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New Method for the Determination of Benzoic and Sorbic Acids in Commercial Orange Juices Based on Second-Order Spectrophotometric Data Generated by a pH Gradient Flow Injection Technique

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Two widely employed antimicrobials, benzoic and sorbic acids, were simultaneously determined in commercial orange juices employing a combination of a flow injection system with pH gradient generation, diode array spectrophotometric detection, and chemometric processing of the recorded second-order data. Parallel factor analysis and multivariate curve resolution−alternating least-squares were used for obtaining the spectral profiles of sample components and concentration profiles as a function of pH, including provisions for managing rank-deficient data sets. An appropriately designed calibration with a nine-sample set of binary mixtures of standards, coupled to the use of the second-order advantage offered by the applied chemometric techniques, allowed quantitation of the analytes in synthetic test samples and also in commercial orange juices, even in the presence of unmodeled interferents (with relative prediction errors of 8.7% for benzoic acid and 2.5% for sorbic acid). No prior separation or sample pretreatment steps were required. The comparison of results concerning commercial samples with a laborious reference technique yielded satisfactory statistical indicators (recoveries were 99.0% for benzoic acid and 101.4% for sorbic acid).

KEYWORDS: Flow injection analysis; pH gradient; parallel factor analysis; multivariate curve resolution; benzoic acid; sorbic acid; food analysis

INTRODUCTION

Benzoic acid (BEN) and sorbic acid (SOR) are regularly employed as antimicrobials in a great variety of foods, namely, fruit products, jams, relishes, beverages, dressings, salads, pie and pastry fillings, icings, olives, and sauerkraut. They are indicated against yeasts and some molds and bacteria (foodborne pathogens but not spoilage bacteria) (*1*−*3*).

Several methods have been reported for the quantitative determination of BEN and SOR in foods: high-performance liquid and thin-layer chromatographies (*3*−*6*), capillary electrophoresis (with the micellar electrokinetic capillary variant being the most usually applied) (*3*, *7*−*10*), second-order derivative spectrophotometry after solvent extraction (*11*), chemometrics-enhanced spectrophotometry (*12*), polarography (*13*), and enzymatic determination (*14*). The AOAC official methods for analyzing sorbic acid in beverages involve (1) steam distillation followed by UV absorption at 260 nm or (2) reaction with thiobarbituric acid and colorimetry at 532 nm (*15*, *16*).

Although most of the above-cited determinations are accurate and sensitive, sample derivatization or extraction is usually required, making the analytical procedures complex and time-consuming. Therefore, there is a constant need to improve these methods, to obtain better analytical figures of merit and/or to shorten the time required for the analysis.

Multivariate calibration is gaining popularity for the simultaneous determination of multicomponent mixtures in several fields (*17*). It is mainly based on spectroscopic data, although any kind of first-order data may be used (first-order refers to data presented in vectorized format, i.e., a spectrum per sample). Full-spectrum multivariate calibration methods offer the advantage of speed in the determination of the components of

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interest, avoiding separation steps in the analytical procedures. Partial least-squares (PLS) has become the usual first-order multivariate tool because of the quality of the obtained calibration models, the ease of its implementation, and the availability of software (*18*). An interesting characteristics of these multivariate methods is that calibration can be performed by ignoring the concentrations of all other components except the analyte of interest. In the food analysis field, a recent study employed PLS and other first-order multivariate methods for the spectrophotometric determination of colorants in a soft drink powder (*19*).

However, all first-order methods, of which PLS is no exception, are sensitive to the presence of unmodeled interferents, that is, compounds occurring in new samples which have not been included during the training step of the multivariate model. This situation is encountered when natural samples of complex composition, such as the presently studied orange juices, are examined. The problem can be alleviated, in part, thanks to the implementation of wavelength selection procedures, which are able to "filter", to some extent, the spectral regions where serious interferences occur, leaving adequate spectral windows for successful application of multivariate models (*20*). A good example is the previous determinations of BEN and SOR in juices by employing a net-analyte-based methodology (*12*). In general, however, no first-order multivariate model can be made immune to unexpected interferents with spectral overlap over the whole useful spectral region where the analyte absorbs.

