



# Technical Specification

**ISO/TS 16465**

## **Animal and vegetable fats and oils — Determination of phthalates in vegetable oils**

*Corps gras d'origines animale et végétale — Dosage des phtalates  
dans les huiles végétales*

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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](http://www.iso.org/directives)).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see [www.iso.org/iso/foreword.html](http://www.iso.org/iso/foreword.html).

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](http://www.iso.org/members.html).

# Animal and vegetable fats and oils — Determination of phthalates in vegetable oils

## 1 Scope

This document specifies two methods for the quantitative determination of phthalates in vegetable oils by gas chromatography-mass spectrometry (GC-MS):

- Part A for the determination of di-2-ethylhexyl phthalate (DEHP).
- Part B for the determination of eight phthalates: dimethyl phthalate (DMP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), dibutyl phthalate (DBP), benzylbutyl phthalate (BBP), di-2-ethylhexyl phthalate (DEHP), di-isononyl phthalate (DINP), di-isodecyl phthalate (DIDP).

Both methods are applicable for all vegetable oils, including crude, refined and virgin.

## 2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 661, *Animal and vegetable fats and oils — Preparation of test sample*

## 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 Part A - Determination of DEHP

### 4.1 Principle

Phthalates are first extracted from oil with acetonitrile followed by dispersive-solid-phase extraction clean-up (dispersive SPE), which is done to remove organic acids, excess water and other components using a combination of primary secondary amine (PSA) sorbent and C18 bonded phase. Finally, the extract is concentrated and analysed by gas chromatography-mass spectrometry (GC-MS).

### 4.2 Reagents

**WARNING — Attention is drawn to national regulations that specify the handling of hazardous substances, and users' obligations thereunder. Technical, organizational and personal safety measures shall be followed.**

Unless otherwise specified, use only reagents of recognized analytical grade.

#### 4.2.1 Toluene, purity 99 % min.

4.2.2 **Acetonitrile**, trace organic analysis grade, purity 99 % min.

4.2.3 **n-Hexane**, trace organic analysis grade, purity 99 % min.

4.2.4 **Chromabond adsorbent Diamino (PSA), Chromabond adsorbent C18<sup>1)</sup>**.

NOTE These powders can be sources of contamination. To remove the contaminants, the powders can be purified using Soxhlet extraction by extracting twice with 200 ml of n-hexane during a six-hour period.

4.2.5 **n-Dodecane**, purity 99 % min.

4.2.6 **Standards**

[Table 1](#) provides the CAS number of phthalates.

**Table 1 — CAS number of phthalates**

Phthalates	Synonym	CAS Registry Number <sup>(2)</sup>
di-2-ethylhexyl phthalate	DEHP	117-81-7
deuterated di-2-ethylhexyl phthalate	DEHP-d4	93951-87-2

4.2.6.1 **Solution of DEHP-d4 internal standard**, mass concentration  $\rho = 50 \mu\text{g/ml}$ .

As an example, in a volumetric flask of 100 ml, weigh to the nearest mg, approximately 50 mg of DEHP-d4 and dilute to 100 ml of toluene and then proceed with a second dilution of this mixture of 10 ml → 100 ml with toluene.

4.2.6.2 **DEHP calibration solutions**

As an example, in a volumetric flask of 100 ml, weigh to the nearest mg, approximately 50 mg of DEHP and dilute to 100 ml of toluene (SM= 500  $\mu\text{g/ml}$ ). Then prepare:

- 20  $\mu\text{l}$  of SM and dilute to 10 ml of acetonitrile: SF1 = 1  $\mu\text{g/ml}$
- 200  $\mu\text{l}$  of SM and dilute to 10 ml of acetonitrile: SF2 = 10  $\mu\text{g/ml}$
- 2 ml of SM and dilute to 10 ml of acetonitrile: SF3 = 100  $\mu\text{g/ml}$

Then prepare a calibration range in acetonitrile following [Table 2](#), using glass syringes washed 10 times with toluene and five times with related solutions.

1) Chromabond sorbent C18 ref 730611 and Chromabond sorbent Diamino (PSA) ref 730611 from Macherey Nagel ([www.mn-net.com](http://www.mn-net.com)) have proved to be free of phthalate contamination. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

2) CAS Registry Number<sup>®</sup> is a trademark of the American Chemical Society (ACS). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Table 2 — Example of preparation for DEHP calibration

	[Concentration] DEHP $C_{\text{cal-ph}}$ $\mu\text{g/ml}$	Volume ( $\mu\text{l}$ )				[Concentration] DEHP-d4 $C_{\text{cal-dph}}$ $\mu\text{g/ml}$	Solution of DEHP-d4 internal standard ( $\mu\text{l}$ )	Volume of acetonitrile to be added ( $\mu\text{l}$ )	Total volume ( $\mu\text{l}$ )
		SF1	SF2	SF3	SM				
G0	0	-	-	-	-	4	80	920	1 000
G1	0,02	20	-	-	-	4	80	900	1 000
G2	0,05	50	-	-	-	4	80	870	1 000
G3	0,1	-	10	-	-	4	80	910	1 000
G4	0,5	-	50	-	-	4	80	870	1 000
G5	1	-	-	10	-	4	80	910	1 000
G6	2	-	-	20	-	4	80	900	1 000
G7	5	-	-	-	10	4	80	910	1 000
G8	10	-	-	-	20	4	80	900	1 000

Depending on the apparatus used, it is not possible to be linear from G0 to G8. In this case, it is necessary to increase the volume of acetonitrile of recovery in order to be linear over the greatest possible range while being able to see the level G1.

### 4.3 Apparatus

Glassware used for the determination shall be thoroughly cleaned at 550 °C during 6 h, such as Pasteur pipette, vial, conical glass sample vial, 10 ml and 15 ml capacity.

4.3.1 Conical glass sample vials, 10 ml capacity.

4.3.2 Conical glass sample vials, 15 ml capacity.

4.3.3 Glass syringe, 10  $\mu\text{l}$ , 20  $\mu\text{l}$ , 50  $\mu\text{l}$ , 100  $\mu\text{l}$ , 250  $\mu\text{l}$  and 500  $\mu\text{l}$  capacity.

4.3.4 Automatic evaporator, for 10 ml tube (optional), recommended operating conditions: temperature of the water bath = 40 °C, nitrogen pressure = 5 psi.

4.3.5 Conical glass sample vials, 2 ml capacity.

4.3.6 Gas chromatograph, suitable for use with capillary column, equipped with an injector split-splitless or equivalent device, a temperature-programmable oven and mass detector with electron ionization source (ionization energy of 70 eV) and SIM (single ion monitoring) mode.

