Method of Test for Sweeteners in Foods - Multiple Analysis

1. Scope

This method is applicable to the determination of 13 sweeteners (acesulfame potassium etc. listed in the attached table) in foods.

2. Method

After extraction, analytes are determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

2.1. Equipment

2.1.1. Liquid chromatograph/tandem mass spectrometer

2.1.1.1. Ion source: electrospray ionization, ESI.

2.1.1.2. Column: Eclipse Plus Phenyl-Hexyl, 3.5 μm, 2.1 mm i.d. × 15 cm, or an equivalent product.

2.1.2. Homogenizer.

2.1.3. Ultrasonicator.

2.1.4. Centrifuge: centrifugal force > 2200 ×g.

2.2. Chemicals

Methanol, HPLC grade;

Ammonium formate, reagent grade;

Deionized water, resistivity ≥ 18 MΩ · cm (at 25℃);

Acesulfame potassium, alitame, aspartame, dulcin, glycyrrhizin, neohesperidin dihydrochalcone (NHDC), neotame, rebaudioside A, rebaudioside B, sodium cyclamate, sodium saccharin, stevioside and sucralose, reference standards.

2.3. Apparatus

2.3.1. Volumetric flask: 10 mL, 50 mL, and 100 mL.

2.3.2. Centrifuge tube: 15 mL and 50 mL, PP.

2.3.3. Membrane filter: 0.22 μm, PVDF.

2.4. Reagents

2.4.1. 0.01 M ammonium formate

Dissolve and dilute 6.3 g of ammonium formate with deionized water to 100 mL. Transfer 10 mL and dilute with deionized water to 1000 mL.

2.4.2. 50% methanol

Dilute 500 mL of methanol with deionized water to 1000 mL.

2.5. Mobile phase

2.5.1. Solvent A

Mix 0.01 M ammonium formate and methanol at the ratio of 9:1 (v/v). Filter with a membrane filter.

2.5.2. Solvent B:

Mix 0.01 M ammonium formate and methanol at the ratio of 1:9 (v/v). Filter with a membrane filter.

2.6. Standard solution preparation

Accurately weigh equivalent 10 mg of cyclamate and saccharin reference standards and 10 mg of acesulfame potassium, alitame, aspartame, dulcin, glycyrrhizin, NHDC, neotame, rebaudioside A, rebaudioside B, stevioside, sucralose reference standards separately into a 10-mL volumetric flask. Dissolve and dilute with 50% methanol to volume as the standard stock solutions. Freeze-storage. When to use, mix appropriate volume of each standard stock solution, and dilute with 10% methanol to 1000 ng/mL as the standard solution.

2.7. Sample solution preparation

2.7.1. Normal sample

2.7.1.1. Liquid sample

Transfer about 1 g of homogenized sample accurately weighed into a 100-mL volumetric flask. Dilute with 50% methanol to volume. Mix well and filter with a membrane filter as the sample stock solution. Transfer 100 μL (a) of sample stock solution and dilute with 10% methanol to 1000 μL (b) as the sample solution.

2.7.1.2. Solid sample:

Transfer about 1 g of fine cut and homogenized sample accurately weighed into a 50-mL centrifuge tube. Add 40 mL of 50% methanol and ultrasonicate for 15 min. Centrifuge at 2200 ×g for 10 min and collect the supernatant. Add 40 mL of 50% methanol to the residue and repeat the extraction once. Combine the supernatant into a 100-mL volumetric flask, mix with 50% methanol to volume. Filter with a membrane filter as the sample stock solution. Transfer 100 μL (a) of sample stock solution and dilute with 10% methanol to 1000 μL (b) as the sample solution.

2.7.2. High fat sample:

2.7.2.1. Liquid sample:

Transfer about 1 g of homogenized sample accurately weighed into a 100-mL volumetric flask. Dilute with 50% methanol to volume. Mix well and filter with a membrane filter. Transfer 2 mL of the solution into a centrifuge tube, add 2 mL of hexane and mix well. Centrifuge at 2000 ×g for 1 min and collect the lower layer as the sample stock solution. Transfer 100 μL (a) of sample stock solution and dilute with 10% methanol to 1000 μL (b) as the sample solution.

2.7.2.2. Solid sample:

Transfer about 1 g of fine cut and homogenized sample accurately weighed into a 50-mL centrifuge tube. Add 40 mL of 50% methanol and ultrasonicate for 15 min. Centrifuge at 2200 ×g for 10 min and collect the supernatant. Add 40 mL of 50% methanol to the residue and repeat the extraction once. Combine the supernatant into a 100-mL volumetric flask, mix with 50% methanol to volume. Filter with a membrane filter. Transfer 2 mL of the solution into a centrifuge tube, add 2 mL of hexane and mix well. Centrifuge at 2000 ×g for 1 min and collect the lower layer as the sample stock solution. Transfer 100 μL (a) of sample stock solution and dilute with 10% methanol to 1000 μL (b) as the sample solution.