A good alternative to the above-discussed problem is to move to high-order data, which are particularly useful for the quantitative analysis of complex multicomponent samples. Specifically, second-order data, in which each sample produces a data matrix, are gaining widespread analytical acceptance (*21*, *22*).

In this paper, we discuss the simultaneous determination of BEN and SOR in real juice samples using second-order data provided by diode array spectrophotometric detection in a flow injection system with an imposed double-pH gradient, analyzed by both parallel factor analysis (PARAFAC) and multivariate curve resolution−alternating least-squares (MCR-ALS) models. A comparison with the official method was performed.

EXPERIMENTAL PROCEDURES

Apparatus. Spectrophotometric measurements were performed on a Hewlett-Packard 8452A spectrophotometer with a diode array detector and a Hellma 178-010-QS flow cell with an inner volume of 18 μL. A Gilson Minipuls 3 peristaltic pump was used as propulsion device. A Rheodyne 5041 injection valve was used. The coil, loop, and transmission lines were made with i.d 0.5 mm PTFE tubing.

Reagents. All solutions were prepared from analytical grade chemicals and purified water (18 mΩ) by using a B-pure system. The acid Britton−Robinson solution (BRH) was prepared by mixing 0.0400 mol L^{-1} phosphoric acid (Mallinckrodt), 0.0400 mol L^{-1} boric acid (Anedra, Buenos Aires, Argentina), and 0.0400 mol L^{-1} acetic acid (Mallinckrodt). The basic Britton−Robinson solution (BROH) was 0.2 mol L^{-1} NaOH (Merck, Darmstadt, Germany). Sodium benzoate and sorbate stock solutions were prepared by dissolving 100.0 mg of each compound (both purchased from Sigma, Milwaukee, WI) in water, completing to 1000.00 mL in a volumetric flask. Working standard solutions of benzoic and sorbic acids in the range of 0.00−12.0 mg L^{-1} were prepared by diluting the above-mentioned stock solution with the BRH solution. Bromocresol green (BCG) purchased from Sigma was employed for the optimization of the flow injection analysis (FIA) system.

For application of the official method of determination, the following reagents were employed: tartaric acid, magnesium sulfate heptahydrate, 0.1 N hydrochloric acid, and copper sulfate (alkaline solution prepared from 1 mL of 0.1% copper sulfate heptahydrate, 0.5 g of sodium carbonate, and distilled water to complete a 1000 mL flask to the mark). All reagents were obtained from Merck.

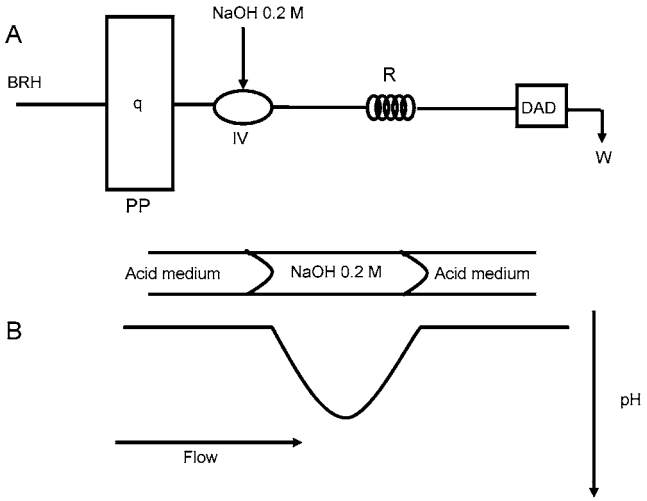

Figure 1. (A) Flow injection system used for the generation of a double-pH gradient: BRH, acid Britton–Robinson solution; IV, injection valve; R, reactor; DAD, diode array detector; W, waste; q, 0.56 mL min^{-1}. (B) Scheme of the pH gradient profile.

