BRITISH STANDARD

BS EN
1528-2 : 1997

Fatty food — Determination of pesticides and polychlorinated biphenyls (PCBs)

Part 2. Extraction of fat, pesticides and PCBs, and determination of fat content

The European Standard EN 1528-2 : 1996 has the status of a British Standard

ICS 67.040

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Committees responsible for this British Standard

The preparation of this British Standard was entrusted to Technical Panel AW/-/3, Food analysis — Horizontal methods, upon which the following bodies were represented:

Association of Public Analysts
Department of Trade and Industry (Laboratory of the Government Chemist)
Food and Drink Federation
Institute of Food Science and Technology
Ministry of Agriculture Fisheries and Food
Royal Society of Chemistry

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National foreword

This British Standard has been prepared by Technical Committee AW/-/3 and is the English language version of EN 1528-2 : 1996 *Fatty food — Determination of pesticides and polychlorinated biphenyls (PCBs) Part 2 : Extraction of fat, pesticides and PCBs, and determination of fat content* published by the European Committee for Standardization (CEN). EN 1528-2 was produced as a result of international discussions in which the United Kingdom took an active part.

Cross-references

Publication referred to	Corresponding British Standard
EN 1528-1 : 1996	BS EN 1528-1 : 1997 *Fatty food — Determination of pesticides and polychlorinated biphenyls (PCBs) Part 1 : General*
EN 1528-3 : 1996	BS EN 1528-3 : 1997 *Fatty food — Determination of pesticides and polychlorinated biphenyls (PCBs) Part 3 : Clean-up methods*
EN 1528-4 : 1996	BS EN 1528-4 : 1997 *Fatty food — Determination of pesticides and polychlorinated biphenyls (PCBs) Part 4 : Determination, confirmatory tests, miscellaneous*

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Summary of pages

This document comprises a front cover, an inside front cover, pages i and ii, the EN title page, pages 2 to 8, an inside back cover and a back cover.

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EUROPEAN STANDARD
NORME EUROPÉENNE
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Descriptors: Food products, edible fats, chemical analysis, determination of content, pesticides, polychlorobiphenyl, fats, extraction

English version

Fatty food — Determination of pesticides and polychlorinated biphenyls (PCBs) — Part 2 : Extraction of fat, pesticides and PCBs, and determination of fat content

Aliments gras — Dosage des pesticides et des polychlorobiphényles (PCB) — Partie 2 : Extraction de la matière grasse, des pesticides et des PCB, et détermination de la teneur en matière grasse

Fettreiche Lebensmittel — Bestimmung von Pestiziden und polychlorierten Biphenylen (PCB) — Teil 2 : Extraktion des Fettes, der Pestizide und PCB und Bestimmung des Fettgehaltes

This European Standard was approved by CEN on 1996-10-27. CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration.

Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the Central Secretariat or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the Central Secretariat has the same status as the official versions.

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CEN

European Committee for Standardization
Comité Européen de Normalisation
Europäisches Komitee für Normung

Central Secretariat: rue de Stassart 36, B-1050 Brussels

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Foreword

This European Standard has been prepared by Technical Committee CEN/TC 275, Food analysis, horizontal methods', the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 1997, and conflicting national standards shall be withdrawn at the latest by May 1997.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

This European Standard consists of the following Parts.

– Part 1 *General* presents the scope of the standard and describes general considerations with regard to reagents, apparatus, gas chromatography etc., applying to each of the analytical methods selected.

– Part 2 *Extraction of fat, pesticides and PCBs, and determination of fat content* presents a range of analytical procedures for extracting the fat portion containing the pesticide and PCB residues from different groups of fat-containing foodstuffs.

– Part 3 *Clean-up methods* presents the details of methods A to H for the clean-up of fats and oils or the isolated fat portion, respectively, using techniques such as liquid–liquid partition, adsorption or gel permeation column chromatography.

