JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Simultaneous Determination of Nonnutritive Sweeteners in Foods by HPLC/ESI-MS

DA-JIN YANG[†,‡] AND BO CHEN*[,†]

Key Laboratory of Chemical Biology and Traditional Chinese Medicine Research, Ministry of Education, Hunan Normal University, Changsha 410081, China, and National Institute for Nutrition and Food Safety, Chinese Center for Disease Control and Prevention, Beijing 100021, China

Nonnutritive sweeteners are the low calorie substances used to replace sugar and other caloric ones. Determination of these sweetners in foods is important to ensure consistency in product quality. In this study, seven artificial (aspartame, saccharin, acesulfame-K, neotame, sucralose, cyclamate, and alitame) and one natural sweetener (stevioside) were simultaneously determined in different foods using high performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometric detection (ESI-MS). The target compounds were quantified using a selective ionization recording (SIR) at *m*/*z* 178, 397, 377, 293, 641, 312, 162, and 182 to cyclamate, sucralose, neotame, aspartame, stevioside, alitame, acesulfame-K, and saccharin, respectively, with warfarin sodium (SIR *m*/*z* 307) being used as an internal standard. The correlation coefficient of the calibration curve was better than 0.998 ($n = 6$), in the range of 0.05 to 5.00 μg/mL for cyclamate, 0.30 to 30.0 μg/mL for sucralose, 0.10 to 10.0 μg/mL for neotame, 0.20 to 20.0 μg/mL for aspartame, 0.50 to 15.0 μg/mL for stevioside, 0.08 to 8.00 μg/mL for alitame, 0.10 to 15.0 μg/mL for acesulfame-K, and 0.05 to 5.00 μg/mL for saccharin. The limits of detection (LODs) were below 0.10 μg/mL, whereas the limits of quantification (LOQs) were below 0.30 μg/mL. It is concluded that the method has merits such as high sensitivity, specificity, and simplicity versus the those of the other methods reported in the literature.

KEYWORDS: Nonnutritive sweetener; aspartame; saccharin; acesulfame-K; neotame; sucralose; cyclamate; alitame; stevioside; warfarin sodium; HPLC/ESI-MS

INTRODUCTION

Nonnutritive sweeteners are used to replace sugar in foods. These sweeteners are helpful in controlling body weight and insulin levels as they provide no or little calories. There are five artificial sweeteners on the market that have been approved by the US FDA, namely, aspartame, saccharin, acesulfame-K, neotame, and sucralose. Their benefits and safety remain controversial. Stevioside is a sweetener from South America that is gradually becoming more popular in the US. Because different nonnutritive sweeteners can elicit different sweet taste quality, 2 or 3 different artificial sweeteners such as cyclamate, saccharin, and acesulfame-K are always added in one food simultaneously to elicit a good sweetener taste. The legal content limits of the different sweeteners in the different types of food are significantly different. In general, the content range of the sweeteners is 10−1000 mg/kg. However, the sweeteners are prohibited to be added in some foods, especially in the foods for infants. For example, the content limit of aspartame in canned or bottled fruit (energy-reduced or with no added sugar) is 1000 mg/kg, that in the beer with a minimum acidity of 30 milli-equivalents expressed as NaOH is 600 mg/mL (European Union standard), whereas the addition of aspartame in cream, reduced cream, and light cream is prohibited (Food Standards Australia New Zealand). Therefore, the high sensitive and specific simultaneous determination of different nonnutritive sweeteners is necessary to the quality control of foods.