Table 1. Composition of the Calibration and Test Samples for Application of the PARAFAC and MCR-ALS Models

	calibration (mg L^{-1})			test (mg L^{-1})	
sample	BEN	SOR	sample	BEN	SOR
C1	1.80	1.80	T1	2.00	2.00
C2	1.80	10.00	T2	6.70	2.00
C3	10.00	1.80	T3	10.70	2.00
C4	10.00	10.00	T4	2.00	6.70
C5	6.00	0.00	T5	6.70	6.70
C6	6.00	12.00	T6	10.70	6.70
C7	0.00	6.00	T7	2.00	10.70
C8	12.00	6.00	T8	6.70	10.70
C9	6.00	6.00	T9	10.70	10.70

Flow Injection Methodology. A simple FIA system with a single channel was designed to generate the pH gradients (**Figure 1A**), with a carrier stream of BRH pumped continuously into the system. When an appropriate volume of BROH is injected, the FIA signal recorded at 270 nm has a shape that is pictorially shown in **Figure 1B**. The baseline pH before the peak appears is acid, and the injected volume of BROH is responsible for the generation of a double-pH gradient (*23*). The pH increases from the front of the flow injection peak, reaching its maximum value at the center of the peak, and then progressively decreases to reach its baseline value.

Samples were prepared in BRH, introduced into the FIA system as a carrier stream, and BROH was injected, creating the necessary pH gradient close to the center of the sample. For each FIA peak registered, spectra were collected in the range of 200−300 nm each 2 nm, and the pH gradient produced 33 equally spaced data points, making a total of $51 \times 33 = 1683$ data points for each sample matrix.

Calibration and Test Sets. A nine-sample set was built for calibration with the employed multivariate models. The analyte concentrations corresponded to a central composite design (samples C1−C9 in **Table 1**). The extreme concentrations for the design were 0.00 and 12.00 mg L^{-1} for both BEN and SOR. They were obtained starting from the stock solutions (see above). Their total spectral−pH evolutions were measured in random order, after injection into the above FIA system.

Additionally, nine binary samples (T1−T9, **Table 1**) were built with analyte concentrations different from those employed for calibration, but within their corresponding calibration ranges and following a full-

factorial design. All of these samples contained freshly squeezed orange juice, obtained from different fruits for each sample, keeping the proportion between juice and the analytes at a level compatible with that present in commercial samples. Total spectral evolutions were measured in random order and on different days from those corresponding to calibration.

Real Juice Samples. Real juice samples were M1, Mocoreta (concentrated orange juice with added vitamin C); M2, Cotti (orange juice); M3, Miju (concentrated orange juice); and M4, Hi-C (orange juice with added vitamin C). The samples were prepared by placing 0.25 mL of commercial juice in a 25.00 mL flask and completing to the mark with distilled water, so that the final analyte concentrations were within the calibration ranges (commercial juices are known to contain ~0.5 g L^{-1} of the analytes). Their total spectral evolutions were registered in random order and on different days from the calibration/test samples.

Official Method. Details on the implementation of the official methodology for the determination of BEN and SOR in juices can be found in the literature (*15*, *16*). Briefly, 20.00 mL of sample is placed in a flask together with tartaric acid (2 g) and magnesium sulfate heptahydrate (50 g). Steam distillation is performed until 350−400 mL is obtained, diluted to 500.00 mL, and filtered. To a volume of 5.00 mL of the filtrate is added 25.00 mL of the alkaline copper sulfate solution, and the resulting mixture is shaken and treated with 5.00 mL of HCl solution. The absorbances of the final solution are measured at 230 and 263 nm using distilled water as blank. The concentrations of sorbic and benzoic acids are then calculated through

$$\text{sorbic acid (g L}^{-1}) = 500(A_2 - 0.0827A_1)/(32V) \quad (1)$$

$$\text{benzoic acid (g L}^{-1}) = 500(A_1 - 0.0060C_1)/(13.4V) \quad (2)$$

where A_1 is the absorbance measured at 230 nm, A_2 is that recorded at 263 nm, C_1 is the concentration of sorbic acid, and V is the sample volume.

