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APPLICATION OF LIQUID CHROMATOGRAPHIC AND SPECTROSCOPIC METHODS FOR THE CHARACTERISATION OF FATTY ACID ANILIDES IN CONTAMINATED COOKING OILS

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SUMMARY

High-performance liquid chromatographic and spectroscopic (mass spectrometric and infrared spectroscopic) methods were used to characterise the presence of oleic, linoleic, linolenic and stearic anilides in contaminated cooking oils from Spain. The procedures provide the basis for a screening technique to ensure the absence of such components in oils.

INTRODUCTION

The toxic effects of cooking oil sold in Spain during 1981 produced widespread illness and death[1]. The Department of Forensic Medicine at the University of Seville asked this Laboratory to assist their investigation by carrying out some trace metal analyses. However, it seemed worthwhile extending the examination to other possible toxic components. For this purpose it is necessary to use a primary screening technique that would allow a comprehensive examination to be made. The procedure that most nearly approaches the ideal for this is size exclusion chromatography which has been used as a forensic screening technique for some years[2], albeit with UV detection (*i.e.*, a selective rather than a universal detector). Comparison of the size exclusion chromatograms of control oils with those from Spanish cooking oils indicated that the latter contained components that were unusual. These components could be extracted from the oil with acetonitrile, and spectroscopic (mass spectrometric, MS, and infrared, IR, spectroscopic) examination of the extract indicated that they were fatty acid anilides. A series of standard anilides were prepared and it was found that they could be separated well under reversed-phase high-performance liquid chromatographic (HPLC) conditions and could be readily monitored by their UV absorbance. Comparison of the retention time data of standard anilides and the extracted compounds before and after bromination enabled a tentative identification to be made. Confirmation was achieved by using the HPLC system in a preparative mode and subjecting individual fractions to spectroscopic examination. The reversed-phase HPLC procedure was also used to confirm that the compounds characterised could be produced by heating aniline with rapeseed oil.

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This paper describes the procedures used in comparing suspect and control oils, isolation of the unknown components and their characterisation. In addition chromatographic and IR spectroscopic screening methods are proposed.

EXPERIMENTAL

Six samples of Spanish cooking-oil were examined and the following authenticated vegetable oils were tested for control purposes: olive oil (five samples), rapeseed oil (two samples), safflower oil, palm oil, palm kernel oil, cotton seed oil, soyabean oil, groundnut oil, castor oil, coconut oil, sunflower-seed oil, grape-seed oil, maize oil and sesame oil. In appearance and odour the Spanish oils did not differ markedly from many of the authenticated vegetable oils.

Size exclusion chromatography

Conditions very similar to those previously reported[2] were used in this study: column, 25 cm × 8 mm I.D. stainless steel; packing, a microparticulate silica of *ca.* 5 μm particle size, with the pore size 130 Å, pore volume 1.25 ml/g, and surface area 320 m^2/g; eluent, tetrahydrofuran (HPLC grade, Fisons)–water (99:1); flow-rate, 2 ml/min; detection, UV absorbance at 254 nm (SpectroMonitor III, LDC); injection, Rheodyne valve (Model 7125) with 20-μl loop.

A 0.2-g amount of each oil was dissolved in 5 ml of eluent and 20 μl of the solution were injected. A molecular weight (size) *versus* retention volume calibration curve was produced by injecting polystyrene standards.

Solvent extraction and spectroscopic examination

A 50-ml volume of oil was extracted with 2 × 50 ml of acetonitrile (Laboratory grade, May & Baker). The combined extracts were washed with 2 × 100 ml of pentane (Distol grade, Fisons) and the acetonitrile solution was evaporated to dryness at 70°C using a rotary evaporator. The residue was dissolved in 5 ml of diethyl ether. A 100-μl volume of this extract was streaked on to a silica thin-layer chromatography plate and developed with chloroform. The compounds of interest ran with an R_F of about 0.7 and this portion of the plate was removed and extracted with diethyl ether. The cleaned sample was then examined by IR spectroscopy and MS using the conditions described below.

IR spectroscopy. The IR spectra were recorded on a Nicolet MX-1 Fourier transform infrared spectrometer employing a Nicolet 7000 MX (× 4) mirror beam condenser when appropriate. The oil extract was evaporated on to KBr powder, pressed into 1.5-mm discs using an ultra-micro die (Perkin-Elmer) and spectra were acquired for 16 min (*i.e.*, 512 scans).

Mass spectrometry. Mass spectra were obtained using a direct insertion probe on a VG 12-12F quadrupole mass spectrometer (VG Analytical) linked to a 2050 Data system (VG Analytical). Electron impact (EI) and chemical ionisation spectra were recorded under the following experimental conditions: *electron impact:* source temperature, 200°C; electron energy, 70 eV; emission current, 500 μA; mass range, 35–535 a.m.u.; scan rate, 6 sec; and *chemical ionisation:* source temperature, 160°C; electron energy, 50 eV; emission current, 1000 μA; reagent gas, isobutane at $5 \cdot 10^{-5}$ Torr at the source housing; mass range, 200–500 a.m.u.; scan rate, 6 sec.

