INTERNATIONAL STANDARD

ISO 3890-2

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Milk and milk products — Determination of residues of organochlorine compounds (pesticides) —

Part 2: Test methods for crude extract purification and confirmation

Lait et produits laitiers — Détermination des résidus de composés organochlorés (pesticides) —

Partie 2: Méthodes d'essai pour la purification des extraits bruts et tests de confirmation

Reference number
ISO 3890-2:2000(E)

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this part of ISO 3890 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 3890-2 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 5, *Milk and milk products*, in collaboration with the International Dairy Federation (IDF) and AOAC International, and will also be published by these organizations.

ISO 3890 consists of the following parts, under the general title *Milk and milk products — Determination of residues of organochlorine compounds (pesticides)*:

— *Part 1: General considerations and extraction methods*

— *Part 2: Test methods for crude extract purification and confirmation*

Milk and milk products — Determination of residues of organochlorine compounds (pesticides) —

Part 2:
Test methods for crude extract purification and confirmation

WARNING — The use of this part of ISO 3890 may involve hazardous materials, operations and equipment. This standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this standard to establish safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This part of ISO 3890 specifies test methods for the purification of the crude extracts obtained by the general methods given in ISO 3890-1. It also gives recommended methods for the determination of the residues of organochlorine compounds in milk and milk products, together with confirmatory tests and clean-up procedures.

2 Normative reference

The following normative document contains provisions which, through reference in this text, constitute provisions of this part of ISO 3890. For dated references, subsequent amendments to, or revisions of, this publication do not apply. However, parties to agreement based on this part of ISO 3890 are encouraged to investigate the possibility of applying the most recent edition of the normative document indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 3890-1:2000, *Milk and milk products — Determination of residues of organochlorine compounds (pesticides) — Part 1: General considerations and extraction methods.*

3 Method A: Liquid-liquid partitioning with acetonitrile and clean-up on a Florisil column (see reference [3])

3.1 Principle

The organochlorine compounds, together with the fat, are extracted from the sample by one of the procedures described in ISO 3890-1. The extract is concentrated almost to dryness, then redissolved in light petroleum and the organochlorine compounds are partitioned into acetonitrile. After mixing the acetonitrile with an excess of water, the organochlorine compounds are partitioned into light petroleum. This organic phase is purified chromatographically on a Florisil column using light petroleum/diethyl ether as eluting solvent. The eluates are concentrated then examined by GLC.

A special method is described for cheese.

3.2 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

3.2.1 Light petroleum, boiling range 40 °C to 60 °C.

Distil over potassium hydroxide or sodium hydroxide pellets.

3.2.2 Acetonitrile (CH_3CN), saturated with light petroleum.

To purify, mix 4 l of acetonitrile with 1 ml of orthophosphoric acid and 30 g of phosphorus pentoxide in a round-bottomed glass flask. Add glass beads and distil at a temperature of between 81 °C and 82 °C. Do not allow the temperature to exceed 82 °C.

Mix the purified acetonitrile with light petroleum until phase separation just occurs.

3.2.3 Diethyl ether ($C_2H_5OC_2H_5$), peroxide-free.

Distil and stabilize with 2,0 % of its volume of absolute ethanol (C_2H_5OH).

3.2.4 Eluting solvent A: mixture of diethyl ether (3.2.3) and light petroleum (3.2.1) (6:94 by volume).

Dry over 10 g to 25 g of anhydrous sodium sulfate (3.2.6).

3.2.5 Eluting solvent B: mixture of diethyl ether (3.2.3) and light petroleum (3.2.1) (15:85 by volume).

Dry over 10 g to 25 g of anhydrous sodium sulfate (3.2.6).

3.2.6 Sodium sulfate (Na_2SO_4), granular, anhydrous.

Heat at 500 °C ± 25 °C for 4 h. Cool and store in a stoppered bottle.

3.2.7 Adsorbent: Florisil (Floridin Co[1)]), 60 to 100 mesh.

Activate by heating at 650 °C ± 25 °C for 4 h and immediately pour the adsorbent into well-stoppered bottles and store in the dark. Before use, heat to 130 °C for at least 5 h.

The adsorbent should be stored either at 130 °C ± 2 °C or at room temperature in a desiccator. In the latter case it should, however, be heated to 130 °C ± 2 °C every 2 days.

Each batch of adsorbent should be checked from time to time as follows.

Pass 1 ml of a standard hexane solution containing 0,1 mg/l of lindane, heptachlor epoxide, aldrin and dieldrin, and 0,3 mg/l of endrin through the adsorption column (see ISO 3890-1:2000, 9.3). Elute and concentrate as described in 3.4.3. Determine by gas chromatography.

The adsorbent is satisfactory if lindane, heptachlor, aldrin and heptachlor epoxide are found quantitatively in the eluting solvent A (3.2.4) and dieldrin and endrin in the eluting solvent B (3.2.5).

3.2.8 Sodium chloride solution (NaCl), 2 % solution.

Heat solid sodium chloride at 500 °C ± 25 °C for 4 h before making up the solution.

3.2.9 Ethanol (C_2H_5OH), absolute.

3.2.10 Sodium oxalate ($Na_2C_2O_4$) or **potassium oxalate** ($K_2C_2O_4$)

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1) Floridin Co is an example of suitable a product available commercially. This information is given for the convenience of users of this part of ISO 3890 and does not constitute an endorsement by ISO of this product.

3.3 Apparatus

Usual laboratory apparatus and, in particular, the following.

3.3.1 Chromatographic columns, of internal diameter 20 mm and length 300 mm, and with PTFE stopcocks and sintered glass discs or glass wool plugs.

3.3.2 Rotary evaporator (Kuderna-Danish[2)] or equivalent), with flask of capacity 500 ml, and with graduated tube attached.

3.3.3 High-speed blender

3.3.4 Separating funnels, of capacities 125 ml and 1 000 ml.

3.4 Procedure

3.4.1 Extraction of fat and organochlorine compounds

3.4.1.1 General methods.

See ISO 3890-1:2000, annex A.

3.4.1.2 Special method for cheese

Place enough diced sample (to provide 3 g of fat), about 2 g of sodium or potassium oxalate (3.2.10) and 100 ml of ethanol (3.2.9) in a high-speed blender (3.3.3) and blend for 2 min to 3 min. (If experience with the product indicates that emulsions will not be broken by centrifuging, add 1 ml of water per 2 g of sample before blending.) Pour the homogenized slurry into a 500 ml centrifuge bottle, add 50 ml of diethyl ether (3.2.3), and shake vigorously for 1 min. Add 50 ml of light petroleum (3.2.1) and shake vigorously for 1 min to 2 min (or divide between two 250 ml bottles and extract each by shaking vigorously for 1 min to 2 min with 25 ml of light petroleum. Proceed as in ISO 3890-1:2000, A.6.3.3.

3.4.2 Liquid-liquid partitioning

Weigh, to the nearest 0,01 g, 1 g to 3 g of the extracted fat into a 125 ml separating funnel (3.3.4) and dissolve in 15 ml of light petroleum (3.2.1). Add 30 ml of acetonitrile saturated with light petroleum (3.2.2) and shake vigorously for 1 min to 2 min. After phase separation, run the lower acetonitrile layer into a 1 000 ml separating funnel (3.3.4) containing 700 ml of sodium chloride solution (3.2.8) and 100 ml of light petroleum (3.2.1). Vigorously shake the light petroleum layer left in the 125 ml separating funnel three times with 30 ml portions of the acetonitrile (3.2.2).

