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DETECTION OF OLIVE OIL ADULTERATION WITH LINOLEIC ACID-RICH OILS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Adulteration of olive oil with very low levels (1–2%) of linoleic acid-rich oils can be unequivocally detected by reversed-phase high-performance liquid chromatography on columns packed with C_{18} alkyl bonded-phase particles. Triglyceride fractionation according to their equivalent carbon numbers is effected in 22–25 min by eluting the column with a non-aqueous mobile phase (acetonitrile–absolute ethanol–isopropanol, 72:18:10), compatible with UV-detector systems.

INTRODUCTION

The wide range of naturally occurring fatty acid and sterol compositions in different olive oils is a limiting factor in the effectiveness of gas–liquid chromatographic (GLC) analyses to reveal the presence of low levels of linoleic acid-rich oils in olive oil. These difficulties were overcome[1] by taking advantage of the predictions of the "even"[2] and "restricted random"[3,4] distribution theories, according to which the qualitative triglyceride composition of each kind of vegetable oil is not affected by natural quantitative variations in its individual fatty acid contents, *i.e.*, in spite of the latter variations, the triglyceride species characteristic for olive oil, *e.g.*, OOO, POO, or for linoleic acid-rich oils, *e.g.*, OLL, PLL, LLL, persist as their respective "fingerprints" (see Fig. 1 for abbreviations).

Previous methods taking advantage of these properties were based on the isolation of triglyceride fractions enriched in the aforementioned unique triglyceride species of linoleic acid-rich oils by either low temperature crystallization[1,5] or by argentation chromatography[6,7]; in both cases the method involves two steps, *i.e.*, the oil fractionation technique needs to be combined with GLC analysis of the fatty acid methyl esters of the isolated triglyceride fraction.

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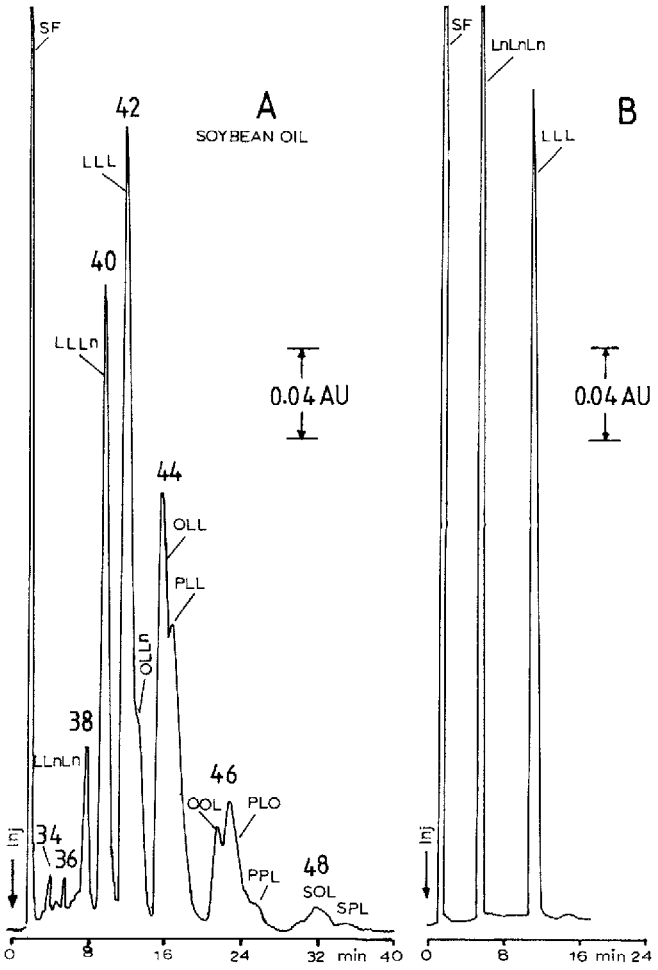

Fig. 1. HPLC patterns of (A) soy bean oil and (B) trilinolein–trilinolenin standards, with acetonitrile–absolute ethanol–isopropanol (78:20:2) as mobile phase. Abbreviations: OOO, POO, etc., denote triglyceride species containing the fatty acyl groups indicated by their symbols S, P, O, L, Ln (stearoyl, palmitoyl, oleoyl, linoleoyl, linolenoyl respectively), regardless of their positions in the glycerol moiety. The numbers, *e.g.*, 48, denote equivalent carbon numbers (ECN), where ECN = CN − 2*n*, CN being the sum of the carbon numbers of the fatty acyl groups and *n* the sum of the double bonds in a triglyceride molecule. All the triglyceride critical pairs having the same ECN, *e.g.*, 48 or the SOL and SPL, are denoted as "fraction ECN-48", etc., in the text. SF = Solvent front (chloroform).