4.3.7 Data acquisition system, with the possibility of manual integration.

4.3.8 Capillary column, capable of being programmed up to 400 °C ("high temperature" type) for which the following characteristics are advised: 95 % dimethyl/5 % diphenyl polysiloxane stationary phase, length of 15 m, internal diameter of 0,25 mm, film thickness of 0,1  $\mu\text{m}$  or length of 30 m, internal diameter of 0,25 mm, 0,25  $\mu\text{m}$  film thickness.

4.3.9 Microsyringe, 5  $\mu\text{l}$  to 10  $\mu\text{l}$  capacity, suitable for split less injection in gas chromatography.

4.3.10 Analytical balance, reading accuracy 0,001 g.

4.3.11 Pasteur pipette, in glass.

**4.3.12 Centrifuge**, capable of attaining at least 3 000 min<sup>-1</sup>, suitable for 10 ml tubes.

**4.3.13 Pipettes**, capable of pipetting volumes up to 10 ml.

#### 4.4 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this document. A recommended sampling method is given in ISO 5555.<sup>[1]</sup>

Analyse only sample aliquots packaged in glass bottles with suitable lids (the sample contact material from the lid should be, for example, polytetrafluoroethylene (PTFE), aluminium, rubber but not plastic nor soft seal) to avoid further contamination. Plastic packaging is not suitable.

Due to the presence of phthalates in the environment, the analysis of these compounds requires precautions throughout the analysis:

- avoid contact with plastic material,
- test the solvents/materials used for phthalates,
- a blank sample should be analysed every four samples,
- glassware that cannot be baked needs to be rinsed with a suitable solvent (acetone),
- avoid contamination from the septum of the injection vials.

#### 4.5 Preparation of the test sample

Prepare the test sample in accordance with ISO 661.

#### 4.6 Procedure

##### 4.6.1 Phthalates extraction from oil

Weigh to the nearest mg, in a conical glass sample vial of 10 ml, 1 g of oil and add 40 µl of DEHP-d4 solution at 50 µg/ml (4.2.6.1). Shake with a vortex for 15 s.

Add 6 ml of acetonitrile and shake with a vortex for 1 min (or 5 min with an automatic vortex).

Centrifuge the conical glass for 2 min at 3 000 rpm.

Transfer the upper layer in a conical glass of 15 ml containing 400 mg of C18 (4.2.4) and 400 mg of PSA (4.2.4).

Shake with a vortex for 1 min.

Transfer the upper layer in a conical glass of 10 ml.

Add 50 µl of n-dodecane.

Evaporate acetonitrile at 40 °C to reach 500 µl ± 50 µl.

There remains a droplet of fat at the bottom of the tube from the dodecane.

Shake with a vortex for 15 s (or 2 min with an automatic vortex).

Centrifuge the conical glass for 2 min at 3 000 rpm.

Transfer the solvent layer avoiding the fat droplet in an injection vial and inject into GC-MS.

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In order to measure the level of contamination of the laboratory, prepare a blank sample. For blank sample, add only 40 µl of DEHP-d4 internal standard solution (4.2.6.1) in a conical glass sample vial of 10 ml and follow the same procedure as for classic sample. (Values of 0,05 mg/kg for DEHP should not be exceeded).

In order to avoid contamination from the septum of the vial (DMP), use a septum with polytetrafluoroethylene (PTFE), otherwise a small piece of aluminium sheet between the cork and the vial can be used.

NOTE Depending on the apparatus used, the concentration of the extract can be modified and adapted according to the system.

### 4.6.2 Gas Chromatography

#### 4.6.2.1 Gas chromatography setup

Install the column (4.3.8) in the gas chromatograph (4.3.6) and check the working conditions by injecting the solvent, acetonitrile. The baseline should be straight with a small positive drift. If the drift is high, proceed to condition the column, for a negative drift check the connections of the column.

If the column is used for the first time, it is necessary to condition the column by heating it in the column oven using a temperature gradient up to 370 °C (depending on the oven temperature chosen for the analysis) in 4 h, maintain the temperature for 2 h.

#### 4.6.2.2 Working conditions for gas chromatography analysis

The working conditions as shown in Table 3 and Table 4 have proved to be adapted to the analysis.

These conditions can be adjusted in accordance with the characteristics of the gas chromatograph and the mass detector apparatus and the column.

Table 3 — GC conditions

Column	DB5 HT (15 m – 0,25 mm – 0,10 µm film thickness or 30 m – 0,25 mm – 0,25 µm film thickness)
Injector	Liner, tapered, deactivated liner with glass wool
Injection port temperature	350 °C
Injection temperature program	Splitless in pressure pulse (12 psi) for 1 min 1 min after the injection, split at 150 ml/min 3 min after the injection, split at 20 ml/min
Injection volume	1,0 µl
Oven temperature	50 °C for 1 min, ramp at 15 °C/min to 100 °C, ramp at 40 °C/min to 290 °C, ramp at 60 °C/min to 350 °C, hold for 2 min
Carrier gas	Helium/hydrogen constant flow rate, 1,2 ml/min
Syringe solvent clean-up	Acetonitrile

Table 4 — MS conditions

Ion source temperature	300 °C				
Quadrupole temperature	150 °C				
Transfer line temperature	325 °C				
Parameters for SIM mode	Phthalates	Quantifier ion	Qualifier ion	Qualifier ion	Qualifier ion
	DEHP	149	167	279	132
	DEHP d4	153	171	283	136

Check that the volume of acetonitrile when injected at 350 °C is not greater than the volume of the liner.

## 4.7 Quantitative determination

### 4.7.1 Calibration curve

The calibration curve of DEHP is of type  $y = a * x$

$$\frac{A_{ph}}{A_{dph}} = a * \frac{C_{cal-ph}}{C_{cal-dph}}$$

The calibration curve is determined as follows:

$$a = \frac{A_{ph} * C_{cal-dph}}{A_{dph} * C_{cal-ph}}$$

where

$a$  is the slope;

$A_{ph}$  is the peak area of DEHP in the calibration solution;

$A_{dph}$  is the peak area of the DEHP-d4 internal standard in the calibration solution;

$C_{cal-dph}$  is the concentration of the DEHP-d4 internal standard in the calibration solution, in micrograms per millilitres (see [Table 2](#));

$C_{cal-ph}$  is the concentration of DEHP in the calibration solution, in micrograms per millilitres (see [Table 2](#)).

