Spanish Toxic Oil Syndrome (1981): Progress in the Identification of Suspected Toxic Components in Simulated Oils

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The Spanish toxic oil syndrome (TOS) caused a unique illness, including acute respiratory effects, eosinophilia, and muscle wasting, in 20 000 people in 1981; epidemiological evidence linked the disease with the consumption of rapeseed oils that had been denatured with aniline. In the study reported here, rapeseed oils were adulterated with aniline and processed to simulate the oils involved in TOS. The aniline-adulterated oils were investigated using radiotracer techniques, advanced sample preparation techniques, including preparative HPLC, and *in vitro* cell culture toxicity tests. HPLC and GC–MS analysis of the aniline-adulterated oils showed a profile similar to that of "case-related" oils (implicated in TOS), particularly with respect to the anilide contents. The *in vitro* cell toxicity tests carried out on fractions separated by preparative HPLC showed that aniline-adulterated oils could reproducibly be distinguished from control oils. The toxic effects were found in only 2 of the 10 fractions. The fraction most consistently toxic was shown to contain largely the C18:3 anilide, as well as some C16:1 anilide.

Keywords: *Toxic oil syndrome; anilides; preparative HPLC; cell toxicity tests*

INTRODUCTION

The Spanish toxic oil syndrome (TOS) was probably the worst outbreak of epidemic food poisoning the world has ever known. Initially the disease centered in Madrid, and by the end of 1981 20 000 cases had been reported and 450 deaths had occurred. The events that occurred have been well documented in the World Health Organization (WHO) Official Report (WHO, 1984) and a review of current knowledge of TOS is also available (WHO, 1992).

There was strong circumstantial evidence that 97% of the people affected by the disease had consumed a product sold as pure olive oil. The suspect product was found to contain rapeseed oil that had been denatured with 2% aniline to downgrade it for industrial use. This oil was then illegally processed and blended with animal and vegetable oils and fats.

A review of the work carried out by many researchers into the TOS syndrome is given by Gillatt (1989). He relates how these workers had been unable to identify either the mechanism by which TOS developed or the compounds resulting from treatment with aniline that caused the disease. This lack of knowledge prevents measures from being taken to avoid a recurrence of this syndrome. A review of the main features of the disease and outlines of past and current commissioned research are also given by Aldridge (1992). He discusses how attempts to produce the disease or some aspect of it reproducibly in experimental animals had so far failed, thus hindering further research in this area.

The objective of the current investigation was to produce oils adulterated with aniline that would simulate those involved in the TOS syndrome at the time the incident occurred. Much previous work had involved examination of "case-related" oils—thought to be implicated in the syndrome—which were in some instances then more than 10 years old. These oils would therefore be subject to the effects of aging and poor initial storage conditions.

The investigation reported in this paper takes into account certain hypotheses about the likely nature of the toxin. The toxin will derive from the aniline reaction with rapeseed oil. It will be dispersed possibly at low concentration within a predominantly triglyceride matrix. Digestion of the matrix will therefore probably be required to release the toxin for absorption into the gut and entry into cells of target organs. Triglycerides are not absorbed, and digestion will liberate more polar lipids, which can be fatty acid mono- or diglycerides. This means that to be absorbed, the toxin will probably need to be a polar lipid material. It must also survive the digestion process.

After adulteration with aniline, the simulated TOS oils were investigated using radiotracer techniques, advanced sample separation techniques, including preparative HPLC, and *in vitro* cell culture toxicity tests. Basically, the aniline derivatives were concentrated to reduce the proportion of triglyceride before being subjected to an *in vitro* digestion procedure. Biologically active fractions of the aniline-treated oil were further investigated.

The results reported demonstrate how simulated TOS oil was produced and indicate the progress being made in the separation of the toxic components in the oil and the effectiveness of *in vitro* cell toxicity tests.

EXPERIMENTAL PROCEDURES

Crude Rapeseed Oil. Two sources of crude rapeseed oil were used for processing. Oil prepared from Jet Neuf variety was supplied by Beoco Ltd., and single-zero rapeseed oil (a type similar to Jet Neuf) was obtained from Berlin University.

Preparation of Simulated TOS Oils. A standardized protocol is given below. The oils were treated with either cold or ring-labeled [^{3}H]aniline, to give a total of 2% m/m aniline. The oils were then placed in 20 and 50 °C incubators and stored for 1 and 7 days before processing.

Processing. The conditions employed in processing the crude rapeseed oils were chosen to mimic those thought to have been employed in Spain at the time of the TOS incident. The free fatty acid content of the oil was determined prior to processing. The same crude oils without the addition of aniline were also processed to provide control oils for all future work.

