INTERNATIONAL STANDARD

ISO 4389

Second edition
1997-12-15

Tobacco — Determination of organochlorine pesticide residues — Gas chromatographic method

Tabac — Dosage des résidus de pesticides organochlorés — Méthode par chromatographie en phase gazeuse

Reference number
ISO 4389:1997(E)

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.

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.

International Standard ISO 4389 was prepared by Technical Committee ISO/TC 126, *Tobacco and tobacco products*.

This second edition cancels and replaces the first edition (ISO 4389:1981) which has been technically revised as a result of extensive examination by members of the CORESTA Pesticide Task Force.

Advances have been made and procedures changed in order to use toluene and *n*-hexane rather than benzene and acetonitrile. Lower detection limits are obtainable for many of the compounds quoted in table 1. A 12-laboratory collaborative study has yielded data for repeatability and reproducibility and spiked standard recovery. Such data were not available in the first edition.

For leaf tobacco, the method has been shown to be free of interfering chromatogram peaks originating from non-organochlorine pesticide substances. However, because it cannot be assumed that interference does not arise in the analysis of tobacco products, it will be seen that the scope has been limited to leaf tobacco.

The method can be used on tobacco products if the analyst is able to recognize chromatogram interference and to investigate the chemical structure of interfering compounds by the use of a mass-spectrometric method. Appropriate procedures for this type of analysis may not be readily

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available to users of this International Standard and have not, therefore, been included.

There is clearly a need for a method which is formally applicable to both leaf tobacco and tobacco products. Research is continuing which may result in a third edition with such a scope.

Annexes A to C of this International Standard are for information only.

Tobacco — Determination of organochlorine pesticide residues — Gas chromatographic method

1 Scope

This International Standard specifies a method for the gas chromatographic determination of pesticide residues in tobacco including leaf tobacco.

The method is applicable to the determination in leaf tobacco of the organochlorine pesticides listed in table 1.

The method is particularly recommended for determination of the substances within the detection limits listed in table 1.

NOTE — ISO 1750 contains the systematic chemical names and structures corresponding to the common names in table 1.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 648:1977, *Laboratory glassware — One-mark pipettes*.

ISO 1042:1983, *Laboratory glassware — One-mark volumetric flasks.*

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*.

ISO 4874:1981, *Tobacco — Sampling of batches of raw material — General principles.*

3 Principle

Extraction of the pesticide residues from a dried and milled sample, mixed with Florisil®, by *n*-hexane in a special Soxhlet extractor. Determination of pesticide residues by gas chromatography equipped with electron-capture detector without any further clean-up.

4 Reagents

4.1 General

All the reagents shall be suitable for pesticide residue analysis. All solvents shall be checked for purity before use by carrying out a blank determination using exactly the same procedure (extraction and gas chromatography) as used for the test sample. The chromatogram obtained from the solvents shall have a baseline without noticeable peaks that could interfere with those from the pesticide residues being determined.

Use only degassed water in accordance with at least grade 2 of ISO 3696.

Table 1 — List of substances with detection limits

Substance	ISO 1750 common name	Detection limit µg/g
aldrin	aldrin	0,02
trans-chlordane	chlordane	0,02
p,p'-DDE	—	0,02
o,p'-DDT	—	0,04
p,p'-DDT	DDT	0,06
dieldrin	dieldrin	0,02
α-endosulfan	endosulfan	0,03
HCB	hexachlorobenzene	0,02
α-HCH or α-BHC	HCH or BHC	0,02
β-HCH or β-BHC	HCH or BHC	0,02
γ-HCH or γ-BHC	gamma-HCH (Lindane) or gamma-BHC	0,01
δ-HCH or δ-BHC	HCH or BHC	0,02
heptachlor	heptachlor	0,02
heptachlor epoxide	-	0,02
o,p'-TDE or *o,p'*-DDD	-	0,03
p,p'-TDE or *p,p'*-DDD	TDE	0,02
o,p'-DDE	-	0,03

4.2 *n*-Hexane

4.3 Reference substances

Certified reference materials of minimum purity 95 % (*m*/*m*) of the substances listed in table 1.

NOTE — *trans*-Chlordane is used as an indicator for chlordane (technical mixture). If α-endosulfan is detected by this method, it is advisable to determine residues of the sum of α-endosulfan, β-endosulfan and endosulfan sulfate by a method suitable for such determinations.