Liquid chromatography (LC) has been the most popular choice for the determination of the sweeteners (*1*−*6*). But only a few are suitable for the simultaneous determination of several sweeteners. Because the physicochemical, electrochemical, and spectral properties of the nonnutritive sweeteners are significantly different, a method for the simultaneous determination of the components is always restricted by the absence of simultaneous separation and detection. From the separation point of view, most HPLC procedures are based on isocratic or gradient reverse-phase (RP) chromatographic separation. Derivative gas chromatography (DGC), RP ion-pair chromatography, ion chromatography (IC), and capillary electrophoresis are also used for the simultaneous determination of nonnutritive sweeteners (*1*−*19*). Although the separation can be completed by different strategies, the different electrochemical and spectral properties of sweeteners affect the detection for simultaneous

* Corresponding author. Tel: +86 731 8865515. Fax: +86 731 8865515. E-mail: dr-chenpo@vip.sina.com.
† Hunan Normal University.
‡ Chinese Center for Disease Control and Prevention.

10.1021/jf803988u CCC: $40.75
Published on Web 03/10/2009

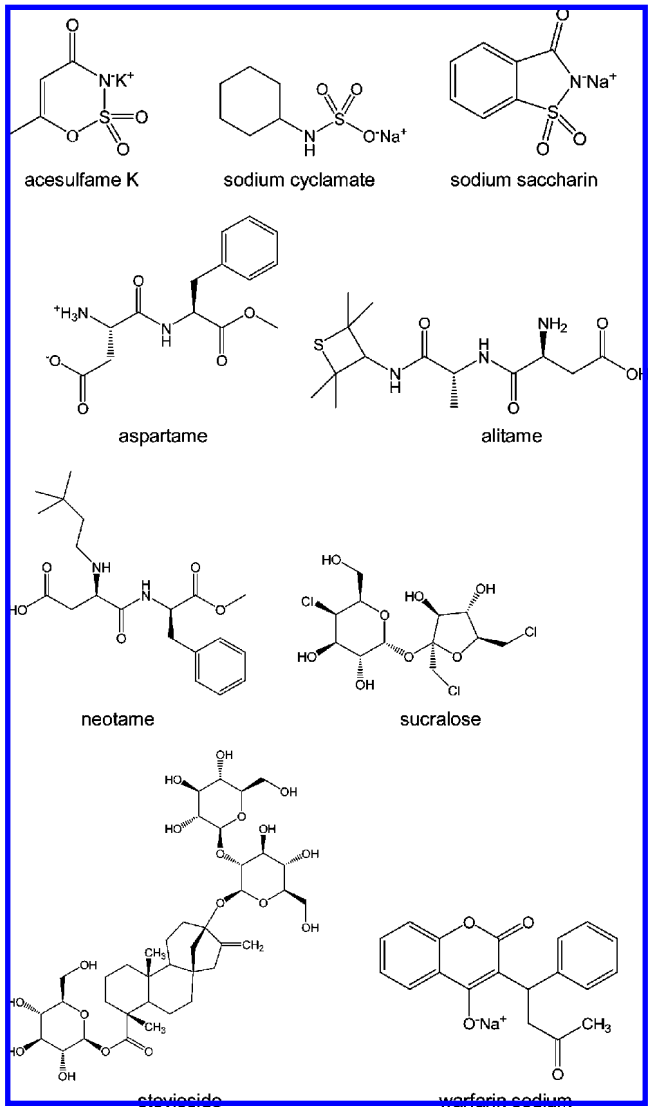

Figure 1. Structures of the investigated sweeteners and internal standard.

determination. Nonnutritive sweeteners are all high intensity sweeteners, and therefore, only smaller amounts are present in foods. From the detection point of view, the development of an analytical method with a high sensitivity is deemed necessary. Various techniques including ultraviolet (UV) absorbance detection, electrochemical amperometric detection, potentiometric detection, flame ionization detection (FID), and ESI-MS, and so forth have been used to detect nonnutritive sweeteners (*1*−*19*). Koyama et al. (*20*) first developed a method for the simultaneous determination of nine types of sweeteners (acesulfame-K, sucralose, saccharin, cyclamate, aspartame, dulcin, glycyrrhizinic acid, stevioside, and rebaudioside A) in various foods by HPLC electrospray mass spectrometry. However, the quantification of the sweeteners without an internal standard was a disadvantage to the ESI/MS detection. Wasik et al. (*21*) reported an HPLC method to analyze nine sweeteners (acesulfame-K, alitame, aspartame, cyclamic acid, dulcin, neotame, neohesperidine, dihydrochalcone, saccharin, and sucralose) simultaneously, with an evaporative light scatting detector being used. Although the HPLC methods have high separation efficiency, their detection sensitivity is compromised.