We have found that recent significant progress in separating triglyceride mixtures by reversed-phase high-performance liquid chromatography (RP-HPLC) offers a powerful tool for the rapid detection of olive oil adulteration by linoleic acid-rich oils in a single step and with increased sensitivity, albeit on the same principles as mentioned above.

The advent of RP-HPLC has revolutionized the separation and analysis of triglyceride mixtures of natural oils and fats[8–42]. Plattner and co-workers[11] showed

the ability of alkyl bonded-phase columns, together with non-aqueous eluents, to resolve triglyceride mixtures according to the chain lengths and the degrees of total unsaturation; *i.e.*, in agreement with previous experience with fatty acid methyl esters[43,44], each double bond of a triglyceride was found to be almost equivalent to the loss of two methylene units from the molecule, this being expressed by the equivalent carbon number (ECN) of each triglyceride species (see Fig. 1). As shown later, by increasing the selectivity of the system, the actual retention time is also affected by the nature of the individual unsaturated fatty acyl moieties contributing to the total unsaturation. This led Perkins and El-Hamdy[21] to the concept of the theoretical carbon number (TCN), which is derived from ECN by subtracting the sum of the numerical factors specific for each individual unsaturated fatty acyl moiety on the triglyceride molecule.

Improved separations of critical triglyceride pairs according to TCN were achieved by lowering the column temperature[23] or using short columns (100 × 4.6 mm) packed with 3-μm particles[32], but full exploitation of the existing possibilities for quantitative analysis is still hindered by specific limitations[37,42] of each type of detector system available, *e.g.*, incompatibility with gradient elution or with some solvents, poor sensitivity, mechanical complexity, high cost, etc.

Nevertheless, as shown in this report, incomplete separation of triglyceride critical pairs, *i.e.*, simple separation according to ECN, is sufficient —if not preferable— for the rapid detection of olive oil adulterations by low proportions of linoleic acid-rich oils. Such separations can easily be achieved with solvents compatible with the most popular UV detector systems, which combine low cost with excellent performance. Inasmuch as highly unsaturated triglyceride species are predominant in the adulterant linoleic acid-rich oils, the normal disadvantage of the UV detector systems in enhancing the peaks corresponding to these triglycerides contributes to the sensitivity of the present method.

EXPERIMENTAL

Materials

Virgin olive oil samples of guaranteed purity were obtained from the chemical laboratory of the Greek Ministry of Commerce. The samples of refined linoleic acid-rich oils (corn, cottonseed, soy bean and sunflower oils) were taken from commercial sources.

Authentic standards for HPLC, GLC and thin-layer chromatographic (TLC) analyses were purchased from Supelco (Bellefonte, PA, U.S.A.) and PolyScience (Niles, IL, U.S.A.). HPLC solvents were purchased from Rathburn Chemicals (Walkerburn, Peebleshire, U.K.). All other reagents were analytical grade, supplied by Merck (Darmstadt, F.R.G.).

Instruments

The HPLC unit used for triglyceride analyses was a liquid chromatograph, Series 3B (Perkin-Elmer, Norwalk, CT, U.S.A.), equipped with a Rheodyne Model 7105 valve loop injector. The detector was a Perkin-Elmer Model 551 dual-beam UV–VIS spectrophotometer, equipped with 8-μl special flow microcells.

The LC columns used were prepacked MicroPak MCH-10 stainless-steel col-

umns, 30 cm × 4 mm I.D., packed with 10-μm C_{18} bonded-phase particles, purchased from Varian (Palo Alto, CA, U.S.A.).

Analyses of fatty acid methyl esters were performed using a Perkin-Elmer Model Sigma 3 gas chromatograph, equipped with a flame ionization detector. The glass columns (1.80 m × 2.2 mm I.D.) were packed with GP 10% SP-2330 on Chromosorb W AW (100–120 mesh) (Supelco).

