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Toxic oil syndrome
Current knowledge and future perspectives

WHO Regional Publications, European Series, No. 42

CORRIGENDA

Page 84, Table 10. Footnote \(^b\) should read: \(^b\) From Table 8.

Page 87, Fig. 17. The formulae for OZT and IZT should appear as follows:

![Formulae for OZT and IZT](attachment://formulae.png)

Page 130, para. 5. The first sentence should read:

The microbiological results of the studies carried out with samples obtained from biopsies or necropsies of the TOS patients were negative or not different from the control population for several species of viruses, bacteria, chlamydiae, rickettsiae, mycoplasmas, fungi and eukaryotic parasites (84).
Toxic oil syndrome

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The views expressed in this publication are those of the contributors and do not necessarily represent the decisions or the stated policy of the World Health Organization.
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Note on Terminology

The policy of the World Health Organization in respect of terminology is to follow the official recommendations of authoritative international bodies such as the International Organization for Standardization (ISO). Accordingly, SI units (Système international d’unités) are used in this volume wherever possible. However, the non-SI measure of parts per million (ppm) is used instead of µmol/g when specific anilides have not been measured and/or when it is not known whether the concentration is expressed per unit of weight or of volume.
Foodborne diseases, especially those with fatal consequences, cut deep into one of the basic tenets of human health and wellbeing – the need for safe, uncontaminated food. The right of the consumer to healthy and safe food is one of the strategic elements of the WHO health for all policy, endorsed by the governments of the Member States of the European Region.

The toxic oil syndrome (TOS) highlights clearly – and tragically – the crucial importance of food safety in daily life. It points out, once again, the need for surveillance and control of food at all stages of production, storage, distribution, sale and use. This and other actions, such as integrating food safety into a comprehensive food quality policy, are needed to prevent the biological and chemical contamination of food that can lead to foodborne diseases. Only then can the countries of Europe succeed in significantly reducing risks to health from food contamination, a fundamental requirement for health for all.

In the case of TOS, the Government of Spain moved swiftly to find the cause of the disease and to prevent further illness. Researchers in Spain began immediately to search for the etiological agent, for its identification could provide the key to treating those affected by TOS. The research quickly broadened to include epidemiology, pathology and clinical aspects, and scientists from other countries joined the effort. At the request of the Government of Spain, the WHO Regional Office for Europe has been involved with TOS since the early stages of the outbreak, providing international expertise in epidemiology, toxicology and clinical medicine.

To further the research effort, the Regional Office and the Spanish Government signed a memorandum of agreement in 1987 that formalized
this collaboration and laid the foundation for an intensive international research programme. As described more thoroughly in the Introduction, the research programme has been directed by an international group of experts in the relevant fields. An important aspect of this programme has been the strengthening of scientific capability in Spain.

This volume brings together the results of many studies on TOS and outlines the directions that future research on this disease will follow. It provides an update on what has been learned about TOS since the first WHO publication in 1984, entitled Toxic oil syndrome: mass food poisoning in Spain.

We wish to acknowledge the efforts made by the authors and to thank them for their contributions. We should also like to express our deepest gratitude to the members of the Joint WHO/FIS Scientific Committee for the Toxic Oil Syndrome, whose names are listed in the front of this volume, for their unstinting dedication towards resolving the complex research issues that surround this perplexing disease.

The research on TOS continues, and it is our deepest hope that the results of this work will benefit those individuals with TOS by providing answers to the questions that remain.

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Stanislaw Tarkowski  
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WHO Regional Office for Europe
Foreword

Just about ten years ago, a strange and terrifying epidemic made its sudden appearance in Madrid and some of its environs. Presenting in most instances as a pulmonary disorder, an infective cause was first suspected, but exhaustive investigations failed to confirm this. What was more, the pathognomy proved bizarre, with other organs being affected. Within months some 20,000 cases were known. The dramatic and overwhelming pressure on the medical services within and around the Spanish capital permitted no time to organize systematic record-keeping, purposeful investigations, or truly rational treatment and management. Extemporization, as for any emergency, was the order of the day, with hospitals overcrowded and physicians struggling with the day-to-day care of pathetically ill patients.

Only when attention was drawn to the association between the illness and the consumption of what was ostensibly cooking oil, sold cheaply by street-corner salesmen or at weekly markets in unlabelled 5-litre plastic containers, could any effective measures be taken to abate the calamity. The authorities at once publicized this finding, warned people not to use any more of this oil, and advised them to return any remaining stocks to depots officially set up for this purpose. Such was the state of panic that, in organizing this collection and identifying each consignment with the home and family from which it had been recovered, the details noted were not as reliable as they might, in retrospect, have been.

In practical terms, the effect of this forthright policy was to bring the epidemic to a virtual end, but not before some hundreds of victims had died in the acute stage, hundreds more were to succumb thereafter, and thousands were to remain permanently incapacitated.
Over the years that have now elapsed, very little progress has been made in elucidating the fundamental etiology of this enigmatic syndrome. There were those who contended that oil was not to blame, but instead that a chemical pesticide was the cause. But the most comprehensive and exacting epidemiological studies, subjected to critical independent assessment, leave practically no doubt that this oil was at least the vehicle by which the disease was conveyed.

It may be pathologically significant that, whereas various organs of the body might be involved, the consistent basic change in all of them was a non-necrotizing arteritis, with its main impact on the endothelium. It might be toxic or immunological in origin.

Since 1981, the authorities in Spain, along with the WHO Regional Office for Europe, have spared neither effort nor expense in pursuing the etiological trail, handicapped though they were by the absence of any analogous disease already recognized in humans and by the lack of any animal model in which the condition could be experimentally induced.

Turning first to a toxic mechanism, numerous projects have been encouraged and funded in various countries with, to date, almost entirely negative results. On the immunological front, few advances have been made. More work needs to be carried out along these lines.

What intensifies the mystery is the ignorance that still exists about the substance(s) that persisted in the oil, as marketed, to render it so fiercely pathogenic. Basically the product was rapeseed oil, exported from France and destined for industrial outlets. As such it was denatured by the simple addition of 2% aniline. Thereafter in Spain it underwent processing, with some additions and admixture, in a manner that is still somewhat obscure. The aniline itself could not have been responsible, for the toxicity of this chemical has been well documented and is quite distinct. Instead, presumably, some chemical changes were brought about in the material that led to the formation of the noxious component(s). That these are somehow related to the aniline is suggested by the higher content of anilides in the unequivocally case-related oil samples. More research is now being directed to detecting those peculiar constituents, natural or exotic, that are to be found in case-related oils, as well as those that may be artificially contrived in rapeseed oil that has undergone processing similar to that believed to have been followed with the original material, before it was put on the market. Then, with the separation, identification and perhaps synthesis of these entities, it may be possible to demonstrate their toxic or immunogenic characteristics in some biological system.
All of this is not to be dismissed as an academic exercise in retrospect. Granted, the toxic oil syndrome as seen in Spain has never been encountered elsewhere, before or since. Yet rapeseed oil is extensively produced and widely used, in different forms, as a human food-stuff. If, therefore, adequate precautions are to be taken to avoid another disaster of this kind, the complicated etiology of the toxic oil syndrome must be unravelled. To this end, the Joint WHO/FIS Scientific Committee for the Toxic Oil Syndrome is designing an overall research strategy, within which contributory projects, wherever they might be inspired, will be promoted and financed.

Roy Goulding
Chairman
Joint WHO/FIS Scientific Committee
for the Toxic Oil Syndrome
Introduction

Elaine C. Grandjean

In May 1981, the issue of food safety took on added importance when a new foodborne disease broke out in epidemic proportions in Spain. The disease, which came to be called the toxic oil syndrome (TOS), developed in people who consumed adulterated rapeseed oil sold as cooking oil. TOS affected more than 20,000 people, of whom several hundred died within the first year. To date, most of those affected continue to display symptoms to varying degrees and more than 800 have died.

During the years since the outbreak, TOS has proven to be a disease of surprises in terms of development. It has three phases which, while showing some overlap, also have distinct clinical profiles. The acute phase, which lasted about two months, was dominated by eosinophilia, pulmonary oedema, myalgias, fever and rash. The intermediate phase featured myalgias, weight loss, nonpitting oedema, hepatopathy and sicca syndrome. The chronic phase is characterized by peripheral neuropathy, hepatopathy, scleroderma and pulmonary hypertension. Most survivors show progressive clinical improvement; nevertheless, new cases of pulmonary hypertension and chronic hepatitis are still being diagnosed.

While the clinical picture of the disease is clear, the precise etiological agent that caused it has not been identified. Nonetheless, the epidemiological and analytical evidence, as presented in the respective
chapters, points overwhelmingly to the ingestion of rapeseed oil denatured with aniline as the vehicle of the etiological agent. This oil was sold illicitly as cooking oil by travelling salesmen or at local weekly markets, primarily in Madrid and in the provinces northwest of Madrid. While hypotheses regarding other possible vehicles of the etiological agent have been brought forward through the years, none has been convincingly substantiated.

What Has Been Done?

International coordination
From the early days of the epidemic, the WHO Regional Office for Europe has worked closely with the Spanish authorities, at their request. In March 1983, a WHO Working Group on the Toxic Oil Syndrome met in Madrid to assess the background information and the situation, to identify gaps in knowledge and priority issues, and to recommend future preventive action (1). Of utmost importance was the need for research in epidemiology, toxicology and pathology to establish beyond doubt the etiology of TOS, to find effective treatments for TOS victims, and to help prevent similar epidemics from occurring in the future.

One of the major recommendations of the Working Group was to establish an international steering committee to evaluate the status of current research and coordinate research activities; to give guidance for future studies and the selection of oil samples for continued storage and further testing; to promote multicentre studies and develop joint protocols; and to arrange the peer review of experimental results.

The WHO Scientific Steering Committee
As a follow-up to this recommendation, the WHO Scientific Steering Committee for the Toxic Oil Syndrome was established in 1984 at a joint meeting of the the Spanish National Plan for the Toxic Syndrome (NPTS) and the WHO Regional Office for Europe. The Steering Committee consisted of nine international experts in pertinent scientific disciplines. In addition to acting as an advisory body to the Regional Office and NPTS on scientific issues related to TOS, the Steering Committee was to review progress and the significance of TOS findings in regard to etiology, make recommendations for future research, establish research priorities, and advise and assist in establishing a network for international collaboration.
The Steering Committee met in 1984 and again in 1985. By the second meeting it was clear that the magnitude of the research programme required the establishment of a Liaison Group to coordinate action between the Regional Office and the Spanish authorities responsible for TOS, on behalf of the Steering Committee. The Liaison Group was formally established in 1986, initially consisting of the Chairman and two members of the Steering Committee.

In 1987, a memorandum of understanding was signed between the Spanish Social Security Health Research Fund (FIS), which took over responsibilities for TOS matters from the NPTS in 1986, and the WHO Regional Office for Europe covering cooperation on the study of TOS. From 1987 to 1990 the Liaison Group held meetings with FIS twice a year.

Call for research proposals
In 1987, an international announcement was issued in major journals inviting research proposals along the following lines:

- search for compounds involved in the pathogenesis of TOS;
- animal toxicity studies;
- pharmaco/toxicokinetic studies of possible etiological compounds;
- biochemical abnormalities found in TOS;
- clinical and in vitro studies of immunological abnormalities related to TOS; and
- clinico-epidemiological studies of the long-term evolution of TOS.

Between 1987 and 1990, 31 research projects along the above lines were funded. The results of many of these, as well as of independent studies, are included in this volume. Nearly 700 research articles are available in the open literature (2).

Recent developments
In May 1990, the WHO Scientific Steering Committee for the Toxic Oil Syndrome met to review progress made since the last meeting in 1985 and to set the directions for future research. It recommended that the reviews of research in epidemiology, clinical aspects, pathology, and experimental and analytical studies be made available to the scientific community and government decision-makers in book form. It also set

aWHO Scientific Steering Committee for the Toxic Oil Syndrome: summary report on the third meeting. Copenhagen, WHO Regional Office for Europe, 1990 (unpublished document EUR/SPA/PCS 010/B(s)).
the research priorities and made recommendations regarding future work, which are also included in this volume.

At the May meeting, the Steering Committee and Liaison Group were reformed, with some alteration in membership, into the Joint WHO/FIS Scientific Committee for the Toxic Oil Syndrome. The list of its members is included in this volume. The Scientific Committee will continue to meet at least twice a year to evaluate progress of funded studies and to discuss research directions.

**Future Research**

The problem areas that need investigation are set out in this volume. In general, the epidemiology of TOS has been well covered, with relatively few new studies needed. Clinical and clinical epidemiological studies on the evolution of TOS over time will, of course, continue to be needed. Experimentally, the search for an animal model will continue, though results to date have not been encouraging. Work in analytical toxicology will move firmly into the area of producing simulated oil and administering it to animals in an attempt to reproduce the disease. Immunological research is an area of growing interest.

Some clinical similarities between TOS and the recently described eosinophilia–myalgia syndrome (EMS) have added a new dimension to the study of TOS. EMS is associated with ingestion of manufactured l-tryptophan. An outbreak of this disease occurred in the United States in the autumn of 1989 and according to official records affects some 1500 individuals, of whom 28 had died as of November 1990. The real incidence has been estimated as high as 5000 cases. Cases have also been recorded in Germany, the United Kingdom and other countries. Both syndromes share such features as severe myalgia, intense eosinophilia and multisystem involvement. As EMS develops, it shows further similarities to TOS in terms of long-term sequelae such as scleroderma-like changes and neuropathy. A coordination of research on both syndromes is being explored.

**References**


Epidemiological studies

E. M. Kilbourne, M. Posada de la Paz
& I. Abaitua Borda

A detailed summary of information on the epidemiology of TOS was presented in a 1984 publication of the World Health Organization (1). The purpose of this chapter is to provide updated information on the most important recent accomplishments and activities in the epidemiology of TOS. Developments include projects at all stages of completion, including papers that have already been published, studies currently under way (including preliminary results where available) and epidemiological work being contemplated for the future.

Epidemiological studies on TOS are divided into two major groups: etiological studies and follow-up studies. For purposes of this discussion, etiological studies are those that attempt to elucidate the nature of the causal agent of TOS, the manner in which people were exposed to it, and the possible influence of factors that may have modified the risk of illness. Follow-up epidemiological studies are those dedicated to a systematic assessment of the manifestations of illness among affected persons. Thus, within the context of this chapter, the term follow-up is used in a somewhat broader sense than many epidemiologists are accustomed to. For example, a cross-sectional study on the prevalence of a particular clinical manifestation among a group of TOS patients would be considered here as a type of follow-up (as opposed to etiological) epidemiological study.
Etiological Studies

Epidemiological studies of the etiology of TOS have largely been of four types: (a) studies on the frequency of specific risk factors for illness in TOS case series; (b) case-referent studies; (c) reports on certain “special” studies or “special situations”, the details of which have important implications regarding TOS etiology; and (d) “toxico-epidemiological” studies involving joint statistical analysis of data on the chemical composition of oils, and historical and clinical information on those who consumed them.

The focus of etiological studies has gradually shifted over time. Initially, such studies were directed towards identifying the vehicle that transmitted the etiological agent. Evidence from these studies continues to implicate aniline-denatured rapeseed oil, supposedly destined for industrial use, as the vehicle of the TOS agent. Thus, those new studies that serve only to demonstrate the oil–illness link are of decreasing interest. Nevertheless, because of past controversy about whether consumption of denatured rapeseed oil caused TOS, proper documentation (i.e. peer-reviewed, published papers) of studies for which data collection was already complete has been important. Moreover, since analytical chemical investigations and experimental toxicological studies have so far failed to identify specifically the TOS agent, etiological epidemiological studies that shed additional light on the nature of that agent are of continuing interest.

In the following descriptions of studies, frequent reference is made to “suspect oil”. The precise definition of suspect oil differs slightly from study to study, but in general refers to oil sold as supposedly pure olive oil in 5-litre plastic containers that were usually unlabelled and marketed by travelling salesmen who either sold door-to-door or in weekly open-air markets (mercadillos). In his report on the etiology of TOS, Sir Richard Doll refers to such oil as “street oil” (2).

Studies of case series
Possibly the most widely cited TOS case series of etiological importance is Tabuenca’s 1981 report (3) that all of the TOS patients he had studied had consumed a suspect oil, characterized as having been sold in unlabelled 5-litre plastic containers by door-to-door salesmen. This report served to focus attention on food oil as the likely vehicle of the etiological agent. Tabuenca’s paper also presented chemical analytical data showing contamination of some oils with rapeseed oil denatured with aniline.
Case-referent studies
A number of studies have compared the exposures of TOS patients with those of unaffected persons, and have provided important information on the association of suspect oil with illness. However, a number of the case-referent studies for which data were collected at the time of the epidemic were never written up for the peer-reviewed literature. Thus, completion and publication of such studies has been an important aspect of epidemiological work on TOS undertaken in recent years. Only two case-referent studies appeared in the open literature prior to 1985 (4, 5). Two more have since been published (6, 7), one of which had previously been summarized by Rigau Pérez (8) as part of a review of epidemiological data on TOS. These four published case-referent studies are summarized here.

A study at the Niño Jesús Paediatric Hospital in Madrid (4) compared the food exposures of 62 TOS patients with those of 62 patients with other pathology. All of the children with TOS had consumed suspect oil whereas only 6% of the children being treated for other problems had done so ($P < 0.001$). Doll, in his report on the etiology of TOS (2), draws attention to the fact that the groups also differed with respect to consumption of other foods, although these other differences were not as striking as the difference between the groups regarding consumption of suspect oil.

Rigau Pérez et al. (5) reported the findings of a series of studies in the small town of Las Navas del Marqués (Avila Province). These investigators found a strong association of illness with the consumption of suspect oil. All 27 families in which TOS cases occurred had consumed such oil, while only 13 (24%) of 54 size-matched and 17 (31%) of 54 randomly chosen control families had done so. Estimating individual oil consumption as a family's total oil consumption divided by the number of persons in the family, Rigau's group initially found evidence of an increased risk of illness with increasing amount of oil consumed. Among case families, 26% of family members consuming 90 – 250 ml weekly became ill, as compared with 53% of those consuming 251 – 500 ml weekly and 71% of persons consuming 501 – 850 ml weekly. The investigators attempted to reproduce this finding as part of a further questionnaire study involving 32 cases but were unable to do so, despite the fact that 16 of the case families were the same in both studies. Moreover, in an analysis limited to these 16 families, the investigators were unable to demonstrate a dose–response association of oil ingestion with illness when the oil consumption data from the
earlier questionnaire were related to the presumably more accurate data on presence or absence of illness derived from the later questionnaire. Thus, while this series of studies showed a strong association of TOS with ingestion of suspect oil, a dose–response effect was not conclusively demonstrated.

The studies done by Rigau's group highlight another important finding in the TOS epidemic: marked differences in clinical outcome were seen in different family members who consumed similar quantities of the same oils (5). Because immunological reactivity to a given substance may vary considerably from person to person, the apparent differences between individuals in susceptibility to the TOS agent may indicate that the agent caused the illness through an immunological mechanism. Differences in immunological function between species can be even greater than those between individuals of the same species. Thus, some species specificity in the nature of the response to the TOS agent might be anticipated on the basis of the data from Rigau's study. In this regard, the fact that oils that must have contained the TOS agent have so far failed to produce TOS-like illness in experimental animals is not a totally unexpected finding.

Cañas & Kilbourne (6) reported the results of a door-to-door questionnaire study conducted in 1981 in the working-class neighbourhood of Orcasur in Madrid. Participants in the study were asked about the types and sources of oil they consumed. TOS cases were present in 5 of the 212 households for which interviews were conducted. All the affected families had consumed oil bought from travelling salesmen, whereas only 71 (34%) of the 207 unaffected families had done so (P = 0.005).

Considering only the 76 families who had bought oil from travelling salesmen, all 5 affected families had bought suspect oil from travelling salesmen in the local mercadillo. Only 27 (38%) of the 71 unaffected families who had bought oil from travelling salesmen had done so in the mercadillo (P = 0.01). On the other hand, only 1 (20%) of the 5 affected families had bought suspect oil from a door-to-door salesman, although 45 (63%) of the 71 unaffected families had done so. Thus, in the Orcasur neighbourhood, truly etiological oil appeared to have been bought in the local mercadillo, although data were insufficient to clearly implicate any specific salesman who sold oil there. The Orcasur study showed no significant association of illness with oils bought from the following sources: grocery stores or supermarkets; molinos (small oil pressing facilities); almacenes (establishments whose principal business is to sell oil in large quantities to small vendors but
which occasionally sell to the public at wholesale prices) or a granel dealers (persons other than those associated with molinos who sell oil to the public but who avoid the cost of bottling their product by pumping measured quantities into containers brought by the buyer).

In 1987, Díaz de Rojas et al. (7) published data on the distribution of TOS cases in two convents of the same religious order, located side by side in Madrid. The residents of the convents were nuns, novices and laywomen. Each convent had a kitchen in which food for all of its residents was prepared. However, the laywomen in each convent ate in a dining room separate from the dining room for nuns and novices. All residents at each convent thus ate the same foods, with the exception that the oil at the tables used as a condiment for salads and vegetable dishes differed: the laywomen at both convents had to use soya oil as a condiment whereas the nuns and novices were permitted the use of olive oil. The “olive oil” used in each convent at the time of the epidemic was store-bought, but came in 5-litre plastic containers. In one convent, symptoms of TOS occurred in 23 (66%) of the 35 nuns and novices but in none of the 56 laywomen. In the other convent, 42 (98%) of the 43 nuns became ill while all of approximately 70 laywomen living there stayed well.

Essentially all of the information from peer-reviewed, published case-referent studies supports the view that denatured rapeseed oil was the vehicle of the TOS agent. Nevertheless, these studies offer relatively little information regarding the nature or identity of the TOS agent itself.

Special studies and special situations
Some of the most important evidence linking aniline-denatured rapeseed oil with the TOS epidemic has come from detailed investigation of the occurrence of TOS under unusual circumstances. Some 99% of officially registered TOS cases occurred in a 14-province area in central and northwestern Spain, some 300 km from Seville (1).

Posada et al. (9) conducted a detailed investigation of TOS cases in the only four families known to have been affected in Seville. Two of the families had consumed food from the epidemic area, and were presumably exposed thereby to the TOS etiological agent. Nevertheless, the other two families had no history of ingesting foods acquired in the affected regions. The only evident link between these latter families and the epidemic was the fact that the heads of both households worked at a Seville oil refinery that processed rapeseed oil for an oil distributor
in the epidemic area. Moreover, both heads of household brought rapeseed oil home for family consumption. Thus, if rapeseed oil were not the vehicle of the etiological agent, the occurrence of TOS cases in these two families is difficult to explain. The report of cases in these families thus provides strong support for the causal role of oil ingestion in the epidemic (2, 9).

In a recent report, Posada et al. (10) describe the occurrence of cases of TOS late in the course of the epidemic. The great majority of TOS cases occurred from April through June 1981. Nevertheless, four of five family members in a province directly adjacent to the 14-province area to which reference is made above became ill with clear-cut findings of TOS during December 1981. The family had acquired two 5-litre containers of oil from an itinerant vendor during the time when most cases occurred. When made aware of the possible danger associated with ingesting this sort of oil, the family brought some oil from one of the containers to the provincial health authorities for analysis. A test for "aromatic amines" was negative, and the family was told that the oil was safe to use. They began to use it in November 1981. When they became ill in December 1981, a second oil analysis done at a reference laboratory in Madrid showed that the oil contained fatty acid anilides (amides, not amines), a finding consistent with the oil having been contaminated with the etiological agent of TOS. The agent thus appeared to have persisted in an active form in the oil for some six months.