– Part 4 *Determination, confirmatory tests, miscellaneous* gives guidance on some recommended techniques for the determination of pesticides and PCBs in fatty foodstuffs and on confirmatory tests, and lists a clean-up procedure for the removal of the bulk of lipids when analysing large quantities of fat.

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Introduction

This European Standard comprises a range of multi-residue methods of equal status: no single method can be identified as the prime method because, in this field, methods are continuously developing. The methods selected for inclusion in this standard have been validated and are widely used throughout Europe. Any variation in the methods used should be shown to give comparable results.

The residues to be analysed in this European Standard are associated with the fat portion of the samples.

In many cases, the residues are expressed in milligrams of pesticide per kilogram of fat (see clause **11** of EN 1528-1 : 1996). In such cases, it is not necessary to determine the fat content of the product, but to measure the residues in a known mass of extracted fat. With all other products, residue levels are reported on a whole product basis and therefore it is necessary to determine the percentage of fat in the product.

1 Scope

This Part of EN 1528 specifies a range of analytical procedures for extracting the fat portion containing the pesticide and polychlorinated biphenyl (PCB) residues from different groups of fat-containing foodstuffs.

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies.

EN 1528-1:1996	*Fatty food — Determination of pesticides and polychlorinated biphenyls (PCBs) — Part 1 : General*
EN 1528-3:1996	*Fatty food — Determination of pesticides and polychlorinated biphenyls (PCBs) — Part 3 : Clean-up methods*
EN 1528-4:1996	*Fatty food — Determination of pesticides and polychlorinated biphenyls (PCBs) — Part 4 : Determination, confirmatory tests, miscellaneous*

3 Principle

Extraction of the residues from the sample matrix by the use of appropriate solvents, so as to obtain the maximum efficiency of extraction of the residue and minimum co-extraction of any substances which can give rise to interferences in the determination. Removal of the solvents by evaporation and, optionally, determination of the fat content by weighing out the mass of the remainder.

4 Reagents and materials

All reagents and materials used shall be suitable for the analysis of residues of pesticides and PCBs and shall be in accordance with **4** in EN 1528-1 : 1996. If purification is necessary, the procedures given in annex A are appropriate.

4.1 *Acetone.*

4.2 *Acetonitrile.*

4.3 *Diethyl ether*, peroxide free.

4.4 *Dichloromethane.*

4.5 *Extraction mixture*, acetonitrile (**4.2**) + dichloromethane (**4.4**) 75 : 25 (*V/V*).

4.6 *Light petroleum*, having a boiling range from 40 °C to 60 °C.

4.7 *Methanol* or *ethanol.*

4.8 *n-hexane.*

4.9 *Enzyme suspension*, phospholipase C suspension, 800 IU/ml[1)] , in ammonium sulfate solution (3,2 mol/l). Store at 1 °C to 4 °C (do not freeze).

4.10 *Glycine buffer solution*, 0,2 mol/l of glycine containing 0,1 g/l of zinc sulfate.

4.11 *Sodium sulfate solution*, 2 g/100 ml.

4.12 *Sodium chloride solution*, saturated.

4.13 *Sodium oxalate*, or potassium oxalate.

4.14 *Sodium sulfate*, granular, anhydrous. Before use, heat at 500 °C or 550 °C for at least 4 h and then allow to cool in a desiccator.

4.15 *Filter aid*, for example Celite®545[2)]. Before use, heat at 400 °C for at least 4 h, allow to cool in a desiccator and store in an airtight bottle.

4.16 *Sea sand*, acid washed. Before use, heat at 400 °C for at least 4 h and allow to cool in a desiccator.

[1)] IU (often called the International Unit or standard unit) is defined as the amount of enzyme which will catalyse the transformation of 1 μmol substrate per minute under standard conditions.

[2)] Celite ®545 is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named.

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5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 *Analytical balance*, suitable for weighings in the range 0,01 g up to 1000 g.

5.2 *Analytical balance*, suitable for weighings in the range 0,1 mg up to 1 g.