RESULTS AND DISCUSSION

Multivariate Analysis. Interestingly, the decomposition of a three-way data array (obtained when second-order data for a set of samples are grouped) is often unique, allowing relative concentrations and spectral profiles of individual sample components to be extracted directly. This permits correction for uncalibrated sample constituents, a property that has been named the "second-order advantage" (*22*, *24*). Three-way data are currently available to the analyst thanks to the implementation of hyphenated analytical techniques, although they can also be produced in a single instrument, such as excitation−emission fluorescence matrices in a spectrofluorometer and absorbance−time measurements in a diode array detector (*21*). In the latter case, time variations of the analytical signal may be produced by a chemical reaction or by changes in the pH of the solution with time.

When pH is selected as modulation for the second data dimension, advantage is taken of the acid−base properties of the analyte, assuming that an observable change in spectra with the pH takes place. FIA systems are well-suited for generating reproducible pH gradients and, combined with diode array UV−visible detection, they provide valuable second-order data. A peculiar characteristic of the data produced in this way is that they are classified, from a mathematical point of view, as "rank-deficient", meaning that the overall rank of the measured data is not equal to the sum of the ranks of the individual species contributions. This phenomenon is the consequence of the correlation of the species concentrations related by proton transfer reactions along the dimension of the pH gradient. Rank-deficient systems have been extensively analyzed with methods such as MCR-ALS (*25*−*28*) and also, to a lesser extent, by constrained PARAFAC (*29*) and residual bilinearization (RBL) (*30*). From the analytical point of view, the interesting fact is that they conveniently exploit the second-order advantage. Finally, the multiway partial least-squares (*N*-PLS) method is also available for application to second-order data (*31*). Although it maintains the matrix structure of the employed data and is able to handle rank-deficient systems, it does not share the second-order advantage.

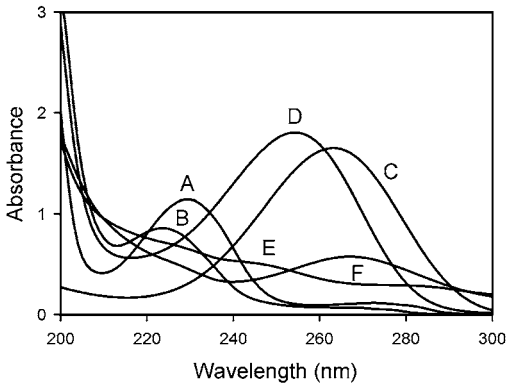

Figure 2. Absorption spectra of the studied analytes and the background signal produced by a freshly squeezed orange juice at two pH values: (A, B) benzoic acid, 10 mg L^{-1}; (C, D) sorbic acid, 10 mg L^{-1}; (E, F) orange juice. The pH values were 2.0 for spectra A, C, and E and 6.0 for spectra B, D, and F.

Spectral and pH Behavior of Analytes. Figure 2 shows the absorption spectra of BEN and SOR at pH values where they are known to exist in their acid and basic forms, together with the spectrum of a typical freshly prepared orange juice at the same pH values. As can be seen, intense overlap occurs among all spectra, a fact that complicates direct analysis by first-order multivariate techniques, unless appropriate wavelength regions are selected where the effect of background components is minimal.