The TOS case with the latest known date of symptom onset is described in the same report (10) and occurred in a middle-aged businessman who used a food oil he found in his mother's house, which he occupied intermittently from April 1982. Although the patient did not report ever having developed the respiratory symptoms of TOS, he did develop clear-cut clinical findings of chronic TOS, resulting in his admission to hospital in the autumn of 1982. He had not paid particular attention to the oil he had been using and at first denied exposure to oil in the plastic 5-litre containers in which suspect oil was usually sold, as this was not the type of container in which his mother usually kept oil. However, because the signs of TOS were so evident, a family member went to the mother's house and found that the patient had indeed been using oil from a container characteristic of the epidemic. This patient had no other history suggesting the ingestion of oil associated with the epidemic. Thus, he was apparently made ill by etiological oil that had been stored for approximately one year.
Taken together, these late cases of TOS have important implications for further study of the TOS agent in the laboratory. The TOS agent appears not to be extremely chemically labile, as it apparently remained pathogenic over a period of many months.

So-called early TOS cases have been under study by Abaitua and others. Activities to date have involved careful investigation of "potential early cases" whose date of onset was listed as prior to 1 May 1981 in any of three databases, including two versions of the official TOS census (one more up to date than the other) and an early line listing of "atypical pneumonia" cases developed by epidemiologists of the Ministry of Health and Consumer Affairs in 1981. Preliminary data from this study identified 155 cases whose recorded date of symptom onset was prior to 1 May. Review of clinical records, and in some cases telephone interviews, established that in the great majority of cases the true date of onset was after 1 May 1981. However, some 27 cases had confirmed onset before that date, although none occurred prior to 23 April 1981. Because rapeseed oil from the Seville oil refinery (mentioned earlier) was potentially available for distribution and consumption several days prior to the first confirmed onset date (11), these preliminary findings suggest that the initial course of the epidemic is consistent with other data regarding the distribution of implicated oil.

Interpretation of data from the early cases project is nevertheless problematic in certain respects. While results obtained to date may be valid within the scope of work originally outlined for the study, it is not clear that currently available data truly answer the question of when the TOS epidemic began. Although overlap in the groups of early cases ascertained via the two versions of the TOS census was substantial, only five (29%) of 17 early cases found through review of the Ministry of Health's pneumonia list were also found through review of one or another of the two versions of the census. Similarly, the same five cases constituted only 33% of the 15 total early cases found through the census reviews. The small degree of overlap between the sets of early cases ascertained through two distinct data sources strongly suggests the existence of other early cases that could not be studied as part of this project and whose symptom onset dates therefore remain unknown. The ongoing TOS register project (see below), involving a comprehensive review of medical records of TOS patients for summary data, and for which data collection is still incomplete, may eventually allow a more comprehensive basis for ascertaining potential early TOS cases and for more definitive data on the onset of the epidemic.
The credibility of current data from the early cases project also suffers from the failure to ascertain, through its data sources, a group of potential early cases among the nuns of a convent in Toledo Province (8). These nuns have been the subject of controversy because they reported having what may have been mild symptoms of TOS (primarily respiratory complaints) as early as February 1981. Although they did not develop florid and clear-cut symptoms of TOS until May 1981, they denied having bought or acquired any oil or oil-containing product during the period from February through the time when florid TOS symptoms appeared among them (8). Three possible explanations can be offered for these findings.

1. Oil was not the vehicle of the TOS etiological agent, an explanation considered unlikely in view of the great weight of other evidence in favour of the oil–illness association.

2. Suspect oil of which the nuns were unaware, or about which they somehow forgot, found its way into the convent’s food supply at the time the epidemic occurred in the general population.

3. One of the firms handling suspect oil sold a relatively small amount to the nuns a few months prior to selling the great bulk of it.

The last explanation receives circumstantial support from documents showing that one firm implicated in the epidemic returned all but a few hundred litres of one batch of potentially suspect oil to its supplier at about the time the nuns report making their final oil purchase prior to the epidemic (11).

An investigation in 1987 involving visits to the two oil-producing French firms known to have been exporting denatured rapeseed oil to Spain immediately prior to the epidemic yielded particularly interesting findings (12). The oil from both firms came from stock that was later distributed for consumption in France with no apparent deleterious effects on consumers’ health. In one company, rapeseed oil was mixed in a tank with aniline sufficient to yield a concentration of 2% prior to its being sent via tanker-truck to Spain. At the other exporter, aniline sufficient to form 2% of the load by weight was added to tanker-trucks, and the oil was then poured on top of the denaturant. Of particular interest is the fact that one French company hired tanker-trucks from two French transport companies to carry its oil into Spain; these trucks ordinarily carried industrial chemicals. Since Spanish customs officials recorded the license plate numbers of the trucks bringing in the denatured
rapeseed oils, the possible toxicants that may have remained in the trucks from previous loads are potentially identifiable through company records. Information on what these trucks carried immediately prior to transporting rapeseed oil has been requested by Spanish Government officials from the two French transport companies through diplomatic and administrative channels, but as of November 1991, this information has not been received.

**Toxico-epidemiological studies**
The term “toxico-epidemiological” is used here to refer to studies in which conclusions are drawn from an epidemiological analysis of measurements of specific chemical analytes in oils.

*First study (13)*
One such published study compared the chemical analyses of oils from partially full (and therefore, probably partially consumed) containers of a “typical” shape and bearing red caps. A total of 29 such oils handed in from households with at least one affected person were compared with 64 similar oils handed in by unaffected families. The “case oils” were substantially richer than the “control oils” in the fatty acids and sterols known to occur in particularly high concentrations in rapeseed oil. Moreover, many case oils were highly contaminated with fatty acid anilides and aniline, whereas such contamination in control oils was substantially less frequent and, when present, tended to be substantially less in quantity. A clear-cut dose–response relationship was seen between the level of aniline and anilide contamination and the risk of a family having been affected by TOS, as demonstrated by the virtually linear relationship between the log of the odds ratio and the level of oil contamination.

The results of this study provide strong additional evidence that oil carried the TOS agent. They also justify, at least in the population of oils studied (those handed in in the towns of Alcorcón and Leganés in Madrid Province), the use of measurements showing a high content of fatty acid anilides as appropriate selection criteria for oil specimens presumably containing the TOS agent. Such oils are particularly appropriate for use in experimental toxicological studies.

Unfortunately, the toxico-epidemiological study design does not permit calculation of the exact probability of an individual oil containing the TOS etiological agent. Nevertheless, the study showed an extremely strong and consistent pattern of increasing likelihood of an
oil coming from the case group as concentrations of aniline and anilide contaminants increased. Moreover, all oils contaminated above a certain level came from case families. Thus, the most highly contaminated case oils have an extremely high probability of containing (or having contained) the TOS agent. When such oils are mixed, even less possibility exists that the TOS agent is absent from the mixture.

Thus, mixtures of highly contaminated case oils from this initial toxico-epidemiological study are being used in some experiments that aim to detect specific toxicity or biological activity in oils. However, until recently only limited quantities of oils authenticated by toxico-epidemiological analysis were available.

Second study
A second toxico-epidemiological study has been conducted to provide more general validation of the use of anilides as an oil selection criterion and to identify additional case oils with a high probability of containing or having contained the etiological agent of TOS. This second study was facilitated by the recent cataloguing of the tens of thousands of oils removed from the market by the Spanish Government in 1981 and stored in a warehouse in the town of Arganda, southwest of Madrid. Each oil’s specimen number and storage location, and the name of the person who handed it in, had been entered into a computerized database in such a way that the oil corresponding to any particular exchange could be found with relatively little difficulty.

The second toxico-epidemiological study involved computerized comparison of names of persons who had exchanged oil with the names of affected persons in the official TOS census. From this comparison, potential case oils were identified for chemical analysis. A random sample of other oils from Arganda (which, in general, came from unaffected families) was also selected. These “case” and “control” specimens were submitted, blind-coded, to an analytical chemistry laboratory in Spain. The corresponding families were interviewed and their case or control status checked through telephone interviews and reviews of medical records.

The results of this study again show clear differences in the levels of fatty acid anilide contamination of case and control oils. These differences are quantitatively similar to those seen in the initial toxico-epidemiological study. Moreover, a strong dose–response effect was again demonstrated, with increasing anilide contamination of an oil associated with increasing probability of the corresponding family
having been affected by TOS. Since oils in this second study came from a large number of collection centres, the use of anilides of fatty acids as an indicator that an oil contained the TOS agent was validated more generally than in the first study, particularly with respect to possible geographic variation in the strength of the TOS–anilide association. Moreover, a substantial quantity of oil with known high anilide content from case families in the second study has been stored and is thus available for use in further analytical chemical and experimental toxicological investigations of TOS etiology.

Follow-up Studies

Although a number of qualitative clinical descriptions of TOS as it affects various organ systems over its course have been published, relatively few studies systematically and quantitatively document the incidence and/or prevalence over time of specific disease manifestations. Such data are not only of scientific and academic interest but may also have important implications for the care of those suffering from TOS. If a late complication (e.g. an increased incidence of a certain tumour) were found to occur particularly frequently, the physicians caring for TOS patients could look specifically for it, possibly leading to its identification at a relatively early and perhaps more treatable stage. Nevertheless, a cohort-wide screening programme for a specific late complication of TOS should be instituted only where a review of all relevant data suggests that such a programme would be appropriate.

A series of systematic follow-up studies have begun or are being initiated by the TOS investigation team at FIS. The goals and methods of these projects are outlined in the following sections. However, the possibility that new follow-up projects might be added to those mentioned below, or that some of the existing projects might be amplified, has by no means been ruled out.

Long-term detailed clinical follow-up

One or more studies are clearly needed to document the long-term evolution of the clinical picture of TOS in the approximately 20 000 affected patients. Investigators at FIS and their epidemiological consultants have given substantial consideration to the feasibility of such studies. They have decided that a very detailed clinico-epidemiological follow-up of all TOS patients would involve great expense and enormous logistical problems in coordinating the collection of appropriately standardized data from the large number of health care providers attending
these patients. Nevertheless, the possibility of collecting less detailed data on all or a major portion of the TOS cohort continues to receive attention.

The “sample of 500” study
While detailed prospective clinical study of the entire cohort may not be possible, a study of some 500 patients, a systematically selected 2.5% sample of the cohort, nevertheless seems both feasible and appropriate. The ultimate intent of this project is to collect relatively detailed data in a systematic manner so that high-incidence complications can be recognized. A second goal is to develop data on which a valid description of the long-term evolution of TOS can be based.

Using the official TOS census as a sampling frame, systematic, representative samples of approximately 100 patients each have been drawn from areas surrounding two hospitals in Madrid and from the provinces served by each of three provincial hospitals. In each hospital, one specialist in internal medicine is to be responsible for seeing each patient approximately once every six months. The patient’s history of any intercurrent medical events is to be taken in a standardized way and a standardized physical examination done at each visit. In addition, a standard battery of laboratory tests is planned, including blood counts and serum chemistries. To avoid an excessive burden both for the patients and the health care system, as well as to avoid undue risk to the patients, other procedures (e.g. chest radiography, echocardiography, cardiac catheterization) will be done only if clinically indicated. If any particular clinical event appears to be occurring with unusually high frequency, the statistical significance of its rate of occurrence can be checked against other data on the incidence of that particular type of health problem in other populations.

The investigators in this project are aware of the potential problems posed by inter-observer differences in data gathered by physicians in the course of the medical history and physical examination. Efforts are being made to minimize such differences. A standard data collection form will be used, and written guidelines for filling out the form have been developed and discussed with the physicians responsible for data collection. As the project gets under way, inter-observer variation and overall quality control will be further checked by means of periodic between-physician statistical comparisons, although the interpretation of any differences found is complicated by the possible contribution of real differences between the patient populations of different areas.
Since the laboratory measurements for each subsample of 100 patients are to be conducted at each of five different hospital laboratories, the investigators will have to deal with questions regarding the reliability of those laboratories and possible interlaboratory variation. These issues are difficult to resolve fully before beginning the study. Early in the project, the investigators will have to look for possible systematic differences in the results from different laboratories for patients who are otherwise clinically similar. Any questions of reliability arising from such periodic statistical reviews can be addressed whenever they occur.

The participating institutions and investigators have been selected, and lists of patients who will be invited to participate have been developed. The protocol for data collection has had extensive review and has been discussed thoroughly with the participating physicians, and data collection is beginning.

**Considerations of design and statistical power**

While the sample of 500 approach may be highly appropriate for evaluating certain aspects of the long-term evolution of TOS and for assessing the significance of complications with high baseline frequency in the general population, the study has insufficient statistical power to identify effectively even large increases in the relative risk of adverse health outcomes whose incidence is low.

To illustrate the extent of this problem, and to assist the reader in assessing its implications for both the sample of 500 and other follow-up studies of TOS patients, a number of specific power calculations for the sample of 500 study have been performed (Table 1). The methods used are outlined by Breslow & Day (14). Data on the age- and sex-specific incidence of lung cancer and of the leukemias in the general population were drawn from a report from the cancer registry of Navarra (15). Similar data on pleural cancer were taken from 1982 incidence data reported from the cancer registry in the Principality of Asturias (16). A total of 100 individuals were randomly sampled from each of the five systematically drawn samples (corresponding to each of the five participating institutions) of patients to be invited to participate in the sample of 500 study. The age- and sex-specific rates from the cancer registries were applied to this "potential" sample of 500 patients (whose age and sex distribution should approximate that of the final sample of 500 after all invitations to participate in that study have been extended and accepted) to arrive at the expected numbers of cases of specific tumour types during the three-year period of 1990–1992.
Table 1. Expected numbers of new cases of selected tumours during 1990–1992 and study power for specific levels of relative risk in the sample of 500a

<table>
<thead>
<tr>
<th>Health outcome</th>
<th>Expected new cases 1990–1992</th>
<th>Relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Lung cancer, all typesb</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>32</td>
</tr>
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<td></td>
<td>46</td>
<td>72</td>
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<td>88</td>
<td>98</td>
</tr>
<tr>
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<td>100</td>
<td></td>
</tr>
<tr>
<td>Leukemia, all typesb</td>
<td>0.16</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>34</td>
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<td></td>
<td>48</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Pleural cancerc</td>
<td>0.02</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>8</td>
</tr>
<tr>
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<td></td>
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<td>26</td>
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<tr>
<td></td>
<td>33</td>
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</tbody>
</table>

a Expected numbers of cases were calculated on the basis of the age and sex distribution of affected persons and assumed that the age- and sex-specific rates of certain referent populations would apply. Tabled values are the statistical power (in percent) to detect a given relative risk with a one-tailed alpha of 0.05.

b Source: Abad Vicente et al.(15).

c Source: Echeverría Rodríguez et al. (16).

For most epidemiological purposes, statistical power can be defined as the probability that study results will reveal a statistically significant increase in the incidence of a health outcome, given that the risk of that outcome is truly increased to a particular extent in study subjects. Detecting an increased risk becomes more probable as the magnitude of that risk increases (Table 1). Statistical power is of particular importance in the design of epidemiological studies from which some reassurance can hopefully be gained about the absence of risk if the study yields a negative result.

Such reassurance for pleural cancer, an extremely rare tumour, will be virtually impossible to gain from the sample of 500 study. The power to establish a significant increase in this tumour is only 6%, assuming that having been affected by TOS causes a three-fold increase in risk, a level often considered clinically significant. Put another way,
the probability of failing to identify a true increase by three in the risk of pleural cancer in this study over the period 1990–1992 is 94%. Even if the relative risk were 20, a value seen only in some of the very strongest exposure–disease associations, the study would have only a one-in-three chance of detecting a significant increase. In the case of lung cancer, one of the most common malignant tumours, the chances of identifying a three-fold increase in risk in TOS patients as both present and statistically significant from 1990–1992 are only about one in five.

While the conventions for the probability level constituting acceptable statistical power are by no means as universally accepted as those for statistical significance, epidemiologists sometimes calculate required sample sizes on the basis of statistical power of 80% or greater, and values of 90%, 95% or even higher are not unusual. Thus, the power values listed in Table 1 are not reasonable for the disease outcomes under consideration, unless one is concerned only with extraordinarily high relative risks for the two most common categories of cancer listed. The power to detect other low-incidence health outcomes in the sample of 500 is similarly small.

**Feasibility of follow-up by letter-questionnaire**

Because of a priori uncertainty regarding the feasibility of undertaking certain aspects of the follow-up of the entire cohort of 20,000 persons affected by TOS by means of a letter-questionnaire, a pilot study was conducted on 1032 censused persons (a 5% sample). Preliminary data from this study indicate that the response to mailing a simple, one-page questionnaire was poor, with only 662 (65.9%) of the 1004 persons for whom mailing addresses were available responding after three mailings. However, telephone follow-up of non-responders was impressively successful. Ultimately, responses were obtained for all but 3 of the 1004 patients. The information sought was very general in nature and had to do principally with any admissions to hospital, surgical procedures and/or biopsies the patient had undergone (an attempt by the investigators to identify sensitively, albeit nonspecifically, possible incident cancer cases) and the births of any children. One of the most important conclusions of this study was that the telephone interview is well accepted and may be extremely useful in Spain for ascertaining certain of the data required for follow-up studies. Telephone interviews may well be a key element in attempts to follow the TOS cohort over the long term, although other options are under consideration as well.
Survival among TOS patients
One of the most basic points of information that must be ascertained periodically in the ongoing follow-up of any cohort is the vital status (alive or dead) of its members. A number of attempts have been made to determine age- and sex-adjusted mortality rates among censused persons over time. So far, only one of these has met with success.

The pilot letter-questionnaire study mentioned above is currently the best source of data on total mortality (all causes) over time among the censused TOS cohort. The vital status as of 7 March 1988 was ascertained for 1008 persons. Survival from 1 May 1981 through 7 March 1988 has been analysed. Rates of mortality by year with age and sex adjustment by the direct method have been calculated, as have standard mortality ratios (SMRs) with 95% confidence limits. Spanish national population and death data for 1981 and stratified by sex and five-year age groups were used as the standard for these calculations (17).

In 1981, excess mortality in the sample was substantial and statistically significant. In 1982 and 1983, mortality still appeared high, although the 95% confidence interval of the SMR included unity. From 1984 through 7 March 1988, the SMR was less than unity, although not significantly so. Thus, the 95% confidence intervals of the SMR have spanned unity since 1982, indicating that more or less the expected number of deaths occurred. However, these confidence intervals are wide owing to the relatively small sample size. Moreover, although the yearly SMRs for deaths from all causes were not significantly elevated after 1981, a statistically significant trend towards death at a younger-than-expected age was seen.

A cause-specific analysis of mortality has not been done because cause-of-death data are not available for all deaths in the sample. Nevertheless, the fact that cohort deaths were close to the level expected from 1982 onwards, even though these deaths included a number of TOS fatalities, suggests the possibility that causes of death other than TOS may have been less in the TOS cohort than in the general Spanish population during the years following the epidemic. In any event, no striking excess of late deaths in the TOS cohort is apparent in the period prior to March 1988.

\[a\] Includes 1001 persons responding to mail/telephone questionnaire, 2 for whom mailing addresses were available but who did not respond, and 5 found despite the absence of a mailing address.
TOS register

Owing to the lack of a more valid or systematically compiled register of TOS cases the Spanish Government’s official census of TOS cases, developed largely for administrative rather than epidemiological purposes, has had to serve as a sampling frame for several follow-up studies (e.g. the sample of 500 and the 5% sample studies). The inadvertent inclusion of some persons in this census who did not fit a well constructed and consistently applied case definition of TOS may tend to bias these studies. However, as the major source of potential selection bias appears to be the possible inclusion of some unaffected persons, any resulting bias may well tend to be conservative.

The lack of reliable clinical data, collected in a standardized and uniform manner, has substantially impeded attempts to study specific subgroups of interest among TOS patients (e.g. persons with scleroderma, documented pulmonary hypertension or chronic hepatopathy). A team of physicians is currently reviewing all clinical histories of TOS patients. The review is being done with a standardized chart abstraction form, a standardized set of instructions for filling out these forms, training in the application of those instructions in potentially ambiguous situations, and supervision by epidemiologists at FIS. The records of most censused patients have been found and reviewed, and a preliminary review of early results indicates that about 75% of them appear to meet a reasonably specific case definition of TOS. Upon completion, the TOS register will become available for use as the new sampling frame for further follow-up studies of the TOS cohort.

Potential increase in cancer incidence

The possibility of a long-term increase in cancer incidence in TOS patients is an important concern that has not been addressed well by studies done to date. A brief review of incident tumours reported to the former National Plan for the Toxic Syndrome (NPTS) among the TOS cohort showed that either TOS was highly protective against a broad variety of tumours or that new cases of cancers in general had been substantially under-ascertained. Given the passive nature of the surveillance conducted at NPTS, the latter seems the more likely of the two possibilities.

The problem of cancer follow-up could be substantially simplified if a viable and operative cancer registry existed for Madrid Province, where some three quarters of TOS cases occurred. TOS patients could be sought among the reports of patients with new cases of tumours, and any increased relative risk of a particular tumour type among TOS patients would
become apparent. Although no Madrid cancer registry exists at present, serious consideration is being given to its creation, in part because of the potential help in identifying new cases of malignancy among TOS patients.

Another possibility is to follow the implicit strategy of the 5% sample study described above. In the questionnaire for this study, patients were asked about sentinel events (i.e. biopsy, surgery and hospitalization) that were considered sensitive (though not specific) indicators of possible new cases of malignancy. The principal problem in detecting tumours in the cohort by this method is the amount of person-hours of work required to acquire and review the medical records corresponding to every positive response. About 10% of the sample had had at least one of the sentinel events in the last year. Thus, the follow-up of sentinel events would require reviewing about 2000 medical records annually.

As high as this figure of 10% may sound, one cannot assume that it represents a clear increase over the proportion of persons experiencing such sentinel events in the general Spanish population, since the equivalent population-based data required to make such a comparison are lacking.

In summary, a feasible way of following this cohort for malignancies with a reasonable degree of statistical power has not yet been developed. If TOS causes an extremely large relative increase in the risk of some particular tumour type as, for example, asbestos does for mesothelioma, then the sample of 500 might be sensitive enough to detect that increase. However, even relatively substantial increases in common tumours could go unrecognized in that study, because of its low statistical power (Table 1).

Potential teratogenic effects
NPTS accumulated passive reports of children born to couples of which one or both members were affected by TOS. The possibility of implementing a project involving the close examination of these children for developmental abnormalities is currently under review. This project has not yet been implemented, nor have estimates of its statistical power been calculated.

Conclusions
While epidemiologically based analyses of data obtained from chemical analyses of oils will continue to be necessary, relatively little remains to
be done with regard to field epidemiological studies of TOS etiology. Such studies have already yielded important findings. Contaminated rapeseed oil has been unequivocally identified as the vehicle of the causal agent, and oil specimens appropriate for further laboratory work have been identified.

Much of the remaining epidemiological work pertains to the ongoing follow-up of the cohort of affected persons. Current studies have given indications regarding the pattern of mortality that occurred in the cohort in the years subsequent to the initiation of the epidemic, and have shown that telephone communication with affected persons is an effective means of gathering data. A new TOS register is being compiled, one that will include useful clinical data that will help describe the course of the epidemic prior to the end of 1987. Nevertheless, an effective approach to the identification of any increases in the incidence of specific cancers and many other long-term health problems that may occur among TOS patients has not yet been developed. Follow-up efforts could be further amplified, but only with a corresponding increase in administrative, logistical and economic support for such an effort.

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Clinical findings

I. Abaitua Borda
&M. Posada de la Paz

In spring 1981, the sudden and massive epidemic of TOS struck Spain, mainly in the central and northwestern areas of the country. The majority of cases were diagnosed during May, June and July of that year, with only a few documented at later dates, including one as long as a year after the epidemic outbreak (1). Neither clinically nor pathologically had any such disease been previously recognized, nor had its characteristics been seen in other animals.

An infective etiology was first suspected, but exhaustive laboratory tests proved negative (Centers for Disease Control, Atlanta, USA, unpublished observations, 1981). Then, epidemiological studies pointed to its cause being rapeseed oil denatured with 2% aniline and further processed before being illicitly sold in 5-litre plastic containers by itinerant traders (2).

Incidence reached a peak about one month after the first case was reported, then rapidly declined. This decline could be attributed to two factors: first, the official warning about this particular oil and its subsequent withdrawal from the market; and second, the dilution of this oil by the purveyors a few days before the warning was issued.

