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Improved Analysis of Fatty Acid Anilides: Application to Toxic and to Aniline and Citric Acid-Containing Oils

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The Spanish Toxic Oil Syndrome (TOS) was traced to the consumption of some cheap oils sold at retail by street salesmen (Tabuenca 1981; Kilbourne et al. 1992). These oils contained different proportions of a refined batch of rapeseed oil initially denatured with 2% of aniline and imported from France for industrial use (Tabuenca 1981). However, although most of the free aniline was effectively extracted during the refining processes, a significant part of it reacted with fatty acids yielding the corresponding fatty acid anilides (FAAs) (Guitart and Gelpí 1992). Although toxicological data on FAAs are very controversial, they had been proposed as the causative agents of the illness or, at least, are considered as the best markers of toxic oils (Bernert Jr. et al. 1987; Kilbourne et al. 1991).

Four gas chromatographic methods have been published for the analysis of FAAs in oils (Diachenko et al. 1982; Vázquez Roncero et al. 1983; Artigas et al. 1983; Balley et al. 1983). The chemical similarity with the methyl esters of fatty acids makes gas-liquid chromatography (GLC) the method of choice for the analysis of FAAs, although their higher molecular weights limited up to now the use of the more resolutive polar stationary phases. Since the introduction of cross-bonded polar phases which can be run at higher temperatures than columns available in the last decade, the possibility to obtain a better separation of FAAs is raised. This paper reports the development of such a method and its application to 10 case oil samples and to the study of the generation rate of FAAs in aniline-denatured rapeseed oils containing citric acid. This additive was used in some refineries that handled the TOS-suspected oils in 1981 as a stabilizer, preservative and antioxidant, and was added normally before the deodorizing process at a l-2% concentration (Special Investigation Team 1981).

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MATERIALS AND METHODS

All the solvents and reagents used (analytical, HPLC or pesticide grade) were purchased from Scharlau (Barcelona, Spain), Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Synthesis of the palmitic (16:0), margaric (17:0) and stearic (18:0) FAAs were carried out following the method of Wheals et al. (1982), and purified by HPLC on a Cl8 semipreparative column (Econosphere) (Alltech, Deerfield, IL, USA). The anilides of palmitic (16:0), oleic (18:l n-9), linoleic (18:2 n-6), linolenic (18:3 n-3), gondoic (20:l n-9), gadoleic (20:l n-11), behenic (22:0) and erucic (22:1 n-9), all of purity >98% and prepared according to the method of Ferrer et al. (1993), were a generous gift from Drs. A. Messeguer and J. Casas from the CID-CSIC in Barcelona, Spain.

Authentic 10 case samples of TOS-associated oils (Bernert Jr. et al. 1987) were kindly provided by Drs. M. Posada de la Paz and I. Abaitua, of the Fund for Health Research in Madrid, Spain. A bottle of commercially available rapeseed oil, purchased in a French market in 1992, was used for recovery and FAA-production assays. For the latter, after preparing 50 mL of a 2% (v/v) aniline denatured oil, 8 mL were distributed in 5 Pyrex tubes (capacity 10 mL) fitted with seal tight teflon caps. To each of these tubes 0, 50, 100, 150 and 200 mg of anhydrous citric acid (samples coded throughout this paper as samples B, C, D, E and F, respectively) were added. After mixing, the tubes were placed in an oven with the temperature set at 80±2°C. Another tube containing 8 mL of non-denatured rapeseed oil was also placed in the oven (sample coded as A). Each of the six samples were analyzed daily from days 1 to 10, and on day 15.

Before analysis the tubes containing the oils were mixed and centrifuged. From each tube, 50 mg of oil were taken from the upper part of the tube and deposited on polypropylene Eppendorf tubes. The 17:0 FAA (10-100 µg) as internal standard (IS) was added at this point. The FAAs were extracted by vigorous shaking of the oils during 2 min with 1 mL of methanol, and centrifugating at 2000 g and 10°C for 15 min (Omnifuge 2.0RS) (Heraeus, Osterode, Germany). Immediately, 900 µL of the upper phase were withdrawn and deposited on 5 mL glass-conic vials with teflon caps. The content of the vials were concentrated by a gentle stream of N_2 in a water bath at 40°C, resuspended in 30 µL of ethyl acetate, and vigorously mixed.

