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Identification of Fatty Acid Anilides in Adulterated Spanish Cooking Oils

G. W. Diachenko, M. P. Yurawecz, B. J. Puma, P. A. Dreifuss*, J. T. Chen*, I. Egry, and R. Carver

*Division of Chemical Technology, *Division of Chemistry and Physics, Food and Drug Administration, Washington, DC 20204*

In the spring of 1981, Spanish authorities uncovered an epidemic of atypical pneumonia which has since claimed at least 200 lives and caused the hospitalization of more than 10,000 people in the vicinity of Madrid (TRENCH 1981). The disease has been attributed to consumption of adulterated cooking oils sold as "olive oil" by door-to-door salesmen (TABUENCA 1981). Most of the subject oils have been retrieved through a Spanish program which allowed free exchange for virgin olive oil.

In July 1981, via the Centers for Disease Control (CDC), Atlanta, GA, we received several oil samples suspected to have been implicated in the epidemic. The oils were analyzed for a wide variety of pesticides, industrial chemicals, and toxic elements. In general, only "usual" or trace levels of those elements or chemicals which are ordinarily determined by FDA procedures were found. We did, however, find high levels of fatty acid anilides in two of the suspect cooking oils and elevated levels of chlorine and bromine in another. This paper reports only the detection and identification of the fatty acid anilides.

After completion of this work, it was reported (TABUENCA 1981) that the oils associated with the illness contained large amounts of rapeseed oil, other oils, and animal fats. The rapeseed oil component had been denatured with aniline, which was detected at low levels (1-50 ppm). It was also reported that 1500-2000 ppm of oleanilide and lower concentrations of azobenzene, acetanilide, and quinoline were present in some of the adulterated oils.

In this paper, we report independent corroborative findings of oleanilide and mass spectrometric (MS) data supporting the presence of other fatty acid anilides in two samples of the adulterated oils.

MATERIALS AND METHODS

Samples. All oil samples were collected on June 16, 1981, in Navas del Mareques, Avila, Spain. Samples were sent to the CDC, who then forwarded portions to us. In this report, "case"

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oils refer to those obtained from families experiencing at least one incidence of atypical pneumonia, whereas "control" oils were obtained from unaffected families; 10 case and four control oils were received.

Analysis. Three-gram portions of selected samples were analyzed by the method of the Association of Official Analytical Chemists for the determination of pesticides in fatty foods (OFFICIAL METHODS OF ANALYSIS 1980). The oil was dissolved in petroleum ether (PE) and then partitioned with acetonitrile. After dilution with water, the acetonitrile fraction was extracted with PE. This extract was cleaned up by Florisil column chromatography by eluting with successive 200 mL portions of 6, 15, 50, and 100% ethyl ether (EE) in PE; the fatty acid anilides eluted in the 50% EE/PE fraction. The eluates were concentrated to 3-8 mL for determination by gas-liquid chromatography (GLC).

Ten percent solutions of all case and control oils in pentane were also analyzed directly by GLC (no sample cleanup).

GLC. The Florisil eluates were analyzed for nitrogen- and phosphorus-containing compounds by using a Hewlett-Packard 5710A gas chromatograph equipped with a nitrogen/phosphorus (N/P) detector. The GLC parameters were those used by LOMBARDO and EGRY (1979). A 1.2 m x 2 mm i.d. glass column packed with 3% OV-101 on 80-100 mesh Chromosorb W(HP) was operated at 230°C with injector temperature 250°C and helium carrier flow rate 30 mL/min. The detector was operated at 300°C and 16 V with 60 mL/min air and 3 mL/min hydrogen flow rates. Under these conditions, triphenyl phosphate elutes in about 1.6 min.

Diluted oils were analyzed by using a Varian 3700 gas chromatograph equipped with a flame ionization detector (FID) and temperature programming (from 80 to 240°C at 40°C/min, then hold for 25 min) on a 1.8 m x 2 mm i.d. glass column packed with 3% OV-101 on 80-100 mesh Chromosorb W(HP), nitrogen carrier flow rate 30 mL/min, detector temperature 300°C, and injector temperature 250°C. Quantitation was performed by comparison of peak area to that of an oleanilide standard.

Infrared (IR) Analysis. Micro KBr discs were prepared by the method of CHEN (1965). IR spectra were obtained with a Perkin-Elmer 180 grating spectrophotometer equipped with 6X dual beam condensing accessory.

