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DETERMINATION OF WAX CONTENT BY CAPILLARY COLUMN GAS-LIQUID CHROMATOGRAPHY

1. Scope

This method describes a procedure for the determination of the wax content of certain fats and oils, under the test conditions.

It may be used in particular to distinguish between olive oil obtained by pressing and that obtained by extraction (olive-pomace oil).

2. Principle

Addition of a suitable internal standard to the fat or oil, then fractionation by chromatography on a hydrated silica gel column. Recovery of the fraction eluted first under the test conditions (whose polarity is less than that of the triglycerides), then direct analysis by capillary column gas-liquid chromatography.

3. Apparatus

3.1. 25-ml Erlenmeyer flask.

3.2. Glass column for gas-liquid chromatography, 15 mm internal diameter and 30 -40 cm long.

3.3. Suitable gas-liquid chromatograph for working with a capillary column, equipped with a system for direct introduction into the column comprising the following:

3.3.1. Thermostat-controlled oven for the columns, capable of maintaining the desired temperature to within 1º C.

3.3.2. Cold injector for direct introduction into the column.

3.3.3. Flame-ionization detector and converter-amplifier.

3.3.4. Recorder-integrator capable of working with the converter-amplifier (3.3.3.), rate of response below 1 second, with variable paper speed.

3.3.5. Capillary column, glass or fused silica, 10-15m long, 0.25-0.32 mm internal diameter, internally covered with SE-52 or SE-54 liquid, or equivalents, to a uniform thickness of 0.10 - 0.30 µm.

3.4. Microsyringe with facilities for on-column injection, capacity 10 µ1, equipped with a case-hardened needle.

4. Reagents

4.1 Silica gel, 70-230 mesh, art. 7754 Merck.

Place the gel in the oven at 500º C for 4 hours. Allow to cool, then add 2% water. Shake well to homogenize slurry. Keep in darkness for at least 12 hours prior to use.

4.2. n-hexane, for chromatography.

4.3. Ethyl ether, for chromatography.

4.4. n-heptane, for chromatography.

4.5. Standard solution of lauryl arachidate, at 0.1% (m/v) in hexane (internal standard).

4.6. Carrier gas: hydrogen, pure, for gas-liquid chromatography.

4.7. Auxiliary gases:

- Hydrogen, pure, for gas-liquid chromatography.
- Air, pure, for gas-liquid chromatography.

5. Procedure

5.1. Separation of the wax fraction

5.1.1. Preparation of the chromatographic column

Suspend 15 g of silica gel hydrated at 2%, in anhydrous n-hexane and introduce into the column.

Allow to settle spontaneously. Complete settling with the aid of an electric shaker to make the chromatographic band more homogeneous. Percolate 30-ml n-hexane to remove any impurities.

5.1.2. Column chromatography

Weigh exactly 500 mg of the sample into a 25-ml flask, and add a suitable amount of internal standard, depending on the assumed wax content, e.g. add 0.1 mg of lauryl arachidate in the case of olive oil, and 0.25-0.5 mg in the case of olive-pomace oil.

Transfer the prepared sample to the chromatographic column, prepared according to 5.1., with the aid of two 2-ml portions of n-hexane.

Allow the solvent to flow to 1mm above the upper level of the absorbent. Then start chromatographic elution; collect 140-ml of the n-hexane/ethyl ether mixture, at 99:1, at a flow of about 15 drops every 10 seconds (2.1 ml/minute).

Dry the resultant fraction in a rotary evaporator until almost all the solvent is eliminated. Remove the last 2 or 3m1 of solvent with the help of a weak current of nitrogen, then add l0-ml n-heptane.

5.2. Gas-liquid chromatographic analysis

5.2.1. Preliminary procedure, conditioning of column

5.2.1.1. Fit the column to the gas-liquid chromatograph, connecting the inlet port to the on-column system and the outlet port to the detector.

