Discrimination between Orange Juice and Pulp Wash by ^{1}H Nuclear Magnetic Resonance Spectroscopy: Identification of Marker Compounds

Gwénaëlle Le Gall, Max Puaud, and Ian J. Colquhoun*

Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, United Kingdom

The potential of NMR spectroscopy and multivariate analysis methods to detect the adulteration of orange juice with pulp wash is demonstrated. Principal component analysis has been applied to ^{1}H NMR spectra of >300 orange and pulp wash juices, and stepwise linear discriminant analysis was used to classify the samples. A model with six principal components gave a high success rate of classification (94%) for both training and validation sets. An important principal component loading showed that dimethylproline played a key role in the discrimination between the two types of juice, with higher levels in pulp wash. Dimethylproline was not previously known as a marker compound for orange juice adulteration. An ANOVA test revealed at least 21 other NMR signals that differed significantly between the authentic and pulp wash groups. The compounds they represent could be seen as potential marker compounds in addition to dimethylproline. This makes NMR with chemometrics an attractive screening tool with advantages in terms of rapidity, simplicity, and diversity of information provided.

Keywords: *NMR; orange juice; pulp wash; authenticity; adulteration; multivariate analysis*

INTRODUCTION

Orange juice is a high-value product traded on a massive scale (>20 L per capita was consumed in the United States in 1996). World demand has been growing during the 1990s, and expectations are that it will continue to grow for the foreseeable future. According to U.S. Food and Drug Administration (FDA) investigations, some companies are known to have made millions of dollars selling fraudulent orange juice (*1*). Adulteration of orange juice may be done by the addition of water, sugars, pulp wash, or other citrus fruit juices such as grapefruit. Pulp wash is a second extract obtained by washing the separated pulp with water after the first pressing of the orange juice. Its chemical composition is similar in many respects to that of orange juice but it is paler, more bitter, and regarded as a lower quality product. Addition of "in-line" or "off-line" pulp wash to orange juice is at present forbidden in the EU, although the regulations are to change following adoption of a new Fruit Juice Directive. According to U.S. federal regulations frozen concentrated orange juice may contain "in line" pulp wash from the same oranges used to make the juice concentrate. "In line" addition of pulp wash to fresh and pasteurized juices is not permitted, nor is addition of "off line" pulp wash to any category of orange juice. The adulteration can also be quite elaborate, with the addition of citric acid, amino acids, or trace metals as well as sugars to mimic the chemical profile of the authentic orange juice.

Many techniques have been investigated to tackle the problem of orange juice adulteration. Several reviews (*2*, *3*) have listed the major techniques employed for this purpose, such as isotopic and chromatographic analyses. Most of the chromatographic methods are based on the study of one specific family of compounds (e.g., sugars). Instead of considering only one type of compound, spectroscopic methods such as NMR spectroscopy have the potential to monitor a wide range of chemicals (sugars, organic and amino acids, phenolics, etc.) in a single spectrum. Those methods also offer advantages in terms of rapidity and simplicity of sample preparation. The richness of information, however, makes the spectra too complex to be analyzed or compared by eye. Multivariate analysis (MVA) is therefore applied directly to the spectral data to extract the useful information. A few papers have previously been published concerning the application of MVA to chemical data for orange juice authentication (*4*−*6*).

The present work shows the potential of NMR to discriminate orange juice from pulp wash using principal component analysis (PCA) followed by linear discriminant analysis (LDA) (*7*, *8*). One of the advantages of NMR is that the chemical origins of the discrimination may be interpretable through an ANOVA test or through comparison of the principal component loadings with the spectra. This permits identification of compounds involved in the differentiation of orange juice and pulp wash and may uncover novel marker compounds. The ultimate aim would obviously be to distinguish authentic samples from ones adulterated by pulp wash and to measure the amount of pulp wash added. Because the two orange products are chemically very similar and pulp wash adulteration is difficult to detect, the present work was aimed at detecting differences between the two "pure" products. The sample set is intended to be sufficiently large to cover the range of natural variability likely to be encountered in practice.

* Corresponding author (telephone +44 1603 255353; fax +44 1603 507723; e-mail ian.colquhoun@bbsrc.ac.uk).

10.1021/jf001046e CCC: $20.00
Published on Web 01/13/2001

Table 1. Summary of the Orange Juices and Pulp Washes Studied

samples	263 pure orange juices plus 50 pulp washes
countries	16 different countries
variety	>25 varieties
°Brix	1.5 < °Brix < 15
year	samples collected from 1991 until 1998
processing	pure samples: 100 hand squeezed, 49 single strength, 114 concentrated pulp washes: 39 concentrated and 11 single strength

MATERIALS AND METHODS

Materials. Table 1 summarizes the details of the 313 samples collected for this study. The samples came from 16 different countries, mainly Brazil (50% of the data set), followed by Israel (10%) and the United States (Florida), Morocco, and Cuba (6% each). The varieties included Valencia (20%), Pera, and Navel. For 45% of the samples, the variety was unknown. The majority of the pulp washes were from Brazil (39 of 50) with others from Florida (6), Argentina (1), or unknown (4). The concentrated samples were diluted to 11−12 °Brix prior to measurement (see Methods). However, not all of the samples were standardized to the same Brix value prior to measurement because some, as received, already had a lower refractometer reading than this. Thus, the single-strength samples were measured as they were received. Samples with 11−12 °Brix formed 50% of the data set, with 35% having <11 °Brix and 15% having >12 °Brix. Samples were collected between 1991 and 1998, the years 1995−1998 accounting for 80% of the data set. The sample collection included hand-squeezed juices (laboratory prepared from whole fruit) as well as commercial single-strength and concentrated juices. Many of the juices were collected directly from producers in the country of origin; the remainder had been authenticated according to the currently adopted isotopic and chromatographic procedures mentioned above.

