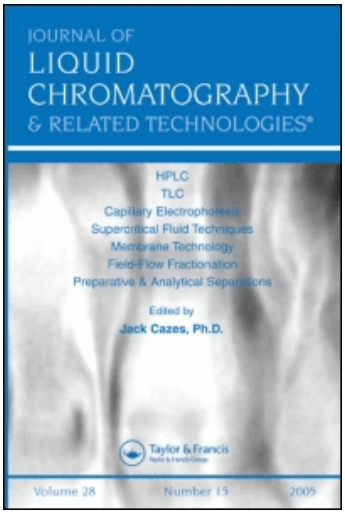

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Intense Sweetener Mixture Resolution by Flow Injection Method with On-Line Monolithic Element

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Abstract: A multi-analyte flow through method is proposed for the simultaneous determination of aspartame (AS), acesulfame-K (AK), and saccharin (SA) in several food and soft drink samples. The procedure is based on the transient retention of the three sweeteners in a commercial quaternary amine ion exchanger monolithic column, placed in its specific holder, and allocated in a monochannel FIA setup using pH 9.0 Tris buffer 0.03 M, NaCl 0,4 M, and $NaClO_4$ 0,005 M as carrier. In these conditions AS is very weakly retained, while AK and SA are more strongly retained, making it possible to measure the intrinsic UV absorbance of, first, AS and then AK and SA after desorption by the carrier itself. The applicable concentration range, the detection limit, and the relative standard deviation were the following: for AS, from 9.5 to 130.0 $\mu g \cdot mL^{-1}$; 2.87 $\mu g \cdot mL^{-1}$; 1.46% (at 65 $\mu g \cdot mL^{-1}$); for AK, between 2.2 and 600.0 $\mu g \cdot mL^{-1}$; 1.0 $\mu g \cdot mL^{-1}$, and 0.08% (at 300 $\mu g \cdot mL^{-1}$); and for SA, between 3.0 and 600.0 $\mu g \cdot mL^{-1}$; 0.9 $\mu g \cdot mL^{-1}$, and 0.09% (at 300 $\mu g \cdot mL^{-1}$). The method was applied and validated satisfactorily for the determination of AS, AK, and SA in foods and soft drink samples, comparing the results against an HPLC reference method.

Keywords: Acesulfame, Aspartame, Flow through optical, Foods and soft drinks, Monolithic minicolumn, Saccharin mixture, Sweetener determination

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INTRODUCTION

Flow injection analysis (FIA) methods present many advantages versus other methods of analysis such us speed, simplicity, cost, and analytical figures. FIA methods can include separative steps to improve their capabilities.[1] Commonly, the separative FIA systems based on solid phase supports can only separate binary mixtures by different ways: 1) the use of microcolumns to retain one analyte, although both analytes are transiently retained in the solid support that fills the flow cell after they pass or are eluted;[2–4] 2) the use of microcolumns to retain one analyte which is also retained in the solid support placed in the flow cell, while the other analyte is measured when it flows amid the interstitial solution through the particles;[5] 3) the use of the differences in the transient retention of both analytes in the flow cell. The transient retention of one analyte in the upper part of the flow cell, away from the measuring area, makes it possible to measure the one that is less retained and, thus its resolution;[6,7] 4) the use of a chemometric approach without separation of the analytes prior to the detection step.[8,9] These systems have many problems such as irregular packing, high back pressure and low flow rates. One solution to these problems could be the use of monolithic columns. Monoliths are continuous stationary phases that are cast as a homogeneous column in a single piece and prepared in various dimensions with agglomeration type or fibrous microstructures. These relatively new types of separative columns are based on a high cross linked porous monolithic polymer, with well defined, bimodal pore size distribution, providing excellent separation power and offering exceptional chemical stability and flow characteristics. They are characterized by flow independent resolution separations and a flow independent binding capacity, which allows high flow rates. They exhibit low backpressure, even at high flow rates.[10–13]

The separation of common inorganic anions has been achieved using several reverse phase monolithic systems in low pressure ion chromatography systems.[10,14,15]

The aim of this work is to develop a new procedure for the determination of three intense sweeteners: aspartame (AS), acesulfame-K (AK), and sodium saccharine (SA), using an anionic monolithic disk embedded in a flow system.

Intense or high potency sweeteners are used to include the sweetness characteristic in food and beverages with very little or no energy intake. Although there are a high number of organic molecules with the property of stimulating taste receptor cells in the mouth,[16] only a few are allowed for use in food and beverages due to toxicity and other problems they present. A common practice in the food industry is to blend intense sweeteners, bringing about a synergy of sweetness, which

makes lower usage levels and a lower cost possible and can improve the overall sweet taste.