Preparation of fatty acid anilides

A 1-g amount of each of the following fatty acids was weighed into test tubes (Soveril tubes): oleic, linoleic, linolenic, lauric, myristic, palmitic, stearic and erucic acid. A 2-ml volume of aniline was added to each sample and the stoppered test tube was heated in an oven at 150°C for 18 h. The contents of each tube were dissolved in 50 ml of diethyl ether and extracted with 2 × 50 ml of 10% hydrochloric acid to remove excess aniline. The ether solution was neutralised by adding it to a beaker containing solid sodium bicarbonate (*ca.* 5 g) and the solution was then filtered and evaporated to dryness. The products were recrystalised from methanol or methanol–water. With the exception of the anilides of oleic, linoleic and linolenic acids which formed as oils, all other products were crystalline.

Reversed-phase HPLC of fatty acid anilides and oil extracts

A packing material made in the Laboratory was used in these studies. The preparation was as follows: 50 g of the silica described above under *Size exclusion chromatography* was oven dried at 150°C for 24 h, and was refluxed for 1 h with 250 ml of xylene containing 25 ml of octadecyltrichlorosilane. After this period 25 ml of trimethylchlorosilane was added and refluxing was continued for a further hour. The product was filtered and washed in succession with xylene, acetone and methanol, and was then vacuum dried at 75°C. This packing material is a typical end-capped C_{18} modified silica and gave a weight loss of 19.5% on ashing at 600°C. The retention characteristics of this material can be correlated with that of commercially available supports. Thus: Spherisorb 5 μm ODS displays less retention, factor 0.8; Zorbax ODS displays greater retention, factor 1.2–1.7.

The following chromatographic conditions were used: column, 12.5 cm × 5 mm I.D. stainless steel; packing, as described above; eluent, methanol–water (95:5); flow-rate, 1 ml/min; detection, UV absorbance at 254 nm (SpectroMonitor III, LDC); injection, Rheodyne valve (Model 7125) with 20-μl loop.

Standard anilides were dissolved in methanol for injection. Oils were shaken with methanol or acetonitrile (0.2–0.5 g of oil + 5 ml of solvent) and an aliquot of the extract was injected. In addition to running the anilides under the conditions just described samples were also mixed with an equal volume of a brominating solution (20 drops of bromine in 25 ml of acetonitrile). The reaction was allowed to progress for 10 min and the reaction mixture was injected.

Preparative HPLC was carried out on the same column system using a sample which consisted of 1 ml of the ether extract prepared as described above under *Solvent extraction and spectroscopic examination* dried down and redissolved in 400 μl of the eluent. Monitoring at 280 nm three fractions were collected; these fractions corresponding to the three major UV absorbing components. The bulked fractions were added to 50 ml of water and extracted with 4 × 10 ml of ether. The extracts were filtered, dried and examined by chromatography and spectroscopy.

Formation of fatty acid anilides by heating oils with aniline

Rapeseed oil and aniline were mixed in different proportions and heated in sealed test tubes at 150°C for 18 h. Aliquots of the mixtures were dissolved in tetrahydrofuran and examined under the reversed-phase conditions described above.

Screening procedures

Either of the two chromatographic systems can be used to screen oils for the presence of anilides. The optimum monitoring wavelength is about 240 nm and in the case of the reversed-phase system the detection limit for individual anilides is about 1 ng (*i.e.*, injected). Quantitation merely requires the use of stock solutions prepared from the standard anilides.

The following procedure was used when screening by IR: 1 g of oil was dissolved in 2 ml of isooctane and shaken for 5 min with 3 ml of methanol. After centrifugation, the methanol layer was removed and washed with 5 ml of isooctane. The methanol fraction was separated, evaporated to dryness and the oil residue taken up in 500 μl of dry ether. About half of the ether solution was spotted as evenly as possible on to a pre-prepared 13-mm KBr disc. Spectra were acquired for 5 min (*i.e.*, 160 scans).

RESULTS AND DISCUSSION

Size exclusion chromatography

The chromatograms obtained with the Spanish cooking-oils and some of the control oils are shown in Fig. 1. The retention volume *versus* molecular weight data for polystyrene standards are shown in Table I.

The Spanish oils and most of the control oils displayed UV absorption at 254 nm and hence the size exclusion chromatograms provided information on how the UV absorbance varied as a function of molecular weight (or size). All oils had a major peak of elution volume 8.2 ml (mol.wt. *ca.* 1780) and the intensity of this peak varied

