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# Detection of Reesterified Oils: Determination of Fatty Acids at Position-2 in the Glycerides of Oils

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## ABSTRACT

The IUPAC method II.D.27 for detection of reesterified oils by analysis of fatty acids at position-2 in fat and oil glyceride is reinvestigated and the effect of storage and different refining processes on the glyceride structure of genuine olive oil and olive-residue oil is studied. The method is unsuitable for oils neutralized by steam distillation because of changes in the triglyceride structure. An improved procedure is proposed in order to minimize erroneous conclusions for several other analyses.

## INTRODUCTION

Reesterified oils are produced by esterification of glycerol and distilled fatty acids or by direct reesterification of oils with high percentages of free fatty acids. For the production of reesterified oils, olive oil or olive-residue oil often is used. The products thus obtained have an identical fatty acid composition to olive oil (fatty acid composition of olive oil and olive-residue oil is practically identical). Since reesterified oils are inexpensive, adulteration of the expensive olive oil or even the less expensive olive-residue oil with these oils is attractive.

Olive oil or olive-residue oil and reesterified oils can be characterized by their triglyceride structures. Saturated fatty acids, i.e., palmitic and stearic acids, are esterified almost exclusively at the combined 1,3-positions of olive oil and olive-residue oil triglycerides, as shown with many other triglycerides of vegetable origin (1,2). However, in reesterified oils, fatty acids have a statistical distribution on the 3-positions of glycerol. Determination of palmitic acid at position-2 of the glycerides of olive oil and olive-residue oil has been used for detecting adulteration by reesterified oils in these products. Published procedures include selective enzymatic hydrolysis by mammalian pancreatic lipase, separation of hydrolytic products by thin layer chromatography (TLC), conversion of isolated 2-monoglycerides to methyl esters, and their fatty acid analyses by gas liquid chromatography (GLC) (3,4).

In this study, a reinvestigation of IUPAC method II.D.27 (5) for the determination of fatty acids at position-2 in the glycerides of oils and fats is undertaken. The method has been provisionally agreed upon by the Codex Alimentarius Committee on Fats and Oils to be forwarded for assessment to the Codex Committee on Methods of Analysis and Sampling (6), and has been adopted by the EEC (7). Furthermore, the effect of storage and different refining processes on the glyceride structure of genuine olive oil and olive-residue oil and, consequently, on the results obtained by this method has been studied and an improved procedure is proposed in order to minimize erroneous conclusions for several analyses.

## EXPERIMENTAL PROCEDURES

### Samples

Samples of genuine virgin olive oil and olive-residue oil were collected from the main oil producing areas of Greece. Industrially processed samples of these oils were supplied by Elais Co., Athens, Greece, and by Avea Co., Crete, Greece.

## MATERIALS AND METHODS

The determination of fatty acids at position-2 of glycerides was performed according to IUPAC method II.D.27 (5), except where indicated. Fatty acid methyl esters were prepared according to AOCS method (8) ($BF_3$ + methanol). Pancreatic lipase was from Sigma Chemical Co., USA. The chromatographic plates were from Macherey-Nagel & Co. (SIL G 50,UV 254). Agitation during hydrolysis was performed either with a magnetic stirrer or with a dental amalgamator. GLC: Varian 2800 or Tracor 550 with FID detectors. Columns: DEGS 15% on Chromosorb AW, 100-120 mesh, and EGGS 10% on Gas Chrom Q, 100-120 mesh, both 1.80 × 3 mm id of stainless steel.

Passage over kieselgel: A chloroform solution of oil (4 g in 66 ml) was percolated through a column (33 × 1.8 cm) with kieselgel 60, 70-230 mesh, (Merck, 30 g) at a rate of ca. 60 drops per min. The triglycerides were eluted with benzene (25 ml) at the same rate. The solvent was removed in a rotatory evaporator under nitrogen.