The samples were purified by injecting the extracts (approximate volume 15 µL each injection) on a guard liquid chromatographic column of 4 mm I.D. and 5 cm length, coated with Nucleosil C8 of 15-25 µm particle size (Macherey-Nagel, Düren, Germany), coupled to a

Knauer (Berlin, Germany) HPLC pump, injector and W detector fixed at 254 nm and connected to a recorder. The mobile phase consisted in acetonitrile:distilled water: ethyleneglycol:acetic acid 70/20/10/0.2 (v/v), run at 1.6 mL/min. The eluates from each sample between 0.75-l and 5-6 minutes (exact time checked daily with the injection of standards) were collected on 15 ml-glass tubes. After addition of 5 mL of distilled water, anilides were extracted by 2 x 2 mL of petroleum ether, vigorous mixing and centrifugation, and transfer of the upper phase to a 5 mL conic glass tubes with teflon caps. After evaporation to dryness under a gentle stream of N_2, the content of the tubes were resuspended in ethyl acetate (10-100 µL) and 0.5-1.0 µL injected in the GC system.

Gas chromatographic analysis of purified extracts of oils were performed at 245°C on a Perkin-Elmer 8500 instrument (Beaconsfield, UK), equipped with a nitrogen-phosphorus detector (NPD), using a cross linked and fused silica 70% cyanopropyl equivalent modified siloxane (BPX70) column of 25 m x 0.53 mm ID and 0.5 µm of film thickness (SGE, Victoria, Australia). Carrier gas (He) flow was set at 3 mL/min, and filters for oxygen, water and hydrocarbons were used to improve its purity. For the NPD, make up gas (He), H_2 (obtained through a hydrogen generator model 9200 from Packard, Downers Grove, IL, USA) and synthetic air flows were optimized to achieve the maximum response for FAAs. Injector and detector temperatures were set at 290°C. The GC was calibrated by injection (x3) of standards of 16:0, 18:l n-9 and 18:2 n-6 FAAs at the following quantities: 50, 250 and 500 ng, and 2.5, 5, 25, 50 and 250 µg, resuspended in 10-100 µL of ethyl acetate and containing the IS. For quantitative purposes, the 16:0 FAA calibration curve was used for all the saturated group of FAAs, the 18:l n-9 FAA for the unsaturated group and the 18:2 n-9 FAA for the polyunsaturated group.

RESULTS AND DISCUSSION

The present method offers a rapid and non-expensive system to purify FAAs from other oil components. For the initial extraction, methanol was selected and used, because other solvents tested were more difficult to evaporate, gave poor recoveries for FAAs or tend to extract higher quantities of undesirable oil components. The use of an HPLC precolumn eluted isocratically was considered the method of choice for the purification of FAAs, because it was rapid, resolutive and with high recoveries, with the additional advantage that every sample could be monitorized individually at 254 nm. Due to the clean methanolic extracts of the oils injected, the precolumn maintained its performance for long time, but it was found convenient to replace the stationary phase every 40-50 samples.

