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Animal and vegetable fats and oils — High-performance liquid chromatography (HPLC) analysis of phenolic antioxidants

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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Introduction

This document describes the analysis of synthetic phenolic antioxidants intentionally added to oils and fats, or inadvertently added during manufacturing processes. Information is also included for estimating the absence of an antioxidant from oils and fats within the limitations of the method. Interference from natural compounds present in rapeseed (canola) oil are also addressed.

~~This document represents AOCs Official Method Ce 6a-2021^[1]. The AOCs revised the original AOCs Ce 6-86^[1] "Antioxidants, Liquid Chromatographic Method" and this document is an adoption of the revised AOCs Ce 6a-2021^[2] "HPLC Analysis of Phenolic Antioxidants" method.~~

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Animal and vegetable fats and oils — High-performance liquid chromatography (HPLC) analysis of phenolic antioxidants

1 Scope

This document specifies a method for the analysis of phenolic antioxidants by high-performance liquid chromatography (HPLC).

It is applicable to quantifying the following synthetic phenolic compounds added to animal and vegetable fats, oils and shortenings as antioxidants, at concentrations normally added to oils:

- propyl gallate (PG);
- octyl gallate (OG);
- dodecyl gallate (also called “lauryl gallate (LG)”);
- 2,4,5-trihydroxybutyrophenone (THBP);
- tert-butylhydroquinone (TBHQ);
- nordihydroguaiaretic acid (NDGA);
- 2- and 3-tert-butyl-4-hydroxyanisole (BHA);
- 2,6-di-tert-butyl-4-(hydroxymethyl)phenol (BHT Alcohol or Ionox-100);
- 2,6-di-tert-butyl-4-hydroxytoluene (BHT).

A method for determining the absence of an antioxidant, or the maximum trace amount, within the limits of the analysis, is given in [Annex B](#).

The issue of canolol, a naturally occurring substance in rapeseed, interfering with the analysis is addressed in [Annex C](#).

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

4 Principle

The sample is diluted in hexane- The antioxidants are extracted into acetonitrile, diluted with isopropanol and analysed by reversed-phase gradient HPLC with ~~ultra violet~~ultraviolet (UV) detection at 280 nm.

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5 Reagents

5.1 **Acetonitrile**, HPLC grade.

5.2 **2-Propanol**, analytical grade.

5.3 **Hexane**, isohexane or similar volatile non-polar solvent, analytical grade.

5.4 **High purity water**.

5.5 **Acetic acid**, > 99,8 %, HPLC Grade, for acidifying mobile phases.

5.6 **HPLC mobile phases:**

- a) Eluent A: Water containing 5 % acetic acid. Add 900 ml high purity water to a 1-l volumetric flask, add 50 ml acetic acid and bring to 1 litre with high purity water.
- b) Eluent B: Acetonitrile containing 5 % acetic acid. Add 50 ml acetic acid to a 1-l volumetric flask, add acetonitrile with mixing to bring to 1 litre.

5.7 **Antioxidant standards:** PG, OG, LG, THBP, TBHQ, NDGA, BHA (mixture of 2- and 3-isomers), Ionox-100 and/or BHT. In practice, only standards for the antioxidants of interest should be prepared.

5.8 **Standard solutions.** Refrigerate all antioxidant solutions out of direct light. Prepare all solutions with 2-propanol + acetonitrile (1:1).

- a) Stock standard (~1 g/l): Accurately weigh and transfer about 50 mg of each antioxidant into a 50 ml volumetric flask, dissolve, dilute to volume and mix.
- b) Working standard, target 80 mg/l: Pipet 4 ml stock solution into a 50 ml volumetric flask, dilute to volume and mix. Other standard concentrations can be prepared if desired.
- c) Calculate the exact standard concentrations as shown by [Formula \(1\)](#):

$$C = m_{AS} \times V \times 0,4$$

where

C is the **concentration of the working standard** in mg/l;

m_{AS} is the mass of the antioxidant standard, in mg, added to make 50 ml stock standard;

V is the volume of stock standard solution used to make the working standard;

0,4 is (1 000 mg/g)/(50 ml stock standard volume ~~×~~ 50 ml working standard volume).

5.9 **Extraction solvents.** Saturate hexane and acetonitrile by mixing and shaking together for 2 min and separate. Unless otherwise specified, use these saturated solvents for the extraction described in [7.1](#). Significant amounts of hexane will dissolve in the acetonitrile; therefore, allow for extra hexane. Determine

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how much acetonitrile and hexane are needed for all samples (each sample will require at least 20 ml hexane and 150 ml acetonitrile).

6 Apparatus

6.1 Gradient HPLC system, consisting of a gradient pumping system, sample injection system, column heater, UV or photodiode array detector and data analysis system. The system shall be capable of pumping at a pressure compatible with an acceptable flow rate for the selected column.

