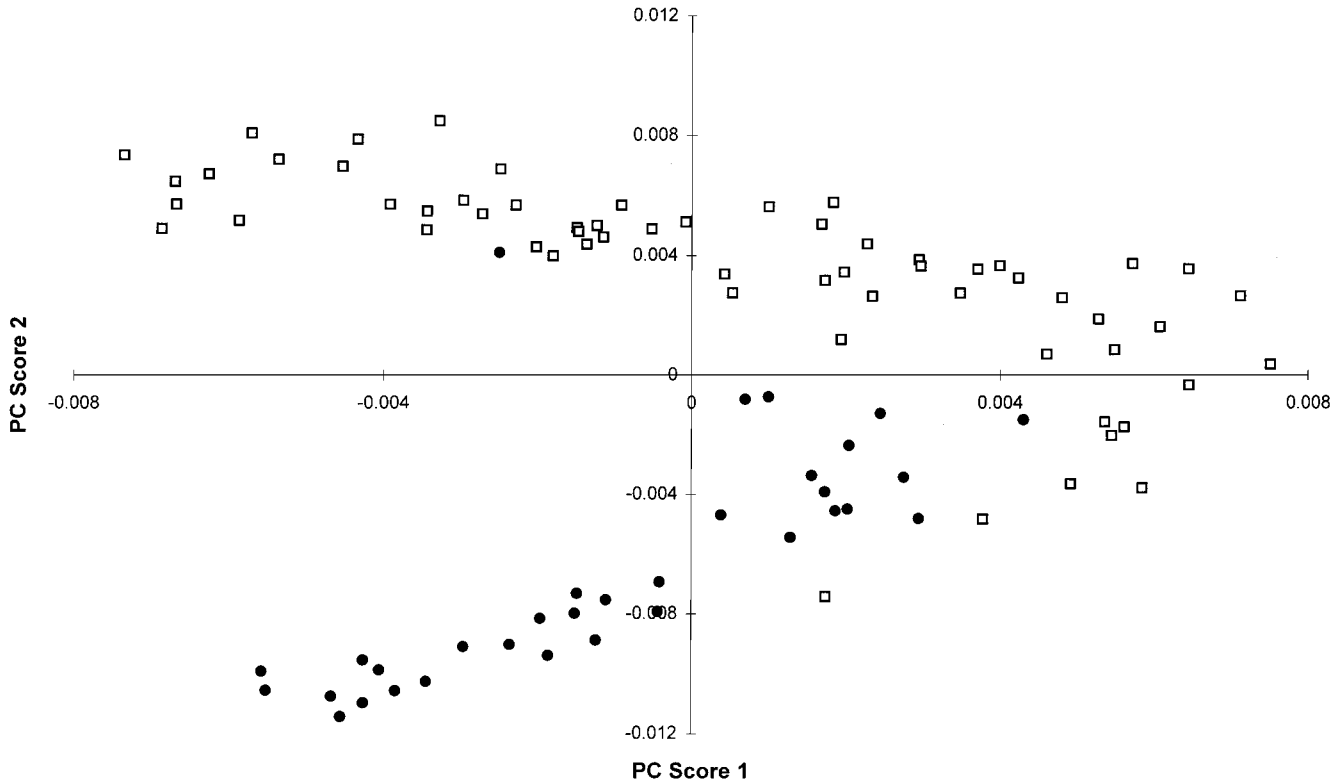

Figure 5. Cluster plot of the first two principal component scores derived from the ^{1}H NMR spectrum of 98 instant coffee samples produced by manufacturer A (□) and by manufacturers B and C (●).

the testing stage of the CVA, using a 7 PC model, 83% of the samples were correctly classified and 17% remained unclassified, with no incorrect predictions. A CVA model containing 7 principal components was therefore used to make predictions about the identities of the blind samples.