The conclusion was that the etiology was toxicologically induced, presumably by some agent in the oil. Isolated cases of unknown etiology but with an analogous clinical presentation in some respects have been
described elsewhere (3, 4) but these have not so far helped to pinpoint the cause of TOS. On the other hand, the relationship between TOS and the eosinophilia–myalgia syndrome (5) makes even more interesting the search for the causal agent of TOS.

Previous clinical accounts of TOS have been derived largely from reports on hospital patients. This chapter summarizes the descriptive epidemiological data, gives an account of a systematic 31-month follow-up of a group of patients that includes other relevant clinical data published since 1983, and discusses the current status of TOS patients.

Incidence and Mortality

A total of 20 643 people were afflicted with the disorder, with a female:male ratio of 1.5:1. This sex difference was more pronounced in those around 40 years of age. When the epidemic began, 21.8% of the cases were in those under 15 years of age (6).

Accurate figures on all deaths from TOS have been impossible to assemble. After 1983, mortality rates seem to be lower than those for the Spanish population at large, but this could be due to under-reporting. To December 1989, the total number of deaths registered among censused TOS patients was 839.

Patients died from several causes. During the acute phase respiratory failure from noncardiogenic oedema was most common, and in the intermediate phase thromboembolic incidents were frequently fatal. In the chronic phase, death has usually been due to respiratory failure, with secondary infectious and/or haemorrhagic complications, as a consequence of gross respiratory insufficiency from nerve and muscle dysfunction. A form of pulmonary hypertension with many clinical and pathological similarities to primary pulmonary hypertension has figured prominently among recent deaths (7).

Evolution of the Disease

Systematic data on the entire affected population are not available at present. Nevertheless, two random samples (one of adults, the other of

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28
children) were selected among those patients diagnosed within the first four months of the epidemic (8). From these samples, 914 patients completed the follow-up programme till month 31 after onset of the disease. Clinical findings are summarized in Tables 2–5. Percentages reported in these tables are estimated from both samples together. They represent averages weighted to the real proportion of affected children and adults in the population.

TOS is a multisystem disease with three successive phases in its evolution but with a common physiopathological link (Table 2) (9–12).

Table 2. Major clinical signs of TOS as it develops

<table>
<thead>
<tr>
<th>Acute phase (months 1–2)</th>
<th>Intermediate phase (months 2–4)</th>
<th>Chronic phase (month 4 onwards)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>Skin oedema</td>
<td>Scleroderma</td>
</tr>
<tr>
<td>Rash</td>
<td>Alopecia</td>
<td>Dysphagia</td>
</tr>
<tr>
<td></td>
<td>Sicca syndrome</td>
<td>Sicca syndrome</td>
</tr>
<tr>
<td>Pulmonary oedema</td>
<td>Pulmonary hypertension</td>
<td>Pulmonary hypertension</td>
</tr>
<tr>
<td>Myalgia</td>
<td>Myalgia</td>
<td>Peripheral neuropathy</td>
</tr>
<tr>
<td></td>
<td>Sensory neuropathy</td>
<td>Weight loss</td>
</tr>
<tr>
<td></td>
<td>Weight loss</td>
<td>Hepatopathy</td>
</tr>
<tr>
<td></td>
<td>Hepatopathy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thromboembolism</td>
<td></td>
</tr>
<tr>
<td>Eosinophilia</td>
<td>Eosinophilia</td>
<td>Eosinophilia (gradually disappears)</td>
</tr>
</tbody>
</table>

**Acute phase**
The notable early symptoms (1–2 months after onset) were nonproductive cough, pain, tightness of the chest and dyspnoea. Radiologically, the lungs showed a widespread, alveolar–interstitial infiltration, unilaterally or bilaterally distributed, with conspicuous Kerley A and B lines and often a pleural effusion, but with a normal cardiac silhouette. Nonrespiratory complaints were general malaise, asthenia, anorexia, headache, tissue oedema, pruritis, a polymorphous rash, arthralgia, myalgia, muscle cramps, and sometimes hepatomegaly and lymphadenopathy. Quite early on, many of these patients suffered from
abdominal pains with and without diarrhoea. The most prevalent clinical signs are given in Table 3.

Only about 1% of patients exhibited central nervous system dysfunction, seen as confusion and stupor and measured by a diffuse slowing of the electroencephalogram. The cerebrospinal fluid remained normal. Resolution of this central nervous system dysfunction was complete.

Haematologically, the eosinophilia in the peripheral blood exceeded 500 cells/mm³, and in a large number of patients it rose to over 3000 cells/mm³. Other laboratory findings were high levels of immunoglobulin E, hypertriglyceridaemia, thrombocytopenia and elevated hepatic transaminase levels.

Table 3. Most prevalent clinical signs during the acute phase (N = 914)

<table>
<thead>
<tr>
<th>Clinical sign</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosinophilia</td>
<td>86.3</td>
</tr>
<tr>
<td>Pulmonary oedema</td>
<td>72.4</td>
</tr>
<tr>
<td>Myalgia</td>
<td>71.5</td>
</tr>
<tr>
<td>Fever</td>
<td>43.9</td>
</tr>
<tr>
<td>Rash</td>
<td>41.5</td>
</tr>
</tbody>
</table>

Intermediate phase
Approximately 59% of these patients developed some of the clinical features of the intermediate phase (2 – 4 months after onset of TOS). This clinical stage was characterized by pulmonary hypertension (13), which was more common among children and young adults, by thromboembolic phenomena (14), and by oedema of the skin progressing to thickening and loss of flexibility, with or without whitish papular lesions interspersed with hyperpigmented areas, to give rise to what has been termed a guttate skin (15). Alopecia and severe myalgia were experienced, together with muscular weakness, hyporeflexia, alterations in superficial and deep sensibility, dysphagia, sicca syndrome, severe weight loss without apparent cause, arterial hypertension in young patients without other contributory factors, Raynaud’s phenomenon, and endocrinological disturbances such as hyperglycaemia, hypertrichosis and amenorrhoea (Table 4).
Table 4. Most prevalent clinical signs during the intermediate phase (N = 914)

<table>
<thead>
<tr>
<th>Clinical sign during month 2 after onset</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myalgia</td>
<td>48.8</td>
</tr>
<tr>
<td>Weight loss</td>
<td>36.3</td>
</tr>
<tr>
<td>Hepatopathy</td>
<td>20.7</td>
</tr>
<tr>
<td>Sicca syndrome</td>
<td>19.6</td>
</tr>
<tr>
<td>Skin oedema</td>
<td>13.6</td>
</tr>
<tr>
<td>Sensory neuropathy</td>
<td>9.6</td>
</tr>
<tr>
<td>Alopecia</td>
<td>9.5</td>
</tr>
<tr>
<td>Pulmonary hypertension</td>
<td>3.1</td>
</tr>
<tr>
<td>Thromboembolism</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Pancreatic enzyme changes were often detected, along with glucose intolerance. Autopsies carried out at this stage showed histological disorganization of the gland.

A number of patients developed slowly progressing dysphagia. Manometry revealed uncoordinated motility in the middle and lower regions of the oesophagus, apparently due to atrophy by denervation and/or fibrosis of the oesophageal wall (16, 17).

At this stage, deaths occurred with manifestations of mesenteric thrombosis and ischaemic colitis. Laboratory tests pointed frequently to hyperglycaemia, hypertriglyceridaemia, hypercholesterolaemia, hypalbuminaemia, thrombocytopenia (or thrombocytosis), unabated eosinophilia, and hepatic enzyme abnormalities with raised transaminases and cholestasis.

**Chronic phase**

The intermediate phase merged almost imperceptibly into the chronic phase between months 4 and 6 after the initial onset of the disease. Percentages of the most prevalent signs among the 914 patients selected for follow-up are given in Table 5. This phase seems to persist indefinitely in many patients, with increasing disability and sometimes death from the incapacitating complications. In others, however, the symptomatology has improved and physical ability has been more or less restored.
Table 5. Most prevalent clinical signs during the chronic phase (N = 914)

<table>
<thead>
<tr>
<th>Clinical sign</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral neuropathy</td>
<td>37</td>
</tr>
<tr>
<td>Hepatopathy</td>
<td>32</td>
</tr>
<tr>
<td>Scleroderma</td>
<td>22</td>
</tr>
<tr>
<td>Pulmonary hypertension</td>
<td>10</td>
</tr>
</tbody>
</table>

Moreover, the significant features of the chronic phase are pulmonary arterial hypertension (18), atrophy and induration of the skin of a sclerodermic nature (19, 20) and muscular wasting and weakness, with hyporeflexia or areflexia and some sensory neuropathy (21). Also seen is respiratory distress arising from neuromuscular deficit, together with limb deformity and muscle retraction (22). Muscle tremor, cramp and myotonia have also been described (23).

Some of the most severely afflicted patients have had a 90% reduction in lung function and required intubation, which has frequently led to complications from infection. At the same time, any eosinophilia that persisted through the intermediate phase gradually disappears in the chronic phase. Some cases have shown marked liver derangement, with chronic hepatitis, cholestasis or nodular regenerative hyperplasia (24). With involvement of the salivary glands the sicca syndrome appears (25), with ensuing dental caries (26). Other secondary features include osteoporosis (27), osteonecrosis (28), carpal tunnel syndrome (29), acro-osteolysis (30) and defective memory (31).

Treatment

None of the treatments tried (e.g. corticosteroids, azathioprine, d-penicillamine, plasmapheresis, vitamin E, superoxide dismutase, vasodilators, analgesics, anti-inflammatoryatories) produced any convincing effect in the control of the disease. Steroids administered during the acute and intermediate phases of the disease did not appear to prevent the chronic phase, although the general thought was that they reduced eosinophil levels, as well as producing an improvement in patients with pulmonary oedema. On the other hand, one well designed study (A. Luengo Dos Santos, unpublished observations, 1985) indicated control of the involuntary muscular movements by diphenylhydantoin (but not valproic acid) as long as the serum levels of the drug were maintained.
The only really effective relief to patients with severe neuromuscular involvement is physical rehabilitation (32).

**Current Status**

**Alimentary system**
While the biochemical parameters of hepatic function have, in general, slowly improved, cholestasis and portal hypertension can persist. Cirrhosis and nodular regenerative hyperplasia are present in some patients (33, 34). Complaints of, for example, pyrosis, dysphagia and intestinal rhythm disorders are probably due to the uncoordinated functioning of the alimentary tract musculature (35). Autopsy findings point to occasional lesions within the pancreas, while hyperglycaemia in some patients may be the result of islet cell damage.

**Cardiorespiratory system**
The interstitial fibrosis seen in most organs has not been found in the lungs. Nevertheless, pulmonary hypertension, with clinical and pathological characteristics similar to those of primary pulmonary hypertension, has been seen in the chronic phase. Some of these patients have exhibited a low carbon monoxide diffusion factor for a few years. Throughout the chronic phase, the incidence of this hypertension has been about 10%, but at present only about 1.2% of the patients are so affected (18). Complaints include palpitations, cardiac arrhythmias and dyspnoea on exertion, though their extent and importance are difficult to determine.

**Nervous system**
In the peripheral nervous system, the neuropathy that was secondary to vascular deprivation proceeded to complete denervation, with cramps and myalgias giving way to muscular atrophy and paresis. Correspondingly, involvement of the sensory nerves is evident as hyperaesthesia, hypoaesthesia and neuritic pain. During the chronic phase there had been some evidence of slow re-innervation in the form of cramps and myalgia (23). Severe disability in the form of headaches, cramps, myalgia, myoclonia, polyneuropathy and strokes nevertheless continues to affect some patients.

**Skeletal system**
In contrast to the acute phase, when arthritis of the large joints was occasionally observed, the chronic phase is characterized dramatically
by musculoskeletal deformities, contractures, arthralgia and spinal pain, upon which demineralization of the bones has become superimposed owing to immobilization (36, 37).

**Dermal system**
Symptoms attributable to the skin changes are mostly in remission, though atrophic areas are still found in the dermis.

**Mental aspects**
Not surprisingly, victims of this devastating disease are commonly beset with mental anxiety and depression, difficulties in adapting to their predicament and, understandably in some instances, compensation neurosis (38, 39). Other psychological symptoms are insomnia, somnolence and memory disorders. These could simply be an expression of the incapacitating physical disease, bearing in mind that organic changes in the central nervous system during the acute phase might be responsible (40).

**Conclusions**
After nine years the survivors are, for the most part, showing progressive clinical improvement. Nevertheless, new cases of pulmonary hypertension, chronic hepatitis and hyperglycaemia are still being diagnosed.

What is more uncertain is the possibility of long-term vascular, neoplastic and psychiatric effects. So far, no indications of excess stillbirths (41) or infantile deformities (42, 43) have been reported. Moreover, within the limitations of the inadequate statistics and the relatively short period of observation, no obviously elevated rate of cancer has yet been noted among these people.

Doubts of this kind necessitate an assiduous follow-up of as large a number of this patient cohort as possible to ascertain the nature of their fate, in addition to offering them the utmost care and assistance, medically and socially, to ameliorate their lot.

**References**
1. **Posada de la Paz, M. et al.** Late cases of toxic-oil syndrome: evidence that the aetiological agent persisted in oil stored for up to one year. *Food and chemical toxicology*, 8: 517–521 (1989).


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As of September 1990, 839 deaths of patients with TOS had been registered by the Spanish National Plan for the Toxic Syndrome (NPTS). The records indicate that an autopsy was performed in more than 60% of the cases.

This chapter is based on information from autopsy studies and from surgical specimens. The information on the autopsy studies came from three main sources. The first was the report of the Pathology Commission of the Ministry of Health (1) on 36 autopsies performed in hospitals in Madrid from 2 May to 20 July 1981, the period here called the first clinical phase, and analysed by a committee of pathologists. The second source was a study of 100 autopsies (2) nonrandomly selected by the Medical Committee of the Toxic Oil Syndrome. This study included 69 autopsies performed in hospitals in Madrid and 31 in hospitals in different provinces affected by TOS (Guadalajara 2, Segovia 5, Avila 2, Salamanca 7, Leon 11, Valladolid 3, and Soria 1) of 69 females and 31 males who died between May 1981 and 13 September 1985. The third source was a study of 39 autopsies performed in the Hospital Universitario 12 de Octubre in Madrid from 23 May 1981 to 7 August 1990. Twelve of these cases have been included in the report of the Pathology Commission and the study of 100 autopsies.
The main source of information on surgical specimens was the report of the Pathology Commission of the Ministry of Health and Consumer Affairs (3) on 648 biopsy and surgical studies performed up to 16 December 1981 in 11 hospitals in Madrid. Of these 648, the breakdown by site was as follows: lung 29, liver 93, skin 218, placenta 60, kidney 6, muscle 118, peripheral nerves 101, and minor salivary glands 23. In addition, several publications that reported on important material were consulted.

The Basic Lesion

Histopathologically, the distinctive feature of TOS is the vascular lesion that is found, with a segmental distribution, in all vessels, arteries, arterioles, capillaries, veins and venules (4). It takes the initial form of a vasculitis or, more specifically, an endovasculitis. The damage progresses from the intima more deeply into the walls of the vessels, without fibrinoid necrosis but with a mixed inflammatory infiltrate and subsequent fibrosis.

Several stages of changes occur. The first features an essentially intimal lesion, with cellular swelling, vacuolization of the endothelium (Fig. 1), subendothelial oedema, exposure of the subendothelial space and cellular necrosis. Some fibrin may also accumulate. Electron microscopy reveals marked cytoplasmic hydropic degeneration and destruction of the membranous organelles. This stage is succeeded by an inflammatory response, in which the affected part of the vessels becomes the site of a mixed infiltration of lymphocytes, histiocytes and, notably in many cases, eosinophils. The media may become involved as well (Fig. 2). Tests for antibodies by immunofluorescence techniques have been consistently negative (2, 4). The lymphocytes are T cells (2). In some cases xanthomatous histiocytes are conspicuous (Fig. 3). Prominent giant cell granulomas in the intima were observed in three cases (2).

Further evolution of this process leads to a fibroblastic proliferation with collagen formation, mainly at a subendothelial level (Fig. 4). Immunocytochemically, it consists of type IV collagen (5). Arteries tend to be affected more than veins, with partial or complete luminal occlusion (Fig. 5) (4, 6). In the final stage, thromboembolic complications result from distortion and narrowing of the vessels (4, 6). As a consequence of this vascular deprivation, the parenchyma of the organs may then be adversely affected.
Fig. 1. Lung, early clinical phase. Endothelial lesion of an artery, characterized by cytoplasmic swelling and vacuolization (haematoxylin–eosin).

Fig. 2. Lung, second clinical phase. Inflammatory infiltrate (lymphocytes and histiocytes) and oedema of the subendothelial space producing a picture of endovasculitis (haematoxylin–eosin).
Fig. 3. Lung, early clinical phase. Vascular lesion showing endothelial changes, foamy macrophages and lymphocytic infiltration of the arterial wall (haematoxylin–eosin).

Fig. 4. Lung, second clinical phase. Medium-sized artery with intimal thickening due to fibroblastic myxoid proliferation (haematoxylin–eosin).
Organ Damage

Lungs
In the first clinical phase the organ predominantly affected was the lung. Thousands of individuals suddenly developed an acute *Mycoplasma*-like pneumonitis (7, 8). The characteristic vascular reactions were diffuse, accompanied by septal oedema, lymphangiectasis, some interstitial infiltration with lymphocytes and monocytes (and, to a lesser degree, with eosinophils) and shedding of desquamated type II pneumocytes and macrophages. In more severely affected people, the alveoli showed conspicuous desquamated pneumocytes or macrophages within the lumina and hyalin in this membrane. Some patients died from acute respiratory failure due to diffuse alveolar damage (1, 4, 6).

Thereafter, some patients developed thromboembolic complications. A few died from pulmonary thromboembolism, a complication that appeared between mid-June and the end of July 1981 (4, 6). About 20% of those admitted to hospital at that time presented with pulmonary hypertension (9–11).
Most of the patients with pulmonary hypertension improved. In 1983, two years after the onset of TOS, the incidence of pulmonary hypertension was 3.5%; by 1985 it was 1.5% (12) and is now even lower. Nevertheless, some patients remain in which pulmonary hypertension has shown a deteriorating course, with an estimated incidence of 1.6 per thousand, leading rapidly to death from right ventricular hypertrophy and cardiac failure. The lungs of these people did not show fibrosis but had the following characteristics (9, 12, 13):

- lesions of thromboembolic hypertension, consisting of thrombi of different ages and abnormal myointimal cellular proliferation, occlusion and recanalization (Fig. 6);

- lesions of primary pulmonary plexogenic arteriopathy, consisting of plexiform lesions and concentric intimal cellular proliferation and fibrosis (Fig. 7); and

- pulmonary veno-occlusive lesions, characterized by intimal fibrosis with formation of frequent intraluminal septae.

Fig. 6. Lung. Late pulmonary lesion in a patient with pulmonary hypertension. Muscular artery with fibrous septa, result of recanalization of an old thrombus (haematoxylin–eosin).
Skin

In the first clinical phase, rashes were morbilliform, scarlatiniform and generalized. Erythema was intense on the face of children, with purpuric macules that coalesced around the mouth. More prominently, generalized purpuric lesions were seen in the flexure creases of the wrist and palm. Erythema multiforme-like lesions were less common on the knees and on the back of the hands (14).

Histologically, the skin lesions of the first clinical phase showed irregular hyperplasia of the epidermis with some elongation of the rete ridges; the papillary dermis was oedematous and superficial, and deep perivascular inflammatory infiltrates were present (Fig. 8). The main pathological changes were found in the blood vessels of the reticular dermis: swollen endothelial cells, haemorrhage, and lymphohistiocytic infiltrates sparse or dense, with a great number of intravascular eosinophils and large mononuclear hyperchromatic cells, that were difficult to classify but could belong to the monocyte or large lymphocyte series. The nerve fibres of the deep dermis were involved partially by inflammatory infiltrates rich in eosinophils (4, 6, 15).
The skin lesions of the second clinical phase were characterized by hard papules and by a sclerodermiform syndrome (14). The papules, 1–3 mm in diameter and deeply infiltrative, were few in number at first but multiplied later. They were present over the entire cutaneous surface except on the palms and soles, and were whitish, pale or dark in colour. Some patients developed sclerodermiform lesions while papules were still present, but no papules were seen in any patient six months later. The sclerodermiform syndrome started with slightly indurated oedematous areas, which became progressively harder. These areas appeared at any site and either remained localized or became generalized. The skin colour was variable, but often dirty brown. The skin lesions were similar in some cases to a localized myxoedema of the lower legs or a patch of morphoea, and in others to an eosinophilic fasciitis on the trunk or to generalized morphoea. The most severe cases resembled systemic scleroderma, with generalized skin sclerosis on the arms and legs, oedematous hardened fingers, sclerodactily and muscle atrophy of the hands. The generalized skin sclerosis was associated with neuromuscular symptoms, and oesophageal involvement with dysphagia resembling scleroderma, but without Raynaud’s syndrome (14). Other authors, however, have reported Raynaud’s syndrome (2, 8).
Histologically, the skin lesions differed progressively from the changes found in the first clinical phase of the disease (4, 15). The epidermis showed slight hyperpigmentation and some exocytosis of the dermal inflammatory cells. The papillary dermis was not oedematous and the endothelial lesion of the papillary vessels, as well as the lymphohistiocytic perivascular infiltrate, were less intensive. On the other hand, fibrotic changes appeared with hyalinization of the dermal papillae. In more advanced lesions, inflammatory cells with lymphangiectasis were absent.

The vascular changes of the dermis persisted in the deep plexus and in the superficial vessels of the panniculus. The muscular vessels showed thickened walls infiltrated by lymphocytes and polymuclear leucocytes. No perivascular inflammation, fibrinoid necrosis, leucocytoclasia or haemorrhage was observed.

In older lesions three types of change were observed.

- Diffuse inflammatory infiltrates were seen, consisting of lymphocytes and hyperchromatic mononuclear cells occasionally mixed with neutrophils and/or eosinophils. In some cases, myofibroblasts were also observed, in three instances cells containing intracytoplasmic eosinophilic inclusion bodies similar to those found in infantile digital fibromatosis (16).

- Nodular deposits of mucin were also seen. These were usually associated with the inflammatory infiltrate, located in mid-dermis between the collagen bundles, which were intensely eosinophilic (4, 15, 17) (Fig. 9).

- Sclerosis was the last change that appeared (4, 15, 17). Intensive eosinophilic broad collagen bundles were present and the panniculus around the secretory coil of the sweat glands disappeared. The hair follicles and sebaceous glands were atrophic, leaving only the arrector pilorum in the sclerotic dermis (Fig. 10). In serial biopsy studies, the inflammatory and mucinous changes reached a peak by November 1981, diminishing thereafter as the sclerotic changes became more important and predominant.

**Neuromuscular system**

The motor involvement included weakness and wasting of skeletal muscles, predominantly of the four limbs, leading to paresis or paralysis according to the degree of muscular atrophy (8, 18). The motor signs were more pronounced than those of the sensory system. However, the
Fig. 9. Skin, second clinical phase. Mucinosis in the dermis (haematoxylin–eosin).

Fig. 10. Skin, second clinical phase. Panoramic view showing marked dermo–hypodermal fibrosis with disappearance of the skin adnexa (haematoxylin–eosin).
upper and lower extremities showed sensory deficits in regard to pain, changes of position and deep vibration. One third of the patients had a peripheral distal distribution of neuromuscular signs and symptoms that was consistent with a diffuse sensory–motor polyneuropathy. In other patients, the nerve involvement was more localized to cutaneous nerves in the form of mononeuropathies. Other manifestations, such as joint fixations or deformities, added to the motor disability, with the joints of the feet, hands and elbows most affected.

The material on which the following descriptions are based consists of 31 muscle biopsies taken during the first weeks or months of neuromuscular symptoms, 73 sural nerve biopsies obtained during the first two years of TOS, and 30 autopsies of patients dying of TOS in Hospital Universitario 12 de Octubre in Madrid over five years (1981–1985). Complete neuropathological examination of the central nervous system was performed in 30 autopsies. Peripheral nerves and samples of skeletal muscle from 20 autopsies were studied.

