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Coupling Proton Transfer Reaction−Mass Spectrometry with Linear Discriminant Analysis: a Case Study

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Proton transfer reaction−mass spectrometry (PTR-MS) measurements on single intact strawberry fruits were combined with an appropriate data analysis based on compression of spectrometric data followed by class modeling. In a first experiment 8 of 9 different strawberry varieties measured on the third to fourth day after harvest could be successfully distinguished by linear discriminant analysis (LDA) on PTR-MS spectra compressed by discriminant partial least squares (dPLS). In a second experiment two varieties were investigated as to whether different growing conditions (open field, tunnel), location, and/or harvesting time can affect the proposed classification method. Internal cross-validation gives 27 successes of 28 tests for the 9 varieties experiment and 100% for the 2 clones experiment (30 samples). For one clone, present in both experiments, the models developed for one experiment were successfully tested with the homogeneous independent data of the other with success rates of 100% (3 of 3) and 93% (14 of 15), respectively. This is an indication that the proposed combination of PTR-MS with discriminant analysis and class modeling provides a new and valuable tool for product classification in agroindustrial applications.

KEYWORDS: Proton transfer reaction−mass spectrometry; *Fragaria* spp.; discriminant analysis

INTRODUCTION

Quality control, variety selection, product development, etc., are typical areas of food production in which it is important to compare samples under study with previously identified references and to monitor whether there exist differences in order to take on site corresponding decisions. This issue can be approached in two different ways depending on goals and the resources available: on the one hand, a few quickly measurable quantities such as, for fruits, sugar content, acidity, and firmness (*1*) or, on the other hand, more advanced and sensitive methods (GC, GC-MS, enzymatic tests, sensory analysis, etc.) yielding more detailed information (*2*). The investment in time and money needed for the latter imposes usually strong restrictions and often limits the applicability to only a few, statistically selected samples. There exist strong efforts in the scientific community to improve this situation by developing fast, sensitive, and nondestructive techniques that can be used routinely for real time evaluation and classification of the food samples (*3*). In this context the evaluation of volatile organic compounds (VOCs) provides a good way to check the samples, because the amount of VOCs is often connected both with their intrinsic properties (e.g., ripening degree, defects, shelf life evolution, effect of treatments) and with the quality perceived by the consumer (see, e.g., ref *4* and reference cited therein). A classical example, in the context of the present studies, is the use of spectroscopic methods (*5*).

Mass spectrometry based on traditional electron impact ionization usually has the disadvantage that the VOC molecules to be analyzed are strongly fragmented in the ionization process and thus the analysis, without additional measures (e.g., separation prior to measurements), is difficult in the case of complex mixtures. Nevertheless, there exist situations in which this method has been considered (*6*). On the other hand, previous investigations demonstrated that on-line analysis of VOCs of potential agroindustrial interest can be successfully performed (*7*, *8*) by the relatively new method of proton-transfer reaction mass spectrometry (PTR-MS) (*9*), and we have shown that a multivariate analysis of PTR-MS spectra allows interesting product discrimination for fruit juices treated with different preserving methods (*10*). The successful use of this method is mainly due to the high sensitivity (*11*), to the fact that sample pretreatment is not necessary (*9*), and, above all, to the fact that fragmentation of the molecules in the mass spectrometric detection process is strongly reduced as compared to that in conventional ionization techniques (*12*). Because of these advantages we extended here our previous PTR-MS studies in

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Table 1. Samples Used for Experiment 1: Comparison of Nine Selections[a]

code	species	variety	parental ♀		parental ♂	no. of measured fruit samples
FB	*Fragaria vesca*					3
CS2	*Fragaria* × *ananassa*	94,568,2	Miss	×	USB 35	3
CS10	*Fragaria* × *ananassa*	96,62,10	91,143,5	×	Miss	4
CS7	*Fragaria* × *ananassa*	96,62,7	91,143,5	×	Miss	3
CS4	*Fragaria* × *ananassa*	97,269,4	Darselect	×	91,143,3	3
VR1	*Fragaria* × *ananassa*	VR 96,57,1	91,143,5	×	89,250,2	3
VR2	*Fragaria* × *ananassa*	VR 96,58,2	91,143,5	×	90,608,1	3
VR5	*Fragaria* × *ananassa*	VR 97,64,5	Darselect	×	89,384,20	3
Patty	*Fragaria* × *ananassa*	Patty (91,290,2)	Marmolada	×	Honeoye	3

[a] The short codes used in the paper are given in the first column. Commercial varieties are indicated by their names; selections under evaluation are indicated by their codes. No variety designation is available for the *F. vesca* fruits.