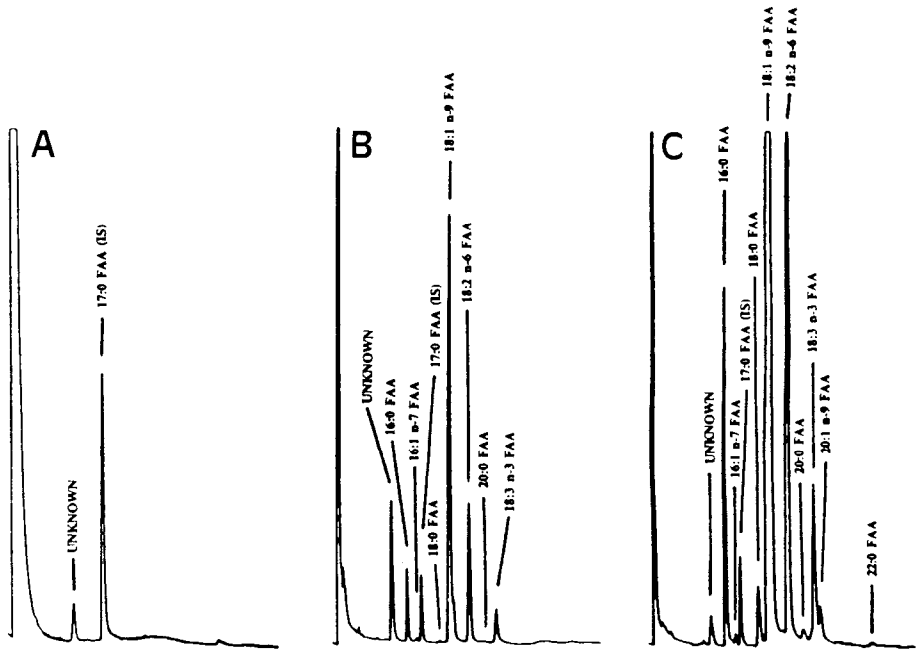

Figure 1. GLC/NPD chromatographic profile obtained with (A) sample A analyzed on day 15, (B) case-TOS oil, and (C) sample F analyzed on day 15.

Despite the general greater sensitivity of detectors used in GC over UV detection in HPLC, and the higher resolution of the GLC capillary columns, only one published method had take advantage of these facts for the analysis of anilides (Bailey et al. 1983), while other three used less resolutive GLC packed columns (Diachenko et al. 1982; Vázquez Roncero et al. 1983; Artigas et al. 1983). However, the non-polar silicone stationary phase used by Balley et al. (1983) is unable to separate the interesting group of polyunsaturated FAAs, which could be resolved on a more polar stationary phase, such as the 70% cyanopropyl column. This column can be raised up to 290°C of temperature without degradation, but the high bleeding on the NPD of this column at high oven temperatures recommended its use in isothermal conditions in order to achieve maximum sensitivities maintaining good peak resolution.

Figure 1 shows the chromatograms of purified extracts of some analyzed samples. As can be observed, controls give very clear extracts on the NPD, with no interfering peaks in the zone of elution of the FAAs. Some FAAs are not fully resolved with this chromatographic conditions:

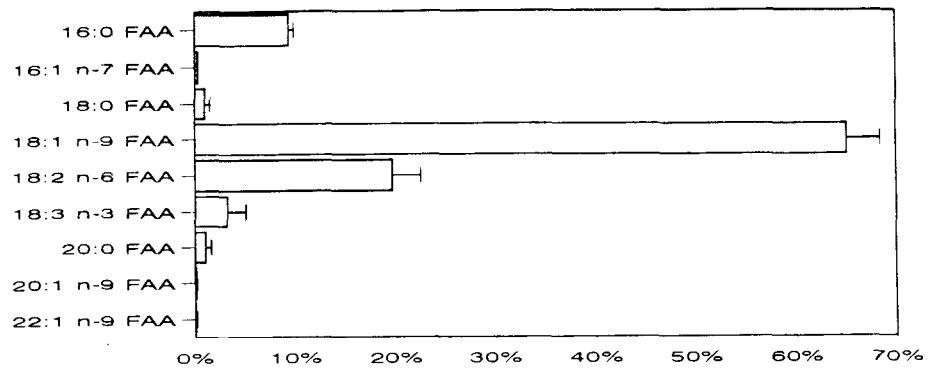

Figure 2. Mean (and SD) composition in anilides of 10 case-oil samples associated with TOS.

this is the case of the 18:l n-7 FAA, eluting close and later than the 18:l n-9 FAA.

Calibration curves were constructed for the 3 most important FAAs (16:0, 18:l n-9 and 18:2 n-9) over a wide range of concentrations. They were linear (r>0.998) between the range 5-2500 ng injected in the GC, and the limit of detection was approximately 2-5 ng, which indicates that the limit of quantification is about 1 ppm, much better than any previously published method. Recoveries for these three FAAs were approximately 90%, with a good reproducibility over the wide range of concentrations assayed (Table 1). Repeated analysis (n=lO) of a 2% aniline denatured rapeseed oil sample containing spontaneously formed FAAs give also a satisfactory reproducibility, with values of coefficient of variation ranging from 6.55% for the 18:0 FAA to 12.11% for the 18:3 n-3 FAA.

Table 1. Percentage of recovery (mean and SD) for some saturated and unsaturated FAAs in fortified samples (50 mg of edible rapeseed oil) (n = 6).

