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Method of Test for Antioxidants in Foods- Multiple Analysis

1. Scope

This method is applicable to the determination of 11 antioxidants (propyl gallate etc. listed in Table 1) in edible oil, cheese, and margarine.

2. Method

After extraction, antioxidants 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: Poroshell 120 EC-C18, 2.7 μm, 3 mm i.d. × 15 cm, or an equivalent product.

2.1.1.3. High speed dispersing device: SPEX SamplePrep 2010 GenoGrinder®, > 1000 rpm, or an equivalent product.

2.1.1.4. Shaker.

2.1.1.5. Centrifuge: centrifugal force＞5000 ×g.

2.2. Chemicals

Isopropanol, HPLC grade;
Acetonitrile, HPLC grade;
Acetic acid, GR grade;
Ascorbic acid, GR grade;
Sodium citrate, AR grade;
Sodium hydrogen citrate, AR grade;
Magnesium sulfate anhydrous, AR grade;
Sodium chloride, AR grade;
Deionized water, resistivity ≥ 18 MΩ•cm (25°C);
Propyl gallate and other 10 antioxidants listed in Table 1, reference standards.

2.3. Apparatus

2.3.1. Volumetric flask (amber): 10 mL.

2.3.2. Centrifuge tube: 50 mL, PP.

2.3.3. Membrane filter: 0.22 μm, PVDF.

2.3.4. Ceramic homogenizer (note 1): Bond Elut QuEChERS P/N 5982-9313, or an equivalent product.

2.3.5. Extraction powder (note 2): containing 1 g of sodium citrate, 0.5 g of sodium hydrogen citrate, 4 g of magnesium sulfate, and 1 g of sodium chloride.

Note: 1. Using the ceramic homogenizer will be dependent on the matrix of the sample.

2. Different extraction kits can be chosen as needed.

2.4. Reagents

2.4.1. 50% acetonitrile containing 1% ascorbic acid.

Dissolve 1 g of ascorbic acid with 50 mL of deionized water, and dilute with acetonitrile to 100 mL.

2.4.1. 2% ascorbic acid solution.

Dissolve 1 g of ascorbic acid with deionized water to 50 mL.

2.5. Mobile phase

2.5.1. Solvent A

Dilute 50 mL of acetic acid with deionized water to 1000 mL, and filter with a membrane filter.

2.5.2. Solvent B

Acetonitrile.

2.6. Standard solution preparation

Accurately weigh equivalent 100 mg of 11 antioxidant reference standards to each 10-mL volumetric flask, dissolve and dilute with isopropanol to volume as standard stock solutions, and then store in the dark at -18℃. When to use, mix appropriate volume of each standard stock solution, and dilute with 50% acetonitrile containing 1% ascorbic acid to 0.25-20 μg/mL for PG, THBP, TBHQ, NDGA, BHA, 4-HR, OG, DG, and BH, and 1-80 μg/mL for ETH and HMBP as the standard solutions.

2.7. Sample solution preparation

Transfer about 0.5 g of the sample accurately weighted into a

centrifuge tube, and add one ceramic homogenizer, 8 mL of deionized water and 10 mL of acetonitrile. Further add extraction powder and vortex several times immediately to avoid salt agglomeration. Shake by the tissue homogenizer at 1000 rpm or by hands for 1 min, and centrifuge at 5000 ×g for 5 min. Dilute 0.5 mL (a) of the supernatant with 2% ascorbic acid to 1 mL (b), mix well and filter with a membrane filter. Take the filtrate as the sample solution (note).

Note: The sample solution needs to analyze immediately.

2.8. Identification and quantification

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

The amount of each antioxidant in the sample (g/kg) $=$

$$\frac{C \times V \times F}{M \times 1000}$$

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

V: the make up volume of acetonitrile (10 mL).

M: the weight of sample (g).

F: the dilution factor, b/a.

HPLC operating conditions (note):

Photodiode array detector: 280 nm.

Column: Poroshell 120 EC-C18, 2.7 μm, 3 mm i.d. × 15 cm.

Oven temperature: 35℃.

Injection volume: 10 μL.

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

Time (min)	A (%)	B (%)
0 → 7	85 → 85	15 → 15
7 → 15	85 → 55	15 → 45
15 → 22	55 → 55	45 → 45
22 → 23	55 → 25	45 → 75
23 → 30	25 → 25	75 → 75
30 → 30.1	25 → 85	75 → 15
30.1 → 35	85 → 85	15 → 15

Flow rate: 0.7 mL/min.

Note: All the parameters can be adjusted according to the equipment used if the above conditions are not applicable.

Remark

1. The limits of quantitation (LOQ) are as follows: 0.01 g/kg for PG, THBP, TBHQ, NDGA, BHA, 4-HR, OG, DG and BHT, and 0.04 g/kg for ETH and HMBP.
2. As confirm by LC/MS/MS, the multiple reaction monitoring (MRM) parameters are shown in Table 1, and maximum permitted tolerances of relative ion intensities are as follows:

Relative ion intensity (% of base peak)	Tolerance (%)
> 50	± 20
> 20～50	± 25
> 10～20	± 30
≤ 10	± 50

3. As confirm by GC/MS, the selected ion monitoring (SIM) parameters are shown in Table 2, and maximum permitted tolerances of relative ion intensities are as follows:

Relative ion intensity (% of base peak)	Tolerance (%)
> 50	± 10
> 20～50	± 15
> 10～20	± 20
≤ 10	± 50

4. Method validation should be performed before quantification with LC/MS/MS or GC/MS.
5. Further validation should be performed when interference compounds appear in samples.