Table 2. Data for the Fruit Samples Used for Experiment 2: Comparison of Two Varieties (CS2 and Miss) from Three Different Batches[a]

batch	harvest date	1st measurement	2nd measurement	cultivation method	produced in
A	April 29, 2002	April 29, 2002	May 4, 2002	tunnel	Verona
B	May 9, 2002	May 10, 2002	May 13, 2002	tunnel	Cesena
C	May 17, 2002	May 17, 2002	May 21, 2002	open field	Cesena

[a] Measurements on days 2, 3, 5, 6, 8, 9, and 12 after harvesting are also available for batch A.

order to further evaluate the potentiality of PTR-MS in the agroindustrial field.

As a case study we evaluate here the possibility to distinguish different strawberry cultivars on the basis of measured PTR-MS spectra analyzed with a particular implementation of a discriminant partial least-squares (dPLS) multivariate analysis. We closely follow the ideas reported by Beebe and Kowalsky (*13*) in the form described by Kemsley (*14*) and implemented in the software WINDAS (*15*). An exhaustive review of these methods can be found in Kemsley's book (*14*) and references cited therein.

The presently introduced technique to carry out measurements and analysis on single fruits (of strawberries) is also of importance, because consumer judgment is on single fruits and not on batch averages (usual quality control methods refer to this). In this sense fruit-by-fruit variability (and thus its control) is crucial for cultivar qualification (*16*). We believe that the fast and individual measurement, the total absence of pretreatment (strawberries are virtually unaffected by the measurement process), and the promising discriminative power of the proposed approach are good bases for the development of an on-line quality/product control method which indeed may be implemented in the agroindustrial processing of fruits and vegetables.

MATERIALS AND METHODS

Samples. In the present study we have carried out two different sets of experiments, that is, one with nine cultivars where only three samples (fruits) per cultivar were collected at the same time, and a second one with two cultivars but from different batches.

Experiment 1. On May 24, 2002, strawberry fruits of nine different clones were collected in the experimental open field of the Istituto Sperimentale per la Frutticoltura (ISF) located in Forlì (Cesena, Italy) and immediately transported to the Agronomic Institute of S. Michele a/A, where measurements took place after 2 days of storage. The samples were stored between harvesting and the actual measurement at 4 °C. When these fruits were picked, no specific criteria for selection of the fruits were applied except for a rough evaluation of a proper ripening and the absence of evident defects or peculiarities. One of the clones was a commercial variety (Patty); the other ones were selections under evaluation by the ISF. The latter ones are here indicated by a short code, that is, CS2, CS10, CS7, CS4, VR1, VR2, and VR5. In **Table 1**, we report corresponding exact codes and the parents of the selected clones; full names indicate commercial varieties. Eight clones were *Fragaria* × *ananassa,* but we also included samples, indicated by FB, of a *Fragaria vesca* clone (closely related to the typical wild strawberry). These latter fruits showed a more pronounced evolution in time (drying) compared to the more stable *Fragaria* × *ananassa*. In contrast to other studies (*17*) that compared different commercial cultivars, we have here (besides Patty and FB) seven genotypes which have a rather close relationship to each other, two of them being even "brothers" (**Table 1**).