### 4.7.2 Quantitative determination of DEHP

$$C_{ph} = \left( \frac{A_{ph} * V_{dph} * C_{dph}}{A_{dph} * M * a} \right) - C_{blank}$$

where

$a$  is the slope;

$A_{ph}$  is the peak area of DEHP;

$A_{dph}$  is the peak area of the DEHP-d4 internal standard;

$C_{dph}$  is the concentration of the DEHP-d4 internal standard solution, in micrograms per millilitres;

$V_{dph}$  is the volume of the DEHP-d4 internal standard solution, in millilitres;

$C_{ph}$  is the concentration of DEHP, in milligrams per kilogram;

$C_{blank}$  is the concentration of DEHP in the blank, in milligrams per kilogram (see [4.6.1](#));

$M$  is the mass of the test portion, in grams.

If the volumes of the internal standard solution indicated in this method are respected, and if the same solution of DEHP-d4 internal standard is used both for the calibration solutions and for the sample preparation, the following simplified calculation formulae can be used for the determination of the calibration curve and the quantification of phthalate:

#### 4.7.2.1 Calibration curve

The calibration curve for DEHP is of type  $y = a * x$

$$\frac{A_{ph}}{A_{dph}} = a * m_{cal-ph} * \frac{40}{80}$$

The calibration curve is determined as follows:

$$a = \frac{A_{ph} * 2}{A_{dph} * m_{cal-ph}}$$

where

- $a$  is the slope from the calibration, using the same DEHP-d4 internal standard solution for the samples and for the calibration;
- $A_{ph}$  is the peak area of DEHP in the calibration solution;
- $A_{dph}$  is the peak area of the DEHP-d4 internal standard in the calibration solution;
- $m_{cal-ph}$  is the mass of DEHP in the solution calibration, in micrograms.

NOTE The coefficient of 2 is added in the formula to consider the ratio of the amount of internal standard between the calibration solutions and the samples (80 µl of the internal standard solution for the calibration and 40 µl of the internal standard solution for the samples).

#### 4.7.2.2 Quantitative determination of DEHP

$$C_{ph} = \left( \frac{A_{ph}}{A_{dph} * M * a} \right) - C_{blank}$$

where

- $C_{ph}$  is the concentration of DEHP, in milligrams per kilogram;
- $a$  is the slope from the calibration, using the same DEHP-d4 internal standard solution for the samples and for the calibration;
- $A_{ph}$  is the peak area of the DEHP;
- $A_{dph}$  is the peak area of the DEHP-d4 internal standard;
- $M$  is the mass of the test portion, in grams;
- $C_{blank}$  is the concentration of DEHP in the blank, in milligrams per kilogram (see [4.6.1](#)).

### 4.8 Precision of the method

#### 4.8.1 Interlaboratory test

Details of the test and the precision of the method are summarized in [Annex A](#). The values derived from this interlaboratory test will possibly not be applicable to concentration ranges and matrices other than those given.

#### 4.8.2 Repeatability

The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, shall in not more than 5 % of cases exceed the value of  $r$  given in [Table A.1](#).

#### 4.8.3 Reproducibility

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories by different operators using different equipment, shall in not more than 5 % of cases exceed the value of  $R$  given in [Table A.1](#).

#### 4.9 Test report

The test report shall contain at least the following information:

- a) all information necessary for the complete identification of the sample;
- b) the sampling method used, if known;
- c) the test method used, with reference to this document, i.e. ISO/TS 16465:2024;
- d) all operating details not specified in this document, or regarded as optional, together with details of any incidents occurred when performing the method, which can have influenced the test result(s);
- e) the test result obtained;
- f) if the repeatability has been checked, the final quoted result obtained.

### 5 Part B - Determination of eight phthalates

#### 5.1 Principle

Phthalates are first extracted from oil with acetonitrile followed by dispersive-solid-phase extraction clean-up (dispersive SPE) which is done to remove organic acids, excess water and other components using a combination of primary secondary amine (PSA) sorbent and C18 bonded phase. Finally, the extract is concentrated and analysed by gas chromatography-mass spectrometry (GC-MS). For refined oil, the acetonitrile extract is directly concentrated without using the dispersive-SPE purification step.

#### 5.2 Reagents

**WARNING — Attention is drawn to national regulations that specify the handling of hazardous substances, and users' obligations thereunder. Technical, organizational and personal safety measures shall be followed.**

Unless otherwise specified, use only reagents of recognized analytical grade.

**5.2.1 Toluene**, purity 99 % min.

**5.2.2 Acetonitrile, trace organic analysis grade**, purity 99 % min.

**5.2.3 n-Hexane, trace organic analysis grade**, purity 99 % min.

**5.2.4 Chromabond adsorbent Diamino (PSA), Chromabond adsorbent C18<sup>3)</sup>**.

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3) Chromabond sorbent C18 ref 730611 and Chromabond sorbent Diamino ref 730611 from Macherey Nagel ([www.mn-net.com](http://www.mn-net.com)) have proved to be free of phthalate contamination. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

NOTE These powders can be sources of contamination. To get rid of them, you can purify the powders with a Soxhlet extraction with two times 200 ml of n-hexane during 6 h.

### 5.2.5 Absolute ethanol.

### 5.2.6 Demineralized water.

### 5.2.7 Standards

The CAS numbers of the phthalate and deuterated phthalate standards are shown in [Tables 5](#) and [6](#), respectively.

**Table 5 — CAS number of phthalates**

Phthalates	Synonym	CAS Registry Number®
dimethyl phthalate	DMP	131-11-3
diethyl phthalate	DEP	84-66-2
Diisobutyl phthalate	DiBP	84-69-5
dibutyl phthalate	DBP	84-74-2
benzylbutyl phthalate	BBP	85-68-7
di-2-ethylhexyl phthalate	DEHP	117-81-7
di-isononyl phthalate	DINP	28553-12-0
di-isodecyl phthalate	DIDP	26761-40-0

**Table 6 — CAS number of deuterated phthalates**

Phthalates	Synonym	CAS Registry Number® for deuterated phthalates
deuterated dimethyl phthalate	DMP-d4	93951-89-4
deuterated diethyl phthalate	DEP-d4	93952-12-6
deuterated diisobutyl phthalate	DiBP-d4	358730-88-8
deuterated dibutyl phthalate	DBP-d4	93952-11-5
deuterated benzylbutyl phthalate	BBP-d4	93951-88-3
deuterated di-2-ethylhexyl phthalate	DEHP-d4	93951-87-2
deuterated di-isononyl phthalate	DINP-d4	1332965-90-8
deuterated di-isodecyl phthalate	DIDP-d4	1346604-79-2

#### 5.2.7.1 Solution of deuterated phthalates internal standards, mass concentration $\rho = 50 \mu\text{g/ml}$

As an example, in a volumetric flask of 100 ml, weigh to the nearest mg, approximately 50 mg of each deuterated phthalate and dilute to 100 ml of toluene and then proceed with a second dilution of this mixture of 10 ml → 100 ml with toluene.