All chromatograms were recorded on a Varian Model 9176 recorder fitted with a Perkin-Elmer Model 2 integrator-calculator-printer.

Chromatographic conditions

The HPLC analyses were performed isocratically at room temperature (25–27°C) at a flow-rate of 2.0 ml/min. The detector was set at 210 nm and the recorder response at 0.4 a.u.f.s. Injections were 20 μl of 5% chloroform solutions of the analyzed oils, except linseed oil, for which 0.5% in chloroform was sufficient. The mobile phase consisted of various combinations of isopropanol with acetonitrile–absolute ethanol (4:1), as shown in the figure legends.

For the analysis of the fatty acid composition of the triglyceride fractions eluted from the LC columns, their fatty acid methyl esters were prepared by transesterification with boron trifluoride–methanol[4,5]. They were finally redissolved in 20–40 μl hexane, and 4 μl were injected into the GC column, under the following conditions: oven temperature, 200°C (isothermal); injector and detector temperatures, 210°C; carrier gas, nitrogen at 20 ml/min.

TLC analyses were performed on glass chromatoplates (20 cm × 20 cm), coated with silica gel G (thickness 0.25 mm) using the solvent light petroleum (b.p. 40–60°C)–diethyl ether–acetic acid (80:20:3). Visualization of spots was accomplished by exposure to iodine vapours.

RESULTS AND DISCUSSION

The HPLC patterns illustrated in Figs. 1–6 are representative examples selected to show the effects of modifying the mobile phase by increasing the proportion of isopropanol added to acetonitrile–absolute ethanol (4:1). The retention time of the triglyceride fraction ECN-48 was reduced from 28–32 min (Figs. 1, 2) to 16–19 min (Figs. 3–6) by increasing the respective isopropanol contents of the mobile phse from 2 to 10%. However, this effect was not accompanied by any appreciable change in resolution of the triglyceride peaks. Therefore, this reversed-phase HPLC system is advantageous for use in routine work on the detection of olive oil adulteration since it is susceptible to wide variations in experimental conditions without accompanying changes in selectivity and sensitivity. In this study, the mobile phase containing 10% isopropanol (Figs. 4–6) was preferred for routine analysis because it enables short analysis times and good resolution.

A general remark is that the sum of the peak areas corresponding to equal amounts of linoleic acid-rich and olive oils is greater in linoleic acid-rich oils, whereas there is no appreciable difference between virgin and refined olive or olive-residue oils (Fig. 5). Therefore, the enhanced intensities of eluted fractions obtained by using a UV detector at 210 nm seem to depend almost only on the total unsaturation of the oil fraction and not on the extinction at 270 nm that is increased in all refined oils.