5.3 *Centrifuge*, explosion proof, provided with glass tubes of capacity 200 ml up to 500 ml, in which the tubes can be spun at a rotational frequency of 1000 min^{-1} to 2000 min^{-1}.

5.4 *Refrigerated centrifuge*, explosion proof, cooled to −15 °C, provided with centrifuge tubes of capacity 50 ml up to 300 ml, in which the tubes can be spun at a rotational frequency of 1000 min^{-1} up to 3000 min^{-1}.

5.5 *Device*, for mincing foodstuffs of animal origin (food chopper).

5.6 *High speed blender*, fitted with a leak proof glass jar and an explosion proof motor, or homogenizer.

5.7 *Vortex*, or test tube mixing apparatus.

5.8 *Drying oven*, capable of being controlled between ambient temperature and 250 °C.

5.9 *Muffle furnace*, capable of being controlled between 400 °C and 600 °C.

5.10 *Microwave oven* (optional).

5.11 *Refrigerator*, spark proof, for storage of sample extracts.

5.12 *Rotary evaporator*, with evaporation flasks of capacity 500 ml and a water bath capable of being controlled between 20 °C and 50 °C.

5.13 *Soxhlet extraction apparatus*, comprising:

a) round-bottomed flask, of capacity 500 ml;

b) extraction chamber, of capacity approximately 200 ml;

c) reflux condenser;

d) heat source (for example a heating mantle).

5.14 *Sand or water bath*, capable of being controlled between ambient temperature and 100 °C.

5.15 *Borosilicate bottles*, 250 ml, glass.

5.16 *Extraction tube*, comprising a glass tube of internal diameter 12 mm and of length 300 mm, having a capillary exit below, and at the upper end a section of length 100 mm of internal diameter 50 mm.

5.17 *Extraction thimbles* (optional). The use of extraction thimbles can often result in the presence of impurities in the sample extracts (interference peaks in the gas chromatogram). They should therefore be pre-extracted with solvent of the highest purity and stored in an all-glass container.

5.18 *Separating funnels*, of capacity 500 ml and 1000 ml.

5.19 *Sintered glass funnels*, of capacity 80 ml, disk diameter 4 cm.

5.20 *Volumetric flasks*, of suitable capacity, e.g. 5 ml, 10 ml.

5.21 *Cotton wool and glass wool*, chemically pure. Before use, extract with *n*-hexane/acetone and store in a well-stoppered flask.

5.22 *Filter paper*, round, of diameter approximately 30 cm, sufficiently solvent washed.

5.23 *Mortar and pestle.*

6 Procedures

6.1 Milk

6.1.1 ***AOAC extraction*** [1], [2]

To 100 ml of fluid milk in a 500 ml centrifuge tube (**5.3**), add 100 ml of ethanol or methanol (**4.7**) and 1 g of sodium oxalate or potassium oxalate (**4.13**) and mix. Add 50 ml of diethyl ether (**4.3**) and shake vigorously for 1 min. Add 50 ml of light petroleum (**4.6**) and shake vigorously for 1 min. Centrifuge for about 5 min at about 1500 min^{-1}. Blow off the solvent layer into a 1 l separating funnel containing 500 ml to 600 ml of water and 30 ml of saturated sodium chloride solution (**4.12**). Re-extract the residue twice, shaking vigorously with 50 ml portions of diethyl ether/light petroleum 1 : 1 (*V/V*). Centrifuge and blow off the solvent layer into the separating funnel after each extraction. Mix the combined extracts and water cautiously. Drain and discard the water layer. Rewash the solvent layer twice with 100 ml portions of water, discarding the water each time. (If emulsions form, add about 5 ml of saturated sodium chloride solution to the solvent layer or include with the water wash.) Pass the solvent solution through a column of anhydrous sodium sulfate (**4.14**), 50 mm × 25 mm outer diameter, and collect the eluate in a 400 ml beaker. Wash the column with small portions of light petroleum and evaporate the solvent from the combined extracts at steam bath temperature under an air current to obtain the fat.