Experiment 2. Here, we collected fruit samples of two cultivar (CS2, same as above, and Miss, a commercial variety) at three different times, grown at two different locations (Cesena, Italy, and Verona, Italy) with two different cultivation methods (tunnel and open field). See **Table 2** for details. The fruit samples were stored between harvesting and the actual measurement at 4 °C. A first set of measurements took place within the first 24 h after harvesting and a second one after 3−4 days; this time delay should represent a typical situation for the fruits to reach, on average, the consumer's table. For one batch (batch A in **Table 2**) we have carried out for both clones additional measurements on days 2, 3, 5, 6, 8, 9, and 12 after harvest.

Measurements. PTR-MS is a mass spectrometric technique based on a particular implementation of chemical ionization using proton transfer from protonated water ions to the volatile substance to be detected. It has been described in many papers (*9*, *18*), and there exists also some literature concerning agroindustial applications [see, e.g., the review of Dunphy (*19*)]. The instrument used here is a standard commercial PTR-MS machine supplied by Ionicon Analytik GmbH, Innsbruck, Austria.

The usual measuring procedure involves first removing the fruits from the 4 °C storage space. After the fruits has remained at room temperature for ∼2 h, one fruit is put for 1 h in a glass vessel (400 mL) provided with two PTFE/silicone septa on opposite sides. After 1 h, the inlet of the PTR-MS was then connected by a $^1/_8$ in. PTFE tube heated to 70 °C with this glass vessel, and the headspace was continuously extracted for 4 min at 9.3 ± 0.1 sccm (corresponding to the acquisition of five complete spectra); the extracted headspace gas was replaced by laboratory air. To avoid possible systematic memory effects from one measurement to the next, the apparatus was flushed with laboratory air for 15 min between measurements, replicate order was randomized, and we used different glass vessels for each fruit. Spectra have been collected between subsequent measurements to control the decay of the signal to the background level.

We consider here spectra from a mass/charge ratio of 29 to 181 amu. We estimated the concentration in parts per billion using the relation (*9*)

$$\text{ppb} = \{1/(kt)\}\{[C^+]/[H_3O^+]\}\{(10^9(K_B)T)/P\} \quad (1)$$

where k is the reaction constant for the proton exchange reaction, t is the drift time in the reaction chamber, $[C^+]$ is the measured ion intensity (counts/s), $[H_3O^+]$ is the intensity of the primary ion beam (counts/s), K_B is the Boltzmann constant, T is the drift tube temperature, and P is the drift tube pressure. We use the same value $k = 2 \times 10^{-9}$ cm^3/s for all masses. This produces a systematic error that is for many compounds <30% (*9*) and not important for the proposed data analysis which requires only that the measuring conditions are constant. For masses 32 and 37 this equation cannot be applied as mass 32 is due to residual oxygen ions and mass 37 corresponds to water dimer ions and thus is, for fixed experimental conditions, a relative indication of the water content of the measured volatile mixture. We did not subtract background signals because the used data analysis is not sensitive to constant signals. We point out that all of the present measurements have been carried out with single, intact, strawberry fruits without any pretreatment.

Data Analysis. The problem of finding significant groups in data and to analyze subsequent measurements in terms of the presence of these groups is important in many fields ranging from quality control to social sciences (*20*). Several techniques have been proposed, but no general criteria can be given for the choice of a particular technique in a specific application. In this work we follow the ideas and notation used by Kemsley (*14*).

For spectroscopic or mass spectrometric data we are usually confronted with high dimensional data sets, that is, with a great number of data points (called here variates) for each sample, that is, the intensity of many peaks in a mass spectrum. Moreover, variates are often strongly intercorrelated. To handle such data and to reduce the intercorrelation among the considered variates, the analysis can be divided into two phases: (a) data compression to reduce the dimensionality using new variates called loading and (b) discriminant analysis to identify groups. For data compression we used principal component analysis (PCA) in both correlation (PCAcor) and covariance (PCAcov) forms (*21*) and discriminant partial least-squares (dPLS) analysis, which is a restriction of the partial least-squares technique of multivariate calibration (*22*). Due to the bias possibly introduced by the group information, dPLS is more likely affected by overfitting, but it is more selective in finding the variates responsible for group differences. Linear discriminat analysis (LDA) was performed by attributing single data points to the closest group. The distance is defined, here, as the distance between the test point under consideration and the center of the group, and we tested three possible distances: Euclidean, Manhattan, and Mahalanobis (*14*).