Acesulfame-K (6-methyl-1,2,3-oxathiazin-4(3H)-one-2,2-dioxide; E-950) (AK) is used as a sweetening agent in the form of potassium salt. It has a synergistic effect when combined with other intense sweeteners, especially in mixtures with aspartame (N-L-α-aspartyl-L-phenylalanine-1-methyl ester; E-951) (AS) (ca. 1:1 by weight) and with sodium cyclamate (ca. 1:5 by weight), whereas only a slight taste enhancement is perceived in mixtures with saccharin. Saccharin (1,2-benzisothiazol-3(2H)-one-1,1-dioxide; E-954) (SA), is used in the form of sodium or calcium salt, a common high potency sweetener. Its bitter metallic aftertaste becomes more noticeable as the concentration increases. At concentrations for normal use, the aftertaste is detectable by about 25% of the population. One choice in order to reduce or eliminate this aftertaste is to blend SA with other sweeteners such as AS or AK.

Although a great variety of methods have been applied to the determination of this sweetener mixture in different matrices, its determination has been mainly by means of separative methods. HPLC[17,18] is the most popular system for separating and analysing these sweetener mixtures, due to its multi-analyte ability and because it adjusts itself better to the physicochemicals in sweeteners, although other chromatographic procedures have been described, such as ionic chromatography and TLC.[19] Different modes of capillary electrophoresis have been applied to achieve the separation these mixtures, and major protocols include CZE[20] and MEKC.[21]

CIM monolithic disks have been used for purification and determination of proteins,[22] for immobilization of enzymes,[23,24] oligo- and polynucleotides,[25] or blood analysis,[26] but to the best or our knowledge never for sweeteners analysis. So, this method contributes to a new application of this type of commercial support. A very simple and cheap FIA scheme is proposed for the analysis of this sweetener mixture without the need for any derivatization reaction. The potential usefulness of the procedure is checked in real samples analysis of food and beverages against an HPLC reference procedure.

EXPERIMENTAL

Reagents and Chemicals

The chemicals used were of analytical reagent grade and all aqueous solutions were prepared using reverse osmosis type quality water produced by a Milli-RO 12 plus Milli-Q purification system (Millipore, Bedford, MA).

Aspartame (AS) (10000.0 $\mu g \cdot mL^{-1}$), saccharin sodium salt (SA) (10000.0 $\mu g \cdot mL^{-1}$) (both from Sigma-Aldrich Química S.A., Madrid, Spain), and acesulfame potassium salt (AK) (10000.0 $\mu g \cdot mL^{-1}$) (Fluka, Madrid, Spain); stock solutions were prepared by exact weighing and dissolution in water. These solutions were spectrophotometrically stable for at least two months stored under refrigeration at 4°C in dark bottles. Solutions of lower concentrations were prepared by dilution with water, maintaining in all instances, the pH constant at 9, 0.4 M in NaCl and $5 \cdot 10^{-3}$ M in $NaClO_4$ (these are the same conditions as the carrier).

The monolithic quaternary ammonium anion exchange (disks 12 mm in diameter and 3 mm thick) based on a rigid highly cross linked monolithic glycidylmethacrylate-co-ethyleneglycoldimethacrylate polymer CIM, with quaternary amine groups supplied by BIA Separations (Ljubljana, Slovenia), was used to achieve the separation.

The disc dimensions were 12 mm in inner diameter and 3 mm in thickness, with a total active bed volume of just 0.34 mL. As carrier solutions, the 1 M buffers boric acid/sodium borate, sodium hydrogen carbonate/sodium carbonate, and tris(hydroxymethyl)aminomethane (Tris) (all from Sigma-Aldrich) were tested. As solid phase extraction cartridges, Isolute SAX (from Biotage, Uppsala, Sweden) and Oasis MAX (from Waters, Milford, MA, US) were used in the treatment of samples.

Apparatus and Software

Absorption measurements were made with a Hewlett Packard HP-8453 diode array spectrophotometer (Norwalk, CT, USA), interfaced to a personal computer via LAN interface, and equipped with a Hellma 178.710-QS flow cell with 10 mm light path. A Hewlett-Packard 1100 liquid chromatograph with DAD detector provided with a C_8 Zorbax column were used to validate the proposed method.

The flow setup consisted of an Ismatec Reglo Digital four channel peristaltic pump working at a constant flow rate and a variable volume Rheodyne 5041 teflon rotary valve controlled electromechanically.[27] The outlet of the injection valve was connected directly to the anion exchange monolithic disk through a disk housing holder that contains the monolithic disk, and connects it to the flow line through standard connectors. Minimum PTFE tubing (Omnifit, Cambridge, England) (0.8 mm i.d. and 1.6 mm o.d.) was used to reduce back pressures and extra column band broadening.