The present study was conducted to develop a HPLC/ESI-MS method for the simultaneous determination of seven artificial sweeteners including aspartame, saccharin, acesulfame-K, neotame, sucralose, cyclamate, alitame, and one natural sweetener, stevioside. This method with obvious merits such as high sensitivity, specificity, and simplicity compared with those of other methods can be used for the routine analyses of nonnutritive sweeteners in foods.

MATERIALS AND METHODS

Chemicals and Reagents. The standards of aspartame, saccharin, acesulfame-K, neotame, sucralose, cyclamate, alitame, stevioside, and warfarin sodium were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Food samples were purchased from a supermarket (Changsha, China). The samples included 13 beverages, i.e., one wine and 2 beers, 5 orange juices, 4 apple juices, and 1 herbal tea, and 11 candied fruits, i.e., three canned peaches, 2 canned mangos, and 6 canned apples, and 8 cakes. HPLC-grade acetone and methanol were obtained from Shanghai Ludu Chemical Plant (Shanghai, China). Ultrapure water was prepared using a Millipore Milli-Q purification system (Millipore, Bedford, MA, USA). Other reagents were of analytical grade. Mobile phases used for HPLC were filtered (0.45 μm) and ultrasonically degassed before use.

Preparation of Standard Solutions. Stock solutions (1.0 mg/mL) of aspartame, saccharin, acesulfame-K, neotame, sucralose, cyclamate, alitame, stevioside, and warfarin sodium (internal standard, I.S.) were prepared in aqueous methanol solution (50:50, v/v). A series of working standard solutions were prepared with a concentration range of 0.20−20.0 μg/mL for aspartame, 0.05−5.00 μg/mL for saccharin, 0.10−15.0 μg/mL for acesulfame-K, 0.10−10.0 μg/mL for neotame, 0.30−30.0 μg/mL for sucralose, 0.05−5.00 μg/mL for cyclamate, 0.08−8.00 μg/mL for alitame, and 0.50−15.0 μg/mL for stevioside. In the working solutions, the concentration of warfarin sodium (internal standard, I.S.) was 1 μg/mL. All stock solutions and working solutions were stored at 4 °C and brought to room temperature before use.

Sample Preparation. Because the sweeteners have good solubility in water or methanol, the food samples were extracted by aqueous methanol solution (50:50, v/v). For the beverage samples, the internal standard solution (warfarin sodium) was added in the sample. The concentration of the internal standard in the sample solution was set at 1 μg/mL. The sample solution was degassed in an ultrasonic bath and filtered through a 0.45 μm syringe filter. The filtrate was directly injected into HPLC. For the solid sample, 10.0 g of sample with the addition of 50 μL of 1.0 mg/mLwarfarin sodium was homogenized and extracted with 20 mL of aqueous methanol solution in an ultrasonic bath for 10 min followed by centrifugation at 3600 rpm for 10 min. The supernatant was transferred into a 50-mL volumetric flask. The precipitate was washed with 10 mL and 10 and 5 mL aqueous methanol solution, respectively. After centrifugation, the supernatants were pooled into the 50-mL volumetric flask. The solution was made up to the 50 mL mark with aqueous methanol solution. The concentration of I.S. in the sample solution was 1 μg/mL. The sample solution was filtered through a 0.45 μm syringe filter. The filtrate was directly injected into HPLC.

HPLC/ESI-MS Analysis. The HPLC system used was an Alliance 2695 module equipped with an autosampler (Waters Inc., Milford, MA, USA). The mass spectrometer used was a Micromass ZQ 2000 (Manchester, UK) equipped with an ESI probe and quadrupole analyzer. The control of system and data acquiring were accomplished using a Masslynx 3.5 workstation (Waters).

