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Analytical Methods

Simultaneous determination of aspartame and acesulfame-K by molecular absorption spectrophotometry using multivariate calibration and validation by high performance liquid chromatography

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ABSTRACT

A new method to determine mixtures of two sweeteners, aspartame and acesulfame-K, in commercial sweeteners is proposed. A classical 5^2 full factorial design for standards was used for calibration in the concentration matrix. Salicylic acid was used as internal standard in order to evaluate the adjustment of the real samples in the PLS-2 model. This model was obtained from UV spectral data, validated by internal cross-validation and was used to find the concentration of analytes in the commercial samples. The PLS-2 method was validated externally by high performance liquid chromatography (HPLC), finding, in all cases, a relative error of less than 10% between the PLS-2 and the HPLC methods. The mean value of recovery degree in real samples was 99.2% with standard deviation of 3.2%. The proposed procedure was applied successfully to the determination of mixtures of aspartame and acesulfame-K in artificial sweeteners.

1. Introduction

The presence in the market of non caloric sugar substitutes has increased because of the demand for new products, which can be used for management diabetic and dietetic reasons as well as in the manufacture of "tooth-friendly" food and pharmaceutical products (Parke, Birch, Portmann, & Kilcast, 1999). Therefore, good analytical methods to assure quality control and product integrity are essential in meeting the needs of this growing market.

Acesulfame-K (3,4-dihydro-6-methyl-1,2,3-oxathiazine-4-one-2,2-dioxide) is a high-intensity and non caloric sweetener. It is not metabolized by the body and is excreted unchanged. Acesulfame-K is currently used in food, beverage, oral hygiene, and pharmaceutical products in about 90 countries; it has about 200 times the sweetening capacity of sugar. The acceptable daily intake (ADI) for acesulfame-K was increased in 1991 to 15 mg kg^{-1} body weight by both, the Joint Expert Committee on Food Additives (JECFA) and the Scientific Committee on Food of the European Commission (SCFEC) (International Sweeteners Association, 2004; Von Rymon Lipinsky, 1991).

Aspartame (N-L-a-aspartyl-L-phenylalanine methyl ester) is between 160 and 220 times sweeter than sugar. It contains two amino acids, aspartic acid and phenylalanine. Studies in a number of animal species indicate that aspartame is quickly and extensively metabolized in its constituent amino acids and methanol. Experimental model systems show that aspartame has low toxicity. In 2000, ADI for aspartame has been set at 40 mg kg^{-1} body weight by both, the JECFA and SCFEC (Homler, Deis, & Shazer, 1991; International Sweeteners Association, 2004; US Department of Health and Human Services, Public Health Service, National Institute of Health, 2003). In recent years, aspartame was studied because it was objected as sweetener and food additive. The safety of aspartame and its metabolic constituents was established through extensive toxicology studies in laboratory animals, using much greater doses than people could possibly consume. Nonetheless, additional research, including evaluations of possible associations between aspartame and headaches, seizures, behaviour, cognition, and mood as well as allergic-type reactions and use by potentially sensitive subpopulations, has continued after approval in 1980. It is clear that aspartame is safe, and there are not unresolved questions regarding its safety under conditions of intended use (Butchko et al., 2002).

A great variety of methods have been applied to the analysis of the aforementioned compounds in foods, but only a few procedures are suitable for the simultaneous determination of aspartame and acesulfame-K in commercial sweeteners. High performance liquid chromatography (HPLC) is the most frequently used technique nowadays (Dossi, Toniolo, Susmel, Pizzariello, &

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Bontempelli, 2006; Kobayashi et al., 1999; Tsang, Clarke, & Parrish 1985; Wasik, McCourt, & Buchgraber, 2007; Wu, Cheng, & Chou, 1995). In addition, ion chromatography (IC) offers an attractive alternative to traditional HPLC methods (Chen & Wang, 2001; Zhu, Guo, Ye, & James, 2005). In the past few years, micellar electrokinetic chromatography (MEKC) and capillary zone electrophoresis (CZE) have been applied to the simultaneous determination of several kinds of sweeteners in foods (Boyce, 1999; Frazier, Inns, Dossi, Ames, & Nursten, 2000; Pesek & Matyska, 1997).

