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Detection and Isolation of Pentachlorophenol in Oil Samples Associated with the Spanish Toxic Oil Syndrome

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May 1981 marks the outbreak in Spain of a unique epidemic disease which by June 10 had been traced to the ingestion of adulterated oils containing various proportions of rapeseed oil denatured with aniline (Tabuenca 1981). Denatured rapeseed oil had been initially imported into Spain for industrial use and later fraudulently refined for human consumption. The disease, known as the Toxic Oil Syndrome (TOS), affected approximately 25,000 people with a mortality of almost 2.5% (Pascual-Castroviejo 1988).

To render it palatable to human consumption the denatured oil was refined and in the process, aniline reacted with free or esterified fatty acids producing the corresponding anilides, which are, until now, the only xenobiotics found at relatively high concentrations in most of these oil samples. Interestingly, the concentration of oleylanilide (the anilide of oleic acid) in oils clearly correlates with toxicity (Kilbourne et al. 1988), but the direct toxic effects of anilides remain controversial, so that the presence of anilides in epidemiologically validated case oils has been considered by the World Health Organization more as a marker of toxic oils than as a product of direct toxicity to humans or animals (Grandjean and Tarkowski 1984). Accordingly, and at different periods, there has been a renewed interest in various other compounds also found in oils (Arribas Jimeno 1982; Gardner et al. 1983; Vioque and Ventura 1984), such as oxidative derivatives of fatty acids, nitrogenous compounds related to aniline, hydrocarbons, pesticides, mycotoxins and metals. However, all of these compounds are present in the samples at low concentrations and/or are considered of low toxicity to implicate them in the etiopathogenesis of TOS.

Although organochlorine residues at unusual concentrations were not detected in oils (Grandjean and Tarkowski

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1984), early reports indicate an abnormally high proportion of chlorine in some samples. Gardner et al (1983) detected mixed fatty acid diesters of 3-chloro-1,2-propanediol in samples of TOS-related oils, and suggested that these compounds could be the origin for their high chlorine content.

During a rutinary analysis with three TOS related oil samples, in one of them we detected polychlorinated biphenyls (PCB) of the Aroclor 1254 type at a level of approximately 3-4 ppm. This fact prompted us to extend the analyses to other TOS related samples, especially to those of the still recent toxic-epidemiologic study of Kilbourne et al. (1988). Surprisingly, analyses showed that, despite of the absence of PCB's in these oils, some of them contained chlorophenols at an abnormally high concentration.

MATERIALS AND METHODS

The oil sample in which PCB's were detected was coded "JEN-B", as obtained in 1982 from Dr. Pestaña, coordinator of the Spanish Research Council studies on TOS. Samples of the toxic-epidemiologic study were kindly provided by Dr. M. Posada, of the Fund for Medical Research in Madrid, who sent us 14 blind coded samples. Some of the samples corresponded to "case" and others to "control" oils, following the classification proposed by Kilbourne et al. (1988). For additional studies, such as blank analyses or calculation of recoveries, samples of edible olive or grapeseed oils, purchased in Spain in 1989, were used. This was done in order to preserve the limited volume available of valuable case and control samples.

All solvents used in the extraction and chromatographic analysis were residue or HPLC grade, and were purchased from E. Merck, Darmstad, FRG, and Scharlau-Ferosa, Barcelona, Spain. Ethereal diazomethane was prepared from N-methyl-N'-nitro-N-nitrosoguanidine (Fluka AG, Buchs, Switzerland). Pentachlorophenol (PCP) (Supelco, Bellefonte, PA), op'DDE (Promochem, Wesel, FRG) and Aroclor 1254 (Alltech, Deerfield, IL), were used as chromatographic and spectrometric standards.

For electron capture detection and quantification of chlorophenols, the method of Veierov and Aharonson (1980) for organochlorine pesticides, involving the purification with sulfuric acid, was used. As internal standard (IS), op'DDE was added from the beginning. Typical recoveries with this method were, for free PCP, near to 100% ($\bar{x}$ = 102%, n = 5).

For mass spectrometric analysis, oil samples were

cleaned up with sulfuric acid and then purified by thin layer chromatography (TLC). Pre-coated analytical silica plates containing fluorescence indicator (E. Merck) were used. Plates were conveniently washed with methanol: acetic acid (98:2 v/v) and activated for 30 min at 100°C before sample deposition. A mobile phase consisting in hexane:diethyl ether:methanol:acetic acid (90:20:3:2) was used. The spot corresponding to PCP in oils (R_f = 0.60) was scrapped off, extracted with acetone, concentrated to dryness and methylated with freshly prepared diazomethane in the presence of a few drops of methanol. Methylated samples were redissolved in heptane for GC-MS determination.