Combine the acetonitrile extracts in the 1 000 ml separating funnel and then shake carefully. Drain the lower, aqueous phase into a second 1000 ml separating funnel and shake it for 12 s with 100 ml of light petroleum. Combine the light petroleum phases from the two 1 000 ml separating funnels. Wash twice with 100 ml portions of water or the sodium chloride solution (3.2.8). Dry over sodium sulfate (3.2.6) and filter into the 500 ml rotary evaporator flask (3.3.2) with attached graduated tube. Rinse the sodium sulfate three times with 10 ml portions of light petroleum (3.2.1). Then concentrate the light petroleum solution to 10 ml using the rotary evaporator (3.3.2).

3.4.3 Clean-up on Florisil

Add to a chromatographic column (3.3.1) a 100 mm layer of adsorbent (3.2.7). Cover with a 10 mm layer of sodium sulfate (3.2.6) and rinse with 40 ml to 50 ml of light petroleum (3.2.1). Pipette 10 ml of the light petroleum concentrate (3.4.2) onto the column (3.3.1), rinsing the container twice with approximately 5 ml portions of light petroleum. Elute into an evaporator flask (3.3.2) with attached graduated tube, using 200 ml of the eluting solvent A (3.2.4). The elution rate should not exceed 5 ml/min. Change the receivers and elute in the same way using 200 ml of the eluting solvent B (3.2.5).

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2) Kuderna-Danish is an example of suitable equipment available commercially. This information is given for the convenience of users of this part of ISO 3890 and does not constitute an endorsement by ISO of this equipment.

Concentrate the two eluates separately to the required small volume using the rotary evaporator (3.3.2). Examine each eluate by GLC. Should further purification be necessary, this can be carried out on a second, freshly prepared adsorbent column or as in ISO 3890-1:2000, annex A.

The first eluate contains any HCB, the HCH isomers, heptachlor, heptachlor epoxide, aldrin, DDE, TDE and DDT. The second eluate contains dieldrin and endrin.

3.5 Gas chromatography

See ISO 3890-1:2000, 6.2. For preliminary tests, etc., see ISO 3890-1:2000, clauses 10 to 14.

4 Method B: Liquid-liquid partitioning with dimethylformamide (DMF) and clean-up on an alumina column (see references [4], [5])

4.1 Principle

The organochlorine compounds, together with the fat, are extracted from the test sample by the procedure described in ISO 3890-1:2000, clause A.6, then the residues are partitioned with dimethylformamide. After addition of sodium sulfate solution, the organochlorine compounds are further partitioned into *n*-hexane. The organic phase is purified by chromatography on neutral aluminium oxide using *n*-hexane as the eluting solvent. The eluate is concentrated then examined by GLC.

Special methods are described for milk and butter.

4.2 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

4.2.1 ***n*-Hexane** [$CH_3(CH_2)_4CH_3$], boiling range 68 °C to 70 °C.

Examine for gas chromatographic purity under working column conditions. Distil over potassium hydroxide, if necessary.

4.2.2 **Acetone** (CH_3COCH_3), general-purpose, reagent grade.

4.2.3 **Dimethylformamide (DMF)**

Examine an *n*-hexane extract of a dilute aqueous solution for interference peaks by GLC. Redistil the solvent, if necessary, and collect the fraction with boiling range 152 °C to 154 °C.

4.2.4 ***n*-Hexane**, saturated with dimethylformamide.

4.2.5 **Dimethylformamide**, saturated with *n*-hexane.

4.2.6 **Sand**, acid washed.

Heat at 500 °C for 4 h, then cool and store in a stoppered bottle.

4.2.7 **Sodium sulfate** (Na_2SO_4), granular, anhydrous.

Heat at 500 °C for 4 h, then cool and store in a stoppered bottle.

4.2.8 **Aluminium oxide** (Al_2O_3), neutral, activated.

Heat aluminium oxide to 500 °C for 4 h, then cool. Carefully add 7 parts of water to 93 parts of aluminium oxide (mass fraction) and mix the solid thoroughly in a closed vessel for at least 90 min. Keep the vessel well stoppered and use the aluminium oxide within 10 days.

4.2.9 Sodium sulfate solution, 2 % solution.

4.3 Apparatus

Usual laboratory apparatus and, in particular, the following.

4.3.1 Soxhlet extraction apparatus

4.3.2 Rotary evaporator (Kuderna-Danish[2)] or equivalent), with flask of capacity 500 ml, and with graduated tube attached.

4.3.3 High-speed blender

4.3.4 Chromatographic columns, of internal diameter 12 mm and length 300 mm, with PTFE stopcocks.

4.3.5 Micro-Snyder[3)] columns

4.4 Procedure

4.4.1 Extraction of fat and organochlorine compounds

4.4.1.1 General methods

See ISO 3890-1:2000, annex A.

4.4.1.2 Special methods

a) Milk

Transfer in the following order, 40 ml of well-mixed milk, 80 ml of acetone (4.2.2) and 80 ml of *n*-hexane (4.2.1) to a 250 ml vortex beaker. Homogenize the mixture for 3 min. Transfer it immediately to a 250 ml centrifuge tube, washing the mixer blades with 10 ml of *n*-hexane, then with 5 ml of water, and add the washings to the tube.

Spin the tube in a centrifuge at a rotational frequency of 2 500 min^{-1} for 5 min. Separate the *n*-hexane solvent layer and pass it through a short column of anhydrous sodium sulfate (4.2.7). Wash the contents of the tube with two successive 25 ml portions of *n*-hexane and run the washings through the column. Reduce the combined extracts to about 15 ml in the rotary evaporator (4.3.2). Transfer the solution to a 100 ml separating funnel graduated at 25 ml and adjust the volume to 25 ml. [See also method E, 7.4.1.2 b) for milk.]

b) Butter

Dissolve 5 g of clarified butterfat (melted and decanted through a filter) in 10 ml of *n*-hexane. Transfer the solution to a 100 ml separating funnel using three successive 5 ml portions of *n*-hexane.

4.4.2 DMF-partitioning of fat and organochlorine compounds

Extract the fat from the 25 ml of hexane solution (4.4.1) with 10 ml of dimethylformamide (DMF) saturated with *n*-hexane (4.2.5), by shaking in a separating funnel. After 2 min to 3 min, run the lower DMF layer into a second

3) Micro-Snyder is an example of suitable equipment available commercially. This information is given for the convenience of users of this part of ISO 3890 and does not constitute an endorsement by ISO of this equipment.

100 ml separating funnel (retaining any interfacial emulsion in the first separating funnel). Repeat the extraction of the *n*-hexane solution with two further 10 ml portions of DMF (4.2.5). Combine the DMF extracts and wash them with 10 ml of *n*-hexane saturated with DMF (4.2.4).

Separate the 10 ml of *n*-hexane and wash with a further 10 ml of DMF (4.2.5). Reject the *n*-hexane and add the washings to the original 30 ml of DMF extract in a 500 ml (or preferably 350 ml) separating funnel. Add 6 ml of *n*-hexane (4.2.1) and shake vigorously for 2 min with 200 ml of sodium sulfate solution (4.2.9).

Allow to stand for 20 min to separate. Collect the *n*-hexane phase by gentle swirling. Drain the aqueous layer to waste, dry the separating funnel with filter paper and drain the *n*-hexane into a graduated tube with a ground-glass neck which will hold 15 ml of solvent. Rinse the separating funnel with small quantities of *n*-hexane and add these to the tube.

