Determination of Pyropheophytin A in Olive Oil

1 Scope and field of application

This method describes a procedure for the determination of the degradation product s of chlorophyll A (pheophytin *A, A*' and pyropheophytin *A*) in olive oil.

1.1 Definitions

The pyropheophtin A content in area % is calculated from the amount of py ropheophytin A as a thermal degradation product of the individual pheophytins and pyropheophytin *A*.

2 Principle of the method

Separation of the pigments (pheophytins, pyropheophytin *A* and chlorophylls) using a miniaturized column chromatography on a silica gel column from the major part of the lipids. The eluate is analysed by RP-18-HPLC and UV-de tection at 410 nm.

4 Reagents

Warning: *Attention is drawn to the regulations which specify the handling of dangerous matter. Technical, organizational and personal safety measurements shall be followed. Unless otherwise stated analytical grade reagents must be used;*

4.1 Chlorophyll A, e.g. Sigma C5753 - 5mg;

4.2 Acetone;

4.3 Methanol;

4.4 Diethyl ether;

4.5 Petroleum ether, boiling range 40 °C to 60 °C;

4.6 Hexane;

4.7 Silica gel cartridge: Strata SI-1(55 µm, 70 A) 1000 mg/ 6 ml supplier: Phenomenex 8B-S012-JCH;

5 Apparatus

5.1 HPLC-system, consisting of a pump, a sample-injecting device (20 µl loop), a VIS-detector for measurements at 410 nm and an evaluation system such as an integrator.

5.2 HPLC column: 250 mm length, 4.0 mm or 4.6 mm internal diameter with reversed-phase type RP 18 filling, of particle size 5 µm; e.g Partisil ODS 3, 5 µm, 250*4.6 mm;

5.3 Taper-shaped flask, of capacity 10 or 25 ml;

5.4 Beakers, in several sizes;

5.5 Volumentric flasks, of capacities 10 ml and 50 ml;

5.3 Autosample vials, of suitable capacity;

5.4 Rotary evaporator, with water bath.

6 Sampling

6.1 Sampling is not part of the method specified in this part. Recommended sampling methods are given in C-I 1 to 5.

6.2 Preparation of test sample

Test samples should be stored cold and light protected. After clean-up the test solutions must be analysed immediately.

7 Procedure

7.1 Weigh, to the nearest 1 mg, about 300 mg of the sample in a small beaker, dissolve it in 1 ml of petroleum ether and pour the solution onto the silica gel column: Rinse the beaker twice with 1 ml portions of petroleum ether.

7.2 As soon as the solvent has drained to the top of column packing elute the non polar substances twice with 5 ml petroleum ether/diethyl ether (90:10; by volume).

7.3 Elute the pheophytin fraction with 5 ml acetone and collect this fraction in a taper-shaped flask (protected from light).

7.4 Evaporate the solvent to dryness on a rotary evaporator at max 20 °C. Dissolve the residue in 200 µl acetone. Use this solution immediately for the HPLC. **Note: pheophytins are very instable at light.**

7.5 High-pressure liquid chromatography (HPLC):

The following conditions have been found to be suitable:

Stationary phase:	Partisil ODS 3, 5 µm, 250* 4.6 mm
Column dimension:	250 mm x 4.6 mm;
Mobile phase:	Water/Metanol/Acetone (4:36:60;by volume)
Flow	1 ml/min
Volume injected	20 µl
Detector:	UV, 410 nm.

8 Results of the determination

8.1 Qualitative analysis:

The chromatographic pattern of the determination may show the peaks of the pigments chlorophyll a, chlorophyll b, the corresponding pheophytins and of pyropheophytin. Reference should me made to the typical chromatogram pre sented in figures 1a and b as the other pigments are not available as stan dards.

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8.2 Quantitative analysis

8.2.1 High performance liquid chromatography

8.2.1.1 Adjust the flow of the mobile phase to 1.0 ml/min and set the detector wavelength to 410 nm.

Inject 20 µl of the sample solution and the standard solutions.

8.2.1.2 I**dentification of the diacylglycerols:**
To identify pheophytin A, pheophytin A' and pyropheophytin A, see the chromatogram of a test sample (Abb. 1a and b).