As software for the acquisition and manipulation of the spectral data, the UV visible Chemstation software package supplied by HP was used. Software programs used for the measurements of FIA peak

and area were: Statgraphics software package, ver. 5.0 STSC Inc. (Statistical Graphics Corporations, Englewood Cliffs, NJ, USA), CSW32 v 1.3.3 1999–2001, and Microsoft Office 2003.

Procedures

Basic Procedure

The sample volume (25 μL) containing between 9.5 and 130.0 $\mu g \cdot L^{-1}$ of AS, between 2.2 and 600.0 $\mu g \cdot L^{-1}$ of AK, and between 3.0 and 600.0 $\mu g \cdot L^{-1}$ of SA, with the same pH as the carrier and adjusted with the same buffer (pH 9.0 Tris buffer 0.03 M, 0.4 M NaCl and $5 \cdot 10^{-3}$ M $NaClO_4$) is inserted into the carrier stream. When the sample arrives at the disk housing holder containing two monolithic disks, the analytes are separated in the order: AS, AK, and SA, being measured in the following conditions: AS at 38 s and 205 nm; AK at 254 s and 226 nm, and SA at 335 s and 205 nm. Then the flow system is conditioned with the carrier up to 500 s to return to baseline. The relationship between the concentration and peak height is established by conventional calibration with external standards.

Reference Procedure

As a reference method, an adaptation of the HPLC-DAD method proposed by Lawrence and Charbonneau[28] was used. A 5 μm C_8 silica in a 150×4.6 mm column was used as a stationary phase, with a mobile phase gradient ranging from 3% acetonitrile in 0.02 M KH_2PO_4 (pH 5) to 20% acetonitrile in 0.02 M KH_2PO_4 (pH 3.5) at a constant flow rate of 1.0 mL min^{-1}. The chromatograms were obtained at a wavelength of 210 nm. In order to obtain the calibration function, 6 different concentration levels and 3 replicates of each one of the standard solutions were analysed using peak area as the analytical parameter.

Treatment of Samples

Before the analysis of the samples, the presence of AS, AK, and SA was tested by an HPLC method[28] comparing the retention times of the problem with those obtained with standard solutions.

For the analysis of beverages in samples containing AK and/or SA, an adequate amount (typically between 5 and 7.5 mL) was taken, degassed, and diluted to 10 mL adjusting the same conditions as the carrier. Finally, they were filtered through a 0.2 μm Millipore filter.

For the analysis in juices, the sample (typically 7.5 mL) was diluted to 10 mL, adjusting the same conditions as the carrier, centrifuged if necessary, and finally filtered through a 0.2 μm Millipore filter. In the case of strawberry sweets, an adequate amount (typically 7 g) was weighed and thoroughly crushed in a glass mortar, then dissolved in water (50 mL) with the aid of an ultrasonic bath, adjusting to the same conditions as the carrier. After that it was centrifuged (4500 rpm for 20 minutes) and filtered through a 0.2 μm Millipore filter. In the case of tomato sauce, the amount (typically 4 g) was suspended in 10 mL of water, and then 6 mL were diluted to 10 mL, centrifuged and adjusted with the same conditions as the carrier, and finally, filtrated through a 0.2-μm Millipore filter. After that, the basic procedure was applied.

For the analysis of samples containing AS, the procedure applied for sample preparation is different because AS is weakly retained, eluting next to the front, where all the substances not retained in the column appear. All analysed samples that contain AS are in mixture with AK. AK content can be determined with the proposed procedure without problems, but the analysis of AS requires a further purification step. Thus, two possible approaches can be used for samples containing AS and AK: 1) The two injection approach: the sample treated as indicated above is injected first, measuring the AK content. Then, a second injection is performed from the cleansed solution to determine AS. The cleanup procedure is: 2 mL of liquid sample (with the same conditions as the carrier) is loaded at a flow rate of 6 mL·min^{-1} in an Oasis MAX cartridge previously conditioned with 2 mL of water and 2 mL of MeOH. The cartridge is then washed by passing 2 mL of water. The AS is eluted with 2 mL of 90% MeOH:water at a flow rate of 6 mL·min^{-1} and collected in a glass vial. The eluate is evaporated to dryness on a hot block at a temperature not exceeding 30°C, under a gentle stream of nitrogen. The residue is solved in 2 mL carrier and analysed, applying the basic procedure. 2) One injection approach: the sample is purified before injection with two solid phase cartridges in series, first an Isolute SAX cartridge and then an Oasis MAX cartridge. The cartridges are conditioned with 2 mL water and 2 mL MeOH. Then, 2 mL of the sample (with the same conditions as the carrier) are passed through the cartridges; the resulting AK is retained in the first cartridge and AS in the second cartridge. After washing the cartridges by passing 2 mL water, the elution is performed with 2 mL carrier (AK is eluted from 1) and 2 mL 90% of MeOH:water (AS is eluted from 2) consecutively. The extract is evaporated to dryness on a hot block at a temperature not exceeding 30°C under a gentle stream of nitrogen, the residue solved in 2 mL carrier and analysed.

