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Pentachlorophenol and Pentachloroanisole in Oil Samples Associated with the Toxic Oil Syndrome

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The etiologic agent in the Toxic Oil Syndrome (TOS) remains still unknown. Most of the initial hypotheses on the origin of TOS were discarded by lack of scientific evidence in their support (Grandjean and Tarkowski, 1984). The strong association of the illness with concentration of oleylanilide and other aniline-derived compounds in some adulterated oils (Hill et al., 1995; Kilbourne et al., 1988), strengthens the first serious hypothesis on the origin of TOS: a rapeseed oil denatured with 2% of aniline and initially imported into Spain for industrial purposes, but later fraudulently refined for human consumption (Tabuenca, 1981). A lot of compounds in oils have been proposed as causative agents of the TOS epidemic (Grandjean and Tarkowski, 1984; Guitart and Gelpí, 1992; Hill et al., 1995), but none has received to date the full consensus of the scientific community.

In a previous work (Guitart et al., 1990) it was described the presence of pentachlorophenol (PCP) in 6 oils from the epidemic period. PCP is a recalcitrant biocide used primarily for wood preservation; it is ubiquitous in the environment, and is considered a priority toxic pollutant (Rao, 1978; WHO, 1987; Hattemer-Frey and Travis, 1989).

The low concentrations detected in TOS-oils and the well known toxic effects of PCP in human beings (Jorens and Schepens, 1993; Rao, 1978; WHO, 1987), suggests a low probability for a direct implication of PCP in the TOS illness (Guitart et al., 1990). However, it was considered of interest to characterize other microcontaminants observed in the contaminated oils and to extend the analysis to other new 138 selected TOS-oil samples, in order to evaluate the potential use of PCP as a marker of toxic oils.

MATERIAL AND METHODS

The study has been carried out following a double-blinded case-control

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design. The oil selection was made using the samples of the Spanish government's oil exchange program, intended to remove potential toxic oils from public consumption. The criteria for the selection of the oils and the classification as "case" or "control" oils was detailed before (Kilbourne et al., 1988). A number of 138 of these samples, known as the Toxico-Epi II oils, were codified and sent to the laboratory in Barcelona to perform the analysis. At the end of the study it was shown that of the 138 samples, 58 belonged to the category of case and 80 to the control group.

Identification of chlorophenols (CPs) and chloroanisoles (CAs) was carried out using the same approach as previously described for PCP (Guitart et al., 1990). Briefly, a pool of positive oils for CPs was dissolved in n-hexane and treated with concentrated sulfuric acid, following the clean-up method of Veierov and Aharonson (1980). The organic layer was then concentrated and the acid-resistant components separated by thin layer chromatography (TLC). Pure standards of PCP, 2,3,5,6-tetrachlorophenol (2,3,5,6-T4CP), 2,3,4- and 2,3,5-trichlorophenol (2,3,4-T3CP and 2,3,5-T3CP, respectively), purchased from Promochem (Wesel, Germany), and the corresponding methylether-derivatives (pentachloroanisole, PCA, tetrachloroanisoles, T4CAs, and trichloroanisoles, T3CAs) obtained by reaction with freshly prepared ethereal diazomethane, were used as chromatographic standards. The bands corresponding to the free and methylated CPs on the plates were scraped off, derivatized with diazomethane if necessary, resuspended in n-hexane and injected in the gas chromatograph-mass spectrometer (Hewlett-Packard model 5995 GC-MS), under the same conditions described in the previous paper (Guitart et al., 1990).

The method for the quantitation of PCP and PCA in the 138 TOS oil samples was also described before (Guitart et al., 1990). Recovery for PCP, 2,3,5,6-T4CP, 2,3,4-T3CP, 2,3,5-T3CP and PCA were found to be quantitative (>78.5%). Calibration curves for PCP and PCA were constructed in order to quantify their concentration in oils, using o,p'-DDE (Promochem, Wesel, Germany) as the internal standard (IS). The 138 oil samples were initially examined to confirm the absence of peaks interfering with the IS. Quantitative analysis were carried out by duplicate, and the results of PCP and PCA concentrations are the mean of both replicates. Apart from PCP and PCA concentrations, for statistical calculations a third variable, CPCPA, was defined as the sum of PCP plus PCA. Other variables, such as oleylanilide, brasicasterol, erucic and gondoic acids, cholesterol and peroxide content of the oils, were supplied by the Fondo de Investigación Sanitaria.