4.4 Internal standard

Use Mirex,[1)] an obsolete pesticide which has been superseded (see reference [2] in annex C).

NOTE — Mirex is a generic name for dodecachloropentacyclo[5.2.1.$0^{2,6}$.$0^{3,9}$.$0^{5,8}$]decane.

4.5 Toluene

4.6 Internal standard stock solution

Weigh, to the nearest 0,0001 g, 0,02 g of Mirex (4.4) into a 100 ml volumetric flask. Dilute to the mark with *n*-hexane (4.2).

4.6.1 Internal standard solution

Pipette 5 ml of the internal standard stock solution (4.6) into a 200 ml volumetric flask and dilute to the mark with *n*-hexane to give a solution containing approximately 5 µg/ml of Mirex. Store the internal standard solution at between 0 °C and +4 °C and exclude light. Under these conditions the internal standard solution is stable for at least 6 months.

4.7 Standard pesticide solutions

Store all pesticide solutions at between 0 °C and +4 °C and exclude light. Under these conditions the solutions are stable for at least 6 months.

4.7.1 Individual standard stock solutions

Weigh, to the nearest 0,0001 g, 0,02 g of each pesticide into individual 100 ml volumetric flasks. Dilute to the mark with *n*-hexane to give individual standard stock solutions containing approximately 200 µg/ml of the individual pesticide.

NOTE — In the case of ß-HCH the standard stock solution should be made by dissolving the pesticide in toluene because of reduced solubility in *n*-hexane.

4.7.2 Mixed stock solution A

Pipette 5 ml of each individual standard stock solution (4.7.1) into a single 200 ml volumetric flask and dilute to the mark with *n*-hexane (or toluene if the conditions of the Note in 4.7.1 are applicable) to give a solution containing approximately 5 µg/ml of each pesticide.

4.7.3 Mixed stock solution B

Pipette 1 ml of mixed stock solution A (4.7.2) into a 10 ml volumetric flask and dilute to the mark with *n*-hexane to give a solution containing approximately 0,5 µg/ml of each pesticide.

4.7.4 Standard calibration solution

Pipette 1 ml of both mixed stock solution A (4.7.2) and the internal standard solution (4.6.1) into a 100 ml volumetric flask and dilute to the mark with *n*-hexane to give a solution containing approximately 0,05 µg/ml of each pesticide and internal standard.

4.8 **Florisil®**[2)], 60 mesh to 100 mesh.

NOTE — Florisil® is a special, selected variety of magnesium silicate. The mesh size range designated as 60 mesh to 100 mesh corresponds to a mesh aperture size range of 250 µm to 150 µm.

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1) Mirex is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

2) Florisil® is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

4.8.1 Requirements

The quality of the Florisil® is one of the most critical features of the test method. The activity of the Florisil® needs to be sufficient to retain impurities present in the extract from the sample while allowing the pesticide residues to be eluted. The Florisil® shall first be pre-treated as described in 4.8.2. Only Florisil® that passes the subsequent verification test described in 4.8.3 shall be used.

4.8.2 Pretreatment

Heat sufficient Florisil® for the verification test for at least 5 h in a quartz crucible in a muffle furnace at 550 °C. Allow the Florisil® to cool in a desiccator that contains no desiccant and transfer to a round-bottom flask. Add 5 ml of water for every 100 g of Florisil®. Mix thoroughly in a rotating flask for approximately 1 h. Allow the Florisil® to equilibrate by storing in a tightly closed glass container for at least 48 h before proceeding as described in 4.8.3.

4.8.3 Verification of activity level

The activity level of the Florisil® is checked by the extraction of dieldrin from n-hexane solution. The solution shall have a concentration equivalent to that of an extract from tobacco containing 1,0 µg/g of this pesticide. The activity level of the pretreated Florisil® is correct if the recovery of dieldrin is better than 95 %.

The activity of the Florisil® shall be checked each time a new portion is prepared.

5 Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastics containers and stopcock grease, otherwise impurities may be introduced into the solvents. All volumetric flasks and pipettes shall comply with class A of ISO 1042 and class A of ISO 648 respectively.

Usual laboratory apparatus and the following items.