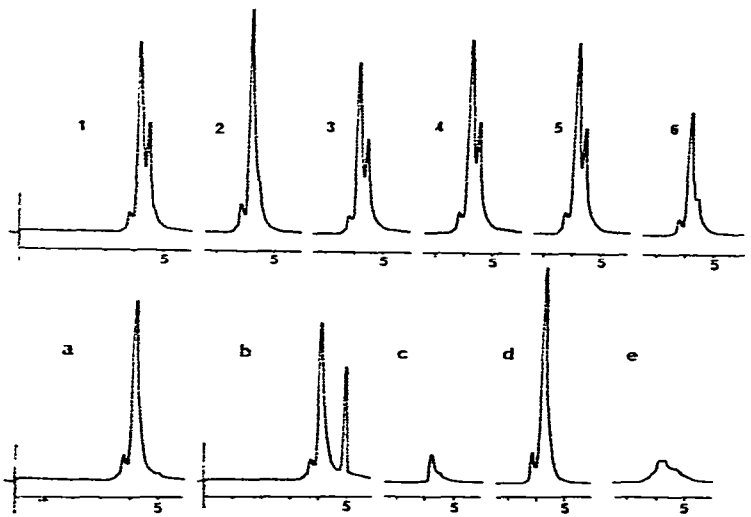


Fig. 1. Size exclusion chromatograms of vegetable oils. Column, 25 cm × 8 mm I.D.; packing, 5 μm silica (see text); eluent, tetrahydrofuran–water (99:1); flow-rate, 2 ml/min; detection, UV at 254 nm; sample concentration, 0.2 g/5 ml of eluent; injection volume, 20 μl. Samples: 1–6 = Spanish cooking oils numbers 1–6, respectively; a = rapeseed oil; b = rapeseed oil + aniline (at 500 ppm); c = olive oil; d = maize oil; e = soya oil. The time scale on these chromatograms is marked in 1-min intervals.

TABLE I

RETENTION VOLUME DATA FOR POLYSTYRENE STANDARDS ON A COLUMN PACKED WITH SILICA OPERATING IN A SIZE EXCLUSION MODE

Column, 25 cm × 8 mm I.D.; packing, 5 μm silica (see text); eluent, tetrahydrofuran–water (99:1).

Molecular weight (polystyrene)	*Retention volume (ml)*
125,000	5.3
25,000	5.9
10,000	6.8
4000	7.7
1400	8.3
650	8.8
92 (toluene)	9.6

considerably in different samples. A feature unique to the Spanish oils, with the exception of sample 2, was a peak of elution volume 9.0 ml (mol.wt. *ca.* 370); this was sufficiently unusual to justify the study that was initiated.

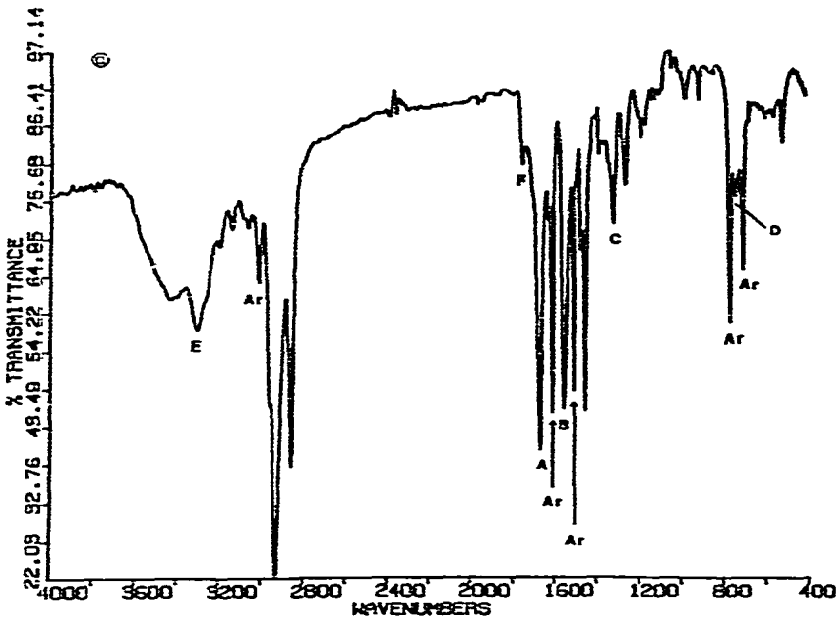
Solvent extraction and spectroscopic examination

By using size exclusion chromatography it was possible to ascertain that the components of molecular weight of about 370 could be extracted from the Spanish oil by acetonitrile. This method of enrichment was used to provide the crude extract suitable for spectroscopic examination. The IR spectrum and the EI mass spectrum of the extract are shown in Fig. 2.

The IR spectrum has strong CH stretching bands in the region of 2850–2950 cm^{-1} together with characteristic features associated with fatty acid anilides[3]. In Fig. 2a the bands at A (1661 cm^{-1}) and B (1546 cm^{-1}) correspond to the amide I (CO stretch) and amide II (NH in plane deformation) modes of a secondary amide. The weaker band C (1310 cm^{-1}) may be attributed to the amide III mode but the amide IV band is obscured by the absorption (D) due to the $(-CH_2-)_n$ rocking mode at 724 cm^{-1}. The band (E) at 3330 cm^{-1} is probably due to the NH stretching mode of a hydrogen bonded secondary amide in the *trans* configuration. The bands (Ar) at 3009, 1601, 1500, 754, and 691 cm^{-1} are all indicative of a monosubstituted benzene ring. The weak band (F) at 1745 cm^{-1} is probably due to the CO stretch of the unreacted glyceride. The EI mass spectrum (Fig. 2b) resembles that of acetanilide in exhibiting a base peak at m/z 93 due to the $(C_6H_5NH_2)^+$ ion and a prominent ion at m/z 135 is attributable to $(C_6H_5NHCOCH_3)^+$. However, the spectrum shows features at masses higher than the molecular weight of acetanilide (*i.e.*, 135), notably an ion at m/z 148. In the case of spectra in which the base peak was well overloaded peaks of low abundance are seen at m/z 355 and 357. The IR and MS data strongly suggested that the extract contained a mixture of fatty acid anilides.