MS Analysis. Electron ionization (EI) GLC/MS spectra were obtained on a Finnigan 3300F EI mass spectrometer with 0.5 mA emission current and 70 eV electron energy. A 0.9 m x 2 mm i.d. glass column packed with 3% SP-2100 on 80-100 mesh Supelcoport was used for GLC. This column was held at 50°C for 5 min after injection, temperature-programmed to 230°C at 8°C/min, and held at 230°C for 50 min. Operating

conditions: injector temperature 230°C; glass jet separator interface temperature 240°C; helium carrier flow rate 30 mL/min.

Chemical ionization (CI) mass spectra were obtained on a Finnigan 3300F by probe insertion with 0.5 mA emission current and 140 eV electron energy. The methane reagent gas pressure in the ion source was 1.0 torr (uncalibrated).

Oleanilide Standard. Oleanilide was synthesized from oleic acid and aniline as described by ROE et al. (1949). The purified product was characterized by GLC/MS.

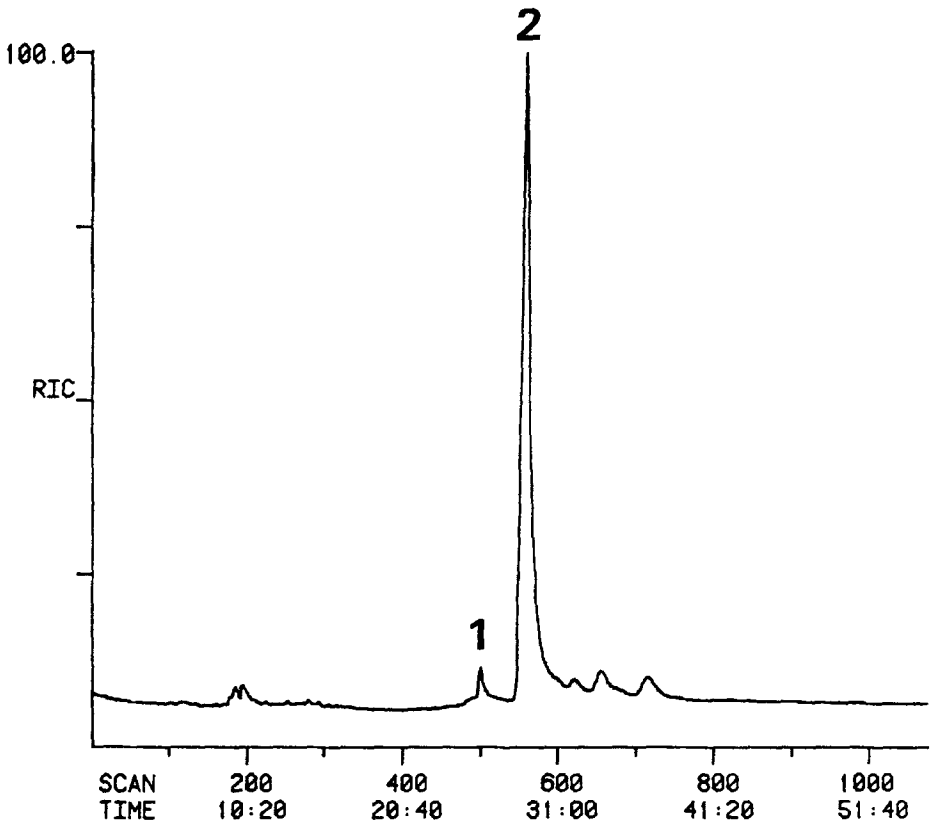

Figure 1. Reconstructed GLC/MS ion chromatogram of 50% EE/PE eluate from an adulterated Spanish cooking oil. GLC conditions: 0.9 m x 2 mm i.d. 3% SP-2100 column, injected at 50°C (held 5 min), programmed to 230°C at 8°C/min, held 50 min. Peak 1 corresponds to the retention time of palmitanilide and peak 2 corresponds to the retention time of oleanilide and other co-eluting fatty acid anilides.

RESULTS AND DISCUSSION

Analytical techniques routinely employed by this laboratory were used to examine the oils for a wide variety of pesticides and organic industrial chemicals; none was found at other than trace or "usual" levels. However, when the 50% EE/PE eluates of two case oils were analyzed for aryl phosphates by the procedure of LOMBARDO and EGRY (1979), large N/P GLC responses were observed. These responses, at retention times of 3.1 and 5.2 relative to triphenyl phosphate (R_{TPP}), had not been previously encountered in our analyses of foods, nor were they detected in a sample of authentic olive oil.