Methods. *NMR Spectroscopy*. Concentrates were diluted with distilled water to 11−12 °Brix using a manual refractometer. The juices were centrifuged, and 750 μL of the supernatant was mixed with 125 μL of D_2O containing 0.14% w/w dimethyl-2-silapentane-5-sulfonate (DSS) as chemical shift reference. The position on the frequency axis (chemical shift) of many NMR signals in orange juice is pH dependent. The chemical shifts of a particular compound can vary from sample to sample because of the natural variation in pH. The pH had to be adjusted because the multivariate analysis procedures described below require corresponding signals in different samples to have the same chemical shift. The pH of all samples was adjusted to 3.74 ± 0.02 by the addition of microliter amounts of 1 M NaOH or 1 M HCl solutions, and 700 μL of the solution was transferred to a 5 mm o.d. NMR tube.

^{1}H NMR spectra were recorded at 27 °C on a 500 MHz Bruker ARX spectrometer fitted with an autosampler. D_2O was used as the internal lock. Each spectrum consisted of 300 scans of 8192 complex data points with a spectral width of 6024 Hz, an acquisition time of 1.36 s, and a recycle delay of 2 s per scan. The NOESY presaturation sequence (*9*) was used to suppress the water signal with low power selective irradiation at the water frequency during both the recycle delay and the mixing time of 0.1 s. Spectra were Fourier transformed with 1 Hz line broadening, phased, and baseline corrected using Felix 97 software running on a Silicon Graphics workstation. The resulting spectra were aligned using the DSS signal as reference, reduced to the real part, saved as ASCII files, and transferred to a personal computer for data analysis.

Multivariate Analysis. A. Principal Component Analysis (PCA). PCA was applied to the data matrix for several reasons. First, it is essential to reduce the number of variables (data points) in order to use the chosen classification method of linear discriminant analysis (LDA). Use of LDA requires that the number of variables should not exceed the number of samples. The PCA calculation produces two matrices called the scores and the loadings. The rows of the scores matrix correspond to the samples (as in the data matrix), whereas the columns are the principal components (PCs): the scores in a given column can be regarded as the samples' coordinates for that PC axis. PCA extracts and preserves the "useful" information in an ordered way. Successive PCs account for decreasing amounts of variance and are uncorrelated in contrast to the original variables. These properties mean that the higher PCs can be discarded, and the number of variables is reduced without losing information. PCA allows data patterns to be more clearly visualised. Plots of the scores for different pairs of PCs can reveal which samples have similar properties: they appear as clusters on the two-dimensional plots. Each column vector of the loadings matrix represents one PC and the elements of the vector relate that PC to the original variables. The column plots have the same form as the NMR spectra and, to some extent, provide interpretable chemical information on the factors responsible for the clustering.

B. Linear Discriminant Analysis (LDA). The LDA classification procedure implemented here takes the scores as inputs and uses the Mahalanobis distance as the measure of distance between samples (*8*). The available samples are divided into training and validation sets. Using the training set, the mean positions of the predefined groups (orange juices and pulp washes) are calculated in the multidimensional space defined by the PCs. Each sample in the training set is then reallocated to the group with the nearest mean position, and the result is compared to the original (correct) assignment. The analyst has to determine how many PCs are required to give the best predictive model. A series of models is examined, starting from one PC and adding one PC at a time. The optimal number of PCs is determined from the classification success rate for the training set. For the model to be acceptable, a comparable success rate has to be obtained when it is applied to the validation set (see Results and Discussion).

Stepwise LDA differs from the classical LDA procedure in the choice of PCs to be used (*10*). For classical LDA, the PCs are taken in order PC1, PC2, PC3, etc. Due to the properties of PCA, PC1 always accounts for the greatest variance followed by PC2, then PC3, etc. In the present case, the spectra of samples from the two groups (orange juice and pulp wash) are so similar that the natural variation in composition within each group of samples is as great as or even greater than the variation observed between the two groups. The samples are clustered in unbiased fashion according to the quantitative differences observed between their spectra. The first few PCs may account for the majority of the variance in the data set, but the information may not be the most relevant for distinguishing orange juice from pulp wash samples. The stepwise LDA procedure picks out, as the first PC in the model, the one that gives the highest classification success rate. The next and subsequent PCs are added to the model using the same criterion regardless of the proportion of variance associated with them. Again, it is important to validate each model by classifying new samples from both groups in a test set.

C. Partial Least Squares (PLS), Canonical Variates Analysis (CVA). The data were also analyzed using PLS for the data reduction step. Two methods were used for the discrimination: LDA and CVA. Model building and LDA are carried out as above with the scores from PLS replacing those from PCA. PLS data reduction is carried out on a training set and requires information on group membership to be supplied with the data matrix. The two classification procedures have different possible outcomes. In LDA the sample has to be assigned to one of the two groups, whereas in CVA it can be assigned to "neither" if it falls outside the tolerance limits determined for each group. Kemsley (*8*) has described PLS/LDA and PLS/CVA procedures in detail.

Software. PCA was carried out in Matlab, version 4.2c.1 (The MathWorks Inc., Natick, MA). LDA and stepwise LDA procedures were carried out with in-house Matlab macros for model building and validation. PLS/LDA and PLS/CVA procedures were carried out using WINDAS software (*8*).