6.1.2 ***Column extraction*** [3]

Thoroughly mix a sufficient quantity (usually 10 ml) of the liquid milk sample in the mortar with sufficient sea sand (**4.16**) and sodium sulfate (**4.14**) (1 + 1 mixture, usually 100 g) to yield a dry friable product.

Transfer the mixture into the extraction tube (**5.16**) previously plugged with glass wool and a 2 cm layer of sodium sulfate. Elute the column with a 2 : 1 (*V/V*) mixture of *n*-hexane and acetone. The quantity of solvent depends on the mass and nature of the sample. Collect the eluate and evaporate it in a rotary evaporator at about 50 °C under reduced pressure. Remove solvent residues by using a gentle stream of nitrogen.

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When extracting dried milk, reconstitute thoroughly by homogenizing 10 g of milk powder with 90 ml of distilled water at 40 °C to 50 °C for 15 min and proceed as described before.

6.1.3 ***Partitioning extraction*** [4]

To 100 g of milk in a 1000 ml beaker, add 500 ml of a 2 : 1 (*V/V*) mixture of *n*-hexane and acetone and homogenize for 4 min. Allow the phases to separate. Decant the upper organic layer into a separating funnel containing 500 ml of sodium sulfate solution (**4.11**). Add a further 50 ml of the *n*-hexane–acetone mixture (2 : 1) into the beaker, and decant into the separating funnel to ensure quantitative transfer of the organic phase. Shake the separating funnel for 30 s. Allow the phases to separate and discard the lower aqueous phase.

Shake the organic layer with a further 500 ml of the sodium sulfate solution. Drain the lower layer as before, but leave approximately 2 ml remaining in the funnel. Rotate the separating funnel about its axis to remove all the water from the sides of the vessel. When all the water has settled, run off the remaining aqueous phase and discard it. Put approximately 20 g of sodium sulfate (**4.14**) in a sintered glass funnel (**5.19**) and run the organic phase through the sodium sulfate into a round-bottomed flask. Evaporate the solution in a rotary evaporator at about 50 °C under reduced pressure. Remove solvent residues by using a gentle stream of nitrogen.

6.1.4 ***Cold centrifugation extraction*** [5]

6.1.4.1 *Extraction of fat*

6.1.4.1.1 *Dry milk and heat treated milk*

Take 30 ml of milk or suspend 20 g of milk powder in 30 ml water and allow to stand for 4 h. Add 50 ml of acetone (**4.1**) and homogenize for 2 min. Centrifuge for 5 min at 1500 min^{-1}. Transfer the upper phase into a funnel (**5.18**) and repeat the extraction with 35 ml acetone. Centrifuge again, combine the upper phases, add 70 ml of *n*-hexane (**4.8**) and mix.

Rotary evaporate the *n*-hexane phase in a tared flask at 35 °C to approximately 1 ml and remove solvent residues using a gentle stream of nitrogen.

6.1.4.1.2 *Raw milk*

Centrifuge 30 ml of raw milk for 10 min at 2500 min^{-1} and transfer the cream into a beaker containing 6 g of sodium sulfate (**4.14**). Add 30 ml of *n*-hexane (**4.8**) and homogenize carefully for 10 min. Filter the hexane phase through a glass wool plug covered with sodium sulfate. Rotary evaporate the *n*-hexane layer at 35 °C to approximately 1 ml and remove solvent residues by using a gentle stream of nitrogen.

6.1.4.2 *Extraction of pesticides and PCBs*

Weigh two portions of fat (maximum 0,5 g) in centrifuge tubes, add 3 ml of extraction mixture (**4.5**), and mix with vortex. Centrifuge at 3000 min^{-1} for 20 min at −15 °C. Separate the phases by decanting the upper phase into a test tube. Warm the remaining fat at the bottom of the centrifuge tube gently until melting by using a microwave oven or a water bath and repeat the extraction using 3 ml of extraction mixture. Collect the organic phases and remove solvent residues at 35 °C using a gentle stream of nitrogen.