The sweeteners were separated on a spherigel analytical column (Johnson Inc., Dalian, China), which was packed with 5 μm C18 sillica (250 mm × 4.5 mm i.d.). A buffer solution was prepared by dissolving 0.8 mL of formic acid and 1.5 mL of triethylamine in 1 L of water. The HPLC mobile phase A was prepared by mixing methanol with buffer solution and acetone (69:24:7, v/v/v), whereas the HPLC mobile phase B was prepared by mixing methanol with buffer solution and acetone (11:82:7, v/v/v). The gradient elution was programmed as previously described (*21*), with the initial mobile phase at 0% A, 100% B held for 4 min, ramped to 53% A, 47% B at 11 min. At 23 min, the mobile phase was ramped to 100% A, 0% B and held until 24 min. The column was washed with 100% methanol for 5 min after gradient elution and then equilibrated for 10 min with the initial mobile phase

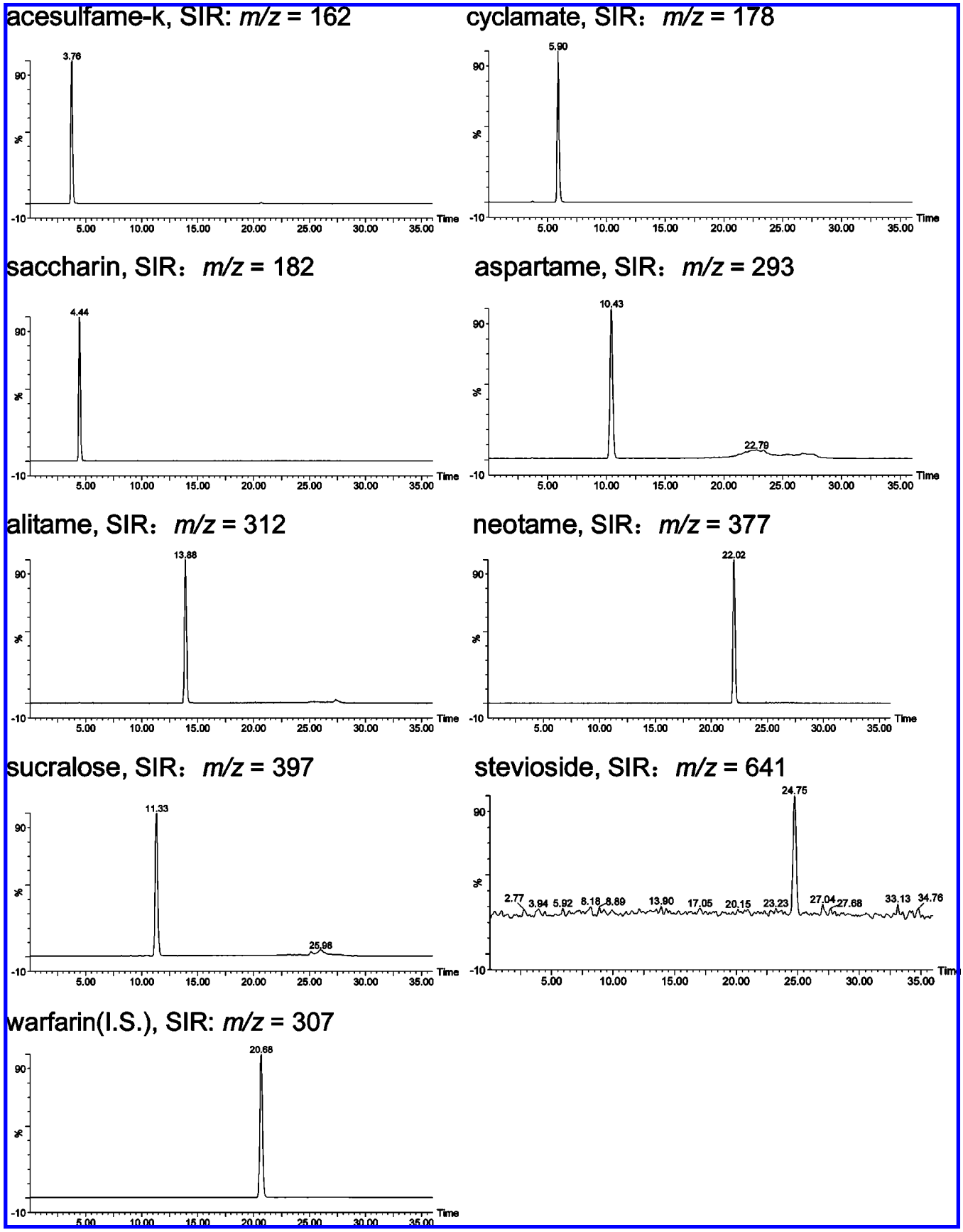

Figure 2. SIR chromatogram of sweetener standards.

for the next injection. The flow rate was kept at 1 mL/min. Injection volume was 10 μL. The outlet of the column was split, and only the 0.2 mL/min portion of the column effluent was delivered into the ion source of MS.

Electrospray ionization was operated in negative ion mode to generate quasimolecular ions. The voltage of capillary, cone, extractor, and RF lens was set at 3.2 kV and 30, 4, and 0.5 V, respectively. The temperature was maintained at 105 and 300 °C for source and

Table 1. Comparison of MS Response of Sweetener in Standard Solution with That in Sample Solution without HPLC Separation

sweetener[a]	relative values (%) (tea beverage)	relative values (%) (cake)	relative values (%)[b] (preserved apple)
acesulfame-K	57.8 ± 9.4	66.1 ± 12.2	72.4 ± 7.3
saccharin	50.1 ± 15.3	70.4 ± 10.5	68.2 ± 11.4
cyclamate	71.2 ± 9.7	79.6 ± 6.4	76.7 ± 10.2
aspartame	47.2 ± 13.5	62.8 ± 11.6	52.5 ± 15.6