Neutralization and Degumming. Crude oil (between 500 and 1000 g) was placed in a water-jacketed vessel. The oil was stirred and its temperature raised to 80 °C. The headspace of the vessel was flushed with oxygen-free nitrogen.

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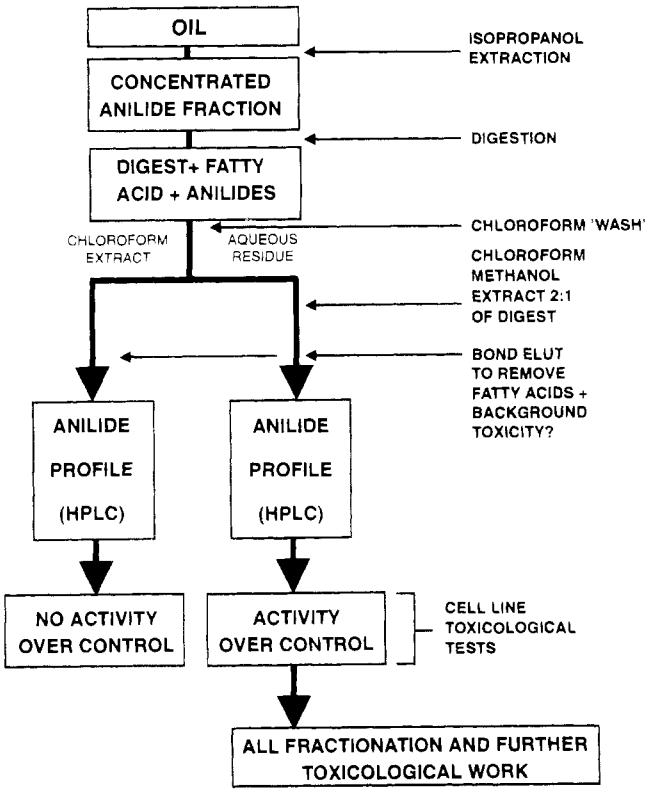


Figure 1. Summary of extraction, digestion, and cleanup procedure prior to fractionation and toxicity testing.

Phosphoric acid (88%) was added dropwise to the oil with continued stirring such that its final concentration was 0.2% by weight. The oil was neutralized with sodium hydroxide and washed with distilled water. The oil and aqueous phases were separated by centrifugation.

Drying and Bleaching. The oil was dried by heating to 90 °C and then bleached with acid-activating bleaching earth at 110 °C.

Deodorization. The oil was heated to 230 °C under a vacuum of 7–10 mmHg for 4.5 h with small quantities of steam derived from distilled water being swept through the product to remove volatile components.

The oil was allowed to cool to ambient temperature under oxygen-free nitrogen and stored at 4 °C.

Extraction of Oil and Digestion Procedure. A combination of solvent extraction and enzyme digestion of the oil was used to concentrate the compounds of interest and provide extracts suitable for *in vitro* toxicity testing. The enzyme digestion procedure was a simulated gut digestion, which the suspect oils would have been subjected to after consumption. In summary, portions of the processed oil were extracted with anhydrous propan-2-ol at low temperature. The propan-2-ol layer was collected and evaporated to an oily residue. Distilled water was added to the residue and the pH adjusted to 1.5. Pepsin was added and the mixture incubated in a shaking water bath at 37 °C for 3 h. In later experiments, this step was omitted, as it was not essential to the digestion of the oils and had no apparent effect on the aniline-related compounds. Following adjustment of the pH to 7.5, porcine pancreatin and sodium taurocholate were added. The mixture was then incubated at 37 °C for 16 h. If digestion was successful, the oil then appeared as an emulsion.

The emulsion was then sequentially extracted with chloroform and chloroform/methanol (2:1). Solvent extracts were separately evaporated to dryness. The entire chloroform/methanol extract was subjected to a further cleanup stage with Bond-Elut columns. This procedure is summarized in Figure 1.

During the extraction and digestion procedure, the yields of product were calculated where possible. In the case of radiolabeled oils, the radioactivity was measured throughout the process.

A portion of the extract (normally 25%) was removed for cell toxicity testing and the remainder reserved for HPLC fractionation. In later studies, the whole extract was subject to HPLC fractionation before cell testing—which was performed on the fractions only. It was found that the HPLC separation removed a substantial amount of interfering material from the oil extracts (also present in the control oils), and the cell test results were easier to interpret.