## RESULTS AND DISCUSSION

The percentage of palmitic acid at position-2 in the glycerides of 62 samples of genuine virgin olive oil from the main

TABLE I

Percentage of Palmitic Acid at Position-2 in the Glycerides of Genuine Virgin Olive Oil (n = 62) and Genuine Olive-residue Oil (n = 30) from Greece

| | Minimum | Maximum | Mean | Palmitic acid (%) | |
|---|---|---|---|---|---|
| | | | | No. > 1.5[a] | No. > 2.0[b] |
| Olive Oil (48 samples, acidity to 3.3%) | 0.6 | 2.7 | 1.5 | 20 | 3 |
| | | | | No. > 1.8[a] | No. > 2.0[b] |
| Olive oil (14 samples, acidity = 3.7-9.2%) | 1.4 | 3.6 | 2.6 | 9 | 9 |
| | | | | No. > 2.2[a] | No. > 2.0[b] |
| Olive-residue oil (30 samples, crude or neutralized) | 1.2 | 4.0 | 2.4 | 12 | 18 |

[a]Limits proposed by the International Olive Oil Council (IOOC), expressed as saturated acids (9), i.e., palmitic + stearic acids. The percentage of stearic acid is too small to be measured accurately.

[b]Limits proposed by the European Economic Community (EEC).

oil-producing areas of Greece (years of production: 1975, 1976) was determined. Among the 62 samples, 48 were edible olive oil samples, i.e., having free acidity up to 3.3% (as oleic acid), and 14 had free acidity 3.7-9.2% which would be consumed after refining. The determination was also performed on 30 samples of genuine olive-residue oil from Crete and Corfu produced in 1975, 1976 and 1977. The samples of olive-residue oil were either crude or neutralized by alkali, soda or distillation. The results are summarized in Table I.

From the results of Table I, it is seen that the limit set by the EEC (7) may be considered adequate for edible virgin olive oil and that the limit proposed by the IOOC for this oil is too low. More interesting is the observation that for oils with higher than 3% acidities, which, according to II.D.27 have to be neutralized with alkali in the presence of a solvent prior to hydrolysis, and for oils which have been neutralized in industry the limits appear too low. According to this observation, we have investigated the effect of the neutralization steps described by II.D.27 on the glyceride structure of oils in order to evaluate the method.

IUPAC method II.D.27 includes the following steps: (a) neutralization with alkali in the presence of a solvent for oils having acidities higher than 3%; (b) passage through a column with activated alumina to purify the samples and also to neutralize oils having acidities up to 3%; (c) hydrolysis with pancreatic lipase; (d) isolation of the 2-monoglycerides by TLC; (e) conversion of the 2-monoglycerides to methyl esters; (f) GLC analysis of the methyl esters; (g) expression of results as saturated acids at position-2 of glycerides.

### Steps a and b

Several samples of olive oil and olive-residue oil with acidities ranging from 3.3-35.5% were neutralized with alkali according to step a, with soda, over alumina according to step b, and over kieselgel. Using the column with kieselgel as described in Experimental Procedures, oils with acidities up to 35% may be neutralized. The results shown in Table II indicate that steps a and b clearly affect the glyceride structure, probably because some hydrolysis of ester bonds followed by acyl migration is taking place. The effect is stronger with alkali, but alumina also results in higher percentages of palmitic acid in position-2 when compared to kieselgel. It has been reported that with alumina, some hydrolysis of triglycerides ester bonds may occur (10). In order to verify the effect of alkali, 3 samples were left with the neutralizing solution for 3 hr; the results indicate it may be preferable to achieve neutralization of oils prior to hydrolysis by kieselgel and eliminate both steps a and b.

### Step c

Concerning enzymatic hydrolysis (step c), method II.D.27 states the tube containing the hydrolysis mixture should remain in a thermostat at 40 ± 0.5 C for 1 min (under agitation) after which it is removed and agitated for another