sucralose	52.4 ± 8.3	73.5 ± 11.3	64.2 ± 8.3
alitame	31.9 ± 15.7	54.7 ± 16.2	42.5 ± 12.6
neotame	5.1 ± 3.7	34.6 ± 10.7	21.4 ± 13.2
stevioside	15.8 ± 10.3	31.2 ± 22.3	11.5 ± 8.6

[a] The concentrations of the sweeteners in the sample solutions are 1.00 μg/mL. [b] The response with the sample solution added standard compared to that of the standard alone (mean value ± RSD (n = 8); injection volume = 10 μL).

desolvation, respectively. The gas flow rate for desolvation and cone was set at 280 and 60 L/h, respectively. The full scan mass spectrum was acquired over a range of m/z 150−900.

Linearity, Limit of Detection, and Limit of Quantification. Calibration curves ($y = ax + b$) were represented by plotting the peak area ratios (y) of sweeteners to warfarin sodium versus the concentrations (x) of the calibration standards. Calibration curves were obtained from weighted ($1/x^2$) least-squares linear regression analysis of the data.

The limit of detection (LOD) was evaluated as the mass giving a signal equal to three times of noise ($S/N = 3$), the limit of quantification (LOQ) was determined as the mass giving a signal equal to ten times of noise ($S/N = 10$).

Precision and Accuracy. Precision of the method was evaluated according to relative standard deviation (RSD). Six blank samples (preserved apple), which were added to standards at low, medium, and high levels were prepared. Consecutive injections for six samples per low, medium, and high levels in one day gave the precision of intraday, and repetitive injections for six samples per low, medium, and high levels in six days gave the precision of interday.

Accuracy of the method was studied by calculating the mean recovery of the sweeteners after adding standards to 10.0 g of blank samples (preserved apple) at low (100 μg), medium (300 μg), and high levels (1000 μg). Each sample of the same concentration was injected five times.

