Detection of Adulteration of Olive Oil by Argentation Thin Layer Chromatography

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Abstract

Minute amounts of adulterant seed oils in olive oil can be detected by GLC analysis of fatty acids of the polyunsaturated triglyceride fraction, obtained by TLC on silver nitrate impregnated silica gel. Every possible effort was made to avoid any critical or time consuming manipulation in this method in order to develop it as a routine testing procedure. A complete analysis is possible in less than 2 hr and the detection of as low as 2% adulteration by other seed oils is accurate and reliable.

Introduction

THE DIFFICULTIES OF TESTING olive oil for adulteration by other seed oils in small amounts (below 20%) using color reactions or estimations of physical and chemical constants of the overall sample have already been discussed (1). A reliable method described for this purpose (1) is based on GLC analysis of a fraction isolated by low temperature crystallization, which is enriched in triglycerides containing at least one saturated acyl residue per molecule. The fatty acid composition of such fractions isolated from virgin olive oils of either very high or very low linoleic acid content is quite constant as they contain mainly SOO[2] accompanied by some SSO. Adulteration by seed oils is identified by a significant change in the fatty acid pattern due to the presence of SSL and SLL, i.e., the linoleic acid analogs which are widespread in seed oils. However, although the original procedures of low temperature crystallization (2) were significantly simplified for this application, the method still requires relatively long time and is difficult for use as a routine testing procedure. For this reason, a much faster and simpler method was devised in this laboratory. It involves GLC analysis of the fatty acids (as methyl esters) of a selected triglyceride fraction, namely the fraction of triglycerides with four or more double bonds per molecule, which is isolated by argentation TLC.

Although separation of triglycerides in classes of the same degree of unsaturation by argentation TLC has proved to be extremely useful in the determination of the triglyceride composition of natural fats and oils (3–7), the method has some minor or serious disadvantages. For example, the recognized incapability of complete resolution of mixtures rich in polyunsaturated triglycerides in a single run, the varying mobilities of individual triglycerides according to their relative amounts in the mixture or to the amount applied for separation, and finally problems related to the nondestructive location of bands with appreciable sensitivity, since iodine vapors, extremely useful agents, do not react in presence of the silver ion.

In the present adaptation of the method aimed at detecting variable small amounts of seed oils in olive oil such problems did naturally arise, and they were carefully considered in order to achieve maximum reliability and reproducibility along with a minimum time requirement for complete analysis.

Experimental Procedures

Materials

Samples of virgin olive oils of guaranteed purity were obtained from the Special Experimental Laboratory of the Greek Ministry of Commerce. Samples of seed oils were obtained from commercial sources. Boron trifluoride, 14% in methanol, was obtained from Applied Sciences Laboratory, State College, Pa. Hexane was redistilled over potassium permanganate. All other common reagents and solvents were used as supplied with no further purification.

Preparation of Chromatoplates

Wood and Snyder (8) reported that plates impregnated with ammoniacal silver ion rather than with silver nitrate are superior in resistance to darkening on storage and that separation of fatty acid methyl esters is better. The plates used in this investigation were prepared by slurrying 20 g of commercial silica gel G with 60 ml of 12.5% silver nitrate containing enough ammonia to stoichiometrically redissolve the silver oxide. (Dissolve 7.5 g of silver nitrate in 60 ml of 3.5% ammonium hydroxide and add conc ammonium hydroxide dropwise until complete solubilization of residual silver hydroxide is obtained). Uniform 0.25 mm, or 1 mm layers were then spead on 5×20 cm and 10×20 cm glass plates, dried in air for 30 min, activated at 110C for 1 hr, and stored in a desiccator without any precaution from light. Such plates were stored for weeks without any appreciable darkening, and did not show any difference in separating capacity of triglyceride mixtures when compared with plates impregnated with plain silver nitrate.