Fit the tube with a micro-Snyder column (4.3.5) and concentrate the *n*-hexane extract to about 2 ml.

4.4.3 Clean-up on aluminium oxide with *n*-hexane

Pour a slurry of 5 g of prepared aluminium oxide (4.2.8) in *n*-hexane (4.2.1) into a chromatographic column (4.3.4) containing a solvent-washed cotton wool plug (ISO 3890-1:2000, A.5.15). Allow the aluminium oxide to settle and cover it with a 30 mm layer of anhydrous sodium sulfate (4.2.7). Drain the *n*-hexane until the meniscus reaches the top of the sodium sulfate layer. Add the *n*-hexane extract (4.4.2) and wash into the column with 2 ml portions of *n*-hexane.

Elute at a flow rate not exceeding 5 ml/min with 50 ml of *n*-hexane (4.2.1), collecting the eluate in the rotary evaporator (4.3.2). Concentrate the eluate to approximately 5 ml. Detach the graduated tube, fit a micro-Snyder column (4.3.5) and concentrate the eluate to 1 ml.

4.5 Gas chromatography

See ISO 3890-1:2000, 6.2. For preliminary tests, etc., see ISO 3890-1:2000, clauses 10 to 14.

5 Method C: Liquid-liquid partitioning with dimethylformamide (DMF) and clean-up on a Florisil column (see reference [6])

5.1 Principle

The organochlorine compounds, together with the fat, are extracted from the sample by the procedure described in 5.4.1. The extract is concentrated almost to dryness, then dissolved in light petroleum. The organochlorine compounds are partitioned into dimethylformamide. After addition of sodium sulfate solution, the organochlorine compounds are further partitioned into light petroleum.

The organic phase is purified by chromatography on Florisil, using light petroleum/diethyl ether as the eluting solvent. The eluate is concentrated then examined by GLC.

5.2 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

5.2.1 **Light petroleum**, boiling range 30 °C to 40 °C, redistilled.

5.2.2 **Diethyl ether** ($C_2H_5OC_2H_5$), peroxide free.

5.2.3 **Light petroleum**, boiling range 60 °C to 80 °C, redistilled.

5.2.4 **Eluting solvent**, mixture of diethyl ether (5.2.2) and light petroleum (5.2.1) (6:94 by volume).

5.2.5 Adsorbent: Florisil (Floridin Co[1)]) 60 to100 mesh.

Heat the adsorbent at 650 °C for 2 h in a muffle furnace. Cool to 130 °C and keep for 5 h at this temperature in a drying oven. Afterwards, allow to cool to room temperature in a desiccator then transfer to an airtight, stoppered jar. Add 5 parts of distilled water to 95 parts of the adsorbent (by volume) and shake until free of lumps. Allow to stand for 24 h and shake before use.

5.2.6 Sodium sulfate (Na_2SO_4), granular, anhydrous.

Heat at 500 °C ± 25 °C for 4 h. Cool and store in a stoppered bottle.

5.2.7 Dimethylformamide (DMF), saturated with light petroleum.

Distil DMF and collect the fraction with boiling range 152 °C to 154 °C. Saturate this with light petroleum (5.2.1).

5.2.8 Light petroleum (5.2.1), saturated with dimethylformamide.

5.2.9 Sodium sulfate solution, 2 % solution.

5.2.10 *n*-Hexane [$CH_3(CH_2)_4CH_3$]

5.3 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.3.1 High-speed blender

5.3.2 Rotary evaporator (Kuderna-Danish[2)] or equivalent), with flask of capacity 500 ml, and with graduated tube attached.

5.3.3 Chromatographic column, of internal diameter 20 mm and length 300 mm, with a sintered glass plate and a PTFE stopcock.

5.4 Procedure

5.4.1 Extraction of fat and organochlorine compounds

For general methods, see ISO 3890-1:2000, annex A.

5.4.2 DMF-partitioning of fat and pesticides

Dissolve the sample extract, containing 2 g to 5 g of fat, in 25 ml of light petroleum saturated with DMF (5.2.8). Transfer to a 250 ml separating funnel. Extract with small portions of a measured quantity (e.g. 75 ml) of DMF saturated with light petroleum (5.2.7) added to the separating funnel. Shake vigorously for 1 min to 2 min each time and drain the DMF-phase into a 500 ml separating funnel. Mix the combined phases with 200 ml of sodium sulfate solution (5.2.9) and shake for 1 min to 2 min each with one 40 ml and three 25 ml portions of light petroleum (5.2.3). Collect the light petroleum phases and wash with about 10 ml of water. Dry over sodium sulfate (5.2.6) and filter through a plug of cotton wool. After addition of about 5 ml of *n*-hexane (5.2.10) through the cotton wool plug, reduce to a volume of about 5 ml in the rotary evaporator (5.3.2).

5.4.3 Clean-up on Florisil with light petroleum

Half-fill the chromatographic column (5.3.3) with light petroleum (5.2.3). Add 20 g of deactivated adsorbent (5.2.5) in small portions through a funnel, keeping the PTFE stopcock partly open and gently tapping the column. Use only columns free from visible inclusions of air. Cover with a 20 mm layer of anhydrous sodium sulfate (5.2.6) and allow the light petroleum to drain to the surface of the column filling.

Transfer the sample extract onto the column with several millilitres of the eluting solvent (5.2.4). Allow it to enter into the column, filling by opening the tap until the meniscus reaches the layer of sodium sulfate (5.2.6).

Wash the original container with several millilitres of eluting solvent (5.2.4) and proceed as above. Elute the column with 200 ml of the eluting solvent into a 500 ml round-bottomed flask at a flow rate not exceeding 5 ml/min. Concentrate the eluate in a rotary evaporator (5.3.2) to 5 ml. Transfer the concentrated extract to a graduated tube with diethyl ether (5.2.2) and dilute to a definite volume (10 ml to 20 ml) with diethyl ether.

5.5 Gas chromatography

See ISO 3890-1:2000, 6.2. For preliminary tests, etc., see ISO 3890-1:2000, clauses 10 to 14.

6 Method D: Column chromatography on aluminium oxide of precisely defined activity (see reference [7])

6.1 Principle

The organochlorine compounds are extracted from the sample using acetone/*n*-hexane. The acetone is partitioned into aqueous sodium sulfate. The *n*-hexane is dried and concentrated. The specified amount of fat extract is purified by chromatography on neutral aluminium oxide of precisely defined activity using *n*-hexane as the eluting solvent.

The eluate is concentrated then examined by GLC.

6.2 Reagents

Use only reagents of recognized analytical grade unless otherwise specified and distilled or demineralized water or water of equivalent purity.

6.2.1 Acetone (CH_3COCH_3)

6.2.2 *n*-Hexane ($CH_3(CH_2)_4CH_3$)

6.2.3 Sodium sulfate (Na_2SO_4), granular, anhydrous.

Heat at 500 °C for 4 h, then cool and store in a stoppered bottle.