RESULTS AND DISCUSSION

The chemical structures of the sweeteners are shown in **Figure 1**. Upon direct infusion of 5 μg/mL solutions of each of the analytes, it was determined that the sweeteners and the warfarin internal standard responded best in negative ion mode. For the analytes, a single mass spectral peak corresponding to the $[M - H]^-$ ion was observed with no detectible adduct formation except for stevioside. Accordingly, the $[M - H]^-$ ions were used as precursor ions for the sweenteners and the internal standard except for stevioside. For stevioside, the fragmentation was investigated using different cone voltages, i.e., 15, 25, 35, 50, 60, 90, 110, and 120 V. At 15 V, a single mass spectral peak corresponding to the $[M - H]^-$ ion (m/z = 803) was observed. With the increase of cone voltage, the abundance of the fragment ion corresponding to the [M-Glc-H]$^-$ (m/z = 641) increased. When the cone voltage was higher than 60 V, the abundance of the fragment ion corresponding to the [M-Glc-Glc-H]$^-$ (m/z = 479) increased and that of [M-Glc-H]$^-$ decreased. After comparing the ratio of signal versus noise, the fragment ion of [M-Glc-H]$^-$ (m/z = 641) owned the highest ratio of signal versus noise. It was the best choice as a quantification ion.

The ESI process is highly complex. Several characteristics of the solvents and additives, such as volatility, viscosity, and so forth, could influence the ionization process and thereby the signal response. A free selection of mobile-phase composition in LC/ESI-MS is not possible since only polar solvents and volatile additives can be used in practice. The selection of the mobile phase in the development of an LC/ESI-MS method often must be balanced between ESI response and LC separation efficiency.

After comparing different mobile phases and gradient elution profiles (*21*), the optimized elution condition was confirmed as described in the Materials and Methods section. Under the conditions, the sweeteners and I.S. were separated completely (**Figure 2**). When using acetonitrile as the organic modifier of the mobile phase, the separation of the sweeteners was not as good as that using methanol. In the buffer−methanol mobile phase system, the addition of acetone could increase the ESI response of the sweeteners. For the ESI response, once the initial charged droplet has been formed, the efficiency of a droplet to emit gas-phase ions is dependent on the surface tension and volatility of the solvent. Because the surface tension and viscosity of acetone are 23.7 mN/m and 0.316 mPa·S (25 °C), respectively, much lower than that of methanol (45.1 mN/m and 0.595 mPa·S (25 °C)), the addition of acetone was beneficial for increasing the ionization efficiency.

Ionization Suppression Investigations in Sample Matrices. The ionization suppression of analytes is a major source of imprecision for bioanalysis using HPLC-ESI/MS. The main problem source commonly reported is the presence of endogenous substances, i.e., organic or inorganic molecules present in the final extract. In this method, the sample was extracted by only methanol. No further purifying step was used. We analyzed three typical blank samples, i.e., a tea beverage, a preserved apple, and a cake, in our laboratory to investigate the effect of ion suppression of the sample matrix. The sample solution with addition of standards and a standard solution were directly infused into the mass spectrometer interface without an HPLC separation. The comparison of ESI/MS responses between the standard solution and the standard in the sample matrix was accomplished (**Table 1**). Results showed there was significant ion suppression to the sweeteners in the sample matrix. After separation by HPLC using the mobile phase conditions described in the HPLC-MS analysis section, the ion suppression by the sample matrix was reduced dramatically. The response of the analyte between the standard solution and sample solution including the same concentration standard had not shown a significant difference. However, when increasing the ratio of methanol in the mobile phase, the retention time of the sweeteners was shortened. When the analytes were eluted near the dead time of the column, the ion suppression by the sample matrix increased significantly, implying that the strong polar components in the sample matrix intensively suppressed the ionization of the analytes.