Figure 6 shows the CV scores for each of the blind coffee samples. The horizontal line indicates the center of group 1, while the center of group 2 is at approximately −5. The proximity of each sample to both of the group centers is the basis for the classification of the coffees into each group. The CVA model with 7 PCs correctly classified 21 out of 22 of the coffees produced by manufacturers A, B, or C and failed to assign just one of the population A_2 samples to either group 1 or group 2. However, it was not possible to identify all of the samples from other sources. Five of these samples were not classified into either group 1 or group 2, two were misclassified as group 2 samples, and seven were misclassified as group 1 samples.

For the training stage of the SIMCA analysis of the population A samples no more than 2 samples were rejected from the model using 3 to 10 principal components. For the UNEQ model no more than 3 samples were rejected using the same number of PCs, and the model incorporating 5 PCs accepted all of samples produced by manufacturer A. The results of the testing stage for both of the class modeling methods showed that there was a significant incidence of incorrectly assigned population B and C samples. For SIMCA, 15 to 41% of the manufacturer B and C samples were accepted into the model, while 6 to 37% were included in the UNEQ model. For both SIMCA and UNEQ a model using at least 7 PCs had the least number of incorrect inclusions into the model.

The predictions made by the UNEQ and SIMCA models did not match the correct classification of the blind samples (**Table 1**). However, **Figure 7** shows the F ratios calculated from a SIMCA model using 7 PCs, and this indicates that coffees in the blind sample set produced by manufacturer A had F ratios that were among the lowest calculated for all of the blind samples. The coffees with the 14 lowest F ratios (i.e., those with NMR spectra that were most similar to the samples from manufacturer A) were all produced by manufacturer A, and the 20 samples with the lowest F ratios contained all 17 of the manufacturer A samples. This demonstrates that the value of the F ratio chosen as a cut-off point for acceptance or rejection into a group is subjective.

Figure 6 clearly shows that sample 17 has a CV score that is not indicative of either a group 1 or a group 2 coffee and therefore has an NMR spectrum that is significantly different from that of any of the coffees produced by the three manufacturers. Using an LDA model containing 7 PCs, the squared Mahalanobis distance of sample 17 from both group 1 and 2 was found to be approximately 4 times longer than that for any of the other samples. Sample 17 was also consistently rejected from both the SIMCA (**Figure 7**) and UNEQ models. Sample 17 was the only product that was produced with the addition of chicory. Chicory is a cheaper alternative to coffee and is therefore a potential adulterant. It is probable that the addition of chicory to coffee could be detected using ^{1}H NMR and multivariate statistics.

The ^{1}H NMR spectrum of a complex mixture such as coffee can be difficult to interpret. By employing multivariate statistical techniques it is possible to simplify the spectral data into a series of principal components that contain almost all of the spectral variability. This allows rapid comparisons to be made between many spectra, and differences within or between sample sets to be identified from the NMR data. Both LDA and CVA, when applied to NMR data compressed using PCA, are able to distinguish between instant coffees produced by different manufacturers. When the predictions made by both LDA and CVA are compared, a high degree of consistency between equivalent models is observed. All of the blind samples that

Table 1. Summary of the Predictions Made by the 2 Discriminant Analysis and the 2 Class Modeling Methods about the Identity of the Manufacturer of the Blind Instant Coffees

sample number	sample[a] classification	LDA[b] prediction	CVA[c] prediction	SIMCA[d] prediction	UNEQ[d] prediction
1	other	1	N	reject	reject
2	other	1	1	reject	reject
3	other	1	1	reject	reject
4	manufacturer A	1	1	reject	accept
5	other	2	N	reject	reject
6	manufacturer A	1	1	reject	reject
7	manufacturer A	1	1	reject	reject
8	manufacturer A	1	1	reject	reject
9	manufacturer A	1	1	reject	reject
10	other	1	1	reject	reject
11	manufacturer A	1	1	reject	reject
12	manufacturer A	1	1	reject	reject
13	other	2	2	reject	reject
14	other	1	1	accept	accept
15	other	1	1	reject	reject
16	manufacturer B	2	2	reject	reject
17	other	2	N	reject	reject
18	manufacturer B	2	2	reject	reject
19	other	2	2	reject	reject
20	manufacturer B	2	2	reject	reject
21	other	1	N	reject	reject
22	manufacturer A	1	1	reject	reject
23	other	1	N	reject	reject
24	manufacturer B	2	2	reject	reject
25	manufacturer A	1	1	reject	reject
26	manufacturer A	1	1	reject	reject
27	Manufacturer C	2	2	reject	reject
28	Other	1	1	reject	reject
29	Other	1	1	reject	reject
30	Manufacturer A	1	N	reject	reject
31	Manufacturer A	1	1	reject	reject
32	Manufacturer A	1	1	reject	reject
33	Manufacturer A	1	1	reject	reject
34	Manufacturer A	1	1	reject	reject
35	Manufacturer A	1	1	reject	reject
36	Manufacturer A	1	1	reject	reject