#### 5.2.7.2 Phthalates calibration solutions

##### 5.2.7.2.1 Light phthalates (DMP, DEP, DiBP, DBP, BBP, DEHP)

As an example, in a volumetric flask of 100 ml, weigh to the nearest mg, approximately 50 mg of each light phthalate and dilute to 100 ml of toluene (SMLP= 500  $\mu\text{g/ml}$ ). Then prepare:

- 20  $\mu\text{l}$  of SMLP and dilute to 10 ml of acetonitrile: SFLP1= 1  $\mu\text{g/ml}$

- 200 µl of SMLP and dilute to 10 ml of acetonitrile: SFLP2= 10 µg/ml
- 2 ml of SMLP and dilute to 10 ml of acetonitrile: SFLP3= 100 µg/ml

### 5.2.7.2.2 Heavy phthalates (DINP, DIDP)

As an example, in a volumetric flask of 100 ml, weigh to the nearest mg, approximately 50 mg of each heavy phthalate and dilute to 100 ml of toluene (SMHP= 500 µg/ml). Then prepare:

- 200 µl of SMHP and dilute to 10 ml of acetonitrile: SFHP1= 10 µg/ml
- 2 ml of SMHP and dilute to 10 ml of acetonitrile: SFHP2= 100 µg/ml

Then prepare a calibration range in acetonitrile following [Table 7](#), using glass syringes washed 10 times with toluene and five times with related solutions.

**Table 7 — Example of preparation for light and heavy phthalates**

	Light phthalates					Heavy phthalates				[Concentration] Deuterated Phthalates $C_{cal-dph}$ µg/ml	Solution of deuterated phthalates internal standards (µl)	Volume of acetonitrile to be added (µl)	Total volume (µl)
	[Concentration] Light phthalates $C_{cal-ph}$ µg/ml	Volume (µl)				[Concentration] Heavy phthalates $C_{cal-ph}$ µg/ml	SFHP1	SFHP2	SMHP				
		SFLP1	SFLP2	SFLP3	SMLP								
G0	0	-	-	-	-	0	-	-	-	4	80	920	1 000
G1	0,02	20	-	-	-	0,1	10	-	-	4	80	890	1 000
G2	0,05	50	-	-	-	0,2	20	-	-	4	80	850	1 000
G3	0,1	-	10	-	-	0,5	50	-	-	4	80	860	1 000
G4	0,5	-	50	-	-	1	-	10	-	4	80	860	1 000
G5	1	-	-	10	-	2	-	20	-	4	80	890	1 000
G6	2	-	-	20	-	5	-	50	-	4	80	850	1 000
G7	5	-	-	-	10	15	-	-	30	4	80	880	1 000
G8	10	-	-	-	20	30	-	-	60	4	80	840	1 000

Depending on the apparatus used, it is not possible to be linear from G0 to G8. In this case, it is necessary to increase the volume of acetonitrile of recovery in order to be linear over the greatest possible range while being able to see the level G1.

### 5.2.8 Chloroperbenzoic acid (CPBA), purity ≤ 77 %.

#### 5.2.9 CPBA solution, $\rho = 0,2$ g/ml in ethanol.

As an example, in a volumetric flask of 25 ml, 5 g of CPBA and dilute to 25 ml of absolute ethanol.

Prepare the solution freshly every day as CPBA solution in ethanol is not stable.

#### 5.2.10 Sodium carbonate solution, $\rho = 0,1$ g/ml in water.

As an example, in a volumetric flask of 100 ml, 10 g of sodium carbonate and dilute to 100 ml of water.

## 5.3 Apparatus

Glassware used for the determination shall be thoroughly cleaned at 550 °C during 6 h, such as Pasteur pipette, vial, conical glass sample vial, 10 ml and 15 ml capacity.

### 5.3.1 Conical glass sample vials, 10 ml capacity.

### 5.3.2 Conical glass sample vials, 15 ml capacity.

**5.3.3 Glass syringe**, 10 µl, 20 µl, 100 µl, 250 µl, 500 µl capacity.

**5.3.4 Automatic evaporator**, for 10 ml tube (optional), recommended operating conditions: temperature of water bath = 40 °C, nitrogen pressure = 5 psi.

**5.3.5 Conical glass sample vials**, 2 ml capacity.

**5.3.6 Insert vial**, 100 µl capacity.

**5.3.7 Gas chromatograph**, suitable for use with capillary column, equipped with an injector split-splitless or equivalent device, a temperature-programmable oven and mass detector with electron ionization source (ionization energy of 70 eV) and SIM (single ion monitoring) mode.

**5.3.8 Data acquisition system**, with the possibility of manual integration.

**5.3.9 Capillary column**, capable of being programmed up to 400 °C ("high temperature" type) for which the following characteristics are advised: 95 % dimethyl/5 % diphenyl polysiloxane stationary phase, length of 15 m, internal diameter of 0,25 mm, film thickness of 0,1 µm or length of 30 m, internal diameter of 0,25 mm, 0,25 µm film thickness.

**5.3.10 Microsyringe**, 5 µl to 10 µl capacity, suitable for split less injection in gas chromatography.

**5.3.11 Analytical balance**, reading accuracy 0,001 g.

**5.3.12 Pasteur pipette**, in glass.

**5.3.13 Centrifuge**, capable of attaining at least 3 000 min<sup>-1</sup>, suitable for 10 ml tubes.

**5.3.14 Pipettes**, capable of pipetting volumes up to 10 ml.

## 5.4 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this document. A recommended sampling method is given in ISO 5555.<sup>[1]</sup>

Analyse only sample aliquots packaged in glass bottles with suitable lids (the sample contact material from the lid should be, for example, polytetrafluoroethylene (PTFE), aluminium, rubber but not plastic nor soft seal) to avoid further contamination. Plastic packaging is not suitable.

Due to the presence of phthalates in the environment, the analysis of these compounds requires precautions throughout the analysis:

- avoid contact with plastic material,
- test the solvents used for phthalates,
- a blank sample should be analysed before analysing samples,
- glassware that cannot be baked needs to be rinsed with a suitable solvent (acetone),
- avoid contamination from the septum of the injection vials.

## 5.5 Preparation of the test sample

Prepare the test sample in accordance with ISO 661.