6.2.4 Sodium sulfate solution, 2 % solution.

6.2.5 Aluminium oxide (Al_2O_3), neutral (Woelm W 200[4)], Activity Grade Super 1 or equivalent).

Preheat the material as supplied at 500 °C ± 25 °C for 3 h to 4 h to remove any moisture and any interfering organic material and cool over phosphorus pentoxide. Deactivate a portion by adding approximately 10 ml of water to 90 g of aluminium oxide, in portions of 2 ml to 3 ml while swirling the flask or bottle. Stopper firmly and shake or place on rollers to mix thoroughly. Leave to equilibrate for 24 h in closed vessels at ambient temperature before use. Standardize the material as follows. Weigh 22,0 g of the aluminium oxide. Prepare a slurry in a small volume of *n*-hexane (6.2.2) and transfer it to a chromatography column (6.3.2). Add a 10 mm layer of anhydrous sodium sulfate (6.2.3) and wash the column with 15 ml to 20 ml of *n*-hexane. Adjust the level of the *n*-hexane layer to just below the top of the sodium sulfate layer.

4) Woelm W 200 is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 3890 and does not constitute an endorsement by ISO of this product.

Place a suitable receiver (of at least 250 ml capacity) under the column. Pipette a small volume of *n*-hexane solution containing 1 g of oil or animal fat onto the top of the column, taking care that the pipette is well drained and that the oil is not allowed to run down the side of the column. Allow the level of the oil in *n*-hexane solution to run down to the top of the sodium sulfate layer. Add 2 ml of *n*-hexane and again allow the level to run down.

Elute the column with 150 ml of *n*-hexane. Evaporate the eluate to a small volume in the evaporator (6.3.3) and transfer it quantitatively into a weighing bottle that has previously been heated to 110 °C, cooled and then weighed. Remove the remaining solvent by heating under a gentle stream of nitrogen. Dry in an oven at 110 °C for 5 min. Cool in a desiccator and weigh to the nearest 0,01 g. Ensure that the fat has reached constant mass. Let the mass of the fat be A.

Pipette a further equal volume of the initial *n*-hexane solution into another preweighed bottle, evaporate, dry and weigh as before. Let the mass of the fat be B.

The fat capacity of the column is equal to $(B - A)$ g expressed to the nearest 0,01 g. Adjust the activity of the aluminium oxide, if necessary in several stages, so that the fat capacity of a column is 0,62 g ± 0,02 g of animal fat or refined vegetable oil or 0,52 g ± 0,02 g of butterfat.

Deactivate enough aluminium oxide for a series of analyses (e.g. the remaining contents of a 500 g bottle) to the defined fat capacity.

6.3 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.3.1 High-speed blender

6.3.2 Chromatography column, of internal diameter 20 mm and length 300 mm, with a PTFE stopcock.

6.3.3 Rotary evaporator (Kuderna-Danish[2)] or equivalent), with flask of capacity 500 ml, and with graduated tube attached.

6.3.4 Glass wool, washed with light petroleum.

6.4 Procedure

6.4.1 General methods

For general methods, see ISO 3890-1:2000, annex A.

6.4.2 Test portion

Weigh, to the nearest 0,01 g, sufficient sample to provide approximately 0,7 g of fat and, in the case of solid products, chop finely.

6.4.3 Extraction of fat and organochlorine compounds

Blend the test portion with 50 ml of acetone (6.2.1) in a high-speed blender (6.3.1) for 2 min. Add 200 ml of *n*-hexane (6.2.2) and continue blending until complete breakdown of the sample is achieved. Allow the phases to separate. Decant the maximum volume of the extract into a 1 l separating funnel through a filter of anhydrous sodium sulfate (6.2.3) on glass wool (6.3.4). Wash the extract twice with 500 ml of sodium sulfate solution (6.2.4), discarding the lower aqueous layers together with a little of the *n*-hexane layer.

Transfer 10 ml of *n*-hexane extract to a preweighed weighing bottle and determine the extracted fat according to 6.4.4. Based upon the calculation in 6.4.4, transfer a volume of *n*-hexane extract containing 0,40 g of milk, cheese or butterfat to the rotary evaporator (6.3.3). Concentrate the transferred amount to 5 ml to 8 ml, disconnect and continue the evaporation to 1 ml to 2 ml on a water bath at between 60 °C to 70 °C under a stream of nitrogen.

6.4.4 Determination of extracted fat

Transfer 10 ml of the hexane extract into a preweighed weighing bottle. Evaporate to dryness at 60 °C under a stream of nitrogen. Dry the residue in an oven at 105 °C for 15 min, cool and weigh the bottle and its contents. Repeat the drying process to constant mass of the bottle and its contents. Calculate the amount of fat in grams per 10 ml of hexane extract by taking the difference between the final mass (in grams) after drying and the mass (in grams) of the preweighed weighing bottle. Do not use this fat determination for the calculation of organochlorine residue contents.

6.4.5 Clean-up

Prepare a slurry of 22 g of deactivated aluminium oxide (6.2.5), having a suitable fat capacity, in a small volume of *n*-hexane and transfer to a chromatography column (6.3.2). Add a 10 mm layer of anhydrous sodium sulfate (6.2.3) to the top of the column and wash the column with 15 ml to 20 ml of *n*-hexane. Adjust the level of *n*-hexane to just below the top of the sodium sulfate layer. Transfer an aliquot of concentrated *n*-hexane solution containing 0,50 g of fat (0,40 g from butter, milk or cheese), prepared as described in 6.4.3, to the top of the column. Elute the column with 150 ml of *n*-hexane, and concentrate the eluate to approximately 8 ml in the rotary evaporator (6.3.3).

Transfer, if necessary, to a graduated tube and continue the evaporation in a water bath at 50 °C under a stream of nitrogen to a volume of 2 ml to 3 ml. Remove the tube with the eluate from the water bath and continue the evaporation at ambient temperature to a final volume of 1,0 ml, then stopper the tube.

6.5 Gas chromatography

See ISO 3890-1:2000, 6.2. For preliminary tests, etc., see ISO 3890-1:2000, clauses 10 to 14.

7 Method E: Column chromatography on alumina column (see reference [8])

7.1 Principle

The organochlorine compounds are extracted from the sample using light petroleum. The specified amount of fat extract is purified by chromatography on basic aluminium oxide of precisely defined activity, using light petroleum as eluting solvent. The eluate is concentrated then examined by GLC.

7.2 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

7.2.1 Light petroleum, boiling range 40 °C to 60 °C, distilled.

7.2.2 Acetone (CH_3COCH_3)

7.2.3 Sodium sulfate (Na_2SO_4), granular, anhydrous.

Heat at 500 °C for 4 h, then cool and store in a stoppered bottle.

7.2.4 Sand, acid washed.

Heat at 500 °C ± 25 °C for 4 h, then cool and store in a stoppered bottle.

7.2.5 Aluminium oxide (Al_2O_3), basic (Woelm W 200[4)], Activity Grade Super I, or equivalent).

Weigh an unopened bottle. Add quickly to the contents (usually 515 g) 27 g of water. Close the bottle immediately. Shake vigorously and allow to stand for 24 h. Transfer the contents to a well-stoppered bottle.

Weigh the empty original bottle and calculate the water content of the deactivated aluminium oxide. The ratio of the obtained proportions shall be 9,5:0,5 (by volume). Adjust if necessary. Check the activity of the aluminium oxide by chromatographing a standard solution of β-HCH with blank fat according to the procedure described below. The recovery of β-HCH, as well as the fat retention, shall be more than 95 %. If the recovery of β-HCH is too low, increase the water content of the aluminium oxide gradually by mixing 0,2 parts of distilled water to 99,8 parts of aluminium oxide (by mass).

7.3 Apparatus

Usual laboratory apparatus and, in particular, the following.

7.3.1 Soxhlet apparatus

7.3.2 Rotary evaporator (Kuderna-Danish[2)] or equivalent), with flask of capacity 500 ml, and with graduated tube attached.