To circumvent these difficulties, a suitable methodology for producing second-order data was designed, combining a flow injection system in which a double-pH gradient is created and diode array spectrophotometric detection (see below). This type of data permits quantitation even in the presence of unexpected interferents, thanks to the implementation of the powerful second-order advantage. Consideration of the spectra shown in **Figure 2** allows setting approximate pH functions for inserting into both PARAFAC and MCR-ALS programs, to provide profile estimations for the initialization of the multivariate programs.

Optimization of the FIA System. The optimization of the FIA system was done using BCG. This reagent has a pK_a value (4.66) similar to those of the analytes (sorbic acid, 4.76; and benzoic acid, 4.19). BCG was prepared in buffer solutions of different pH values, and the FIA signals were obtained at the wavelength at which the basic form of the BCG shows maximum absorbance. Then, BCG prepared in BRH solution was pumped into the system, and a volume of BROH was injected. In this manner, a calibration of the different pH values along the FIA signal was performed, implying that a correspondence could be established between different times along the FIA peak and the different pH values. This procedure helped to ensure that a pH gradient was created in the range from 2.5 to 6.0, which is known to include the pK_a values for both analytes.

All of the variables were optimized as a compromise between the width and height of the FIA peak, because the dispersion

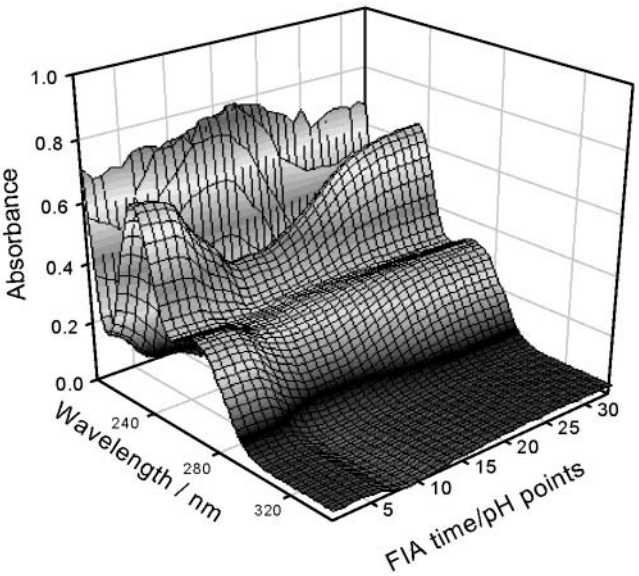

Figure 3. Three-dimensional plot of the spectral–pH evolution for the calibration sample C4, after injection into the FIA system.

Table 2. Prediction Results in the Set of Synthetic Samples

	N-PLS[a]		PARAFAC[a]		MCR-ALS[a]	
sample[b]	BEN	SOR	BEN	SOR	BEN	SOR
T1	4.50 (3)	1.91 (2)	2.75 (2)	2.10 (3)	3.70 (3)	2.71 (2)
T2	6.81 (2)	1.92 (3)	5.95 (2)	2.01 (2)	4.93 (3)	1.83 (2)
T3	9.03 (2)	2.02 (2)	9.66 (4)	1.95 (3)	8.61 (3)	1.75 (2)
T4	4.65 (2)	6.04 (2)	2.13 (2)	6.93 (3)	1.15 (2)	7.52 (4)
T5	7.04 (3)	6.57 (4)	7.02 (3)	6.82 (3)	6.03 (4)	6.73 (3)
T6	9.13 (3)	7.33 (5)	11.15 (3)	6.74 (3)	10.12 (4)	6.71 (3)
T7	4.83 (4)	10.75 (4)	1.93 (2)	11.03 (4)	1.84 (2)	10.84 (3)
T8	6.85 (3)	10.12 (5)	6.74 (2)	10.81 (3)	6.71 (3)	10.46 (3)
T9	9.01 (4)	10.04 (5)	10.82 (3)	10.61 (3)	10.85 (4)	10.23 (3)
RMSE	1.92	0.45	0.56	0.16	1.22	0.44
REP	29.7	7.0	8.7	2.5	18.9	6.84