NOTE. Recent experiences have shown that centrifuging at −10 °C is more appropriate.

6.2 Butter

6.2.1 ***AOAC extraction*** [1]

Warm the sample in a beaker at 50 °C to 60 °C until the fat clearly separates. Decant the melted fat through a dry filter paper or a small glass wool plug.

6.2.2 ***Partitioning extraction*** [4]

Homogenize 20 g of the sample with 250 ml of a 3 : 1 (*V/V*) mixture of *n*-hexane and acetone. Shake the organic phase in a separating funnel with 250 ml of the sodium sulfate solution (**4.11**). Transfer and evaporate the organic phase in a rotary evaporator at about 50 °C under reduced pressure. Remove solvent residues by using a gentle stream of nitrogen.

6.2.3 ***Cold centrifugation extraction*** [5]

Warm the sample in a beaker at approximately 40 °C and centrifuge at 1000 min^{-1}. Decant the melted fat through a dry filter paper.

Proceed as described in **6.1.4.2.**

6.3 Cheese, milk products

6.3.1 ***Soxhlet extraction*** [2], [3]

Heat a 500 ml round-bottomed flask containing five glass beads to 105 °C for 30 min in the drying oven, allow to cool in a desiccator and weigh. Repeat until constant mass is obtained, i.e. until two consecutive weighings differ by no more than 0,01 g.

Grate cheese well and weigh milk products directly on a watchglass.

Place the sample (usually 10 g) in a mortar and grind well with either Celite®545 (**4.15**) or a 1 : 1 mixture (usually 40 g) of sea sand (**4.16**) and sodium sulfate (**4.14**) to yield a dry friable powder. The amount of sodium sulfate/sand required depends on the quantity and water content of the foodstuff. Transfer the powder quantitatively into a fluted filter paper (**5.22**).

Wipe the mortar, pestle and watchglass with a wad of cotton wool (**5.21**) moistened with light petroleum (**4.6**) (see note). Put the cotton wool also in the filter paper and insert the latter (closed up) into the chamber of a Soxhlet extraction apparatus.

Fill the weighed 500 ml flask with 250 ml of light petroleum (see note) and extract the sample for 6 h under reflux. Remove the solvent in a rotary evaporator at about 50 °C under reduced pressure. Remove solvent residues by using a gentle stream of nitrogen.

NOTE. If, in addition to organochlorine pesticides and PCB congeners, lipid soluble organophosphorus pesticides are to be analysed, then diethyl ether should be used instead of light petroleum for the Soxhlet extraction.

SEE SAFETY ASPECTS IN **4.3** OF EN 1528-1 : 1996.

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6.3.2 ***AOAC extraction*** [1]

Place 25 g to 100 g of diced sample (to provide 3 g of fat), 2 g of sodium oxalate or potassium oxalate (**4.13**), and 100 ml of ethanol or methanol (**4.7**) in a high speed blender and blend for 2 min to 3 min. (If experience with the product indicates emulsions will not be broken by centrifuging, add 1 ml of water per 2 g of the sample before blending.) Pour into a 500 ml centrifuge bottle, add 50 ml of diethyl ether (**4.3**) and shake vigorously for 1 min. Then add 50 ml of light petroleum (**4.6**) and shake vigorously for 1 min (or divide between two 250 ml bottles and extract each by shaking vigorously for 1 min with 25 ml of each solvent in turn). Centrifuge for about 5 min at about 1500 min^{-1} and proceed as described in **6.1.1**.