7.3.3 High-speed blender

7.3.4 Centrifuge, capable of rotating at frequency of 2 500 min^{-1}.

7.3.5 Quartz wool

7.3.6 Chromatographic column, of internal diameter 6 mm and length 175 mm, having an outlet of internal diameter 1 mm and length 40 mm, and a solvent reservoir of internal diameter 70 mm and length 125 mm.

7.3.7 Graduated tubes, of capacity 25 ml.

7.4 Procedure

7.4.1 Extraction of fat and organochlorine compounds

7.4.1.1 General methods

See ISO 3890-1:2000, annex A.

7.4.1.2 Special methods

a) Anhydrous butterfat

Heat the fat to about 50 °C and decant through a dry, warm filter. Dissolve the fat in light petroleum (7.2.1) to obtain a solution containing 35 mg/ml to 50 mg/ml. Use 2 ml for the analysis.

b) Milk

Transfer in the following order 40 ml of milk, 80 ml of acetone (7.2.2) and 80 ml of light petroleum (7.2.1) in a high-speed blender (7.3.3). Blend it and centrifuge the mixture at a rotational frequency of 2500 min^{-1} for 5 min. Take an aliquot, for example 10 ml, from the (upper) light petroleum layer. Dry by filtration through sodium sulfate (7.2.3) and wash the sodium sulfate with a little light petroleum. Remove the bulk of the light petroleum in the rotary evaporator (7.3.2). Adjust the volume so that a concentration of 35 mg/ml to 50 mg/ml of fat is obtained. Use 2 ml for the analysis. [See also method B, 4.4.1.2 a).]

c) Cheese

Blend 20 g to 25 g of the sample in a high-speed blender (7.3.3) with 50 ml of light petroleum (7.2.1). Dry by filtration through sodium sulfate (7.2.3) and wash the sodium sulfate with a little light petroleum. Remove the bulk of the light petroleum in the rotary evaporator (7.3.2).

Adjust the volume so that a concentration of 35 mg/ml to 50 mg/ml of fat is obtained. Use 2 ml for the analysis.

7.4.2 Column chromatography

Place a small wad of quartz wool (7.3.5) in the outlet of the chromatographic column (7.3.6). Weigh 4,0 g of deactivated aluminium oxide (7.2.5) and transfer it to the column. Ensure good packing by tapping the column walls. Transfer 2 ml of the solution, obtained according to 7.4.1, to the column. Rinse the inside walls of the column with three 1 ml portions of light petroleum (7.2.1). Elute with 25 ml of light petroleum, collecting all the eluate in a graduated tube (7.3.7) and concentrate to a suitable volume.

In order to obtain a good recovery, particularly of β-HCH, it is essential to use at least 70 mg of fat on a column of 4,0 g of aluminium oxide. This column can also easily retain 100 mg of fat. If larger amounts of fat are to be investigated (for example, when the sensitivity of the detectors is not sufficient), the above-mentioned amounts of aluminium oxide and light petroleum shall be increased accordingly. The clean-up step can easily be scaled-up to accommodate 250 mg of fat.

7.5 Gas chromatography

See ISO 3890-1:2000, 6.2. For preliminary tests, etc., see ISO 3890-1:2000, clauses 10 to 14.

8 Method F: Column chromatography on partially deactivated Florisil
(see references [9], [10], [11])

8.1 Principle

The organochlorine compounds, together with the fat, are extracted from the test sample by the procedure described in ISO 3890-1:2000, A.6. The extract is concentrated almost to dryness and redissolved in light petroleum. The specified amount of fat extract is purified by chromatography on Florisil using light petroleum/dichloromethane as eluting solvent. The eluate is concentrated almost to dryness, then redissolved in light petroleum for examination by GLC.

A special method is described for milk, unsweetened condensed milk and sweetened condensed milk.

8.2 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

8.2.1 **Light petroleum**, boiling range 40 °C to 60 °C, redistilled.

8.2.2 **Dichloromethane**, distilled over sodium hydroxide pellets (boiling point 39 °C).

8.2.3 **Eluting solvent**: mixture of light petroleum (8.2.1) and dichloromethane (8.2.2) (4:1 by volume).

8.2.4 **Adsorbent: Florisil** (Floridin Co[1)]), 60 to 100 mesh.

Heat at 550 °C ± 25 °C overnight. Cool and store in well-sealed container.

Before use, heat at 130 °C ± 2 °C for at least 5 h and add 3 parts of distilled water to 97 parts of the adsorbent (by mass). Shake the mixture for at least 20 min and then store in well-sealed container for 10 h to 12 h to ensure uniform distribution of water. Use within 3 days.

8.3 Apparatus

Usual laboratory apparatus and, in particular, the following.

8.3.1 Rotary evaporator (Kuderna-Danish[2)] or equivalent), with flask of capacity 500 ml, and with graduated tube attached.

8.3.2 Chromatographic columns, of internal diameter 22 mm and length 600 mm, with PTFE stopcocks.

8.4 Procedure

8.4.1 Extraction of fat and organochlorine compounds

8.4.1.1 General methods

See ISO 3890-1:2000, annex A.

8.4.1.2 Special methods

a) Milk and unsweetened condensed milk

Weigh 10 g of the test sample in a 250 ml beaker and add 25 g of adsorbent (8.2.4), stirring with a glass rod. It is essential to have a lump-free powder, able to run through a narrow funnel.

b) Sweetened condensed milk

Weigh 10 g of the test sample in a 250 ml beaker. Mix thoroughly with 5 ml of distilled water. Add 25 g of adsorbent (8.2.4) and mix as above.

8.4.2 Clean-up on Florisil

Plug a chromatographic column (8.3.2) with glass wool. Pour in 100 ml of light petroleum (8.2.1) and add 25 g of adsorbent (8.2.4). After the column filling has settled, allow the solvent to run through to a height 10 mm above the adsorbent layer. Dissolve 0,5 g to 1 g of the extracted fat in 10 ml of light petroleum and transfer it quantitatively onto the adsorbent in the column.

When investigating milk or condensed milk (see 8.4.1.2), add the adsorbent, soaked with substrate, through a narrow funnel onto the column of adsorbent (8.2.4) prepared as described above. Allow to settle under vibration. Elute with 300 ml of the eluting solvent (8.2.3). The elution rate should not exceed 5 ml/min. Concentrate the eluate to 2 ml in the rotary evaporator (8.3.1) under reduced pressure at 40 °C. Remove the remaining solvent using a stream of air and transfer the residue to a measuring tube with small portions of light petroleum (8.2.1) and dilute to a volume of 5 ml.

NOTE 1 Absolute dryness after application of air or nitrogen is not necessary. A last trace of light petroleum will not affect the result, as dichloromethane is nearly completely evaporated during the procedure.

For reasons of economy, the above-mentioned procedure may be modified to a small-scale procedure that uses only 10 % of the high purity adsorbent and solvents as follows. Weigh, to the nearest 0,01 g, 1,00 g of extracted fat into a 20 ml one-mark volumetric flask. Dilute to the 20 ml mark with light petroleum and shake well to mix.

Tamp a small plug of glass wool into a chromatographic column with diameter 8 mm x 200 mm, fitted with an outlet stopcock and having a 30 ml reservoir at the upper end. Introduce 15 ml of light petroleum onto the column and add slowly 3,0 g of standardized Florisil (8.2.4). Ensure tight packing of the adsorbent by gently tapping the sides of the column with a glass rod. When the Florisil has settled, allow the solvent to run through to about 10 mm from the top of the column.