TABLE II

Effect of Neutralization Steps on the Percentage of Palmitic Acid in Position-2 in the Glycerides of Olive Oil and Olive-residue Oil

| Sample | Acidity (%) | Alkali | Soda | Alumina | Alkali (3 hr) | Kieselgel |
|---|---|---|---|---|---|---|
| Olive oil | 3.3 | 2.0 | - | 1.4 | - | - |
| | 4.4 | 3.4 | 2.6 | 2.6 | - | 1.8 |
| | 4.8 | 1.4 | - | 1.1 | - | 1.0 |
| | 5.5 | 2.9 | 2.3 | 1.5 | - | 1.2 |
| | 5.6 | 1.6 | - | 1.0 | - | - |
| | 5.9 | 2.8 | 2.1 | 1.4 | - | - |
| | 7.0 | 1.5 | - | 0.9 | - | - |
| | 8.0 | 2.1 | - | 1.1 | - | - |
| | 9.2 | 3.1 | 2.3 | 1.4 | - | 1.1 |
| Olive-residue oil | 16.6 | 3.9 | - | 1.8 | 8.0 | 1.4 |
| | 18.8 | 3.5 | 2.7 | 2.0 | 5.9 | 1.4 |
| | 19.1 | 2.6 | - | 1.6 | 5.7 | 1.2 |
| | 22.5 | 2.7 | 2.1 | - | - | - |
| | 35.5 | 4.0 | 3.4 | - | - | - |

2 min at room temperature, and then cooled under running water before 1 ml of 6 N hydrochloric acid is added to stop hydrolysis. We measured the temperature of the tube content after 0.5 and 1 min during the hydrolysis for 1 min in the thermostat and it was 32.5 and 37 C, respectively. Thus, the duration of hydrolysis is extended to more than 3 min, which is considered long, whereas the temperature for a fraction of a min only reaches 37 C. The hydrolysis conditions should be chosen to achieve deacylation as rapidly as possible and to minimize undesirable acyl migration in the partial glycerides. Optimal reaction conditions are considered, i.e., reaction temperatures 37-40 C and duration of hydrolysis < 90 sec (11). The Luddy et al. (3) semimicro procedure is recommended by which the tube and contents are first warmed at 40 C for 1 min without shaking, followed by vigorous shaking for 1 min at the same temperature.

Luddy et al. (3) report that the degree of hydrolysis with their procedure (enzyme/sample ratio is similar in the 2 methods) reaches 50 ± 5%. Using the II.D.27 procedure, we found that, by weighing the fatty acids isolated by TLC, the hydrolysis did not exceed 22%.

### Step d

After hydrolysis is stopped with 6 N hydrochloric acid, the hydrolysis products are extracted with diethyl ether. According to step d, the 2-monoglycerides are then isolated by TLC with a developing solvent consisting of hexane/diethyl ether/formic acid, 70:30:1 (v/v/v). Under these conditions, the $R_f$ of 2-monoglycerides is ca. 0.035, which is low. The amount of formic acid in the developing mixture should be increased to 2-3% in order to double the $R_f$ value and avoid removal of components remaining on the baseline.

### Step g

The IOOC has proposed to the Codex Committee on Fats and Oils that results be expressed as saturated acids in position-2 of glycerides (i.e., palmitic + stearic acids). Stearic acid in the fatty acids of olive oil does not exceed 2.5-3%, thus the percentage of stearic acid in position-2 is too small to be calculated accurately. We recommend that results be expressed as percent palmitic acid, which has been adopted already by the EEC (7).

### Storage

We investigated the effect of storage on the glyceride structure—especially of oils with high acidities resulting in considerable partial glyceride content.

Samples of crude olive-residue oil, with acidities ranging from 10.2-35.5% as oleic acid stored for 1-2 years, have shown considerable increases in the percentage of palmitic acid at position-2 of their glycerides when examined by method II.D.27. On the contrary, 2 samples of olive oil with acidities 0.9% and 2.1% have shown no alteration.

Since the structure of partial glycerides is expected to be altered with time, palmitic acid at position-2 is also determined in the pure triglycerides of the oils. Columns with kieselgel are used for neutralization purposes, but in this case, also for isolating the pure triglycerides of the oils (12). The results of Table III suggest that the enzymatic hydrolysis should be performed on the pure triglycerides, at least for oils containing considerable percentages of partial glycerides in which the structure is altered during storage. This is a second reason that chromatography over kieselgel is preferred versus neutralization with alkali and/or over alumina of oils prior to hydrolysis.