5.1 Rotary evaporator.

5.2 Tobacco mill, with 2 mm mesh**.**

5.3 Oven, with ventilation**.**

5.4 Muffle furnace.

5.5 Heating mantles.

5.6 Soxhlet extractor, for continuous extraction (see figure 1).

5.7 Reflux condenser.

5.8 Desiccator.

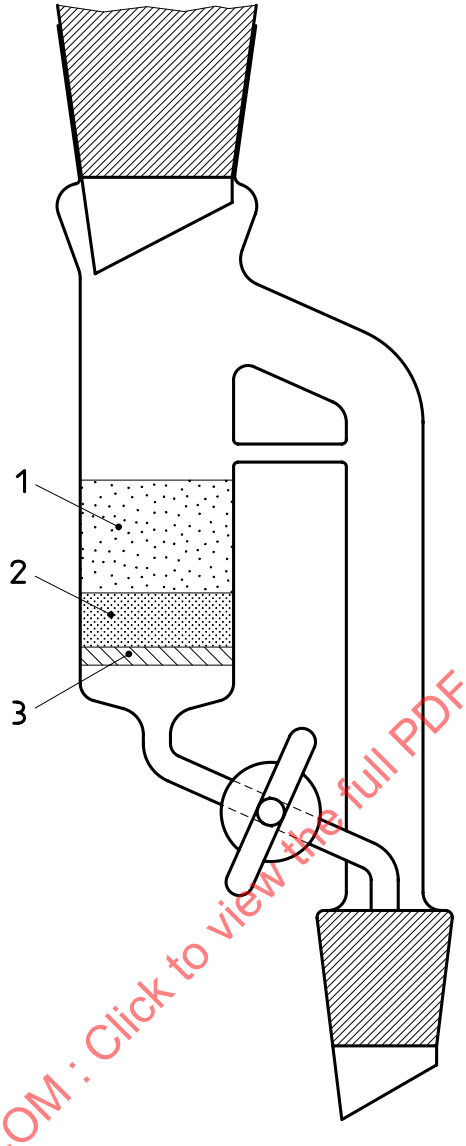
5.9 Quartz crucible.

5.10 Gas chromatograph.

5.10.1 Basic requirements

Operate the gas chromatograph in accordance with the manufacturer's instructions. The injection port, oven and detector shall each be equipped with a separate heating unit.

The conditions given in 5.10.2 to 5.10.7 have been found to be satisfactory on a particular make of instrument and are given for guidance. If other conditions are used they should be validated prior to use.



Key

1 Tobacco + Florisil®
2 Florisil®
3 Filter disk porosity 1

Figure 1 — Apparatus used for tobacco extraction

5.10.2 Temperatures

The injection port temperature shall be between 180 °C and 210 °C. The detector temperature shall be between 290 °C and 340 °C. If any other conditions are used they shall be sufficient to achieve satisfactory separation of all components and similar to that given in the specimen chromatogram (see figure A.1).

A suitable temperature programme is

- initial temperature 40 °C
- initial time 2 min
- temperature profile 1 20 °C/min from 40 °C to 150 °C
- temperature profile 2 3 °C/min from 150 °C to 270 °C
- final time 15 min at 270 °C
- total GC run time 62,5 min

5.10.3 Gas flow rates

Gas flow rates should be set according to the instrument manufacturer's guidance and the analyst's experience.

Suitable gas flow conditions are

- carrier gas helium, 4 ml/min
- make-up gas nitrogen, 30 ml/min
- septum purge 5 ml/min
- split vent 30 ml/min

5.10.4 Injection mode

Use 2 µl splitless with split valve closed for 1 min after injection.

5.10.5 Injection device

Use an automated injector or any suitable alternative means of injection.

For manual injection, the use of a microsyringe capable of injecting 1 µl to 5 µl portions is recommended. Before solutions are injected with the syringe, rinse it at least ten times with *n*-hexane then five times with the solution. After injection rinse the syringe five times with *n*-hexane.

5.10.6 Column

A fused silica capillary column of length 30 m and of internal diameter 0,32 mm is recommended; stationary phase DB-5[3] (5 % methyl phenyl silicone); thickness of the stationary phase 0,25 µm. The performance of the column should be sufficient to achieve satisfactory separation of all components and similar to that given in the specimen chromatogram (see figure A.1).

5.10.7 Detector

An electron-capture detector shall be used with a sensitivity sufficient to detect (twice baseline noise) a 2 µl injection of a 0,001 5 µg/ml *pp*'-DDT solution.

6 Sampling and preparation of test sample

6.1 Sampling

Sample the tobacco in accordance with ISO 4874. Pay particular attention to ensuring that the test sample is representative of the product as received.