In order to confirm the presence in oil samples of PCP by an alternative method not involving the use of sulfuric acid, oil was dissolved in hexane and fractioned by TLC, using the same chromatographic conditions stated above. Selected zones were scrapped off and analyzed by GC-ECD.

Quantitative analyses were carried out on a Perkin-Elmer model 8500 gas chromatograph. A 0.53 mm I.D. and 30 m long open tubular column, coated with a 5% diphenyl-95% dimethyl stationary phase (Rt_{x}-5, 1 µm film thickness) (Restek, Bellefonte, PA), coupled to an ECD, was used. Initial oven temperature was 150°C, maintained for 3.4 min, and programmed to 300°C at 9°C/min. Carrier and make-up gas (nitrogen) flows were 4 and 55 ml/min, respectively. Injector and detector temperatures were 320 and 340°C. Calculation of chlorophenol contents in oil samples was made by peak height relative to the IS. Confirmative studies were made injecting the extracts on a SPB-608 capillary column (Supelco), 15 m long and 0.53 mm ID, under identical chromatographic conditions.

MS analyses were carried out on a Hewlett-Packard model 5995 GC-MS, equipped with a cross-linked HP-1 capillary column (30 m x 0.2 mm ID, 0.33 µm film thickness). A temperature program from 70 to 115°C at 10°C/min and then to 285°C at 4°C/min was used. Injection port and transfer line temperatures were set at 240 and 280°C. Ion source and mass analyzer were set at 200 and 190°C, respectively. Spectra were obtained at 70 eV.

RESULTS AND DISCUSSION

Figure 1 shows the PCB profile found in the JEN-B sample, which was the origin of our investigations. The human toxicity of that sample is uncertain, but it contains rapeseed oil and 336 ppm of anilides (Guitart, 1984, unpublished data). The fact that these chlorine compounds were not detected in any of the other oil samples further analysed, makes the detection of PCB

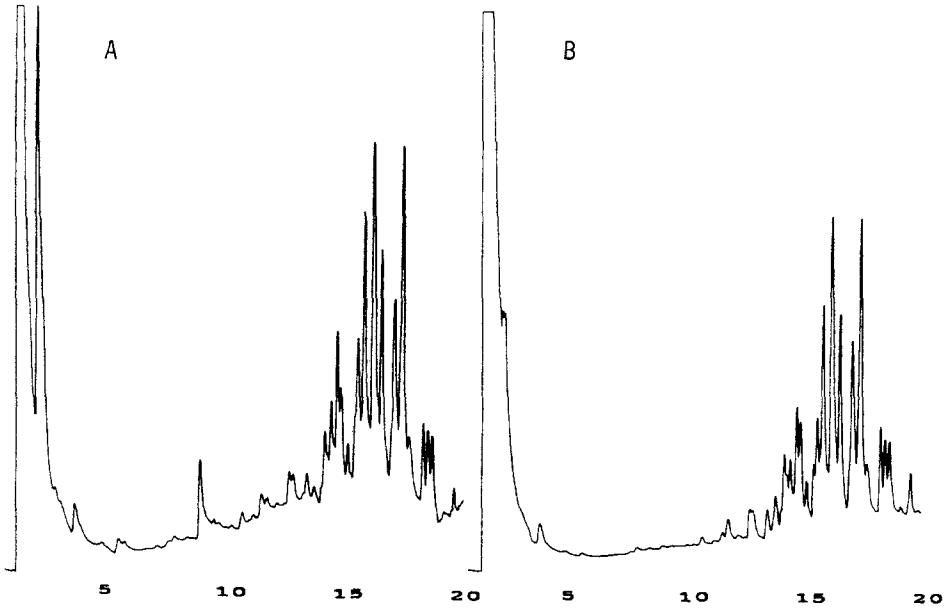

Figure 1. GLC-ECD profile on a SPB-608 column of a JEN-B extract (A) and pure Aroclor 1254 standard (B). The peak in A eluting at 8.6 min corresponds to HCB.

probably not important from the toxicological point of view. However, this finding could have a bearing on the differential history of the batches in which the original aniline-denatured rapeseed oil was divided. External contamination with different xenobiotics, PCB's among them, could have happened in any of those batches.

Most of the 14 samples of the toxic-epidemiologic study showed only low quantities of hexachlorobenzene (HCB), and α- and τ-hexachlorocyclohexane (HCH) (Figure 2A). However, 6 of the 14 samples presented a characteristic profile in which four peaks were clearly distinctive (Figure 2B). One of them, corresponding to the major and later eluting peak, had the same retention time of pure underivatized PCP both on SPB-608 and $Rt_{\infty}5$ stationary phases. This fact, together with its resistance to sulfuric acid, ECD response and TLC behavior, led us to tentatively confirm the presence of free PCP in some of the samples. The definitive confirmation came from the identical fragmentation pattern obtained by GC-MS after derivatization with diazomethane (Figure 3).