The potential danger of the described approaches is that the models could force the data fitting (overfitting) using not significant fluctuation or differences. This can be often the case in multivariate analysis because the number of observed variables (here the intensities of the spectral peaks) is usually much higher than the number of observations (*14*).

An important question in this context is, how many PLS scores (or PCA scores) should we use to obtain as much information as possible without overfitting the model? Statistical considerations can give an estimate of the confidence of the model, but they rely on assumptions (normality) that may not be correct or are difficult to check in the case one does not have many measurements. In ref *14* a decision tree is proposed that, on the basis of the number of variates (here the number of mass/charge ratios in the considered spectrum), the number of observations, and the number of groups, allows the analyst to understand if the model is likely affected by overfitting or not; the answer is, however, only indicative, and further tests are necessary to confirm whether overfitting is present or not. A safe approach, which assumes only data independence, is to use internal cross-validation: all samples but one are used to build the model, and the remaining sample is used to test it; the process is repeated for all samples in a row. The percentage of success gives a confidence number for the group structure introduced by the analysis as PLS input. We will use this approach.

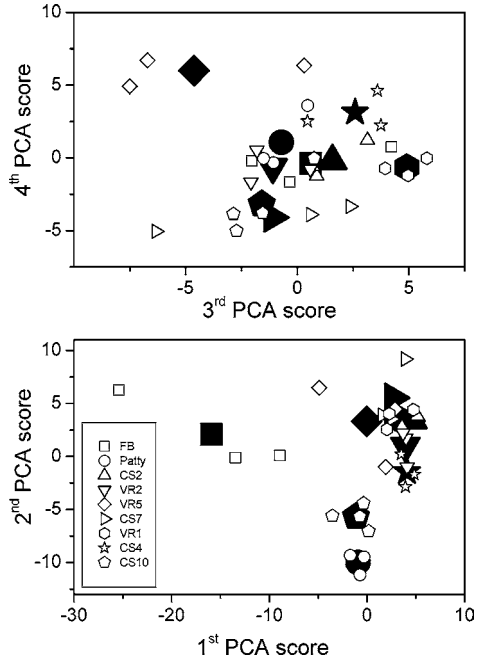

Figure 1. First four PCA (correlation mode) scores for analysis of nine strawberry genotypes. Different symbols indicate different clones—open points for the single data and bigger solid points for their average.

Our previous experiments (*10*) showed better performances of discriminant analysis for PTR-MS spectra if the data were normalized to unit area before further treatment. This is reasonable because this can easily take account of effects related, for example, to fruit size and surface, and this turned out to be true also here, so we will skip the discussion and presentation of data not normalized. Most of the data analysis has been performed by the software WINDAS (*15*) and partly by other statistical software [Statistica (*23*)] and standard data sheet and visualization software.

RESULTS AND DISCUSSION

We started with a simple data exploration by looking at the first four scores for all of the samples of experiment 1 with all three methods implemented in the WINDAS program: PCA correlation mode (**Figure 1**), PCA covariance mode (**Figure 2**), and dPLS (**Figure 3**); different symbols indicate different groups; solid bigger points indicate the group center (this point corresponds to the average of the single measurements for that group), and smaller open points indicate single measurements. Some features of the data are evident and suggest that we have a good basis for further dicriminant analysis. *F. vesca* samples (squares) are well separated by the first two scores in dPLS and PCAcor showing, however, poor clustering, whereas in PCAcov they are better separated by the third score and well clustered by the second and first scores. Patty (circle) and CS10 (pentagons) form also clear clusters: the various measurements are close together and well separated from other varieties that seem to belong to a single cluster if we consider only the first two scores. Using however more scores, other groups can be separated as well, for example, CS4 (stars) in PLS1 × PLS2 overlap with VR2 (down triangles), but using PLS3 × PLS4, we notice a clear separation of these two clones. The same holds for several other groups.