Preparative-Scale HPLC of Digests (Monitored at 254 nm). Digest/extracts prepared above were homogenized with 75% v/v methanol to form an emulsion, which was immediately injected onto the preparative HPLC (Waters Delta Prep 4000 system). A gradient of 82% methanol (in water) to 100% methanol was used, followed by a dichloromethane wash. Initially, 10 fractions were collected, of which fraction 1 was mostly the void volume of the column and fraction 10 was principally the dichloromethane wash. Fractions 2–9 were each 10-min collection windows.

Later work collected fractions 1–5 only, and the dichloromethane wash was brought in at the equivalent of fraction 6, which was then the final fraction. At this stage fraction 4 was subdivided into three further fractions (denoted A, B, and C) for collection.

All fractions were rotary-evaporated to dryness.

Procedure for *in Vitro* Biological Screening (Cell-Line Toxicity Testing). Cell lines used for the testing were chosen to give a range of different cell types. They were purchased from the European Collection of Animal Cell Cultures as follows: (i) rat liver epithelial cells, clone 9 (ECACC No. 88072203); (ii) mouse neuroblastoma, Neuro 2a (ECACC No. 89121404); (iii) bovine lung endothelial cells, BAOEC (ECACC No. 86123102). The cells were maintained at 37 °C in a CO_2 incubator in the following media: (i) and (ii) minimum essential medium (MEM) with Earle's salts containing 10% (v/v) fetal calf serum, 20 mM HEPES buffer, and 10% v/v antibiotic solution containing penicillin (100 units/mL medium) and streptomycin (100 μg/mL medium); (iii) medium 199, with 0.125% w/v sodium bicarbonate, 15% fetal calf serum, 20 mM HEPES buffer, and 10% v/v antibiotic solution containing penicillin (100 units/mL medium) and streptomycin (100 μg/mL medium).

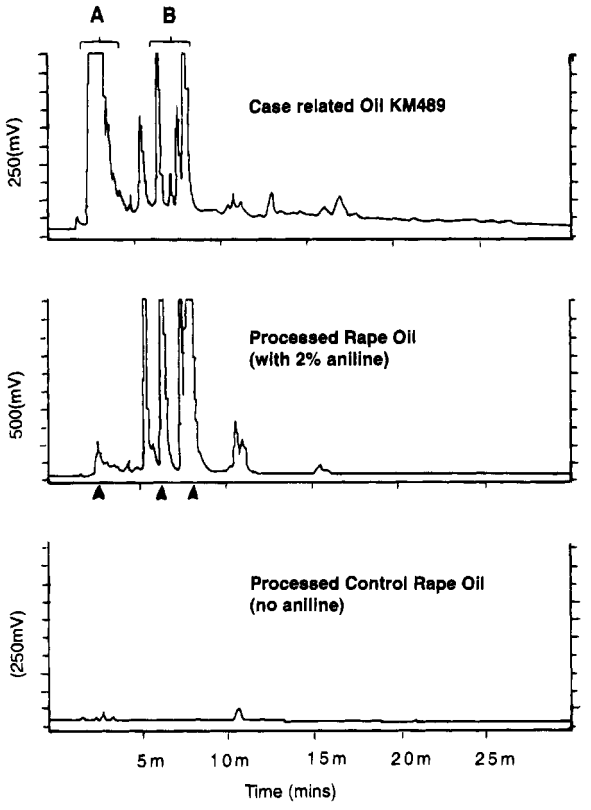
The cells were plated in complete medium at a density of 2×10^4/mL; 1 mL was added to each well of a number of 24-well multidish plates. The cells were examined microscopically to ensure normal growth before addition of any of the test extracts. All test extracts were inoculated into duplicate wells, and each multidish included duplicate untreated and solvent controls, control (unadulterated) oil extracts, and a positive toxin control.

Toxic effects were assessed initially by visual examination with phase contrast microscopy. In the later stages of the project, this assessment was refined by the use of vital dyes. The method used was based on that of Riddell *et al.* (1986) and involved the use of neutral red dye (uptake into lysosomes/endosomes and vacuoles of living cells gives a quantitative indication of cell number and viability) and Coomassie Blue dye (measures total cell protein). Growth curves of the cells could be plotted and control and aniline test extracts compared.

It was noted that the bovine lung endothelial cells were more sensitive than the other two cell lines to the aniline-adulterated oil extracts. This is in keeping with the fact that lung biopsy material of TOS victims showed a primary lesion in the endothelial cells (Aldridge, 1992). This paper summarizes the toxicity of the bovine lung endothelial cells as the most sensitive of the cell line toxicity tests.

