International Standard

ISO 5132

First edition
2024-10

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

Reference number
ISO 5132:2024(en)

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Published in Switzerland

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Foreword

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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.

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].

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 ultraviolet (UV) detection at 280 nm.

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):

$$C = m_{AS} \times V \times 0{,}4 \qquad (1)$$

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 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.

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.

For standards and samples, run a linear gradient from 30 % eluent B to 100 % eluent B over 10 min and hold at 100 % eluent B for 4 min. The gradient can be adjusted if necessary to aid the separation of interfering compounds.

Use UV detection at 280 nm. Collect data from 0 min to 14 min.

For samples, regenerate the column more rapidly by increasing the flow rate by 50 % and hold at 100 % eluent B for 5 min, or until non-polar lipids are eluted. Re-equilibrate the column at 70 % eluent A, 30 % eluent B at the recommended elution flow rate for 5 min.

Identify peaks by comparison with the retention times of the standards. Determine the peak areas.

For the background check, carry out a reagent blank determination, substituting 25 ml hexane for the hexane-oil mixture. Extract the hexane as given in 7.1.2 to 7.1.4. If small peaks that cannot be eliminated are consistently present in the reagent blank, subtract the background peak areas.

8 Calculations

Calculate concentrations of each antioxidant in mg/kg as shown by Formula (2):

$$AO = \frac{(A_S - A_B) \times C \times 10}{A_{ST} \times m_S} \times \frac{C}{C_R} \quad (2)$$

where

AO is the antioxidant in mg/kg;

A_S is the peak area from the sample;

A_B is the peak area of any background that shall be subtracted;

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

10 is the final dilution volume of 10 ml;

A_{ST} is the peak area from the standard;

m_S is the mass of sample in g;

C_R is the concentration of the working standard recovered after evaporation.
Expected recoveries are between 90 % and 95 % for TBHQ, BHT and BHA, and 98 % for propyl, octyl and lauryl gallate. Recovery precision should be < 1 % of the coefficient of variation for identical evaporation conditions.

9 Validation

The results of the collaborative study that was carried out between April and July 2021 are given in Annex A.

Annex A
(informative)

Validation study

A.1 General

A collaborative study was carried out between April and July 2021 by ANSI. Sixteen laboratories from nine countries [Australia (1), China (3), France (1), Indonesia (1), Italy (1), Malaysia (1), the Kingdom of the Netherlands (2), the United States of America (5), Vietnam (1)] agreed to participate. Fourteen laboratories returned usable data. Not all laboratories analysed all the antioxidants in the study.

A.2 Collaborative study method

Twelve collaborative samples containing between four and nine antioxidants each were sent to the collaborating laboratories for analysis: a total of 55 analyses for each laboratory that analysed all the antioxidants.

All the data received were subjected to statistical outlier tests. The Cochran, Single Grubbs and Double Grubbs outlier tests were used, in that order.

Outliers were sequentially removed: Cochran first, then single Grubbs, then double Grubbs, until either the data had no significant outliers or the number of outliers removed exceeded the allowable limit (2 data points out of 9).

A.3 Collaborative study results

The collaborative study results are given in Tables A.1 to A.6.

Table A.1 — Refined, bleached, deodorized (RBD) soybean oil with added antioxidants

Parameter	PG	TBHQ	BHA	BHT	LG
Total number of laboratories	12	13	14	14	13
Total number of replicates (after outlier removal)	24	25	27	28	25
Overall mean of all data (grand mean), mg/kg	169,4	88,8	50,4	103,7	148,5
Repeatability standard deviation, s_r (mg/kg)	3,87	2,74	0,74	3,38	2,75
Reproducibility standard deviation, s_R (mg/kg)	9,38	9,04	3,25	15,14	7,95
Coefficient of variation, repeatability, $C_{V,r}$ (%)	2,28	2,97	1,46	3,26	1,85
Coefficient of variation, reproducibility, $C_{V,R}$ (%)	5,54	9,79	6,46	14,6	5,35

Table A.2 — RBD soybean oil with added antioxidants

Parameter	THBP	NDGA	Ion	OG
Total number of laboratories	6	10	7	13
Total number of replicates	12	20	14	26
Overall mean of all data (grand mean), mg/kg	172,6	51,8	106,9	48,3
Repeatability standard deviation, s_r (mg/kg)	3,86	5	2,5	2,08
Reproducibility standard deviation, s_R (mg/kg)	9,69	18,82	10,65	6,91
Coefficient of variation, repeatability, $C_{V,r}$ (%)	2,24	9,65	2,34	4,31
Coefficient of variation, reproducibility, $C_{V,R}$ (%)	5,61	36,32	9,96	14,32