Thin-Layer Chromatography

The chromatoplates were divided with a pencil into four 1 cm wide lanes and 400 μg of oil sample (8 λ of 5% solution in hexane) were applied to each lane at a distance of 2.5 cm from the edge. The plates were then developed in the system benzene-chloroform-ethanol (180:20:1 v/v/v) for approx 30 min which are required for the solvent to reach the upper edge of the plate.

Larger amounts of lipid fractions that are required for GLC analysis with a thermal conductivity detector can be obtained by fractionating 50 mg of oil sample applied as a continuous line on a 10×20 cm chromatoplate of 1 mm thickness.

For the complete analyses of oil samples depicted in Table I, the triglyceride spots were located under

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[2] Designations such as SSO, SOO, etc. represent triglyceride species without regard to position of fatty acids in the molecule, where S, U, O, L stand for saturated, unsaturated, oleic and linoleic acid residues respectively.

TABLE I

Fatty Acid Composition (Mole %) of Triglyceride Fractions Isolated by TLC Fractionation of Natural Oils
(The relative amount of each fraction (Mole %) is given in parentheses.)

	Fraction isolated by TLC				
	No. 1 (SSO)	No. 2 (SOO, SSL)	No. 3 (OOO, SOL)	No. 4 (OOL, SLL)	No. 5 (OLL, LLL, etc.)
Olive oil	(5.3)	(26.2)	(43.6)	(16.1)	(9.8)
Saturated acids	68.0	34.5	10.4	9.3	6.5
Monoenoic acids	32.0	64.9	82.6	57.3	56.5
Linoleic acid	.....	0.6	7.0	33.0	37.0
Cottonseed oil	(5.3)	(19.2)	(20.7)	(26.0)	(28.8)
Saturated acids	66.0	54.5	26.7	23.1	3.2
Monoenoic acids	34.0	24.9	46.2	16.9	14.3
Linoleic acid	.....	20.6	27.1	60.0	82.5
Corn oil	(2.4)	(7.6)	(16.9)	(27.6)	(45.5)
Saturated acids	66.5	49.5	24.3	16.2	2.4
Monoenoic acids	33.5	32.8	51.2	24.2	10.3
Linoleic acid	.....	17.7	24.5	59.6	87.1
Sesame oil	(3.1)	(1.4)	(36.5)	(18.7)	(40.3)
Saturated acids	66.4	63.7	29.8	7.2	1.7
Monoenoic acids	33.6	2.2	34.7	52.7	24.9
Linoleic acid	.....	34.1	36.5	40.1	73.4
Soybean oil	(1.9)	(6.8)	(11.3)	(24.1)	(55.9)
Saturated acids	66.7	50.3	28.9	20.8	3.1
Monoenoic acids	33.3	32.7	42.1	25.2	16.3
Linoleic acid	.....	17.0	28.5	53.7	69.4
Linolenic acid	.....	.....	0.3	0.3	11.2

UV light after spraying with 0.05% ethanolic dichlorofluorescein.

Inasmuch as the technique finally adopted for detecting adulteration of olive oil involves a fatty acid methyl ester analysis of the polyenoic triglyceride fraction only, the step of locating the triglyceride spots prior to transesterification may be omitted as well. The high reproducibility of the mobility of this fraction, combined with the fact that incomplete recovery would have no noticeable effect on its composition (see below), has proved this modification quite possible provided that the chromatographic pattern is checked from time to time on reference plates, using the charring procedure (3). Furthermore, the reliability of the results obtained by applying this simplification may obviously be secured each time by charring the same chromatoplate used for analysis, after scraping off the band of polyenoic fraction.

A purification step prior to TLC fractionation for reducing the free acidity of the sample was also adopted in the final method for testing adulteration as it was established that free fatty acid may noticeably alter the composition of the polyenoic triglycerides fraction (see below). Removal of free fatty acids is accomplished by shaking a 5% hexane solution of the oil sample with an equal volume of 0.5 N sodium carbonate in 50% methanol. The hexane layer is then washed twice with 50% methanol and brought to its original volume with hexane prior to application on the chromatoplate.