Reversed-phase HPLC of fatty acid anilides and oil extracts

The chromatograms obtained with methanol extracts of Spanish oils and solutions of standard anilides are shown in Fig. 3. It will be seen that with the exception of sample 2, each oil extract contained the same components in the same relative ratios and that the intensity of these peaks in the chromatograms correlated well with the



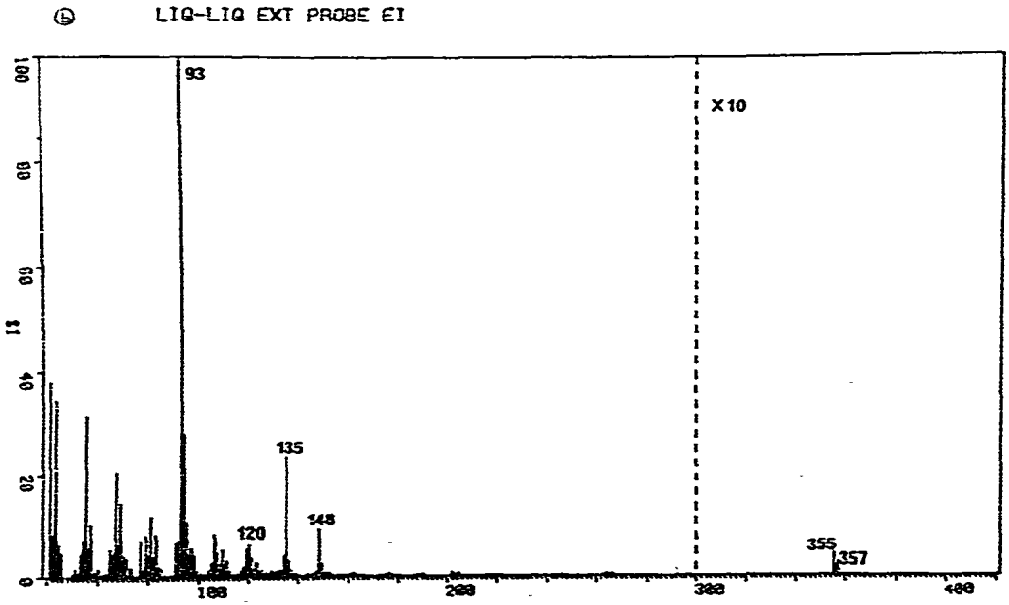


Fig. 2. (a) IR spectrum and (b) EI mass spectrum of an acetonitrile extract of contaminated Spanish cooking oil.