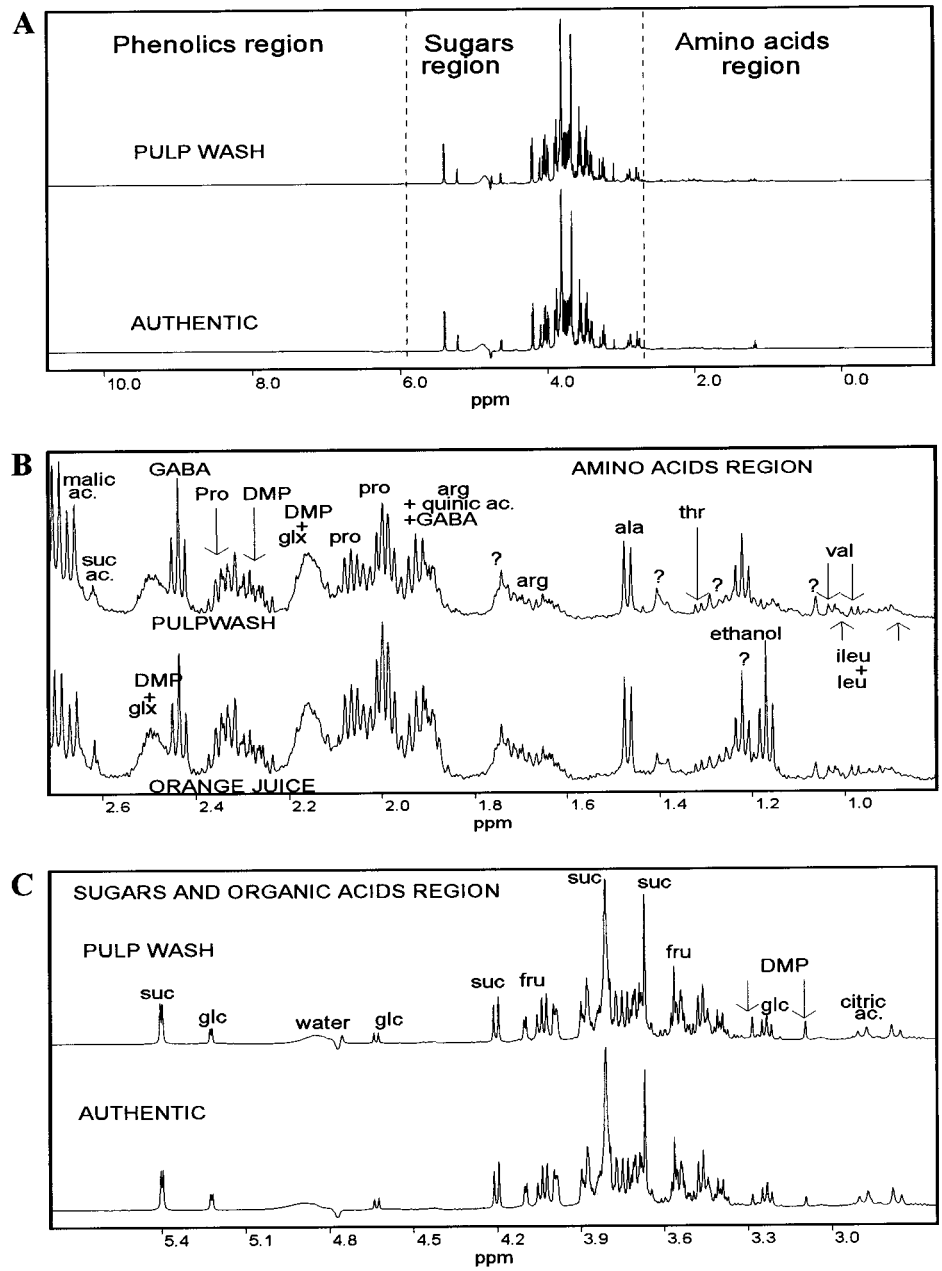


Figure 1. (A) 500 MHz ^{1}H NMR spectra of typical orange juice and pulp wash samples (overall view); (B) expanded view, high-field region (key: suc. ac., succinic acid; GABA, γ-aminobutyric acid; glx, glutamine/glutamic acid; DMP, dimethylproline); (C) midfield region (key: suc, sucrose; glc, glucose; fru, fructose).

For each spectrum, 4140 points were extracted from the original 8192 points using a PASCAL program written in-house. The regions containing water and ethanol signals as well as parts of the spectrum that did not contain any signals were excluded. Two-thirds of the samples made up the training set, and the remaining one-third constituted the validation set. The samples in the data matrix were ordered so that the authentic juices came first followed by the pulp washes. Within these groups there was a further division of samples into "single strength" and "originally from concentrate". The order of samples was otherwise random. Every third sample (3, 6, 9, etc.) was placed in the validation set. Essentially the same results as those described here were obtained when samples 1, 4, 7, etc., or samples 2, 5, 8, etc., were chosen as the validation set.

The effects of various data processing treatments (normalization, scaling) on the MVA were determined. The data matrix was normalized by adjusting the total intensity of each spectrum (row) to unity. In principle this should remove the variation resulting from use of samples with different Brix values, but in fact normalization had little effect on the discrimination success rate. PCA was carried out using both the covariance and correlation methods (*8*). In the covariance method the data matrix is mean-centered; in the correlation method the matrix is mean-centered and scaled so that all columns have a mean of zero and variance of unity. Results presented here are from correlation method PCA as this was found to give slightly better discrimination.

F values and box plots were calculated using the Matlab macro "anova1" (Statistics toolbox). The macro was modified to allow calculation of the *F* value for every point across the spectrum.

RESULTS AND DISCUSSION

Signal Assignments. Figure 1A shows typical orange juice and pulp wash ^{1}H NMR spectra. After water suppression, the spectrum was dominated by signals from the main sugars and acids. The ^{1}H NMR spectra

Table 2. ^{1}H Chemical Shifts of Compounds in Orange Juice and Pulp Washes from 1D and 2D (COSY, HOHAHA) NMR

compound	chemical shifts (ppm)							
Val	0.98	1.03	2.26					
ethanol	1.17	3.65						
?	1.22	3.72	3.95					
?	1.26	3.03						
Thr	1.32		4.26					
Ala	1.47	3.79						
Arg	1.68	1.9	3.25	3.76	7.22			
Orn?	1.74	3.03						
quinic acid	1.88	2.09	4.02					
GABA	1.93	2.45	3.03					
Pro	1.99	2.07	2.33	3.34	3.41	4.12		
DMP	2.15	2.28	2.49	3.09	3.28	3.53	3.69	4.07
Glx	2.15	2.49						
?	2.32	2.96						
?	2.42	2.63	4.23					
malic acid	2.68	2.85	4.42					
citric acid	2.76	2.82						
Asx	2.85	2.95	4.00					
succinic acid	2.63							
β-Fru	4.10							
β-Glc	4.62							
α-Glc	5.23							
Suc	5.41	4.20						
narirutin?	6.25	6.97	7.44					
Tyr	6.89	7.18						
Phe	7.32	7.42						
His	8.65							

are conveniently viewed as three separate regions because of the very large range of signal intensities between regions. For ease of reference the regions are named in Figure 1A according to the category of compound that gives rise to the majority of signals for each region (phenolics, sugars, amino acids). Parts B and C of Figure 1 depict in detail the signals observed in two of the regions. The amount of information is so great that, for any systematic comparison of a large number of samples, the need for multivariate analysis becomes obvious. Apart from the presence of the ethanol triplet at 1.17 ppm in the orange juice spectrum (Figure 1B), there is little obvious difference between the orange juice and pulp wash spectra. In fact, the orange juice shown was a single-strength sample, whereas the pulp wash was from a concentrate. Ethanol was found only in single-strength and hand-squeezed samples and was absent from concentrates, whether orange juice or pulp wash. As it was obviously not a differentiating factor between juice and pulp wash, the ethanol regions were excluded from the data matrix. The signal-to-noise ratio in the phenolic region is quite poor, and this part of the spectrum remains largely unassigned mainly because of a lack of relevant reference compounds. However, it is an important region for discrimination and will be discussed later.

