Should 100% methyl malvalate or sterculate be desired, further separation from the accompanying common fatty esters can be achieved by standard procedures. Both cyclopropenoid esters can be precipitated from linoleate during a low temperature crystallization and palmitate can be separated by the urea complex technique (16,17).

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Detection of Adulteration of Olive Oil with Seed Oils by a Combination of Column and Gas Liquid Chromatography[1]

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ABSTRACT

Samples of virgin olive oil and refined seed oils, as well as mixtures of olive oil with 10 and 5% seed oils were fractionated by column chromatography on silicic acid impregnated with ammoniacal silver nitrate. It was possible to isolate a characteristic fraction enriched in polyunsaturated triglycerides. Its linoleic acid content in pure olive oil never exceeds 9.3%, whereas in pure seed oils, it varies between 38.1 and 70.1%; in mixtures of olive oil with 10 and 5% of seed oils, the respective values are 22.3-38.2% and 15.6-32.1%. The oleic-to-linoleic acid ratios of the same fraction are more than 7.6 (olive oil), 0.2-0.8 (seed oils), 1.1-2.0 (olive oil with 10% seed oils) and 1.4-3.6 (olive oil with 5% seed oils). These analytical values may be used as a safe criterion for the eventual adulteration of olive oil with seed oils.

INTRODUCTION

Olive oil is frequently adulterated by other vegetable oils of a lower commercial price. Official methods for purity control of olive oil include the old classic criteria (physical and chemical constants [1]) in combination with the determination of the specific extinction coefficient in the ultraviolet (UV) (2) and the fatty acid composition by gas liquid chromatography (GLC) (3-5). However, it is largely recognized that when the level of the adulteration is lower than 10-15%, these criteria are insufficient to give reliable conclusions.

The GLC analysis of the fatty acid composition of a natural oil does not always lead to safe indications regarding its purity, since most natural oils—mainly the vegetable oils—contain the same fatty acids in variable amounts. Thus, in adulterating an oil with low proportions of one or more other oils, the determination of the overall fatty acid composition is not sufficient to reveal the admixture.

For this reason, a deeper insight into the structural features of the natural oils is required to solve this problem, based on the composition of certain characteristic glyceride fractions (6). This approach is based on the "even" (7) and the "restricted random" (8) distribution theories which are believed to describe with a satisfactory approximation the rules governing the distribution of individual fatty acids into glycerides' molecular species in most natural oils.

On the other hand, the degree of unsaturation of triglycerides (i.e., the presence in their molecule of one or more double bonds) is related to their polarity and therefore, by the use of suitable analytical techniques they may be separated into groups containing triglycerides of several types, e.g., saturated (of the type SSS), monounsaturated (of the type SSO), diunsaturated (of the types SOO and SSL), where S, O, L, denote, respectively, saturated acyl-, oleoyl- and linoleoyl- moieties of triglycerides. These groups have a characteristic and almost constant relative amount of fatty acids, which is very different from oil to oil.

For the separation of triglycerides on the basis of their degree of unsaturation most suitable seems to be the thin layer chromatographic (TLC) technique on silica gel impregnated with silver nitrate, initially used by Barret et al. (9) in order to study their structure.

[1] This work was taken in part from the doctoral dissertation of S. Passaloglou-Emmanouilidou.

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Applying this technique improved by the use of the ammoniacal silver complex ion (10), in combination with the GLC determination of fatty acids, Galanos et al. (11) achieved the isolation of a characteristic polyunsaturated glyceride fraction, which proved very useful for the detection of olive oil adulteration by small percentages (2.5% or less) of other vegetable oils.

In this investigation, we attempted to devise a more reliable method for the detection of the admixture of olive oil with low percentages of refined seed oils by substituting the thin layer by column chromatographic isolation of a fraction enriched in characteristic polyunsaturated triglycerides, and their species were then revealed by GLC analysis of fatty acid methyl esters.

Column chromatography has three fundamental advantages over TLC which affect the reliability of the results: (a) more flexibility in eluting systems, (b) considerably larger sample quantities, and (c) direct collection of eluting fractions. In the final stages of this investigation, special care was taken to simplify the overall analytical procedure in order to increase its utility in routine laboratory work.

EXPERIMENTAL METHODS

Materials

Virgin olive oil samples of guaranteed purity with a free fatty acid content less than 1% (expressed as oleic acid) were taken from the chemical laboratory of the Greek Ministry of Commerce or from various oil-producing industries.

The samples of refined seed oils (corn oil, cottonseed oil and soybean oil) were taken from commercial sources. All the olive oils used were of Greek origin, whereas the seed oils were of domestic or foreign production.

