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Gas Chromatographic/Mass Spectrometric Determination of Aniline in Food Oils Associated with the Spanish Toxic Oil Syndrome

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In 1981, a new disease, known today as the toxic oil syndrome (TOS), descended upon the people of Spain (Grandjean and Tarkowski 1984). A strong association between TOS and contaminated food oil was established early (Tabuenca 1981). Subsequent investigations implicated food oils containing rapeseed oil denatured with aniline (Ventura Diaz 1982; Posada et al. 1987). However, little aniline was found in the oils; some other etiologic agent in the oil had apparently produced the illness. Many researchers have investigated these oils, but the specific etiologic agent has not been identified.

Significant progress in this research has been hampered by the difficulty in identifying the specific oil samples that produced illness in specific TOS cases. Moreover, the disease has not been reliably reproduced in experimental animals (Grandjean and Tarkowski 1984). In 1981, all suspect oils were recalled by the Spanish Government and exchanged for pure olive oil. Because many of the exchanged oil samples were probably unrelated to TOS, it has been extremely difficult to identify specific oils within the recalled oil collection that must have contained the causical agent. This lack of certainty regarding which oils contained the etiologic agent has greatly impeded progress toward identification of the causal agent and has complicated the interpretation of many of the available toxicological data.

In 1984, the Spanish Government invited the Centers for Disease Control (CDC) to participate in its research efforts to study the TOS problem. One of us (E.K) was detailed to Spain to assist in the study of the illness. Part of our work in this area has been an attempt in our laboratories to classify a group of "blind-coded" case and control oils according to selected chemical measurements. We report here a newly developed method for determining aniline in these oils and the results of these analyses. Most results of the epidemiological part of the study are described elsewhere (Kilbourne et al. 1987).

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MATERIALS AND METHODS

The 195 oil specimens used in this study were collected during the 1981 oil recall/exchange program and stored in two unheated and nonair-conditioned storage areas in Madrid province. In the summer of 1985, these oils were selected, blind-coded, and shipped to our laboratory for selected analysis. Details of sample selection and case/control identification are described elsewhere (Kilbourne et al. 1987). Two aniline (J.T. Baker Chemical Co., Phillipsburg, NJ) in benzene solutions, 40 and 10 μg/mL, were used to fortify (20 μL) Goya Spanish Olive oil (Goya EN Espana, S.A., Sevilla, Spain--purchased at an Atlanta market) for use as aniline-fortified-in-oil standards of 800 and 200 ppb, respectively. An aniline U-$^{13}C_6$ (MSD Isotopes, Montreal, Canada) in benzene solution (25 μg/mL) was used as an internal standard to fortify (20 μL) all samples and aniline-fortified-in-oil standards. The aniline U-$^{13}C_6$ contained less than 1% ^{12}C-aniline. Heptafluorobutyric anhydride (HFBA) (Pierce Chemical Co., Rockford, IL) was the derivatizing agent. Borate buffer, pH 9.2, was prepared by adding 1.24-g of boric acid (Malinckrodt, Paris, KY) and 53.4 mL of 0.2N NaOH to a 200-mL volumetric flask and diluting to volume with deionized water. The purified HFBA derivative of aniline was synthesized by a modification of the method of Walle and Ehrsson (1970). The white crystalline product was recrystallized from hexane/benzene and had a melting point of 94.5-95°C.