6.3.3 ***Extraction under reflux*** [6]

Thoroughly mix 10 g to 30 g (according to fat content) of the finely grated or otherwise comminuted cheese sample with a two or three times larger amount by mass of sodium sulfate (**4.14**). Transfer the mixture to a 250 ml Erlenmeyer flask, and extract successively with four 100 ml portions of a 2 : 1 (*V/V*) mixture of dichloromethane (**4.4**) and acetone (**4.1**) by heating for 15 min under reflux. Evaporate the combined extracts. Dissolve the residue remaining after evaporation in 20 ml of light petroleum (**4.6**), decant the solution carefully through a glass wool plug into a 50 ml round-bottomed flask, and evaporate the solution at about 50 °C under reduced pressure. Remove solvent residues by using a gentle stream of nitrogen.

6.3.4 ***Column extraction*** [3]

Proceed as described in **6.1.2.**

6.3.5 ***Cold centrifugation extraction*** [5]

To 10 g of cheese add 10 g of sodium sulfate (**4.14**) and 50 ml of *n*-hexane (**4.8**). Homogenize and centrifuge for 5 min at 1500 min^{-1}. Decant the supernatant solution and repeat the extraction with 50 ml of *n*-hexane. Combine both extracts, rotary evaporate the *n*-hexane layer at 35 °C to approximately 1 ml and remove solvent residues using a gentle stream of nitrogen.

Proceed as described in **6.1.4.2** .

6.4 Meat, meat products, fish, fish products

6.4.1 ***Column extraction*** [3]

Proceed as described in **6.1.2**.

6.4.2 ***Soxhlet extraction*** [2], [3]

Proceed as described in **6.3.1**.

6.4.3 ***Extraction under reflux*** [6]

Triturate 25 g of the coarsely comminuted sample with 100 g sodium sulfate (**4.14**) in a mortar and transfer it into a round-bottomed flask with a ground joint. Extract the mixture successively with four 100 ml volumes of boiling light petroleum (**4.6**) for 10 min under reflux. Evaporate the combined extracts in a rotary evaporator at about 50 °C under reduced pressure. Remove solvent residues by using a gentle stream of nitrogen.

6.4.4 ***Partitioning extraction for meat products*** [4]

Chop and mince the sample in a food chopper. Transfer 30 g of the sample into a 500 ml beaker and add enough sodium sulfate (**4.14**) to give a friable mixture. Add 300 ml of a 2 : 1 (*V/V*) mixture of *n*-hexane (**4.8**) and acetone (**4.1**), transfer to a blender cup and blend the mixture for 3 min. Decant the extract through a funnel containing a plug of cotton wool into a 1000 ml separating funnel (**5.18**). Re-blend the sample residue with a further 150 ml portion of the 2 : 1 *n*-hexane–acetone mixture and decant through the cotton wool plug into the separating funnel. Add 250 ml of sodium sulfate solution (**4.11**) and shake the funnel for 30 s. Allow the layers to separate and discard the lower, aqueous layer. Wash the upper layer in the separating funnel with another 250 ml portion of sodium sulfate solution. Pass the *n*-hexane layer through a sintered glass funnel (**5.19**) containing approximately 20 g of sodium sulfate into a round-bottomed flask, and evaporate the solution in a rotary evaporator at about 50 °C under reduced pressure. Remove solvent residues by using a gentle stream of nitrogen.

6.4.5 ***Partitioning extraction for fish and crabs*** [4]

Macerate the crab pancreatic tissue or fish entrails sample in a blender to mix the sample thoroughly. Weigh out approximately 25 g of crab or 100 g of fish tissue. Add 200 g of sodium sulfate (**4.14**) and mix with a stirring rod until a friable mixture is obtained. To the sample mass, add 200 ml of a 3 : 1 (*V/V*) mixture of *n*-hexane (**4.8**) and acetone (**4.1**) and heat on the water bath under reflux for 20 min with constant stirring. Pour the solvent into a separating funnel containing 500 ml of the sodium sulfate solution (**4.11**). Carry out the extraction two more times, each with a 150 ml portion of the 3 : 1 *n*-hexane–acetone mixture. Combine all the extracts in the separating funnel. Shake the funnel for 30 s, and allow the two phases to separate. Drain the lower aqueous layer and discard it. Add a further 500 ml of the sodium sulfate solution and repeat the washing procedure. Run the remaining organic layer through a sintered glass funnel (**5.19**) containing approximately 15 g of sodium sulfate into a round-bottomed flask and evaporate the solution in a rotary evaporator at about 50 °C under reduced pressure. Remove solvent residues by using a gentle stream of nitrogen.