## 5.6 Procedure

### 5.6.1 Phthalates extraction from oil

#### 5.6.1.1 Refined oil

Weigh to the nearest mg, in a conical glass sample vial of 10 ml, 1 g of oil and add 40 µl of deuterated phthalates solution at 50 µg/ml (5.2.7.1). Shake with a vortex for 15 s.

At the first extraction, add 6 ml of acetonitrile and shake with a vortex for 1 min (or 5 min with an automatic vortex).

Centrifuge the conical glass for 2 min at 3 000 rpm.

Transfer the upper layer in a conical glass sample vial of 15 ml.

At the second extraction, add a second time, 6 ml of acetonitrile in the conical glass containing the oil and shake with a vortex for 1 min (or 5 min with an automatic vortex).

Centrifuge the conical glass for 2 min at 3 000 rpm.

Transfer the upper layer in the conical glass sample vial of 15 ml containing the first extraction (gather the two extracts).

Evaporate acetonitrile at 40 °C to reach 500 µl ± 50 µl.

Shake with a vortex for 15 s (or 2 min with an automatic vortex).

Centrifuge the conical glass for 2 min at 3 000 rpm if there is a fat droplet on the bottom of the tube.

Transfer the solvent layer avoiding the fat droplet in an injection vial and inject into GC-MS.

In order to measure the level of contamination of the laboratory, prepare a blank sample. For blank sample, add only 40 µl of deuterated phthalates internal standards solution (5.2.7.1) in a conical glass sample vial of 10 ml and follow the same procedure as for classic sample. (Values of 0,05 mg/kg for the light phthalates and 0,25 mg/kg for the heavy Phthalates should not be exceeded).

In order to avoid contamination from the septum of the vial (DMP), use a septum with polytetrafluoroethylene (PTFE), otherwise a small piece of aluminium sheet between the cork and the vial can be used.

NOTE Depending on the apparatus used, the concentration of the extract can be modified and adapted according to the system.

#### 5.6.1.2 Crude or virgin oil

Weigh to the nearest mg, in a conical glass sample vial of 10 ml, 1 g of oil and add 40 µl of deuterated phthalates solution at 50 µg/ml (5.2.7.1). Shake with a vortex for 15 s.

At the first extraction, add 6 ml of acetonitrile and shake with a vortex for 1 min (or 5 min with an automatic vortex).

Centrifuge the conical glass for 2 min at 3 000 rpm.

Transfer the upper layer in a conical glass of 15 ml containing 400 mg of C18 (5.2.4) and 400 mg of PSA (5.2.4).

At the second extraction, add a second time, 6 ml of acetonitrile in the conical glass containing the oil and shake with a vortex for 1 min (or 5 min with an automatic vortex).

Centrifuge the conical glass for 2 min at 3 000 rpm.

Transfer the upper layer in the conical glass of 15 ml containing C18 and PSA and the first extraction (gather the two extracts).

Shake with a vortex for 1 min (or 5 min with an automatic vortex).

Centrifuge the conical glass for 2 min at 3 000 rpm.

Transfer the upper layer in a conical glass of 15 ml.

Evaporate acetonitrile at 40 °C to reach 500 µl ± 50 µl.

Shake with vortex for 15 s (or 2 min with an automatic vortex).

Centrifuge the conical glass for 2 min at 3 000 rpm if there is a fat droplet on the bottom of the tube.

Transfer the solvent layer avoiding the fat droplet in an injection vial and inject into GC-MS.

### 5.6.1.3 Clean-up for heavy phthalates with epoxidation (optional)

Sometimes, we can observe interferences (e.g. squalene in olive oil) that prevents the identification of the heavy phthalates with certainty. In this case, it is possible to clean up the extract obtained in [5.6.1.1](#) and [5.6.1.2](#) with an epoxidation step. During this step, the light phthalates may be lost because of the two complete evaporations. This preparation is therefore only valid for the quantification of DINP and DIDP.

Evaporate completely the extract obtained in [5.6.1.1](#) or [5.6.1.2](#).

Add 600 µl of n-hexane and vortex 15 s.

Add 500 µl of CPBA solution ([5.2.9](#)) and shake for 15 min at room temperature with an automatic vortex.

Add immediately to stop the reaction 3 ml of sodium carbonate solution ([5.2.10](#)), shake for 2 min.

Centrifuge at 2 500 rpm for 2 min.

Recover the n-hexane phase in a conical evaporation tube.

Remove n-hexane under a nitrogen flow at 40 °C.

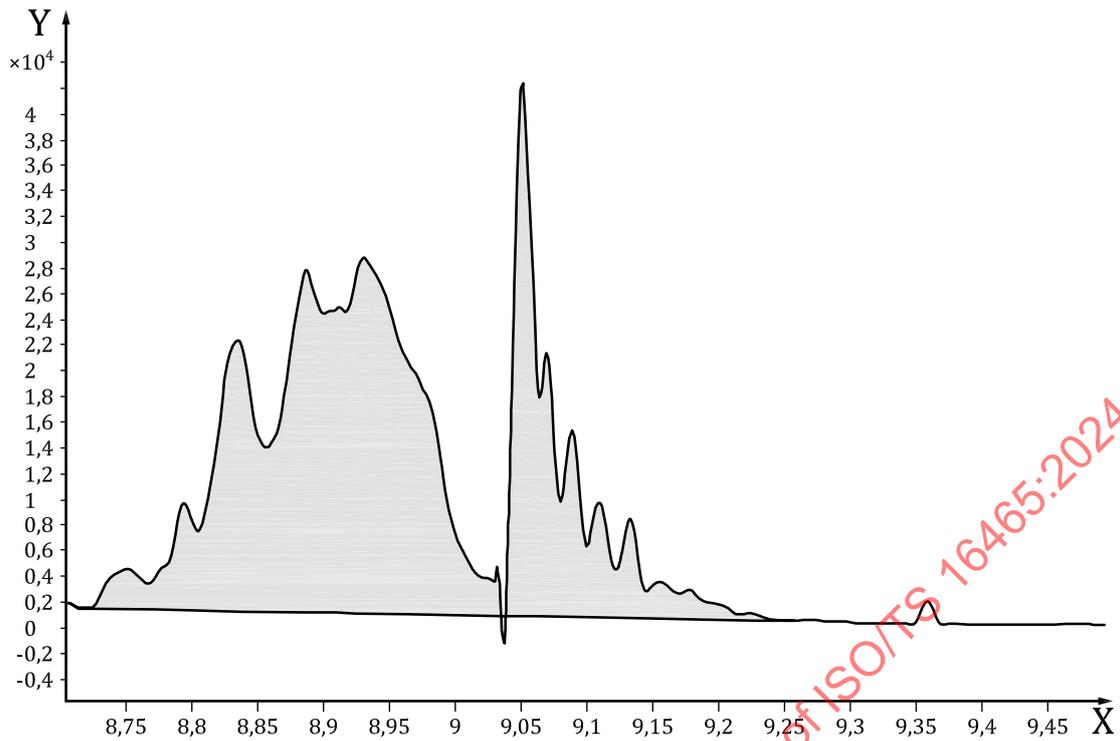
Add 300 µl of acetonitrile and vortex.