6.2 C18 Reversed-phase HPLC column, available from a wide variety of manufacturers. Most C18 columns will be capable of the needed separation. A guard column is highly recommended to protect the analytical column. Narrow diameter columns consume less solvent and also have a higher response. Smaller particle size improves resolution, but also increases back pressure.

6.3 Borosilicate beakers, 50 ml and 150 ml.

6.4 Separatory funnels, 125 ml and 250 ml.

6.5 Volumetric flasks, 50 ml.

6.6 Class A volumetric pipet, 4 ml.

6.7 Graduated glass cylinders, with ground-glass stoppers, 10 ml.

6.8 Graduated cylinders, 50 ml and 1 litre.

6.9 Solvent evaporation system: either a multi-vessel nitrogen blow-down evaporator such as TurboVap (Biotage, Uppsala, Sweden)¹⁾ or a rotary evaporator.

6.10 Appropriate evaporation vessels: either vessels for the nitrogen evaporation system (e.g. TurboVap vials, 250 ml) or round-bottomed flasks, 250 ml, for the rotary evaporator.

6.11 Analytical balance, capable of weighing to the nearest 0,001 g.

7 Procedure

7.1 Extraction of liquid oils, animal fats and shortenings

7.1.1 Weigh, to the nearest 0,01 g, approximately 4 g of oil into a 50 ml beaker. Quantitatively transfer to a 125 ml separatory funnel using about 20 ml hexane saturated with acetonitrile and rinse the beaker with saturated hexane. Close and shake the separatory funnel to completely dissolve (for solid fats) and mix.

7.1.2 Extract the oil-hexane mixture with 50 ml of acetonitrile saturated with hexane. If an emulsion forms, break by holding the 125 ml separatory funnel under hot tap water for 5 s to 10 s. Collect the extract (bottom phase) in a 250 ml separatory funnel and repeat the extraction with 50 ml acetonitrile saturated with hexane twice. Drain the combined extracts into a 250 ml round-bottomed flask or TurboVap vial (see 7.1.3). The draining shall be carried out slowly to prevent the inclusion of hexane-oil droplets.

The 150 ml acetonitrile extract may be stored overnight under refrigeration and protected from light if necessary.

¹⁾ TurboVap (Biotage, Uppsala, Sweden) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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7.1.3 For evaporation, use one of the following two options:

- a) Option 1: Nitrogen evaporation. Collect the samples in appropriate evaporation flasks. To an additional evaporation flask, add 10,0 ml of the working standard and approximately 140 ml acetonitrile. If there are many samples, prepare one flask containing the working standard to run with each evaporation batch. Place the samples plus one flask containing the working standard in the evaporator set to 40 °C and start the nitrogen flow. Evaporate until there is less than 4 ml volume remaining, but not to dryness, to obtain concentrated acetonitrile extracts. Correct the antioxidant results for the recovery of the working standard (see [Clause 8](#)).
- b) Option 2: Rotary Evaporation: Collect the samples in 250 ml round-bottom flasks and evaporate the acetonitrile extract to 3 ml to 4 ml; the temperature of the water bath should be no more than 40 °C. Evaporation should be completed in less than 10 min. To an additional evaporation flask, add 10,0 ml of the working standard and approximately 140 ml acetonitrile. Evaporate as with the samples until there is less than 4 ml volume remaining, but not to dryness, to obtain concentrated acetonitrile extracts. Correct the antioxidant results for the recovery of the working standard (see [Clause 8](#)).

7.1.4 Using a disposable pipet, transfer the concentrated acetonitrile extract to a 10 ml graduated cylinder. Rinse the flask with small portions of acetonitrile and transfer the rinses to the graduated cylinder with a disposable pipet until 5 ml is collected. Rinse the disposable pipet and rinse the flask with small portions of 2-propanol, transferring all the rinses to the graduated cylinder until exactly 10 ml is collected. Mix the contents of the graduated cylinder.

Samples containing TBHQ, BHT or BHA should be analysed within 4 h to avoid losses.

7.2 Chromatography

7.2.1 HPLC column

A C18 reversed-phase HPLC column of the following column dimensions is essentially equivalent for the separation:

- 4,6 mm diameter × 250 mm length with 5 µm packing, recommended flow rate 1,5 ml/min;
- 3,0 mm diameter × 150 mm length with 3 µm packing, recommended flow rate 0,65 ml/min;
- 2,0 mm diameter × 100 mm length, with 2 µm packing, recommended flow rate 0,2 ml/min.

Flow rates can require adjustment to accommodate the pressure limitations of the HPLC system. Other diameters and column particle sizes may be used providing the baseline separation of the required antioxidants and contaminants can be obtained.

7.2.2 HPLC conditions

The HPLC conditions are as follows:

- Column temperature: 40 °C.
- Starting conditions: 70 % eluent A, 30 % eluent B.
- Injection volume: 5 µl, which may be adjusted based on column size.
- Flow rate appropriate to the column.