Other methods less commonly used for aspartame and acesulfame-K determination are FT-Raman spectroscopy (Armenta, Garrigues, & De La Guardia, 2004), FT-Raman spectroscopy with chemometric analysis (Khurana, Cho, Shim, Li, & Jun, 2008), amperometric method based on the use of bilayer lipid membranes (Nikolelis & Pantoulias, 2000) spectrophotometry based on the complexation with Cu (Fatibello-Filho, Marcolino-Junior, & Pereira, 1999), biosensors for aspartame determination (Odaci, Timur, & Telefoncu, 2004), sequential flow injection coupled with enzymic detection (Pena, Lima, Saraiva, & Lucia, 2004) and flow injection sensor with on-line solid-phase extraction (Vallvey, Valencia, Nicolás, & García-Jiménez, 2006).

The instrumental methods above mentioned are important referential methods, but are based on expensive analytical instruments, which are not available for laboratories of routine in quality control and low resources. On this basis, a new, fast and inexpensive method for the simultaneous determination of mixtures of aspartame and acesulfame-K using the partial least square (PLS-2) multivariate calibration, is proposed in this work. The quality of analytical results obtained in five real samples of commercial sweeteners by PLS-2, was validated by high performance liquid chromatography (HPLC) method. The behaviour of the model in the real samples was evaluated using salicylic acids as internal standard, which was incorporated in the PLS-2 model.

The partial least square (PLS) regression method is a multivariate calibration tool that was used in a large number of previous works to determine the concentration of several analytes in a wide variety of samples, in combination with different spectroscopic techniques (Brereton, 2000; Galeano Diaz, Guiberteau, Ortiz Burguillos, & Salinas, 1997; Lozano, Camiña, Boeris, & Marchesvky, 2007; Ni, Zhang, & Kokot, 2005). PLS uses the original spectroscopic variables, which allows to evaluate the concentration of mixtures of analytes (Beebe, Pell, & Seasholtz, 1998; Massart et al., 1997). The PLS-2 method is a multivariate calibration tool that uses a unique model to predict simultaneously the concentration of analytes in real samples (Martens & Naes, 1996).

2. Experimental

2.1. Instrumental

UV spectral measurements were taken using an Ocean Optics model CHEMUSB4 UV–vis spectrophotometer with linear CCD array detector (Duiven, The Netherlands). The spectrophotometric measures were carried out at room temperature (about a mean of 20 °C). pH measurements were taken with a pH metre HORIBA F42 (Tokio, Japan). The HPLC data were obtained by KONIK KNK-500-A Series (Miami, FL, USA). A 25 cm C-18 column Lichrosorb RP18 (USA) was used with KONIK UV detector (Miami, FL, USA).

The PLS-2 data analysis was carried out using the Unscrumbler 6.11 software (CAMO ASA, Trondheim, Norway).

2.2. Reagents

The water used in all studies was ultrapure water (18.2 MΩ cm) obtained from a Barnstead Easy pure RF compact ultrapure water system (Dubuque, IO, USA). Acesulfame-K and aspartame, chloride and salicylic acids, potassium dihydrogen phosphate and potassium chloride, were purchased from Sigma (St. Louis, MO, USA). Acetonitrile HPLC grade was obtained from Merck (Durmsted, Germany).

2.3. Standard solutions

For the PLS-2 method, aspartame, acesulfame-K and salicylic acid stock solutions were prepared weighing accurately 0.5000, 0.5000 and 0.2500 g respectively, dissolving and diluted to the mark whit ultrapure water into a 1000 ml volumetric flasks. The buffer solution of pH 2.0 was prepared using chloride acid and potassium chloride, following pH values by Clarck and Lubs (Meites, 1963). The concentration matrix for calibration step was obtained with a suitable amount of stock solution (mixture of sweeteners and internal standard) and transferred to a 25 ml volumetric flask. Five millilitres of chloride acid buffer solution pH 2.0 was added and diluted to the mark with ultrapure water. The absorbance of these mixtures was measured in 10 mm quartz cells between 200 and 330 nm at 0.2 nm intervals with respect to a blank of chloride acid buffer solution pH 2.0.