Isolation of Methyl Esters

The marked bands of silica gel were scraped off the plate and transferred into screw capped tubes. Benzene (0.5 ml) and methanolic 14% boron trifluoride reagent (2.0 ml) were added into each sample and the tubes placed in a boiling water bath for 20 min. After cooling, 4 ml hexane and 1 ml water were added, the contents shaken well and the aqueous layer removed with a pipet. The hexane layer was washed with 2 ml of 50% methanol, the methanolic layer removed as above and another 2-ml portion of 50% methanol added. After mixing and complete separation of the phases the hexane layer was withdrawn with a clean pipet, transferred in a small tube, evaporated to dryness under nitrogen and redissolved in 100 λ of redistilled hexane just prior to injection into the gas chromatograph.

To determine the relative amounts of the triglyceride fractions during the complete analysis of oil samples (Table I), the same known amount of methyl myristate was added as an internal standard to all tubes before extraction of the methyl esters from the transesterification mixture. Corrections for small amounts of naturally occurring myristate were necessary in some cottonseed oil fractions which therefore were analyzed with and without added methyl myristate.

Feldman and Rouser (9) showed also that direct transesterification without prior elution of the lipids is a reliable technique. The efficiency of the present transesterification method performed in presence of dichlorofluorescein was confirmed using a modification of the procedure described by Litchfield et al (6). The scraped silica gel bands of duplicate fractions were transferred into a filter funnel bearing a cotton plug near the lower end and containing activated silicic acid up to the middle of the stem. The mixed micro column thus formed was eluted with 10 ml of dry diethyl ether and the recovered triglycerides were then transesterified using the boron trifluoride method of Morrison and Smith (10). Gas-liquid chromatographic analysis showed both procedures equally effective.

Gas Liquid Chromatography

The analysis of fatty acid methyl esters was carried out on a 6 ft × ¼-in. U-shaped glass column of 15% ethylene glycol succinate on Anakrom AK, 60–70 mesh (Analab) in a Barber-Colman Model 10 argon chromatograph with an argon ionization detector. The column temperature was maintained at 178 C, the injector at 230 C, the detector at 240 C and the inlet pressure was maintained at 12 psi.

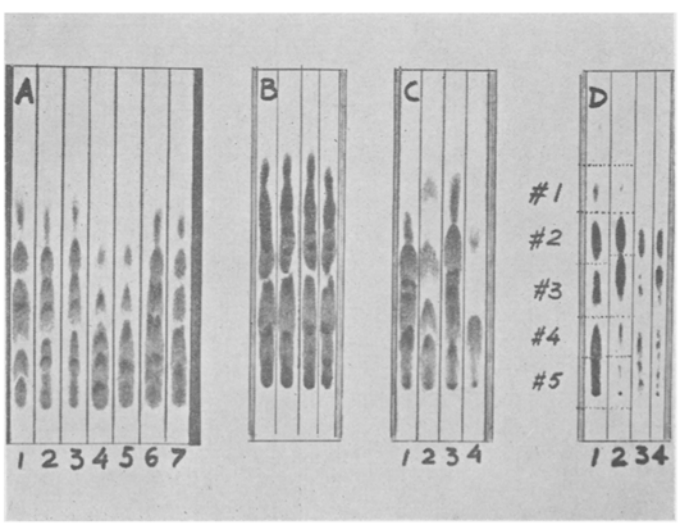

FIG. 1. Argentation TLC of plant oil samples in benzene-chloroform-ethanol (180:20:1 v/v/v). Plate A: 1- Olive kernel oil (400 μg); 2- Olive oil (500 μg); 3- Olive oil adulterated with 10% corn oil (450 μg); 4- Soybean oil (400 μg); 5- Corn oil (400 μg); 6- Cottonseed oil (700 μg); 7- Cottonseed oil (400 μg). Plate B: Cottonseed oil (850 μg in each lane). Plate C: 1- Olive oil (500 μg); 2- Cottonseed oil (250 μg); 3- Olive oil (700 μg); 4- Triolein (40 μg). Plate D: 1- Cottonseed oil (300 μg); 2- Olive oil (300 μg); 3- Cottonseed oil (150 μg); 4- Olive oil (150 μg); No. 1 to No. 5- Bands analyzed by GLC.