Mass Spectrometry. *Probe. (1) Oil Samples and Fractions.* Small aliquots were placed inside a sample tube (1 cm, 2 mm i.d., 4 mm o.d.) as solutions in hexane. The solvent evaporated almost instantaneously.

Tubes were placed in the end of the solid probe, and the whole assembly was introduced into the mass spectrometer. Mass spectra were acquired as the probe was slowly heated from ambient to 400°C. These spectra reflect the sequential distillation of individual compounds, each occurring at a well-defined temperature.



A: Corresponds to fraction 2 on gradient runs
B: Corresponds to fraction 4 on gradient runs

Figure 2. HPLC chromatograms of a case-related oil, aniline-adulterated oil, and control rapeseed oil.

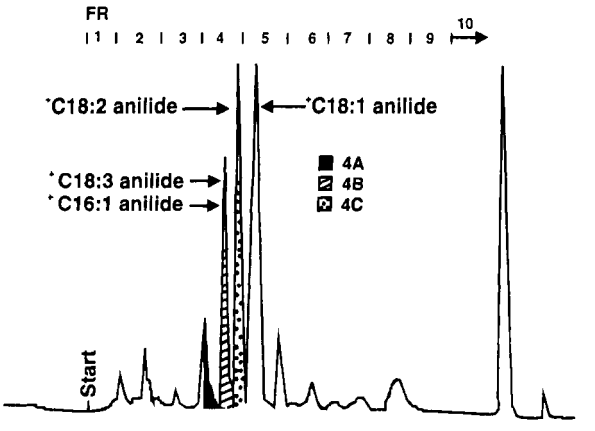
(2) Mass Spectrometer Settings: scan width, 650 to 20 amu; scan rate, 1 s/decade; other parameters, 70 eV, 100 μA beam current; temperature, 200 °C.

GC–MS. Samples were also examined by GC–MS with the following chromatographic conditions: column, 25 M OV101, 025 μm film, 0.32 mm i.d.; program, 50 °C for 2 min, 10 °C/min to 280 °C, then hold.

RESULTS

HPLC chromatograms of a case-related oil (KM 489), an aniline-adulterated processed rape oil, and a control unadulterated processed rape oil are shown in Figure 2. (The oils were extracted with methanol, but not subjected to the digestion procedure, prior to injection on the HPLC.) It can be seen that similar profiles are shown by the aniline-adulterated oil and the case-related oil, particularly with respect to the anilide contents. Rapeseed oils stored with aniline for 1 or 7 days at 20 or 50 °C and then processed were submitted to the Spanish authorities for anilide determinations, for comparison with case-related oils. Studies in Spain had indicated that anilides were the only reliable marker for relative toxicity in case-related oils (Guitart and Gelpi, 1992). This, however, did not necessarily mean that the anilides were responsible for the TOS syndrome. The case-related oils were approximately 10 years old when profiled and as such were highly oxidized and difficult to extract. The aniline-treated oils were therefore used for fractionation and toxicity studies, in preference to the case-related oils.

The highest levels of anilides were found after 7 days of storage at 50 °C; these levels were in the same order as the 2000 mg/kg said to be typical for case-related oils. Subsequent work therefore concentrated on oils that were stored at 50 °C for 7 days with aniline before processing.



*Component anilides tentatively identified by probe and GC-MS

Figure 3. Initial fractionation pattern devised for preparative HPLC.

A number of the control and aniline-adulterated oils were analyzed by probe mass spectrometry. All oils showed a series of ions mostly derived from the volatile unsaturated acid parts of the triglyceride molecules. However, the aniline-adulterated oils contained a series of compounds with a base peak at *m/z* 93. With an eight-peak index, these were broadly identified as fatty acid anilides. Examination of the molecular ions of these anilides showed that these compounds broadly matched the fatty acid composition of the oil.

After extraction and digestion there was little change in the profile of the aniline-adulterated oil by HPLC, indicating that this procedure had not removed the compounds of interest while providing a considerable cleanup of the oils. This profile is shown in Figure 3, which also highlights the fractions collected and shows the subfractionation of fraction 4 into 4A, 4B, and 4C and the identification of peaks by GC–MS. The C18:1 anilide was identified in fraction 5, and the C16:1, C18:2, and C18:3 anilides were identified in fraction 4. Radioactivity measurements of individual fractions showed that the dichloromethane wash was the most radioactive, followed by (in decreasing order) fractions 5, 4, 2, and 3.