Just by looking at these graphs we cannot easily derive the minimum number of loadings needed to have a maximum efficiency of the model without entering the overfitting region. Scree plots (plot of explained variance as a function of the number of used loadings) give a first insight into this problem:

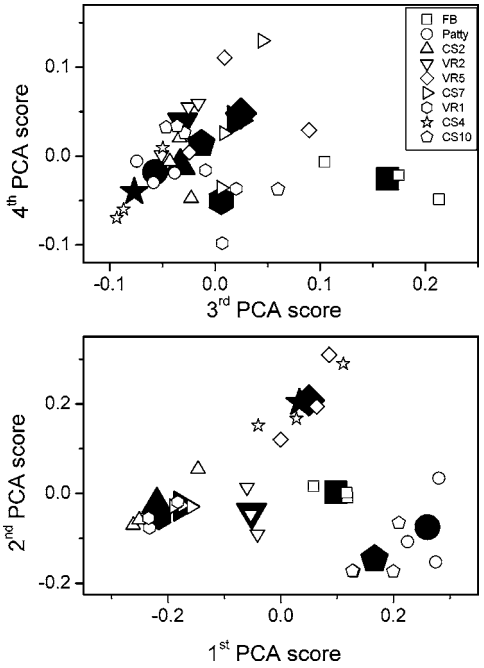

Figure 2. First four PCA (covariance mode) scores for analysis of nine strawberry genotypes. Different symbols indicate different clones—open points for the single data and bigger solid points for their average.

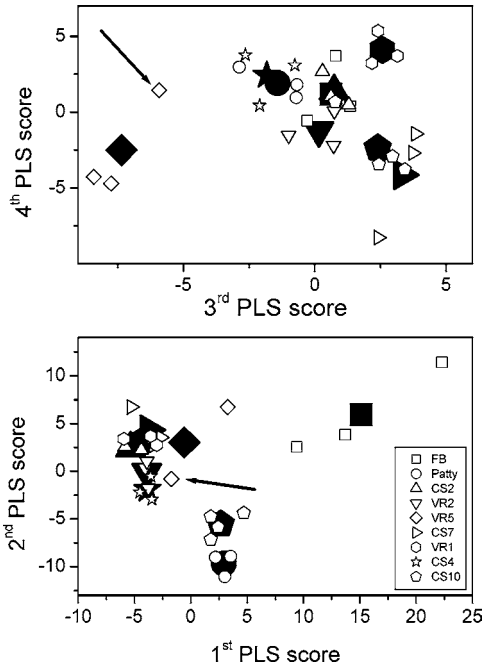

Figure 3. First four PLS scores for analysis of nine strawberry genotypes. Different symbols indicate different genotypes—open points for the single data and bigger solid points for their average. The arrows indicate the only sample that is not correctly attributed both in the training and in the test phase.

that is, the points where the graph slope changes give a rough indication of the number of dimensions that one has to take into account (*24*). **Figure 4** shows that for PCA (covariance mode) the first 5 or 6 loadings contain all of the information (but in this case small but accurate signals can be lost); on the contrary, dPLS and PCAcor give similar results and indicate that it can be reasonable to go up to 8−10 loadings.

Other information on this point can be obtained by plotting the percentage of successful attributions of measurements to the right group versus the number of scores used. We did this

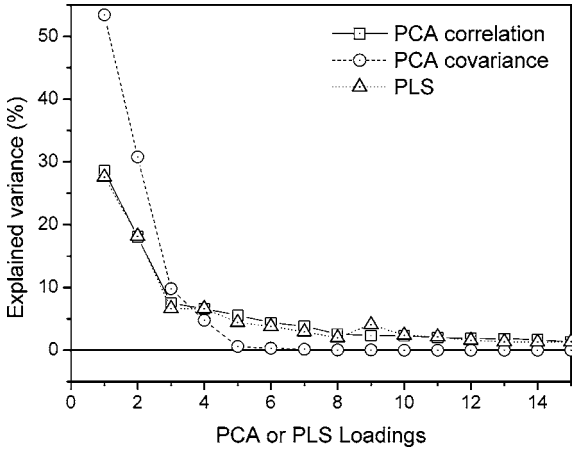

Figure 4. Scree plot for the three data compression methods used.

for the three possible compression methods (PCAcov, PCAcor, and dPLS) and for three different ways for measuring distances (Euclidean, Manhattan, and Mahalanobis). The first four scores seem to do most of the work for all compression methods, but again up to eight or more are needed to reach a maximum. Euclidean distances are less effective, and all methods, even if a high number of dimensions are used, fail in one (PCAcor and dPLS) or two cases (PCAcov).