6.2 Preparation of test sample

Dry the tobacco in the oven (5.3) set at 50 °C for 2 h to a water content of approximately 5 % (*m*/*m*) after drying.

Grind the tobacco through a 2 mm mesh (5.2) taking care to avoid heating above 50 °C. Alternatively the tobacco may be received in a milled form in which case ensure that the moisture content is less than 10 % (*m*/*m*).

Store the tobacco in sealed containers and exclude light. If samples are kept for longer than one month prior to analysis, they shall be stored in a freezer at a temperature below −8 °C.

[3] DB-5 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

7 Procedure

7.1 Test portions

Weigh, to the nearest 0,01 g, 5 g tobacco test portions into 50 ml beakers. Add 5 g of pretreated Florisil® (4.8) and mix thoroughly. Carry out the procedure described in 7.2 and 7.3.

7.2 Extraction

Add 5 g of pretreated Florisil® (4.8) to a Soxhlet extractor. Transfer the test portion as prepared in 7.1 without mixing so that two separate layers are formed.

NOTE — For recovery determinations, appropriate pesticide standard solutions should be added at this stage, by means of a pipette, to the top of the test portion layer.

Transfer 60 ml of the *n*-hexane (4.2) and 1 ml of the internal standard solution (4.6.1) to a suitable round-bottom flask of capacity 150 ml to 250 ml.

Assemble the extraction apparatus, ensuring good seals at all joints and turn on the heating mantles (5.5).

Regulate the heating element and the tap on the Soxhlet extractor to give a distillation rate of at least 200 ml per hour. The level of *n*-hexane above the tobacco shall be kept constant by adjusting the tap on the Soxhlet extractor. Do not allow the round-bottom flask to become dry. Total extraction time is 4 h 30 min.

After extraction, allow to cool for at least 30 min and take an aliquot portion of the extract for analysis by gas chromatography. No volumetric adjustment is made.

7.3 Linearity

Pipette aliquot portions of 10 ml, 5 ml and 1 ml of mixed stock solution A (4.7.2), into three individual 100 ml volumetric flasks. Add 1 ml of the internal standard solution (4.6.1) to each volumetric flask and dilute to the mark with *n*-hexane (4.2). Pipette an aliquot portion of 1 ml of mixed stock solution B (4.7.3) into two individual volumetric flasks of capacities 100 ml and 200 ml. Add 1 ml internal standard solution (4.6.1) to the 100 ml volumetric flask and 2 ml of the internal standard solution to the 200 ml volumetric flask and dilute both up to the mark with *n*-hexane. This procedure provides pesticide concentrations of approximately 0,5 µg/ml, 0,25 µg/ml, 0,05 µg/ml, 0,005 µg/ml and 0,002 5 µg/ml.

These solutions shall be used to check the linearity of electron-capture detector response. This need only be checked when using a detector for the first time, or after any servicing of the detector or associated electronic circuitry.

7.4 Calibration

If the detector response was found to be linear, a single-level calibration may be used. For single-level calibration, the standard calibration solution (4.7.4) should be used.

7.5 Gas chromatography

Set up the gas chromatograph and equilibrate the system. Check that reproducible results are obtained from triplicate injections of the standard calibration solution (4.7.4). The single value shall not differ from the mean value by more than ± 5 %. Carry out duplicate injections of each sample bracketed by single injections of the standard calibration solution and calculate the mean values.

Specimen chromatograms of the standard calibration solution and a tobacco extract are given in figures A.1 and A.2.

8 Expression of results

The amounts of pesticides are determined by the internal standard method.

The response factor E_p for the respective pesticide is given by the equation

$$E_p = \frac{c_{pst}}{A_{pst}} \times \frac{A_{ist}}{c_{ist}}$$

where

A_{pst} is the peak area or the peak height of the respective pesticide in the standard calibration solution (4.7.4);

A_{ist} is the peak area or the peak height of the internal standard (Mirex) in the standard calibration solution;

c_{pst} is the concentration, in micrograms per millilitre, of the respective pesticide in the standard calibration solution;

c_{ist} is the concentration, in micrograms per millilitre, of the internal standard solution in the standard calibration solution.