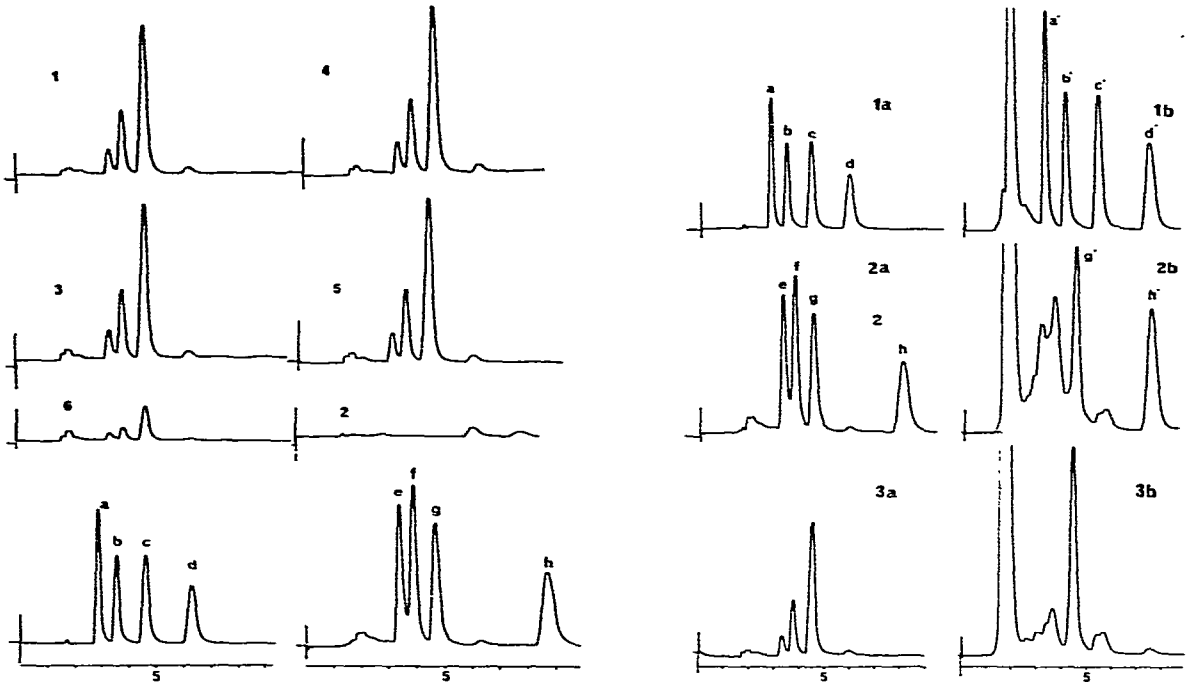


Fig. 3. Reversed-phase liquid chromatograms of oil extracts and standard anilides. Column, 12.5 cm × 5 mm I.D.; packing, C_{18} modified silica (see text); eluent, methanol–water (95:5); detection, UV at 254 nm, sensitivity 1.0; injection volume, 20 μl. Samples: 1–6 = Spanish cooking oils extracts in 5 ml of methanol (sample weights were 348, 484, 399, 464, 373, and 446 mg, respectively); a = lauric anilide; b = myristic anilide; c = palmitic anilide; d = stearic anilide; e = linolenic anilide; f = linoleic anilide; g = oleic anilide; h = erucic anilide. The time scale on these chromatograms is marked in 1-min intervals.

Fig. 4. Reversed-phase liquid chromatograms of an oil extract and standard anilides before and after bromination. Conditions as in Fig. 3. Samples: 1a = anilides of saturated fatty acids; 1b = the same mixture after bromination; 2a = anilides of unsaturated fatty acids; 2b = the same mixture after bromination; 3a = a methanol extract of Spanish oil 1; 3b = same sample after bromination. The compounds are designated in the same coding as in Fig. 3. The peaks shown with a suffixed letter correspond to the bromination products formed from the parent anilide.

intensity of the suspect peak in the size exclusion chromatograms. In Fig. 4 are shown chromatograms run under the same conditions before and after bromination. With the exception of oil 2 all the Spanish oil extracts behaved in the same way on bromination and hence the chromatograms illustrated (*i.e.*, 3a and 3b in Fig. 4 derived from oil 1) are typical of all the results. The retention volume data are summarised in Table II.

The retention of fatty acid anilides under reversed-phase conditions is largely determined by differences in the chain length and degree of unsaturation of the fatty acid moiety. Bromination provides a convenient method of producing additional qualitative information, with anilides of saturated fatty acids yielding a single product (probably the monobromo derivative) of slightly longer retention and greater UV absorbance than the parent compound. The unsaturated fatty acid anilides display a somewhat different pattern after bromination —oleic and erucic anilides, with

TABLE II

RETENTION VOLUMES OF STANDARD ANILIDES, EXTRACTS OF SPANISH COOKING OIL AND THEIR BROMINATION PRODUCTS ON A COLUMN PACKED WITH A C_{18} MODIFIED SILICA

Column, 12.5 cm × 5 mm I.D.; packing, C_{18} modified silica (see text); eluent, methanol–water (95:5) at 1 ml/min.

Compound	*Retention volume (ml)*	*Retention volume after bromination (ml)*
Lauric anilide	2.8	3.2
Myristic anilide	3.5	4.0
Palmitic anilide	4.5	5.3
Stearic anilide	6.3	7.4
Linolenic anilide	3.3	*
Linoleic anilide	3.7	*
Oleic anilide	4.6	4.6
Erucic anilide	8.8	8.3
Peak 1	3.3	*
Peak 2	3.7	*
Peak 3	4.6	4.6
Peak 4	6.3	7.4

* A mixture of several products formed

one double bond in the aliphatic side chain yield major products of slightly lower retention volume and slightly greater UV absorbance than the parent anilide. In contrast linoleic and linolenic anilides which have a greater level of unsaturation, yield several products none of which produce chromatographic peaks of an intensity greater than that of the starting product. Thus a comparison of chromatograms run before and after bromination can give additional evidence to aid in the characterisation of individual anilides.

The retention characteristics and bromination patterns of the components extracted from contaminated Spanish cooking oils supported the conclusions derived from the spectroscopic interpretation. Thus it seems that these oils contain oleic, linoleic and linolenic anilides together with a smaller proportion of stearic anilide. Oil sample 2 was not found to contain anilides.

Fig. 3 indicates that the relative proportion of the four anilides in the Spanish oil extracts is constant and on a peak area basis corresponds to: linolenic anilide, 8%; linoleic anilide, 24%; oleic anilide, 64%; and stearic anilide, 4%. It should be appreciated that these values apply only to the extracts and will only relate to the levels in the oil if extraction does not lead to preferential enrichment of any of these compounds.

To obtain some idea of the levels of anilides actually present in the oil samples, a control oil was fortified with known amounts of oleic anilide, and was then examined by both chromatographic methods. The failure to obtain a crystalline oleic anilide prevented accurate quantitation from being performed, but the results suggested that four of the samples (*i.e.*, 1, 3, 4 and 5) contain about 5 mg of anilide per g of oil; that sample 6 contained about 1 mg/g and that sample 2 was anilide-free.