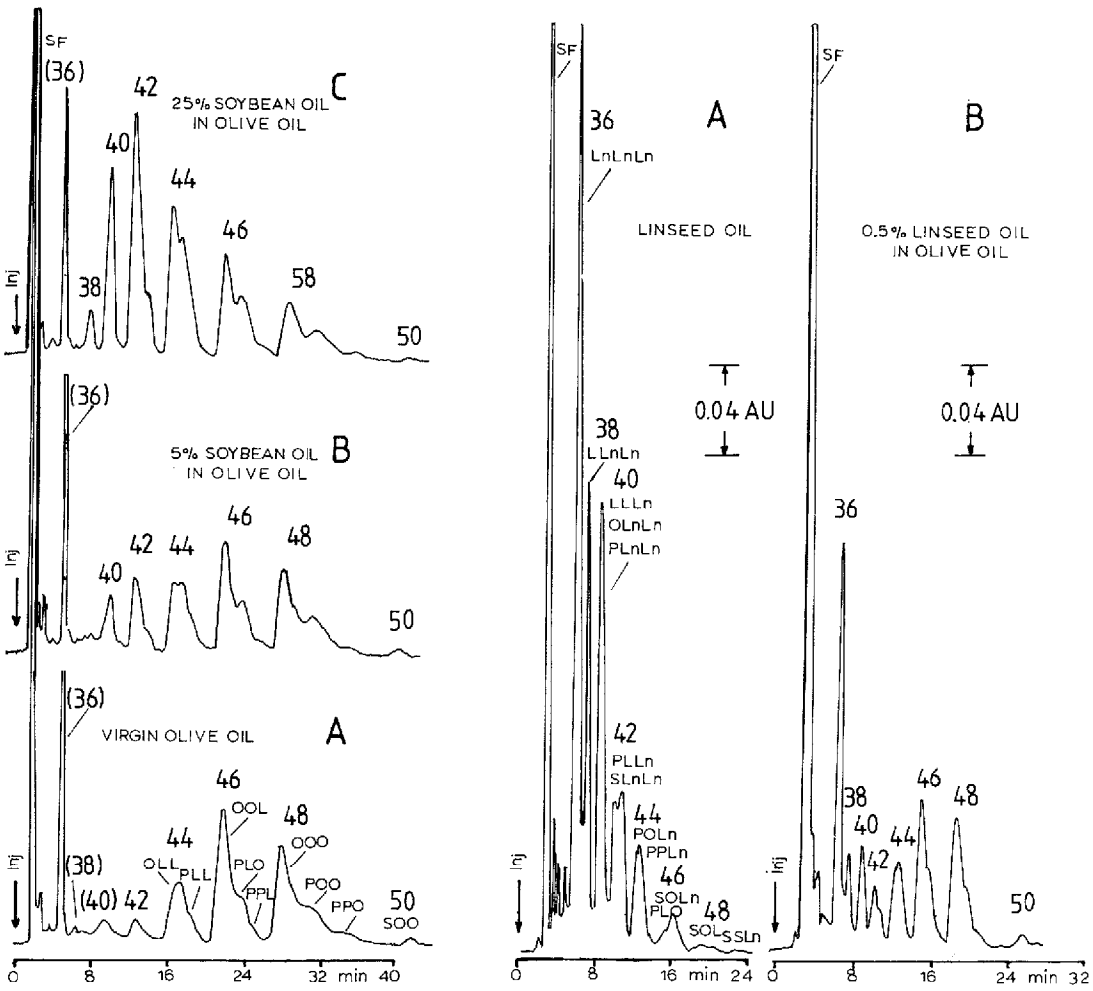

Fig. 2. HPLC patterns of (A) virgin olive oil, (B) 5% soy bean oil and (C) 25% soy bean oil in olive oil (A). Mobile phase and abbreviations as in Fig. 1.

Fig. 3. HPLC patterns of (A) linseed oil and (B) 0.5% linseed oil in olive oil. Mobile phase: acetonitrile–absolute ethanol–isopropanol (75:19:6). Abbreviations as in Fig. 1.

Lipid fractions corresponding to ECN-40 or lower values are of limited importance for detecting the presence of linoleic acid-rich oils in olive oil, although they represent the most characteristic components of the linolenic acid-rich oils such as linseed oil (Fig. 3A). As shown in Fig. 3B, the presence of even 0.5% linseed oil in olive oil may be disclosed by the HPLC pattern of fractions ECN-36 to 42, although this is not possible by GLC analysis of the fatty acid composition. Linseed oil is not a common adulterant of olive oil and in this study it was used mainly for identification purposes. Thus, it is obvious that the olive oil fractions marked ECN-36 to 42 (Fig. 5) coincide with the respective triglyceride fractions of linseed oil (Fig. 3B).