Muscle pathology
The main pathological findings in muscle were inflammatory cell infiltration, muscle atrophy and endomysial fibrosis (4, 19). The inflammatory cell infiltration was evident in the first group of biopsies performed and reached a maximum between weeks 3 and 15 after onset of TOS. The cell infiltrates consisted mainly of mononuclear cells with occasional neutrophils. Eosinophils could be found in the vicinity of blood vessels. The inflammatory cell infiltrates were diffuse in the epimysium and perimysium (Fig. 11) and were rarely found in the endomysium. The capsules of muscle spindles and coverings of small nerve twigs in the muscle were very frequently infiltrated. Vasculitis with infiltration of the vessel wall was present in 35% of the cases after week 7 following onset of the disease. The vasculitis involved mainly the veins and capillaries, and became more apparent in the chronic cases (after week 12) when the interstitial inflammation had nearly disappeared.

In addition to the inflammatory myopathy described above, all patients showed initial neurogenic atrophy. The affected fibres were type II and presented inversion of the reaction to oxidative enzymes. The degree of neurogenic atrophy increased intensely with time and was very clear at 18 months after onset of TOS. Its effect on muscle fascicles varied (Fig. 12). At this chronic stage, endomysial fibrosis was marked. Fascicles of fibrous tissue in an eosinophilic matrix were seen between the atrophic fibres.
Fig. 11. Cross-section of muscle showing inflammatory cell infiltration of the perimysium and of a muscle spindle (haematoxylin–eosin).

Fig. 12. Severe denervation atrophy in skeletal muscle. One fascicle in the right lower corner is totally atrophic. Other fascicles show groups of atrophic fibres among normal fibres (haematoxylin–eosin).
At autopsy, muscles had the characteristic lesions described in biopsies; the inflammatory cell infiltration was moderate to severe in perimysial veins and capillaries. All cases showed severe neurogenic atrophy affecting whole fascicles and with severe endomysial fibrosis. Distal muscles were more affected than those of the upper extremities. Intercostal muscles and diaphragm muscles were affected moderately.

Peripheral nervous system
The earliest and most frequent findings (93% of cases) were epineurial inflammatory cell infiltrates around veins and capillaries (4, 18). A large number of cases (60%) showed mononuclear cell infiltration of the perineurium, often starting focally and later extending to the whole circumference. Within the same nerve trunk, each nerve fascicle was involved to a different degree. Very rarely was an endoneurial capillary involved. In addition to perineuritis, perineurial fibrosis was seen in 40% of the cases (Fig. 13). The fibroblastic proliferation of the perineurium varied in intensity from one fascicle to another. In chronic cases, severe perineurial fibrosis was present without perineurial inflammatory infiltrates. In 15% of the cases, the epineurial artery was semi-obliterated owing to subendothelial fibrosis and endothelial swelling.

Fig. 13. Second clinical phase. Transverse section of sural nerve with inflammatory cell infiltration and fibrosis of the perineurium (haematoxylin–eosin).
The nerve fibres showed abnormalities in all cases. Degeneration of myelinated fibres was present in isolated nerve fibre preparations and in semi-thin sections of Epon-embedded nerves. The myelin sheathes appeared homogenized, with dense axoplasm. Degenerating fibres were frequently distributed under affected areas of the perineurium. Unmyelinated fibres were relatively preserved. Endoneurial macrophages were present in many samples.

At autopsy, abnormalities in both sensory and motor nerves were seen with the most severe damage in nerves in the distal portions of the lower extremities. The damage varied in intensity from one case to another. The histopathological changes were similar to those described previously in the biopsies. They consisted of inflammatory cell infiltrates around capillaries and veins of the epineurium and endoneurium. Focal and diffuse perineuritis was associated in some cases with severe perineurial fibrosis. Parenchymal lesions of the nerve with fibre degeneration were always present.

Central nervous system

The only important abnormality was the presence of enlarged, often vacuolated neurons exhibiting central chromatolysis (20). Of TOS-related deaths, 90% of patients with the neuromuscular syndrome had prominent chromatolytic neurons in the anterior horns of the spinal cord. In addition to spinal motor neurons, the same abnormality was present in symmetrical groups of neurons of the brain stem (reticular substance, cuneate and gracile nuclei, grey nuclei of the basic pons, loculus coeruleus and, occasionally, the trigeminal and vestibular nuclei).

Central chromatolysis in patients who died from TOS was studied by electron microscopy, morphometry and immunocytochemistry. The conclusion from this work is that central chromatolysis in TOS is probably a primary toxic neuronal response, and is different from axonal response or nutritional deficiencies. A “dying-back” component to distal degeneration of the nerve fibres played a role in TOS neuropathy.

Liver

The study of 842 patients with TOS admitted to Hospital Universitario 12 de Octubre in Madrid between 1 May 1981 and 31 December 1982 (3, 21, 22) provides a comprehensive overview of the frequency and anatomopathological features of the hepatopathy of TOS. The prevalence of the hepatopathy was about 5000 cases among the approximately 20 000 people affected by TOS.
The hepatopathy had the following characteristics: non-age specific, but with a higher incidence in patients between 30 and 40 years of age (median 42 years); a female:male ratio of 1.5:1; barely symptomatic; no parallelism between the general clinical picture and the degree of hepatopathy; and a good correlation between the biochemical and clinical findings.

In the study of 842 patients a liver biopsy was performed on 75 patients. In eight of them a second biopsy was performed. Furthermore, a careful anatomopathological study of the liver was carried out during 23 autopsies.

Histologically, the liver presented portal and lobular lesions. The portal areas showed inflammatory infiltrates of variable intensity by lymphocytes, histiocytes, polynuclear leucocytes and eosinophils. The ductal epithelium showed nuclear stratification, cytoplasmic vacuolization and inflammatory exocytosis (Fig. 14). Furthermore, vasculitis of the portal vein and endothelial lesions of the portal arteries were detected.

The lobular lesions consisted mainly of eosinophilic degeneration of scattered hepatocytes and diffuse mixed inflammatory infiltrates of

Fig. 14. Liver, second clinical phase. Portal area with lymphohistiocytic inflammatory infiltrates and lesions of the epithelial cells of the ductal epithelium (haematoxylin–eosin).
variable intensity. In many patients, conspicuous mitotic figures of hepatocytes and disperse eosinophils were observed. In addition, slight or more intense cholestasis was common. Overall, the histological picture in the liver was heterogeneous, with microscopic cholestasis with very little inflammatory and degenerative changes on the one hand and, on the other, major inflammatory and degenerative features with little or no biliary pigment.

The vascular changes observed in the liver showed similar features to those observed in other organs. In one autopsy study of 100 cases (2), vascular changes were found in 42%. The predominant lesion was slight intimal fibrosis of the central lobular veins, with no consequences on the surrounding liver parenchyma. Nevertheless, veno-occlusive disease in an acute phase (with almost complete obliteration of branches of the central lobular veins by scleroderma) was observed in four patients who died between September 1981 and February 1982 (2, 23).

Furthermore, intracytoplasmic PAS-positive inclusions were observed in 7 of the 100 autopsies (2). Ultrastructurally, they corresponded to round vesicles of the endoplasmic reticulum, and immunocytochemical analysis showed that they contained IgG, IgA and fibrinogen. These changes have been related to the vascular lesion and secondary increased permeability of the vascular walls.

Taking into account the clinical, biochemical and morphological features of the hepatopathy in TOS, the hepatopathy can be defined as a “toxic cholestatic hepatitis” (21) that has undergone spontaneous clinical remission in most cases. Nevertheless, patients with no clinical symptoms still show nonspecific histological changes in late repeated liver biopsies.

Patients with long-standing liver disease presented several pathological features. In a study of 124 patients followed from 1981 to 1986, Solis et al. (22, 24) found the following pathological changes: toxic cholestatic hepatitis in 14 and chronic active hepatitis in 13; nonalcoholic liver cirrhosis in four, one of which presented features similar to those of primary biliary cirrhosis (a similar case has also been reported by Faro Leal et al. (25)); nonalcoholic steatohepatitis in eight alcoholics and 11 nonalcoholics; hepatocellular adenoma measuring 0.5 cm in one; and diffuse nodular regenerative hyperplasia (DNRH) (Fig. 15) in eight. All these late liver lesions are scarce and are most probably coincidental, with the exception of the DNRH of the liver (25–27).

In one study (26), 24 cases of DNRH were seen over a period of 9 years with a prevalence of 3.1 per 100,000 and an incidence of
0.34 per 100,000. In 15 cases (62.5%) previous intake of drugs or exposure to possibly hepatotoxic substances was found. Seven of these patients had TOPO, indicating that the causal agent of this syndrome might also be responsible for the hyperplastic nodular growth (26). The lesions of endovasculitis may have impaired the liver blood flow, resulting in hepatocellular damage in central lobular areas and reactive regenerative proliferation of hepatocytes in paraportal areas.

**Pancreas**

This organ was also affected in many cases. Aguilera Tapia (2) studied the pancreases in 83 of 100 autopsies. Chronic pancreatitis was observed in 18 cases (5 men and 13 women). The most severe lesions were observed in patients who died between August 1981 and August 1982. Taking into account the number of patients who died during this period, 33% of the pancreases showed the histological picture of chronic pancreatitis. Previous to this period, isolated cases of acute pancreatitis had been reported (4, 6).

The cases with chronic pancreatitis were characterized histologically by an intensive fibrosis. In some cases the fibrosis showed a diffuse pattern and in other cases an irregular or focal one. The fibrosis
was associated with atrophy of the acini. In a few cases the fibrosis and atrophy of the exocrine parenchyma was so severe that only the islets of Langerhans of the endocrine pancreas were present. The fibrosis and atrophy were associated with diffuse or multifocal lymphoplasmocytic inflammatory infiltrates. In some cases the inflammatory infiltrates were predominantly periductal and perivascular, not affecting much or at all the exocrine parenchyma that showed fibrosis and atrophy. Immunocytochemical examination showed that the inflammatory infiltrates were built up by T cells (2). These 18 cases showed severe vascular lesions. The vascular lesions were more frequent and more intensive in the arteries than in the veins. As in other organs, the vascular lesion was predominantly intimal, showing an intensive obliterating fibrosis that frequently occluded completely the vascular lumen. A conspicuous subintimal xanthomatosis associated with T cells was observed in 13 cases. Usually the media of the arteries was not affected. However, the inflammatory infiltration affected the entire thickness of the walls of the pancreatic veins.

In 14 additional cases, less intensive vascular lesions were observed and no chronic pancreatitis was present. Furthermore, three cases showed amyloidosis of the islets of Langerhans, and four other cases vascular lesions consisting of cavities in the islets of Langerhans, filled with blood. These cavities were not delimited by an endothelium, since the cells were immunocytochemically negative for factor VIII-associated antigen, but by endocrine cells that were positive for insulin after immunocytochemical reactions. These lesions affected 10% of the islets of Langerhans in two cases; in the other two the lesion was less frequent.

In this autopsy series (2), seven of the patients had developed hyperglycaemia after onset of TOS and seven had presented with sicca syndrome (mainly oral dryness and dysphagia).

Salivary glands
Biopsies of 11 patients who developed sicca syndrome (28) showed dilatation of the intercalary ducts and progressive acinar atrophy; in two specimens, epidermoid metaplasia associated with inflammatory infiltrates of lymphocytes, plasmacytes, histiocytes and mast cells was observed. No vascular lesions were detected. The intimal alteration was considered a lymphoepithelial lesion of T cells (UCHL 1 positive), which showed ultrastructurally digitiform projections directed towards the junctions of the ductal and myoepithelial cells.
A total of 21 salivary glands were histologically investigated in a study of 100 autopsies (2). One case from the acute phase showed only minimal inflammatory infiltration. Of 20 cases from the intermediate phase, two showed normal glands but 18 showed fibrosis and atrophy of the glandular parenchyma of varying intensity, being very severe in seven cases, a feature that has also been reported by others (4, 6). They showed lymphoplasmocytic inflammatory infiltrate, in isolated cases associated with mast cells or eosinophils, of minimal or moderate intensity. The vascular lesion in the salivary glands was always minimal or absent.

**Digestive tract**
The most prevalent finding has been mesenteric thrombosis (1, 4, 6), which appeared by the end of the acute phase followed by ischaemic enterocolitis at the beginning of the intermediate phase (16 August–27 October 1981). Furthermore mixed inflammatory infiltrates, sometimes rich in eosinophils, and vascular lesions of variable intensity were present. The ischaemic enterocolitis was probably a consequence of the obliterating vascular lesion.

**Heart**
The hearts of 10 patients showed non-infective thrombotic endocarditis (NITE) (2, 4, 6). The endocardium presented lesions with features similar to those of the endothelial lesion in the vessels. NITE affected the right chambers of the heart in five cases. Furthermore, myocarditis was observed in an important number of cases (2, 4, 6). The inflammatory infiltrates were predominantly lymphocytic and occasionally of the mixed type; in a few cases eosinophilic leucocytes were conspicuous.

A most striking finding has been myocardial infarct in a few young patients with TOS (2) due to an almost complete obliteration of the coronary arteries by a tremendous thickening of the intima because of fibrotic proliferation (Fig. 15). These lesions are similar to the advanced lesions of atherosclerosis.

Many patients with TOS developed a sclerodermiform picture (8). Because scleroderma is associated with cardiac disorders, the hearts of eight victims who died from TOS were studied systematically and completely (29). Dense fibrosis of the sinus node in two hearts resembled changes found in scleroderma. Atrionodal junctional haemorrhage and cystic degeneration of the sinus node in the other six hearts resembled changes found in lupus erythematosus. Small and
large branches of the coronary arteries exhibited typical lesions of TOS, consisting of thickening of the intima followed by proliferation of myointimal cells, oedema and fibrosis. These lesions were associated with a sloughing off of the inner wall and embolization of the detached fragment downstream in the same coronary artery. Every heart had many degenerative lesions within the nerves, ganglia and the coronary chemoreceptors. Both arterial and neural abnormalities prominently involved the conduction system.

Kidneys
Interstitial inflammatory infiltrates of the mixed type were commonly observed in the autopsies (1, 4, 6) and in some cases eosinophils were conspicuous. Vascular lesions (mainly endovasculitis) were also common, but the most significant vascular lesion in this organ was intimal fibrosis of the interlobular and arcuate arteries. Furthermore, fresh and old thromboemboli were detected. However, no clinical manifestations have been reported in TOS patients with the exception of six cases of glomerulonephritis (30). In these cases there was most probably no relationship between the glomerular lesions and TOS.

Lymphatic system
In the first clinical phase, lymph nodes showed a reactive lymphadenitis with dilatation of the sinuses in 66% of 36 autopsies (1), erythrophagocytosis in 3 cases, and cortical microinfarcts caused by venous vascular lesions in another 3 cases. Autopsy studies performed later did not find any pathology.

No pathological findings related to TOS have been reported in the spleen (1, 2, 4, 6).

In the thymus, only one autopsy revealed diffuse and intensive lesions, consisting of severe fibrosis, xanthomatosis and lymphocytic infiltration of the intima of blood vessels (2).

Other sites
Only minimal vascular lesions of the bone marrow, in isolated cases, have been reported (2), and no bone lesions have been reported. Osteoporosis has been mentioned in an unspecified number of cases (2).

In a few cases, severe interstitial fibrosis associated with atrophy of the lobules and ducts of the breast has been reported in women aged 18–38 years (2).

No conspicuous vascular lesions were observed in the endocrine system and the male and female genital organs (1, 2, 4, 6).
The placenta of mothers with TOS showed no pathological changes, except one which showed intensive inflammatory infiltration by eosinophils (a patient sent for consultation to F.J. Martínez-Tello).

Pathogenesis

As soon as a relationship between the anilides found in the oil and TOS was established (8), speculation was raised that they were responsible for the lesion of the endothelium. However, although Kilbourne et al. (31) demonstrated an evident dose–response relationship, with increasing concentrations of aniline and anilides associated with increasing risk of TOS, no one has been able to reproduce in experimental animals the lesions found in TOS. In fact, the toxic reactions produced by those substances are quite different from those seen in TOS (32). It could be speculated that TOS was caused by free radicals, since these have been implicated in similar lesions such as those produced by radiation or nitrofurantoin (33, 34), or after ingestion of diets rich in polyunsaturated fatty acids (35). The release of free peroxide radicals induced by oleoyl anilides could act on different cell membranes and provoke structural and enzymatic alterations leading to endothelial damage.

In our opinion, the primary vascular lesion caused increased capillary permeability that, together with the diffuse alveolar damage, was responsible for the respiratory illness and pulmonary X-ray features observed in the early phase of the disease.

The observed endothelial lesion could also have induced the thrombosis found in many cases in different vessels and organs, mainly during a certain period of the evolution of the disease. On the other hand, protective enzymes in lung endothelial cells, such as carboxypeptidase N, are able to interact with substances circulating within the pulmonary system, playing an important role at the experimental level in the acute respiratory distress syndrome (ARDS) (36). The toxic substances in TOS patients could have reached the pulmonary circulation and triggered this mechanism, since many patients with TOS in the acute phase showed features of ARDS. Thereafter, the thrombotic phenomena could be due to the interaction between vessel endothelium and circulating clotting factors, and would contribute to perpetuating the vessel injury in the late stages of the disease. Lesions similar to those of TOS have been seen in radiation vasculitis (34, 37). Another possible
explanation could be the action of free radicals by direct activation of thrombocytes (38). The thromboembolic complications could have been caused by a disseminated intravascular coagulation, detected in a number of patients.

Certain aspects of TOS suggest that immunological mechanisms could be involved in the pathogenesis of some lesions, such as the abundance of lymphocytes and/or eosinophils in the inflammatory infiltrates. However, no evidence for humoral immunomechanisms has been found: vascular or tissual immunocomplexes have not been observed and, with the exception of two instances (a personal case and one of Aguilera (2)), no fibrinoid necrosis has been observed in the vascular lesions. Nevertheless, in the chronic phase, TOS shows striking clinical and pathological similarities to diseases in which immunopathological mechanisms are well known to operate, such as progressive systemic sclerosis, Sjögren’s syndrome, rejected organs and graft-versus-host disease (28, 37, 39–42). That only a minority of patients showed a progression of the vascular lesion points to one or more predisposing factors also involved in the pathogenesis whose mechanisms, such as histocompatibility antigens, are not yet clear. The lymphocytic infiltrates in lesions of TOS in different organs have been demonstrated to be T cells, which also indicates that a cell-mediated immunopathogenic mechanism could be implicated.

Recently the eosinophil–myalgia syndrome (EMS), associated with the ingestion of L-tryptophan, has been described. It is similar to TOS in some clinical and pathological features (43, 44). However, as in TOS, the pathogenesis of EMS is not known at present.

Conclusions

TOS is a new multisystemic disease whose etiology and pathogenesis remain unknown. The basic lesion is a peculiar non-necrotizing vasculitis that affects mainly the intima (an “endovasculitis”) and involves vessels of every type and size, in nearly every organ. The endothelial injury is considered the first lesion. This lesion was associated in many cases with thrombotic phenomena, which also contributed to perpetuating the vascular lesion and ischaemia and/or the parenchymal atrophy of several organs. The initial lesions of the lung corresponded to ARDS, death in this phase being due to respiratory failure. Death late in the first clinical phase was due mainly to thromboembolic complications.
In the second clinical phase, some patients presented a neuromuscular syndrome due to an inflammatory neuropathy with a lymphocytic perineuritis that led to perineural fibrosis with secondary axonal degeneration. The muscle presented an interstitial inflammatory myopathy, followed by a neurogenic muscular atrophy. Many patients showed a sclerodermiform clinical picture. The skin lesions consisted of dermal and subdermal fibrosclerosis, with vasculitis of the small arteries in the lower dermis. A few young patients had myocardial infarctions because of coronary lesions; scleroderma-like lesions were also observed in the heart. A minority of patients with long-standing disease developed pulmonary hypertension due to a plexogenic pulmonary arteriopathy that led to death because of right ventricle heart failure. About one quarter of the affected population had hepatopathy defined as a toxic cholestatic hepatitis. Most such patients showed a favourable clinical course, but a few developed DNRH. In addition, in a certain period of the second phase, 33% of the patients died with chronic pancreatitis. Finally, a few patients in the second clinical phase developed sicca syndrome. The salivary gland of these patients showed features compatible with those of Sjögren’s syndrome.

In our opinion, the first step in this process is the endothelial lesion, probably a consequence of direct aggression by the toxic substances and/or of other pathogenic mechanisms such as free radicals. However, since a small number of patients showed progression of the vascular lesion and of the disease, it is reasonable to think that other individual predisposing factors could have played an important role, such as the immunopathological mechanisms of delayed hypersensitivity.

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Experimental studies

W.N. Aldridge

The outbreak of TOS was of such magnitude and explosiveness that the authorities were overwhelmed by the medical emergencies, and the aspects requiring epidemiological and toxicological expertise had to take second place. The initial disease presented as a respiratory illness with, as is now clear, a short delay between the consumption of the oil and the appearance of the symptoms. In any episode of poisoning in these circumstances, obtaining case-related oil samples will obviously be difficult and especially in Spain where the consumption of oil in the average family is very high. Initially, the oil samples were collected by individual scientists and then officially in large numbers, labelled by source, after public announcement that the toxin was in the oil. Whatever the technical merit of a particular toxicological investigation, the unknown authenticity of the oils has nearly always thrown doubt on the relevance of the results.

In 1983 a WHO meeting recommended, based on the evidence available, that oils tested for toxicity and/or subjected to detailed analysis should contain at least 700 μg/g of fatty acid anilides (1), equivalent to 2 μmol/g of oleoyl anilide. Since June 1981, when a very large collection of oils had been amassed, the validity of the ad hoc decision that fatty acid anilide content could be used as a marker of case oils has
been reinvestigated. The total number of aniline derivatives in these oils is still unknown, though others in addition to the anilides have been found in some samples of oil (see Chapter 5).

Chemical constituents unconnected with aniline have been postulated to have caused the outbreak — notably paraquat and the organophosphorus pesticides fenamiphos and isofenphos. However, the oil hypothesis, which may now be regarded as confirmed (see below and Chapter 1) has no place for these substances on epidemiological, clinical, analytical or toxicological grounds.

The validity of the oil hypothesis (i.e. the toxin being consumed in a purported edible oil originating from rapeseed oil denatured for industrial use by the addition of aniline) has now received convincing support from a series of epidemiological studies (2–4). A particularly important investigation (5) has combined a very careful re-examination of case records, selection of the stored oils and their chemical analysis. Extracts of results published in this paper are given in Table 6. A comparison of case and control oils shows that constituents expected to be found in rapeseed oil were at higher concentrations in the case oils and those expected to be lower were lower. The rapeseed oil was correlated with the presence of aniline and fatty acid anilides of rapeseed oil. Although case and control oils cannot be absolutely differentiated by their anilide content, the probability of an oil containing 600 µg/g (1.7 µmol/g) oleoyl anilide and not being a case oil in this series is very small. In the series studied, 3 out of 64 control and 13 out of 29 case oils contained such concentrations. Preliminary results from a current examination of case and control oils collected mainly in another district provide similar results (M. Posada & A. Abaitua, unpublished observations, 1989). Therefore, the oil hypothesis is very firm and case oils can now be selected with a high probability of success by their high content of fatty acid anilides. Such oils may be used for further study, recognizing that they are more than eight years old; mixing together oils with high anilide content should minimize possible error from an assumption that every oil with a high anilide content is a case oil.