The median values of parameters measured in case and control groups were compared; p values for differences between groups were calculated by the Mann-Whitney test. A number of backward and forward stepwise strategies for variable selection was employed. The χ^2 statistics (calculated as twice the difference in model log-likelihoods) was used to determine the p values corresponding to differences between models. A p value of 0.05 or less was considered statistically significant. A previous analysis on variables was performed in order to determine if there existed collinearity among them (Belsley, 1991).

RESULTS AND DISCUSSION

The mass spectra of the unknown peaks observed in the ECD profile of the oils were compared with those of the available authentic standards of CPs and CAs, and they were identified as PCA, T4CP and T4CA, and T3CP and T3CA (Figure 1); no attempt was made to characterize the type of isomers of the lower polychlorinated phenols and anisoles. This confirms the tentative identification described in the previous paper (Guitart et al., 1990), based in the same retention time on a 0.53 mm open tubular column coated with 5% diphenyl-dimethylsilicone stationary phase, and the same retention factor on TLC of the compounds detected in the oils and the free and methylated standards of CPS.

The number of samples testing positive for PCP and PCA was 121 (87.68%), higher than the previously published value (42.86%) based on the analyses of 14 samples (Guitart et al., 1990). The concentrations ranged from ND to 38.5 mg/g for PCP, and from ND to 3.8 mg/g for PCA. None of these samples contained PCP or PCA alone: all of the samples positive for PCP were also positive for PCA and the other CPs and CAs. The pattern of the positive samples was in fact very similar, and the concentrations of CPs and derivatives was, for the major part of samples, PCP>PCA>T4CP>T4CA>T3CP>T3CA. The ratio PCP/PCA was variable between samples (range 0.7 to 64.2), and their respective concentrations do not show any correlation ($r < 0.2$). The lack of correlation between PCP and PCA concentrations should not be interpreted as indicative of a different origin. As they always appear together, it is more logical to believe that this could be explained by their different solubility in oils, or by its different reactivity with other components of the oils or containers.

As it is shown in Table 1, variables such as the concentration of oleylanilide, brasicasterol, erucic and gondoic acids, and cholesterol, were significantly higher in case than in control oils, whereas values of peroxides, PCP, PCA and CPCPA were randomly distributed among

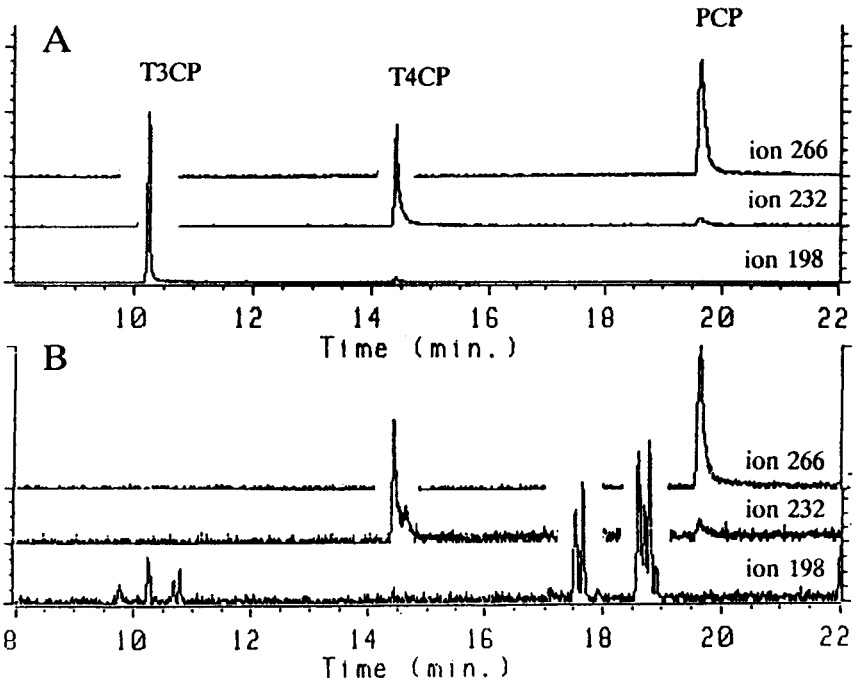

Figure 1. Selected ion monitoring of standards of chlorophenols (A) and chlorophenols purified from a TOS-case related oil (B).

samples. On the other hand, final results from the model of logistic regression were based only in two variables, namely the presence of oleylanilide and gondoic acid.