[a] Coffees not produced by manufacturers A, B, or C are labeled "other". [b] 1 and 2 indicate membership of group 1 (manufacturer A) or group 2 (manufacturer B or C). [c] As above. N indicates that the sample could not be ascribed to either group 1 or group 2. [d] Accept indicates acceptance to group 1 and reject indicates rejection to that group.

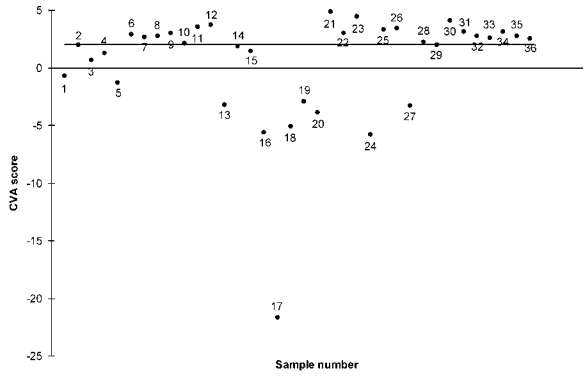

Figure 6. Canonical variates scores calculated from the first 7 principal components of the blind samples plotted against the NMR experiment number. The dashed line indicates the center of group 1.

were classified by CVA were placed in the same group by LDA using the 7 PC model. However, it is only possible to identify the manufacturer of a coffee if data from that manufacturer are included in the original model. Coffees cannot generally be rejected if they are from sources that were not included during the model-building stage. SIMCA may be able to determine whether a coffee was produced by a single manufacturer, but the cut-off point for acceptance into the group defining the manufacturer should be set by rigorously testing the model with coffees from many sources.

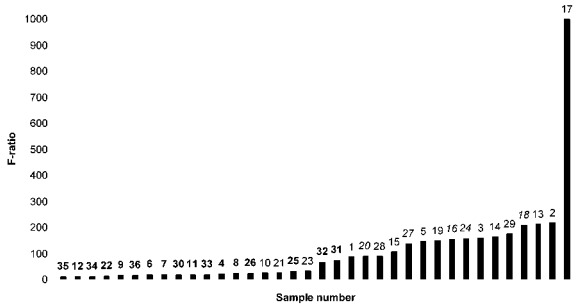

Figure 7. F ratio calculated for the blind coffee samples using a SIMCA model containing 7 principal components.

We have demonstrated, using statistical methods, the presence of inherent differences between coffees produced by different manufacturers, and even between those produced by the same manufacturer, by identifying 5-(hydroxymethyl)-2-furaldehyde as a marker compound using the structural characteristics determined by NMR. The methods described here have wide applications in the fields of food authenticity and quality assurance. The origin of chemical variability within a food can be quickly assessed using these methods, and the inherent ability of NMR to provide information about the chemical composition of a sample will allow the source of variation to be identified. This study has focused on differences in the chemical composition of instant coffees due to variations in the manufacturing process, but these methods could easily be applied to classification and labeling problems relating to geographical and botanical origin.

ABBREVIATIONS USED

CVA, Canonical variates analysis; FID, free induction decay; LDA, linear discriminant analysis; PC, principal component; PCA, principal components analysis; PTFE, poly(tetrafluoroethylene); SIMCA, soft independent modeling of class analogy; TOCSY, total correlation spectroscopy; TSP, sodium 3-(trimethylsilyl) propionate-d_4; UNEQ, unequal dispersed classes.

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