The solvents and reagents used were of the maximal purity available commercially and some of them, e.g., petroleum ether and benzene, were purified by distillation.

Column Chromatography

The adsorbent for column chromatography consisting of silica gel impregnated with ammoniacal silver nitrate was prepared as follows: 20 g of silicic acid (previously purified and activated) is mixed with 60 ml of ammoniacal silver nitrate solution (7.2 g $AgNO_3$ in 20 ml of 10% NH_4OH; concentrated ammonia is added by drops until the complete solubilization of Ag(OH) occurs and then diluted to 60 ml with acetone). The mixture is heated in a water bath at 70-80 C until it reaches a pulpy consistency and finally, it is oven-dried at 110 C for 12 hr.

This material can be stored for several weeks in a dessicator, even unprotected from light, because it resists darkening considerably and does not make any difference in the separating capacity of triglyceride mixtures.

Column chromatographic fractionations were accomplished on a standard-joint glass column (40 cm long and 10 mm id) fitted with a 50-ml separatory funnel on its upper end, and bearing a porous diaphragm and a stop-cock in its lower part. The eluates were collected in a 50-ml vacuum filtering flask fitted with drying $CaCl_2$-tube in its side arm, and placed in an ice bath. The separatory funnel was closed with a curved glass tube filled with glass-wool.

Twelve g of adsorbent prepared as already described was slurried with 50 ml of the first eluent and transferred into the chromatographic column. The oil samples (80-90 mg dissolved in 2-3-ml of the first eluting solvent) were pipetted into the column, taking care not to disturb the adsorbent; during the fractionation, the temperature of the column was maintained at 15-20C by the room air conditioning. If the room temperature is above 20 C, a jacketed column is necessary (12).

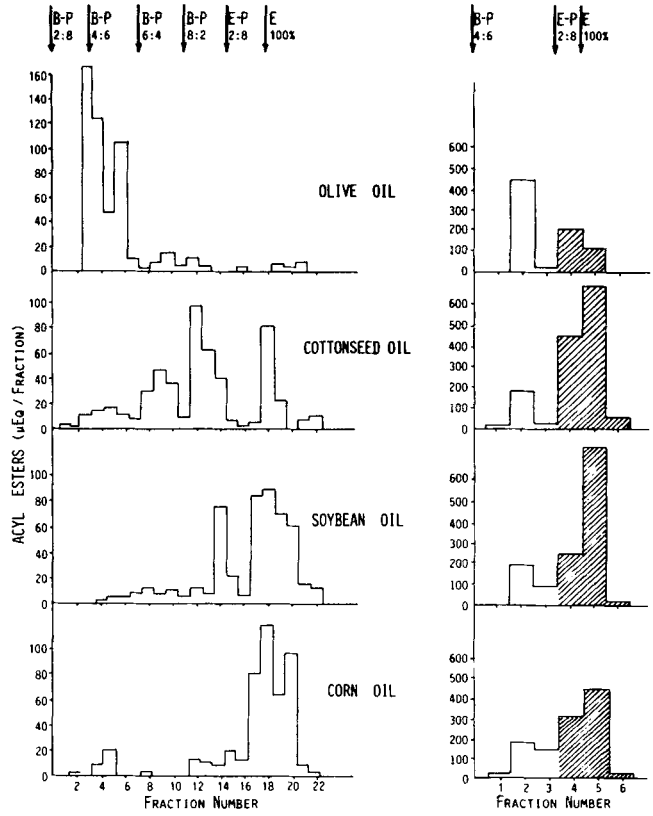

FIG. 1. Comparative fractionation data of virgin olive oil and seed oils by argentation column chromatography. The bulk-fractionation (second-stage) scheme indicated on the right was based on the elution patterns of the first-stage experiments depicted on the left. Open and dashed areas of the second-stage patterns (right) represent the two final combined-bulk fractions collected in the routine method. Elution was accomplished with mixtures of benzene (B), petroleum ether (P) and diethylether (E), as indicated in the upper edge. Fraction volumes in the left-side patterns were 10 ml, whereas in the right-side patterns they were 20 ml for fractions 1-3 and 30 ml for fractions 4-6.

As eluents, various combinations of benzene, petroleum ether and ethyl ether were used in order to provide a progressively increasing polarity as specified in Figure 1.

The percentage of triglycerides recovered after fractionation of the four oils described amounts to 90.9-96.8% of the sample quantity.

The triglyceride content of the fractions collected was assayed by spectophotometric determination of their ester contents (13).

Gas Chromatographic Analysis

Fatty acid methyl esters were prepared according to Morrison and Smith (14).