The calibration of peak height to quantity of methyl ester was carried out using a standard mixture of methyl esters of known composition.

Results

The five major triglyceride fractions isolated by TLC fractionation were labelled as fractions 1–5 as shown in Fig. 1, plate D. They represent the mono-, di-, tri-, tetra- and penta- (or more) unsaturated triglycerides respectively. The fatty acid composition results obtained and the relative amounts of these fractions in the overall oil samples (Table I) were in agreement with data reported by other investigators using similar techniques (3,5).

Fraction 1 contains only SSO and is found in appreciable amounts only in olive and cottonseed oils.

Fraction 2 of olive oil, as expected, was found to contain only trace amounts of linoleic acid and an O/L molar ratio higher than 100. The same fraction of all seed oils contains very high linoleic and saturated acid contents (Table I) and an O/L ratio varying only between 0.1 and 2.0 due to the presence of high amounts of SSL which is absent in olive oil. However, the relative quantity of fraction 2 in olive oil is much higher than in seed oils and detection of adulteration via GLC analysis of this fraction, although possible, cannot reach the

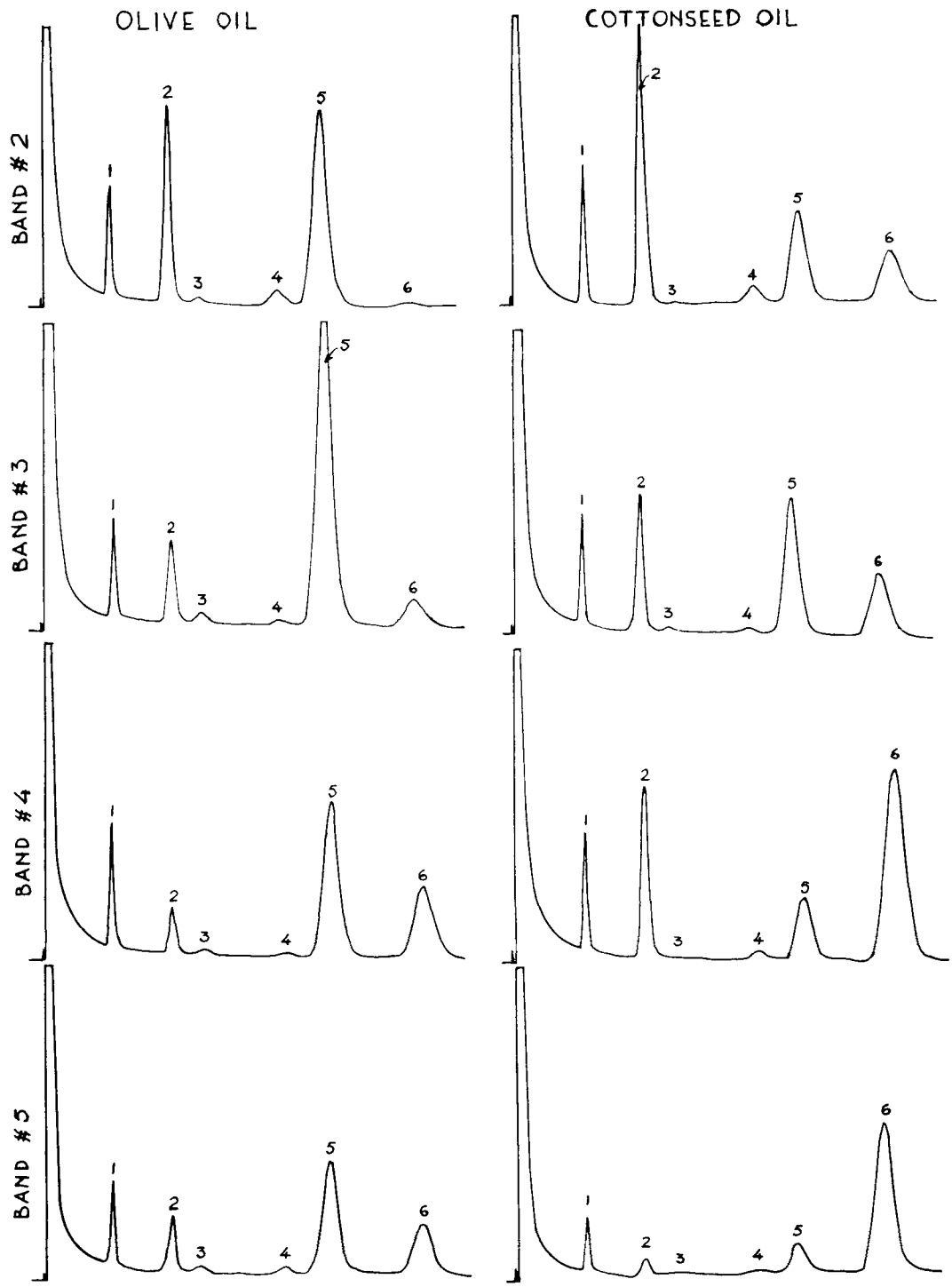

FIG. 2. Representative GLC chromatograms of triglyceride fractions of olive and cottonseed oils, as marked on plate D (Fig. 1). Peak 1- methyl myristate (internal standard); 2- methylpalmitate; 3- methyl palmitoleate; 4- methyl stearate; 5- methyl oleate; 6- methyl linoleate.

sensitivity obtained by analysis of the polyunsaturated fraction as described below.

Fraction 3, containing the species OOO and SOL, is even less characteristic. Although the SOL content in the trienoic fraction of seed oils is much higher than in olive oil, the latter contains SOL in amounts that vary extensively from sample to sample depending on the overall linoleic acid content. In addition, trienoic triglycerides represent the predominant fraction in olive oil which is a disadvantage related to the sensitivity of detecting adulteration as mentioned above.