[a] Values are expressed in mg L^{-1} and correspond to mean of three replicates. Values in parentheses correspond to standard deviations $\times 10^2$. [b] See nominal concentrations in **Table 1**. RMSE = root-mean-square error (mg L^{-1}); REP = relative error of prediction (%).

of the injected volume was responsible for the generation of the pH gradient. The volume of the alkaline solution was tested between 50 and 150 μL, and the selected value was 50 μL. The length of the reactor was checked between 1300 and 1900 mm, and no substantial differences were noticed; hence, a reactor of 1300 mm was selected. The flow rate was varied in the range of 0.35−0.84 mL min^{-1}, and the optimum value was 0.56 mL min^{-1}.

In **Figure 3** the spectral−pH evolution of one of the calibration mixtures is shown, as obtained from the corresponding FIA peak. As can be seen in this figure, changes in absorption spectra are observed that are the expected ones when the pH of the sample is increased close to its center.

Synthetic Samples. Analysis of the set of synthetic samples was first carried out by *N*-PLS. Leave-one-out cross-validation was first applied to obtain the optimum number of factors for the calibration set of samples. Application of Haaland's criterion (*18*) led to the conclusion that this number is three. Subsequent prediction on the synthetic set with this three-factor *N*-PLS model yielded the prediction results quoted in **Table 2**. The results indicate rather large errors for benzoic acid, presumably due to the effect of unmodeled background juice, which is not taken into account by the calibration set.

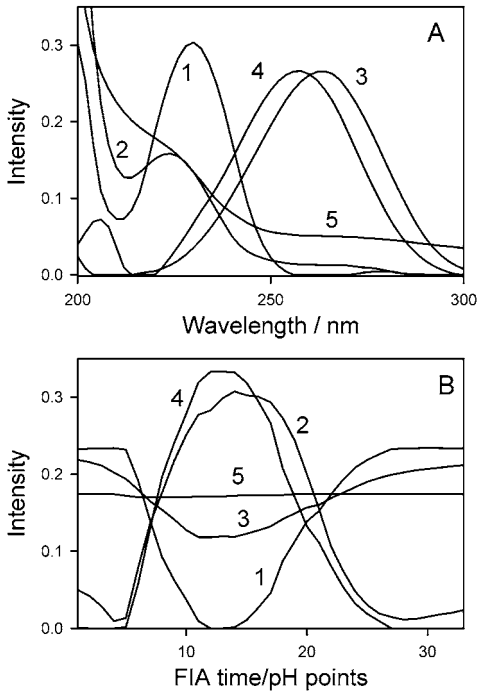

Figure 4. (A) Normalized spectral profiles provided by the five-component PARAFAC model when processing a synthetic test sample. (B) Normalized pH profiles. In both cases, the numbering corresponds to the order assigned by the model in terms of the contribution to the overall spectral variance. Normalization is carried out to unit length. The numbers correspond to the following compounds: 1, benzoic acid; 2, benzoate; 3, sorbic acid; 4, sorbate; 5, average background stemming from the fruit juice.

The PARAFAC and MCR-ALS methodologies were implemented for the synthetic set of samples in the manner described in **Table 1** of the Supporting Information. In both cases, the construction of five-component models proved to be satisfactory for all synthetic samples: these five components were sorbic acid, sorbate, benzoic acid, benzoate, and an average background contribution from the fruit juice. PARAFAC processing of sample T1, for example, furnishes the spectral profiles shown in **Figure 4A** and the pH profiles plotted in **Figure 4B**. Agreement between spectral profiles of the calibrated components and pure spectra of standards is seen to be satisfactory. Indeed, comparison with the spectra shown in **Figure 2** allows one to match component 1 in **Figure 4A** with benzoic acid, component 2 with benzoate, component 3 with sorbic acid, and component 4 with sorbate, leaving component 5 to be identified as the average background stemming from the fruit juice. The pH profiles shown in **Figure 4B** are the expected ones on the basis of the known spectral−pH behavior of the analytes; notice that the intersection between the profiles for benzoic acid and benzoate (components 1 and 2) occurs at a higher pH as compared with that for sorbic acid and sorbate (components 3 and 4), in agreement with the order of the pK_a values for these components. Similar spectral and pH profiles were obtained when MCR-ALS was applied to the synthetic set.