8.3 **Calculation of the percentage of pyropheophytin A (*w*) in % (A/A):**

8.3.1 Use the peak areas to calculate the concentration of the analytes in the sample solution. For the purpose of this method it is assumed that the response factors of all pigments are equal. The amount of pyropheo phytin A in area % *(w)* has to be calculated using the formula:

$$W = A_{pppA} * 100 / (A_{pp\,A} + A_{ppA'} + A_{pppA})$$

Where:

A_{pppA} is the peak area of pyropheophytin A

$A_{pp\,A}$ is the peak are of pheophytin A

$A_{ppA'}$ is the peak are of pheophytin A'

Give the result to one decimal place.

8.4 Precision

The precision of the method is the result of interlaboratory study organized by the "Joint Committee for the Analysis of Fats, Oils, Fat Products, Related Products and Raw Materials (GA Fett)" on an international basis[*)]. The study was carried out in 2005 on five samples. The results are given in table 1.

Table 1: Summary of statistical results

Sample	A	B	C	D	E
Number of participating	16	16	16	16	16

[*)] Evaluated according to DGF Standard Method A-II 1 "Execution and evaluation of ring tests" and "ISO 5725:1994, Precision of test methods - Determination of repeatability and reproducibility for a standard test method by interlaboratory tests".

laboratories (N) Number of laboratories retained after eliminating outliers (n) Number of individual test results of all laboratories on each sample (z)	12 24	12 24	14 28	15 30	15 30
Mean value (m), %	28,86	34,010	5,63	6,26	84,75
Repeatability standard deviation (s_r) Repeatability coefficient of variation (RCV_r), % Repeatability limit (r)	0,529 1,8 1,48	0,609 1,8 1,704	0,592 1035 1,658	0,363 5,8 1,015	1,087 1,3 3,043
Reproducibility standard deviation (s_R) Reproducibility coefficient of variation (RCV_R), % Reproducibility limit (R)	1,78 6,2 4,981	2,08 6,1 5,814	0,79 14,1 2,219	1,29 20,7 3,622	3,06 3,6 8,554

8.5 **Repeatability limit (*r*):**

The repeatability limit (*r*) is the value less than or equal to the absolute difference of two test results which can be expected with a probability of 95 %, under repeatability conditions.

Repeatability conditions are defined as conditions under which test results are obtained with the same method, on identical test material, in the same laboratory, by the same operator, using the same equipment and reagents, within a short interval of time.

8.6 **Reproducibility limit (*R*):**

The reproducibility limit (*R*) is the value less than or equal to the absolute difference of two test results which can be expected with a probability of 95 %, under reproducibility conditions.

Reproducibility conditions are defined as conditions under which test results are obtained with the same method, on identical test material, in different laboratories, by different operators, using different equipment and reagents, within a short interval of time.

9 Literatur:

9.1 A.Serani, D.Piacenti (2001) Sistema analitico per l'identificazione di oli deodorati in oli vergini di oliva Nota 1 – Analisi di pigmenti clorofilliani in oli vergini di oliva, La Rivista Italiana delle Sostanze Grasse Vol. LXXVIII 459-463