RESULTS AND DISCUSSION

Variables

The three sweeteners under study, AS, AK, and SA show very similar absorption spectra with maximums near 200 nm and similar molar absorptivities (AS: maximum absorption in water: 195 nm, molar absorptivity ε: $1.67 \cdot 10^4$ L·mol^{-1}·cm^{-1}, and a shoulder at 205 nm, ε: $9.99 \cdot 10^3$ L·mol^{-1}·cm^{-1}; AK: 227 in water, ε: $5.23 \cdot 10^3$ L·mol^{-1}·cm^{-1}, and SA: 205 nm, ε: $1.54 \cdot 10^2$ L·mol^{-1}·cm^{-1}. This means they must be separated to resolve the mixture. We previously studied the resolution of the sweetener mixture of AS and SA including a mini-column filled with C_{18} silica (300 mg, bead size 20–80 μm) in the flow setup,[4] but this has the disadvantages of high back pressure and low separative power. The aim of this study is to explore the possibilities offered by commercial ion-exchange monolithic disks for the separation of organic compounds using an FIA scheme. As monolithic disk minicolumn, we selected a CIM® disk made up of a glycidylmethacrylate-co-ethyleneglycoldimethacrylate polymer (12 mm in diameter and 3 mm thick) with quaternary amine groups QA). Working with this very short separative element, we were able to solve the usual problem when using low pressure pumps, such as peristaltic pumps[14] as is our case, for separations on monolithic columns. In this case, the system back pressure is reduced using tubing with 0.8 mm i.d. and the maximum flow rate attained was 2.0 mL·min^{-1}.

Eluent Conditions

The eluent composition is studied together with the length of the column because both are interdependent. The working methodology was always the same: first, test separations with one monolithic disc in the minicolumn holder and next with two discs.

The composition and pH of the carrier used affect the retention and resolution of the sweeteners on the QA disk mini-column. The carrier pH is the key factor for separation; at a pH value higher than 6, AS retention is increased in the QA disk in accordance with the pK_a values of AS dipeptide (pK_1 3.18; pK_2 7.82; isoelectric point 5.25). The retention of SA (pK_a 1.6) and AK (pK_a −0.28) are not pH dependent. The retention time of AS on the QA disk (Figure 1) increases at pH values higher than 7 up to pH 9, as is predictable. At pH values higher than 10, two peaks appear in the diagram, probably due to the partial cyclodehydration of AS to form a diketopiperazine.[16] Consequently, we selected pH 9 as the as working pH, because it permits the retention of AS in the disk and its separation from the other sweeteners without any degradation.

This pH value is available with monolithic disks because its working range pH is between 2 and 13.

The data in Figure 1 were obtained by using one monolithic disk. With two discs the curve is similar, but with higher retention times, as could be expected because of the longer column length.

The working pH was adjusted with a buffer, although the simple use of a buffer did not enable the resolution of the AK/SA mixture; even with a 1 M buffer concentration, the analytes did not leave the column in 2000 s, either with one or two monolithic discs. Consequently, we adjusted the pH with the minimum amount required of buffer containing $NaClO_4$ (0.01 M) in order to facilitate the elution of analytes. As a buffer we tested pH 9 borate, carbonate, and TRIS with a concentration ranging between 0.01 and 0.05 M (5 levels). Finally, we selected TRIS as a buffer because the analytical signals for the three sweeteners studied were, on average, 15% higher than the signals with the other buffers. Additionally, the buffer concentration selected was 0.03 M because it was the minimum concentration that shows good buffering capacity.

As noted before, it is not possible to resolve the AK/SA couple with buffer alone. Consequently, we tried to improve the resolution, testing different eluent anions by adding several salts to the buffer, namely Na_2SO_4, $NaNO_3$, NaCl, and $NaClO_4$. With Na_2SO_4 we did not obtain good results; we tested different concentration levels and the sweeteners