An inspection of the compositions and relative amounts of the polyenoic fractions 4 and 5 (Table I) suggested their prominent importance in detecting adulteration of olive oil by seed oils. The fatty acid compositions of fractions 4 and 5 of olive oil (Table I) appear very close to each other. This indicates that incomplete resolution between tetra- and pentaenoic triglycerides occurs. This result should be expected since the tetraenoic triglyceride (OOL) content of the triglyceride mixture of olive oil is much higher than its content in pentaenoic triglyceride (OLL) and separating polyunsaturated triglycerides by this technique is very difficult. Furthermore, the only conclusion that can be derived by inspecting the composition of these fractions is that their saturated fatty acid belongs to SOL streaking back from the major fraction 3 rather than due to presence of SLL in olive oil.

To check the possibility that small amounts of free fatty acids, diglycerides and oxidized triglycerides are contaminating these fractions, thus altering their fatty acid composition, several olive oil samples were examined by this technique: without purification of the triglycerides, after previous purification of the triglyceride fraction via silicic acid chromatography or preparative TLC and after washing with aqueous sodium carbonate in order to remove the fatty acids. Only slight differences were observed between purified and nonpurified samples. The fatty acid compositions of fractions 5 of the aliquots purified via either chromatography or sodium carbonate wash were in all cases quite similar, thus indicating that the acidity of the olive oil samples is the only reason for a noticeable influence on the composition of the polyenoic fraction. The sodium carbonate washing step was therefore included in the procedure finally adopted thus improving the sensitivity and reliability of the test.