Investigations of fraction 4B, with a radiodetector, allowed on-line monitoring of HPLC chromatograms to establish which peaks were radiolabeled. An example of the radiochromatogram is shown in Figure 4. This shows a double peak, which roughly corresponds to one of the UV 254 nm absorbing peaks. The second peak of the double peak is not apparent in the UV chromatogram; this may be due to a lack of sensitivity. It could also suggest an aniline derivative that does not have a benzene ring or possibly contains a ring that has been substituted.

The HPLC profile of the prepared aniline-adulterated oil was consistent with respect to the anilide components in a number of different preparations of processed oil. The only variable component was the material visible in the fraction 2 area of the chromatogram. It was seen that the age of the oils before extraction affected the amount of material visible in fraction 2; oils extracted soon after processing contained much less fraction 2 material than those that had been stored for several months. A comparison of the HPLC chromatograms of a freshly prepared and extracted oil and an oil stored for 10 months before extraction is shown in Figure 5.

An accelerated aging test was devised to simulate such aging effects. Storage of aniline-adulterated oil

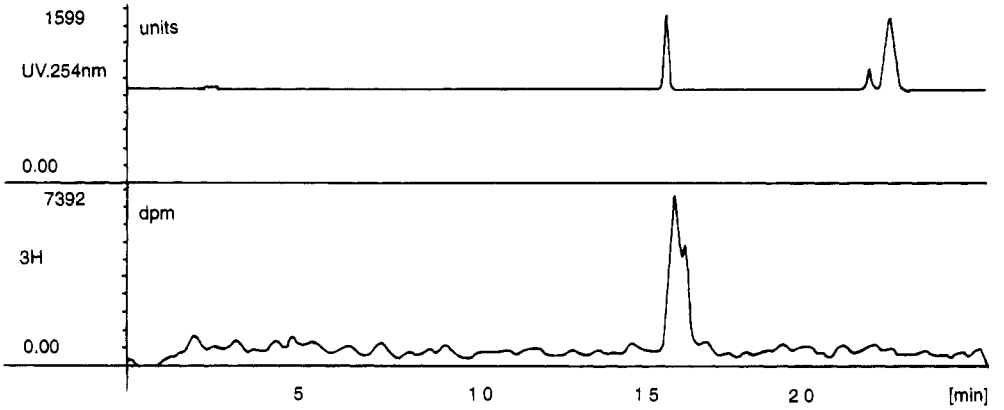


Figure 4. HPLC chromatogram of fraction 4B, with on-line detection of tritium.

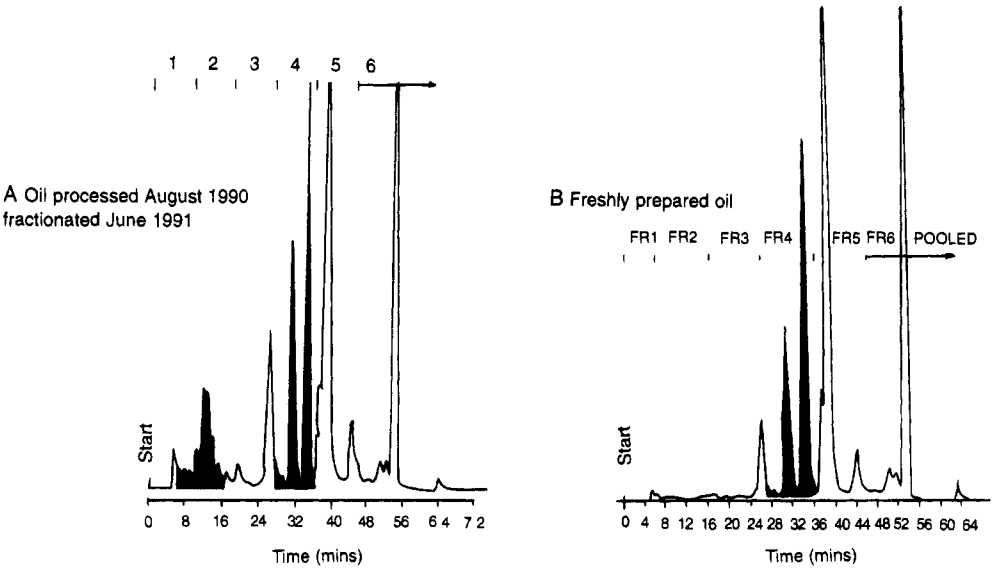


Figure 5. HPLC chromatograms of aniline-adulterated oils: (A) stored for 10 months before extraction; (B) extracted immediately after processing (fractions 2 and 4 are shaded).

extracts (with air space over the sample) for 1 week at 4 and 37 °C showed that fraction 2 components increased, with a subsequent decrease in fraction 4 components, at 37 °C only. This suggested that components in fraction 4 might be oxidizing to form more polar compounds that elute in fraction 2.