Table 1 shows the free PCP content of the 6 oils in which this pesticide was detected. Correlation between

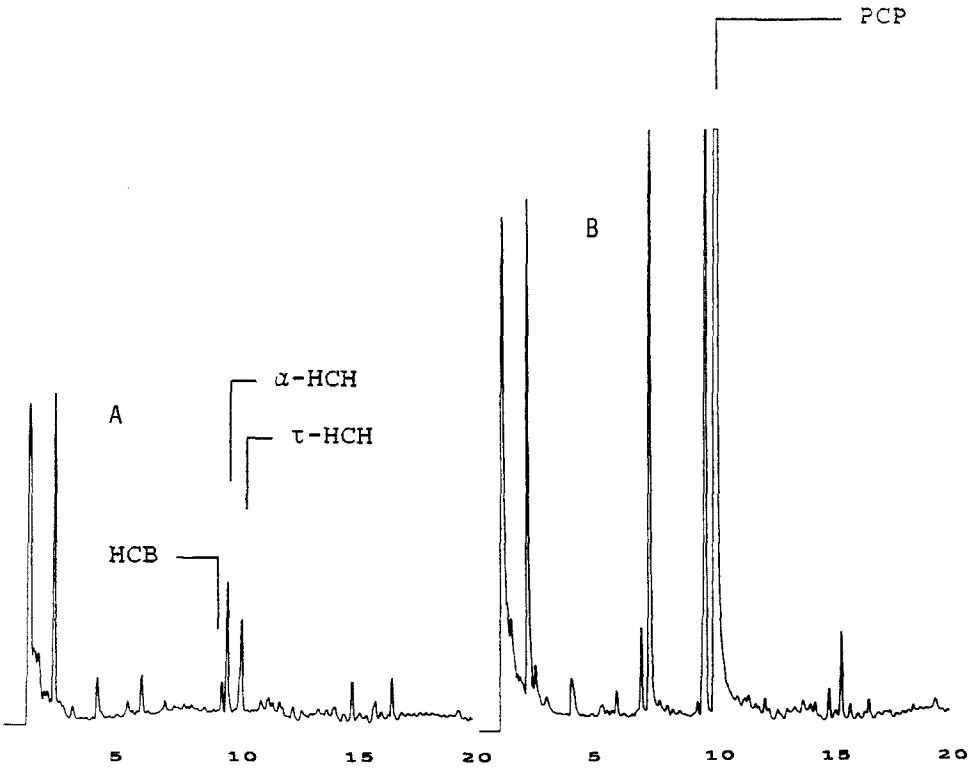

Figure 2.- Typical pattern obtained on a Rt_{α}-5 column, in identical chromatographic conditions, with one group of samples (A), containing mainly HCB, and α- and τ-HCH, and the other group (B), containing chlorophenols.

Table 1. Concentration of free PCP (µg/g oil) and anilides[1] (µg/g) in TOS-associated oil samples.

SAMPLE	PCP	ANILIDES	SAMPLE	PCP	ANILIDES
A+	ND	517	B+	ND	660
C+	ND	760	D+	ND	1105
E+	ND	1118	F+	ND	1154
G+	ND	1209	H+	ND	1246
I+	2.3	1456	J	2.1	ND
K	1.5	ND	L	1.4	ND
M	0.3	ND	N	0.2	ND

1) Values determined by Dr. Bernert Jr. and coworkers at the CDC, Atlanta, GA, and kindly provided by Dr. Posada.
+ Indicates a "case" oil sample, as defined in the toxic-epidemiologic study of Kilbourne et al. (1988).
ND = Not detected.

PCP and anilide concentrations was non statistically significative because in eight case oils PCP was not present and, inversely, the five control oils contained PCP.

The peak eluting just before free PCP (Figure 2B) in oils has also the same t_R in both columns and the same R_f of methylated PCP, so that it tentatively can be ascribed to that compound. The origin of the methylated PCP in oils is unclear, but could probably be formed during the refining process. Both free and methylated PCP were found to be present as such in oils by direct TLC fractionation. If we assume that the other two minor and yet unidentified peaks are also chlorophenols, the total content of such compounds in the 6 oils would be approximately of 0.5-4 µg/g. Finally, it is known that PCP can react with fatty acids giving the corresponding PCP-fatty acid esters (Leighty and Fentiman 1982), so if this reaction had occurred in oils (as it happened between aniline and fatty acids), the total sum of PCP in oil samples could be still much higher.