With a volumetric pipette transfer 2,00 ml (equivalent to 100 mg of sample) of the fat solution obtained by either the normal or the more economical method to the Florisil column. Allow the solution to run through and elute organochlorine compounds with 30 ml of the eluting solvent (8.2.3). Collect the eluate in a 100 ml round-bottomed flask. Complete elution should not take less than 15 min.

Concentrate the eluate in the rotary evaporator (8.3.1) to about 2 ml. Remove any remaining solvent by gently blowing with a stream of clean air. Add 2,00 ml of isooctane to the invisible residue, stopper and swirl the flask. Use this concentrate for gas chromatographic analysis.

8.5 Gas chromatography

See ISO 3890-1:2000, 6.2. For preliminary tests, etc., see ISO 3890-1:2000, clauses 10 to 14.

9 Method G: Column chromatography on partially deactivated silica gel (see reference [12])

9.1 Principle

The organochlorine compounds, together with the fat, are extracted from the test sample. The extract is purified by chromatography on a silica gel column, with elution with light petroleum/dichloromethane (80:20 by volume). The eluate is concentrated then examined by GLC.

9.2 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

9.2.1 Light petroleum, boiling range 40 °C to 60 °C.

Distil, if necessary, over a Raschig column of at least 500 mm length.

9.2.2 Dichloromethane, distilled over sodium hydroxide (NaOH) pellets (boiling point 39 °C).

9.2.3 Eluting solvent: mixture of light petroleum (9.2.1) and dichloromethane (9.2.2) (4:1 by volume).

9.2.4 Silica gel, 70 to 230 mesh.[5)]

Activate by heating at 450 °C ± 25 °C for 3 h. Cool and store in well-sealed container.

9.2.5 Deactivated silica gel

Mix 90 parts of active silica gel (9.2.4) with 10 parts of water (by volume). Shake the mixture for at least 20 min then store in a well-sealed container for at least 10 h to 12 h to ensure uniform distribution of water.

9.2.6 *n*-Hexane [$CH_3(CH_2)_4CH_3$], or ***n*-heptane** or **isooctane**

Distil, if necessary, over a Raschig column of at least 500 mm length.

9.3 Apparatus

Usual laboratory apparatus and, in particular, the following.

9.3.1 Analytical balance, capable of weighing to the nearest 0,01 g.

5) The product No. 7734 from Merck, Darmstadt, Germany, has proved to be suitable. The amount and the analytical procedure have been tested using this product. This information is given for the convenience of users of this part of ISO 3890 and does not constitute an endorsement by ISO of this product.

9.3.2 Rotary evaporator (Kuderna-Danish[2)] or equivalent), with flask of capacity 500 ml, and with graduated tube attached.

9.3.3 Chromatographic columns, of internal diameter 22 mm, with PTFE stopcocks.

Columns of 250 mm length with an extension at the upper end have proved to be satisfactory.

9.3.4 Evaporation flasks, of capacity 500 ml.

9.3.5 Beakers, of various sizes.

9.3.6 Pipettes

9.3.7 Funnels

9.3.8 Glass wool, washed with light petroleum (9.2.1).

9.3.9 Glass rods, of diameter 8 mm.

9.3.10 Water vacuum pump

9.4 Procedure

9.4.1 Extraction of fat and organochlorine compounds

For general methods, see ISO 3890-1:2000, annex A.

9.4.2 Clean-up

9.4.2.1 Fat and organochlorine compounds

Plug a chromatographic column (9.3.3) with glass wool (9.3.8) and fill it by thorough vibration with 15 g of deactivated silica gel (9.2.5). Dissolve 0,5 g of the extracted fat (9.4.1) in 5 ml of light petroleum (9.2.1) and transfer it quantitatively onto the top of the silica gel column using a suitable pipette.

Elute with 130 ml of the eluting solvent (9.2.3). Add 1 ml of internal standard solution to the eluate. Concentrate the eluate slowly under reduced pressure to about 1 ml, using the rotary evaporator (9.3.2) at 40 °C and the water pump (9.3.10). Remove the remaining solvent with a stream of air and transfer the residue to a measuring tube with small portions of *n*-hexane (9.2.6). Dilute to a volume of 1 ml.

9.4.2.2 Short procedure for milk, evaporated milk, dried milk and cheese (see reference [13])

Mix 10 g of milk, or 10 g of evaporated milk diluted with water according to the concentration factor, or 2 g to 4 g of dried milk reconstituted with 10 ml of water at 40 °C or 2 g to 5 g of cheese together with 8 ml to 9 ml of water with 15 g of activated silica gel (9.2.4) in a beaker. Stir continuously with a glass rod to obtain a free-flowing powder without lumps.

In accordance with the fat capacity of the “separating” silica gel layer with 10 % of water (below), the mass of fat in the sample shall be between 0,3 g and 0,8 g.

The mass of water in the sample shall be about 10 g. If not, add an appropriate amount of water. Only a water load of 40 % deactivates the silica gel in such a way that the organochlorine compounds are transported with the solvent front to the top of the “separating” silica gel layer with 10 % of water below.

Fill the chromatographic column (9.3.3) with 30 g of deactivated silica gel (9.2.5) and (on top of it) the above-described mixture of the sample with silica gel. Rinse the beaker with two portions of 50 ml of the eluting solvent (9.2.3) and add these portions carefully on top of the column. Add a further 300 ml of the eluting solvent (9.2.3) to

the column and elute with a flow rate not exceeding 5 ml/min. Add internal standard solution to the eluate. Concentrate the eluate slowly under reduced pressure to about 1 ml using, for example, a rotary evaporator (9.3.2) at 30 °C. Carefully evaporate the remaining solvent at atmospheric pressure and room temperature to (nearly) dryness. Make up the residue to a volume of 2 ml using *n*-hexane or isooctane (9.2.6).

NOTE The procedure described can be scaled down by a factor of 2 by reducing the amounts of sample and reagents accordingly.

9.5 Gas chromatography

See ISO 3890-1:2000, 6.2. For preliminary tests, etc., see ISO 3890-1:2000, clauses 10 to 14.

10 Method H: Gel-permeation chromatography

10.1 Principle

The organochlorine compounds are extracted from the test sample and the extracting solvent is evaporated to low volume. The extracts in are dissolved ethyl acetate/cyclohexane and purified by chromatography using a gel-permeation column, with ethyl acetate/cyclohexane as the eluting solvent. The eluate is concentrated then examined by GLC.

10.2 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

10.2.1 Cyclohexane (C_6H_{12}), distilled over sodium paraffin dispersion.

10.2.2 Ethyl acetate ($CH_3CO_2C_2H_5$), distilled.

10.2.3 Ethanol (C_2H_5OH), distilled.

10.2.4 Diethyl ether ($C_2H_5OC_2H_5$), peroxide-free, distilled over calcium chloride.

10.2.5 Sodium sulfate (Na_2SO_4), granular, anhydrous.

Heat at 500 °C for 4 h, then cool and store in stoppered bottle.

10.2.6 Sodium sulfate solution, 2 % solution in doubly distilled water.

10.2.7 Light petroleum, boiling range 40 °C to 60 °C, redistilled.

10.2.8 Chromatography gel (Biorad, Bio-beads S-X 3, 200-400 mesh or equivalent).[6)]

10.2.9 Eluting solvent: mixture of ethyl acetate (10.2.2) and cyclohexane (10.2.1) (1:1 by volume).

10.3 Apparatus

Usual laboratory apparatus and, in particular, the following.