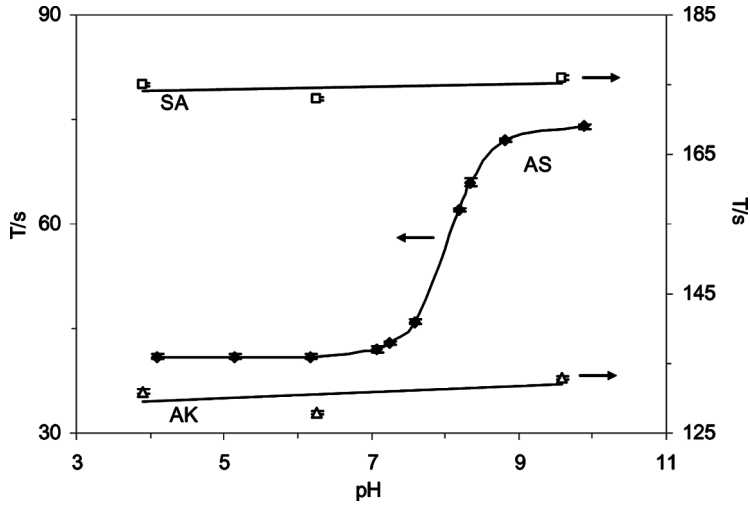

Figure 1. pH dependence on the retention time of AS. Samples have the same conditions as the carrier. Conditions: HCl at different pH, sample loop 125 μL, flow rate 1.0 mL·min^{-1}, AS concentration 10 μg·L^{-1}, $NaClO_4$ concentration 0.01 M. Error bars coming from three replicate measurements.

did not elute up to 2000 s. Similar results were obtained with $NaNO_3$. Better results were obtained with NaCl. Figure 2A shows the AK-SA resolution with the NaCl concentration. The minimum resolution (around 0.8) was achieved using one monolithic disc at a low NaCl concentration, but the analysis time increased up to unacceptable values (up to 3000 s). By using two discs, similar behaviours were encountered at higher NaCl concentrations; thus, with 1 M NaCl the analysis time was higher than 1000 s and thus, it was not useful.

The influence of $NaClO_4$ (Figure 2B) on AK-SA resolution is higher than chloride, as can be expected considering its higher size. However, the analysis time was, as with chloride, very high (for example, with $NaClO_4$ 0.01 M the analysis time is greater than 1200 s with resolution around 0.8) using one disc. With two discs, the analysis time is shortened but even very high. Better results were obtained using binary mixtures of NaCl and $NaClO_4$. To select the best eluent, we studied carriers containing $NaClO_4$ ranging from 0.001 M to 0.05 M (5 concentration levels), because at higher concentration levels the resolution of AK and SA dropped (see Figure 2B). At all these $NaClO_4$ levels, we tested several concentrations of NaCl (5 levels between 0.1 and 0.6 M), but using only one disc it was not possible to achieve enough resolution to measure them and

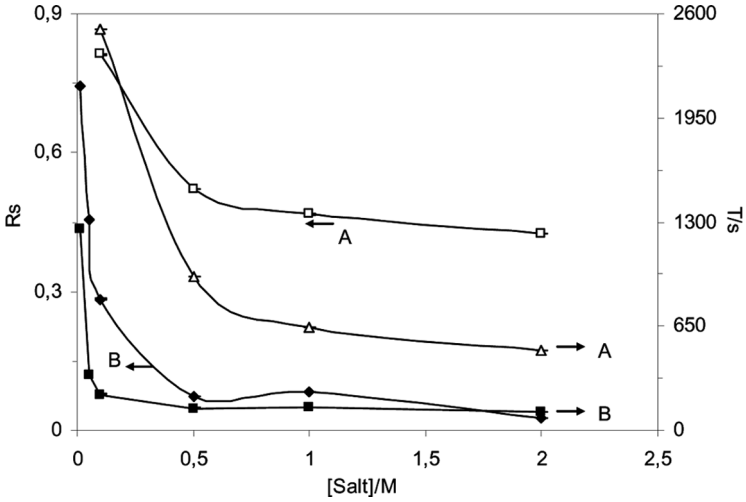

Figure 2. (a) Evolution of the resolution of AK-SA and the analysis time with the NaCl concentration. Conditions: pH 9.0 Tris buffer 0.03 M, NaCl variable, analyte concentration 10 $\mu g \cdot L^{-1}$. (b) Evolution of the resolution of AK-SA and the analysis time with the $NaClO_4$ concentration. Conditions: pH 9.0 TRIS buffer 0.03 M, $NaClO_4$ variable, analyte concentration 10 $\mu g \cdot L^{-1}$. Error bars coming from three replicate measurements.

achieve an acceptable analysis time. However, the use of two discs improved the AK/SA separation and it was possible to achieve a compromise between analysis time and resolution. The optimum composition was 0.005 M $NaClO_4$ and 0.4 M NaCl plus the buffer; under these conditions, the resolution was sufficient (0.8) to measure FIA peaks in height with an acceptable analysis time (around 800 s). Under these conditions, the sweetener AS is weakly retained and is eluted near the front because composition is eluted quickly with this carrier (average retention time 30 s).