Spectroscopic confirmation

Confirmation of the identity of the three major UV absorbing components in the Spanish oils was achieved by the spectroscopic examination of the fractions corresponding to the three major peaks on the chromatograms shown in Fig. 3. The IR spectra of the three fractions showed only minor differences between them and were very similar to that given by the crude extract as shown in Fig. 2. The EI

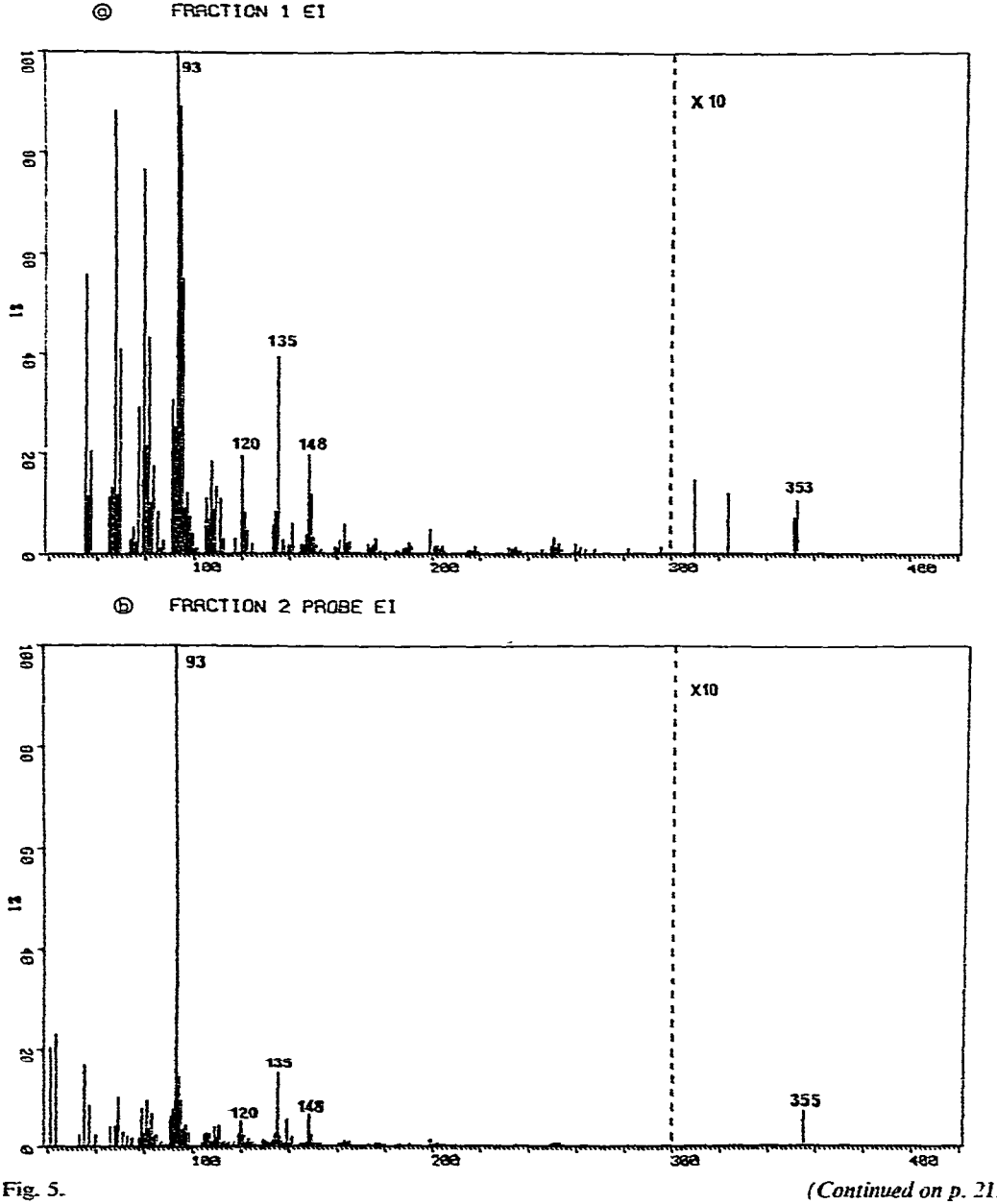


Fig. 5.