A 1-g oil sample (or an aniline-fortified-in-oil standard) was fortified with 20 μL of internal standard. One mL of isooctane and 2 mL of 1N H_2SO_4 were added to the sample, and the tube was vortexed vigorously and centrifuged. The top layer was discarded, and 3 mL of hexane was added. The tube was vortexed and centrifuged, and the top layer of the mixture was discarded. The aqueous solution was saturated with Na_2SO_4, and 3 mL of hexane was added. The tube was vortexed and centrifuged, and the top layer was discarded. The aqueous solution was made alkaline (pH 9.2) with 0.5 mL of NaOH, and 3 mL of benzene was added. Following vortexing and centrifuging, 2 mL of the top layer was transferred to a clean tube, and 50 μL of pyridine and then 50 μL of HFBA were added. The tube was capped, vortexed, and placed in a water bath at 45°C for 15 min. The tube was removed and cooled. The benzene layer was washed by addition of 4 mL of borate buffer; the tube was vortexed and centrifuged. The benzene layer was transferred to another tube, and the washing step was repeated. Finally, the benzene layer was transferred to a clean tube, and Na_2SO_4 was added. The sample was stored in a refrigerator at 12°C until it was analyzed. One mL of the benzene solution was transferred to an autosampler vial before the analysis. A Varian Autoinjector, Series 8000, injected 3 μL of the benzene solutions into a Finnigan MAT 1020, gas chromatograph equipped with a mass spectrometric detector (GC/MS) operated in the positive chemical ionization mode. The methane gas pressure was 0.13 torr, and

the source temperature was 80°C. The GC/MS was operated in the full-scan mode from 250-350 amu at a rate of 0.9 sec/scan. Gas chromatography was performed on a 30-m fused-silica capillary column bonded with a 1.0 μm film of Durabond-5 (J & W Scientific, Folsom, CA). The injector, detector, and separator temperatures were all 250°C. The oven temperature was initially 70°C for 2 min, and then was programmed at 20°C/min to 270°C. The GC automatically recycled when the oven temperature reached 270°C. Quantitation was accomplished by measuring the areas of the 290 and 296 amu ions of aniline and ^{13}C-aniline peaks, respectively, at 8.3 min and calculating the ratio of these areas. The mean response factors of the 800 ppb and 200 ppb aniline-fortified-in-oil standards were used to calculate the concentration of aniline in each sample.

RESULTS AND DISCUSSION

As an integral part of the joint CDC/Spanish Government study of the TOS, a toxico-epidemiological study involved measuring several selected analytes in a set of blind-coded oil samples. The goal was to attempt to identify chemical measurements that could be used for pattern recognition of case-associated oils. The link between aniline-denatured rapeseed oil and TOS necessitated the measurement of aniline in this study. Several other chemical measurements were also made, and these are described elsewhere (Kilbourne et al. 1987; Bernert et al. 1987). Initially, we used a sample preparation procedure with a toluidine internal standard and HFBA derivatization similar to that described above, followed by analysis by capillary gas chromatography with electron-capture detection (GC/ECD). We found interference in the GC/ECD analysis by this procedure that was seemingly related to peroxidation products. To find an interference-free analysis, we chose an isotope-dilution, mass-spectrometric procedure using positive chemical ionization. We used the same sample preparation procedure in this new method but used U-$^{13}C_6$ aniline as the internal standard. The selectivity of positive chemical ionization made this an attractive method of choice. The positive chemical ionization (PCI) mass spectrum of the purified HFBA derivative showed the dominant $(M+H)^+$ion at 290 amu (100% relative abundance [RA]), and four other ions, all less than 8% RA, at 270, 291, 318, and 330 amu. Because of the limited amount of U-$^{13}C_6$-aniline, the HFBA derivative was not synthesized. The PCI mass spectrum of the aniline U-$^{13}C_6$ HFBA derivative generated by using an extract prepared from an aniline-fortified-in-oil sample with the aniline U-$^{13}C_6$ internal standard was quite similar to the ^{12}C-aniline derivative and had a $(M+H)^+$ion at 296 amu (100% RA) and four other minor ions at 276, 297, 324 and 336 amu (all less than 10% RA). Olive oil aniline-fortified-in-oil standards fortified with various amounts of aniline produced a linear response over the range of 100 to 1,200 ppb. The upper range limit was not determined. The limit of quantitation was 100 ppb for a 1-g sample, although concentrations below 100 ppb could be detected. The recoveries of aniline added into olive

oil at 200 and 800 ppb were 64 and 65%, respectively. The recovery was determined by comparing the aniline-fortified-in-oil standards to a benzene solution of the purified HFBA aniline derivative. The precision for 200 and 800 ppb aniline-fortified oils was reflected by the coefficients of variations (CV) of 9.9 and 9.2%, respectively, for 24 runs analyzed over 40 days. This method was found to be both selective and sensitive for determining aniline in oil samples.