Method Validation. *Method Selectivity.* Foods have very complex composition. A valid method should ensure that complex matrices cannot interfere with the determination of targets. Considering the different matrixes of different foods, five different blank samples, i.e., a wine, an orange juice, an apple juice, a canned peach, and a cake, were determined for the selectivity of the proposed method. There was no interference from experiments (figure is not shown). The selectivity met the requirements.

Linearity, Limit of Detection, and Limit of Quantification. Linearity was obtained over the different concentration ranges for the different sweeteners (**Table 2**). The eight target sweeteners exhibited different responses to MS-SIR. Results demon-

Table 2. Calibration Curve, Limit of Detection (LOD), Limit of Quantification (LOQ) of the Eight Sweeteners

sweetener	conc. range (μg/mL)	calibration curve	correlation coefficient	LOD (μg/mL)	LOQ (μg/mL)
acesulfame-K	0.10−5.0	$Y = 0.0108x + 0.0277$	0.9994	0.02	0.05
saccharin	0.05−5.00	$Y = 0.0217x + 0.009$	0.9992	0.01	0.03
cyclamate	0.05−5.00	$Y = 0.0274x - 0.0191$	0.9995	0.01	0.03
aspartame	0.20−20.0	$Y = 0.0033x - 0.0258$	0.9980	0.02	0.05
sucralose	0.30−30.0	$Y = 0.0017x + 0.0021$	0.9992	0.04	0.10
Alitame	0.08−8.00	$Y = 0.0105x + 0.0311$	0.9986	0.02	0.04
neotame	0.10−10.0	$Y = 0.0143x - 0.041$	0.9986	0.02	0.05
stevioside	0.50−15.0	$Y = 0.00005x + 0.0005$	0.9995	0.10	0.30

Table 3. Accuracy of the Analytical Method ($n = 5$)

sweetener		acesulfame-K	saccharin	cyclamate	aspartame	sucralose	alitame	neotame	stevioside
low level	added (μg)	100	100	100	100	100	100	100	100
	found (μg)	99.8 ± 2.3	102.4 ± 1.2	99.6 ± 3.3	96.5 ± 1.6	98.4 ± 0.6	97.4 ± 1.2	99.2 ± 3.5	98.0 ± 3.6
	recovery (%)	99.8 ± 2.3	102.4 ± 1.2	99.6 ± 3.3	96.5 ± 1.7	98.4 ± 0.6	97.4 ± 1.2	99.2 ± 3.5	98.0 ± 3.7
medium level	added (μg)	300	300	300	300	300	300	300	300
	found (μg)	293 ± 5.3	297 ± 2.4	288 ± 8.9	298 ± 11.9	311 ± 6.5	293 ± 7.3	313 ± 9.7	309 ± 2.8
	recovery (%)	97.7 ± 1.8	99.0 ± 0.8	96.0 ± 3.1	99.3 ± 4.0	103.7 ± 2.1	97.7 ± 2.5	104.3 ± 3.1	103.0 ± 0.9
high level	added (μg)	1000	1000	1000	1000	1000	1000	1000	1000
	found (μg)	1012 ± 29.3	997 ± 42.9	954 ± 13.4	1023 ± 8.2	1018 ± 21.4	984 ± 33.4	1032 ± 16.5	983 ± 36.4
	recovery (%)	101.2 ± 2.9	99.7 ± 4.3	95.4 ± 1.4	102.3 ± 0.8	101.8 ± 2.1	98.4 ± 3.4	103.2 ± 1.6	98.3 ± 3.7

strated that the LODs and LOQs of different sweeteners were significantly different. However, the LODs and LOQs were lower than those of the reported methods in the literature.

Method Precision and Accuracy. The intraday precision of analytes ranged from 1.45 to 7.23%, and the interday precision ranged from 1.77 to 9.41% (**Table 3**). The present results indicated that the recovery was 95.4−104.3% for the analytes. Because the quantification of the target compounds was based on the internal standard, the extraction recovery of I.S. must be investigated. After comparing the peak area of the added known amount of I.S. in the blank samples (preserved apple) with that of I.S. in the standard solution, it could be found that the extraction recovery was 98.7 ± 2.1% ($n = 8$).