The FIA variables studied were flow rate and loop volume. The influence of the flow rate on the signal was studied between 1.1 and 2.0 mL min^{-1}, because at lower flow rates, the analysis time increased up to 800 s, and using a peristaltic pump and two monolithic disks the maximum flow rate attained was 2.0 mL·min^{-1}. The main factors in selecting the optimum flow rate were the resolution and the analysis time. Resolution, as can be expected with monolithic columns, is independent of the flow rate, while the analysis time increases with the decrease in flow rate (Figure 3). Thus, the maximum flow rate tested–the maximum flow rate that the system is able to support–was selected as optimum (2.0 mL min^{-1}) in order to minimise the analysis time (500 s).

An increase in the sample volume between 25 and 500 μL increases (as can be expected) the analytical signal as a result of the larger amount

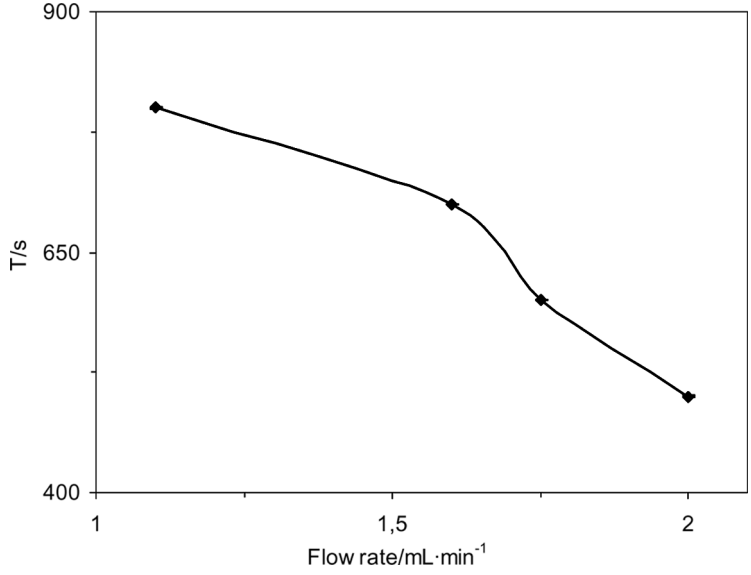

Figure 3. Optimisation of flow rate: evolution of analysis time. Conditions: pH 9.0 Tris buffer 0.03 M, 0.4 M NaCl, 0.005 M $NaClO_4$, analyte concentration 10 μg·L^{-1}, flow rate variable. Error bars coming from three replicate measurements.

of analyte in the flow system. The main factor selecting the appropriate loop volume was the resolution between AK and SA. Increasing loop volume increases the analytical signal but decreases the resolution of the peaks. Better resolution was achieved with the minimum loop volume, 25 μL, due to the lower bandwidth ($\omega_{\frac{1}{2}}$.), thus, 25 μL was selected as the optimum loop volume (Figure 4).

Analytical Features

The peak height was used as the analytical signal because the resolution of AK and SA is not sufficient to use the area as signal. For AS, the calibration function was linear between 9.5 and 130.0 $\mu g \cdot L^{-1}$, for AK from 2.2 to 600.0 $\mu g \cdot L^{-1}$, and for SA from 3.0 to 600.0 $\mu g \cdot L^{-1}$, these being the analytical signals collected at the wavelength of the maxima for each sweetener. The adjustment of the analytical data was carried out by linear regression, using the lack of fit test to test the linearity (7 replicates of each standard and 5 standards for each calibration function). The standard deviation of the background signal measured for the blank, which is necessary for the estimation of the IUPAC detection limit (K = 3) and the quantification limit (K = 10)[29], was taken as the average of ten determinations and noted as RSD units. Table 1 shows these and

