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MOHWA0020.03

Method of Test for Preservatives in Foods

1. Scope

This method is applicable to the determination of 12 preservatives (benzoic acid etc. listed in the attached table) in foods.

2. Method

After extraction, preservatives are determined by high performance liquid chromatography (HPLC).

2.1. Equipment

2.1.1. High performance liquid chromatograph.

2.1.1.1. Detector: photodiode array detector.

2.1.1.2. Column: ACE C18-AR, 5 μm, 4.6 mm i.d. × 25 cm, or an equivalent product.

2.1.2. Centrifuge: centrifugal force＞3500 ×g.

2.1.3. Ultrasonicator.

2.1.4. Distillation apparatus.

2.2. Chemicals

Sodium hydroxide, AR grade;

Citric acid ($C_6H_8O_7 \cdot H_2O$), AR grade;

Trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), AR grade;

Tartaric acid, AR grade;

Sodium chloride, AR grade;

Methanol, HPLC grade;

Acetonitrile, HPLC grade;

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

Benzoic acid and other 11 preservatives listed in the attached table, reference standards.

2.3. Apparatus

2.3.1. Distilling flask: 500 mL.

2.3.2. Centrifuge tube: 50 mL, PP.

2.3.3. Volumetric flask: 50 mL, 100 mL, 200 mL, 500 mL and 1000 mL.

2.3.4. Membrane filter: 0.45 μm, PVDF.

2.4. Reagents

2.4.1. 50% methanol.

Dilute 500 mL of methanol with deionized water to1000 mL.

2.4.2. 0.1 N sodium hydroxide.

Dissolve and dilute 0.4 g of sodium hydroxide with deionized water to 100 mL.

2.4.3. 5 mM citrate buffer solution.

Dissolve and dilute 7.0 g of citric acid and 6.0 g of trisodium citrate with deionized water to 1000 mL. Dilute the above solution 10 times with deionized water, and filter with a membrane filter.

2.4.4. 15% tartaric acid.

Dissolve and dilute 15 g of tartaric acid with deionized water to 1000 mL.

2.5. Mobile phase

2.5.1. Solvent A

5 mM citrate buffer solution.

2.5.2. Solvent B

Mix methanol and acetonitrile at the ratio of 1:2 (v/v), and filter with a membrane filter.

2.5.3. Solvent C

Mix methanol, acetonitrile and 5 mM citrate buffer solution at the ratio of 1:2:7 (v/v/v), and filter with a membrane filter.

2.5.4. Solvent D

Mix methanol and 5 mM citrate buffer solution at the ratio of 6:4 (v/v), and filter with a membrane filter.

2.6. Standard solution preparation

2.6.1. Accurately weigh equivalent 50 mg of benzoic acid, sorbic acid, dehydroacetic acid, *p*-hydroxybenzoic acid and salicylic acid reference standards to each 100-mL volumetric flask, dissolve with 5 mL of 0.1 N sodium hydroxide, and dilute with deionized water to volume as standard stock solutions. When to use, mix appropriate volume of each standard stock solution and dilute with 50% methanol to 0.25～100 μg/mL as the standard solutions of acid preservatives.

2.6.2. Accurately weigh equivalent 50 mg of methyl *p*-hydroxybenzoate, ethyl *p*-hydroxybenzoate, isopropyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate, secbutyl *p*-hydroxybenzoate, isobutyl *p*-hydroxybenzoate and butyl *p*-hydroxybenzoate reference standards to each 100-mL volumetric flask, and dissolve and dilute with 50% methanol

to volume as standard stock solution. When to use, mix appropriate volume of each standard stock solution and dilute with 50% methanol to 0.25～100 μg/mL as the standard solutions of aryl ester of *p*-hydroxybenzoic acid.

2.7. Sample solution preparation

2.7.1. Direct dilution (liquid sample)

Degas the carbonated samples at room temperature in an ultrasonicator until there is no further effervescence. Transfer about 5 g of the degassed sample accurately weighted into a 100-mL volumetric flask and dilute with 50% methanol to volume. Filter with a membrane filter after 10 min standing time. Take the filtrate as the sample solution.

2.7.2. Extraction

Transfer about 5 g of the well-mixed sample accurately weighted into a glass flask, add 50 mL of 50% methanol, and ultrasonicate at room temperature for 30 min. Transfer into a 100-mL volumetric flask, dilute with 50% methanol solution to volume, and centrifuge at 3500 rpm for 10 min. Collect the supernatant, and filter with a membrane filter. Take the filtrate as the sample solution.

2.7.3. Distillation

Transfer about 25 g of the well-mixed sample accurately weighted into a distilling flask, and add 15 mL of 15% tartaric acid, 60 g sodium chloride and 150 mL deionized water. Distill and collect about 490 mL of the distillate at the speed of 10 mL/min. Transfer the distillate into a 500-mL volumetric flask, dilute with deionized water to volume, and filter with a membrane filter. Take the filtrate as the sample solution.