By analysis of 17 samples of pure olive oil of varying acidities (0.50–4.30%) and linoleic acid contents (4.10–17.80%) after sodium carbonate washing, it was confirmed that the linoleic acid content of the combined polyenoic fractions (4 and 5) varies between very narrow limits (35.2–37.0 moles%), the O/L molar ratio lies always between 1.52 and 1.58, and the quantity of the fraction represents in all cases approximately 15–25% of the entire olive oil sample.

On the other hand, the respective polyenoic fractions (combined), of seed oils, represent 55–80% of the weight of the sample, containing 65–75 moles % of linoleic acid and yielding an O/L molar ratio as low as 0.14 to 0.42.

Discussion

The qualitative examination of the chromatographic pattern of an olive oil using argentation TLC has proved to be of dubious usefulness as a sensitive and reliable test for the detection of probable adulteration of olive oil by seed oils, although the individual chromatographic patterns of olive and seed oils appear distinctively different (Fig. 1). Its limited success is attributed mainly to the well known difficulty in locating the lipid spots on such chromatoplates. The presence of the silver ion extensively decreases the sensitivity of the charring procedure so that to distinguish all the triglyceride fractions of plant oils it is often necessary to examine the patterns of more than one concentration of the same sample, since some fractions can be detected only by overloading the plates (Fig. 1). However, this introduces additional problems as the R_f values of individual triglyceride fractions are highly affected by their concentration and relative proportions as shown in Fig. 1.

In the present approach such disadvantages are overcome by using a technique as sensitive and reliable as the GLC analysis for the identification of triglyceride species characteristic of all seed oils but absent in olive oil. This is accomplished after previous TLC fractionation of the triglycerides of the olive oil suspected for adulteration.

A reliable method for the detection of such adulteration should be based obviously on the analysis of triglyceride fractions possessing the following properties: A more or less constant composition for all olive oils, distinctively different compositions when isolated from olive or seed oils, and their relative amount with respect to the overall oil sample should be low for olive oils and high for seed oils, in order to increase the sensitivity. All these requirements are fulfilled by the polyenoic triglyceride fraction with four or more double bonds per triglyceride molecule as already mentioned (see Results).

The accuracy and sensitivity of detecting adulteration of olive oil by seed oils using the present method is therefore self evident. Representative results are given in Table II. As low as 2% of such adulteration can be detected by this method with maximum reliability, as was indicated by testing numerous admixtures of olive oil with several commercial samples of seed oils.

It should be emphasized that the present method, by its nature, is applicable particularly for the detection of adulteration by seed oils of high linoleic acid content. Although at present all commercial seed oils belong to this class, it is very probable that the continuous search for new edible fats and oils will make commercially available products resemble olive oil in composition, as for instance the oil of safflower seed variety UC-1 (11) which is now in the way of commercialization (12). This high oleic acid variety of safflower oil, containing approx 80%

TABLE II

Application of the Present Method for Testing Adulteration of Olive Oil

Seed oil added to olive oil	Linoleic acid in polyenoic fraction (Mole %)	Molar ratio O/L in polyenoic fraction
None	35.2–37.0	1.52–1.58
Cottonseed oil, 10%	43.5	1.13
Cottonseed oil, 5%	40.0	1.34
Cottonseed oil, 2.5%	38.4	1.42
Corn oil, 10%	47.0	1.04
Corn oil, 5%	41.7	1.26
Corn oil, 2.5%	38.9	1.42
Sesame oil, 10%	42.1	1.24
Sesame oil, 5%	39.5	1.38
Sesame oil, 2.5%	38.0	1.43

oleic acid and only 12% linoleic acid, was recently proved to possess considerable stability towards oxygen and excellent physical properties to be used as a high stability edible oil (13). If the triglyceride composition of this oil proves comparable to that of olive oil, its detection in admixtures with the latter will obviously be impossible by any of these techniques.

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