SPIKED QUANTITY	16:0 FAA	18:l FAA	18:2 FAA
0.25 μg	87.7±13.2	92.8±14.0	92.7±9.8
2.5 μg	88.6±7.26	95.7±5.31	89.9±13.3
25 μg	90.6±6.36	88.3±7.68	88.2±7.63
250 μg	86.1±7.93	87.0±11.4	84.7±12.3

Figure 2 shows the concentration (in %) of FAAs in the 10 TOS-case oils analyzed by triplicate. The similar proportion of FAAs in these samples suggest that only one source of denatured rapeseed oil was used, but the different total concentration (mean ± SD = 956±469 μg/g,

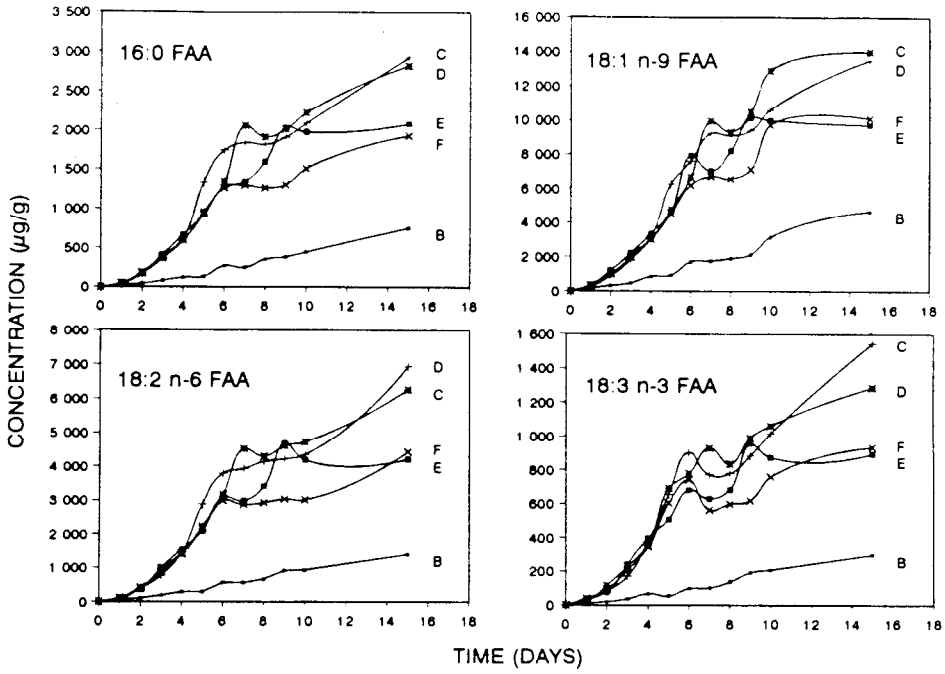

Figure 3. Kinetics of generation of the 16:0, 18:l n-9, 18:2 n-6 and 18:3 n-3 FAAs in heated (80°C) 2% aniline-denatured rapeseed oils containing different proportions of citric acid: B = 0%, C = 0.625%, D = 1.25%, E = 1.875% and F = 2.5% (w/v).

range 223-1607 µg/g) confirms that the original aniline-denatured oil was mixed in different proportions with other oils before distribution to the consumers (Guitart and Gelpí 1992). TOS-case samples were also analyzed by Bernert Jr. et al. (1987) by an HPLC-UV method, but they could only quantify the 16:0, 18:l n-9 and 18:2 n-6 FAAs due to interferences in some samples with other oil components, so Figure 2 provides new information on the composition of FAAs in case-oils.

The preparative and analytical method was also applied to study the effect of the presence of different proportions of citric acid on the generation of FAAs in a 2% aniline-containing rapeseed oil. This additive was used for some of the refineries that handled the aniline-denatured oil, and initially it was believed that its presence had no effect on the refined product obtained (Special Investigation Team 1981). However, results obtained in the present study clearly indicated that the addition of this chemical had an unnoticed effect on the total quantity of FAAs formed, which are dramatically increased (Figure 3). It should be pointed out that the oils containing citric acid became turbidous and darken with time. The synthesis

of FAAs increased greatly until day 6 in samples containing citric acid, and from then the speed was reduced; this effect of "saturation" was more manifest with the samples containing higher concentration of citric acid. In our opinion, this acid-catalyzed and other possible effects or synthesized by-products derived from the presence of citric acid in the refining process of aniline-denatured oils needs to be deeply explored from the analytical and toxicological point of view.

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