This new method was applied to the analysis of the 195 blind-coded oil samples. The results are shown in Table 1. Forty-two (21.5%) of the 195 oil samples contained aniline at 100 ppb or greater. Measurable concentrations of aniline ranged from 110 to 1,300 ppb. Epidemiological investigation of the 195 oils involved the comparison of 29 case oils and 64 control oils in this set (Kilbourne et al. 1987). Statistical analysis of the aniline data showed a highly significant difference ($p < 0.0001$) between the concentration in case oils and control oils.

Although aniline-denatured rapeseed oil was clearly associated with TOS, no health significance has been attributed to the small amount of free aniline present in these oils, which generally contained less than 30 ppm (Pestana and Munoz 1982; Grandjean and Tarkowski 1984; Ventura Diaz 1982).

Table 1. Results of aniline analyses of Spanish oil samples

	All Oils	Case Oils*	Control Oils*
Total Number of oil samples	195	29	64
Mean Aniline Concentration (ppb) ± Standard Deviation	77±173	284±329	40±130
Median Aniline Concentration (ppb)	0	220	0
Number of Oils with > 100 ppb Aniline	42	16	9

*Comparison of case and control oils gave Wilcoxon p-value < 0.0001.

Results of previous studies of aniline-exposed industrial workers and animal toxicity testing show that clinical signs and symptoms of aniline intoxication are quite different from TOS (Beard and Noe 1981). These studies provide evidence that aniline is not, itself, the toxic agent in the oil. Nevertheless, we have found that aniline may be a useful indicator of the presence of the TOS agent (Kilbourne et al. 1987; Bernert et al. 1987). The association of aniline with the occurrence of TOS cases supports the hypothesis that TOS was associated with consumption of aniline-denatured rapeseed oil.

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REFERENCES

Beard RR, Noe JT (1981) Aromatic nitro and amino compounds. In: Clayton GD, Clayton FE (eds) Patty's Industrial Hygiene and Toxicology, Vol 2A. John Wiley & Sons, New York pp 2413-2489.

Bernert JT, Kilbourne EM, Akins JR, Meredith NK, Wages S (1987) Compositional analysis of oil samples implicated in the Spanish toxic oil syndrome. In press, J of Food Science.

Grandjean P, Tarkowski S (eds) (1984) Toxic oil syndrome. Mass poisoning in Spain. World Health Organization, Copenhagen, Denmark.

Kilbourne EM, Bernert JT, Posada de la Paz M, Hill RH, Borda IA, Kilbourne BW, Zack MW, Toxico-Epidemiologic Study Group (1987) Chemical correlates of pathogenicity of oils related to the toxic-oil syndrome epidemic in Spain. In press, Am J Epidiem.

Pestana A, Munoz E (1982) Anilides and the Spanish toxic oil syndrome. Nature 298:608.

Posada M, Castro M, Kilbourne EM, Diaz de Rojas F, Abaitua I, Tabuenca JM, Vioque A (1987) Toxic-oil syndrome case reports associated with the I.T.H. oil refinery in Sevilla. Food Chem. Toxicol. 25:87-90.

Tabuenca JM (1981) Toxic-allergic syndrome caused by ingestion of rapeseed oil denatured with aniline. Lancet 2:567-568.

Ventura Diaz LM (1982) Analisis convencionàl de las muestras disponibles deaceites supuestament toxicos. Grasas y Aceites 33:73-77.

Walle T, Ehrsson H (1970) Quantitative gas chromatographic determination of picogram quantities of amino and alcoholic compounds by electron capture detection. I. Preparation and properties of the heptafluorobutyrl derivatives. Acta Pharm Suecica 7:389-406.

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