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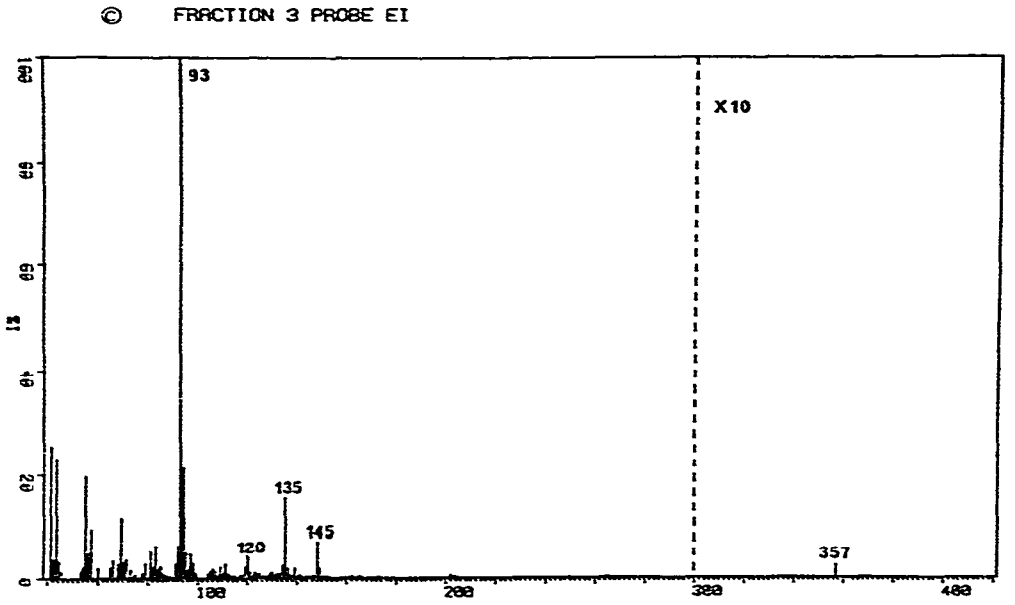

Fig. 5. EI mass spectra of preparative HPLC fractions. (a) Fraction 1 (*i.e.*, the first major retained peak on the chromatogram); (b) fraction 2 (*i.e.*, the second major retained peak); (c) fraction 3 (*i.e.*, the third major retained peak).

mass spectra of the fractions (see Fig. 5) were also similar to each other and that of the crude extract in that the base peak of *m/z* 93 and ions of *m/z* 135 and 148 were common to all. Weak molecular ions were observed in overloaded spectra at *m/z* 353, 355, and 357 for fractions 1 to 3 inclusive. These ions could be assigned to linolenic, linoleic and oleic anilides. Further confirmation was obtained from the isobutane chemical ionisation spectra which exhibited as base peaks the pseudo molecular $(M + 1)^+$ ions at *m/z* 354, 356, and 358 respectively. In the third fraction there was also evidence of the presence of stearic anilide from the $(M + 1)^+$ ion at *m/z* 360.

Heating experiments

The earlier studies showed that all but one of the Spanish oil samples submitted to our Laboratory were contaminated with fatty acid anilides. Although we cannot be certain that these compounds gave rise to the observed poisoning outbreak in Spain it cannot be ruled out, and hence we felt that it was necessary to establish that they could be formed in processes through which the oil had been taken prior to human consumption. The only information available were a few press reports, and it was not possible to simulate exactly the way in which they became contaminated. Apparently vegetable oils not destined for human consumption are sometimes denatured by heating with aniline. This process causes the oils to become darkly coloured and obviously unpalatable. In the case of the Spanish oil poisoning it seems likely that the colour and odour was removed from the denatured oil by some process that failed to remove the anilides that were also present.

The results obtained by heating a control rapeseed oil with aniline in different

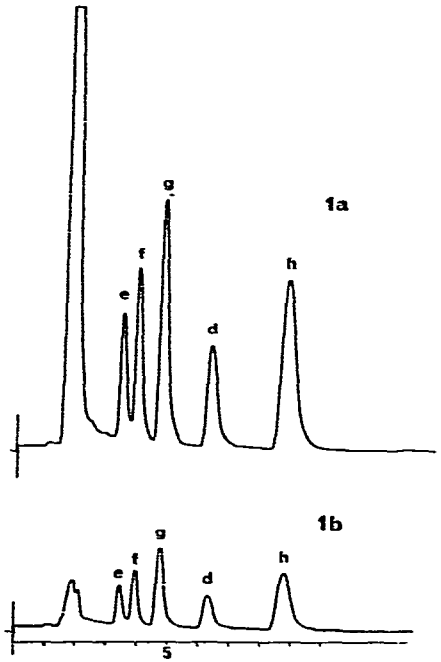

Fig. 6. Reversed-phase liquid chromatograms of a rapeseed oil heated with aniline at 150°C. Conditions as in Fig. 3. Samples: 1a = 0.4 g of rapeseed oil and 0.4 g of aniline heated for 18 h at 150°C. The sample was then dissolved in 5 ml of tetrahydrofuran and 100 μl of this solution was diluted with 5 ml of eluent to provide the solution for injection; 1b = 0.4 g of rapeseed oil and 0.03 g of aniline heated for 18 h at 150°C. Sample dilution as for 1a. The anilide peaks are coded as in Fig. 3.

proportions are typified by the chromatograms shown in Fig. 6. Regardless of whether the oil or the aniline were in excess, heating resulted in a blackening of the oils and the chromatograms show that a mixture of anilides of constant relative proportion were formed. Present in the mixture were four of the anilides identified in the Spanish cooking oils, but a comparison of Figs. 3 and 6 shows that in the latter the levels of stearic and erucic anilides formed by heating give the chromatograms a quite different appearance. This may reflect a difference in the composition of the oils prior to the formation of the anilides or could possibly result from the treatment used in removing the dark colour from the Spanish oils.