10.3.1 High-speed blender

6) Biorad and Bio-beads are examples of suitable products available commercially. This information is given for the convenience of users of this part of ISO 3890 and does not constitute an endorsement by ISO of these products.

10.3.2 Tumbling apparatus

10.3.3 Rotary evaporator (Kuderna-Danish[2)] or equivalent), with flask of capacity 500 ml, and with graduated tube attached.

10.3.4 Freeze drier

10.3.5 Soxhlet extraction apparatus

10.3.6 Gel-permeation chromatograph (commercially available or laboratory built).

10.3.7 Separating funnel, of capacity 500 ml.

10.4 Procedure

10.4.1 Extraction of fat and organochlorine compounds

For general methods see ISO 3890-1:2000, annex A.

10.4.2 Clean-up by gel-permeation chromatography

Weigh, to the nearest 0,01 g, exactly 3 g of fat, (obtained according to ISO 3890-1:2000, clause A.3). Dissolve in eluting solvent (10.2.9) and dilute to 50 ml with this solvent. Apply 5 ml of this solution into the loop of the gel-permeation chromatograph (10.3.6).

Separate the fat from the residues using a 320 mm column of chromatography gel (10.2.8), presoaked in the eluting solvent (10.2.9), packed in a tube of diameter 25 mm × 400 mm.

Adjust the elution rate to 5 ml/min by applying light pressure to the column (the pressure shall not exceed 0,5 bar) and collect 100 ml to 160 ml of eluate, which contains the fat, in a round-bottomed flask. Concentrate the eluate to 5 ml in the rotary evaporator (10.3.3) under reduced pressure at 40 °C.

After each sample, wash the column for 3 min with eluting solvent (10.2.9).

If a laboratory-built apparatus is used, the amounts of sample and reagent should be adjusted accordingly.

10.5 Gas chromatography

See ISO 3890-1:2000, 6.2. For preliminary tests, etc., see ISO 3890-1:2000, clauses 10 to 14.

11 Confirmatory tests and additional clean-up procedure

11.1 Confirmatory test A: Determination of organochlorine compounds with glass-capillary gas chromatography (see references [14], [15], [16])

11.1.1 Apparatus

Usual laboratory apparatus and, in particular, the following.

11.1.1.1 Gas chromatograph, with electron-capture detector, provided with a capillary injection system.

11.1.1.2 Capillary column, with the following characteristics:

— length at least 25 m;

— stationary phase CP-Sil 7, SE 30, OV1 or equivalent;

— film thickness 0,1 μm to 0,4 μm;

— inner diameter 0,1 mm to 0,4 mm;

— pressure-controlled mobile phase (carrier gas: helium or hydrogen);

— linear velocity 200 mm/s to 400 mm/s;

— make-up gas (nitrogen or argon/methane) flow rate about 20 ml/min;

— detector purge about 30 ml/min (nitrogen or argon/methane);

— injector temperature 210 °C;

— detector temperature 300 °C to 350 °C; temperature programming depends on the injection technique (see below).

11.1.2 Injection technique

11.1.2.1 Splitless injector

Close the splitter and septum purge if present.

Inject 1 μl to 5 μl (splitless) when the column temperature is 100 °C. When solvents more volatile than isooctane are used, it is advisable to lower the temperature of the column to 90 °C or even 80 °C.

Open the splitter (and septum purge if present) a short time (0,25 min to 3 min) after the injection. The time must be determined experimentally.

One minute after opening the splitter, start the temperature programming. Programme at a rate between 5 °C/min and 40 °C/min. End the programme with the temperature between 210 °C and 230 °C.

The final holding time depends on the temperature programming rate, but will be in the order of 15 min to 30 min.

Cool to initial temperature.

Close the splitter (and septum purge).

Inject the next sample.

11.1.2.2 All-glass solid injector

With this device 1 μl or 2 μl can be injected in a compact plug into the capillary. Because the solvent has already been evaporated, isothermal analysis can be carried out, making superfluous the cooling of the gas chromatographic system at the end of the analysis.

11.1.3 Testing the whole system

11.1.3.1 Test the capillary column (commercially obtained or laboratory-made) regularly under standard conditions with the compounds of interest.

11.1.3.2 Inject an organochlorine pesticide mixture, containing β-HCH, dieldrin, endrin and *p*,*p'*-DDT, at 210 °C with a small split (1:20). For dieldrin (capacity ratio > 5) the theoretical plate number should be 80 000 at least.

11.1.3.3 Equal amounts of endrin and dieldrin injected splitless under the conditions given in 11.1.2 should result in a peak height ratio of at least 65 %. If not, adsorption prevents normal elution of endrin.

11.1.3.4 *p*,*p'*-DDT, which is very sensitive to degradation, is suitable as a test compound. With the conditions mentioned in 11.1.2.1 there is a long residence time in the injection port. Particularly where the glass liner is not sufficiently deactivated, degradation occurs. Isothermal (210 °C) injection of *p*,*p'*-DDT with split injection (short residence time), with split on cold columns (volumes and temperatures as in 11.1.2.1) and splitless injection under the conditions of 11.1.2.1 give information on where degradation occurs and to what extent.

11.1.3.5 Linearity tests should be run at regular intervals. With use, the quality of the column decreases and adsorption increases, especially at lower concentrations.

11.1.4 Application and analysis of samples

Organochlorine compounds are extracted from the test samples according to one of the methods mentioned in this part of ISO 3890. Particularly with extracts from fatty samples, higher-boiling substances also are injected together with the organochlorine compounds. These higher-boiling compounds will contaminate the glass liner in the injection system (or the all-glass solid injector), resulting in adsorption of the compounds of interest. Regular cleaning is necessary. In the case of the glass liner, it is possible to work with a pre-column instead of a glass liner. Special attention shall be paid to degradation of *p*,*p'*-DDT.

11.2 Confirmatory test B: Thin-layer chromatography of organochlorine compounds (see reference [9])

11.2.1 Principle

An aliquot of a purified sample extract is applied to a thin layer of aluminium oxide, together with a series of reference compounds. The chromatogram is developed by ascending migration using light petroleum as a mobile phase and the separated compounds are made visible by spraying with silver nitrate solution and subsequent exposure to a strong ultraviolet lamp.

11.2.2 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

11.2.2.1 **Light petroleum**, boiling range 40 °C to 60 °C, refluxed over sodium hydroxide pellets and distilled.

11.2.2.2 **Silver nitrate solution** (spraying reagent).

Dissolve 0,5 g of silver nitrate ($AgNO_3$) in about 1 ml of water. Add 99 ml of 95 % ethanol (C_2H_5OH) and mix.

11.2.2.3 **Reference solutions** of organochlorine compounds, in isooctane, containing 0,05 μg/μl.

11.2.2.4 **Precoated TLC plates**, aluminium oxide, type E (neutral), F254, alufoil sheets Merck No. 5550[7].

11.2.3 Apparatus

Usual laboratory apparatus and, in particular, the following.

11.2.3.1 **Ultraviolet lamp**, for photochemical detection of organochlorine compounds on TLC.

7) Merck No. 5550 is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 3890 and does not constitute an endorsement by ISO of this product.

Quarzbrenner für photochemische Zwecke from Philips, type HPK 125 W/L[8)] with a VGI/HP 125 W transformer is recommended.