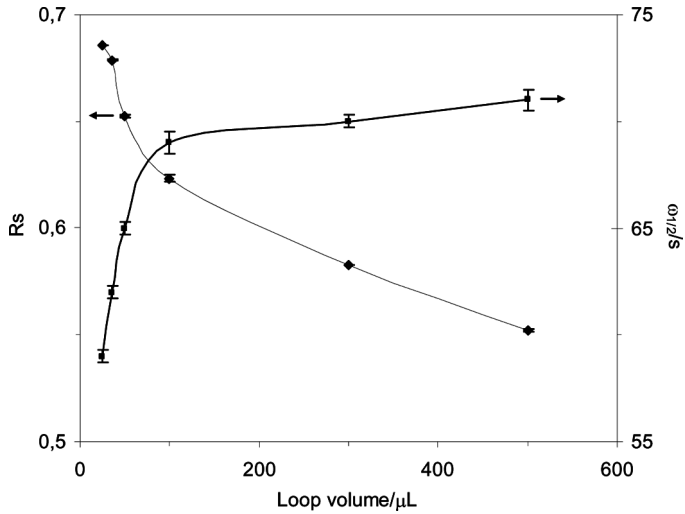

Figure 4. Evolution of the resolution (Rs) of AK and SA and the bandwidth ($\omega_{1/2}$) with the loop volume. Conditions: pH 9.0 Tris buffer 0.03 M, 0.4 M NaCl, 0.005 M $NaClO_4$, analyte concentration 10 $\mu g \cdot L^{-1}$, flow rate 2.0 $mL \cdot min^{-1}$, loop volume variable. Error bars coming from three replicate measurements.

Table 1. Analytical performance for AS, AK and SA flow methods

		AS	AK	SA
Parameters	Units	205 nm	226 nm	205 nm
B	$mL \cdot \mu g^{-1}$	$6.26 \cdot 10^{-3}$	$2.91 \cdot 10^{-3}$	$3.42 \cdot 10^{-3}$
S_b	$mL \cdot \mu g^{-1}$	$9.12 \cdot 10^{-5}$	$2.22 \cdot 10^{-6}$	$3.24 \cdot 10^{-6}$
A	–	$2.58 \cdot 10^{-2}$	$3.62 \cdot 10^{-3}$	$-3.63 \cdot 10^{-3}$
S_a	–	$6.08 \cdot 10^{-3}$	$7.43 \cdot 10^{-4}$	$1.17 \cdot 10^{-3}$
R^2	–	99.83	99.99	99.99
LRD	$\mu g \cdot mL^{-1}$	9.5–130.0	2.2–600.0	3.0–600.0
DL	$\mu g \cdot mL^{-1}$	2.9	1.0	0.9
QL	$\mu g \cdot mL^{-1}$	9.5	0.7	3.0
RSD	%	1.46	0.08	0.09

b: slope; S_b: standard deviation slope; a: intercept; S_a: standard deviation intercept; r: correlation coefficient; PL: Probability level lack-of-fit test; LRD: Linear range dynamic; DL: Detection limit; QL: Quantification limit; RSD: Relative standard deviation.

analytical parameters. The sampling frequency is $7\,h^{-1}$. The proposed method shows satisfactory results for quantification of each sweetener in the presence of different ratios of the co-existing sweeteners. Six sweetener mixtures with ratios in the range 1:20 to 20:1 for AS, between 1:120 to 120:1 for AK and SA were added at levels of 10 to 130 $\mu g \cdot L^{-1}$ for AS, at levels of 2 to 600 $\mu g \cdot L^{-1}$ for AK, and at levels of 3 to 600 $\mu g \cdot L^{-1}$ for SA. Recovery of sweeteners was in the range of 103.8% to 101.4% for AS, 98.2% to 102.5% for AK, and of 97.6% to 103.4% for SA, with mean recovery of 102.6% for AS, 100.4% for AK, and 100.5% for SA.

Analytical Applications

The proposed method was applied to different beverages and food samples, which contain the sweeteners under study, commonly found in different supermarkets in the town of Granada (Spain). Some of the commercial products contain the three sweeteners, but most commonly, the samples contained only one of them or at most, binary mixtures. We chose these samples in order to evaluate all the possibilities in binary mixtures and, when possible, the ternary sweetener mixtures. The presence of the sweeteners is indicated on the label, and demonstrated by the comparison with the HPLC retention times.

When AS was not present in the sample, the treatment of sample was very simple (see Treatment of Samples). When AS was present in the sample, we applied an extraction method developed by us. The sample needs to be purified in order to obtain a clean FIA record. Because AS

is weakly retained, it is eluted next to the front, and, consequently, non-retained substances present in the sample cause interference with the AS signal. Therefore, samples with AS need to be purified. As is usual with HPLC, it is not necessary to purify the sample (most papers only degas and dilute the sample)[18,30] and we have developed a cleanup procedure for samples containing AS. The cleanup is based on solid phase extraction. Different SPF cartridges were tested for retention of AS and AK, but we were not able to find one cartridge for this purpose that shows good recoveries. In the end, we selected two different cartridges, one for each sweetener: Namely with the anion exchange cartridges Oasis MAX (hydrophilic-lipophilic-balanced water-wettable copolymer) for the retention of AS, although not for AK; and Isolute SAX 500 mg (silica quaternary amine based) in which AK is strongly retained while AS is weakly retained. For both cartridges we worked in conditions to obtain a good anionic retention (samples were conditioned with the same conditions as the FIA carrier). For the elution of analytes from cartridges, several eluents were tested in order to obtain the correct eluent. For Isolute SAX, water did not elute AK and so, it was selected as the cleaning solution, while the carrier did so because of its composition, and consequently, was selected as the eluent for AK. For AS, several hydro-methanolic carriers (from 10% to 100%) were tested in order to study the AS elution behaviour. Water did not elute it; and then it was also selected as cleaning solution. An increase in the MeOH percentage, increases the elution power for AS, finding that MeOH 90% elutes AS from the column completely, and so it was used as eluent. Working in these conditions, the recovery found for both sweeteners was near 100%. It is possible to use both cartridges in series in order to shorten the analysis time.