For HPLC method, aspartamo and acesulfame-K stock solutions were prepared weighing accurately 0.1000 g l^{-1} for both cases. The mobile phase was prepared with 10% (v/v) acetonitrile and 90% (v/v) 0.02 mol l^{-1} potassium dihydrogen phosphate solution. Standard solutions were obtained diluting adequate volumes of aspartame and acesulfame-K stock solutions with a mobile phase into 10 ml volumetric flasks.

2.4. Real samples preparation

For the PLS-2 method, five solid (powder) commercial sweeteners were prepared weighing (0.8000 ± 0.0001) g of sweeteners, which were directly dissolved in ultrapure water and transferred into 100 ml volumetric flasks. From this solution, 2 ml were transferred to 250 ml volumetric flasks and added with a buffer solution pH 2 to mark. For the HPLC method, samples were prepared diluting adequate volumes of the above mentioned sweeteners solutions with a mobile phase: 10% (v/v) acetonitrile and 90% (v/v) 0.02 mol l^{-1} potassium dihydrogen phosphate solution. These sample solutions were filtered before injection.

2.5. HPLC procedure

Seven standard solutions and five replicates were prepared for both, aspartame and acesulfame-K, with concentrations of 3.0×10^{-3}, 4.0×10^{-3}, 5.0×10^{-3}, 6.0×10^{-3}, 7.0×10^{-3}, 8.0×10^{-3} and 9.0×10^{-3} (g l^{-1}). The HPLC conditions were: carrier flow 0.75 ml min^{-1}, absorbance measure using a single wavelength at 205 nm, similar to those stated by (Armenta et al., 2004). The obtained calibration curves yielded a R^2 regression coefficient of 0.9992 and 0.9988 for aspartame and acesulfame-K respectively, and were used to validate the PLS-2 method. The repeatability of HPLC system was evaluated performing successive injection ($n = 6$) 5.0×10^{-3} g l^{-1}, for aspartame and saccharin in combined standard solution. The Relative Standard Deviation (RSD) values were better than 0.3% for aspartame and 0.1% for acesulfame-K.

3. Results and discussion

3.1. The PLS model

A full factorial design 5^2 was used to build the calibration matrix, with five levels of concentration and two variables. The PLS-

Table 1
Concentration matrix for the PLS-2 model.

Std.	Aspartame[a]	Acesulfame-K[a]	Internal Std[a]
1	0.0000	0.0000	0.0000
2	0.0010	0.0000	0.0010
3	0.0020	0.0000	0.0040
4	0.0040	0.0000	0.0020
5	0.0080	0.0000	0.0000
6	0.0000	0.0004	0.0080
7	0.0010	0.0004	0.0010
8	0.0020	0.0004	0.0020
9	0.0040	0.0004	0.0040
10	0.0080	0.0004	0.0000
11	0.0000	0.0008	0.0080
12	0.0010	0.0008	0.0040
13	0.0020	0.0008	0.0020
14	0.0040	0.0008	0.0010
15	0.0080	0.0008	0.0000
16	0.0000	0.0020	0.0080
17	0.0010	0.0020	0.0020
18	0.0020	0.0020	0.0010
19	0.0040	0.0020	0.0040
20	0.0080	0.0020	0.0020
21	0.0000	0.0040	0.0080
22	0.0010	0.0040	0.0000
23	0.0020	0.0040	0.0010
24	0.0040	0.0040	0.0040
25	0.0080	0.0040	0.0080

[a] Concentration expressed in g l^{-1}.

2 model was obtained using a total of 25 standard solutions, which were obtained adding adequate volumes of aspartame and acesulfame-K stock solution and random volumes of internal standard stock solution, in similar concentration that the analytes. Table 1 show the concentration matrix used in the calibration step. Absorbance readings were carried out in different days in order to take into account instrumental variations, due to the fact that the PLS-2 uses full-spectra data (Lozano et al., 2007).