Transfer 200 µl of supernatant acetonitrile to an injection vial, avoiding any residues in the lower phase.

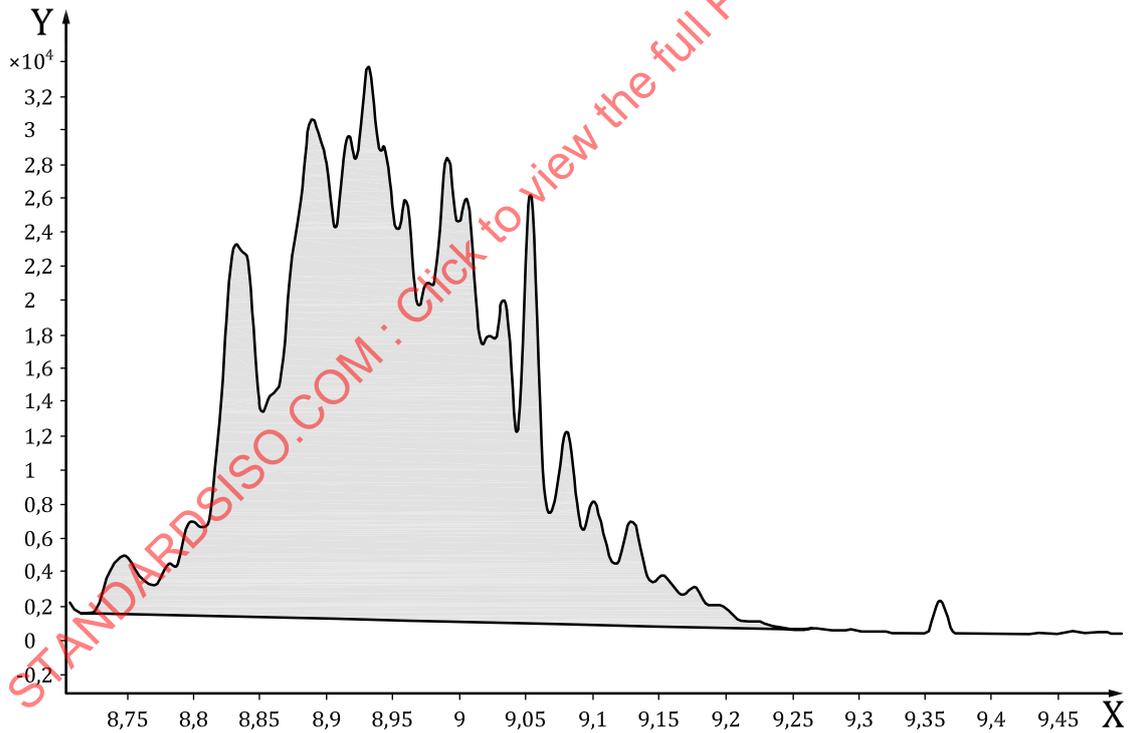
Inject in GC-MS.

NOTE Depending on the apparatus used, the concentration of the extract can be modified and adapted according to the system.

The impact of epoxidation on the retention time of DINP can be seen in the chromatograms shown in [Figure 1](#) where a) is before epoxidation and b) after epoxidation.



a) DINP chromatogram before epoxidation



b) DINP chromatogram after epoxidation

**Key**

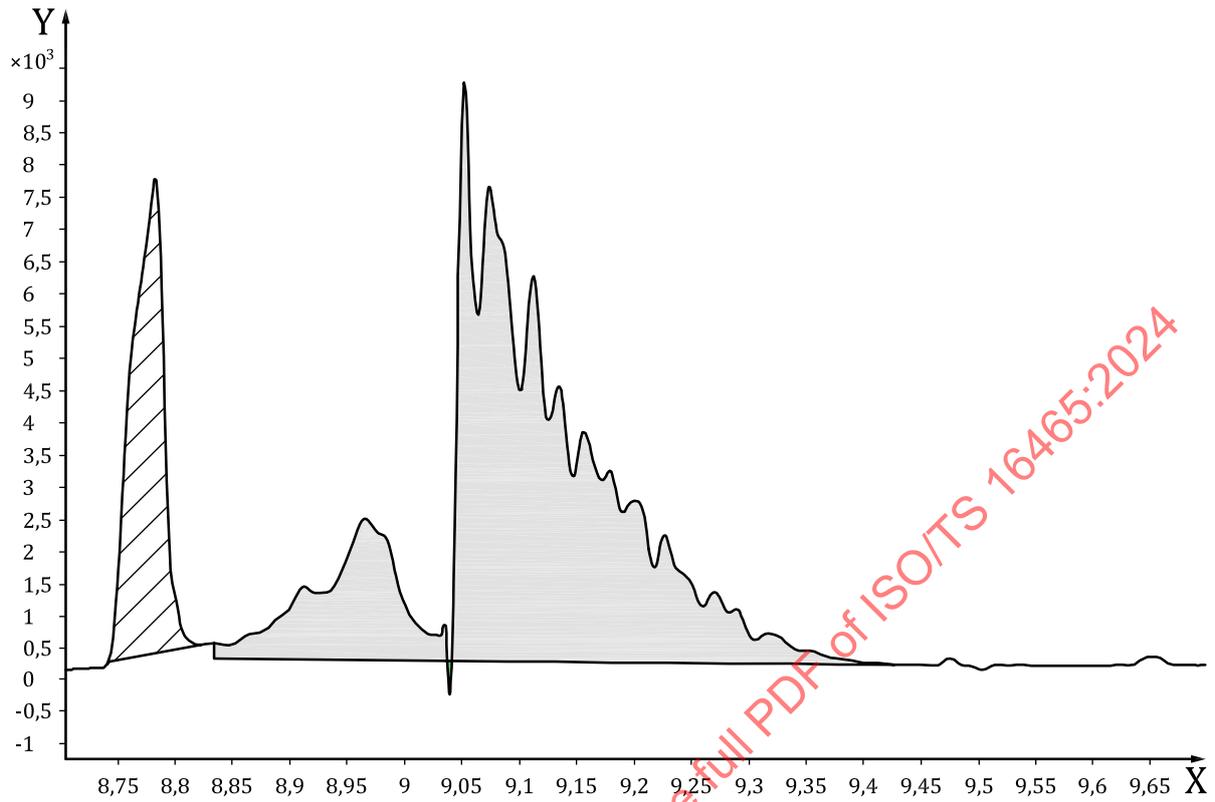
X acquisition time (min)