From an epidemiological and toxicological point of view, and according to the data obtained and the statistical analyses performed, we can conclude that PCP or PCA (and for extension the rest of CPs and CAs) have had no direct influence in the poisonig outbreak. Moreover, their concentrations in oils related to the epidemic period seemed to be too low to produce special toxic effects, and only could account for an increased exposure to these compounds. The effects and toxicity of PCP and other CPs are well documented (Jorens and Schepens, 1993; WHO, 1987; WHO, 1989), and although little is known on PCA, its oral toxicity (expressed as lethal dose 50) on mice is still lower than that of PCP: 318-331 mg/kg body weight (bw) for PCA versus 129-134 mg/kg bw for PCP (Renner et al., 1986).

Another question is how to justify the presence of the methylether derivatives of PCP, T4CP and TBCP, in the oils. They have never been described as contaminants of pure or technical PCP (Rao, 1978; WHO,

Table 1. Statistical analysis performed on 138 selected oils. Results are expressed as medians and percentiles 10-90.

Parameter	Case oils	Control oils	p (Mann-Whitney)
Oleylanilide (μg/g)	59.55 (0-1021)	0.00 (0-45.05)	0.0001
Brasicasterol (μg/g)	0.22 (0-0.49)	0.01 (0-0.27)	0.0001
Erucic acid (μg/g)	3.1 (0-6.5)	0.2 (0-4.1)	0.0001
Gondoic acid (μg/g)	8.7 (1.9-12.3)	2.4 (1.1-8.8)	0.0001
Cholesterol (μg/g)	0.095 (0-0.63)	0.025 (0-0.42)	0.0009
Peroxides (meq/kg)	225.3 (169-317)	246.8 (154-339)	0.18
PCP (μg/g)	1.75 (0-7.1)	1.9 (1-6.1)	0.075
PCA (μg/g)	0.6 (0-1.2)	0.7 (0.33-1.75)	0.070
ΣPCPA (μg/g)	2.4 (0-8.3)	2.75 (1.4-7.0)	0.067

1987). A chemical origin is difficult to sustain because it is not known how the CPs would enter in contact with a methylating agent and/or a methyl donor. Wherever the case, those methylethers should have been formed or introduced before oil refining, because this process did not include the contact with a methyl donor (Grandjean and Tarkowski, 1984). Moreover, the presence of the lower CPs suggested that PCP was of the technical grade, because traces of T4CP and T3CP in technical grade PCP had been reported as typical contaminants of this pesticide (Rao, 1978; WHO, 1987). This idea was further reinforced by the analysis of the content of polychloro-dibenzo-p-dioxins (PCDDs) and the polychloro-dibenzofurans (PCDFs) in the oils (Guitart et al., 1993), that were detected at percentages similar to those reported for some technical grade PCP.

Microbiological degradation of PCP also produces T4CP and T3CP by a dechlorination process (WHO, 1987), and it has been demonstrated that both bacteria (Häggblom et al., 1989; Neilson et al., 1983) and fungi (Cserjesi and Johnson, 1972; Curtis et al., 1972) are capable to methylate CP compounds to the corresponding anisoles. Thus, part of the free lower CPs and all of the anisoles could have been originated by a microbial process, which probably is a more plausible explanation for the presence of PCA and the other anisoles than the formation by a simple chemical reaction. If this hypothesis is correct, it should represent the first direct confirmation of the contact of the oils with something that underwent microbiological growth and metabolism.

With the present data it is not possible to establish when and how this contamination occurred, but it could be hypothetized that: 1) the fact that some oils containing PCP are oleylanilide-free, and viceversa, indicate that CPs and CAs were introduced by an oil different of the aniline-denatured rapeseed; 2) previous episodes with toxic fat

(Firestone, 1973) suggest that the vehicle was an animal esterified oil (or fat) which was present in 77.8% of the TOS-related oil samples (Guitart and Gelpí, 1992); and 3) although bacteria and fungi are known to be able to grow in a vegetable oil, especially when it contains moisture (Okpokwasili and Williams, 1991), it is more plausible to believe that CAs were formed from the CPs in a mattrix other than the oils.

In conclusion, this study indicates that, at the time of the epidemic, the oils could have been exposed to several foreign potentially toxic substances other than aniline and related compounds, although PCP and PCA do not seem to be directly related with the disease.