The results obtained from the cell toxicity tests showed that both whole oil digests and fractions prepared by HPLC of aniline-adulterated oil were consistently more cytotoxic than control oil preparations. The bovine endothelial cells were found to be the most sensitive, and an example of the effect of whole oil digests on these cells is shown in Figure 6. This shows the growth and survival of these cells after exposure to various dilutions of aniline-adulterated and control oil digests. The effects were measured spectrophotometrically after the cells were stained with Neutral Red (taken up by living cells only) and Coomassie Blue (which gives a measure of total cell protein). The highest optical density figure correlates with maximum cell growth. It can be seen that 4 mg of control oil (equivalent weight of oil) had no effect on the cells, whereas the aniline-adulterated oil destroyed the cells at this concentration. The cells were only able to survive when the aniline-adulterated oil was diluted to 0.25 mg.

The cytotoxic effect of the HPLC fractions is shown in Figures 7 and 8. It can be seen that in the five oil preparations represented in the figures, no cytotoxic effect was caused by fractions 1, 3, and 5–10 in any of

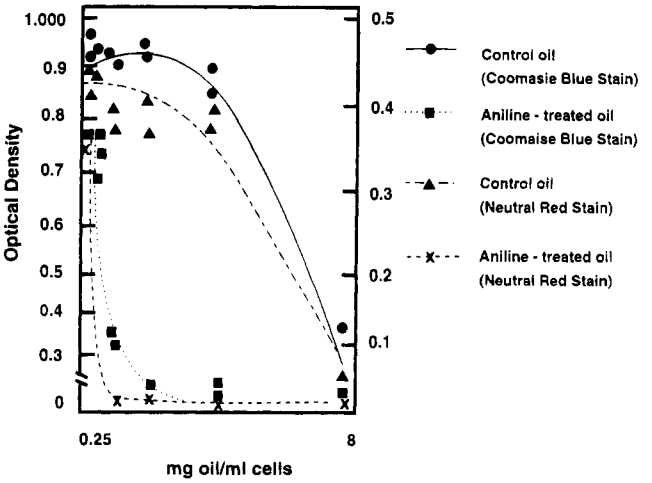


Figure 6. Effect of control and aniline-adulterated extracts/digests on bovine lung endothelium cells.

these preparations. Fraction 4 was consistently cytotoxic, and fraction 2 had variable toxicity. In the examples represented here, the cytotoxicity of control extracts of some of the fraction 2 preparations was high, which therefore canceled out the aniline-related toxicity. In earlier preparations, there was a significant cytotoxicity caused by fraction 2 of aniline-adulterated oils.

The cytotoxicity detected in fraction 4 was always related to the 4B part of the fraction (see Figure 3). In addition, some preparations showed toxicity in the 4C

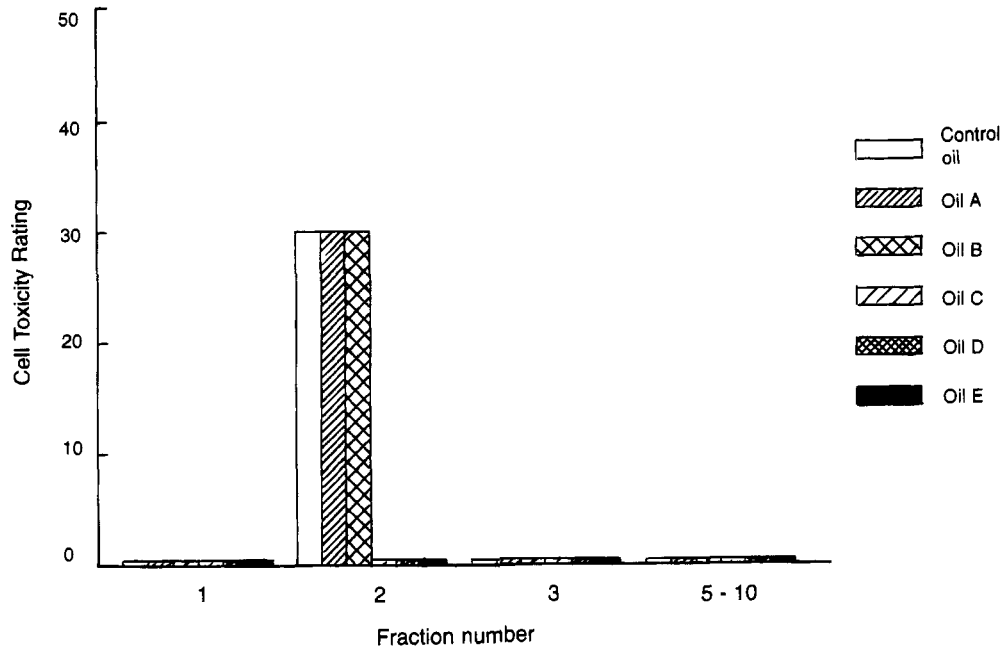


Figure 7. Toxicity testing of fractions (excluding fraction 4) of control oils and aniline-adulterated oils A–E.