This first data exploration indicates that all methods used show a similar performance, in particular PCAcor and dPLS (**Figures 1** and **3**), indicating that the total variance (used by PCAcor) is induced mainly by the difference among groups (enlightened by dPLS).

The key check is a cross-validation test that we performed by LDA analysis on 10 PLS scores and Euclidean distances, the more conservative approach. We are surely in a region where overfitting can be a problem, and only the results of the test will say if this is the case. The classes are here the nine different genotypes. Every strawberry was attributed to the right group except one VR5 sample, giving a success percentage of 96.4% (27 of 28). The wrongly assigned sample is the same one that was wrongly attributed in the training phase, even with a high number of scores, and is indicated by an arrow in **Figure 3**. There is obviously no reason to expect better results in the test phase than in the training phase. It is, however, interesting to notice that this sample is not "wrong enough" to avoid proper classification when used in the training phase.

Are the observed differences due to the genetic and a really phenotypic expression of the different clones? Or are they attributable, having measurements on only a few samples, to differences in ripening degree, size, physiological and pathological conditions, etc.? The second presented experiment tries to answer this question. Now we consider only two genotypes, Miss and CS2, but three different batches collected at different times, in different places, and produced in different ways (**Table 2**). Moreover, we measured the strawberry after harvesting and then again after 4 days (3 days at 5 °C and 1 day at room temperature). For one batch we have also measurements for additional days after the harvest. Every measurement is on a different single intact fruit and done under the same experimental condition as in the previous experiment. The goal is to have the maximum variability that we can expect for commercial fruits produced in a certain region (Po Valley, northern Italy) and see if even in this case the promising results of the first experiment are confirmed.

It turns out that all three data compression methods discriminate unambiguously the two varieties (CS2 and Miss) using just

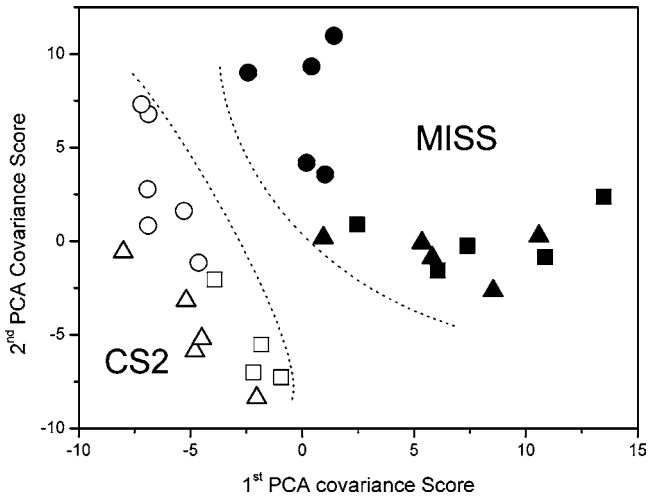

Figure 5. First versus second PCA score (correlation mode) for the measurements on the first day for Miss (solid points) and CS2 (open points). Different symbols indicate the three different production batches.