The residue content R_p of the respective pesticide, expressed in micrograms per gram dried tobacco, is given by the equation

$$R_p = \frac{A_p \times E_p \times Q_{ist} \times 100}{A_i \times m \times (100 - w)}$$

where

A_p is the peak area or peak height of the respective pesticide in the sample extract;

A_i is the peak area or peak height of the internal standard in the sample extract;

Q_{ist} is the quantity of internal standard added to the extraction solution, in micrograms (approximately 5 µg);

m is the mass of the tobacco test portion (7.1), in grams;

w is the moisture content of dried tobacco (6.2), as a percentage by mass.

9 Repeatability, reproducibility and recovery

Details of a collaborative study on the precision of the method are given in annex B.

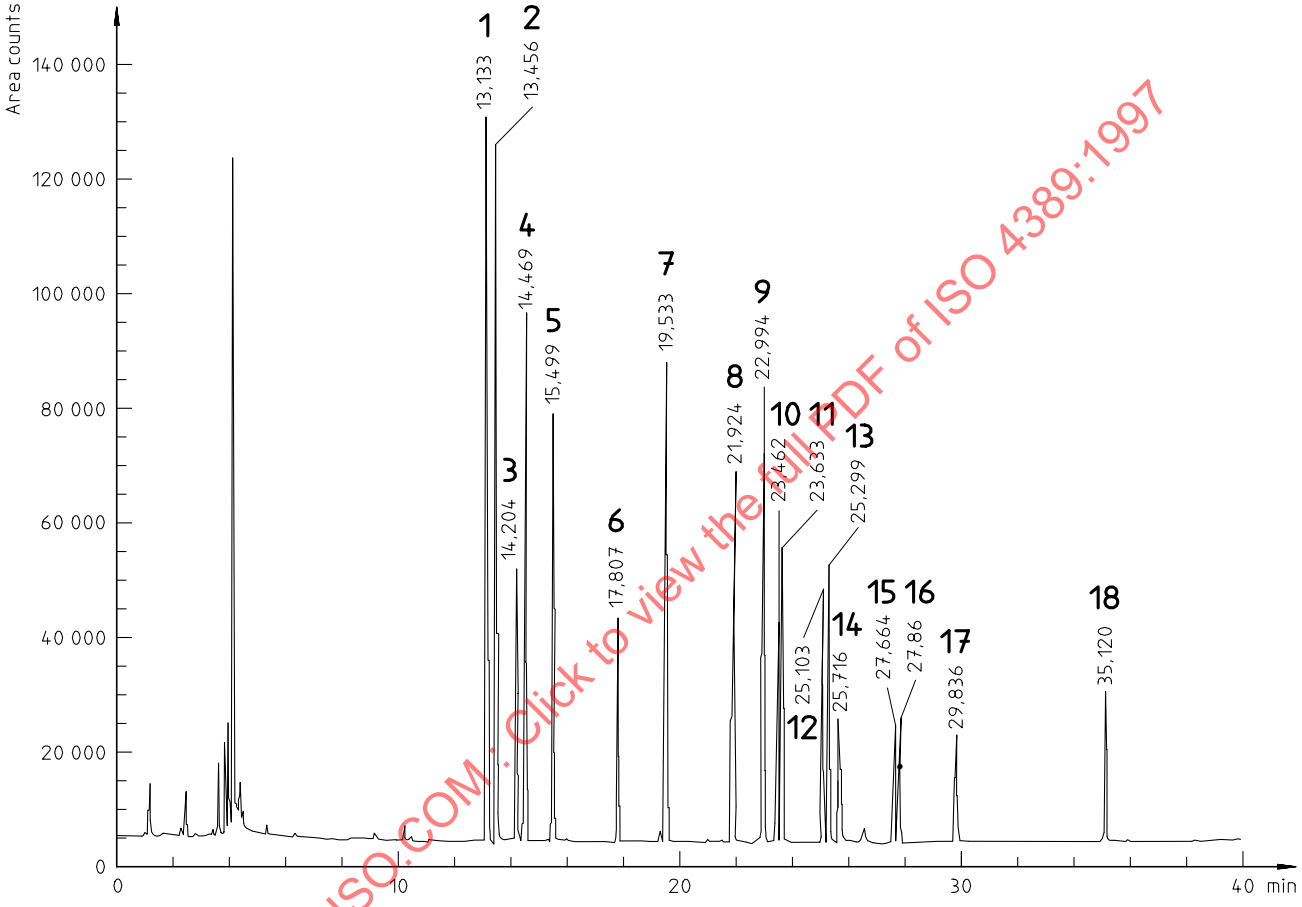
10 Test report

The test report shall show the method used and the results obtained, in micrograms per gram of dried tobacco. It shall indicate the amounts of each of the individual pesticide residues identified. It shall also mention any operating conditions not specified in this International Standard or regarded as optional, as well as any circumstances that may have influenced the results.