Table A.3 — RBD rapeseed oil with added antioxidants

Parameter	PG	BHA	BHT	LG
Total number of laboratories	12	14	13	13
Total number of replicates	23	27	25	25
Overall mean of all data (grand mean), mg/kg	90,4	202,1	44,7	88,7
Repeatability standard deviation, s_r (mg/kg)	5,24	8,46	2,17	2,90
Reproducibility standard deviation, s_R (mg/kg)	5,68	17,3	3,70	5,45
Coefficient of variation, repeatability, $C_{V,r}$ (%)	5,79	4,19	5,06	3,26
Coefficient of variation, reproducibility, $C_{V,R}$ (%)	6,28	8,56	8,27	6,14

Table A.4 — RBD rapeseed oil with added antioxidants

Parameter	THBP	NDGA	Ion	OG
Total number of laboratories	6	10	7	13
Total number of replicates	12	20	14	25
Overall mean of all data (grand mean), mg/kg	86,2	36,5	95,5	189,8
Repeatability standard deviation, s_r (mg/kg)	2,85	2,16	3,62	4,19
Reproducibility standard deviation, s_R (mg/kg)	3,98	4,88	9,14	9,77
Coefficient of variation, repeatability, $C_{V,r}$ (%)	3,31	5,91	3,79	2,19
Coefficient of variation, reproducibility, $C_{V,R}$ (%)	4,62	13,4	9,57	5,11

Table A.5 — RBD corn (maize) oil with added antioxidants

Parameter	PG	TBHQ	BHA	BHT	LG
Total number of laboratories	12	14	14	13	13
Total number of replicates	23	27	28	25	26
Overall mean of all data (grand mean), mg/kg	42,1	140,0	111,8	170,4	29,8
Repeatability standard deviation, s_r (mg/kg)	1,18	7,16	3,04	5,22	0,86
Reproducibility standard deviation, s_R (mg/kg)	3,03	28,56	8,4	14,0	2,9
Coefficient of variation, repeatability, $C_{V,r}$ (%)	2,82	5,03	2,72	4,73	2,87
Coefficient of variation, reproducibility, $C_{V,R}$ (%)	7,25	20,05	7,51	8,68	9,73

Table A.6 — Palm olein with added antioxidants

Parameter	PG	THBP	TBHQ	NDGA	BHA	Ion	OG	BHT	LG
Total number of laboratories	11	6	14	10	14	7	11	14	13
Total number of replicates	21	12	27	19	27	14	21	28	25
Overall mean of all data (grand mean), mg/kg	99,9	96,5	85,5	108,2	90,81	65,5	90,4	91,0	81,8
Repeatability standard deviation, s_r (mg/kg)	2,00	4,13	3,43	2,75	2,14	1,69	2,28	4,38	1,41
Reproducibility standard deviation, s_R (mg/kg)	4,18	6,32	24,16	9,85	4,62	3,68	4,16	7,89	5,88
Coefficient of variation, repeatability, $C_{V,r}$ (%)	1,94	4,28	3,96	2,52	2,34	2,58	2,51	4,81	1,72
Coefficient of variation, reproducibility, $C_{V,R}$ (%)	4,04	6,55	27,89	9,02	5,06	5,62	4,58	8,67	7,14

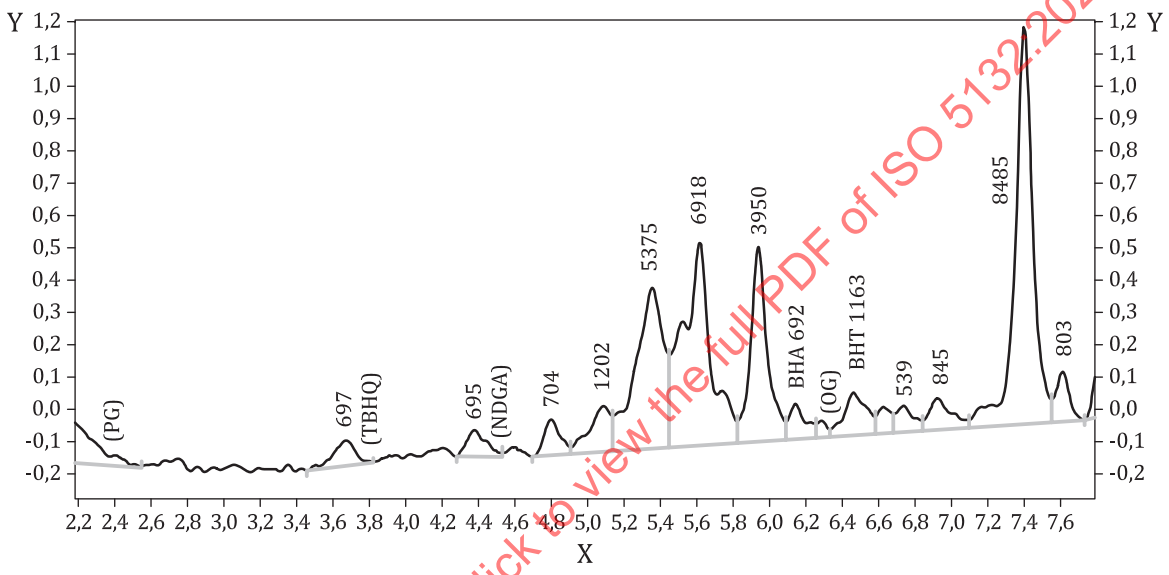
Annex B
(informative)