Reference

1. Page, B. D. 1993. Liquid chromatographic method for the determination of nine phenolic antioxidants in butter oil: collaborative study. J. AOAC Int. 76: 765-779.
2. Jia, W., Ling, Y., Lin, Y., Chang, J. and Chu, X. 2014. Analysis of additives in dairy products by liquid chromatography coupled to quadrupole-orbitrap mass spectrometry. J. Chromatogr. A 1336: 67-75.

Reference chromatogram

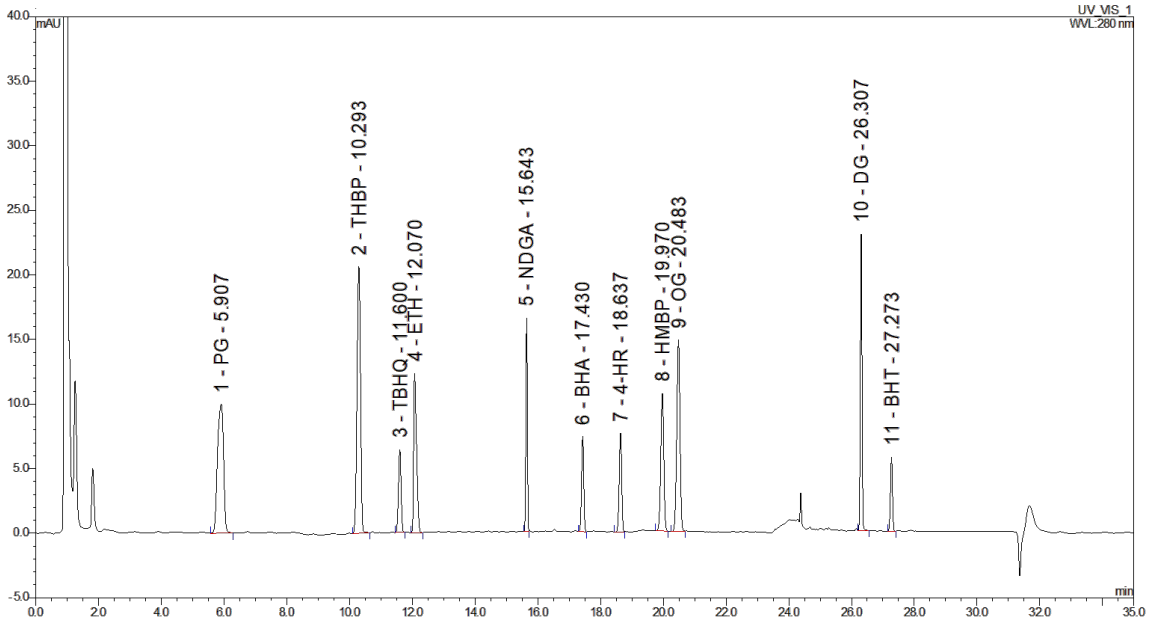

Figure. The chromatogram of PG and other 10 antioxidant standards by HPLC.

Table 1. The MRM parameters of 11 antioxidants by LC/MS/MS

Analyte	Ionization mode	Detect ion: Precursor ion (*m/z*)> product ion (*m/z*)	Declustering potential (V)	Collision energy (eV)
Propyl gallate (PG)	ESI^-	211 > 169*	-55	-21
		211 > 124	-55	-34
2,4,5-Trihydroxybutyrophenone (THBP)	ESI^-	195 > 125*	-50	-28
		195 > 166	-50	-28
tert-Butyl hydroquinone (TBHQ)	ESI^-	165 > 149*	-53	-30
		165 > 108	-53	-31
Ethoxyquin (ETH)	ESI^+	218 > 174*	44	40
		218 > 160	44	48
Nordihydroguaiaretic acid (NDGA)	ESI^-	301 > 122*	-60	-37
		301 > 273	-60	-25
Butyl hydroxy anisole (BHA)	ESI^-	179 > 164*	-33	-20
		179 > 149	-33	-35
4-Hexyl resorcinol (4-HR)	ESI^-	193 > 149*	-48	-20
		193 > 122	-48	-27
4-Hydroxymethyl-2,6-di-*tert*-butylphenol (HMBP)	ESI^-	235 > 217*	-50	-30
		235 > 160	-50	-36
Octyl gallate (OG)	ESI^-	281 > 124*	-80	-42
		281 > 169	-80	-29
Dodecyl gallate (DG)	ESI^-	337 > 124*	-110	-52
		337 > 169	-110	-36
Dibutyl hydroxy toluene (BHT)	ESI^-	219 > 203*	-60	-35
		219 > 163	-60	-20

*quantitation ion.

Note: All the parameters can be adjusted according to the equipment used if above conditions are not applicable.

Table 2. The SIM parameters of 8 antioxidants by GC/MS

Analyte	Ionization mode	quantitation ion (*m/z*)	quantitation ion (*m/z*)
Propyl gallate (PG)	EI	170	153, 212
2,4,5-Trihydroxy butyrophenone (THBP)	EI	153	181, 196
tert-Butylhydroquinone (TBHQ)	EI	123	151, 166
Ethoxyquin (ETH)	EI	202	174, 217
Butyl hydroxyl anisole (BHA)	EI	137	165, 180
4-Hexylresorcinol (4-HR)	EI	123	125, 136
4-Hydroxymethyl-2,6-di-*tert*-butylphenol (HMBP)	EI	221	161, 236
Dibutyl hydroxyl toluene (BHT)	EI	205	145, 220

Note: All the parameters can be adjusted according to the equipment used if above conditions are not applicable.