11.2.4 Procedure

Concentrate the sample and blank extracts to a suitable volume in a graduated tube or Kontes vial. By means of a micropipette, place a volume of sample extract, containing sufficient organochlorine compounds to give a spot containing between 0,025 μg and 0,25 μg, on a precoated aluminium oxide E plate. Do the same with an equal volume of the blank.

Apply standard solutions to procedure spots containing 0,025 μg, 0,05 μg, 0,10 μg, 0,15 μg, 0,20 μg and 0,25 μg of organochlorine compounds respectively. For best results keep the size (spot diameter) of the spotted sample aliquots and standards as small as possible.

Develop the chromatogram over a distance of about 150 mm by ascending migration in a pre-saturated tank using light petroleum (11.2.2.1) as a mobile phase. When the mobile phase has reached the front line, remove the plate from the tank and let the adherent solvent evaporate.

Spray abundantly with the ethanolic silver nitrate solution (11.2.2.2). Insufficient spraying will result in poor sensitivity. After spraying, wait 10 min and examine the plate carefully. If, at this time, brown or black spots have appeared, they are not due to organochlorine compounds. Sometimes, both blank and sample exhibit a yellow-brownish zone at an Rf of 0,70.

Mark the position of the spot(s) with a pencil and place the chromatogram under the ultraviolet lamp. Irradiate for 10 min. Remove the plate from the lamp and spray lightly with distilled water until the chromatogram is just moistened. Expose the plate again to the ultraviolet lamp. The organochlorine compounds should now appear as violet black spots in 1 min to 2 min.

If the visibility of the spots is not satisfactory, irradiate for another 10 min. Moisten them again and continue irradiation for 1 min to 2 min until even the lowest concentration of the standards is clearly discernible. If Rf-values are all rather low and separation is poor, repeat the chromatography using light petroleum containing 1 % acetone.

CAUTION: For successful detection of organochlorine compounds on thin layers, the laboratory atmosphere should not contain traces of hydrochloric acid, chlorine or sulfurous compounds. Even vapours of halogenated solvents, such as chloroform, cause considerable background darkening and consequently lower the detection sensitivity.

11.2.5 Evaluation of the chromatogram

Identify the spot(s) in the sample aliquot by comparison with those given by the reference compounds. Take only those spots into account that are not present in the blank. Rf-values for chlorinated pesticides are given in Table 1.

Estimate the approximate quantity of the pesticide in the sample aliquot by comparison with the spots given by different quantities of the reference compounds. Ideally, TLC and GLC should give qualitatively and quantitatively the same results. But, since TLC estimations are relatively inaccurate, appreciable differences can be expected.

Common sense should be the guide in these cases. If, for example, GLC indicates 500 μg/kg and TLC indicates 350 μg/kg, consider these results as satisfactory. On the other hand, if the results for GLC are 500 μg/kg and for TLC are 150 μg/kg respectively, it is recommended to repeat the analyses.

8) Philips, type HPK 125 W/L is an example of a suitable apparatus available commercially. This information is given for the convenience of users of this part of ISO 3890 and does not constitute an endorsement by ISO of this product.

11.3 Confirmatory test C: Chemical modifications (see references [17], [18], [19], [20], [21])

11.3.1 General

Many organochlorine compounds can be converted into different compounds by means of chemical reactions. The derivatizing reaction is carried out on both the sample extract containing the tentatively identified residue(s) and a suitable amount of the reference compound(s).

Comparison of the chemical and chromatographic behavior of the reaction product(s) from the sample extract and the reference compound(s) provides useful additional evidence for the confirmation of the presence of the tentatively identified residues in the sample.

Among various chemical systems developed for this purpose, derivative formation in solid matrix is recommended because it is specific, sensitive and the most simple to carry out.

Four solid matrix chemical derivatization techniques for the confirmation of the identity of various organochlorine compounds are described below. No such technique exists for HCB and, consequently, a wet derivatization confirmatory procedure is given for this compound.

Table 1 — Rf-values of organochlorine compounds and their degradation products in the system Al_2O_3E/light petroleum

Compound	Rf-value
Methoxychlor	0,00
δ-HCH	0,00
Endosulfan B	0,02
β-HCH	0,03
ε-HCH	0,11
Dieldrin	0,12
Endrin	0,16
Heptachlor epoxide	0,18
γ-HCH	0,20
Pentachloroaniline	0,21
p,p'-TDE	0,23
Endosulfan A	0,23
o,p'-TDE	0,26
α-BHC	0,31
γ-Chlordane	0,39
α-Chlordane	0,45
p,p'-DDT	0,48
Oxychlordane	0,54
o,p'-DDT	0,57
Heptachlor	0,60
p,p'-DDE	0,65
Pentachloronitrobenzene	0,68

Table 1 — Rf-values of organochlorine compounds and their degradation products in the system Al_2O_3E/light petroleum (*continued*)

Compound	Rf-value
Aldrin	0,72
Pentachlorobenzene	0,76
HCB	0,77
Mirex	0,80
Toxaphene (streak)	0,00 to 0,70
NOTE PCB: all Aroclors yield not clearly separated spots with Rf-values of between 0,65 and 0,75.	

11.3.2 Derivative formation in solid matrix

11.3.2.1 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

11.3.2.1.1 Aluminium oxide 60 (Al_2O_3), active basic type E, activity I, Merck 1067[9].

11.3.2.1.2 Aluminium oxide 90 (Al_2O_3), activated, acidic, activity I, Merck 1078[9].

Check the purity of both adsorbents by shaking 0,5 g with 2 ml of pure toluene. Allow the solids to settle and inject an aliquot of the supernatant into the GLC apparatus under the same conditions as used for the pesticide analysis. If peaks are observed, purify by heating at 550 °C ± 25 °C for at least 3 h.

11.3.2.1.3 Toluene, ethyl acetate, or **isooctane** (2,2,4-trimethylpentane), suitable for residue analysis.

11.3.2.1.4 Sulfuric acid, $c(H_2SO_4)$ = 95 % to 97 % solution (mass fraction).

11.3.2.1.5 Hydrochloric acid (HCl), fuming (mass fraction of at least 37 %).

11.3.2.1.6 Zinc chloride ($ZnCl_2$), anhydrous, for example Merck 8816[9].

11.3.2.1.7 Solid matrix, for micro-scale alkali treatment (alkaline alumina).

Dissolve 5 g of potassium hydroxide (KOH) pellets in 4 ml of water in a 400 ml glass beaker. Add in small portions, while thoroughly mixing with a glass rod, 50 g of basic aluminium oxide (11.3.2.1.1). Transfer to a 500 ml flask and shake well. Store in a desiccator until used. It is stable for more than 6 months if kept dry.

11.3.2.1.8 Solid matrix, to confirm identity of endrin (acidic aluminium oxide).

Add cautiously 5 ml of sulfuric acid (11.3.2.1.4) to 2,5 ml of water. Cool in an ice bath for 20 min to 30 min. In a pre-cooled mortar grind rapidly with a pestle 50 g of ice-cold pure acidic alumina (11.3.2.1.2) with the diluted sulfuric acid. Transfer the mixture to a glass-stoppered flask and shake it for 2 h in a shaking machine. Keep in a tightly closed container in a desiccator. The solid matrix thus prepared is active for more than 1 year.

11.3.2.1.9 Solid matrix, to confirm the identity of endosulfan (strongly acidic aluminium oxide).

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9) Merck 1067, 1078 and 8816 are examples of suitable products available commercially. This information is given for the convenience of users of this part of ISO 3890 and does not constitute an endorsement by ISO of these products.