Estimation of the absence of an antioxidant

B.1 General

Analyse the sample as indicated in the method, including analysis of a standard of the antioxidant of interest.

Expand the baseline of the chromatogram so very small peaks are visible, as shown in Figure B.1.



Key

X	minutes
Y	mVolts
PG	propyl gallate
TBHQ	tert-butylhydroquinone
NDGA	nordihydroguaiaretic acid
BHA	2- and 3-tert-butyl-4-hydroxyanisole
OG	octyl gallate
BHT	2,6-di-tert-butyl-4-hydroxytoluene
Numerical annotations	peak areas

Figure B.1 — Expanded baseline for maize (corn) oil

The absence of an antioxidant may be determined when only baseline noise, with no real peak, is present at the retention time of the antioxidant of interest.

In the example given in Figure B.1, it can be determined that TBHQ is absent, but it cannot be determined whether BHT or BHA are absent. Most vegetable oils contain minor components that co-elute with BHT and BHA.

To determine the limits of the estimate, find a small peak approximately three times the height of the baseline noise in the chromatogram. This will be the surrogate peak for the antioxidant. Determine the area of that peak and divide that area by the response factor of the antioxidant of interest and correct for dilution.

This number represents an estimate slightly higher than the maximum amount of the antioxidant that could be present.

The calculation as shown by Formula (B.1) is identical to the calculation used in Formula (2) for the samples, except that instead of using the area of the same peak, the area of the surrogate peak chosen is used:

$$AO_{\mathrm{LD}} = \frac{A_{\mathrm{X}} \times C \times 10}{A_{\mathrm{ST}} \times m_{\mathrm{S}}} \tag{B.1}$$

where

AO_{LD} is the antioxidant's limit of detection in mg/kg;

A_{X} is the peak area from the surrogate peak in the chromatogram;

C is the concentration of standard in mg/l;

10 is the final dilution volume of 10 ml;

A_{ST} is the peak area from the standard;

m_{S} is the mass of sample in g.

EXAMPLE In the example given in Figure B.1, the surrogate peak area (A_{X}) is 697, the concentration of the standard (C) is 79,4 mg/l and the peak area (A_{ST}) is 630 538 (obtained from a separate standard calibration chromatogram): the sample mass (m_{S}) is 4,03 g, giving the TBHQ limit of detection, in mg/kg, as shown in the following formula:

$$\text{TBHQ limit of detection} = \frac{697 \times 79{,}4 \times 10}{630\,538 \times 4{,}03} = 0{,}218$$

$$AO_{\text{LD-TBHQ}} = \frac{697 \times 79{,}4 \times 10}{630\,538 \times 4{,}03} = 0{,}218$$

where $AO_{\text{LD-TBHQ}}$ is the limit of detection for TBHQ in mg/kg.

The results are reported as: "TBHQ not detected. LOD = 0,2 mg/kg".

If a peak is present at the retention time of an antioxidant, the HPLC-UV data cannot be used to determine the absence of an antioxidant. The amount calculated from the peak eluting at that retention time can be reported as the maximum amount that can be present.

B.2 Collaborative study results

In a collaborative study carried out in 2021, laboratories were asked to use the procedure outlined in Clause B.1 to determine the limit of detection for TBHQ in soybean oil that contained no antioxidants.

Nine laboratories returned data. In most cases, the laboratories were able to obtain estimated limits of quantitation below 1 mg/kg, as shown in Table B.1.

Table B.1 — Estimated limit of quantitation for TBHQ in soybean oil

Laboratory	Limit of quantitation mg/kg
1	0,09
2	1,39
3	3,2
4	0,1
5	0,17
6	0,1
7	0,3
8	0,089
9	0,312