NMR assignment is essential to determine the origin of the signals that are shown by MVA to be responsible for the discrimination between different groups of products. To assist the assignment, ^{1}H NMR spectra of standards of amino acids, organic acids, sugars, etc., known to be present in orange juice (*11*) have been run under the same analytical and spectrometric conditions as the juice samples. Another way to assign the juice spectra is through the use of 2D NMR experiments: COSY or HOHAHA for ^{1}H−^{1}H correlation and HMQC (or HMBC) for ^{1}H−^{13}C correlation, although the last two methods were not used in this case. COSY spectra of orange juice and pulp wash were obtained and assigned

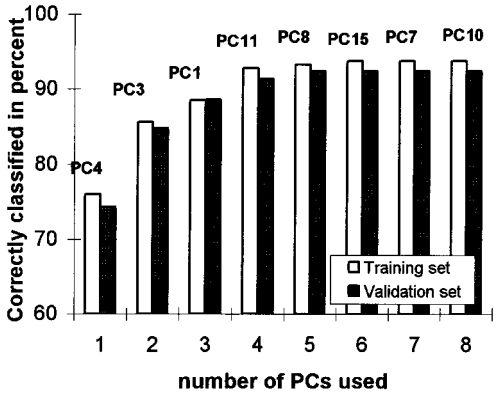


Figure 2. Dependence of LDA classification success rates on number of PCs included in the model. The PC added at each stage of the stepwise LDA procedure is indicated. Correlation method PCA was used.

as described previously for other fruit juices (*12*, *13*). Table 2 summarizes the chemical shift information available for orange juice from the 2D spectrum and the reference standards.

Discrimination between Orange Juice and Pulp Wash. The data set comprised 263 authentic and 50 pulp wash samples each represented by a ^{1}H NMR spectrum of 4140 points. PCA was carried out on the training set samples, and the scores were used to create classification models based on different numbers of PCs as described. The validation set samples were projected into the multidimensional space of each model, and the reclassification success rate was determined using the criterion of least distance from the group mean position (determined from the training set). The classification success rate was compared to that of the training set samples for each model.

Figure 2 presents the results of the classification for the two types of orange product. It shows the percentage of correctly classified samples versus the number of PCs included in the model. Comparison of results for training and validation sets showed no evidence of overfitting as similar rates of success were seen for reclassification (training set) and assignment (validation set). Nor was there evidence of underfitting as the success rate climbed rapidly to 88% using the first three PCs but then increased slowly for each subsequent PC. Models based on five and six PCs were almost equally good with only two fewer samples of the training set correctly identified by the five-PC model. The model with six PCs reached 94% correctly classified samples, wrongly assigned samples amounting to 13 of 208 for the training set (10 authentic seen as pulp wash and 3 pulp wash seen as authentic) and 8 of 105 incorrect for the validation set (4 authentic seen as pulp wash and 4 pulp wash seen as authentic). The predictive ability of the six-PC model was good enough to classify correctly 84 of 88 authentic samples and 13 of 17 pulp washes from the test set. The quality of the statistical model is judged by its ability to accommodate the natural variability of orange juice so that few authentic samples are rejected, but at the same time it has to be discriminating enough to reject the pulp wash samples. The six-PC model efficiently met those two requirements. A six-factor LDA model incorporating scores from PLS data reduction identified 87 of 88 orange juices and 16 of 17 pulp washes correctly in the test set. The CVA model, also with six PLS factors, gave only one wrongly identified

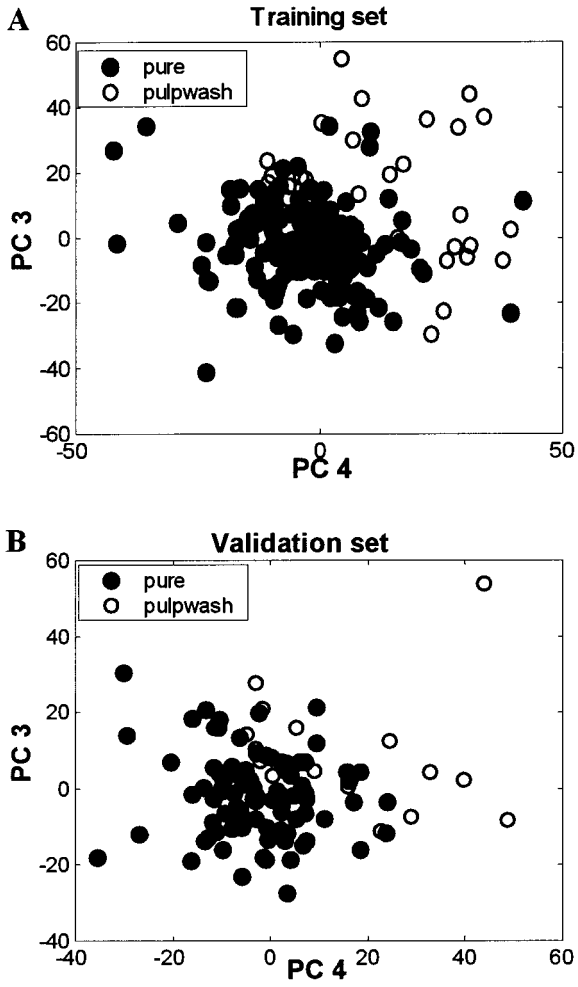


Figure 3. PC scores on the first two PC axes (PC4 and PC3) selected by the stepwise LDA procedure: (A) scores from PCA of the training set spectra; (B) scores for the validation set calculated using the training set PC loadings.