Fig. 1 shows the spectral overlapping of aspartame, acesulfame-K and internal standard in a 200–330 nm range. The calibration step was performed by the combination of the A absorbance matrix (or R response) and the C concentration matrix (Beebe et al., 1998; Esbensen, Schönkopf, & Midtgaard, 1997). The built model was obtained using auto scaled data.

The final model was built using internal validation (cross-validation method), where the model leaves out one standard of the calibration set every time. Then, the calibration and validation model error could be calculated through root mean square of calibration (RMSEC) and root mean square of prediction (RMSEP)

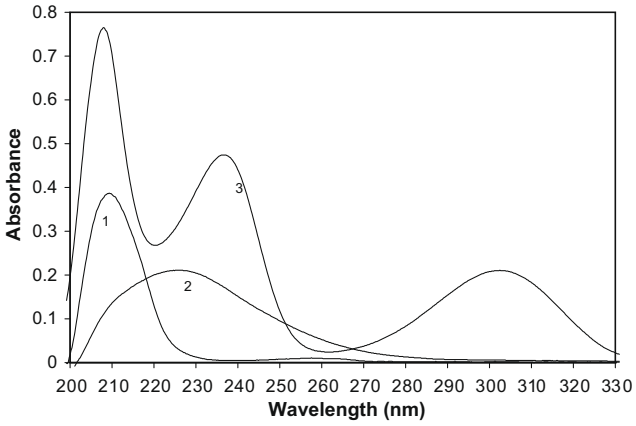

Fig. 1. Spectral curves of: 1 – aspartame (0.008 g l^{-1}), 2 – acesulfame-K (0.004 g l^{-1}) and 3 – internal standard (0.008 g l^{-1}).

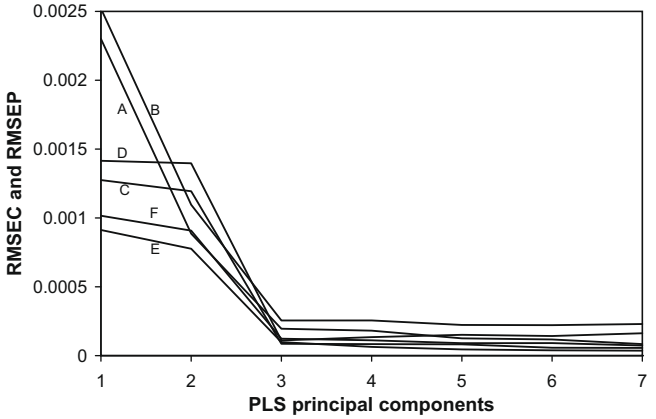

Fig. 2. RMSEC and RMSEP for aspartame, acesulfame-K and internal standard. Aspartame: (A) calibration, (B) validation. Acesulfame-K: (C) calibration, (D) validation. Internal standard: (E) calibration and (F) validation.

(Beebe et al., 1998; Esbensen et al., 1997). Fig. 2 shows RMSEC and RMSEP of the PLS-2 model for aspartame, acesulfame-K and internal standard, which helps to decide the adequate number of PLS components, by election of minimal value, which corresponds to 3th PC in all cases. Fig. 3 shows the explained variance (cumulative percentage) obtained in the calibration and validation processes with the PLS-2 method, which helps to decide the better number of PLS principal components. In Fig. 3, as with RMSEC and RMSEP in Fig. 2, three PLS principal components were needed to explain 99.4%, 99.6% and 99.9% of the original information for, aspartame, acesulfame-K and internal standard, respectively, in the calibration sets, transforming the original 655 wavelength absorbance variables into only 3 PLS principal components (Beebe et al., 1998). With this PLS-2 model, the observed predicted concentration plot was obtained for aspartame, acesulfame-K and salicylic acid (internal standard) where R^2 coefficients were 0.997, 0.999 and 0.998, respectively, suggesting a good fit in the model.