The approach used in this chapter to assess present and future experimental studies is summarized in Fig. 16. This illustrates the connections between various past, present and future studies. In addition to the sections dealing with case oils and their known constituents tested by in vivo and in vitro methods, an additional section covers the biological activity of postulated constituents.
Table 6. Analysis of case and control oils

<table>
<thead>
<tr>
<th>Component</th>
<th>Case (N = 29)</th>
<th>Control (N = 64)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg/g)</td>
<td>(mg/g)</td>
</tr>
<tr>
<td>1. Brassicasterol</td>
<td>0.17 (0.00 - 0.55)</td>
<td>0.00 (0.00 - 0.40)</td>
</tr>
<tr>
<td>2. Campesterol</td>
<td>0.66 (0.15 - 1.77)</td>
<td>0.32 (0.19 - 1.42)</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>389 (255 - 511)</td>
<td>285 (214 - 448)</td>
</tr>
<tr>
<td>cis-Vaccenic acid (18:1)</td>
<td>20.8 (8.8 - 326)</td>
<td>11.8 (7.2 - 29.4)</td>
</tr>
<tr>
<td>Gondoic acid (20:1)</td>
<td>8.4 (1.4 - 13.2)</td>
<td>2.7 (0.6 - 10.7)</td>
</tr>
<tr>
<td>3. Stigmasterol</td>
<td>0.13 (0.00 - 0.24)</td>
<td>0.19 (0.06 - 0.29)</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>79 (66 - 116)</td>
<td>95 (70 - 133)</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>34.6 (20.1 - 45.7)</td>
<td>37.0 (27.0 - 45.4)</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>215 (145 - 422)</td>
<td>406 (140 - 510)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>(µmol/g)</th>
<th>(µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4. Oleoyl anilide</td>
<td>1.01 (0.00 - 3.49)</td>
<td>0.00 (0.00 - 0.60)</td>
</tr>
<tr>
<td>Linoleyl anilide</td>
<td>0.24 (0.00 - 1.12)</td>
<td>0.00 (0.00 - 0.22)</td>
</tr>
<tr>
<td>Palmitoyl anilide</td>
<td>0.12 (0.00 - 0.35)</td>
<td>0.00 (0.00 - 0.06)</td>
</tr>
<tr>
<td>Aniline</td>
<td>0.0024 (0.000 - 0.0064)</td>
<td>0.0000 (0.0000 - 0.0017)</td>
</tr>
<tr>
<td>Total aniline equivalent</td>
<td>1.38</td>
<td>0.00</td>
</tr>
<tr>
<td>Aniline added to denatured rapeseed</td>
<td>236</td>
<td>–</td>
</tr>
</tbody>
</table>

The case and control oils were selected using epidemiological criteria. For details see Kilbourne (5); the analyses are also taken from this source.

1 Constituent specific for rapeseed oil; 2 constituents at higher concentrations in rapeseed oil; 3 constituents at lower concentrations in rapeseed oil.

Results are shown in median (10th - 90th percentile).

Relationship between Clinico-epidemiological and Experimental Studies

The acute respiratory phase in humans is the most likely sign of poisoning to be seen in experimental studies to test the toxicity of case oils. The episode described by Díaz de Rojas (2) provides perhaps the most convincing evidence that, provided the dose of oil ingested was sufficient, the attack rate for dyspnoea and pneumonia was high. In this
episode 23 novices, two nuns, a priest and a superior went on a spiritual retreat, taking with them "olive oil" in 5-litre plastic containers. Very soon after the first consumption of the oil some of the novices did not feel well, and within eight days some had developed dyspnoea. In all, 22 out of 25 (88%) of the nuns and novices developed clinical signs characteristic of early TOS. Thus, a reasonable assumption is that this chemically induced lung disease might be reproducible in some experimental animals, even if large doses may be required.
The other early clinical finding in the majority of cases was a greatly raised peripheral eosinophilia (6–10). In one study (8) all patients had either an abnormal X-ray on admission (95%) or an absolute eosinophil count (automated white cell count and manual differential) of over 500 cells/mm³ at some point during the illness; the eosinophil count exceeded 1500 cells/mm³ for 69% of the patients. A raised circulating eosinophilia is associated with many immunological phenomena but is by no means conclusive evidence that an immune mechanism is involved (11). Nevertheless, as a routine, eosinophil counts should be regularly determined in experimental research to try to establish the etiology of TOS.

Although concentrating on the acute signs of TOS in long-term studies may seem sensible, signs characteristic of the chronic stage might also appear. Current knowledge assumes that the disease is produced by one chemical substance rather than by several, and also that it should be possible to produce the whole disease process in any experimental animal. Even for humans, chronic signs of the disease have still not been inextricably linked to an early acute respiratory illness. One report describes a late case in a patient who presented with the chronic phase signs and symptoms in June 1982 after consuming oil stored at home since the outbreak in April–June 1981; no respiratory symptoms were reported (12). This report indicates that a constituent of the oil capable of causing some aspects of the intermediate and chronic phases of the disease had persisted for one year.

**Toxicity Studies of Case-related Oils in Experimental Animals**

Table 7 summarizes all toxicity studies found in a search of reports and in the literature. With so few positive data, most of these reports are unpublished. Two papers (13, 15) report lung toxicity in rats. The former short paper (13) claims not only that “case oils” containing 2 ppm aniline and 1500 ppm anilides cause lung toxicity, but that a mixture of oleoyl and linoleoyl anilides (1500 ppm) each in olive oil is also effective. No confirmatory full paper from this laboratory has appeared. The more extensive paper (15) claims that “case oil” caused respiratory difficulties, pulmonary oedema and an increase in the size of the thymus and spleen in both acute (single dose of 20 ml/kg on days 1 and 7) and semi-acute (dose of 20 ml/kg twice a week for three weeks) studies in rats. The “case oil” used is said to have caused the death of a child. This oil
<table>
<thead>
<tr>
<th>Oil sample</th>
<th>Anilide content (ppm)</th>
<th>Experimental species</th>
<th>Dose</th>
<th>Effect due to case oil</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>No number</td>
<td>1500</td>
<td>Rat (Wistar)</td>
<td>2 ml/day for 15, 20 and 31 days</td>
<td>Lung lesions</td>
<td>13</td>
</tr>
<tr>
<td>From A. Portera-Sanchez</td>
<td>Oleoyl 560, palmitoyl 35, linoleoyl 45</td>
<td>Rat (Sprague Dawley)</td>
<td>26.7–46.5g/kg for 4 weeks</td>
<td>Nil (no changes in nervous system)</td>
<td>14</td>
</tr>
<tr>
<td>F3</td>
<td>&gt;1000</td>
<td>CD mouse</td>
<td>5 ml/kg daily for 4 weeks</td>
<td>Nil</td>
<td>b</td>
</tr>
<tr>
<td>P1/1695C</td>
<td>&gt;1000</td>
<td>CD mouse</td>
<td>5 ml/kg daily for 4 weeks</td>
<td>Nil</td>
<td>b</td>
</tr>
<tr>
<td>DMT82</td>
<td>1120</td>
<td>Rat – Se + Vit E^c</td>
<td>0.3 ml/day for 3 weeks</td>
<td>Nil</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rat + Se + Vit E^c</td>
<td>0.3 ml/day for 5 days/week</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>ZQ</td>
<td>1000–1500</td>
<td>Rat</td>
<td>0.1 ml for 12 days + 0.5 ml for 7 days</td>
<td>Nil^e</td>
<td>f</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hamster</td>
<td>0.05 ml/day for 15 doses</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mouse</td>
<td>0.025 ml/day or 15 doses</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>Shipment 1, No. 2</td>
<td>Present</td>
<td>Rat</td>
<td>6 ml orally for 7 doses</td>
<td>Nil</td>
<td>g</td>
</tr>
<tr>
<td>-------------------</td>
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<td>-------------------------</td>
<td>-----</td>
<td>---</td>
</tr>
<tr>
<td>47-P1-1868</td>
<td>?</td>
<td>Rat</td>
<td>2 ml (day 1)</td>
<td>Nil</td>
<td>f</td>
</tr>
<tr>
<td>MC-1</td>
<td>?</td>
<td>Rat</td>
<td>2 ml (day 1)</td>
<td>Nil</td>
<td>f</td>
</tr>
<tr>
<td>47-P1-1868</td>
<td>?</td>
<td>(A) Rat + Se + Vit E</td>
<td>0.1 ml/day for 7 days, 0.2 ml/day from day 8 to day 14 and 0.3 ml/day from day 15 to day 21</td>
<td>(A) Nil</td>
<td>f</td>
</tr>
<tr>
<td>(B) Rat + Se + Vit E</td>
<td></td>
<td></td>
<td></td>
<td>(B) Nil</td>
<td></td>
</tr>
<tr>
<td>Shipment 2, No. 68</td>
<td>3000</td>
<td>Rat</td>
<td>5 ml (6 doses over 2 weeks)</td>
<td>Nil</td>
<td>g</td>
</tr>
<tr>
<td>Guinea pig</td>
<td></td>
<td></td>
<td>Intradermal</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Topical</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oral (no dose stated)</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td></td>
<td>Topical (no dose stated)</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td>Intramuscular (no dose stated)</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td></td>
<td></td>
<td>5 ml (6 doses over 3 weeks)</td>
<td>Nil</td>
<td>g</td>
</tr>
<tr>
<td>Oil sample</td>
<td>Aniline content (ppm)</td>
<td>Experimental species</td>
<td>Dose</td>
<td>Effect due to case oil</td>
<td>Reference</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------</td>
<td>----------------------</td>
<td>------</td>
<td>------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>No number</td>
<td>2035</td>
<td>Rat</td>
<td>20 ml/kg (6 doses over 3 weeks)</td>
<td>Lung toxicity, Increase in thymus and spleen</td>
<td>15</td>
</tr>
<tr>
<td>Case, KM-489&lt;sup&gt;h&lt;/sup&gt;</td>
<td>&gt;1200</td>
<td>Rat (Brown Norway)</td>
<td>4 ml/kg (twice weekly for 13 weeks)</td>
<td>Nil&lt;sup&gt;e&lt;/sup&gt;</td>
<td>i</td>
</tr>
<tr>
<td>Control, MB-489&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Nil</td>
<td>Mouse (CBA)</td>
<td>10 ml/kg (twice weekly for 7 weeks)</td>
<td>Nil&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Case, ELC-788&lt;sup&gt;h&lt;/sup&gt;</td>
<td>&gt;1200</td>
<td>Dog (beagle)</td>
<td>3 ml/kg (5 days)</td>
<td>Nil at 61 days</td>
<td>j</td>
</tr>
<tr>
<td>Control, RPF-788&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Nil</td>
<td>Monkey (Cynomolgus)</td>
<td>2 ml/kg (18 days)</td>
<td>Nil at 74 days</td>
<td>k</td>
</tr>
<tr>
<td>Case, NPG-90&lt;sup&gt;h&lt;/sup&gt;</td>
<td>&gt;900</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, CEG-90&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Nil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case oil&lt;sup&gt;h&lt;/sup&gt;</td>
<td>&gt;1200</td>
<td>Dog (beagle)</td>
<td>3 ml/kg for 5 days</td>
<td>Nil (3 weeks)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>l</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guinea pig</td>
<td>5 ml/day for 3 weeks</td>
<td>Nil (7 weeks)&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chicken</td>
<td>2 ml/day + 3 ml/day for 4 days</td>
<td>Nil (7 weeks)</td>
<td></td>
</tr>
</tbody>
</table>
a Nil effect means no effect on growth rate, no sign of poisoning, and sometimes no conventional histology.
c Fed either selenium and vitamin E or vitamin E only.
d Verschoyle, R.D. et al., unpublished results, 1983; see Aldridge & Connors (16).
e No case-oil-related increase in eosinophils.
g Kimbrough, R., unpublished results, 1981.
h Case oils selected by the toxico-epidemiological studies (see text and Kilbourne (5)).
contained 3–4 ppm of aniline, 2035 ppm of oleoyl anilide and 700 ppm of 3-aminophenyl-1,2-propanediol.

Most studies have used rats, and in some cases the oils used have contained substantial amounts of anilides. Fewer studies have been carried out on other species of experimental animal (mouse, guinea pig, hamster and rhesus monkey) but the results obtained have been uniformly negative. Studies on eosinophilia in several species of experimental animal have shown negative results. The toxicity of one oil was studied using two groups of rats, one with a diet deficient in selenium and vitamin E and the other with a diet supplemented with these substances (16). Determinations of selenium and vitamin E in whole blood and plasma, respectively, confirmed that the diet poor in these substances had resulted in a deficiency state that was specifically removed when the diet was supplemented. No differences due to dosing with an assumed case oil containing anilides was found in either group. This experiment was carried out because several suggestions had been made in the literature (unsupported by evidence) that the disease might be caused by free radicals.

What is probably the only report of a long-term (200 days) experiment feeding a “case oil” to rats found an increase in the collagen content of the skin, a late development in TOS (17). The oil used was a sample (No. 39508) obtained from the National Centre for Food and Nutrition in Majadahonda and is said to have been ingested by TOS-affected people who died and to have come from lots seized by the Spanish authorities. No statement is provided as to whether this oil contained fatty acid anilides. Nevertheless, the administration of this oil mixed with standard rat chow in a 2:8 ratio and available ad libitum for 200 days led to a large increase in the collagen content of the skin. On electron microscopic examination, the skin showed a great increase in the number of collagen fibres of uniform diameter arranged in thick bundles. The interstitial space was fully occupied in the treated animals. None of these findings was seen in the rats fed a diet of unadulterated rapeseed oil.

In further studies, shrinkage of skin samples occurred at 64.0 ± 0.5 °C for control animals and at 67.1 ± 0.2 °C for experimental animals. This increased thermal stability could be consistent with the increased collagen content and its arrangement in thick bundles. The elevated temperature of shrinkage of collagen has also been found after treatment with cross-linking reagents (18, 19), though the same effect might be expected from any stabilizing arrangement of collagen fibres. The composition of
amino acids in the collagen and the degree of hydroxylation were the same for both groups of rats and all collagen was type 1. A much larger proportion of the collagen of the treated rats was extractable by acid (35.8%) than for the control rats (8.4%). The \( \text{alpha-chain:beta-component} \) ratio of this acid-extracted collagen was about three times higher in the case-oil treated rats than in the controls. These two observations probably indicate a reduction in the number of cross-links in the collagen. The authors claim that these effects are unlikely to be due to the presence of fatty acid anilides in the case oils because they have not been seen in rats fed oleoyl anilide for other studies (20, 21). These two studies were of shorter duration (10–15 and 30 days, respectively). The authors carried out (with oleoyl anilide) a similar experiment to the above with a “case oil”, but no details of the vehicle in which it was dissolved, the amount of oleoyl anilide or the time of administration are specifically given. With the treated oils, shrinkage of collagen occurred with decreased (i.e. 60.1 ± 0.5 °C) instead of increased temperature. Some doubt about the composition of the anilide used in these experiments has been raised (see below).

In another study, “denatured rapeseed oil” is compared with oleoyl anilide with respect to the fatty acid composition of phosphatidyl ethanolamine and the content of malondialdehyde and glutathione in the kidney, liver and lung of rats (21). For two months, three groups of rats were fed a daily Purina diet (10 g) containing 0.5 ml olive oil, 0.5 ml olive oil containing oleoyl anilide or 0.5 ml “denatured rapeseed oil”. Neither the purity of the oleoyl anilide nor the origin of the denatured rapeseed oil is specified. Malondialdehyde content increased and glutathione decreased in the denatured-oil-treated rats in all tissues, but the changes were most marked in the lungs. No significant changes were found in the anilide-fed rats. The arachidonic acid content of the phosphatidyl ethanolamine was reduced markedly in the tissues of the denatured-oil-fed rats, while linoleic acid increased. Changes in the control or anilide-fed rats were either not present or were very small.

These results with malondialdehyde and glutathione are of potential interest because of the possibility that anilides are bioactivated. Also of potential interest are the changes in arachidonic acid because other studies show changes in its production (see below). However, all these results are difficult to interpret. As mentioned above, the purity of the oleoyl anilide and the origin of the denatured rapeseed oil are not specified; in addition, no control group was included in which the rats were fed only rapeseed oil. Although a few changes are reported in the
anilide-fed rats, the authors raise the possibility that the results shown in the denatured-oil-fed rats could be due to the oleoyl anilide content of the denatured oil. They state: “However, the oleoyl anilide-fed rats did not have results significantly different from the controls; it could be due, at least partially, to insufficient amounts of anilide consumed by the animals to observe an effect”. The anilide content of the denatured rapeseed oil used in these experiments is not specified but, assuming that this is a case oil and that it contained 1000 ppm anilide per ml, then the 0.5 ml supplied to the rats each day would contain 0.5 mg anilide. In this study (21) the rats, fed 2 mg anilide daily, are stated to have consumed about 90% of the diet daily and to have shown no overt signs of toxicity during the two-month feeding schedule.

**Testing Case Oils in in vitro Systems**

The dual difficulties of adding oils to cell cultures without causing secondary problems (e.g. restricting oxygen supply to the cells), and the uncertainty as to whether the early samples were authentic case oils, makes assessment of these studies almost impossible. Some work indicates that normal constituents of rapeseed oil, such as polyunsaturated free fatty acids and their oxidized products, are toxic to cells in culture plates (22–27).

In the years that have elapsed since the poisoning episode, many changes have occurred in the stored oils. Nevertheless, cell lines may well be useful in testing substances isolated from case oils or from oils processed by a simulated “refining” process.

**Toxity of Known Constituents of Case Oils in Experimental Animals**

The association of fatty acid anilides with case oils is now very strong (5) (see Chapter 1) with the major anilide being oleoyl anilide, although on general grounds anilides must be assumed to be formed from the many different fatty acids present in rapeseed oil. However, no firm evidence is available that any anilides have or have not an etiological role in TOS.

When rapeseed oil to which aniline had been added was put through a simulated refining process, several derivatives other than fatty acid anilides were detected but not all have been identified (28, 29). One compound in
supposedly “toxic oils” has been identified as 3-aminophenyl-1,2-propanediol, either free or after liberation from its fatty acid derivatives (30, 31). The parent diol or its mono- or dioleoyl diesters have now been synthesized and the structure of the parent compound established from spectroscopic data (ultraviolet, infrared, electromagnetic), molecular size estimation (exclusion chromatography), nitrogen content (2.0%) and the formation of a magenta colour with p-dimethylaminocinnamaldehyde (32). Thus, although such compounds may well be constituents of case oils, this aspect has not been formally established as it has for the anilides.

An extensive study by Casals et al. (20) showed that the administration of oleoyl anilide to rats results in a profound depression of the systems involved in the synthesis of lipids in the lung and adipose tissue but not in the liver. Rats received 5 mg (25–30 mg/kg) oleoyl anilide in olive oil daily by the oral route for 10–15 days, followed by various periods without dosing before the animals were killed and the tissues fractionated in order to study their capacity to synthesize lipids. No changes in the condition of the rats were reported. The transacylation reaction demonstrated by measuring the incorporation of radiolabelled palmitoyl-coenzyme A into triacylglycerols (triglycerides) was the most affected, with 95% inhibition after dosing for 13 days followed by 4 days without anilide. After 45 days without anilide, lipogenic activity had only partially returned (70% inhibition).

This interesting study (20) presents two problems. First, the method used to synthesize the oleoyl anilide may lead to other products (33), and second, another experiment to check if such an effect on lipid synthesis systems occurred in vivo gave negative results (34). When the condensing agent N,N′-dicyclohexylcarbodiimide is used to assist the reaction of aniline with oleic acid, N-cyclohexyl fatty acid amides are formed as by-products (33). Other methods are available for the synthesis of such anilides that have yielded authentic products (35–37). Therefore, checking the authenticity of the oleoyl anilide used in the experiments of Casals et al. (20) is important; it was synthesized by the reaction of oleic acid and aniline in the presence of equimolar amounts of dimethylaminopropyl ethyl carbodiimide (not with N,N′-dicyclohexylcarbodiimide as in the work of Freixa et al. (33)).

Tucker & Cunningham (34) studied the incorporation of radiolabelled palmitic acid (administered intravenously) into the lipids of the lung, epididymal fat and the liver in rats after oral administration with oleoyl anilide at the same dose (5 mg daily) and for the same period (13 days) as
in the work of Casals et al. (20); the ability of the animals to incorporate palmitic acid into lipids was determined five days later. No changes between the treated and control rats were found, nor were any significant differences seen in the body weight gain or the weight of the epididymal fat bodies. The weight of the perirenal fat was marginally greater in the treated group ($P<0.05$). The oleoyl anilide in this case was synthesized by reaction of oleic acid and aniline in the absence of any condensing agent, and the purity in excess of 99% was established by capillary gas chromatography (37) and mass spectrometry.

This discrepancy between the results from these two laboratories remains unresolved (20, 34). Even if the composition of the "oleoyl anilide" differed and the results may be irrelevant to TOS, from a scientific point of view it will be important to establish what is causing the profound inhibition of lipid synthesis pathways and its precise mechanism.

Two other studies have been carried out on the effect in rats of oleoyl anilide on the concentrations of polyunsaturated fatty acids, malondialdehyde and glutathione in the liver, kidney and lung (21) and on the microviscosity of the isolated liver plasma membranes (38). Minimal changes were found for all these factors.

Oleoyl or linoleoyl anilides, incorporated into phosphatidyl choline liposomes, were administered intraperitoneally to rabbits at a dose of 0.01 mg/kg body weight for three weeks, or by gavage daily for two weeks (39). The authors claimed that this regime led to signs of neurological abnormality after 3–6 weeks (39). Various structural changes in the central nervous system at the light and electron microscopic levels are also summarized. An immunological mechanism was postulated, and a short report has appeared claiming that fatty acid anilides are immunogenic to rabbits (40). No confirmation or full publication of these potentially interesting findings has appeared, and the purity or method of synthesis of the anilides is not known.

The oleoyl esters of 3-aminophenyl-1,2-propanediol, which have been found in some oils, have been synthesized and characterized (31, 32). Table 8 summarizes their toxicity in mice. The most toxic compounds were the parent diol and the mono-oleoyl ester. No mortality was recorded after administration of the diester, though changes in the lung were seen in a few animals. For the monoester administered either orally or intraperitoneally, the most frequent changes at autopsy were in the lung (41). Lung changes were also seen (congestion and infarction) after intraperitoneal injection but not after oral dosing. Organ weights
are provided for the liver, spleen, kidneys, heart and testicles, but unfortunately the weight of the lungs is not recorded. The summary of the histological findings is given in Tables 9 and 10. The findings are given variously as inflammation or congestion, and eosinophils are mentioned as present in some organs. No blood eosinophil count was done. The frequency of changes was highest after treatment with the monoester. The mono- and diesters of 3-aminophenyl-1,2-propanediol can be separated by thin layer chromatography and detected and visualized by spraying the plates with p-dimethylaminocinnamaldehyde. By solvent extraction of several organs from rats previously dosed with the parent diol, and using the above analytical technique, mono- and diesters were found, indicating that the diol can be esterified in vivo. These results are interesting, even though rather large doses of the compounds were required to produce toxicity of either the diol or its mono-oleoyl ester. No other confirmatory studies on these compounds have been published.

Table 8. Toxicity to mice of 3-aminophenyl-1,2-propanediol (diol) and its mono- and dioleoyl esters

<table>
<thead>
<tr>
<th>Compound</th>
<th>Vehicle</th>
<th>No. of doses x mg/kg per day</th>
<th>Sex</th>
<th>Deaths/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diester</td>
<td>Olive oil</td>
<td>8 x 465 orally</td>
<td>M</td>
<td>0/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 x 465 i.p.</td>
<td>M</td>
<td>0/8</td>
</tr>
<tr>
<td>Monoester</td>
<td>Olive oil</td>
<td>8 x 465 orally</td>
<td>M</td>
<td>1/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 x 465 orally</td>
<td>F</td>
<td>0/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 x 465 i.p.</td>
<td>M</td>
<td>2/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 x 465 i.p.</td>
<td>F</td>
<td>4/8</td>
</tr>
<tr>
<td>Diol</td>
<td>Saline solution</td>
<td>11 x 465 orally</td>
<td>M</td>
<td>0/11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11 x 465 orally</td>
<td>F</td>
<td>0/11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 x 465 i.p.</td>
<td>M</td>
<td>10/11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 x 465 i.p.</td>
<td>F</td>
<td>7/8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 x 230 i.p.</td>
<td>M</td>
<td>1/11</td>
</tr>
</tbody>
</table>

*a i.p. = intraperitoneal route.