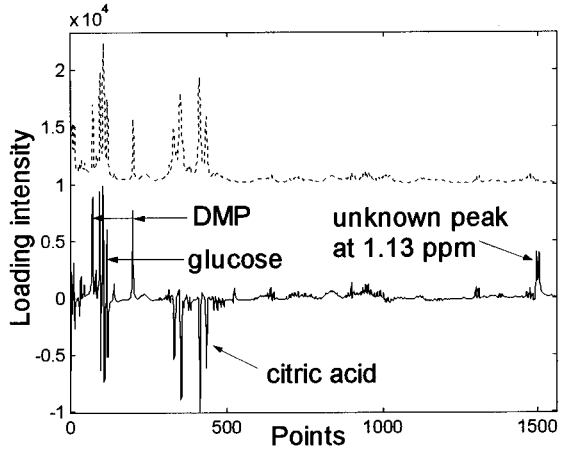
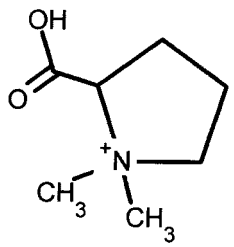


Figure 4. PC4 loading (lower solid line) and mean spectrum of all samples (upper dashed line), high-field region.

sample (pulp wash identified as orange), but 7 of 88 orange juices and 6 of 17 pulp washes were assigned to neither group. This gives a better picture of the number of borderline cases than does LDA.

Although these results are encouraging, other techniques have been reported to detect pulp wash addition at levels of 10% (NIR/PCA with a 20-factor model; *5*) or even less (capillary electrophoresis/neural network; *14*). It was decided not to proceed with NMR experiments on samples at this level of adulteration because, to succeed, one would expect to have complete success in discriminating pulp wash itself from orange juice. NIR spectroscopy is perhaps more successful than NMR because the samples are examined as dried solids and signals may be present from components (insoluble phenolics, pectin) that are not detected by NMR. Howevef, no definite information on which components were responsible for the success of the discrimination could be obtained from the NIR data (*5*). NMR is more promising in this regard.

Stepwise LDA has the advantage of selecting first the PCs that give the best discrimination between the two groups. Figure 3 shows the plot of the sample scores on PC4 versus PC3, the first two PCs selected. It clearly appeared that the "pure" group samples were located on the negative part of PC4, whereas the "pulp wash" ones tended more to the positive side. The chemical origin of the discrimination was partly revealed in the loading of PC4 (Figure 4). For example, the loading at the position of the citric acid signals was negative, which means that the "pure" samples (more negative scores on PC4) on average contained more citric acid than the pulp washes. More interestingly, the loadings corresponding to a set of signals later identified as belonging to dimethylproline (**1**), as well as those of an unknown



compound (1.13 ppm), were positive, meaning that their content appeared to be greater in the pulp wash group than in the pure one. Dimethylproline was not previously reported as a marker compound for the discrimination between orange juice and pulp wash. The identification of this molecule was based on the shifts obtained from the COSY spectrum of an orange juice (Table 2) and by comparing these with the chemical shifts quoted by Blunden et al. (*15*). Dimethylproline signals indeed appeared more prominently in a second COSY spectrum of a pulp wash sample. The most noticeable signals in the 1D spectra were the two methyl singlets at 3.09 and 3.28 ppm, flanking the β-Glc H-2 multiplet at 3.26 ppm. Rapp et al. (*16*) first pointed to the presence of dimethylproline in orange juice in a ^{13}C NMR study, although not in the context of authentication. They reported that the dimethylproline concentration in orange juice was between 240 and 700 mg L^{-1}, making dimethylproline one of the major compounds in orange juice after sugars and organic acids.

Figure 5A presents a histogram summarizing the intensity distribution of one of the methyl signals of dimethylproline (3.28 ppm) in the authentic and pulp wash samples. The height of the methyl signal was obtained after normalization (to a value of 1) of the total spectral intensity for each sample, to compensate for differences in Brix value. The authentic group approximately followed a normal population distribution with a mean intensity of 1.7×10^{-3}. The pulp wash

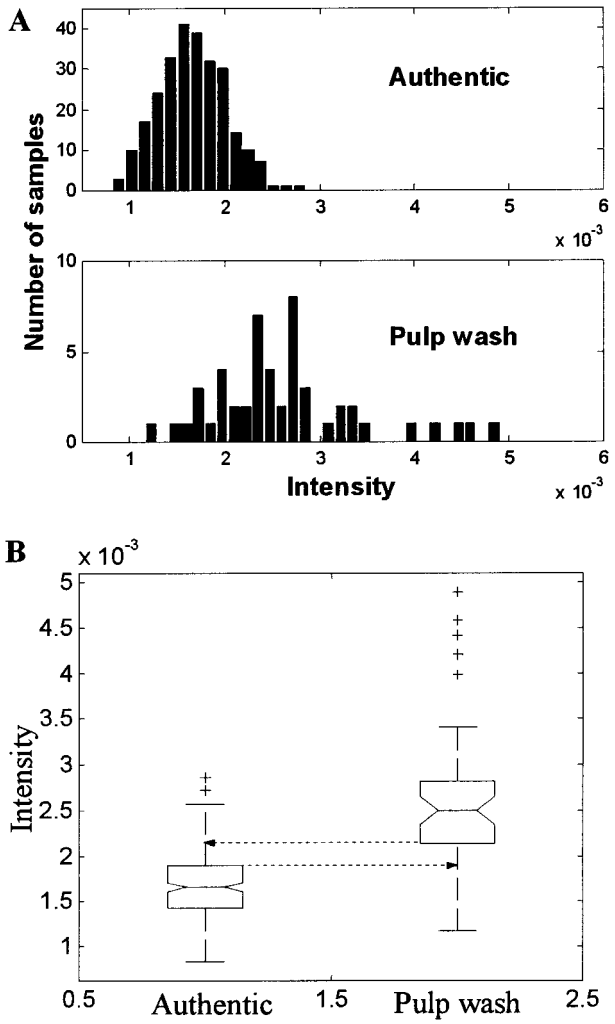


Figure 5. Signal intensity distributions for methyl signal (3.28 ppm) of DMP, comparison of orange juice ("authentic") and pulp wash groups: (A) histograms; (B) box plots showing medians, confidence intervals (notches), range, and outliers (+).