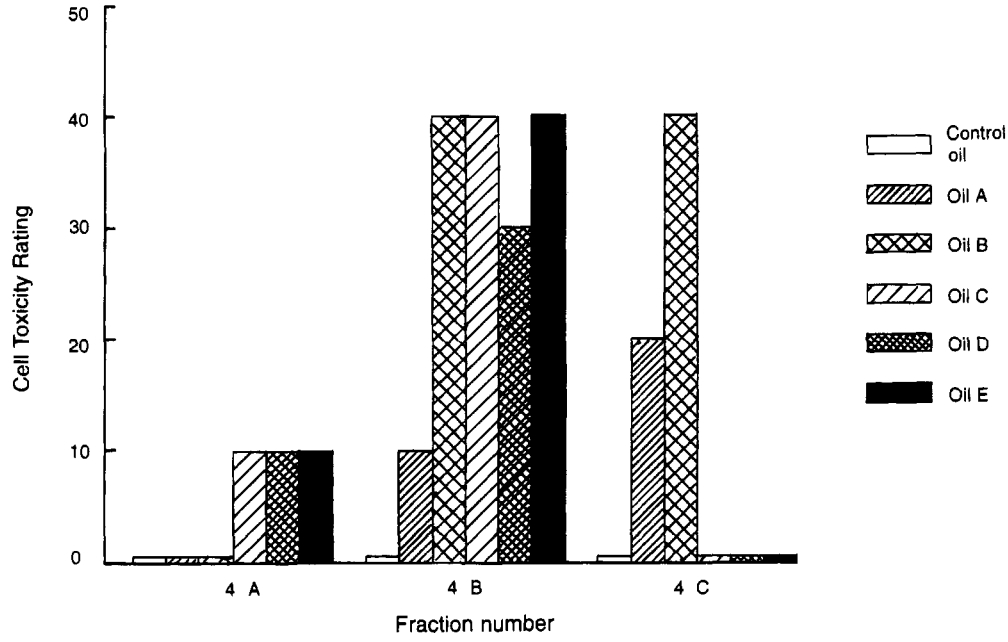


Figure 8. Toxicity testing of fraction 4 (subfractions) of control oils and aniline-adulterated oils A–E.

part of the fraction. The relative cytotoxic effects of fraction 4, prepared from a range of separate oil samples, showed a relationship to the amount of radioactivity detected in the fraction. This is illustrated in Figure 9, which shows the cytotoxicity rating and radioactivity measurements for fraction 4 prepared from these oils. These fractions were prepared individually over a time scale of at least 1 year. In summary, the oil samples containing the highest radioactivity were the most cytotoxic. The amount of radioactivity in each fraction did not automatically determine the toxicity rating of that fraction. For example, fractions 10 and 5 contained the most radioactivity and were consistently nontoxic.

Fractions prepared from control and aniline-adulterated oil were also tested for toxicity with a splenocyte assay, which measured incorporation of tritiated thymidine. This work was performed at BIBRA, Carshalton, U.K. The results showed that there were clear and statistically significant differences in the degree of toxicity between the aniline-adulterated and control oils for fractions 2 and 4.

DISCUSSION

In conclusion, HPLC chromatographic traces have repeatedly differentiated between samples of case-related and control oils provided by FIS. Differences have also been demonstrated chromatographically between processed aniline-treated rapeseed oil of the Jet Neuf variety and that similarly processed but without aniline. A profile similar to that of the case-related oils was produced by the aniline-treated oils, with respect to the anilide contents.