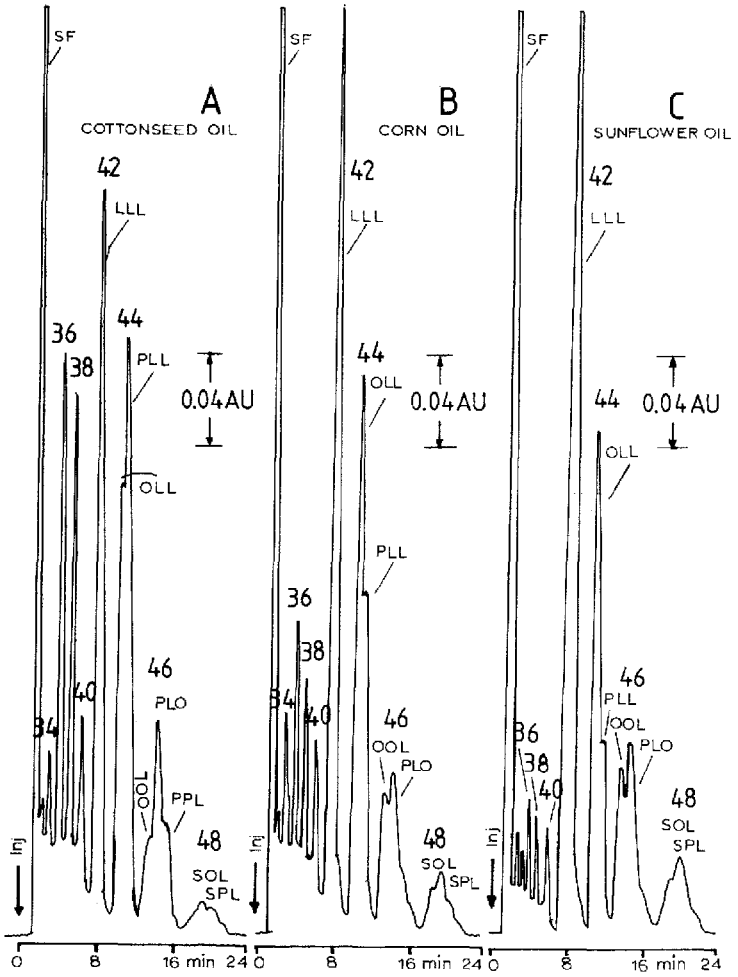

Fig. 4. HPLC patterns of (A) cottonseed oil, (B) corn oil and (C) sunflower oil. Mobile phase: acetonitrile–absolute ethanol–isopropanol (72:18:10). Abbreviations as in Fig. 1.

However, by TLC analysis, the olive oil fraction ECN-36 was found to contain at least six components, of which the major components were much more polar than triglycerides and only one minor component moved close to the spot of the triolein authentic standard. A similar component was also identified in olive oil fractions ECN-38 and 40, but the very small quantities available would not allow the detection of any other minor components possibly present. These findings suggest that the quite sizeable olive oil fraction ECN-36 does not contain considerable amounts of trilinolenin (LnLnLn) or equivalent triglycerides of low-molecular-weight fatty acids, but rather its main components belong to the unsaponifiable matter.

Attempts to eliminate these components by passing the olive oil samples or their hexane solutions through alumina or silica gel columns, as well as by partitioning in hexane–methanol biphasic systems, failed to decrease the size of fraction