The figures of merit (FOM) of this method were calculated: sensibility (SEN_k), limit of detection (LOD), selectivity (SEL_k) and analytical sensibility (γ_k). SEN_k is described as:

$$SEN_k = \frac{1}{\|b_k\|}$$

Where b_k is the vector of regression coefficient for the k analytes. LOD is defined as:

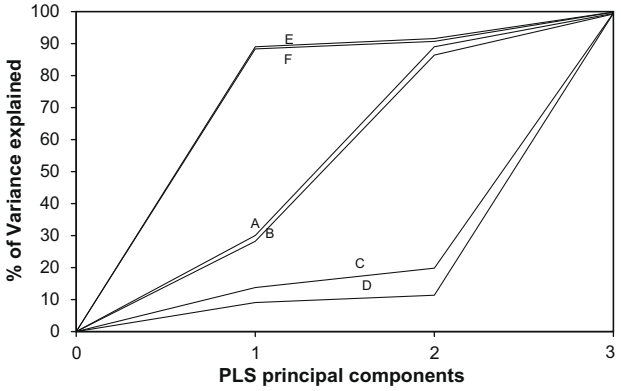

Fig. 3. Percent of explained variance in the calibration and validation model set, for aspartame: (A) calibration, (B) validation. Acesulfame-K: (C) calibration, (D) validation. Internal standard: (E) calibration, (F) validation as function wavelength for first (1), second (2) and third (3) PLS principal components.

$$LOD = 3.3\|\delta_r\|\|b_k\|$$

Where $\|\delta_r\|$ is a measure of instrumental noise. SEL_k is defined as:

$$SEL_k = \frac{\|s_k^*\|}{\|s_k\|}$$

Where s_k is the vector of spectral sensitivities of k components in the pure form, whilst s_k^* in the projection onto the net analyte signal space (Lorber, Faber, & Kowalsky, 1997). Finally, analytical sensibility is defined as:

$$\gamma_k = \frac{SEN_k}{\|\delta_r\|}$$

In this method the following figures of merit were found (a) for aspartame: limit of detection (LOD) = 0.29 μg mL^{-1}, sensibility (SEN) = 1.1 10^{-2} mL μg^{-1}, analytical sensibility (γ_k) = 11 mL μg^{-1} and selectivity (SEL) = 0.058. (b) for acesulfame-K: limit of detection (LOD) = 0.11 μg mL^{-1}, sensibility (SEN) = 3.0 × 10^{-2} mL μg^{-1}, analytical sensibility (γ_k) = 30 mL μg^{-1} and selectivity (SEL) = 0.063 (Llamas, Di Nezio, Palomeque, & Fernández Band, 2008; Lorber et al., 1997).

3.2. Internal standard data in the PLS model for real samples

Salicylic acid was used as internal standard to evaluate the predictive ability of the model in the unknown real samples of sweeteners, to calculate the recovery percentage and estimate possible matrix effects. This evaluation is important to know the behaviour of the model in relation to unknown samples, without external validation. This means that, if the internal standard has a good fit in the PLS model, the same facts will occur with the analytes in real samples. If the predicted concentration of internal standard is the same as the observed concentration in the real samples, it indicates that the matrix effects in the samples do not affect the PLS model. Internal standard was added to real samples in random concentration and levels similar to interesting analytes. Table 2 shows the results of added and predicted concentrations of internal standard in solid real samples (powder) obtained directly from the Argentine Market: A (Hileret), B (Genser), C (Equalsweet), D (Grandietsweet) and E (Tibaldi). In all cases, the predictive ability of internal standard in real samples using the proposed model has a mean value of 99.2% with standard deviation of 3.2%, which indicates that, due to dilution, the matrix effects do not alter the predictive ability of the model and the final results obtained show an excellent recovery degree in all samples.

3.3. Real samples prediction and HPLC validation results

Five real samples of the sweeteners above mentioned, were analysed with five replicates by the PLS-2 model: A (Hileret), B (Genser), C (Equalsweet), D (Grandietsweet) and D (Tibaldi). The components found in all real powder samples were: aspartame, acesulfame-K, carbohydrates, silicon dioxide and phenylalanine.

Table 2
Added-predicted concentrations of internal standard in real sample set to evaluate matrix effects and recovery degree.