PARAFAC and MCR-ALS were applied to the set of calibration samples and each of the test samples using the five-component model described above, providing the prediction results shown in **Table 2**. As can be seen, better statistical indicators are obtained with these two multivariate strategies, in agreement with the above discussion concerning the usefulness of the second-order advantage in the case of PARAFAC and MCR-ALS, which is not exploited by *N*-PLS. However,

Table 3. Prediction Results in the Set of Synthetic Samples

	official method[a,b]		*N*-PLS[a,b]		PARAFAC[a]		MCR-ALS[a,b]	
sample[c]	BEN	SOR	BEN	SOR	BEN	SOR	BEN	SOR
M1	0.69	0.24	0.95	0.17	0.71	0.27	0.64	0.22
M2	0.57	ND	1.10	ND	0.62	0.03	0.62	0.02
M3	0.71	0.24	1.05	0.41	0.70	0.24	0.76	0.23
M4	0.28	0.18	0.12	0.24	0.24	0.25	0.24	0.21

[a] All results are expressed in g L^{-1}. For a paired *t* test between methods, average standard error (SE), 0.03 g L^{-1}; degrees of freedom (DOF), 4; confidence level, 95%; critical *t* value, 2.79. Only for PARAFAC and MCR-ALS were the values of ($\Delta \times$DOF/SE) smaller than the critical *t* (Δ is the difference between the results under comparison). [b] ND = not detected. [c] See text for correspondence between sample code and manufacturer.

the results provided by PARAFAC appear to be better than those furnished by MCR-ALS, indicating that there are no serious deviations from the trilinearity condition required by the former technique.

Real Samples. The analysis of real samples was made in the manner described above for the synthetic set, using *N*-PLS, PARAFAC, and MCR-ALS, and the results were compared with those provided by the official methodology (**Table 3**). *N*-PLS is seen to yield unsatisfactory results, for reasons explained above concerning the second-order advantage, which is unavailable for this methodology. On the other hand, the values obtained with PARAFAC and MCR-ALS are close to those obtained by the official method. Indeed, a paired *t* test indicates no significant difference between the official and the presently proposed methodology: all experimental *t* values are below the critical one (see **Table 3**). This strongly suggests that multivariate analysis using suitable chemometric modeling techniques is a useful alternative for the analysis of commercial juices.

Method Comparison. In comparing the above-employed multivariate methodologies, one should take into account the following characteristics: (1) analytical performance, (2) ease and speed of program operation, and (3) model interpretability.

N-PLS is definitely the easiest of the three multivariate methods, requiring almost no knowledge of the spectral properties of the analytes. However, it provides no discernible physical interpretation as to the chemical components. Perhaps more important is the fact that it does not exploit the second-order advantage and, therefore, from a purely analytical point of view, can be regarded as the weakest methodology.

On the other hand, both PARAFAC and MCR-ALS are able to handle the occurrence of interferences not modeled in the calibration set, a property of immense utility in the analytical context. The main difference lies in the trilinearity requirement for PARAFAC, which is relaxed in the case of MCR-ALS. These two methods also yield a wealth of system properties of useful physical meaning. However, in cases such as the presently studied one, where rank deficiency occurs, the price paid is a somewhat complex program operation, requiring the introduction of initial guesses of component spectra for successful data decomposition. A variety of tools is available to help the analyst in this regard.

Supporting Information Available: Theory of PARAFAC, MCR-ALS, and *N*-PLS methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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