2.8. Matrix-matched calibration curve

Take a blank sample and follow the procedure described in section 2.7 to obtain the blank sample stock solution. Separately mix 100 μL (a) of blank sample stock solution with 10 ~ 150 μL standard solution and dilute with 10% methanol to 1000 μL (b) as the matrix-matched standard solutions. Operate LC-MS/MS according to the following conditions. Establish the matrix-matched calibration curve of each sweetener by the ratios of the peak area vs. the added concentrations (10 ~ 150 ng/mL).

LC-MS/MS operating conditions (note):

Column: Eclipse Plus Phenyl-Hexyl, 3.5 μm, 2.1 mm i.d. × 15 cm.

Column oven temperature: 30℃.

Mobile phase: gradient program of solvent A and solvent B is as follows.

Time (min)	A (%)	B (%)
0.0 → 0.5	90 → 90	10 → 10
0.5 → 4.5	90 → 80	10 → 20

4.5 → 5.0	80 → 60	20 → 40
5.0 → 10.0	60 → 54	40 → 46
10.0 → 11.0	54 → 41	46 → 59
11.0 → 17.0	41 → 32	59 → 68
17.0 → 18.0	32 → 0	68 → 100
18.0 → 18.9	0 → 0	100 → 100
18.9 → 19.0	0 → 90	100 → 10
19.0 → 22.0	90 → 90	10 → 10

Flow rate: 0.35 mL/min.

Inject volume: 5 μL.

Capillary voltage:

ESI positive: 4 kV.

ESI negative: 3.5 kV.

Nozzle voltage:

ESI positive: 0.5 kV.

ESI negative: 1 kV.

Gas temperature: 300℃.

Gas flow: 8 L/min.

Nebulizer gas pressure: 45 psi.

Sheath gas temperature: 350℃.

Sheath gas flow: 11 L/min.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, cone voltage and collision energy are shown in the attached table.

Note: All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.9. Identification and quantification

Accurately inject 5 μL of the sample solution and the matrix matched standard solutions into LC-MS/MS separately. Operate according to the conditions in section 2.8. Identify each sweetener based on the retention time and the relative ion intensities (note). Calculate the amount of each sweetener in the sample using the following formula.

$$\text{The amount of each sweetener in the sample (g/Kg)} = \frac{C \times V \times F}{M} \times 10^{-6}$$

Where,

C: the concentration of each sweetener in the sample solution calculated by the matrix-matched calibration curve (ng/mL)

V: the volume of the final sample solution (100 mL)

M: the weight of the sample (g)

F: the dilution factor, b/a.

Note: Relative ion intensities are calculated by peak areas of quantitative ions divided by peak areas of qualitative ions (≤100%). Maximum permitted tolerances of relative ion intensities are as follows:

Relative ion intensity (%)	Tolerance (%)
> 50	± 20
> 20～50	± 25
> 10～20	± 30
≦ 10	± 50

Remark

1. Limit of quantification (LOQ) for each sweetener (acesulfame potassium etc. in total 13 items) is 0.01 g/Kg.
2. Further validation should be performed when interference compounds appear in samples.

Reference

Koyama, M., Yoshida, K., Uchibori, N., Wada, I., Akiyama, K. and Sasaki, T. 2005. Analysis of nine kinds of sweeteners in foods by LC/MS. J. Food Hyg. Soc. Japan 48: 72-78.

Table. MRM parameters of 13 sweeteners (acesulfame potassium, etc.).

Analyte	CAS #	Ionization mode	Quantitation Ion pair			Qualitation Ion pair		
			Precursor ion (*m/z*) >product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Precursor ion (*m/z*) >product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Acesulfame potassium	55589-62-3	ESI^-	162 > 82	-102	9	162 > 78	-102	37
Alitame	80863-62-3	ESI^+	332 > 129	105	16	332 > 159	105	20
Aspartame	22839-47-0	ESI^+	295 > 120	102	25	295 > 180	102	9
Dulcin	150-69-6	ESI^+	181 > 108	102	25	181 > 136	102	13
Glycyrrhizin	1405-86-3	ESI^-	821.4 > 351	-240	48	821.4 > 113	-240	60
Neohesperidin dihydrochalcone (NHDC)	20702-77-6	ESI^-	611 > 303	-226	33	611 > 125	-226	49
Neotame	165450-17-9	ESI^+	379 > 172	102	21	379 > 319	102	13
Rebaudioside A	58543-16-1	ESI^-	965.4 > 803.5	-190	28	965.4 > 641.4	-190	72
Rebaudioside B	58543-17-2	ESI^-	803.4 > 641.4	-270	56	803.4 > 317	-270	68
Sodium cyclamate	139-05-9	ESI^-	178 > 80	-135	28	178 > 95	-135	36
Sodium saccharin	128-44-9	ESI^-	182 > 42	-160	28	182 > 106	-160	17
Stevioside	57817-89-7	ESI^-	641.4 > 479	-300	45	641.4 > 317	-300	49
Sucralose	56038-13-2	ESI^+	414 > 199	102	9	414 > 216	102	5