Screening methods

For screening purposes either of the HPLC methods described above could be used to provide rapid and sensitive analysis of oils contaminated with anilides. For those laboratories without HPLC equipment the IR screening method offers an alternative approach. Typical spectra derived from contaminated and uncontaminated oils are shown in Fig. 7. Comparison of these spectra and that given in Fig. 2 shows that the simple extraction procedure described is adequate to indicate the presence of anilides in a contaminated sample. The procedure takes about 30 min but could be reduced if smaller volumes of solvent were used, because evaporation of the methanol

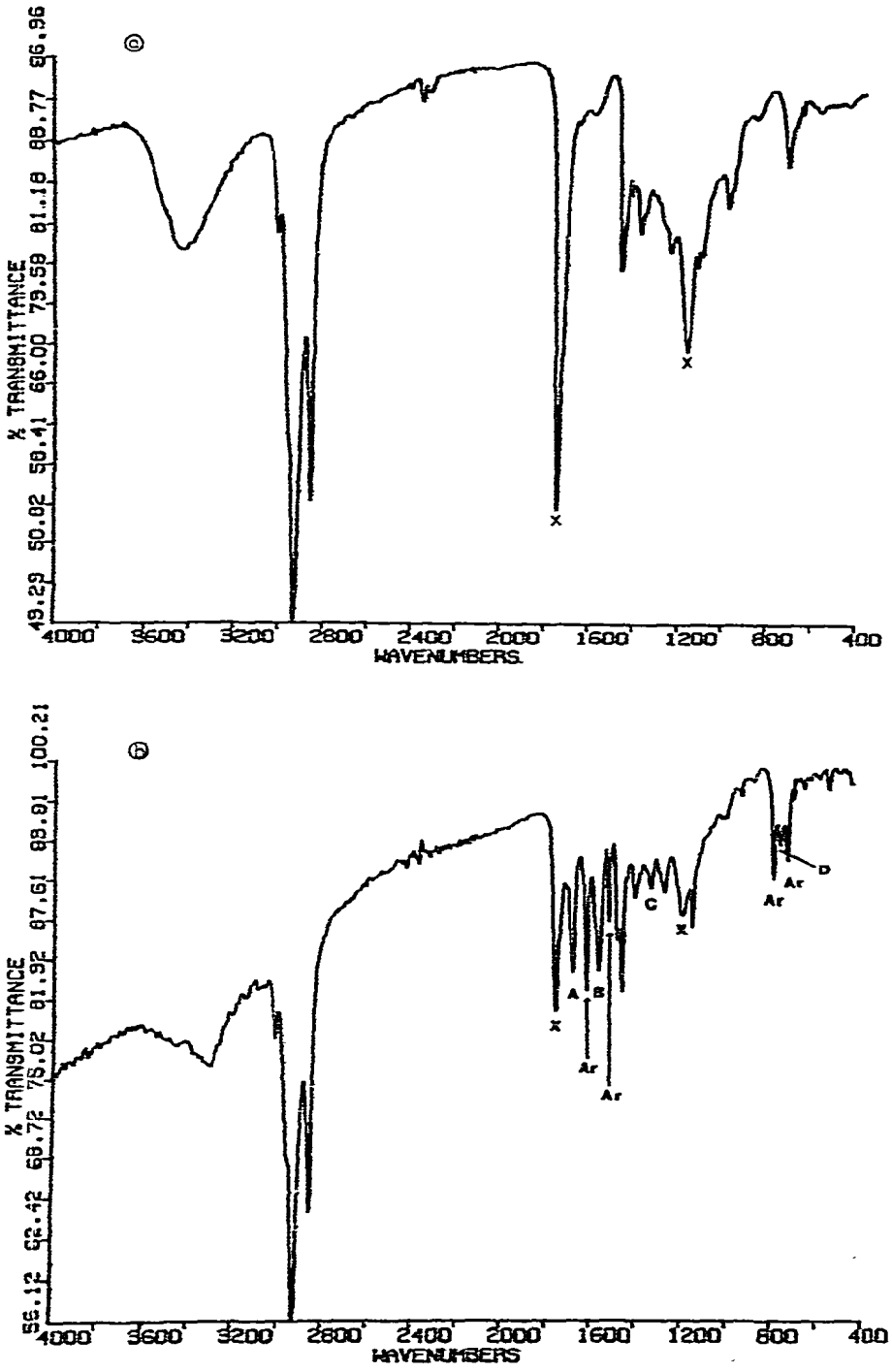

Fig. 7. IR spectra of methanol extracts of (b) contaminated Spanish cooking oil and (a) an uncontaminated rapeseed oil.

extract is the time consuming step. The isooctane wash could also be omitted although the ester bands (X) of the co-extracted glyceride would be very much stronger.

CONCLUSIONS

The analytical study presented in this paper shows that Spanish cooking oils associated with the epidemic in Madrid contain mixtures of oleic, linoleic, linolenic and stearic anilides. Such compounds can be formed by heating aniline with a rapeseed oil and the chromatographic and spectroscopic methods that have been used in this study would provide procedures suitable for screening for the presence of such compounds in contaminated oils.

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