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REFERENCES

Belsley DA (1991) Conditioning diagnostics: Collinearity and weak data in regression. John Wiley and Sons, New York, USA

Cserjesi AJ, Johnson EL (1972) Methylation of pentachlorophenol by *Trichoderma virgatum.* Can J Microbiol 18:45-49

Curtis RF, Land DG, Griffiths NM (1972) 2,3,4,5-Tetrachloroanisole association with musty taint in chickens and microbiological formation. Nature 235:223-224

Firestone D (1973) Etiology of chick edema disease. Environ Health Perspect 5:59-66

Grandjean P, Tarkowski S (eds.) (1984) Toxic Oil Syndrome: Mass Food Poisoning in Spain. World Health Organization, Copenhagen, Denmark

Guitart R, Abian J, Arboix M, Gelpí E (1990) Detection and isolation of pentachlorophenol in oil samples associated with the Spanish toxic oil syndrome. Bull Environ Contam Toxicol 45: 181-188

Guitart R, Gelpí E (1992) Chemical composition of TOS-elated oils. In Toxic Oil Syndrome: Current Knowledge and Future Perspectives, WHO Regional Publications, European Series No. 42. WHO, Copenhagen, Denmark, pp. 99-142

Guitart R, Nørgaard L, Mariani G, Posada P, Abaitua I, Gelpí E, Fanelli R (1993) Analysis of polychlorinated dioxins and furans in samples of the toxic oil syndrome. Hum Exp Toxicol 12:273-278

Häggblom MM, Janke D, Middledorp PJM, Salkinoja-Salonen M (1989) O-methylation of chlorinated phenols in the genus *Rhodococcus.* Arch Microbiol 152:6-g

Hattemer-Frey HA, Travis CC (1989) Pentachlorophenol: Environmental partitioning and human exposure. Arch Environ Contam Toxicol 18:482-489

Hill Jr RH, Schutz HH, Posada de la Paz M, Abaitua Borda I, Philen RM, Kilbourne EM, Head SL, Bailey SL, Driskell WJ, Barr JR, Needham LL (1995) Possible etiologic agents for toxic oil syndrome: Fatty acid esters of 3-(N-phenylamino)-1,2-propanediol. Arch Environ Contam Toxicol 28:259-264

Jorens PG, Schepens PJC (1993) Human pentachlorophenol poisoning. Hum Exp Toxicol 12:479-495

Kilbourne EM, Bernert Jr JT, Posada de la Paz M, Hill Jr RH, Abaitua Borda I, Kilbourne BW, Zack MM, The Toxico-Epidemiologic Study Group (1988) Chemical correlates of pathogenicity of oils related to the toxic oil syndrome epidemic in Spain. Am J Epidemiol 127:1210-1227

Neilson AH, Allard AS, Hynning PA, Remberger M, Landner L (1983) Bacterial methylation of chlorinated phenols and guaiacols: formation of veratroles from guaiacols and high-molecular-weight chlorinated lignin. Appl Environ Microbiol 45: 774-783

Okpokwasili GC, Williams TO (1991) Stability to biodeterioration of vegetable oils. Material Organism 26:53-62

Rao KR (ed.) (1978) Pentachlorophenol: Chemistry, pharmacology, and environmental toxicology. Plenum Press, New York, USA

Renner G, Hopfer C, Gokel JM (1986) Acute toxicities of pentachlorophenol, pentachloroanisole, tetrachlorohydroquinone, tetrachlorocatechol, tetrachlororesorcinol, tetrachlorodimethoxy-benzenes and tetrachlorobenzenediol diacetates administered to mice. Toxicol Environ Chem 11:37-50

Tabuenca JM (1981) Toxic allergic syndrome caused by the ingestion of rapeseed oil denatured with aniline. Lancet ii (8246), 567-568

Veierov D, Aharonson N (1980) Improved cleanup of large lipid samples for electron capture gas chromatographic quantitation and gas chromatographic-mass spectrometric confirmation of organochlo-rine residues. J Assoc Off Anal Chem 63:202-207

WHO (1987) Environmental Health Criteria 71: Pentachlorophenol. World Health Organization, Geneva, Switzerland

WHO (1989) Environmental Health Criteria 93: Chlorophenols other than pentachlorophenol. World Health Organization, Geneva, Switzerland