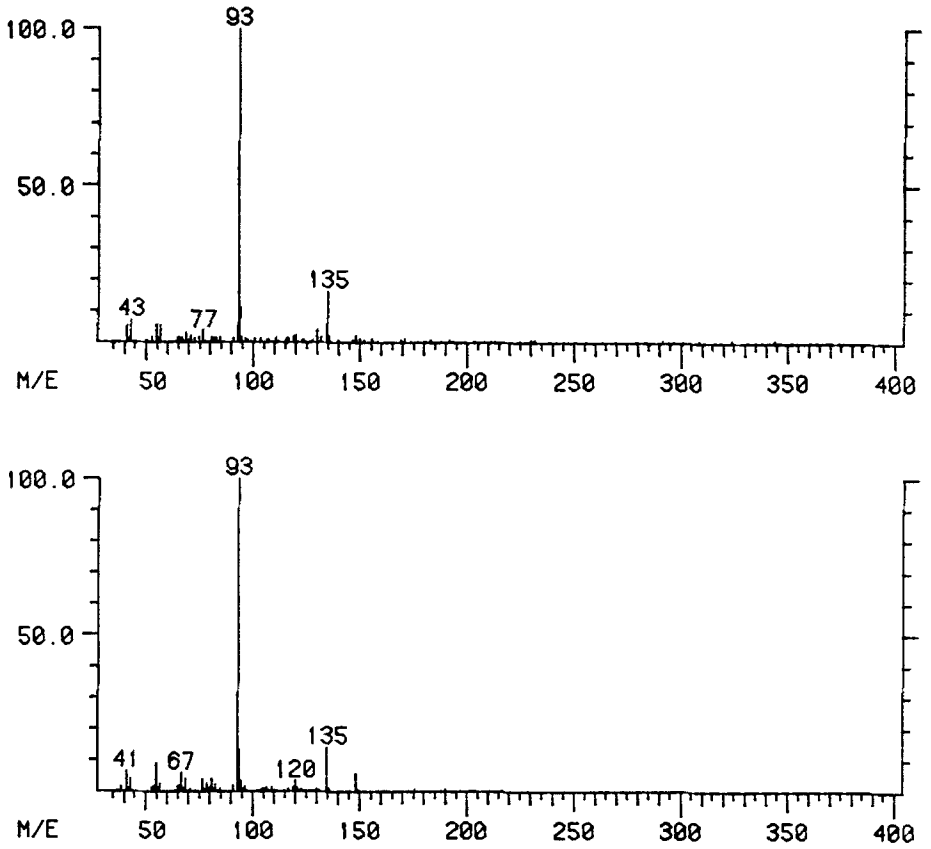

Figure 2. EI mass spectra of substances producing peaks 1 (upper spectrum) and 2. (Both peaks are shown in Figure 1.)

The 50% EE/PE eluates were analyzed further by GLC/MS. Figure 1 is a reconstructed ion chromatogram of one of the case oil samples. Peaks 1 and 2 corresponded in retention time to the major peaks in the N/P GLC chromatograms. Very similar EI mass spectra were obtained for the substances producing both peaks (Figure 2). These spectra, containing peaks for ions at m/z 41, 43, 55, 77, 93 (base), 120, and 135, resembled those of

long chain anilides (TOMER and DJERASSI 1974), but did not provide definitive molecular weight information. The oleanilide standard produced an EI mass spectrum similar to that obtained for the peak 2 component. The substances producing corresponding GLC peaks from a second case oil produced mass spectra nearly identical to those shown in Figure 2.

For IR analysis, the material at the GLC retention time of peak 2 was collected by trapping on KBr powder, and a micro KBr disc was prepared. The IR assignments are listed in Table 1. The data indicate that the unknown substance contains NH, C=O, a monosubstituted benzene ring, and an aliphatic hydrocarbon chain. These data are consistent with an N-phenyl amide of a long chain fatty acid.

The material at the GLC retention time of peak 2 was also trapped on glass beads and examined by CI/MS. The spectrum is shown in Figure 3. The ions at m/z 354, 356, and 358 correspond to $[M + H]^+$ ions produced from a mixture of