Y counts for ion 293,0

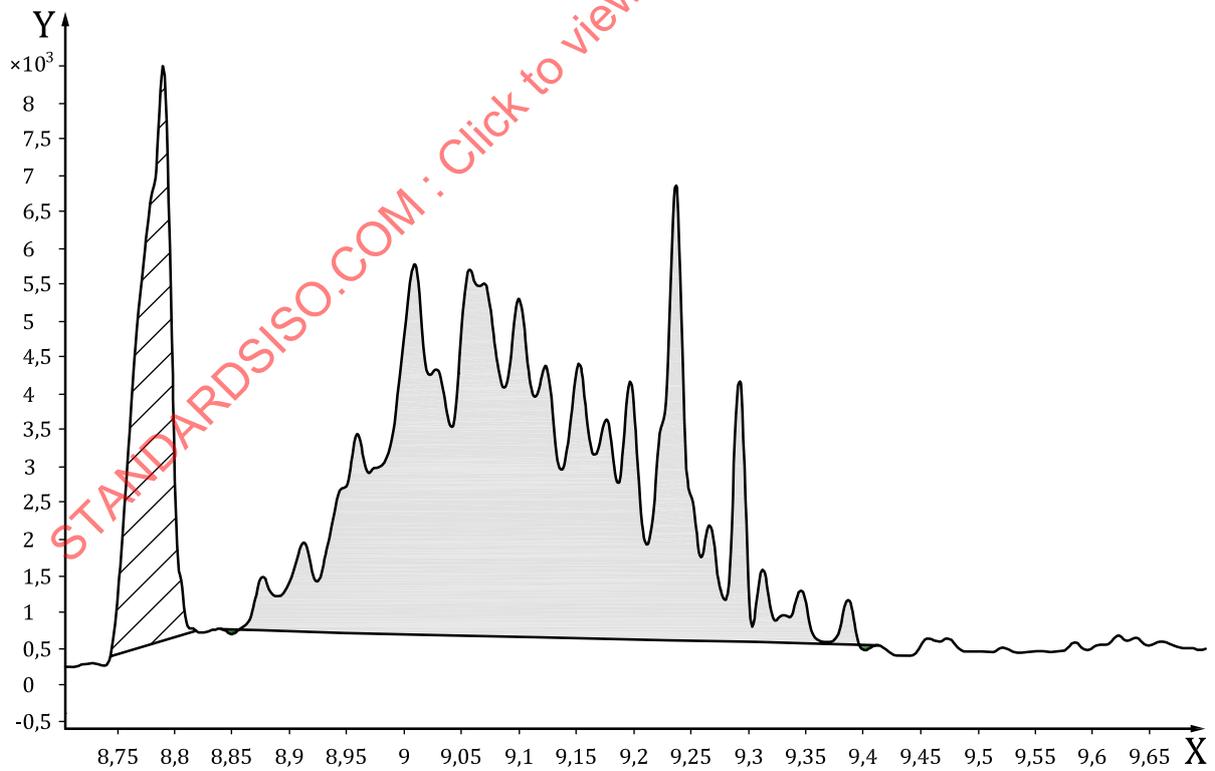
NOTE The signal from 8,75 min to 9,25 min corresponds to DINP.

**Figure 1 — Impact of epoxidation on the retention time of DINP in olive oil**

The impact of epoxidation on the retention time of DIDP can be seen in the chromatograms shown in [Figure 2](#) where a) is before epoxidation and b) after epoxidation.



a) DIDP chromatogram before epoxidation



b) DIDP chromatogram after epoxidation

**Key**

X acquisition time (min)

Y counts for ion 307,0

NOTE The signal from 8,85 min to 9,40 min corresponds to DIDP.

**Figure 2 — Impact of epoxidation on the retention time of DIDP in olive oil****5.6.2 Gas chromatography****5.6.2.1 Gas chromatography setup**

Install the column (5.3.9) in the gas chromatograph (5.3.7) and check the working conditions by injecting the solvent, acetonitrile. The baseline should be straight with a small positive drift. If the drift is high, proceed to condition the column, for a negative drift check the connections of the column.

If the column is used for the first time, it is necessary to condition the column by heating it in the column oven using a temperature gradient up to 370 °C (depending on the oven temperature chosen for the analysis) in 4 h, maintain the temperature for 2 h.

**5.6.2.2 Working conditions for gas chromatography analysis**

The working conditions as shown in Table 8 and Table 9 have proved to be adapted to the analysis.

These conditions can be adjusted in accordance with the characteristics of the gas chromatograph apparatus and the column.

**Table 8 — GC conditions**

Column	DB5 HT (15 m – 0,25 mm – 0,10 µm film thickness or 30 m – 0,25 mm – 0,25 µm film thickness)
Injector	Liner, tapered, deactivated liner with glass wool
Injection port temperature	350 °C
Injection temperature program	Splitless in pressure pulse (12 psi) for 1 min 1 min after the injection, split at 150 ml/min 3 min after the injection, split at 20 ml/min
Injection volume	1,0 µl
Oven temperature	50 °C for 1 min, ramp at 15 °C/min to 100 °C, ramp at 40 °C/min to 290 °C, ramp at 60 °C/min to 350 °C, hold for 2 min
Carrier gas	Helium constant flow rate, 1,2 ml/min
Syringe solvent clean-up	Acetonitrile

Table 9 — MS conditions

Ion source temperature	300 °C				
Quadrupole temperature	150 °C				
Transfer line temperature	325 °C				
Parameters for SIM mode	Phthalates	Quantifier ion	Qualifier ion	Qualifier ion	Qualifier ion
	DMP	163	194	133	135
	DMP d4	167	137	198	139
	DEP	149	177	222	
	DEP d4	153	181	226	
	DIBP	149	167	223	205
	DIBP d4	153	171	227	209
	DBP	149	205	223	
	DBP d4	153	209	227	
	BBP	149	132	206	238
	BBP d4	153	136	210	242
	DEHP	149	167	279	132
	DEHP d4	153	171	283	136
	DINP	293			
	DINP d4	297	153		
DIDP	307				
DIDP d4	311	153			

Check that the volume of acetonitrile when injected at 350 °C is not greater than the volume of the liner.

NOTE The MS conditions and the parameters for SIM mode can be adjusted according to the characteristics of the MS.