Reference chromatograms

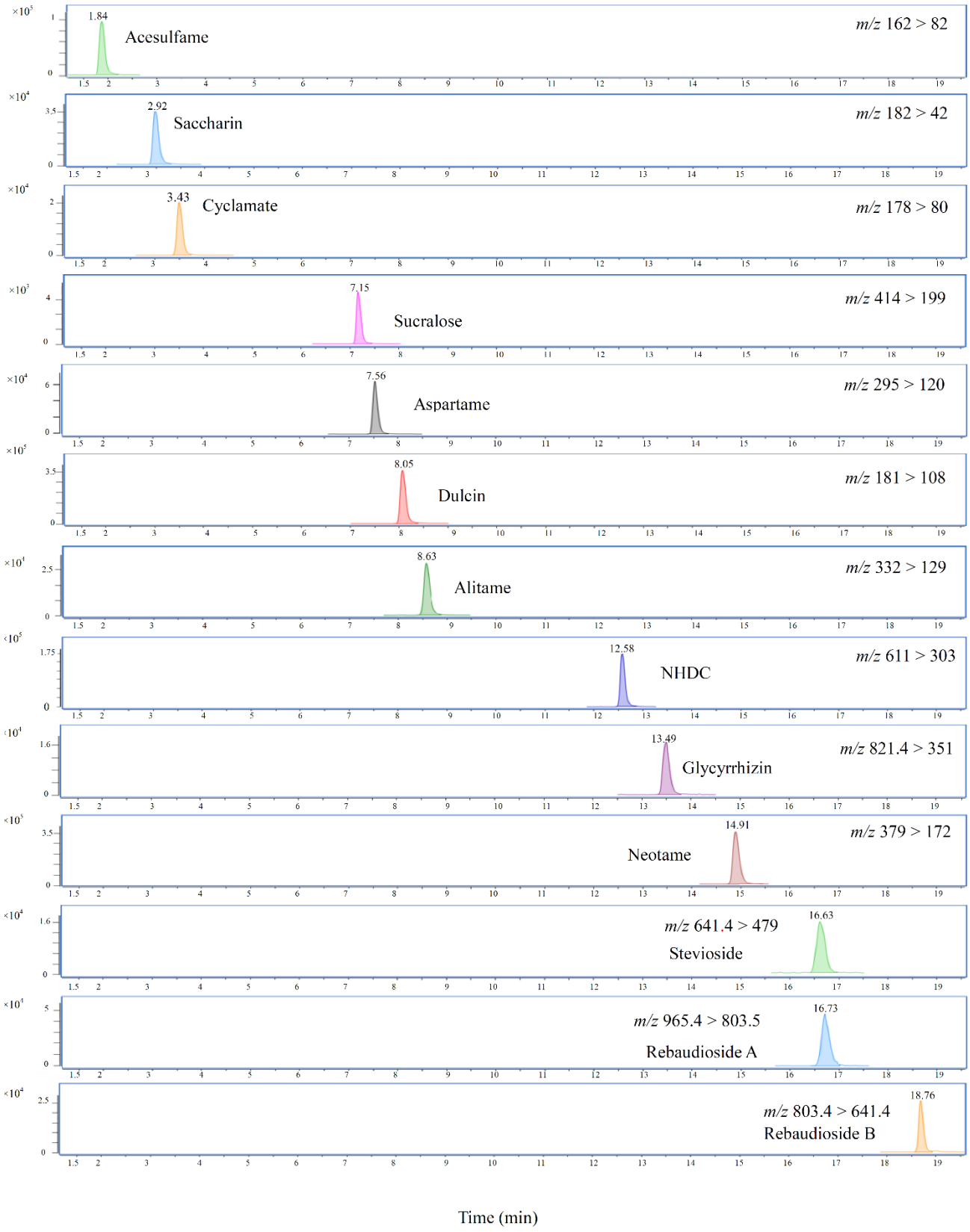

Figure. MRM chromatograms of 13 sweeteners (acesulfame potassium, etc.) analyzed by LC-MS/MS.