For methyl ester analysis, a Hewlett-Packard dual column gas chromatograph was used (Model 5700A, with a differential flame ionization detector and electronic integrator. The columns were of coiled stainless steel, 6 ft long and 1/8 in. id, filled with 10% DEGS on 80-100 mesh WAW-DMCS. Conditions were: column temperature, 184 C (isothermal); detector and injection port temperature, 250 C; carrier gas, helium with a flow rate of 36 ml/min; attenuation, 10 x 32 or 10 x 64; chart speed, 15 in./hr; sample volume, 0.1-1 μl of a 1-2% methyl esters solution.

The identification of peaks was done by comparing them to fatty acid methyl ester mixtures of known composition. The relative amount of fatty acids was calculated from the peak areas by the integrator.

Routine Method for Testing Adulterations

One g of the (olive) oil under examination is dissolved in 20 ml of benzene-petroleum ether (4:6, v/v), and 2 ml of this solution is applied to a column prepared as already described. The column is eluted with 60 ml of the same solvent mixture, and the effluents are discarded. Then, 30 ml of diethyl ether/petroleum ether (2:8, v/v) and 60 ml of plain diethyl ether are passed successivly through the column, and the effluents are collected in one pooled fraction. After evaporation of the solvents, the fatty acid composition of the eluted material is determined by GLC analysis of their methyl esters, prepared as already described.

RESULTS AND DISCUSSION

All the samples of virgin olive oil and refined seed oils examined in the present work, when analyzed for their fatty acid compositions prior to fractionation, were proven to lie within the normal ranges of individual fatty acid contents described in the literature.

The samples of olive oil analyzed, in particular, included extreme cases with respect to their linoleic acid content (close to the lower and higher limits), since it is well known that olive oils poor in linoleic acid may be adulterated with 10-20% of seed oils and still yield an overall linoleic-acid content lower than the highest one for virgin olive oils.

For this reason, the sample of olive oil used as a standard for comparison during our work contained one of the highest linoleic acid (15.8%) and the lowest oleic acid (66.9%) contents.

Therefore, the results in this paper refer to the limiting worst conditions of adulterating olive oil with seed oils.

Elution Patterns of Pure Vegetable Oils

In the first stage of this investigation, pure vegetable oils (virgin olive oil, and refined corn, cottonseed and soybean oils) were fractionated with six eluting solvent mixtures of increasing polarity and their elution patterns were followed in detail by collection 22 10-ml fractions. Each of these 22 fractions was then analyzed for total ester content as a measure of the quantity of the eluted triglycerides.

The results (summarized in the left side of Fig. 1) indicated that the bulk of the triglyceride components from olive oil was eluted with benzene/petroleum ether (2:8 and 4:6, v/v), whereas from the seed oils the bulk of material was eluted with solvent mixtures of higher polarity. These elution patterns are in accordance with the triglyceride species compositions of the fractionated vegetable oils, since the first fractions (up to number 7) contain the mono- and diunsaturated species of the types SSO, SOO and SSL. Elution of tri- and tetraunsaturated triglycerides (OOO, SOL, SSLn and OOL, SLL, SOLn) occurs in the next fractions, whereas the triglycerides with five or more double bonds are eluted in the last fractions (after number 14).

Since each of the aforementioned triglyceride species may be present in more than one of the collected fractions depending on its overall content in the oil tested, we considered that each of the peaks distinguishable in these elution patterns contains mainly triglycerides of the same group (or degree of unsaturation). On this basis, fractions under the same peak were then pooled, and these pooled fractions (6 from olive and corn oils, 5 from cottonseed oil and 4 from soybean oil) were analyzed by GLC for their fatty acid composition.

As expected, the most clear and characteristic differences were observed in the last of these pooled fractions, i.e., (a) its linoleic acid content was only 4.7% for olive oil, whereas it amounted to 68.4, 55.2 and 73.2% for cottonseed, soybean and corn oils, respectively; (b) its ratio of oleic to

TABLE I

Analytical Data of the Combined Second Fraction of Olive Oil, Seed Oils (Fig. 1), and Their Mixtures