## 5.7 Quantitative determination

### 5.7.1 Calibration curve

The calibration curve of each phthalate is of type  $y = a * x$

$$\frac{A_{ph}}{A_{dph}} = a * \frac{C_{cal-ph}}{C_{cal-dph}}$$

The calibration curve is determined as follows:

$$a = \frac{A_{ph} * C_{cal-dph}}{A_{dph} * C_{cal-ph}}$$

where

$a$  is the slope;

$A_{ph}$  is the peak area of the phthalate in the calibration solution;

$A_{dph}$  is the peak area of the deuterated phthalate internal standard in the calibration solution;

$C_{cal-dph}$  is the concentration of deuterated phthalate internal standard in the calibration solution, in micrograms per millilitres ([Table 7](#));

$C_{\text{cal-ph}}$  is the concentration of phthalate in the calibration solution, in micrograms per millilitres (Table 7).

### 5.7.2 Quantitative determination of phthalate

$$C_{\text{ph}} = \left( \frac{A_{\text{ph}} * V_{\text{dph}} * C_{\text{dph}}}{A_{\text{dph}} * M * a} \right) - C_{\text{blank}}$$

where

$a$  is the slope;

$A_{\text{ph}}$  is the peak area of phthalate;

$A_{\text{dph}}$  is the peak area of the internal standard;

$C_{\text{dph}}$  is the concentration of the deuterated phthalate internal standard solution, in micrograms per millilitres;

$V_{\text{dph}}$  is the volume of the deuterated phthalate internal standard solution, in millilitres;

$C_{\text{ph}}$  is the concentration of phthalate, in milligrams per kilogram;

$C_{\text{blank}}$  is the concentration of phthalate in the blank, in milligrams per kilogram (see 5.6.1.1);

$M$  is the mass of the test portion, in grams.

If the volumes of the internal standards solution indicated in this method are respected, and if the same solution of deuterated phthalates internal standards is used both for the calibration solutions and for the sample preparation, the following simplified calculation formulae can be used for the determination of the calibration curve and the quantification of phthalates:

#### 5.7.2.1 Calibration curve

The calibration curve of each phthalate is of type  $y = a * x$

$$\frac{A_{\text{ph}}}{A_{\text{dph}}} = a * m_{\text{cal-ph}} * \frac{40}{80}$$

The calibration curve is determined as follows:

$$a = \frac{A_{\text{ph}} * 2}{A_{\text{dph}} * m_{\text{cal-ph}}}$$

where

$a$  is the slope from the calibration, using the same deuterated phthalate internal standard solution for the samples and for the calibration;

$A_{\text{ph}}$  is the peak area of the phthalate in the calibration solution;

$A_{\text{dph}}$  is the peak area of the internal standard deuterated phthalate in the calibration solution;

$m_{\text{cal-ph}}$  is the mass of phthalate in the solution calibration, in micrograms.

NOTE The coefficient of 2 is added in the formula to consider the ratio of the amount of internal standard between the calibration solutions and the samples (80 µl of the internal standard solution for the calibration and 40 µl of the internal standard solution for the samples).

### 5.7.2.2 Quantitative determination of phthalate

$$C_{\text{ph}} = \left( \frac{A_{\text{ph}}}{A_{\text{dph}} * M * a} \right) - C_{\text{blank}}$$

where

- $C_{\text{ph}}$  is the concentration of phthalate, in milligrams per kilogram;
- $a$  is the slope from the calibration, using the same deuterated phthalate internal standard solution for the samples and for the calibration;
- $A_{\text{ph}}$  is the peak area of the phthalate;
- $A_{\text{dph}}$  is the peak area of the deuterated phthalate internal standard;
- $M$  is the mass of the test portion, in grams;
- $C_{\text{blank}}$  is the concentration of the phthalate in the blank, in milligrams per kilogram (see [5.6.1.1](#)).

## 5.8 Precision of the method

### 5.8.1 Interlaboratory test

Details of the test and the precision of the method are summarized in [Annex B](#). The values derived from this interlaboratory test will possibly not be applicable to concentration ranges and matrices other than those given.

### 5.8.2 Repeatability

The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, shall in not more than 5 % of cases exceed the value of  $r$  given in [Tables B.1](#) to [B.8](#).

### 5.8.3 Reproducibility

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories by different operators using different equipment, shall in not more than 5 % of cases exceed the value of  $R$  given in [Tables B.1](#) to [B.8](#).

## 5.9 Test report

The test report shall contain at least the following information:

- all information necessary for the complete identification of the sample;
- the sampling method used, if known;
- the test method used, with reference to this document, i.e. ISO/TS 16465:2024;
- all operating details not specified in this document, or regarded as optional, together with details of any incidents occurred when performing the method, which can have influenced the test result(s);
- the test result obtained;
- if the repeatability has been checked, the final quoted result obtained.

## Annex A (informative)

### Results of an interlaboratory test — Part A - Determination of DEHP

An international collaborative test involving eight laboratories in seven countries was carried out on five oil samples in 2019. The test was organized by ITERG and LESIEUR and the results obtained were subjected to statistical analysis according to ISO 5725-1<sup>[2]</sup> and ISO 5725-2<sup>[3]</sup> to give the precision data shown in [Table A.1](#).

The five samples were:

- Sample 1: Sunflower oil, refined
- Sample 2: Sunflower oil, refined, spiked
- Sample 3: Sunflower oil, refined, spiked
- Sample 4: Nut oil
- Sample 5: Olive oil, virgin

**Table A.1 — Summary of statistical results of DEHP obtained according to Part A**

DEHP content - Part A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Number of participating laboratories, $n_p$	7	8	8	8	8
Number of laboratories retained after eliminating outliers, $n_p$	7	8	8	8	7
Number of test results in all labs ( $n$ )	14	16	16	16	14
Mean ( $m$ ) mg/kg	-	1,41	4,43	2,63	2,24
Repeatability standard deviation, $s_r$ , mg/kg	-	0,11	0,21	0,10	0,06
Repeatability coefficient of variation, CV, $r$ , %	-	7,4	4,8	3,7	2,5
Repeatability limit, $r$ , ( $= 2,8 \times s_r$ ), mg/kg	-	0,29	0,59	0,27	0,16
Reproducibility standard deviation, $s_R$ , mg/kg	-	0,34	1,01	0,62	0,61
Reproducibility coefficient of variation, CV, $R$ , %	-	24,2	22,7	23,7	27,1
Reproducibility limit, $R$ , ( $= 2,8 \times s_R$ ), mg/kg	-	0,96	2,81	1,75	1,70
- indicates value lower than 0,1 mg/kg					