Source: Maestro Durán et al. (38).
Table 9. Numbers of mice, treated according to the schedules given in Table 8, showning various macroscopic changes at autopsy

<table>
<thead>
<tr>
<th>Autopsy finding</th>
<th>Group</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Diester (oral, M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diester (i.p.,(^b)M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoester (oral, M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoester (oral, F)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoester (i.p., M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoester (i.p., F)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diol (oral, M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diol (oral, F)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diol (i.p., M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Grey-brown colouration</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Congestion</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Perihepatitis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congestion</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Infarction</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Emphysematous zones</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Splenitis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Congestion</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Perisplenitis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulation</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hyaline gout formation</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Except for group L, where the dose was 230 mg/kg body weight per day.

\(^b\) i.p. = intraperitoneal route.

Source: Maestro Durán et al. (38).
Table 10. Histopathological findings in mice treated according to the schedules given in Table 8\textsuperscript{a}

<table>
<thead>
<tr>
<th>Histopathological finding</th>
<th>Control</th>
<th>Group(s)\textsuperscript{b}</th>
<th>C + D</th>
<th>E + F</th>
<th>C</th>
<th>A</th>
<th>B</th>
<th>L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congestion</td>
<td>++40%</td>
<td>+100%</td>
<td>++100%</td>
<td>++100%</td>
<td>+++100%</td>
<td>+50%</td>
<td>+100%</td>
<td>+100%</td>
</tr>
<tr>
<td>Haemorrhage</td>
<td>+50%</td>
<td>100%</td>
<td>+33%</td>
<td>+100%</td>
<td></td>
<td></td>
<td></td>
<td>+25%</td>
</tr>
<tr>
<td>Acute inflammation</td>
<td>++50%</td>
<td>+100%</td>
<td>+50%</td>
<td>+100%</td>
<td></td>
<td></td>
<td></td>
<td>+100%</td>
</tr>
<tr>
<td>Necrosis</td>
<td>+33%</td>
<td>20%</td>
<td>+50%</td>
<td>+20%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>+50%</td>
<td>100%</td>
<td>+20%</td>
<td>+100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortical acute inflammation</td>
<td></td>
<td></td>
<td>++20%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lung</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congestion</td>
<td>++75%</td>
<td>+++100%</td>
<td>++++100%</td>
<td>+++100%</td>
<td>+++100%</td>
<td>+100%</td>
<td>+100%</td>
<td>+100%</td>
</tr>
<tr>
<td>Haemorrhage</td>
<td>+75%</td>
<td>+++19%</td>
<td>+++50%</td>
<td>+100%</td>
<td></td>
<td></td>
<td>+80%</td>
<td>+50%</td>
</tr>
<tr>
<td>Acute inflammation</td>
<td>++50%</td>
<td>+80%</td>
<td>+100%</td>
<td></td>
<td></td>
<td>+50%</td>
<td>+100%</td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>+50%</td>
<td>100%</td>
<td></td>
<td>+100%</td>
<td></td>
<td></td>
<td></td>
<td>+50%</td>
</tr>
<tr>
<td>Fatty vacuoles</td>
<td></td>
<td></td>
<td>++80%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congestion</td>
<td>+60%</td>
<td>++100%</td>
<td></td>
<td>No change</td>
<td></td>
<td>+30%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute inflammation</td>
<td></td>
<td></td>
<td>+100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty necrosis</td>
<td></td>
<td></td>
<td>+100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 10. (contd.)

<table>
<thead>
<tr>
<th>Histopathological finding</th>
<th>Control</th>
<th>Group(s) $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C + D</td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congestion</td>
<td></td>
<td>++100%</td>
</tr>
<tr>
<td>Medullary congestion</td>
<td>+50%</td>
<td></td>
</tr>
<tr>
<td>Corticomedullary congestion</td>
<td>++100%</td>
<td></td>
</tr>
<tr>
<td>Acute inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemorrhage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Necrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty necrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Heart</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Testicle</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Degree of effect: + = minimum; ++ = slight; +++ = moderate; ++++ = heavy.

$^b$ From Table 4.

*Source:* Maestro Durán et al. (38).
Testing Known Constituents of Case Oils in *in vitro* Systems

After rat liver was perfused with a medium containing oleoyl anilide followed by isolation of the plasma membranes, the liver plasma membranes showed a decreased microviscosity, indicating a more fluid environment in them (38). A similar change was found after the addition of oleoyl anilide to a suspension of hepatocytes. The use of dual-labelled anilide established that the majority of the radioactivity in the membranes was due to the intact oleoyl anilide. Assessing the relevance of these findings to TOS is difficult. The amounts of anilide added were large: in the perfusion experiments, 20 mg was added to the perfusate and 0.5 mg anilide was added to the suspension of hepatocytes in 1.5 ml of medium. Linking these findings to those from *in vivo* experiments is also difficult; after oral administration of oleoyl anilide to rats for 30 days, no significant differences from the controls were found (38).

The hypothesis that the inflammatory reactions, mainly shown as a vasculitis, in TOS patients may be mediated by an enhancement of the pathway of synthesis of prostaglandins and leukotrienes has been studied in two laboratories. The addition of oleoyl or linoleoyl anilides to human polymorphonuclear leucocytes induced a time-dependent generation of arachidonic acid (42). Linoleoyl anilide was more effective than oleoyl anilide, with a half-maximal increase being obtained with 0.2 mg per ml cells. The effect was specific since neither an increase in the synthesis of oleic acid nor a change in the incorporation of labelled precursors into several phospholipids was seen. The concentrations of linoleoyl anilide necessary to produce this effect on arachidonic acid synthesis are rather high if considered in the light of the amount of linoleoyl anilide ingested by TOS victims. However, this same paper shows that linoleoyl anilide inhibited the synthesis of triacylglycerol in human polymorphonuclear leucocytes at concentrations of 1 µg/ml. This result should be compared with the effects of oleoyl anilide on triacylglycerol synthesis after its administration to rats (see above).

Other studies using mouse peritoneal macrophages have shown that their exposure to 1.5 mM linoleoyl anilide for two hours increased the synthesis of the prostaglandin 6-oxo-PGF$_1$ and the thromboxane TBX$_2$ (43). Other work has shown that linoleoyl anilide specifically increases the production of 12-hydroxyeicosatetraenoic acid (12-HETE) while 15-HETE was unaltered. An increase was found at a concentration of 0.1 mM linoleoyl anilide in a suspension of 5 x 10$^6$ cells per ml, but 1.0 mM
was required to produce a significant change (G. Bioque et al., unpublished data, 1989).

As stated above (42), the significance of this work for TOS is difficult to assess at present. The results are important because they provide information about the biological activity of known constituents in case oils. If the effects of anilides on these pathways are significant (44) then many chemical structures are possible. Rapeseed contains many fatty acids, both saturated and unsaturated. In addition, the unsaturated fatty acids can yield a variety of oxidized products (45). The validity of the hypothesis that the prostaglandin pathway is involved will depend on a realistic comparison of the in vitro activity of anilides present in case oils and the concentrations likely to be achieved in humans by ingestion of the oils. Their rates and routes of metabolism in the particular species will also be important (36, 46–48).

**Biological Activity of Postulated Derivatives of Aniline**

In 1984, a suggestion was made that the symptomatology of TOS resembled in many respects graft-versus-host disease (GVHD) (49, 50). A possible reaction product formed from aniline and a known constituent of some rapeseed oils were postulated to bear some structural resemblance to drugs that cause a GVHD-like condition in humans. The hypothesis is that goitrin, a glycoside, releases an isothiocyanate that reacts with aniline to produce a thiourea, which under acid conditions in an oil refining process cyclizes to yield a structure resembling the chemical structure of phenytoin (Fig. 17). This substance was postulated to be 1-phenyl-5-vinyl-2-imidazolidinethione (IZT). IZT was synthesized and, when tested by the popliteal lymph node assay, was shown to be active (51). The popliteal lymph node assay has given positive results for compounds that cause some autoimmune diseases in humans (52–54). Besides this autoimmune possibility, another attraction of the postulated reactions is that a substituted thiourea would be formed. Other thioureas are well known to cause lung damage, to be species-specific and to bring about their effects by an attack on the endothelial cells in the lung.

A selection of oils was supplied to Dr Kammüller and Dr Seinen but they were able to find traces of IZT in only one of the oils with their analytical technique (56). Subsequent investigation found that only one or two of the oils supplied could safely be classified as case oils (A. Abaitua & M. Posada, unpublished data, 1988). Other work established that the
Fig. 17. Reactions postulated to take place between aniline and the isothiocyanate derived from progoitrin from rapeseed oil

Source: Kammüller et al. (52) and Bernert et al. (55).
compound said to be IZT was another cyclized product, 5-vinyl-2-thiazolidinephenylamine (5-VTPA) (55, 57) (see Fig. 17); this has now been confirmed (58). In a selection of authentic case oils (5), 5-VTPA has not been found (55). The absence of 5-VTPA in these eight-year-old samples of oil could be because it is not sufficiently stable (about 23 weeks at room temperature) (55). It is uncertain as to whether the rapeseed oil was denatured with aniline after the isothiocyanate-containing glycosides had been removed by a refining process. Therefore, neither 5-VTPA nor IZT (which has not yet been synthesized) seems likely to be involved in TOS, although involvement of an autoimmune mechanism has not been excluded.

Conclusions and Recommendations for Research

Oils can now be selected with a good pedigree for association with cases of TOS, even though they are over nine years old and have often been stored in less than ideal conditions. These oils can be used for experimental studies, though to increase the probability that the sample consists of mainly case-related oils, oils containing greater than 600 μg/g anilides should be mixed together. To assess whether one particular oil is a case oil is still a problem, but epidemiological evidence and the presence of anilides and substantial amounts of rapeseed oil in the sample allow reasonable certainty that the decision will be correct. Reaching decisions about the authenticity of the “case oils” used in early research is bound to be difficult; the least information required is their anilide content.

The only substances for which we have sound epidemiological and analytical evidence of their presence in case oils, and which are reasonably stable, are the anilides of oleic, linoleic and linolenic acids. Other anilides of minority fatty acids would be expected to have similar stabilities but lower concentrations; the stability of their oxidized products is unknown. Several research groups have carried out research on oleoyl and linoleoyl anilides. In one series of studies, a very high activity of both oleoyl and linoleoyl anilides has been demonstrated on the lipid synthetic pathways in rats and in polymorphonuclear leucocytes (20, 42). Experiments designed to test these findings in rats have not provided confirmation (34), and questions have been raised about the chemical authenticity of the anilides used. These inconsistencies must be resolved, if necessary by further research. Because of its scientific interest and irrespective of its relevance to TOS, a substance with such a
high biological activity on lipid synthetic mechanisms both in vivo and in vitro should be identified.

The esters of 3-aminophenyl-1,2-propanediol and the parent compound have been found in some “case oils” and after simulated refining procedures with oils containing aniline. Using the selection of oils made by Kilbourne et al. (5) and by Posada & Abaitua (unpublished observations, 1989) an association of these substances with case oils should be possible to check rather easily. In one toxicological study on these compounds in mice, although rather large doses were required, tissue toxicity was seen and the lung was the organ most frequently attacked (41). This work was not published in a journal specializing in pathology and has not been confirmed; therefore, this work should be repeated in mice and other species. As for the anilides, oxidized chemical structures are also possible. The stability of this whole class of substances in oil should be determined.

For the future, many uncertainties remain that give rise to questions. For example:

- Is the syndrome a single disease entity?
- Is the whole syndrome produced by one or by more than one chemical substance?
- Does one chemical substance have several biological actions that overlap so as to produce the whole syndrome?
- Are the toxic derivative(s) of aniline formed by reaction with “normal” constituents of rapeseed oil or with other added extraneous substance(s)?
- Is the toxic substance(s) a derivative of aniline?
- What are the stabilities of the toxin(s) during storage in the oils?
- Does the whole or part of the syndrome involve a primary immunological mechanism or immunological pathology?

The simplest hypothesis is that in humans, one or more compounds formed by reaction of aniline with constituents of rapeseed oil cause TOS by attacking the endothelial cells of the vasculature. A comfortable hypothesis would be that the whole syndrome is initiated by and follows from such a chemical attack. Although the intermediate and chronic stages of the disease could possibly be brought about by an immunological mechanism, the early effects on the lung occurring a
short time after consumption of the oil are more likely to result from a
direct chemical toxicity to the lung. Whether this early toxicity to the
lung is initiated by the same substance that initiated the late stages is
unknown. Perhaps one relatively unstable substance causes the lung
toxicity and one or more other substances cause the late symptoms.
Unfortunately, no animal model has been found for TOS or parts of it.
Claims that “case oils” cause lung toxicity and increases in skin colla-
gen in rats have not been confirmed. In all these studies no firm
evidence has emerged about the authenticity of the “case oils”, although
in those said to have caused lung toxicity the presence of substantial
amounts of anilides is reported. Since an animal model as a bioassay
system is lacking, the standard procedure of linked bioassay and chemi-
cal separation followed by identification, synthesis and toxicological
research has not yet been started. The very old but authentic samples of
oils now available would not seem likely to lead to an animal model;
the amounts available are also restricted. These oils will be mainly of
analytical value to test for the presence of candidate substances or, more
likely, their breakdown products.

Assuming that aniline derivatives are involved, the best hope for
progress towards the identification of etiological agent(s) lies with the
outcome of a simulated refining process (1) using labelled isotopes so
that a balance sheet for aniline can be determined for all the steps of the
process. Such research has now begun (59). Without doubt, many
derivatives of aniline are theoretically possible (60), but with tech-
niques now available the isolation, identification and synthesis of com-
ponds formed can probably be achieved.

Choosing the methods for biological testing of oil fractions and
isolated and synthesized substances is, at the moment, very difficult.
Methods for the processing of fractions so that they may be added to in
vitro biological systems must be developed. The choice of biological
systems to use for testing is mostly arbitrary. It is difficult to envisage
the identification of etiological agent(s) without an animal model for
the whole syndrome or part of it with which to validate the significance
of positive results in in vitro systems. Although all attempts to establish
an experimental model have so far failed, the testing of fresh fractions
from the simulated refining procedures on a small number of many
animal species seems inescapable. Many in vitro systems, including
those with a logical connection with the disease syndrome or parts of it,
should be incorporated into the testing schedules. However, in the
absence of an experimental model or bioassay system, one cannot
logically and rigorously exclude that one or more of the fatty acid anilides produced TOS in humans. At present the only safe conclusion is that they are markers of case oils; until a bioassay system is established, other aniline derivatives will undoubtedly join that category.

The case oils and their non-case controls resulting from the toxicological and epidemiological studies (5; M. Posada & A. Abaitua, unpublished observations, 1989) are a very valuable collection. Although biological activity relevant to TOS has not been found in these oils, they must still contain the chemical clues to the etiological agent(s). Even if the agents are unstable, establishing their previous presence from breakdown products ought to be possible. Thus, even though this collection of oils is nine years old and may now be biologically inert, they should be stored under conditions that should ensure future stability (i.e. under nitrogen and at 70 °C). With the exception of a small study on cats, no further administration of these oils to experimental animals should be permitted; they should instead be retained for analytical studies as etiological agents are postulated.

References


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Chemical composition of TOS-related oils

R. Guitart & E. Gelpi

Basic and Nontoxic Components of TOS-related Oils

Edible oils can be obtained from different sources. The lipid composition of oils is very similar, but some qualitative and quantitative differences exist as a function of their vegetable (or animal) origin.

From the fatty acid and sterol pattern of approximately 50 samples of TOS-related oils, the Institute of Fats and their Derivatives in Seville (1-4) determined that they were a mixture of various oils of diverse origin; some of the analysed samples contained up to five different components. Ventura Díaz (2, 3) specified that 17.7% of these oils contained three components, 71.1% four components and 11.1% five components (Table 11). The most frequent composition (53.3% of cases) was olive residue oil (the oil obtained by solvent extraction from

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The only accepted “case” and “control” oils are those belonging to the first toxico-epidemiological study of Kilbourne et al. (5), all of which have been compositionally analysed by Bernert et al. (6). Those oils certified in this study, as well as in the second toxico-epidemiological study, as high anilide case oils can thus be appropriately termed “epidemiologically validated case oils”. All other epidemic-related oils herein considered are referred to as “TOS-related” or “TOS-associated oils”, meaning that whereas they might have been case-related, no firm epidemiological data support this fact.
Table 11. Percentages of suspected TOS-related samples that contained each type of oil or fat

<table>
<thead>
<tr>
<th>Oil or fat</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refined olive residue oil</td>
<td>93.3</td>
</tr>
<tr>
<td>Refined rapeseed oil</td>
<td>91.1</td>
</tr>
<tr>
<td>Pig fat and/or esterified oil</td>
<td>77.8</td>
</tr>
<tr>
<td>Refined grapeseed oil</td>
<td>66.7</td>
</tr>
<tr>
<td>Refined sunflower oil</td>
<td>28.9</td>
</tr>
<tr>
<td>Olive oil</td>
<td>26.7</td>
</tr>
<tr>
<td>Refined cottonseed oil</td>
<td>6.7</td>
</tr>
<tr>
<td>Refined soya oil</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Source: Vioque (1) and Ventura Díaz (2–4).

the pressed seed of the olive fruit), rapeseed oil, grapeseed oil, and pig fat and/or esterified oil (2, 3, 7).

Initial reports of the oil composition of suspected TOS oils, made at the National Centre for Food and Nutrition (8), indicated that the basic composition was rapeseed oil and animal fats, with a small quantity of olive residue oil. A later report in May 1982\(^a\) pointed out that the TOS-associated oil samples contained (60–90%) rapeseed oil variously mixed with liquid vegetable oil (10–30%), animal fats (30%) and very poor quality olive oil (0–5%).

The presence of highly unsaturated tung oil (from seeds of *Aleurites fordii*) in TOS-related samples was analytically rejected (9).

**Acylglycerides**
Triglycerides constitute the major fraction of oils. In almost 50 TOS-associated oils analysed by thin layer chromatography (TLC) the Institute of Fats and their Derivatives did not detect esters other than those arising from glycerol and common fatty acids (1, 2, 4, 10).

**Monoglycerides and diglycerides**
The presence in TOS-associated oils (and also in edible oils) of monoglycerides and diglycerides was confirmed by TLC purification and detection with phosphomolybdic acid (11). No attempt was made to study the chemical composition of these fractions.

\(^a\) Report of the results of the National Centre for Food and Nutrition. Copenhagen, WHO Regional Office for Europe, 1983 (unpublished document ICP/RCE 905(1)/35).
Triglycerides
The triglyceride (or triacylglyceride) composition of TOS oils has not been fully determined. However, this fraction has been purified and detected by TLC (11–13) and use of a high performance liquid chromatography (HPLC) method with refractometric detection has also been reported (14).

The Institute of Fats and their Derivatives analysed samples of TOS-related oils for saturated fatty acids in the 2-position of triglycerides (1, 4). From 45 samples, the arithmetic mean and standard deviation of the saturated fatty acids in the 2-position was 5.50 ± 5.00%, with a range of 0.4 to 30.6 (recalculated from Ventura Díaz (2)). In Spain, the highest legally permitted value of this parameter in a vegetable oil is 1.8% for cottonseed oil, as compared to 1.0% and 1.6%, the values permitted for rapeseed and refined olive oils, respectively (15). The high value of saturated acids in the 2-position in TOS-related oils could be explained by the presence of fats or oils of animal origin in the samples, as it is well known that biosynthetic pathways in plants produce mainly triglycerides with an unsaturated fatty acid esterifying the second carbon atom of glycerol. An alternative explanation is a high content of inter-esterified or re-esterified oil.

Guitart (11) determined, by TLC purification and gas-liquid chromatography, the fatty acid composition of the triglyceride fraction of three TOS-associated oils (see below). The composition of oxidized fatty acid-containing triglyceride was also qualitatively analysed by TLC purification and gas chromatography/mass spectrometry (GS/MS) detection (see section on oxidized derivatives of fatty acids).

Fatty acids

Free fatty acids
Free fatty acid content, as the acidity index, was reported by Ventura Díaz (2) for 46 samples. Results range from 0.09% to 5.07%, with the exception of one sample (an oil apparently used to fry fritters) which was 44.03%. Overall, these results are lower than what is legally permitted in Spain for virgin olive oil (≤3%) and pure olive oil (≤1%) but higher than for refined olive and liquid vegetable oils (≤0.2%) (15).

Guitart (11) studied the free fatty acid composition of three TOS-related oils. This fraction was purified by TLC and, after derivatization with diazomethane to the corresponding fatty acid methylester, the samples were injected into a Carbowax 20M capillary column coupled
to a flame ionization detector (FID). The results (as a percentage of total free fatty acids) are shown in Table 12.

Esterified fatty acids
The total fatty acid profile of TOS-related oils has been reported (2, 4, 16). Nevertheless, the application of high resolution methods of analysis, using capillary columns, has been reported in only a few cases (6, 11).

Guitart (11) analysed the total fatty acid composition of five TOS-related oils and one edible rapeseed oil (Table 12). In a nonpolar stationary phase (OV-1 type) some of these oils were found to contain quantities of the trans isomer of oleic acid (i.e. elaidic acid, 2.6–3.8%). The fatty acid profile of the triglyceride fraction was also analysed in three TOS-related oils (Table 12).

Bernert et al. (6) studied the fatty acid profile of an important number of oils. Based on epidemiological data, they made a distinction between case oils (oils from families whose clinical histories met a strict case definition of TOS, and whose containers were typical of oils thought to be etiological for the syndrome and which appeared to have been partially consumed by the family; N = 29) and control oils (oils from families who denied having had TOS or a similar illness but whose oils were exchanged in containers of the typical shape and which appeared to have been partially consumed; N = 64). The analyses were made on a free fatty acid phase capillary column, and the results were expressed in quantitative form using the 19:0 fatty acid as the internal standard. To normalize these two sets of data, the results have been recalculated to percentages (Table 12).

Case-related oils contained low quantities of erucic acid (22:1, n–9) confirming previous reports that the original aniline-denatured rapeseed oil belonged to the “low erucic acid-type” category (3, 4, 10).

Oxidized derivatives of fatty acids
Although oxidized derivatives of fatty acids (hydroperoxides and epoxides) can be considered toxic, they have usually been found at low concentrations in all oil samples, edible or not, because unsaturated fatty acids oxidize extremely easily.

Peroxide index values initially determined in samples from TOS-associated oils were abnormally high, ranging up to 75 with a mean of 28.9. From approximately 50 samples, 91% had a peroxide value higher than authorized for refined oils and 69% higher than authorized for virgin or pure olive oils (4, 17), which is ≤10 and ≤20 meq of active
Table 12. Percentage fatty acid compositions of TOS-related and rapeseed oils and case and control oils

<table>
<thead>
<tr>
<th>Oil Sample</th>
<th>14:0</th>
<th>16:0</th>
<th>16:1 (n-7)</th>
<th>18:0</th>
<th>18:1 (n-9)</th>
<th>18:1 (n-7)</th>
<th>18:2 (n-6)</th>
<th>18:3</th>
<th>20:0 (n-9)</th>
<th>20:1</th>
<th>22:0</th>
<th>22:1 (n-9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOS-related ((N=3))</td>
<td>NQ</td>
<td>12.6</td>
<td>1.5</td>
<td>16.6</td>
<td>45.4</td>
<td>3.1</td>
<td>7.4</td>
<td>0.1</td>
<td>3.0</td>
<td>2.3</td>
<td>5.0</td>
<td>1.4</td>
</tr>
</tbody>
</table>
| Fatty acids in tri-
| glyceride fraction |      |      |              |      |              |              |              |      |              |      |      |              |
| TOS-related (\(N=3\)) | NQ   | 9.5  | 0.5          | 5.6  | 42.6         | 3.2          | 31.9         | 2.1  | 0.7          | 1.2  | 0.5  | 1.3          |
| Total fatty acids   |      |      |              |      |              |              |              |      |              |      |      |              |
| TOS-related (\(N=5\)) | NQ   | 5.4  | 0.2          | 4.0  | 42.9         | 3.0          | 35.8         | 4.2  | 0.7          | 1.4  | 0.6  | 0.9          |
| Rapeseed (\(N=1\)) | NQ   | 4.4  | 0.2          | 2.1  | 55.8         | 3.7          | 23.1         | 6.7  | 0.6          | 1.6  | 0.5  | 0.9          |
| Case (\(N=29\))    | 0.3  | 10.3 | 0.7          | 3.8  | 46.0         | 2.5          | 31.6         | 2.9  | 0.4          | 0.9  | 0.3  | 0.4          |
| Control (\(N=64\)) | 0.3  | 11.0 | 0.6          | 4.1  | 36.8         | 1.7          | 42.1         | 1.9  | 0.3          | 0.5  | 0.4  | 0.2          |

\(^a\)14:0 = myristic acid; 16:0 = palmitic acid; 16:1 \(n-7\) = palmitoleic acid; 18:0 = stearic acid; 18:1 \(n-9\) = oleic acid; 18:1 \(n-7\) = vaccenic acid; 18:2 \(n-6\) = linoleic acid; 18:3 \(n-3\) = linolenic acid; 20:0 = arachidic acid; 20:1 \(n-9\) = eicosenoic acid; 22:0 = behenic acid; 22:1 \(n-9\) = erucic acid.