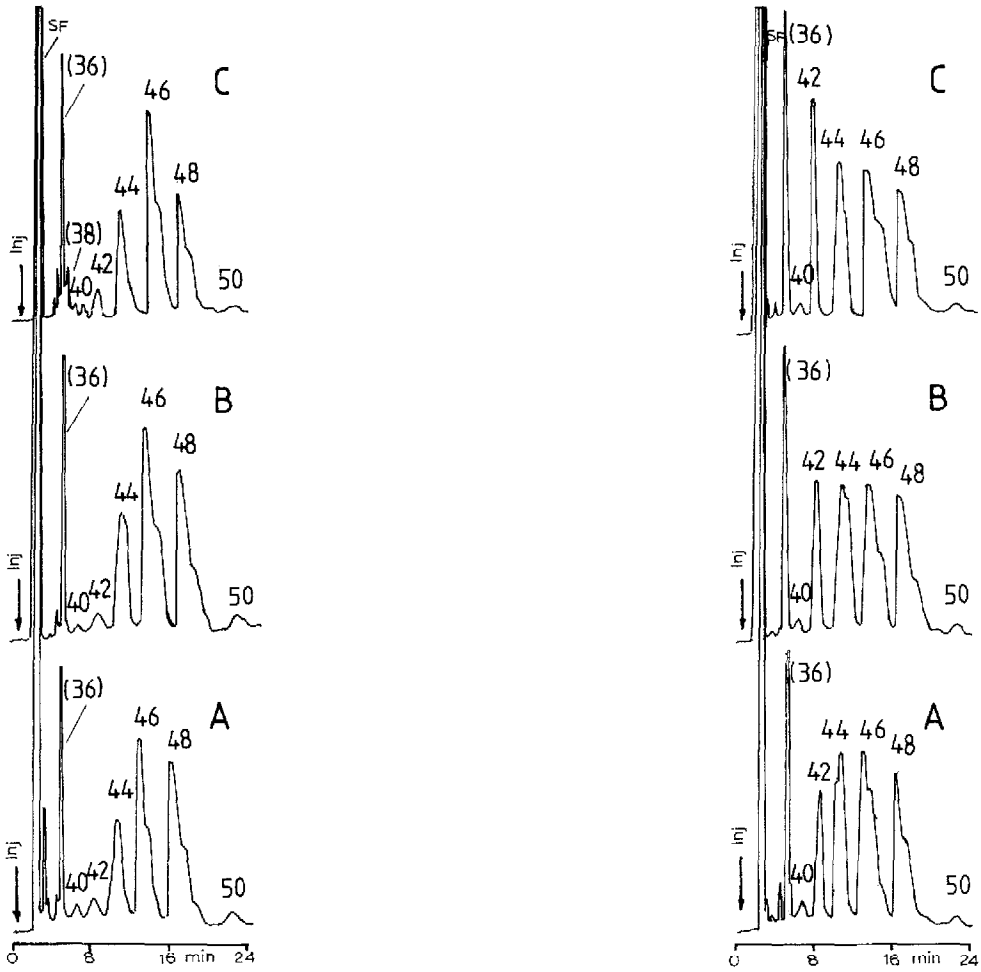

Fig. 5. HPLC patterns of (A) virgin olive oil, (B) refined olive oil and (C) olive-residue oil. Mobile phase as in Fig. 4, abbreviations as in Fig. 1.

Fig. 6. HPLC patterns of the virgin olive oil of Fig. 5A adulterated with 5% of (A) cottonseed oil, (B) corn oil or (C) sunflower oil. Mobile phase as in Fig. 4, abbreviations as in Fig. 1.

ECN-36. Therefore, further characterization of its components was discontinued and little attention was also given to the respective fractions of the linoleic acid-rich oils. Fractions ECN-38 and 40 of soy bean oil (Fig. 1A) are expected to contain triglycerides of linolenic acid (LLLn, LLnLn), while fractions ECN-34 to 40 of other seeds oils (Fig. 4) are expected to contain unsaponifiables mixed with triglycerides of fatty acids of low molecular weight (myristic, lauric, decanoic, etc.).

Features of olive oil

The HPLC patterns illustrated in Figs. 2A and 5A, B, C are representative of a large number of virgin, refined and olive-residue oils with different L contents.

There are three main fractions corresponding to ECN-44, 46 and 48. According to GLC analysis of their fatty acid methyl esters, their main triglyceride components are OLL (faster peak) and PLL in fraction ECN-44, OOL (faster peak) and POL in fraction ECN-46 and OOO (faster peak) and POO, with small amounts of PPO in fraction ECN-48. The minor triglyceride fraction ECN-50 contains mainly SOO with some SPO.

Special attention was given to the other minor fraction ECN-42, which corresponds to triglycerides of the types LLL, LLM, OLLn, etc., not expected to be present in identifiable quantities in olive oils. By TLC analysis it was shown that this fraction consists exclusively of triglycerides, but GLC analysis afforded a rather complicated composition of the fatty acid methyl esters, with peaks corresponding to oleic, palmitic, palmitoleic, myristic, lauric, stearic and linolenic acids (in decreasing order). Therefore, this fraction consists of a complex mixture of mixed triglycerides eluted at this position because they contain several fatty acids of low ECN, either naturally occurring in olive oil, or formed by oxidation. This latter possibility is supported by an observation that in most olive-residue oils analyzed the respective fractions ECN-42 were more intense than in virgin olive oil (compare Fig. 5C and A). In any case, these results demonstrate, as expected, that LLL is not a major component of this fraction in olive oil.

Another interesting feature, important for the detection of olive oil adulterations, was that despite the wide differences in L content of the olive oil samples examined, the relative intensities of the fractions ECN-46 and 44 remain largely unchanged; the peak height of fraction ECN-44, almost half that of fraction ECN-46, is only slightly reduced in olive oil samples of low L content (Fig. 2A), whereas the concomitant relative increase in fraction ECN-48 is quite pronounced, in accordance with the predictions of the even and restricted random distribution theories[2–4].