Fatty acids	Olive oil	Corn oil	Cottonseed oil	Soybean oil	Olive + 10% corn	Olive + 10% cotton	Olive + 10% soyb.	Olive + 5% corn	Olive + 5% cotton	Olive + 5% soyb.
Myristic	--	0.2	0.4	0.4	0.4	0.7	--	--	--	--
Palmitic	14.4	7.6	28.5	9.2	23.5	17.3	8.7	30.6	13.8	14.2
Palmitoleic	1.3	0.4	1.2	1.6	3.2	1.1	1.9	1.5	0.6	1.8
Stearic	3.0	1.4	2.5	3.3	4.2	3.7	2.4	3.6	3.2	3.4
Oleic	70.7	19.0	27.8	36.1	44.6	50.1	42.5	48.7	63.6	45.7
Linoleic	9.3	70.1	38.1	42.4	22.8	25.9	38.2	15.6	17.4	32.1
Linolenic	1.3	1.3	1.5	7.0	1.3	1.2	6.3	1.0	1.4	3.8
Ratio O/L	7.6	0.2	0.7	0.8	2.0	1.9	1.1	3.1	3.6	1.4
Percentage of total triglyceride (w/w)	38.9	62.5	79.2	74.0	51.5	50.2	57.6	48.6	45.1	41.6

linoleic acid content (O/L) was 5.8 for olive oil, whereas in the three seed oils, it ranged between only 0.2 and 0.3; (c) its size was quite different from oil to oil, representing only 2.5% of the overall eluted material in the case of olive oil, and 21.3, 65.6 and 22.8% in the cottonseed, soybean and corn oils, respectively. These great differences in size are quite obviously significant in augmenting the former two features (L content and O/L ratio) of polyunsaturated triglyceride fractions isolated from adulterated olive oils.

Bulk-Fractionation of Pure and Admixed Samples

On the basis of the results just described, which are in accordance with the general features of earlier publications by De Vries (12) and Galanos et al. (11), we proceeded to the second stage of this investigation as follows: the composition and volumes of the eluting agents were modified first, to simplify and shorten the experimental procedure for the routine testing of adulterations, and second, to provide better resolution of the more popular triglycerides.

As summarized in the right side of Figure 1, this "second stage" fractionation scheme involved the collection of three 20-ml fractions eluted with benzene/petroleum ether (4:6, v/v), one 30-ml fraction eluted with diethyl ether/petroleum ether (2:8, v/v) and two 30-ml fractions eluted with plain diethyl ether. The respective elution diagrams depicted in Figure 1 (right side) suggest that these six fractions can be reduced to only two, the fatty acid composition of which should be sufficient to devise the routine method (already described in Experimental Methods) for testing the adulterations of olive oil by seed oils.

This routine method was then applied to samples of the same pure oils (olive oil, corn oil, cottonseed oil and soybean oil) and to mixtures of olive oil with each of the three other seed oils in concentrations of 10 and 5%, as well. Table I shows the results of determination of fatty acid composition of the second (combined) fractions of all the pure oils examined at this stage of this investigation and adulterated samples of olive oil, as well.

The results of the table suggest that this second characteristic fraction isolated from seed oils contains a high percentage (62.5-79.2%) of eluted triglycerides, whereas the fraction from olive oil this fraction amounts to 38.9% of the original sample.

The admixture of the olive oil with 10 or 5% corn, cottonseed and soybean oil increases the amount of triglycerides contained in this fraction, leaving no doubt that it is enriched in polyunsaturated triglycerides.

The linoleic acid content of this fraction does not exceed 9.3% for olive oil, whereas in the other three seed oils, it varies between 38.1 and 70.1%; in olive oil adulterated by 10% of seed oils, it ranges between 22.8 and 38.2% and in adulteration of olive oil by 5% seed oils, the linoleic acid percentage varies between 15.6 and 32.1%.

Another characteristic value of this last fraction is its O/L ratio. Thus, while in pure olive oil this ratio always has a high value (7.6), in pure seed oils the O/L ratio is much lower, lying between 0.2 and 0.8.

When olive oil is mixed with 10% of the oils just described, the O/L ratio increases to a level of 1.1-2.0 and in the 5% admixture, this ratio varies from 1.4 to 3.6, i.e., in all cases, it remains considerably lower than the O/L ratio of pure olive oils.

The linolenic acid content in the respective soybean oil fraction amounts to 7.0%, whereas in the same fraction of olive, corn and cottonseed oils, it varies between 1.3 and 1.5% and does not exceed those limits in mixtures of olive oil with corn and cottonseed oil. On the contrary, in mixtures of olive oil with soybean oil the linolenic acid content of the last fraction reaches a percentage of 6.3 to 3.8% when the level of adulteration is 10 and 5%, respectively. Therefore, the linolenic acid content of this last fraction may be used for the identification of adulteration of olive oil by soybean oil.

The experimental data of this stage are in complete agreement with the results of our preliminary work, as well as with the findings of other investigators (13,14).

On the basis of these observations, we believe that the application of the analytical technique described here leads to safe conclusions with respect to the detection of eventual adulteration of olive oil by seed oils. Important features of this method are simplicity and brevity, permitting its application in the routine analysis of oils.

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