9.2 K. Aitzetmüller (1989) Chlorophyll-Abbauprodukte in pflanzlichen Ölen, Fat Sci. Technol. 91,99-105

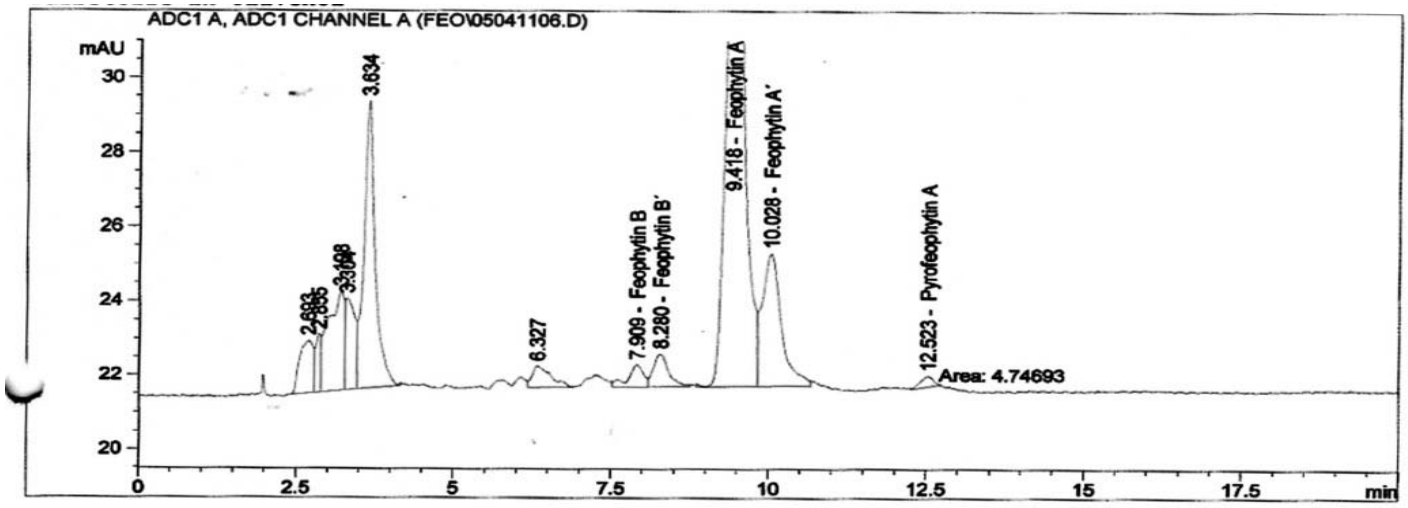

Figure 1a: Olive Oil, extra virgin

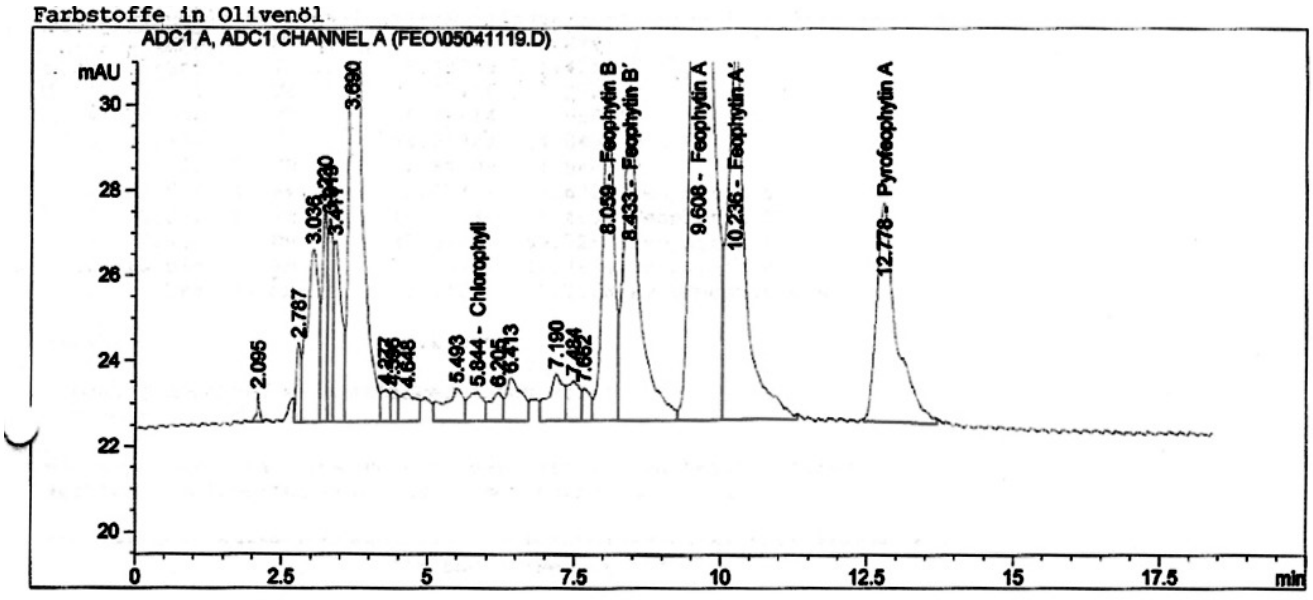

Figure 1b: Olive oil, refined