Features of seed oils

A common feature of the HPLC patterns of all the linoleic acid-rich oils examined in this study (Figs. 1A and 4) is that the fractions ECN-42, corresponding to LLL, give the highest peaks in all cases, while fractions ECN-50 are not identifiable. In contrast to olive oil, fraction ECN-44 of all linoleic acid-rich oils corresponds to the largest peak area, while fractions ECN-46 and 48 are much smaller and of decreasing order of magnitude. It is noteworthy that the absolute intensities of fractions ECN-46 and 48 of linoleic acid-rich oils are very close to those of olive oils and, therefore, addition of other linoleic acid-rich oils in olive oil will not change appreciably the respective part of the HPLC pattern of the adulterated olive oil.

According to the results of GLC analysis of the fatty acid methyl esters, the main triglyceride components of fractions ECN-44 and 46 of linoleic acid-rich oils are the same as in olive oil, although some quantitative relationships between their triglyceride components are inverted. Thus, in cottonseed oil, which is rich in palmitic acid, there is more PLL than OLL in fraction ECN-44 and an extra peak, corresponding to PPL, appears in fraction ECN-46 (Fig. 4A). Also, there is more POL than OOL in fractions ECN-46 of all other linoleic acid-rich oils (Figs. 1A, 4B, 4C).Furthermore, fraction ECN-48 of all linoleic acid-rich oils seems to contain mainly SOL and SPL instead of OOO. The latter is present in negligible amounts, if at all.

Detection of adulteration

From the representative HPLC patterns illustrated in Figs. 2 and 6, it is obvious that the present technique is quite suitable for the rapid and unequivocal detection of the presence of very low amounts of linoleic acid-rich oils in olive oil. Furthermore, it permits a semi-quantitative estimation of the level of adulteration (impossible by other techniques), while at the 4–5% level (or higher) it even allows a tentative identification of the adulterant oil. For instance, the presence of soy bean oil may be detected by the enhancement of peak ECN–40 (Fig, 2B, C), while the relative intensities of the peaks ECN-40, 42 and 44 to each other and to peak ECN-46 may give an estimate of the level of adulteration. Likewise, in the examples of Fig. 6, the relative intensities of the peaks ECN-42 and 44 to each other and to peak ECN-46 clearly reflect the particular features of the respective seeds oils (Fig. 4), thus allowing their tentative recognition in the mixtures and their approximate levels.

Quantitative aspects.

For comparative purposes, the intensity (area or height) of the fraction ECN–46 may be used as the unit of comparison (denoted 100%) since it remains almost unchanged in adulterated olive oil samples, as already mentioned. The intensities of all other column fractions may then be expressed as a percentage of fraction ECN-46.

On the basis of the results obtained from 17 virgin olive oil samples (of low and high L contents), 2–4 samples of each linoleic acid-rich oil and several samples of their mixtures, the following quantitative aspects are of importance.

(1) The relative areas of the peaks ECN-42, 44 and 48 (with respect to that of peak ECN-46) in all the olive oil samples examined were found in the ranges 5–8, 45–50 and 90–130% respectively. Alternatively, with the mobile phase containing 10% isopropanol, the respective peak heights were 8–14, 55–60 and 55–90%.

(2) Addition of every 1% of linoleic acid-rich oil in any olive oil sample causes an increase in the peak area of ECN-42 (as a percentage of the peak area of ECN-46) of 15–18% for sunflower oil, 10–12% for corn oil and 6–8% for soy bean and cottonseed oils. The respective increases in peak heights are 25–30, 16–20 and 10–14%.

(3) By using the sum of the peak areas of ECN-42 and 44, the respective ranges of variation are 45–58% for genuine olive oil and increases of 23–28, 18–20 and 15–18% for each 1% of added sunflower, corn and soy bean or cottonseed oils respectively.

These quantitative figures are cited here to indicate the possibilities of the method. Theoretically, the presence of 1.5% sunflower oil and 2% of other linoleic acid-rich oils in olive oil may be detected according to these figures. In practice, visual inspection of the HPLC patterns obtained can unequivocally reveal the presence of 2.5% sunflower oil and 3–4% of other linoleic acid-rich oils in olive oil.

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* The present list of references does not intend to cover all the pertinent literature in the field of detection of olive oil adulteration by any suitable technique, but to offer a list of the recent advances in triglyceride analysis by reversed-phase HPLC.