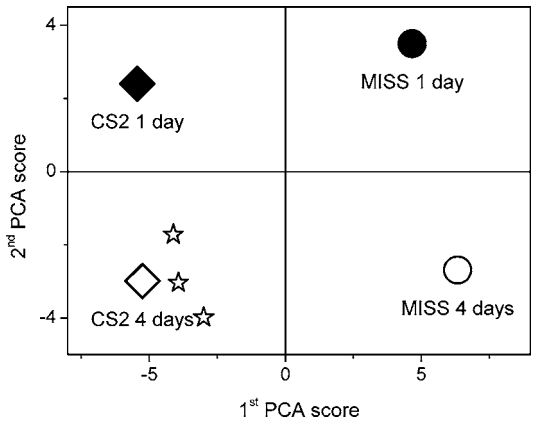

Figure 6. CS2 data of the first experiment used as a test on the model developed in the second experiment (two clones, two times). Centers of trial measurements are indicated by larger symbols: open points for fourth day and solid for the first day. Smaller stars are the test measurement. They are correctly attributed to CS2 on the fourth day (see text).

the first two scores and LDA gives a percentage of right assignment of 100%. Nevertheless, some structures in the data are evidently also related to the different production batches.

In **Figure 5**, for instance, the first two PCA (correlation mode) scores for the three batches at the first day of measurement are reported. A possible structure induced by different batches is confirmed by the following analysis.

For each batch we randomly chose three of five points as training set and use the remaining two data as the test set. The analysis with dPLS and discriminant analysis indicates that (a) we need three PLS scores for completely correct assignment of the measurements of the training set to the right clone and batch, (b) all test measurements are attributed to the right clone starting with five or more PLS scores (LDA, Euclidean distance), and (c) different batches are sometimes mixed. In particular, for the first day batch A (Miss) is not completely distinguished from batch C (Miss), and batch C (CS2) is confused with batch A (CS2). Batches A (for CS2) and B (for both clones) are well separated. In total 3 of 12 tests are wrongly assigned; for the fourth measurement day the different batches are less separated (5 wrong tests of 12). This seems to be a constant in our measurements: we noticed that often measurements on the first day have a rather large variance that tends to decrease in successive days. This could be connected to postharvest stress, to differences in treatments and ripening that are smoothed during preservation, and, in general, with the high metabolic rate observed for strawberry (*16*).

To follow this up we carried out additional measurements on batch A on days 2, 3, 5, 6, 8, 9, and 12 for both clones CS2 and Miss. It is interesting to note that, applying again discriminant analysis on the compressed data (dPLS on all Miss and CS2 data defining four groups: CS2 first day, CS2 fourth day, Miss first day, and Miss fourth day, followed by LDA on five PLS scores), of these independent measurements all of the data are attributed to the right clones but only when using for comparison the data point corresponding to the fourth day. This is first evidence that fresh fruits are systematically different from fruits being stored for >1 day (stored at 4 °C or at room temperature). In other words: the model developed for fruits at the fourth day after harvest works for all other data points except for the data point obtained on the first day, indicating that this data point constitutes its own class.

The results of the second experiment show that even with the high differences in location, harvesting, measuring time, and cultivation methods the two investigated varieties are well separated using only their PTR-MS fingerprint. Differences among batches are also evident but, for the present, they cannot be unambiguously attributed to specific experimental parameters. There is also evidence that measurements on the first day before storage exhibit a greater variance and tend to form a group separated from the other measurements.

As CS2 was present in both experiments, we can perform a last, conclusive, cross-validation test to see if the model developed in one experiment can explain the data collected in the other. In this case the independence of the measurements (training and test) is complete (different harvesting time, different measuring session, different fields, etc.). In **Figure 6** we see that all three data points of experiment 1 for CS2 (small stars) are attributed to the right group and, again, to the group corresponding to measurements after 4 days. We remind the reader that measurements of the first experiment were performed on the third to fourth day after harvesting and the test can be considered completely successful: single fruits are assigned to the right variety.

On the other hand, we can use the CS2 measurements of experiment 2 as a test on the model developed in experiment 1 (LDA with Euclidean distance on 10 PLS scores on the 9 classes defined by the 9 genotypes).