HPLC/ESI-MS Analysis of Samples. Thirty-two samples were analyzed by the proposed method. A typical SIR chromatogram of candied fruit is shown in **Figure 3**. The results (mean ± SD, mg/kg, $n = 5$ each) are listed as follows: Among the 13 different beverage samples measured, 11 samples contained sodium cyclamate (14.5 ± 0.2, 278.3 ± 3.1, 34.7 ± 0.8, 40.2 ± 0.8, 152.1 ± 1.1, 50.3 ± 4.0, 45.7 ± 1.9, 110.0 ± 2.7, 30.4 ± 1.0, 250.3 ± 4.1, and 202.7 ± 7.2), 2 samples contained saccharin (17.1 ± 0.4 and 78.2 ± 6.2). Among the 11 candied fruit samples, 3 samples contained sodium cyclamate (55.2 ± 4.2, 127.4 ± 7.1, and 32.1 ± 1.2), 3 samples contained sucralose (5.7 ± 0.4, 44.3 ± 2.1, and 27.1 ± 0.7), 2 samples had acesulfame-K (152.4 ± 5.1 and 278.6 ± 4.0), 1 samples contained saccharin (110.4 ± 1.0), 1 sample contained cyclamate (90.8 ± 3.7) and sucralose (23.3 ± 1.2), and 1 sample contained no target nonnutritive sweeteners. For the cake samples, 2 samples contained sucralose (17.4 ± 1.2 and 33.0 ± 2.0), 5 samples contained neotame (23.4 ± 1.7, 76.2 ± 2.8, 11.0 ± 0.8, 107.8 ± 5.2, and 283.1 ± 3.7), and 1 sample contained sodium cyclamate (210.2 ± 3.1). From the analytical results of the real samples, sodium cyclamate was a major sweetener used in the foods, especially in beverages, in the Chinese markets. Saccharin is also used in the foods nowadays.

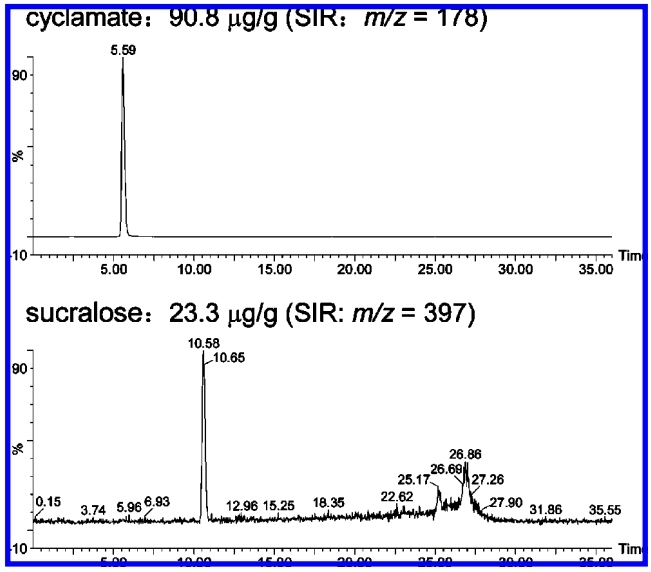

Figure 3. SIR chromatogram of a preserved peach sample.

In summary, high-intensity sweeteners have been widely used in foods, and a reliable analytical method for simultaneous quantification of these sweeteners is necessary for the quality control of foods. Compared to the HPLC-ELSD method (*21*), the present method had a higher sensitivity. In addition, the specificity of the method was higher than that of the ELSD method because of the partial structural identification ability of mass spectrometry.

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Received for review December 23, 2008. Revised manuscript received February 13, 2009. Accepted February 14, 2009. This work was financially supported by the National "973" project (2006CB504701) and the National Natural Science Foundation of China (20875028) and (2007FJ1005).

JF803988U