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6.4.6 ***Cold centrifugation extraction*** [5]

6.4.6.1 *Meat and fish*

To 20 g of meat or fish, add 10 g of sodium sulfate (**4.14**) and 50 ml of *n*-hexane (**4.8**). Homogenize and centrifuge for 5 min at 1500 min^{-1}. Decant the supernatant solution and repeat the extraction with 50 ml of *n*-hexane. Combine both extracts, rotary evaporate the *n*-hexane layer at 35 °C to approximately 1 ml and remove solvent residues using a gentle stream of nitrogen.

Proceed as described in **6.1.4.2.**

6.4.6.2 *Animal fat*

Warm the sample in a beaker at approximately 50 °C. Decant the melted fat through a dry filter paper at approximately 50 °C.

Proceed as described in **6.1.4.2.**

6.5 Eggs

6.5.1 ***Column extraction*** [3]

Shell the eggs into a glass beaker. Discard the shell and homogenize the remainder. Proceed as described in **6.1.2**.

6.5.2 ***Soxhlet extraction*** [2], [3]

Shell the eggs into a glass beaker. Discard the shell and homogenize the remainder. Proceed as described in **6.3.1**.

6.5.3 ***Partitioning extraction including phospholipase C treatment*** [4]

Shell the eggs into a glass beaker. Discard the shell and homogenize the remainder. Accurately weigh a 20 g sample into a borosilicate bottle (**5.15**), add 10 ml of glycine buffer solution (**4.10**) and 50 µl of enzyme suspension (**4.9**). Incubate at (37 ± 1) °C for 2 h, agitating gently. Transfer the sample to a 600 ml beaker, add 250 ml of a 3 : 1 (*V/V*) mixture of *n*-hexane and acetone, and homogenize for 2 min. Allow the phases to separate and decant the solvent mixture through a funnel containing a small plug of cotton wool into a 500 ml separating funnel containing 250 ml of sodium sulfate solution (**4.11**). Add a further 50 ml of the 3 : 1 *n*-hexane–acetone mixture to the beaker, agitating gently, and decant into the separating funnel. Shake for 30 s. Allow the phases to separate. Discard the lower layer, rotating the flask to remove any water adhering to the sides of the flask, and run the upper layer through a sintered glass funnel (**5.19**) containing approximately 20 g of sodium sulfate. Evaporate the solution in a rotary evaporator at about 50 °C under reduced pressure. Remove solvent residues by using a gentle stream of nitrogen.

7 Further processing

Further processing shall be carried out in accordance with EN 1528-3 : 1996 and EN 1528-4 : 1996.

8 Evaluation of results

The results shall be evaluated in accordance with clauses **9** to **11** of EN 1528-1 : 1996.

9 Test report

The results of the tests shall be reported in accordance with clause **12** of EN 1528-1 : 1996.

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Annex A (informative)
Purification of some solvents and reagents

Acetone	Distilled over glass beads.
Acetonitrile	4000 ml of acetonitrile are mixed with 1 ml of orthophosphoric acid and 30 g of phosphorus pentoxide in a round-bottomed glass flask. Glass beads are added and the mixture is distilled at 81 °C to 82 °C (do not allow the temperature to exceed 82 °C).
Diethyl ether	Distilled over glass beads.
Ethanol	Distilled over glass beads.
Light petroleum	Distilled over potassium hydroxide or sodium hydroxide pellets.
Methanol	Distilled over glass beads.
n-hexane	Distilled over sodium hydroxide pellets.
Sodium sulfate	Heated at 500 °C for at least 4 h and cooled in a desiccator.

Annex B (informative)
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List of references

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