2.8. Identification and quantification

Accurately inject 10 μL of the sample solution and the standard solutions into the HPLC separately, and operate according to the following conditions. Identify each preservative based on the retention time and the absorption spectrum. Calculate the amount of each preservative in the sample by the following formula:

$$\text{The amount of each preservative in the sample (g/kg)} = \frac{C \times V \times F}{M \times 1000}$$

where,

C: the concentration of each preservative in the sample solution calculated by the standard curve (μg/mL).

V: the make up volume of sample (mL).

M: the weight of sample (g).

F: conversion factor (note).

Note: The amount of each aryl esters of *p*-hydroxybenzoic acid in the sample is determined as *p*-hydroxybenzoic acid through the conversion factor.

Aryl ester of *p*-hydroxybenzoic acid	Molecular weight	Conversion factor
Methyl *p*-hydroxybenzoate	152.15	0.9077
Ethyl *p*-hydroxybenzoate	166.17	0.8311
Isopropyl *p*-hydroxybenzoate	180.20	0.7664
Propyl *p*-hydroxybenzoate	180.20	0.7664
Secbutyl *p*-hydroxybenzoate	194.23	0.7111
Isobutyl *p*-hydroxybenzoate	194.23	0.7111
Butyl *p*-hydroxybenzoate	194.23	0.7111
p-Hydroxybenzoate	138.12	1

HPLC operating conditions (note):

Column: ACE C18-AR, 5 μm, 4.6 mm i.d. × 25 cm.

Flow rate: 1 mL/min.

Chromatographic parameter 1:

Photodiode array detector: 230 nm for salicylic acid, benzoic acid, and dehydroacetic acid, 256 nm for aryl ester of *p*-hydroxybenzoic acid and 260 nm for sorbic acid.

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

Time (min)	A (%)	B (%)
0 → 5	78 → 73	22 → 27
5 → 20	73 → 63	27 → 37
20 → 50	63 → 33	37 → 67

50 → 65	33 → 78	67 → 22

Chromatographic parameter 2:

Photodiode array detector: 230 nm for salicylic acid, benzoic acid, and dehydroacetic acid, 256 nm for aryl ester of *p*-hydroxybenzoic acid and 260 nm for sorbic acid.

Mobile phase: solvent C.

Chromatographic parameter 3:

Photodiode array detector: 256 nm for aryl esters of *p*-hydroxybenzoic acid.

Mobile phase: solvent D.

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

Remark

1. The limits of quantitation (LOQs) for acid preservatives are 0.02 g/kg, and for aryl esters of *p*-hydroxybenzoic acid are 0.005 g/kg.
2. When the direct dilution method or the extraction method are not applicable to the sample, the distillation method may be applied but limited in the analysis of benzoic acid, sorbic acid, and dehydroacetic acid.
3. As confirm by LC/MS/MS, the multiple reaction monitoring (MRM) parameters are listed in the attached table.
4. Method validation should be performed before quantification with LC/MS/MS.
5. Further validation should be performed when interference compounds appear in samples.

Reference

1. The Pharmaceutical Society of Japan. 2015. Methods of Analysis in Health Science. pp 330-334. KANEHARA & CO., LTD. Tokyo. Japan.
2. Kishi, H. and Yamada, T. 2007. Simultaneous determination of nine kinds of preservatives in foods by HPLC. J. Food Hyg. Soc. Jpn. 48: 58-63.

Table. The MRM parameters of 12 preservatives by LC/MS/MS

Analyte	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)
Benzoic acid	ESI^-	121 > 77	-40	-15	—	—	—
Sorbic acid	ESI^+	113 > 95	21	15	113 > 67	21	21
Dehydroacetic acid	ESI^+	169 > 85	42	27	169 > 127	42	18
p-Hydroxybenzoic acid	ESI^-	137 > 93	-27	-16	137 > 65	-27	-40
Salicylic acid	ESI^-	137 > 93	-27	-26	137 > 65	-27	-41
Methyl *p*-hydroxybenzoate	ESI^-	151 > 92	-37	-29	151 > 136	-37	-20
Ethyl *p*-hydroxybenzoate	ESI^-	165 > 92	-30	-31	165 > 137	-30	-18
Isopropyl *p*-hydroxybenzoate	ESI^-	179 > 92	-38	-32	179 > 137	-38	-19
Propyl *p*-hydroxybenzoate	ESI^-	179 > 92	-38	-32	179 > 137	-38	-20
Secbutyl *p*-hydroxybenzoate	ESI^-	193 > 92	-39	-32	193 > 136	-39	-22
Isobutyl *p*-hydroxybenzoate	ESI^-	193 > 92	-39	-32	193 > 136	-39	-21
Butyl *p*-hydroxybenzoate	ESI^-	193 > 92	-39	-32	193 > 136	-39	-21

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