The free PCP concentrations found are probably too low to elicit the clinical signs associated to this pesticide. However, PCP is not an atoxic compound, and taking into account the heavy consumption of oil by the Spanish population and/or its volatility (when oil is used for friers), the daily dose intake would be probably sufficient to produce the potentiation of toxic effects in very sensitive subjects. In this sense, the interference of chlorophenols on normal detoxification pathways has been described (Arrhenius et al. 1977).

Moreover, the toxicity would be increased if other known contaminants of technical grade PCP were also present, such as dibenzofurans or dioxins (Nilsson et al. 1978). Nevertheless, the detection of chlorophenols in control oils makes the hypothesis of a direct and unique implication of such compounds of low probability.

A more plausible hypothesis would be that PCP could act in a synergistic mode with anilides or aniline, or that PCP would react with aniline and/or their derivatives, giving a highly toxic compound. This fact could explain why in control oils, in which anilides (markers of the original rapeseed denatured oil) are for the most part absent, PCP and other chlorophenols could be present without toxicological consequences.

Analytical, biochemical and toxicological investigations are now undertaken in our laboratories in order to evaluate the possible relevance of these pesticides in the origin of one of the more important massive food intoxications in the recent human history.

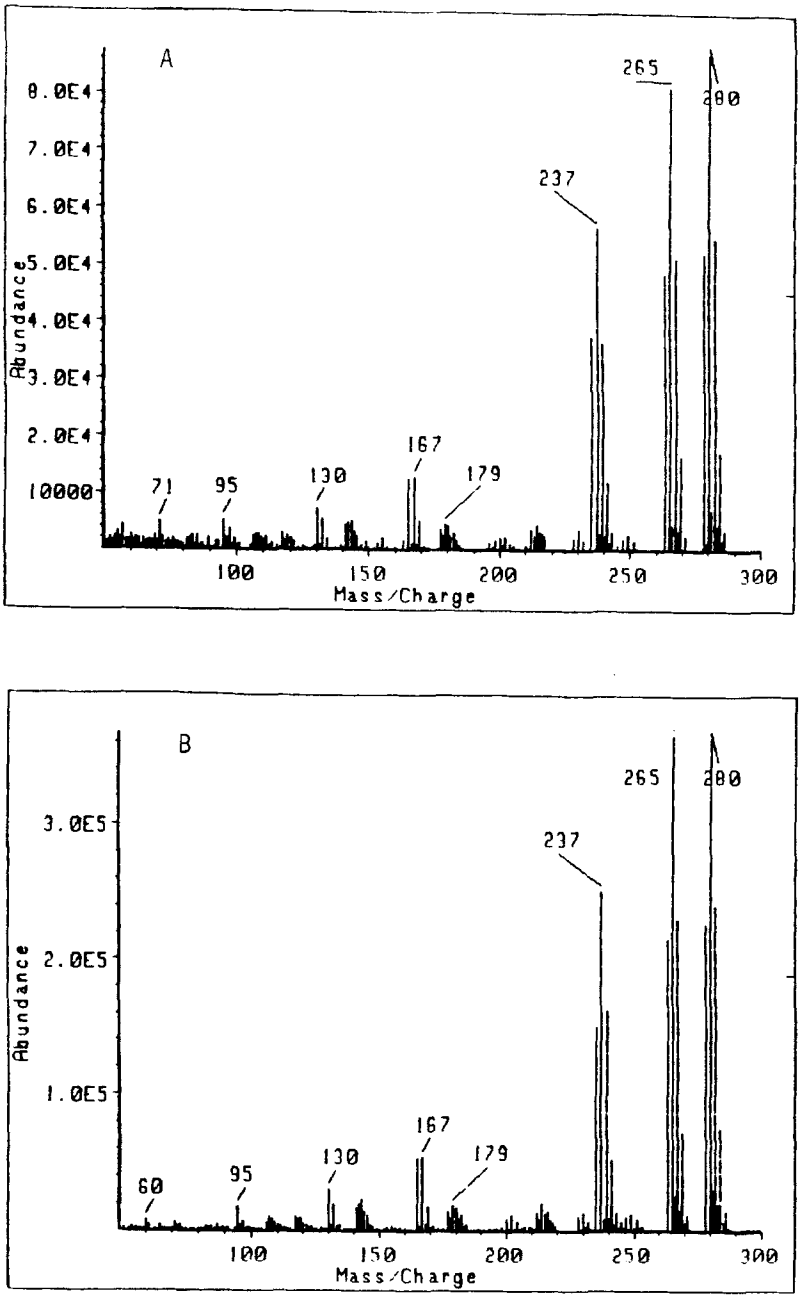

Figure 3. Mass spectra fragmentation pattern of a methylated extract of sample I (A), and pure methylated PCP (B).

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