There is another possibility for measuring samples containing AS and AK using only one cartridge, but two injections were necessary. First, the sample (degassed, diluted, filtered, and conditioned like the carrier) was injected in the flow system in order to measure the AK. Next, the previously injected sample was subject to a further purification to measure AS using an Oasis MAX cartridge (conditioned, cleaned, and eluted as indicated before). Both measurement methods were viable, and tested with good results, but in the end, the first (with one injection and two cartridges) was selected because it is faster.

Additionally, the results obtained by the proposed procedure were validated by statistical comparison with a reference method HPLC method[28] (Table 2). This table includes the mean values from 3 determinations of each sample, standard deviations of these measurements and the probability value (P_{val}) of the test used for the comparison of the results obtained for both methods. As can be observed, the results obtained with both methods are statistically similar.

Table 2. Results obtained for the mixture by reference and proposed methods*

Sample	AS/mg·L^{-1}			AK/mg·L^{-1}			SA/mg·L^{-1}		
	Proposed method*	Reference method*	P-value (%)	Proposed method*	Reference method*	P-value (%)	Proposed method*	Reference method*	P-value (%)
Cola light 1	252 ±7.74	254 ±7.77	79.93	66 ± 1.33	67.2 ± 0.38	6.53			
Cola light 2	62 ± 2.08	58 ± 2.59	13.23	126 ±1.36	125 ±1.30	53.08			
Fruit Juice	144 ±6.08	141 ±4.35	51.69	31 ± 1.58	32.5 ± 0.42	24.47			
Apple Juice	34.5 ± 0.92	34.6 ± 0.13	88.33	30.2 ± 0.58	29.44 ± 0.01	15.92	17.2 ± 0.39	16.81 ± 0.05	30.26
Black tea	31 ± 1.48	33 ± 1.01	62.84	56.6 ± 0.68	56.1 ± 0.11	27.97			
Tonic water				243 ±1.72	245 ±1.04	12.02	57.8 ± 0.40	55 ± 1.98	9.04
Orange drink				118 ±1.90	116.8 ± 0.32	32.34	55.8 ± 0.59	56.10 ± 0.05	41.07
Water				36 ± 1.12	37.3 ± 0.12	22.17			
Orange drink				255 ±1.74	254.2 ± 0.28	62.53			
Soda							93 ± 2.92	92 ± 1.31	61.68
Tomato sauce**				105.4 ± 0.68	106.0 ± 0.47	38.14	54 ± 1.11	54.05 ± 0.05	73.07
Strawberry Sweets**				156 ±2.45	156.9 ± 0.32	50.48	161 ±2.10	161.9 ± 0.49	78.97

*Mean of three determinations ±SD.

**Results are expressed in mg L^{-1}, except in the case of tomato sauce and strawberry sweets which are expressed in mg Kg^{-1}.

The proposed method shows several advantages against the usual HPLC procedure; it uses very simple, fast instrumentation that is cheaper and has similar precision. The disadvantage is that the other procedure has a better resolution than our procedure, as expected with HPLC, although ours is sufficient for determination.

CONCLUSION

An optical flow through multi-analyte method has been developed for aspartame, acesulfame-K, and sodium saccharine mixtures using a commercial ion exchanger monolithic disk in the flow as the basis for separation. A simple FIA configuration with a single channel, with no previous derivatization reaction of the analytes, makes separation possible in disks based on the transient retention of AK and SA while AS is very weakly retained. This paper provides a practical, simple, rapid, and inexpensive method for the simultaneous determination of AS-AK-SA mixtures at $\mu g \cdot mL^{-1}$ levels, which may be employed in routine analysis. The method described here makes it possible to analyse this high potency sweetener mixture in commercial samples. The results were chemometrically validated and compared with an HPLC reference method. The results are good enough, but the method is limited by the choice of monolithic support. The limit is due to fact that the properties of the monolithic support are not the best to couple it to a flow system (such as its dimensions, for example). Currently, we are working on the development of new monolithic supports, with the aid of specialised personnel.

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