group distribution was less well-defined (because of the smaller number of individuals). The spread of the intensities was greater than for the authentic samples, and the mean value was 2.8×10^{-3}. The notched box plot in Figure 5B gives another view of the dimethylproline distribution between the two groups. The three horizontal lines of each box are at (from bottom to top) the 25th percentile, median, and 75th percentile values. The median values clearly differed, whereas the notches, which represent a robust estimate of uncertainty about the median, did not overlap. ANOVA (*17*) gave an F value of 188 (with degrees of freedom of 1 and 311, an F value of 10.8 corresponds to $P < 0.001$), which clearly rejected the null hypothesis that the two populations were not significantly different. However, the range of values (indicated by the vertical lines and outlier symbols) did overlap considerably. In the case of citric acid signals, the medians were also significantly different, but the range for the authentic group was very wide and completely overlapped the rather narrow range for pulp wash. Thus, neither dimethylproline nor citric acid levels on their own can be used to distinguish orange juice from pulp wash, although both play a part in the multivariate discrimination.

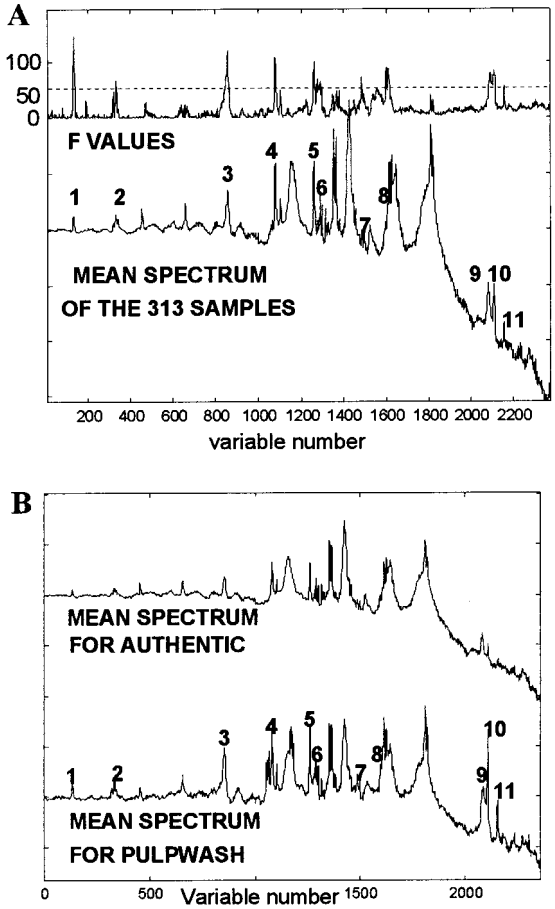


Figure 6. (A) F values and (below) mean spectrum of all samples, low-field ("phenolic") region; (B) mean spectra for authentic and (below) pulp wash samples, phenolic region. Chemical shifts (parts per million) of numbered peaks: 1, 9.12; 2, 8.82; 3, 8.06; 4, 7.73; 5, 7.46; 6, 7.42; 7, 7.13; 8, 6.96; 9, 6.25; 10, 6.21; 11, 6.15.

It was of interest to explore what other signals or compounds might be important for the discrimination so a Matlab macro was written to extend the calculation of the F value to every data point in the spectrum. A plot of the F values thus obtained should reveal the signals of potential marker compounds, that is, those associated with the highest F values. Figure 6A shows the F values compared with the mean spectrum of all samples (authentic plus pulp wash) in the low-field or "phenolic" region. The 11 peaks or multiplets with the highest F values (all with $F > 50$) are indicated on the mean spectrum. Plotting the mean spectrum for each group separately revealed that it was always the pulp wash samples that seemed to contain more of these unknown compounds (Figure 6B). Some differences of pattern between the two mean spectra were not picked up by the ANOVA procedure: the multiplet just to the left of peak 4, for example, appeared in the mean pulp wash spectrum but not at all in the authentic one. However, examination of the individual pulp wash spectra showed that this multiplet had an extremely high value in only one sample and was not characteristic of pulp wash.

The assignment of the unknown 11 signals needs to be done to determine if they are from known or novel marker compounds. This might be achieved by measuring ^{1}H spectra of suspected compounds (if available as reference standards) or by using HPLC NMR. A recent

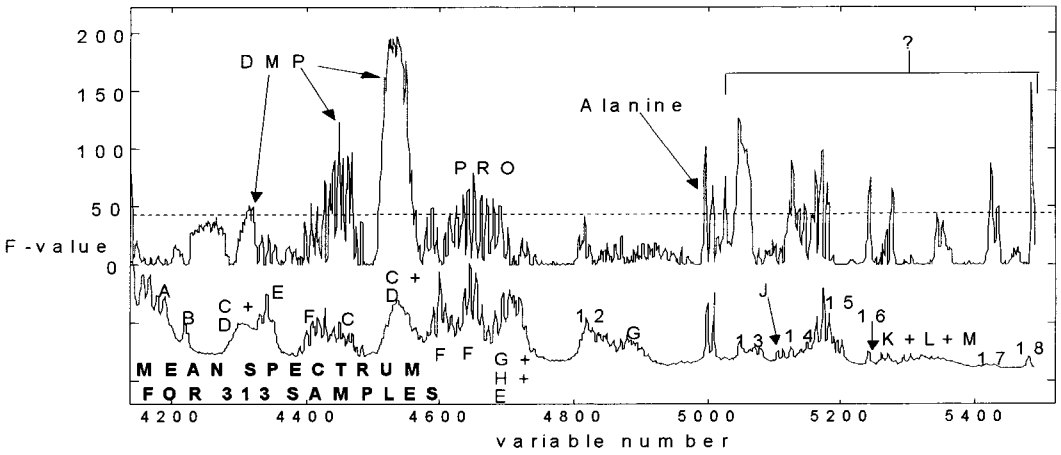


Figure 7. *F* values and (below) mean spectrum in the high-field region (key: A, malic ac.; B, succinic ac.; C, DMP; D, glx; E, GABA; F, pro; G: arg; H, quinic ac.; I, ala; J, thr; K, val; L, ileu; M, leu). Chemical shifts (parts per million) of numbered peaks: 12, 1.75; 13, 1.37; 14, 1.29; 15, 1.22; 16, 1.06; 17, 0.8; 18, 0.71.