Check the gas-liquid chromatography apparatus (operation of gas loops, detector and recorder efficiency, etc.).

5.2.1.2. If the column is being used for the first time, it is advisable to condition it. Run a light flow of gas through the column, then switch on the gas-liquid chromatography apparatus. Gradually heat to a temperature at least 200 C above the operating temperature (note). Maintain this temperature for at least 2 hours, then regulate the apparatus to the operating conditions (regulate gas flow, light flame, connect to electronic recorder, regulate oven temperature for column, regulate detector, etc.). Record the signal at a sensitivity at least twice as high as that required to perform the analysis. The base-line should be linear, with no peaks of any kind, and must not have any deviation.

A negative rectilinear deviation indicates that the column connections are not correct; a positive deviation indicates that the column has not been properly conditioned.

Note: Keep the conditioning temperature at all times at least 20º C below the maximum temperature specified for the eluent employed.

5.2.2. Choice of operating conditions

5.2.2.1.The operating conditions are generally as follows:

- Column temperature: 80º C at first, rising by 30º C/minute to 120º C, then programmed to increase by 5º C/minute up to 340º C.
- Detector temperature: 350º C.
- Linear speed of carrier gas: hydrogen, 20-35 cm/sec.
- Instrument sensitivity: 4-16 times the minimum attenuation.
- Recorder sensitivity: 1-2 mV, from bottom Of scale.
- Paper speed: 30 cm/hour.
- Amount injected: 0.5 - 1 µl solution.

These conditions may be modified to suit the characteristics of the column and the gas-liquid chromatographic apparatus (in order to obtain chromatograms meeting the following conditions: retention time of C32 internal standard must be 25 $\pm$ 2 minutes and the most representative peak of the waxes must lie between 60 and 100% from the bottom of the scale).

5.2.2.2.Determine the peak integration parameters in such a way as to obtain a correct evaluation of the peak areas considered.

5.2.3. Performance of the analysis

5.2.3.1.Take up 1 µl of the solution with the aid of the 10 µl micro-syringe; draw back the piston until the needle is empty. Introduce the needle in the injection system and inject quickly after 1-2 seconds. After about 5 seconds, gently extract the needle.

5.2.3.2. Perform the recording until the waxes are completely eluted.

The base-line must always satisfy the required conditions (5.2.1.2.).

5.2.4. Peak identification

Identify the peaks from the retention times, by comparing them with mixtures of waxes with known retention times, analysed under the same conditions.

Figure 1 gives a wax chromatogram of a virgin olive oil.

5.2.5. Quantitative analysis

5.2.5.1. Determine the areas of the peaks corresponding to the internal standard and the aliphatic esters from C36 to C46 with the aid of the integrator.

5.2.5.2. Determine the wax content of each of the esters, in mg/kg of fat, according to the formula:

$$\text{ester x mg/kg} = \frac{Ax \,.\, ms \,.\, 100}{As \,.\, m}$$

where:

Ax = area corresponding to the peak for each single ester in square millimetres.

As = area corresponding to the lauryl arachidate peak, in square millimetres.

ms = mass of the lauryl arachidate added, in milligrams.

m = mass of the sample taken for determination, in grams.

6. Expression of the results

Give the different wax contents, and the sum of those contents, in mg/kg of fat.

Appendix

Determination of linear gas speed

Inject 1-3 µl of methane (propane) into the gas-liquid chromatographic apparatus, after adjusting it to the normal operating conditions. Measure the time the *gas* takes to run through the column, from the moment it is injected until the peak emerges (tM).

The linear speed in cm/sec. is given by the formula L/tM where L is the length of the column, in cm, and tM *is* the time measured in seconds.