Sample	Internal standard		
	Added[a]	Predicted[b]	Recovery (%)[b]
A	0.0065	0.0066 ± 0.0003	100.9 ± 3.7
B	0.0025	0.0024 ± 0.0002	97.6 ± 8.3
C	0.0045	0.0046 ± 0.0001	103.5 ± 2.5
D	0.0085	0.0084 ± 0.0002	98.3 ± 2.4
E	0.0015	0.0014 ± 0.0002	95.3 ± 12.8

[a] Concentration expressed in g l^{-1}.
[b] Average ± standard deviation of five determinations per sample.

Table 3
Predicted concentrations in real samples by PLS-2 model and validation by HPLC method.

Sample	Label values[a,c]	Aspartame		Label values[a,c]	Acesulfame-K	
		PLS-2[b]	HPLC[b]		PLS-2[b]	HPLC[b]
A[a]	26.5	26.2 ± 0.3	28.33 ± 0.09	26.5	26.8 ± 0.3	26.14 ± 0.04
B[a]	23.2	23.1 ± 0.2	25.28 ± 0.07	10.7	11.2 ± 0.1	12.41 ± 0.02
C[a]	16.1	15.1 ± 0.4	15.47 ± 0.05	16.1	16.4 ± 0.4	15.37 ± 0.04
D[a]	22.5	21.4 ± 0.3	23.60 ± 0.08	7.5	7.3 ± 0.3	8.10 ± 0.03
E[a]	20.0	19.7 ± 0.3	18.81 ± 0.05	13.3	12.5 ± 0.2	13.82 ± 0.03

[a] Concentrations of powder sweeteners expressed in mg g^{-1}.
[b] Average ± standard deviation of five determinations per sample.
[c] Value obtained from the manufacturer.

Results were validated by the HPLC method. The results obtained by the PLS-2 and HPLC methods are shown in Table 3. The relative error between both, PLS-2 and HPLC methods was less than 10% in all cases. This value is similar to that obtained by the PLS method in previous works for other types of samples (Llamas et al., 2008; Lozano et al., 2007; Sikorska et al., 2008).

3.4. Comparative statistical study between PLS-2 and HPLC methods

In order to evaluate the accuracy and precision of the PLS-2 vs HPLC method, the bivariate least square (BLS) calibration method was used (Culzoni, De Zan, Robles, Mantovani, & Goycochea, 2005). In previous works, the BLS was successfully used for evaluate statistically new analytical methods (Acebal, Lista, & Fernandez Band, 2008; Del Río, Riu, & Ruis, 2001; Ferré, Boqué, Fernández-Band, Larrechi, & Rius, 1997; González, Herrador, & Asuero, 1999). The linear model based on the BLS method, take in account the comparable errors in both axes, should fit a straight line where the intercept is not significantly different from 0 and the slope not significantly different from 1 (Riu & Rius, 1996; Riu & Rius, 1997). BLS method was used with the results presented in Table 3. The estimated intercept and slope were compared with their ideal values of 0 and 1 using the elliptical joint confidence region (EJCR) test. If the point (1,0) is inside the EJCR, it can be concluded that constant and proportional biases are absent (Culzoni et al., 2005). The ellipse for the two analytes (aspartame and acesulfame-K) include the theoretical values (1,0) at level of confidence of 95%, which indicates adequate statistical fit of PLS-2 in comparison with HPLC.

4. Conclusions

The results of this work show that the combination of full-spectra spectrophotometric methods with the PLS-2 data analysis, has allowed for the simultaneous determination of aspartame and acesulfame-K in artificial sweeteners. In comparison with others, this method can be used without previous chemical separations using only a UV spectrophotometer: for this reason it is simple, rapid and inexpensive. The recovery study showed an adequate adjust of PLS-2 model by use of an internal standard. The statistical comparative study of validation, demonstrate that PLS-2, it is not significant different from HPLC at 95% level of confidence. For these reasons, the proposed method for the determination of aspartame and acesulfame-K in artificial sweeteners, can be used as an alternative procedure for laboratories of routine analysis and dietetic quality control laboratories of minor complexity.

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