### Refining

In order to compare possible alterations caused by different refining procedures, palmitic acid in position-2 of glycerides has been determined according to II.D.27 and according to the improved procedure proposed here, i.e., neutralization and isolation of pure triglycerides over kieselgel in 3 crude olive-residue oils (acidities 17.5, 21.4 and 24.5%). The determination was repeated in the same oils after one part of each of them was neutralized industrially with soda and the other part by steam distillation, and in their final

TABLE III

Effect of Storage on Percentage of Palmitic Acid at Position-2 in the Glycerides of Olive-residue oil and Virgin Olive Oil

| | Acidity (% oleic acid) | According to II.D.27 (IUPAC) | With neutralization and isolation of pure glycerides over kieselgel |
|---|---|---|---|
| Olive-residue oil produced in 1975 and examined in: | | | |
| 1976 | 10.2 | 2.2 | |
| | 18.9 | 1.8 | |
| 1978 | 15 | 3.1 | 1.5 |
| | 26 | 4.4 | 1.3 |
| Olive-residue oil produced in 1976 and examined in: | | | |
| 1977 | 22.5 | 2.7 | |
| | 18.8 | 3.5 | |
| | 16.6 | 3.9 | |
| | 35.5 | 4.0 | |
| 1978 | 24.6 | 4.5 | 1.4 |
| | 24.2 | 5.8 | 1.3 |
| | 18.6 | 5.4 | 1.8 |
| | 38.5 | 5.0 | 1.7 |
| Virgin olive oil produced in 1976 and examined in: | | | |
| 1977 | 2.1 | 1.5 | |
| | 0.9 | 1.1 | |
| 1978 | 2.2 | 1.6 | 1.4 |
| | 0.9 | 1.2 | 1.1 |

TABLE IV

Effect of Different Refining Processes on the Percentage of Palmitic Acid at Position-2 in the Glycerides of Olive-residue Oil and Olive Oil

| Sample | Crude | Neutralized with soda | Refined | Neutralized by distillation | Refined |
|---|---|---|---|---|---|
| Olive-residue oil I | 4.2[a] | 3.1[a] | 3.3[a] | 5.4[a] | 5.7[a] |
| | 2.0[b] | 1.1[b] | 1.2[b] | 4.0[b] | 3.9[b] |
| Olive-residue oil II | 2.6[a] | 2.9[a] | 2.8[a] | 4.2[a] | 4.4[a] |
| | 1.8[b] | 2.0[b] | 2.0[b] | 3.7[b] | 3.8[b] |
| Olive-residue oil III | 2.9[a] | 2.9[a] | 2.8[a] | 5.8[a] | 5.7[a] |
| | 1.5[b] | 1.8[b] | 1.9[b] | 4.2[b] | 4.2[b] |
| | | | Neutralized with alkali | | |
| Olive oil I | 2.0[a] | 1.4[a] | 1.4[a] | - | - |
| Olive oil II | 1.8[a] | 1.6[a] | 1.6[a] | - | - |
| | 0.9[b] | 0.9[b] | 1.1[b] | - | - |

[a]According to IUPAC method II.D.27.

[b]According to the proposed improved procedure, i.e., neutralization and/or isolation of pure triglycerides over kieselgel.

refined products. Moreover, determinations have been performed on 2 samples of olive oil (acidities 3.5 and 3.8%) before refining, after neutralization with alkali and on the final refined products. The results are shown in Table IV. Again the need to eliminate steps a and b of method II.D.27 and replacement by kieselgel column chromatography are indicated. Furthermore, by comparing results obtained by the 2 different industrial neutralization procedures, it is apparent that steam distillation affects the triglyceride structure of the oils to a considerable extent. Steam distillation had been previously reported to affect the glyceride structure of oils (13). Thus, the method is found to be inapplicable to oils neutralized by steam distillation. Therefore the part of the footnote is necessary which was proposed by an IUPAC observer at the Tenth Session of the Coeex Committee on Fats and Oils (14): and reads: "Certain refining processes may affect the glyceride structure of the oil and lead to the level of palmitic acid in position-2 of genuine oils that have not been subjected to reesterification being above the maxima given." This statement is to be included in our proposed procedure. Otherwise, refining processes do not affect the results obtained to a detectable extent.

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