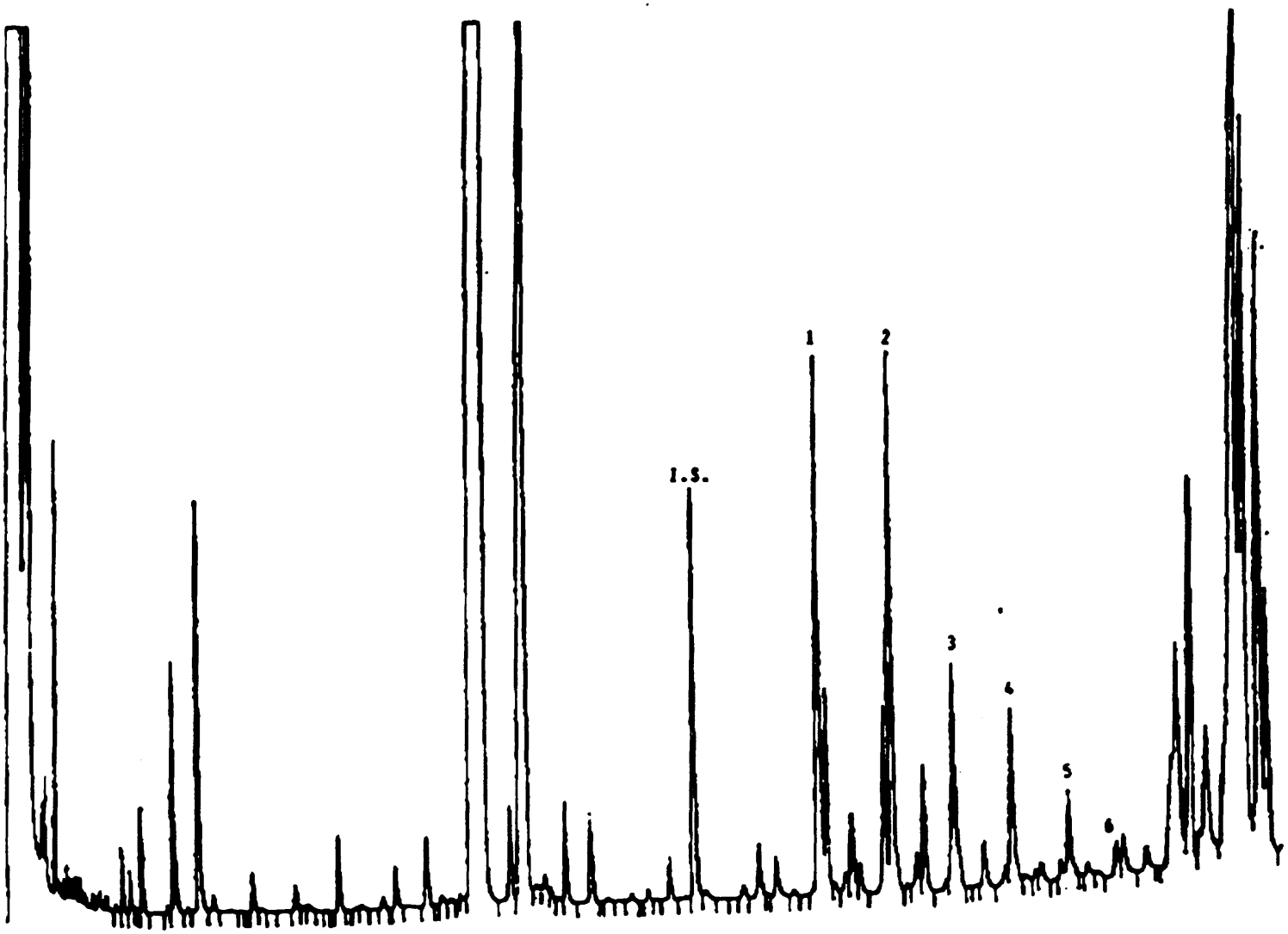

FIGURE 1: Wax chromatogram of a virgin olive oil

I.S. = Internal standard Ester C32
1 = Esters C36
2 = Esters C38
3 = Esters C40
4 = Esters C42
5 = Esters C44
6 = Esters C46