The case-related oils, which had been subjected to prolonged periods of storage since the toxic oil syndrome incident, also contained fraction 2 material (see Figure 2). The case-related oil consumed by the victims of this tragedy would more closely resemble freshly prepared oils from our processing as there is some evidence that

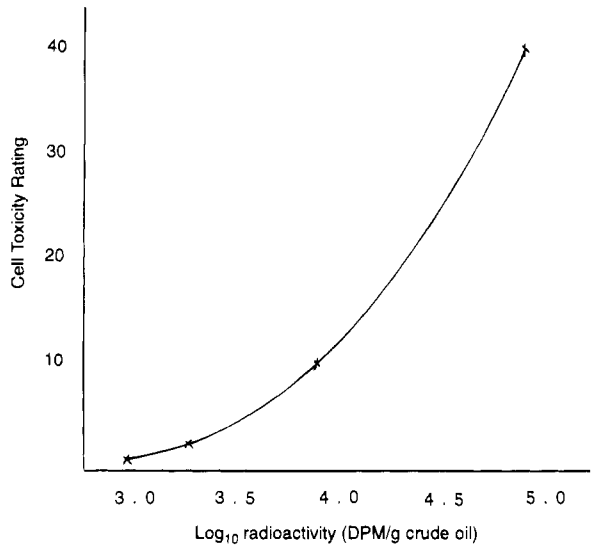


Figure 9. Cell toxicity ratings and radioactivity measurements for fraction 4 prepared from four separate processed oils (aniline-adulterated).

the oils were consumed within a few weeks of adulteration and reprocessing. It therefore seems more likely that the aniline-related toxin in fraction 4 was implicated in the outbreak of the disease rather than the fraction 2 material.

After suitable preparation, the oils treated with aniline have consistently shown cytotoxic effects *in vitro* to a number of cell lines (rat liver epithelial, mouse neuroblastoma, bovine lung endothelial) and rat splenocytes (performed by BIBRA).

Use of radiolabeled aniline has shown that the cytotoxicity is associated with some aniline-derived moieties. The variability in fraction 2 toxicity is thought to be due to the aging process discussed earlier, for two reasons. First, this cytotoxicity was only found in the older oil preparations; it was never detected in freshly prepared oil samples. Second, the cytotoxicity correlated with material detected by HPLC in the fraction 2 region of the older oils, suggesting that "toxic" aniline-related fraction 4 components had oxidized to form more polar "toxic" compounds eluting in fraction 2.

In summary, the cell toxicity tests have shown that aniline-adulterated oils can reproducibly be distinguished from control oils and the cytotoxic effects can be isolated to two fractions only, namely fractions 2 and 4. This study indicates that the toxic components in fraction 4 are most probably formed initially when the oils are stored with aniline but decompose slowly in the processed oils to form components detectable in fraction 2, over months of further storage. This hypothesis would seem to be supported by the fact that fraction 2 material was detected only by HPLC, and corresponding cytotoxic effects observed, in those oils that had been stored for several months or more before extraction.

The fraction that has been most consistently cytotoxic *in vitro* is that designated by preparative HPLC as fraction 4B. With GC–MS this has been shown to contain largely the C18:3 anilide, as well as some C16:1 anilide, and possibly erucamide or another amide. Radiochromatograms demonstrated a double peak in this fraction, which had only a single UV 245 nm counterpart (see Figure 4). This may suggest chemical alteration to the benzene ring in this aniline derivative.

The fraction designated 4C contains the C18:2 anilide and has only sporadically demonstrated aniline-related toxicity. There was no correlation between the toxicity of fraction 4C and the amounts of C18:2 anilide in this fraction. Fraction 5 contains the C18:1 anilide and has been reproducibly nontoxic. No significant toxicity has been demonstrated in any of the remaining fractions separated by HPLC.

The work reported here has established that compounds resulting from treatment with aniline in rapeseed oil have shown reproducible toxicological effects to a range of cell lines. The effects have not yet been isolated to a single compound. Previous workers (Vazquez Roncero *et al.*, 1983) have demonstrated the presence of propanediol compounds, such as 3-(phenylamino)-1,2-propanediol and its ester, in case-related oils. The propanediol compounds did not elute in fractions 2 and 4 with the HPLC conditions employed in this work; they have also failed to demonstrate any toxicity in laboratory feeding experiments to date (Aldridge, 1992). It is possible that these compounds may be altered during digestion in the gut, due to the effects of enzymes such as lipase. Such alterations would also affect the chromatography of these compounds. Further work would be needed on the effects of simulated gut digestion on these compounds before it can be concluded that they were not responsible for the toxicity demonstrated in this current work.

This work has not fully resolved the etiology of the toxic oil syndrome or identified the mechanism by which TOS developed. It is, however, the first reported evidence that *in vitro* toxicity tests can distinguish between aniline-adulterated (simulated TOS oil) and control oil samples. The approach taken highlights the scope for further work in the identification and purification of the toxic component responsible for this disease syndrome. Further ideas are currently being discussed with the Spanish authorities and the WHO Steering Committee.

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