\(^b\)NQ = Not quantified.

Source: Bernert (6) and Guitart (11).
oxygen per kg of fat, respectively (15). In addition, active oxygen method stabilities were low (1–10.5 hours). In other words, these oils are less able to withstand air oxidation than oils with normal concentrations of natural antioxidants.

In line with the hypothesis supporting peroxidative free radical mechanisms in the etiopathogenesis of TOS (5), Roselló et al. (18) identified in suspected oil samples two 18:2 fatty acids hydroxylated in positions 9 and 13 plus the corresponding 11-hydroxy,9,10-epoxide. All of these are consistent with the formation of reactive hydroperoxides from unsaturated fatty acids.

Completing this work, Guitart (11) detected on TLC plates two fractions from some TOS-related oils rich in hydroperoxide-containing compounds. One of these fractions had the Rf value of oxidized free fatty acids, while the other (which gave more intense reaction to specific spray reagents than the former) was tentatively ascribed to a triglyceride containing one oxidized fatty acid. Using gas chromatography/mass spectrometry (GC/MS) 8-, 9-, 10- and 11-hydroxy-18:1 and 9- and 13-hydroxy-18:2 were identified, both in the cis,trans and in the trans,trans forms. These oxidized fatty acids were not detected in samples of edible olive and rapeseed oils, but were detected in two adulterated but non-TOS-related samples.

The peroxidative hypothesis based on the high peroxide values and low oxidative stabilities of adulterated TOS oils also found support in early work carried out by the various groups from the Spanish Research Council (7). In some experiments, the in vitro effects of TOS oils with high peroxide values on the peroxidation of membrane lipids in rat liver homogenates were measured by colorimetric determination of malondialdehyde (Table 13). The data suggest that TOS-related oils stimulate lipid peroxidation without any correlation with anilide content, and that this effect could be inhibited by vitamin E. However, no clear implications have emerged from these data. In this regard the well known antioxidant effects of aniline should be taken into account. Dobarganes García et al. (19) showed that the addition of 2% aniline to various oils contributed to their stability and to a decrease in their peroxide values. In the process, aniline becomes oxidized to different products, some of which will be discussed below (see page 111). In any case, many of the TOS-related oils were highly oxidized, which would be in line with their low residual content of aniline and with the fact that anilides presumably do not have antioxidant activity.
Table 13. Peroxidation of membrane lipids in rat liver homogenates when exposed to different TOS-related and edible oil samples, measured by colorimetric determination of malondialdehyde.

<table>
<thead>
<tr>
<th>Oil sample</th>
<th>Malondialdehyde production$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapeseed</td>
<td>1.84 ± 1.98 (n = 13)</td>
</tr>
<tr>
<td>Olive</td>
<td>2.57 ± 2.38 (n = 7)</td>
</tr>
<tr>
<td>Zrq$^b$</td>
<td>5.25 ± 2.71 (n = 10)</td>
</tr>
<tr>
<td>Ag-Pr$^c$</td>
<td>4.91 ± 2.41 (n = 10)</td>
</tr>
<tr>
<td>JEN b$^b$</td>
<td>3.37 ± 2.45 (n = 5)</td>
</tr>
<tr>
<td>PH 2$^b$</td>
<td>6.16 ± 3.85 (n = 3)</td>
</tr>
<tr>
<td>PH 5$^c$</td>
<td>6.10 ± 4.15 (n = 3)</td>
</tr>
</tbody>
</table>

$^a$ nmol/mg protein produced in 10 minutes at 37°C.

$^b$ Contained anilides.

$^c$ Either did not contain anilides or contained them below the limits of detection.

Source: Vioque et al. (7).

Sterols

Free sterols

Guitart (11) analysed the non-esterified sterol composition of several TOS-related and edible oils (one rapeseed and two adulterated but non-TOS-associated oils). This minority fraction was purified by TLC and analysed, after derivatization to trimethylsilyl ethers, by GC-FID and GC/MS. The results, as a percentage of total free sterols, are shown in Table 14.

Total sterols

The total sterol fraction of the oils has been analysed by several authors (4, 6, 10, 16).

Fractionation by gel filtration of the unsaponifiable components of denatured crude rapeseed oil coded RAPSA B$^a$ (containing 6707 and 36693 ppm aniline and anilides, respectively) yielded only 27.4 mg (9.5%) sterol and other alcoholic terpenic fractions, while the corresponding value for an edible rapeseed oil was 288.5 mg (66.9%) (20). No explanation for this significant difference was given because the

$^a$ RAPSA is the firm in San Sebastián that imported aniline-denatured crude rapeseed oil from France.
authors were searching for hydrocarbons in the unsaponifiable fraction of the RAPSA B sample (see page 122).

Bernert et al. (6) studied the major sterols present in the oils after saponification of the samples and conversion to their trimethylsilyl derivatives. The samples analysed were the 29 case and 64 control oils mentioned before. The results (recalculated to percentages) are shown in Table 14.

Table 14. Percentage sterol composition of TOS-associated and edible oils and case and control oils

<table>
<thead>
<tr>
<th>Oil sample</th>
<th>Cholesterol</th>
<th>Brassicasterol</th>
<th>Campesterol</th>
<th>Stigmasterol</th>
<th>beta-Sitosterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOS-related</td>
<td>5.6</td>
<td>7.6</td>
<td>21.8</td>
<td>10.0</td>
<td>54.5</td>
</tr>
<tr>
<td>(N = 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rapeseed</td>
<td>–</td>
<td>11.7</td>
<td>26.6</td>
<td>2.0</td>
<td>59.0</td>
</tr>
<tr>
<td>(N = 1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MILLAN (a)</td>
<td>22.0</td>
<td>–</td>
<td>6.1</td>
<td>3.4</td>
<td>54.4</td>
</tr>
<tr>
<td>(N = 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Case</td>
<td>4.6</td>
<td>7.1</td>
<td>26.3</td>
<td>3.4</td>
<td>58.6</td>
</tr>
<tr>
<td>(N = 29)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.3</td>
<td>4.0</td>
<td>19.6</td>
<td>6.3</td>
<td>64.8</td>
</tr>
<tr>
<td>(N = 64)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(a\) MILLAN samples either did not contain anilides or contained them below limits of detection. They are two adulterated but non-TOS-associated oils.

Source: Bernert (6) and Guitart (11).

Other minor components

Phospholipids
No attempt to study this fraction of the oils has been undertaken. However, Guitart (11) tentatively detected this fraction on TLC plates after phosphomolybdic acid spraying.

Pigments
The National Centre for Food and Nutrition\(a\) reported that in some cases, chlorophyll was added to the TOS-related oils to achieve the desired colour.

\(a\) Unpublished document ICP/RCE 905(1)/35 (op.cit.).
**Tocopherols**

Although not specifically quantified, the low stability to oxidation of TOS-related oils (4, 7) was interpreted as indicative of poor content of antioxidant, such as vitamin E.

The composition of tocopherols in some samples of TOS-related oils was determined at the Unilever Research Laboratorium at Vlaardingen in the Netherlands (Koch, G.K., personal communication, 1983) using a HPLC titration technique. The results obtained are shown in Table 15.

<table>
<thead>
<tr>
<th>Oil sample</th>
<th>Tocopherol (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>alpha</td>
</tr>
<tr>
<td>DMT 82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60</td>
</tr>
<tr>
<td>M3/763&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70</td>
</tr>
<tr>
<td>M3/957&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
</tr>
<tr>
<td>M3/1050&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
</tr>
<tr>
<td>P1/1555&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
</tr>
<tr>
<td>P1/1695B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31</td>
</tr>
<tr>
<td>P1/1695C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
</tr>
<tr>
<td>P1/1880&lt;sup&gt;b&lt;/sup&gt;</td>
<td>125</td>
</tr>
<tr>
<td>P1/1919&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
</tr>
<tr>
<td>X/82/2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31</td>
</tr>
<tr>
<td>X/82/15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34</td>
</tr>
<tr>
<td>X/82/27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
</tr>
<tr>
<td>XI/82/21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41</td>
</tr>
<tr>
<td>XI/82/23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Contained anilides.

<sup>b</sup> Either did not contain anilides or contained them below the limits of detection.

Source: Koch, G.K. personal communication, 1983.

**Basic Fingerprinting of TOS-related Oils**

This section includes those analyses carried out in an attempt to obtain characteristic profiles that would distinguish between TOS-related and edible oils. The only prerequisite for including a study as valid for
fingerprinting is that the whole oil was used. Alternatively, selected fractions purified in one step would also qualify. Thus, analyses undertaken to study one or more specific fractions of the samples (such as sterols, fatty acids or anilides) can be found elsewhere under the appropriate heading.

Analytical techniques other than chromatography or spectrometry have been evaluated for fingerprinting suspected oil samples (21). However, many of them lack sufficient resolution to obtain valuable data and/or definite conclusions.

**Thin layer chromatography**

TLC has been used extensively by the Institute of Fats and their Derivatives to establish differential patterns between edible and “toxic” oils. Initially (10, 13), conventional detection with ultraviolet radiation at 254 nm or iodine vapour were used, but these methods lacked sufficient sensitivity and sometimes gave unclear results. The use of selective chemical reactions for aniline (or other aromatic amines) with p-dimethylaminocinnamaldehyde, after acid hydrolysis of the anilides on the plate, gave better results and provided a means of distinguishing between TOS-related (defined as containing anilides) and edible oil samples (12, 22, 23). Interestingly, six of the spots detected in TOS-related oils gave aniline after acid hydrolysis, but only one corresponded to the fatty acid anilides; the chemical structure of the other five components remained unclear at the time (13). Soon after, one of these five spots was identified as the oleoyl diester of 3-aminophenyl-1,2-propanediol (12, 13, 24). These authors claim that the other four unidentified spots are probably hydrolysis products, positional isomers or oxidized derivatives of 3-aminophenyl-1,2-propanediol.

Guitart (11) detected several bands in TOS-related oils using phosphomolybdic acid or sulfuric acid spraying. Many of them were identified as the bands corresponding to phosphoglycerides, monoglycerides, di- and monohydroxylated free fatty acids, diglycerides, anilides, free sterols, free fatty acids, triglycerides and esterified sterols. However, no spot was found that was common to all TOS-related oils and that was absent from the edible oils. Some fluorescent compounds were detected on the plates at 360 nm, but again no differential pattern was established. Interestingly, after unsaturated anilides on the plate were exposed to 254 nm of radiation for approximately 20 minutes, a yellow-blue fluorescent compound appeared when the plate was exposed to 360 nm. This phenomenon was observed with pure standards.
of oleoyl, linoleoyl and arachidonyl anilides, and with TOS-associated oils rich in anilides.

High performance liquid chromatography

HPLC techniques, with either ultraviolet (UV) or fluorescence detection, have probably been the most successful and extensively used method for fingerprinting the oils (11, 14, 25–28).

Dabrio (14) assayed and published a method useful for determining the presence of fatty acid anilides by means of a non-aqueous reversed phase HPLC system coupled to a UV detector set at 254 nm. Apparently, the presence of anilide peaks was the only remarkable difference between TOS-related and edible oils.

Grimait & Albaigés (26) assayed different HPLC methods (normal and reversed phase). The reversed phase mode, using an octadecylsilica column and a solvent programme with mixtures of acetonitrile and tetrahydrofurane, and with UV detection at 254 nm, was the best choice for oil fingerprinting. The profile differences between an edible soybean oil and eight TOS-associated oils were concentrated on an early-eluting group of peaks (corresponding to the anilides) and on three major peaks eluting last in the chromatogram (unidentified compounds eluting in the zone corresponding to triglycerides). The eight TOS-related samples presented either one or both of these groups of peaks. Bonded polar phases, such as u-Bondapack-CN and u-Bondapack-NH₂, were also tested but only the first gave reliable results. Using hexane as solvent and the UV detector at 210 nm, Grimait & Albaigés (26) detected saturated and unsaturated glycerides and anilides, with no other trace impurities observed.

Using similar reversed phase conditions, Artigas et al. (25) evaluated more than 30 selected samples using UV at 254 nm and fluorimetric monitoring (313 and 500 nm for excitation and emission wavelengths). They concluded that all of the profiles were similar in pattern and that, in spite of quantitative differences reflecting the different composition of the oil mixtures, the only major additional feature was the extra peak that could be taken as a first indication of the presence of anilides. The study of methanolic extracts of one edible rapeseed oil and three TOS-associated oils, using a fluorimeter fixed at 278 and 335 nm, gave four different patterns, with some fluorescent peaks. They concluded, however, that: "The appearance of these fluorescent components relative to the pure oil sample has to be considered again as representative of compositional differences within the samples rather than indicative of a
poison or toxic substance, as there is no common denominator in the three patterns". The lack of proper reference material was also claimed in the comparison of profiles of a TOS-associated and a mixture of edible oils (sunflower, rapeseed and olive) chromatographed on a normal phase column (u-Porasil) and detected with UV at 254 nm. Guitart (11), using reversed phase HPLC conditions similar to those described by Artigas et al. (25) and analysing several TOS-related and edible oils, confirmed the results and conclusions of these authors. Size exclusion chromatography profiles of control oils compared with contaminated cooking oils indicated the presence of some unusual components, identified by spectroscopic and mass spectrometric techniques as anilides, as reported by Wheals et al. (28).

Gas–liquid chromatography
Grimalt & Albaigés (26) used a high efficiency glass capillary column coated with SE-52 (coupled to a FID), with direct injection of samples, to observe possible differences between oils. They concluded that, after peak-by-peak comparison, only those peaks attributed to the anilides were different between the original and noncommercialized aniline-denatured rapeseed oil (RAPSA) and one edible rapeseed oil. No other samples were reported assayed by this method.

Spectrometry
Ultraviolet and visible light spectrometry (200–800 nm) have been the spectrometric techniques preferred for fingerprinting oils. In an exhaustive study, Alvarez Rodriguez et al. (29, 30) concluded that the application of such techniques to TOS-related and edible oils allowed them to infer whether a vegetable oil was pure, and approximately if an "olive" oil sample could be related to TOS. The last is based on a shift of the maximum absorbance at 666 nm in olive oils (probably due to chlorophylls) to a lower wavelength in oils related to TOS, and also on the increase in absorbance at 230 nm due to the anilides.

Constituents Related to Aniline Denaturation

Aniline
Aniline in trace amounts (<30 ppm) was one of the first extraneous compounds detected in TOS-suspected oil samples (2, 31, 32). As already well accepted, aniline comes from the 2% aniline originally added as a denaturant to the rapeseed oil.
In analyses carried out at the Institute of Fats and their Derivatives (4), all 36 TOS-related oil samples containing anilides in concentrations ranging from 3 to 2037 ppm were found to contain from traces (<0.5 ppm) up to 1.1 ppm free aniline. The same report gives a concentration of 16 069 and 6479 ppm of aniline, respectively, for samples of bulk unrefined denatured rapeseed oil from the RAPSA and ITH refineries. On the other hand, only traces of aniline (<0.5 ppm) remain in the ITH sample after refining.

A study of the analyses of 72 samples of oil collected in the oil exchange operation, carried out by the Central Regional Agricultural Laboratory in Madrid, shows aniline concentrations in these oils ranging from 0.5 to 15 ppm (33). Of the 72 samples, aniline was present in 39, of which 32 were related to families with TOS patients. Of the 33 samples not containing traces of aniline, only nine come from families with patients. Thus, a correlation seems to exist between aniline and TOS.

The low content of aniline in samples of refined oils, and of oils removed from the commercial routes of distribution relative to the 2% added to imported crude rapeseed batches, indicates that aniline was removed both upon refining and later by reactions with suitable oil constituents, as discussed below. It was also diluted upon mixing of refined rapeseed oil with other oils and fats (1), as stated above.

Anilides
The reaction of aniline with the acylglycerides and free fatty acids in rapeseed oil to generate the corresponding fatty acid N-phenylamides, collectively known as anilides, is now well established. These reactions took place spontaneously during transportation and/or storage, as well as in refining (see page 117). Fatty acid anilides, which unlike aniline were present in suspect oil samples in concentrations up to 2000 ppm (4, 7, 34), were considered reliable indicators of toxic oils (31). The crude RAPSA and ITH oils containing 16 069 and 6479 ppm, respectively, of aniline (2) also contained 20 461 and 11 506 ppm, respectively, of anilides. However, the content of anilides in ITH oil samples dropped to 1846 ppm after refining.

The total anilide content of some TOS-related oils is shown in Table 16. As indicated, the anilide content is highly variable within samples. Moreover, the lack of information about the exact origin of samples, especially in the earlier studies, can introduce some confusion.

---

an ITH is the acronym for Industria Trianera de Hidrogenación, a firm in Seville that also handled the oil.
in view of the zero content in anilides in several samples, thus question-
ing whether anilide (used as indicative of the original aniline-denatured
rapeseed oil) is the best marker for the illness. However, a more recent
evaluation of the content of fatty acid anilides in 195 oil samples
implicated in TOS, including the 29 high probability “case” and 64
“control” oils (6), supports the general opinion that anilides are still
the most useful markers of case-related oil samples, having been
detected in 62% of case oils in concentrations ranging from 30 to 2000
ppm.

Guitart (11) and Bernert et al. (6) studied the anilide composition of
TOS-related oil samples in detail. Guitart studied this fraction by GC-
FID on a capillary column coated with OV-1 after TLC purification;
this technique tentatively detected the anilide of the trans isomer of
18:1 n-9 fatty acid. Bernert et al. (10) used a reversed phase HPLC
method with UV detection at 243 nm that allowed the separate quantifi-
cation of the polyunsaturated C18 fatty acid anilides (namely 18:2 and
18:3). In both cases, the 17:0 anilide was used as the internal standard.
The results are shown in Table 17.

Reversed phase HPLC methods were also used by Wheals et al.
(28) to characterize oleic, linoleic, linolenic and stearic anilides in TOS
oils. On the other hand, capillary gas chromatography was used by
Balley et al. (35) for the determination of fatty acid anilides in aniline-
adulterated oils, but with oleoyl-orthotoluidine as the internal standard.
Since the anilides of oleic, linoleic and linolenic acid were found to
co-elute on the capillary column, GC-MS analysis was needed to verify
that oleoyl anilide was the major anilide component. These results
confirmed earlier GC-MS work by Diachenko et al. (36), who extracted
the oil samples according to the method of the Association of Official
Analytical Chemists for pesticides in fatty foods.

From the very first stages of research into the etiology of TOS, an
effort was made to establish the toxicity of anilides both in vitro and in
vivo. For this purpose, the anilides of oleic and linoleic acids were those
preferentially synthesized for toxicological studies. However, since di-
rect reaction of the corresponding free fatty acids with aniline requires
temperatures of the order of 150°C for 24 hours (37), an alternative
procedure involving N,N′-dicyclohexylcarbodiimide (DCC), a condens-
ing agent widely used in the preparation of amides, was favoured by
some authors (38, 39).

Unfortunately, this procedure was later found to be responsible for
the formation of an unsuspected by-product, N-cyclohexyllinoleamide,
### Table 16. Anilide content of TOS-related samples

<table>
<thead>
<tr>
<th>Oil sample</th>
<th>Concentration^a</th>
<th>Oil sample</th>
<th>Concentration^a</th>
<th>Oil sample</th>
<th>Concentration^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
<td>3-J</td>
<td>576</td>
<td>1-1</td>
<td>60,000</td>
</tr>
<tr>
<td>2</td>
<td>38</td>
<td>4-J</td>
<td>3</td>
<td>2-1</td>
<td>66,000</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>5-J</td>
<td>343</td>
<td>1A</td>
<td>55,000</td>
</tr>
<tr>
<td>4</td>
<td>2,037</td>
<td>6-J</td>
<td>165</td>
<td>AG-1</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>305</td>
<td>7-J</td>
<td>708</td>
<td>AG-2</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td>8-J</td>
<td>1,449</td>
<td>AG-3</td>
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</tr>
<tr>
<td>7</td>
<td>95</td>
<td>9-J</td>
<td>5</td>
<td>BDR/81</td>
<td>850</td>
</tr>
<tr>
<td>8</td>
<td>ND</td>
<td>10-J</td>
<td>6</td>
<td>DMT 82</td>
<td>528</td>
</tr>
<tr>
<td>9</td>
<td>70</td>
<td>11-J</td>
<td>ND</td>
<td>F-1</td>
<td>10</td>
</tr>
<tr>
<td>10-A</td>
<td>32</td>
<td>A.V.P</td>
<td>440</td>
<td>F-2</td>
<td>20</td>
</tr>
<tr>
<td>10-B</td>
<td>109</td>
<td>C.B.</td>
<td>1,652</td>
<td>F-3</td>
<td>1,800</td>
</tr>
<tr>
<td>11</td>
<td>614</td>
<td>M.C.</td>
<td>1,350</td>
<td>F-4</td>
<td>1,900</td>
</tr>
<tr>
<td>12-A</td>
<td>1,456</td>
<td>RAPSA</td>
<td>20,461</td>
<td>F-5</td>
<td>10</td>
</tr>
<tr>
<td>12-B</td>
<td>797</td>
<td>ITH-C</td>
<td>11,506</td>
<td>F-6</td>
<td>10</td>
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<tr>
<td>13</td>
<td>581</td>
<td>ITH-R</td>
<td>1,846</td>
<td>JMM</td>
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<tr>
<td>14</td>
<td>1,018</td>
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<td></td>
<td>M3/763</td>
<td>68</td>
</tr>
<tr>
<td>15-A</td>
<td>15</td>
<td></td>
<td></td>
<td>M3/957</td>
<td>971</td>
</tr>
<tr>
<td>15-B</td>
<td>1,228</td>
<td></td>
<td></td>
<td>M3/1050</td>
<td>31</td>
</tr>
<tr>
<td>16</td>
<td>185</td>
<td></td>
<td></td>
<td>M3/1136</td>
<td>10</td>
</tr>
<tr>
<td>17</td>
<td>1,566</td>
<td></td>
<td></td>
<td>P1/1555</td>
<td>90</td>
</tr>
<tr>
<td>18</td>
<td>ND</td>
<td></td>
<td></td>
<td>P1/1695B</td>
<td>&lt;5</td>
</tr>
<tr>
<td>1-C</td>
<td>ND</td>
<td></td>
<td></td>
<td>P1/1695C</td>
<td>2,650</td>
</tr>
<tr>
<td>2-C</td>
<td>ND</td>
<td></td>
<td></td>
<td>P1/1880</td>
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<tr>
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<td>P1/1919</td>
<td>17</td>
</tr>
<tr>
<td>4-C</td>
<td>27</td>
<td></td>
<td></td>
<td>SHG</td>
<td>&lt;5</td>
</tr>
<tr>
<td>5-C</td>
<td>48</td>
<td></td>
<td></td>
<td>X/82/2</td>
<td>2,500</td>
</tr>
<tr>
<td>1-F</td>
<td>27</td>
<td></td>
<td></td>
<td>X/82/6</td>
<td>1,773</td>
</tr>
<tr>
<td>2-F</td>
<td>332</td>
<td></td>
<td></td>
<td>X/82/7</td>
<td>1,315</td>
</tr>
<tr>
<td>3-F</td>
<td>977</td>
<td></td>
<td></td>
<td>X/82/15</td>
<td>875</td>
</tr>
<tr>
<td>4-F</td>
<td>90</td>
<td></td>
<td></td>
<td>X/82/27</td>
<td>1,587</td>
</tr>
<tr>
<td>5-F</td>
<td>104</td>
<td></td>
<td></td>
<td>XI/82/21</td>
<td>&lt;5</td>
</tr>
<tr>
<td>1-J</td>
<td>ND</td>
<td></td>
<td></td>
<td>XI/82/23</td>
<td>1,086</td>
</tr>
<tr>
<td>2-J</td>
<td>613</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Source: Ventura Diaz (4).