For the data of the first day we have only 12 of 16 (75%) successes, but actually in the first experiment we do not have data on fresh fruits and so the model has not been developed on comparable samples. On the contrary, if we consider data of the fourth day we have 14 successes of 15 (95%), and we can consider this test to be successful. In this case, on the basis of the previous analysis we cannot hope to summarize all of the needed information in a few dimensions (the model was developed with 10 PLS scores, and we saw that with fewer than about 8 dimensions we do not expect sufficient discrimination). Nevertheless, an appropriate choice (manually trying various possibilities) of the scores can give a good visualization also in three dimensions: for example, in **Figure 7** we plotted the average of the nine groups of the first experiment (dot-centered hexagons for all groups, and CS2 indicated by a solid bigger circle), and we indicate with smaller open circles the measurements for CS2 on the fourth day of the second experiment (the three plotted PLS scores were chosen because they seemed to give a good visualization of the data). The good agreement is evident, and there is only one class of the model that overlaps

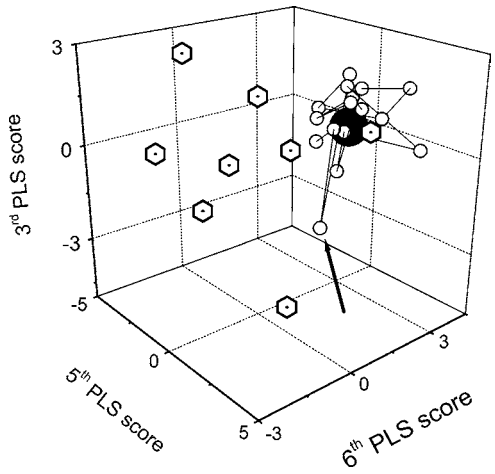

Figure 7. CS2 data of the second experiment used as a test on the model developed in the first experiment (nine varieties). The centers of the groups used in the training phase are indicated by open dot centered hexagons and the one of CS2 by a solid circle. Test measurements are the open smaller circles connected by a line. The arrow indicates the only sample that is wrongly attributed to VR2 instead of CS2.

with CS2 even using only three dimensions. This graph is also interesting because it gives a visualization of the spread of the data (showing only one data point wrongly assigned, designated by the arrow). As the small circles correspond to data of three different batches this seems to be quite a remarkable result.

CONCLUSIONS

A complete method, from sampling to data analysis, for the classification of agroindustrial products has been proposed and tested. We showed that PTR-MS can successfully be coupled with data compression and class modeling methods to provide a fast and sensitive tool for product discrimination based only on nondestructive VOCs measurements. Internal cross-validation and validation between different experiments give high and promising success percentages. Moreover, we found indications that differences due to shelf life and to different production batches can also be determined reliably.

On the basis of our results we believe that the proposed method provides a promising tool for quality, product, or process control not only for breeding and genetics but also in practical, industrial applications.

Vice versa (trusting the technique and analysis), our results suggest a strong effect of the genetics on the volatile compounds profile of strawberries, indicating that a proper control of this point is crucial for the development of new varieties because, whatever a definition of quality could be, surely, for food and, in particular, for strawberry, it should include aroma.

The spectra used for the analysis described include chemical information that can be used to understand the reason of the observed differences. Even if several compounds can be associated with some masses with reasonable accuracy, we prefer, before publishing comments on this point, to extend our experimental database on fragmentation in PTR-MS. Particularly in the PCA covariance mode, the loadings produced by the analysis preserve a reminiscence of the real spectra and can be used to understand which are the compounds/masses that explain the observed differences (*25*). In this work we measured a single fruit for 4 min and allowed some more time to clean gas lines between measurements (15 min for each fruit); moreover, we did not use automatic sampling systems, but an operator had to follow almost continuously the measurements. Nevertheless, the whole procedure could be, in principle, computer controlled from the sampling phase to the discriminant analysis and data representation on the computer monitor. This is one of the most qualifying aspects of the proposed method and could be the basis for a complete automatic system for practical application with a very short time from sample preparation to data displaying. A last development we would like to mention is that it is worth trying to correlate PTR-MS data not only with the variety but also with other data (ripening degree, other chemical analysis, sensory analysis, etc.). If successful, this research will give a fast, nondestructive method not only for variety identification but, more generally, for real-time product evaluation.

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