The report shall include all details required for complete identification of the sample.

Annex A
(informative)

Examples of chromatograms



Key

1 α-HCH
2 HCB
3 β-HCH
4 γ-HCH
5 δ-HCH
6 Heptachlor
7 Aldrin
8 Heptachlor epoxide
9 *trans*-Chlordane
10 *o,p*-DDE
11 α-Endosulfan
12 Dieldrin
13 *p,p*-DDE
14 *o,p*-DDD
15 Endrin
16 β-Endosulfan
17 *o,p*-DDT
18 *p,p*-DDT

Figure A.1 — Chromatogram of standard calibration solution containing 0,05 µg/ml of each pesticide

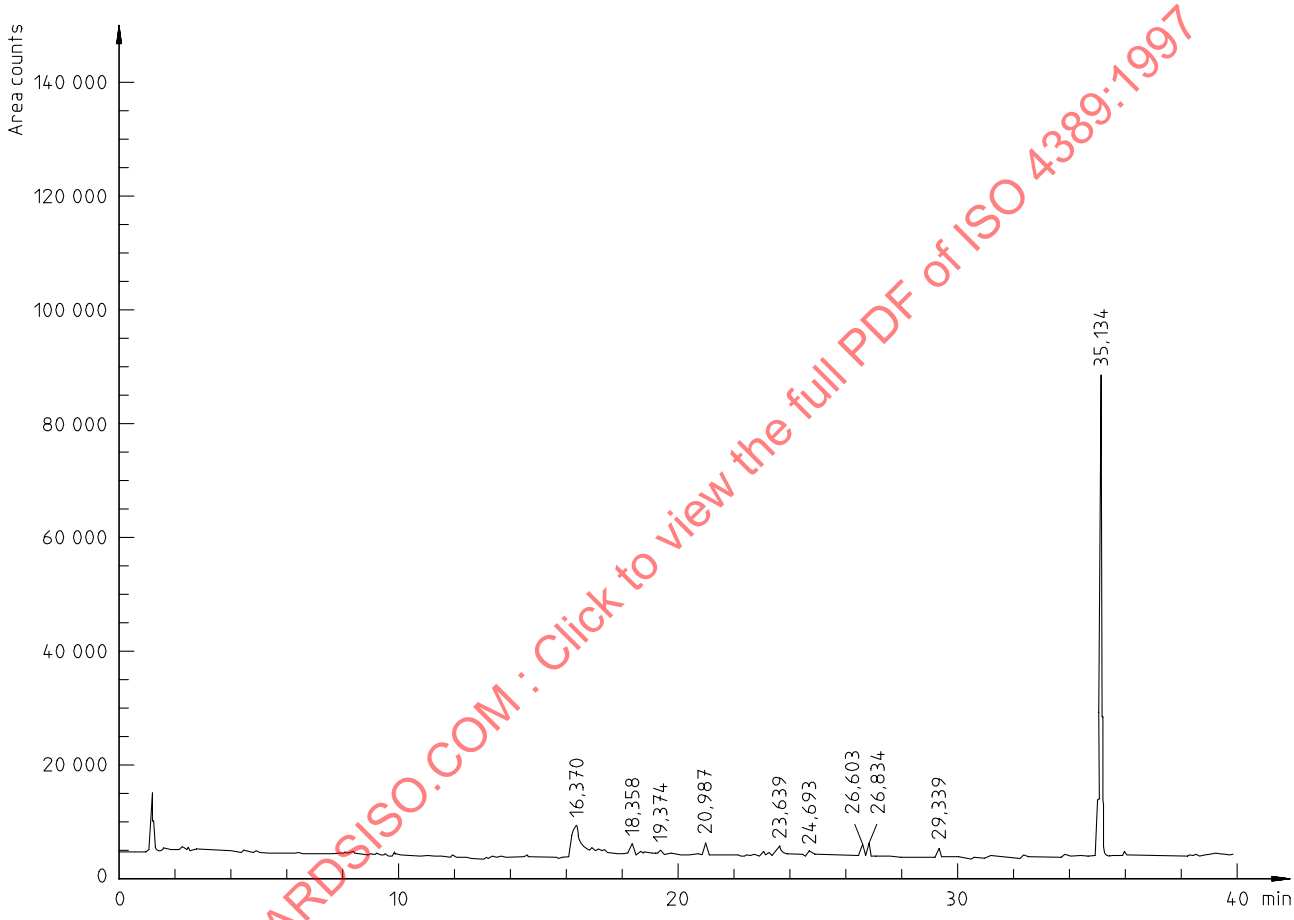


Figure A.2 — Chromatogram of a blank tobacco sample

Annex B
(informative)