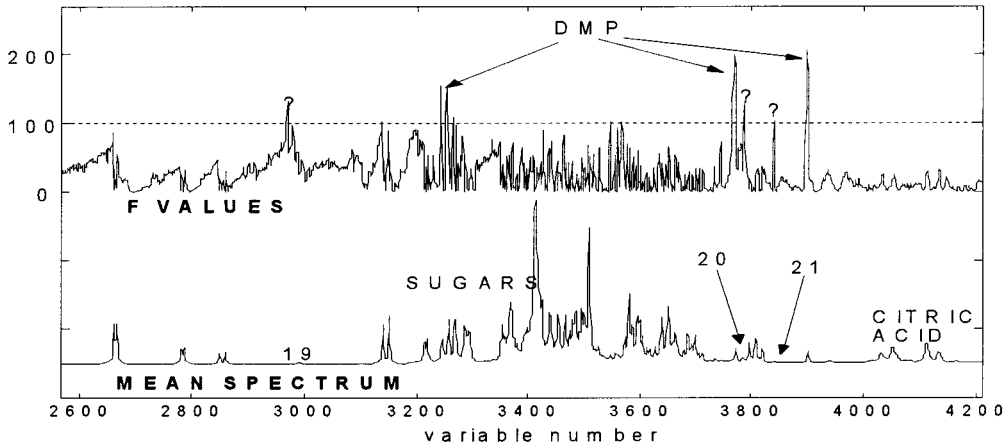


Figure 8. *F* values and (below) mean spectrum in the midfield region. Chemical shifts (parts per million) of numbered peaks: 19, 4.45; 20, 3.27; 21, 3.18.

paper (*14*) has examined the potential of some phenolic compounds as markers for pulp wash adulteration. The study concluded that the three most useful marker compounds were didymin, narirutin, and phlorin. Some of the unknown signals mentioned above, for example, 9, 10, and 11, in Figure 6B, at ~6.2 ppm, are possibly from one or more of these compounds (*18*, *19*), although it is difficult to be certain as the appropriate reference standards were not available. In a previous NMR/PCA study (*6*) orange juices and pulp washes were distinguished on the first PC, although only three pulp wash samples were used. It was proposed that aromatic compounds such as naringenin or hesperidin were partly responsible for the discrimination. Signals of hesperidin could not be positively identified in our spectra despite comparison with a standard, but on the basis of NMR data for naringin (*20*), some possible signals of narirutin were identified (Table 2), including peaks 6 and 8 and one at 6.25 ppm in the region of peaks 9−11. HPLC analysis of two pulp wash samples, one in which NMR signals 9−11 were essentially absent and a second in which they were very prominent, showed an 8-fold increase of one peak in the second sample. It was assigned as a simple phenolic glycoside with a UV spectrum identical to that of phlorin (*19*). The flavanone glycosides increased by only 2-fold at most. Peaks 9−11 were therefore assigned to phlorin or closely related compounds. Hesperidin is present in high total amounts in pulp wash but in a very insoluble form (*2*): stronger NMR signals may result from a more readily soluble compound, such as phlorin, even though its total amount is lower.

In the high-field and midfield regions there is the opportunity to compare the signals picked out with high *F* values with those that were important on the PC4 loading discussed above (Figure 4). Figure 7 shows a plot of the *F* values together with the mean spectrum of all samples in the amino acid region. The ANOVA procedure picked out signals of dimethylproline, proline, and alanine with $F > 50$. Between 1.4 and 0.7 ppm there were a number of unassigned signals (13−18), associated with quite minor features of the mean spectrum (intensities comparable with the methyl signals of valine, leucine, and isoleucine). The most notable with $F > 100$ are the broad signal 13 (δ 1.4) and the singlet 18 (δ 0.7).

The midfield region contained strong signals from the sugars and citric acid. Here the indicator *F* value has been arbitrarily set to 100 because identification of signals with lower *F* values is not easy in the center of the spectrum (Figure 8). The highest *F* values were given by the *N*-methyl signals of dimethylproline plus other more minor peaks (labeled 19−21). The identification of dimethylproline as a marker compound agrees with the conclusion drawn from the PC4 loading, which indicated higher average concentrations of dimethyl-

proline in pulp wash compared with authentic juices. The citric acid was shown in the same loading (Figure 4) to be at lower average concentration in pulp wash than in authentic samples, whereas an unknown compound (doublet at δ 1.13) appeared like dimethylproline to have a higher concentration. In the ANOVA test, however, differences in level of citric acid and the unknown compound between authentic and pulp wash groups were not seen as significant. For citric acid this was probably because it was found at a very wide range of levels across all samples. For the unknown compound, examination of individual spectra showed that it was only found in a few of the hand-squeezed samples but was present in these at a very high level, possibly from a deterioration process. This shows the need for caution in an oversimplified interpretation of information from the PC loadings. The loadings give some novel and valuable chemical information about the underlying difference between the two groups of samples in the case of dimethylproline but may tend to overemphasize the importance of other compounds, particularly if their distribution is somewhat unusual.

This work has shown that NMR combined with multivariate analysis appears to be a suitable tool for orange juice authentication. Classification models were created using a training data set and revealed to be robust using a validation set. Ninety-four percent of the samples were correctly classified using six PCs. The analysis of the loadings also explained that a key to the difference between authentic and pulp wash juices lay, among other things, in the difference in the level of dimethylproline. This compound was not previously known as a marker compound for recognition of pulp wash addition. An ANOVA test performed for authentic and pulp wash groups on each point of the spectrum revealed that the signals of dimethylproline, together with numerous other signals, were significantly different when the two groups were compared. Dimethylproline and several other compounds with NMR signals identified but not yet characterized are therefore shown to be potential marker compounds for the orange juice adulteration.