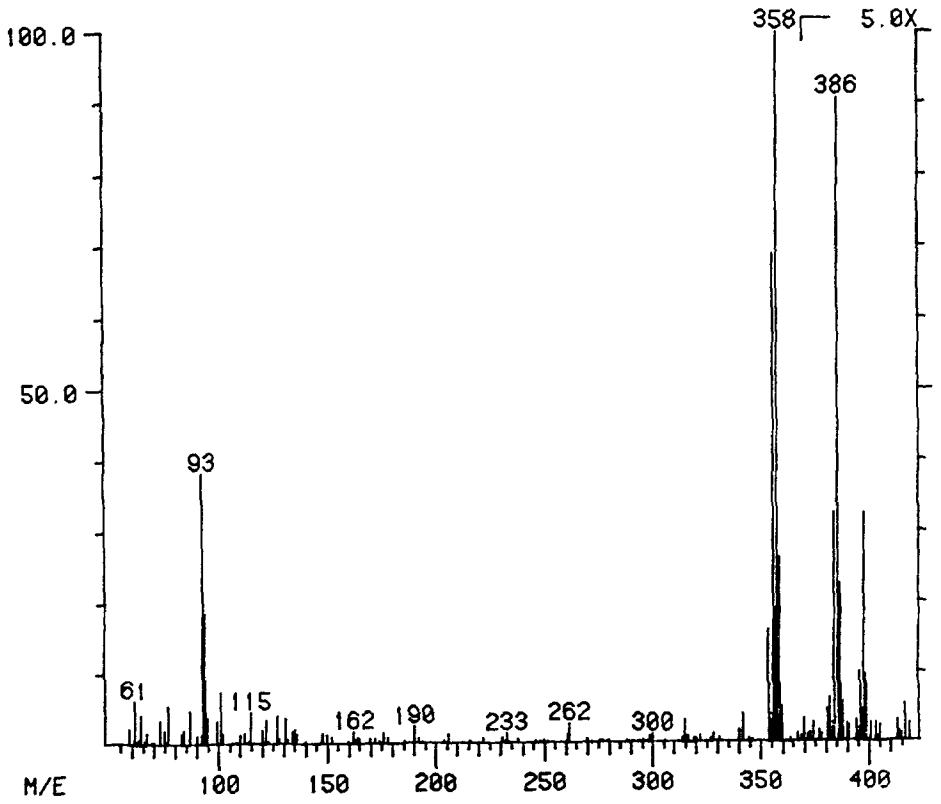

Figure 3. Methane CI probe mass spectrum of fraction corresponding to peak 2. Ion currents at m/z 370 and above have been magnified by a factor of 5.

TABLE 1

Assignments for IR Spectrum of Peak 2 Component (Figure 1)[a]

Wavenumber (cm^{-1})	Functional Group
3300 (wm)	NH st
3000 (vw)	CH st on C=C
2942 (m,sh)	CH_3 asymmetric st
2916 (vs)	CH_2 asymmetric st
2846 (m)	CH_2 symmetric st
1662 (s)	C=O st
1601 (ms)	C=C st of aromatic ring
1542 (s,b)	NH in-plane deformation
1500 (m)	C=C st of aromatic ring
1444 (ms)	CH_3, CH_2 asymmetric in-plane deformation
755 (mw)	5 adjacent free H atoms of aromatic ring, out-of-plane
694 (wm)	deformation

[a]Abbreviations: st = stretching; sh = shoulder; b = broad; v = very; s = strong; m = moderate; w = weak.

compounds of molecular weights 353, 355, and 357. These molecular weight assignments are substantiated by $[M + 29]^+$ and $[M + 41]^+$ adduct ions for the three compounds. The data also indicate that a small amount of a compound with molecular weight 359 may be present. These data, in conjunction with the EI and IR data, are consistent with co-eluting N-phenyl amides of stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids. The relative abundances of the $[M + H]^+$ ions at 354, 356, and 358 suggest that oleanilide is the major component of the mixture.

The GLC retention time of the oleanilide standard (R_{TPP} 5.2) matched that of peak 2 in the case sample. The combined IR, EI/MS, CI/MS, and GLC evidence confirmed the identity of peak 2 as a mixture of oleanilide and co-eluting fatty acid anilides.

The oleanilide standard was also used to estimate total fatty acid anilides in the two case samples by FID/GLC; the fatty acid anilide concentration of each sample was about 2000 ppm. Fatty acid anilides were not detected in the other eight case oils or in the four controls at a detection limit of 150 ppm (relative to oleanilide).

Our independent findings of high fatty acid anilide levels support those reported by Hernando Bolando and colleagues of the Spanish Central Customs Laboratory (TABUENCA 1981). The latter report speculates that the anilides were formed during attempts to refine the oils by distillation of the aniline

(added at 2% as a denaturant for the rapeseed oil). This treatment may also account for peak 1 in Figure 1; the similarity of the EI/MS spectra of the substances producing peaks 1 and 2 suggests that the minor component may also be a fatty acid anilide. The GLC retention time of peak 1 (R_{TPP} 3.1) matched that of a reference standard of palmitanilide (16:0) obtained from CDC. Because of the low level present, no attempt was made to confirm the presence of palmitanilide by CI/MS.

It must be emphasized that the findings of fatty acid anilides do not necessarily imply a causal relationship with the illnesses in Spain. These findings, coupled with those reported by TABUENCA (1981), suggest that fatty acid anilides and/or aniline may serve as indicators of adulterated Spanish cooking oils that have been reported to produce toxic effects in humans.

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