Results of interlaboratory tests

In a collaborative study involving 12 laboratories and samples with four spiking levels (F_1, F_2, F_3, F_4), the values given in table B.1 for mean recovery (Rec), standard deviation of repeatability (s_r) and standard deviation of reproducibility (s_R) were obtained.

Each laboratory investigated two samples at each spiking level. As each sample was determined in duplicate, this represents a total of 48 determinations per pesticide at each level. For further details about the statistical evaluation of results, see reference [3] in annex C. Apart from the 17 pesticides mentioned in table B.2, the possible analysis of endrin and ß-endosulfan was also investigated by the joint experiment. Results given in reference [3] show that these two pesticides cannot be analysed by the method.

Table B.1 — Results of the collaborative study

Pesticide	F_1				F_2				F_3				F_4				Detection limit
	Spiking level µg/g	Rec. %	s_r %	s_R %	Spiking level µg/g	Rec. %	s_r %	s_R %	Spiking level µg/g	Rec. %	s_r %	s_R %	Spiking level µg/g	Rec. %	s_R %	s_R %	µg/g
α-HCH	0,0995	79	12	27	0,4975	102	14	18	0,9950	105	8	13	4,9750	95	11	18	0,02
β-HCH	0,1000	89	17	25	0,5000	99	10	15	1,0000	100	6	11	5,0000	86	5	14	0,02
γ-HCH (Lindane)	0,1005	100	26	34	0,5025	101	15	16	1,0050	109	9	19	5,0250	100	7	29	0,01
δ-HCH	0,1020	62	13	27	0,5100	88	7	18	1,0200	96	9	15	5,1000	90	12	22	0,02
HCB	0,1000	109	9	19	0,5000	105	16	18	1,0000	105	8	13	5,0000	88	8	16	0,02
heptachlor	0,1020	100	13	19	0,5100	105	13	15	1,0200	107	12	16	5,1000	101	13	25	0,02
aldrin	0,1000	83	13	24	0,5000	100	14	14	1,0000	107	8	14	5,0000	103	7	25	0,02
heptachlor epoxide	0,1025	84	15	29	0,5125	89	16	19	1,0250	97	16	18	5,1250	91	11	24	0,02
trans-chlordane	0,0995	86	8	23	0,4975	96	6	14	0,9950	102	4	10	4,9750	99	6	18	0,02
o,p'-DDE	0,1010	105	12	25	0,5050	106	9	11	1,0100	110	4	7	5,0500	101	8	19	0,03
α-endosulfan	0,1010	73	14	36	0,5050	81	9	15	1.0100	83	10	18	5,0500	77	10	23	0,03
dieldrin	0,1015	84	15	34	0,5075	87	23	27	1,0150	93	13	22	5,0750	95	14	31	0,02
p,p'-DDE	0,1000	104	10	21	0,5000	115	6	17	1,0000	120	4	15	5,0000	112	6	21	0,02
endrin	0,0995	53	12	35	0,4975	59	30	31	0,9950	58	19	41	4,9750	78	26	43	0,03
β-endosulfan	0,1000	12	17	24	0,5000	12	19	21	1,0000	14	15	21	5,0000	12	9	22	0,02
o,p'-DDD	0,1005	111	9	20	0,5025	109	6	10	1,0050	114	5	10	5,0250	100	9	12	0,03
p,p'-DDD	0,1010	117	14	29	0,5050	113	8	20	1,0100	116	6	19	5,0500	119	13	25	0,02
o,p'-DDT	0,1015	106	11	27	0,5075	109	10	18	1,0150	109	7	14	5,0750	102	8	10	0,04
p,p'-DDT	0,1005	104	13	24	0,5025	111	8	21	1,0050	117	5	22	5,0250	116	11	21	0,06