Of course, to make the NMR and multivariate analysis method practical, detection of low-level adulteration is required. This could be the object of further developments of the method. However, the advantages in terms of rapidity, simplicity, and diversity of information obtained make the technique very attractive. Although only pulp wash adulteration has been considered here, the spectra of a large number of authentic juices were collected and will provide a database for investigations of other types of adulteration. Similar databases of FTIR spectra have been incorporated in software that is suitable for use in factories (*21*) for the authentication of raspberry and strawberry purees (*22*, *23*).

ABBREVIATIONS USED

CVA, canonical variates analysis; DMP, dimethylproline; LDA, linear discriminant analysis; MVA, multivariate analysis; PCs, principal components; PCA, principal component analysis; PLS, partial least squares

ACKNOWLEDGMENT

We thank CSL (Central Science Laboratory, York, U.K.) for supplying many orange and pulp wash samples, Francesco Casuscelli for initial NMR assignments, and Marianne Defernez for advice and help with Matlab.

LITERATURE CITED

(1) Kurtzweil, P. Fake Food Fight. *FDA Consumer* **1999**, *33* (2, March−April).
(2) Widmer, W. W.; Cancalon, P. F.; Nagy, S. Methods for determining the adulteration of citrus juices. *Trends Food Sci. Technol.* **1992**, *3*, 278−286.
(3) Hammond, D. A. Authenticity of fruit juices, jams and preserves. In *Food Authentication*; Ashurst, P. R., Dennis, M. J., Eds.; Blackie: London, U.K., 1996; Chapter 2, pp 15−59.
(4) Dettmar, H. P.; Barbour, G. S.; Blackwell, K. T.; Vogl, T. P.; Alkon, D. L.; Fry Jr., F. S.; Totah, J. E.; Chambers, T. L. Orange juice classification with a biologically based neural network. *Comput. Chem.* **1996**, *20*, 261−266.
(5) Twomey, M.; Downey, G.; McNulty, P. B. The potential of NIR spectroscopy for the detection of the adulteration of orange juice. *J. Sci. Food Agric.* **1995**, *67*, 77−84.
(6) Vogels, J. T. W. E.; Terwel, L.; Tas, A. C.; Van Der Berg, F.; Dukel, F.; Van Der Greef, J. Detection of adulteration in orange juices by a new screening method using NMR spectroscopy in combination with pattern recognition techniques. *J. Agric. Food Chem.* **1996**, *44*, 175−180.
(7) Krzanowski, W. J. *Principles of Multivariate Analysis*: *A User's Perspective*; Oxford University Press: New York, 1988.
(8) Kemsley, E. K. *Discriminant Analysis and Class Modelling of Spectroscopic Data*; Wiley: Chichester, U.K., 1998.
(9) Hull, W. E. Experimental aspects of two-dimensional NMR. In *Two-Dimensional NMR Spectroscopy. Applications for Chemists and Biochemists*; Croasmun, W. R., Carlson, R. K., Eds.; VCH: New York, 1994; Chapter 2, p 125.
(10) Bertrand, D.; Courcoux, P.; Autran, J.-C.; Meritan, R.; Robert, P. Stepwise canonical discriminant analysis of continuous digitalized signals: application to chromatograms of wheat proteins. *J. Chemom.* **1990**, *4*, 413−427.
(11) VdF (Association of the German Fruit Juice Industry). *RSK-Values, the Complete Manual*; Flüssiges Obst: Schönborn, Germany, 1987.
(12) Belton, P. S.; Delgadillo, I.; Holmes, E.; Nicholls, A.; Nicholson, J. K.; Spraul, M. Use of high-field ^{1}H NMR spectroscopy for the analysis of liquid foods. *J. Agric. Food Chem.* **1996**, *44*, 1483−1487.
(13) Gil, A. M.; Duarte, I. F.; Delgadillo, I.; Colquhoun, I. J.; Casuscelli, F.; Humpfer, E.; Spraul, M. Study of the compositional changes of mango during ripening by use of nuclear magnetic resonance spectroscopy. *J. Agric. Food Chem.* **2000**, *48*, 1524−1536.
(14) Cancalon, P. F. Analytical monitoring of citrus juices by using capillary electrophoresis. *J. AOAC Int.* **1999**, *82*, 95−106.
(15) Blunden, G.; Gordon, S. M. Betaines and their sulphonio analogues in marine algae. *Prog. Phycol. Res.* **1986**, *4*, 39−80.
(16) Rapp, A.; Markowetz, A.; Niebergall, H. Anwendung der ^{13}C NMR spektroskopie in der aminosaureanalytik in wein und fruchtsaften. *Z. Lebensm. Unters. Forsch.* **1991**, *192*, 1−6.
(17) Wonnacott, T. H.; Wonnacott, R. J. *Introductory Statistics*; Wiley: New York, 1990.
(18) Berhow, M.; Tisserat, B.; Kaves, K.; Vandercook, C. *Survey of Phenolic Compounds Produced in Citrus*; USDA Technical Bulletin 1856; U.S. GPO: Washington, DC, 1998.
(19) Louche, L. M.-M.; Gaydou, E. M.; Lesage, J.-C. Determination of phlorin as peel marker in orange (*Citrus*

sinensis) fruits and juices. *J. Agric. Food Chem.* **1998**, *46*, 4193−4197.
(20) Colquhoun, I. J.; Goodfellow, B. J. Chiral recognition by cyclodextrins: the interaction of naringin with β-cyclodextrin, *J. Chem. Soc., Perkin Trans. 2* **1994**, 1803−1807.
(21) Wilson, R. H.; Colquhoun, I. J.; Kemsley, E. K. Screening food products for authenticity and adulteration. *Am. Lab. News* **2000**, in press.
(22) Kemsley, E. K.; Holland, J. K.; Defernez, M.; Wilson, R. H. Detection of adulteration of raspberry purees using infrared spectroscopy and chemometrics. *J. Agric. Food Chem.* **1996**, *44*, 3864−3870.
(23) Holland, J. K.; Kemsley, E. K.; Wilson, R. H. Use of Fourier transform infrared spectroscopy and partial least squares regression for the detection of adulteration of strawberry purees. *J. Sci. Food Agric.* **1998**, *76*, 263−269.

Received for review August 24, 2000. Revised manuscript received November 14, 2000. Accepted November 17, 2000. We thank the U.K. MAFF Link program on Agro-Food Quality for funding (AFQ119).

JF001046E