^b Source: Koch, G.K., personal communication, 1983.

**Note.** The RAPSA and the two ITH samples (and probably also 1-1, 2-1 and 1A) correspond to aniline-denatured rapeseed oil. ITH-C indicated crude oil, while ITH-R indicated refined oil. ND = Not detected.
Table 17. Fatty acid anilide composition (ppm) of some TOS-related and case and control oils

<table>
<thead>
<tr>
<th>Oil sample</th>
<th>Anilide&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:0</td>
</tr>
<tr>
<td><strong>TOS-related</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>JEN B</td>
<td>18</td>
</tr>
<tr>
<td>M3/957</td>
<td>30</td>
</tr>
<tr>
<td>TOX 1</td>
<td>17</td>
</tr>
<tr>
<td>MILLAN-150</td>
<td>0</td>
</tr>
<tr>
<td>MILLAN-300</td>
<td>0</td>
</tr>
<tr>
<td>TOX 2</td>
<td>0</td>
</tr>
<tr>
<td><strong>Case and control</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Case (N = 29)</td>
<td>54.2</td>
</tr>
<tr>
<td>Control (N = 64)</td>
<td>9.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> cis 18:1 isomer = oleic acid; trans 18:1 isomer = elaidic acid.

<sup>b</sup> Source: Guitart (11).

<sup>c</sup> Source: Bernert (6).

<sup>d</sup> NQ = Not quantified.

of unknown toxicity in itself, which could lead to confusion in the interpretation of toxicity tests (40). Some batches of anilides used in toxicity screening procedures contained this substance in concentrations of 5–25%. Thus, the purity of any toxicant used for toxicological screening should be ascertained prior to experimental work to ensure meaningful results, even though in this case indirect evidence indicated that this particular by-product would not be toxic to rats (41).

To avoid discrepancies in reported values from different laboratories, analytical procedures should be properly validated. For instance, two different oil samples analysed independently for anilides at the Unilever Research Laboratorium, The British Industrial Biological Research Association (BIBRA) and the National Centre for Food and Nutrition were found to contain <5 and 2650 ppm, 0 and 1750 ppm, and 1600 and 4500 ppm, respectively. For three other samples in which anilides were not detected at BIBRA, analyses carried out at Unilever showed 0, <5 and 1587 ppm. Chromatographic interferences in the determination of fatty acid anilides by GC in soya oil samples were acknowledged by the staff at the National Centre for Food and Nutrition (42). In view of these results, measurement of fatty acid anilides in TOS oil samples should preferentially include pre-extraction and concentration
steps by solid-phase extraction procedures, such as silica (43) or reversed phase cartridges (6), prior to HPLC or GC analysis.

Other detected or hypothetical products
Aside from aniline and the fatty acid anilides, several other nitrogenous and non-nitrogenous compounds, in principle extraneous to an edible oil, have been detected in TOS oil samples. Most of these are found at trace levels, and in several cases their presence can be related to the use of aniline as an oil denaturant. The list includes azobenzene, methylanilines, bromoaniline, formylanilide, acetylanilide, nitrobenzene, quinolines, bromodialkylbenezene, iminoquinones and phenylisocyanates (34, 44, 45). From the toxicological point of view, however, directly implicating them in the etiopathogenesis of TOS would be difficult as their toxicity in rats is known to be low (LD₅₀ values ranging from 500 to 1300 mg/kg).

Preliminary toxicological data are also available for compounds such as the oleoyl diester of 3-aminophenyl-1,2-propanediol, which was synthesized together with the corresponding mono- and dioleoyl esters (46) and which was toxic to rats when administered intraperitoneally.

Air oxidation of aniline at room temperature gives rise to 2,5-dianilino-N-phenyl-p-benzoquinoneimine and 2-amino-5-anilino-N-phenyl-p-benzoquinoneimine (47). At higher temperatures (110–180 °C) azobenzene is also formed. All of these oxidation products are produced in greater quantities under the concurrent effect of UV radiation, and have been identified in denatured rapeseed and TOS oil samples (19, 48). These authors also reported on the tentative mass spectrometric identification of a further aniline-oxidized product that could have the structure of N′-(N,N′-diphenyl-p-benzoquinoneimine)-p-phenylenediamine, with a molecular weight of 531. Iminoquinone derivatives were considered an important associated pathogenic factor by Pestana & Munoz (32).

The formation of various types of oxidation derivatives of aniline could account for that fraction of combined aniline not found as fatty acid anilides, as reported by Vázquez Roncero et al. (13, 22, 49) in a detailed study of the reactions of free aniline during refining, storage and transport of aniline denatured oils discussed below.

Based on the known chemistry of sulfur compounds in rapeseed oil (50, 51), Kammüller et al. (52) proposed that 2-hydroxy-3-butenyl isothiocyanate, generated from the glucosinolate progoitrin (5-vinyl-2-oxazolidinethione), may have reacted instead with the aniline present in
TOS oils, producing the corresponding \(N\)-(2-hydroxy-3-butenyl)-\(N'\)-phenylthiourea. The latter might cyclize during refining of oil to 1-phenyl-5-vinyl-2-imidazolidinethione (IZT) and, as the authors claim, this compound could be a potential inducer of allergic and autoimmune-like diseases. The whole hypothesis seemed to be supported by their identification of IZT in one out of fourteen “case-related” oils, but in this case concentrations of IZT were only about 1 ppm. Two major weaknesses of the hypothesis, aside from the low occurrence and concentration of IZT in TOS oil samples, rest on both epidemiological and chemical grounds. From the epidemiological point of view, Abaitua & Posada at FIS in Madrid have established that the oil samples used in this study were not valid for drawing conclusions about the presence of IZT in case-related oils. On the other hand, Bernert and co-workers at the Centers for Disease Control in Atlanta, USA, came to the conclusion that the product characterized as IZT by Kammüller et al. was in fact 2-anilino-5-vinyl-2-thiazoline (AVT), which they have been unable to detect at the 2.5 ppm level in 13 samples from the epidemiologically defined “case” group (53).

In line with these observations, the sulfur-containing compounds appear to be present in very low amounts and thus could not account for any process of direct toxicity. Furthermore, in accordance with the stoichiometry of thiourea formation, only approximately 10% of the aniline nitrogen would be chemically combined with sulfur, leaving the major part of aniline free to undergo anilide formation and/or oxidative reactions of the type mentioned above.

Evidence of hydroperoxides from the fatty acid anilides had also been suggested by Dobarganes García & Gutiérrez González-Quijano (19), Vioque & Vioque (34) and Guitart (11), and recently unequivocally confirmed with mass spectrometric data (43). In the latter case, two epoxy-hydroxylated derivatives of the anilide of linoleic acid were obtained using a model of accelerated oxidation in a stream of air. These were identified as \(N\)-phenyl-9,10-epoxy-11-hydroxy-12-octadecenamide and \(N\)-phenyl-12,13-epoxy-11-hydroxy-9-octadecenamide. The same compounds were identified in rapeseed oil samples supplemented with the anilide of linoleic acid, \(N\)-phenyllinoleamide, and then submitted to accelerated oxidation. Likewise, the accelerated oxidation of linoleic acid, one of the main constituents of the oil samples, gave rise to the 9,10- and 12,13-epoxy isomers of 11-hydroxy-12-octadecenoic acid. Interestingly, both groups of derivatives, with and without the \(N\)-phenyl moiety, are structurally
related to a neutrophil-derived leukotoxin such as 9,10-epoxy-12-octadecenoate (55).

**Aniline reactions during oil transport, storage and/or refining**

From the point of view of chemical reactivity, aniline in oil would react preferentially with the triglyceride constituents of an oil sample to give the corresponding fatty acid anilides. This would be a more favourable and thus higher yield process than direct reaction with free fatty acids, which requires temperatures well above those occurring in transportation or storage (37). This is one of the reasons why condensing agents such as DCC, as mentioned above, were initially used in synthesis procedures. Along these lines, and on account of the high anilide content (up to 60 000 ppm) in some of the crude rapeseed oil samples, spontaneous formation of fatty acid anilides during transportation and storage would indeed occur readily at ambient temperatures, if given enough time. This is confirmed, for instance, by analyses of four imported crude rapeseed oils denatured with aniline, as shown in the data in Table 18, which are taken from the work of Vázquez Roncero et al. (22).

Data given in Table 18 indicate that in about eight months, the concentration of anilides increased by a factor of 2.5–2.8 while aniline content concomitantly decreased. At the relatively low temperatures (30–50 °C) and given sufficient time (6–8 months) up to 33% of added aniline could react to produce anilides in a concentration of 25%.

At higher temperatures, detectable amounts of anilides can be obtained in a matter of hours. After subjecting a sample of refined rapeseed oil denatured with 2% aniline to 190 °C for 6 hours, Vázquez Roncero et al. (22) detected 2693 ppm anilides; that is, 3.5% of added aniline was in the form of anilides. The rest (18 548 ppm) remained as free aniline. Confirmation of spontaneous anilide formation was obtained by the same authors in both static and stirring tests, as well as in tests carried out with labelled aniline. In one case, mixing refined rapeseed oil with 2% free fatty acids and 2% aniline in a glass container at 37 °C for 10 days produced 1098 ppm anilide, whereas only traces were reported under the same conditions but in the absence of the 2% free fatty acids (neutral refined oils). When the temperature was raised to 50 °C, 18 265 ppm was produced in only seven days. The authors conclude that anilides are easily formed at temperatures of 30–50 °C by reaction of free fatty acids with aniline. However, as stated above, direct reaction with free fatty acids is not a very favourable chemical
Table 18. Spontaneous formation of anilides in oil samples

<table>
<thead>
<tr>
<th>Oil sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Date analysed</th>
<th>Aniline (ppm)</th>
<th>Anilide (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude ITH</td>
<td>9 September 1981</td>
<td>6 480</td>
<td>13 000</td>
</tr>
<tr>
<td>RAPSA 1</td>
<td>12 November 1981</td>
<td>16 070</td>
<td>24 900</td>
</tr>
<tr>
<td>RAPSA 2A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20 May 1982</td>
<td>10 060</td>
<td>33 080</td>
</tr>
<tr>
<td>RAPSA 2B&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20 May 1982</td>
<td>6 700</td>
<td>36 690</td>
</tr>
</tbody>
</table>

<sup>a</sup> Import records indicated that the four samples belonged to a single batch stored in June 1981 at RAPSA warehouses.

<sup>b</sup> Kept in iron containers.

<sup>c</sup> Kept in underground cement depots.

process, so that the reaction is most likely based on a process of acid-catalysed anilinolysis. In this regard, data by Koch (34) indicate that 90% of the anilides were derived from triglycerides and only 10% from free fatty acids.

A study on the fatty acid and anilide composition of TOS oil samples (22) showed a higher proportion of oleic acid than linoleic acid in anilides, in line with the observations of Bernert et al. (6). However, in work mimicking storage, transport and refining conditions, anilides seemed to contain more linoleic acid than the original acids (22), so that the unsaturated acids are those preferentially reacting with aniline. In general, as stated by Bernert et al., the fatty acid ratios in anilides appeared consistent with the formation of these products in crude rapeseed oils, with the admixture of other oils occurring later. Not only can these reactions be spontaneous during transport and/or storage, but they can certainly also take place during processing, as shown independently by Vázquez Roncero et al. (22) and Koch (34). In this regard, Vázquez Roncero et al. (22) showed that during neutralization and bleaching, the concentration of aniline gradually decreases while that of anilides stays more or less constant or even increases slightly, reflecting the balance between formation de novo and loss through processing. During deodorization at 220–280 °C, aniline is almost completely removed and anilides are reduced to 15% of their original concentrations.

Early work on simulating conventional refining procedures carried out at Unilever Research Laboratorium confirmed these observations (34). In this case, using <sup>3</sup>H-labelled aniline added to oil samples, neutralization at 80–85 °C by aqueous washings removed 20% of the added aniline, while bleaching at about 80–120 °C under negative pressure.
plus filtration removed most of the radioactivity, with only 7.4% remaining in the oil. This corresponded to 1500 ppm radioactive aniline. Further deodorization at 220 °C and 1–3 mmHg for three hours, as usually carried out in conventional refining of edible oils, did not remove any more aniline. On the other hand, direct deodorization was found 100% effective in removing all free aniline, indicating that the 7.0% radioactivity remaining when deodorization is preceded by bleaching should be accounted for by reaction products of aniline.

However, depending on the facilities and conditions in a given oil-processing plant, complete elimination of all volatile substances might not be achieved in the deodorization step. According to the information gathered by the Special Investigation Team of the Interministerial Commission (33), the various refineries involved in the processing of crude rapeseed oil samples for the distributors effected deodorization at temperatures from 170 to 230 °C under 2–20 mmHg negative pressure, except in one case where the operation took place at <1 mmHg (Table 19). Thus, a few parts per million of aniline in the refined oils would not be totally unexpected. For instance, the aniline content of refined rapeseed oil in some tests was 80 ppm at Danesa-Bau (a firm in Madrid that handled denatured rapeseed oil) and 40 ppm at ITH, a result consistent with the higher temperature, lower pressure and longer deodorization time at ITH.

In another test, the same two plants receiving crude rapeseed oils from RAPSA produced refined oils with 53.5 and 25.9 ppm aniline equivalents (aniline plus anilide derivatives), respectively. In addition, a sample taken at the bleaching stage at ITH had 6.7 ppm anilide and 24,636 ppm oleoyl anilide. Comparing these figures with the values of fully refined oil, it is evident that whereas most of the aniline is removed upon bleaching, the anilide content stays relatively high, probably reflecting the balance between removal and new synthesis. Finally, deodorization drastically reduced the concentration of anilides.

**Constituents other than Aniline and Aniline Derivatives**

Many other constituents have been reported either present or absent by a number of investigative groups. However, in the majority of these reports the problems arising from the small number and uncertified origin of the oil samples make the interpretation of many such findings more difficult than in reports dealing with normal constituents, fingerprinting, or aniline and its derivatives. In addition, lack of detail on the
Table 19. Synopsis of the process of refining crude rapeseed oil

<table>
<thead>
<tr>
<th>Operation</th>
<th>Parameter</th>
<th>Danesa-Bau&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ITH&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Giresa</th>
<th>M. De Pablos</th>
<th>P. Alonso</th>
<th>A. Tarrega&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Valencia</th>
<th>Sabater&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degumming, purification</td>
<td>Temperature</td>
<td>85°C</td>
<td>80°C</td>
<td>6-8°C</td>
<td>80°C</td>
<td>900°C</td>
<td>-</td>
<td>70°C</td>
<td>40°C</td>
</tr>
<tr>
<td></td>
<td>Concentration, PO</td>
<td>0.1%</td>
<td>0.15%</td>
<td>0.2%</td>
<td>0.1-0.2%</td>
<td>-</td>
<td>-</td>
<td>0.1-0.2%</td>
<td>0.1%</td>
</tr>
<tr>
<td></td>
<td>and H3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutralization</td>
<td>Temperature</td>
<td>90°C</td>
<td>80°C</td>
<td>10-80°C</td>
<td>85°C</td>
<td>70°C</td>
<td>70°C</td>
<td>80°C</td>
<td>80°C</td>
</tr>
<tr>
<td></td>
<td>Concentration, NaOH</td>
<td>20°Be&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20°Be</td>
<td>20°Be</td>
<td>16-20°Be</td>
<td>18-20°Be</td>
<td>18-26°Be</td>
<td>20°Be</td>
<td>20-22°Be</td>
</tr>
<tr>
<td>Decolorization, bleeding</td>
<td>Temperature</td>
<td>90-100°C</td>
<td>110°C</td>
<td>110°C</td>
<td>100-120°C</td>
<td>80°C</td>
<td>80-120°C</td>
<td>90-100°C</td>
<td>90°C</td>
</tr>
<tr>
<td></td>
<td>Pressure Earth</td>
<td>20-25 Torr</td>
<td>60 Torr</td>
<td>20 Torr</td>
<td>20 Torr</td>
<td>3%</td>
<td>3%</td>
<td>3-5%</td>
<td>1.5-2%</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>2%</td>
<td>1-3%</td>
<td></td>
<td>0.5-1%</td>
<td>3%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deodorization, Time</td>
<td>Temperature</td>
<td>190-200°C</td>
<td>230°C</td>
<td>170-200°C</td>
<td>200-220°C</td>
<td>170-18°C</td>
<td>180°C</td>
<td>220°C</td>
<td>190°C</td>
</tr>
<tr>
<td></td>
<td>Pressure Time</td>
<td>20 Torr</td>
<td>7-10 Torr</td>
<td>0 Torr</td>
<td>2-3 Torr</td>
<td>5 Torr</td>
<td>8 Torr</td>
<td>7 Torr</td>
<td>4 Torr</td>
</tr>
<tr>
<td></td>
<td>4 h</td>
<td>4½ h</td>
<td>12 h</td>
<td>2 h</td>
<td>5 h</td>
<td>4-6 h</td>
<td>4-5 h</td>
<td>4 h</td>
<td></td>
</tr>
<tr>
<td>Winterization</td>
<td></td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

<sup>a</sup> Not so clearly case-related.
<sup>b</sup> Most clearly case-related.
<sup>c</sup> No relation with TOS cases (Catalonia).
<sup>d</sup> Be = Baumé, an arbitrary scale of specific gravities.
<sup>e</sup> Torr = unit of pressure in measuring partial vacuum, equal to 1/760 of standard atmosphere.
detection limits of the specific laboratory procedures used in some cases enhance this problem.

For the record, and even though the reliability of some of the reports on other extraneous constituents is at best dubious, a summary of the data published on several classes of compounds is included below. In certain of these cases the data reported can be considered valid, while in other cases they would require validation by an independent laboratory.

**Pesticides and related compounds**

The presence of pesticides (no specifications given about the type or number of samples analysed) could not be established at the National Centre for Food and Nutrition in Majadahonda (56).

**Herbicides**

TOS-related oil samples have been analysed for diquateryninary paraquat, which is very poorly soluble in oil. This herbicide was not detected by the US Federal Drug Administration (detection limit of 1 ppm) in five TOS-associated samples analysed in 1981 (57). The report of the National Institute of Toxicology in Seville (58), covering an unspecified number of oils and without mention of the limit of detection, was negative for paraquat. Kimbrough reached similar conclusions (59). Artigas et al. (60) analysed three oils using HPLC (UV detection) and GC/MS. The concentration of paraquat, if present, was lower than 0.5 ppm, and such low levels could not explain the toxic effects observed in humans. Paraquat was not present in toxicologically significant concentrations in four samples of blood, serum and urine taken from TOS patients (61).

**Organochlorine compounds**

The detectable amounts of organochlorine pesticides found by the National Centre for Food and Nutrition (62, 63) were considered normal for this kind of sample (64).

Hexachlorobenzene (HCB) was tentatively identified and quantified in five samples of TOS-related oils (57). However, the concentrations detected were low, ranging from <0.005 to 0.1 ppm.

Beaubernard et al. (65) found no pesticides in a TOS-associated oil and in an edible rapeseed oil, apart from lindane at low concentration (<0.1 ppm) in both samples.

Polychlorinated biphenyls (PCBs) were said to have been searched for in TOS oil samples but not detected (66). In a recent study, Guitart et al. (67) detected PCBs of the Aroclor 1254 type in one TOS-related
sample at concentrations of 3–4 ppm. More importantly, the analyses for PCBs carried out on 14 case and control samples detected free pentachlorophenol (PCP) in some of the oils at concentrations ranging from 0.3 to 2.3 ppm. Methylated PCP was also tentatively identified in those samples. Rather surprisingly, the five control and one of nine case oils contained these chlorophenols, which complicates the interpretation of this finding.

**Organophosphorus compounds**

No organophosphorus insecticides were detected by the National Centre for Food and Nutrition (62, 63). An earlier report mentioned that the residue levels of these compounds in samples of oils were “normal” (56).

Orthocresolphosphate (triorthocresyl phosphate, TOCP) was not found in TOS-related oils (9, 31, 68).

Early studies on cholinesterase activity in TOS patients at Niño Jesús Hospital in Madrid found normal levels, thus eliminating organophosphorus insecticides as the cause of the disease (9). Organophosphorus insecticides were not found at toxicologically significant concentrations in more than 20 biological samples from TOS patients (61).

In an assay of four oils – a TOS-associated oil containing 700 ppm anilides, an olive oil, an oil mixture and an oil mixture containing 700 ppm anilides – no statistical difference in inhibition of acetylcholinesterase activity was found between them (69).

**Hydrocarbons**

Mineral oil was analytically confirmed to be absent in the unsaponifiable fraction of several TOS-related samples (9, 56, 62–64, 66). No lubricating oil was detected in a TOS-related oil sample by Beaubemard et al. (65). Cutting oils were also absent (9).

Vioque et al. (20) detected an abnormally high unsaponifiable content in one noncommercialized aniline-denatured rapeseed oil (RAPSA B, from a subterranean vitrified concrete container at San Sebastián). After careful study of this fraction by TLC, GC, elemental analysis and infrared spectrophotometry, they concluded that the sample contained approximately 3–4% mineral oil. Another sample (RAPSA A, stored in metal containers) also contained mineral oil but at a lower, unspecified concentration. Afterwards, Vioque & Ventura (70) analysed the mineral oil content of commercial samples of TOS-associated oils. The lubricating oils Ertex 21, Ertex 31 and Spindle 80/90 (from RAPSA at
San Sebastián) were used as standards (Table 20). They tentatively concluded that Ertex 21 was the mineral oil present in the TOS samples.

Unilever Research Laboratorium (Koch, G.K., personal communication, 1983) determined mineral oil content only in those samples of TOS-related oils that contained more than 1000 ppm anilides (Table 21). Using the three Spanish mineral oils mentioned above as standards, they found that the contamination detected in some samples was of the heavy lubricating oil type. These concentrations agree with the values obtained by the Institute of Fats and their Derivatives.

The results of Vioque & Ventura and Koch introduced more confusion about the origin of samples, as no clear correlation between concentrations of anilides and mineral oil was found. Thus, samples with high concentrations of anilides (e.g. sample P-1/1.695C, with 2265 ppm) contained no detectable amounts of mineral oil, while others that did not contain anilides (e.g. sample VLL-82) contained 1000 ppm mineral oil.

Using GC/MS, Artigas et al. (25, 71) also identified some straight-chain saturated hydrocarbons (from 19 to 23 carbon atoms) in two of three TOS-related samples investigated. However, these compounds were not quantified.

**Mycotoxins**

Mycotoxins originating from grapeseed (one of the most typical oils in the TOS samples, see Table 11) were rejected as a possible cause of the illness (7).

Borregón Martínez (56), Vioque (1) and Tabuenca Oliver (9) reported that aflatoxins were not present in oils (unspecified number of samples analysed).

Beaubernard et al. (65) reported that mycotoxins (no specification about the compound(s) analysed) were not present in a TOS oil sample.

The WHO Working Group on the Toxic Oil Syndrome (31) reported that studies on the possible presence of mycotoxins (mainly trichotheccenes and cytochalasins) were undertaken at that time, but indicated that preliminary findings were negative.

**Metals and nonmetals**

Except for iron, metals were not found in suspected samples (no specification of the number of samples analysed) by the National Centre for Food and Nutrition (56, 62). Using atomic absorption spectrometry, the Centre found no evidence of arsenic, calcium, chromium, copper, lead,
### Table 20. Concentrations of mineral oil in samples of TOS-related oils containing anilides (left column) and in samples not containing anilides or containing them below the level of detection (right column)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mineral oil (ppm)</th>
<th>Sample</th>
<th>Mineral oil (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Fr</td>
<td>900</td>
<td>3-OV</td>
<td>900</td>
</tr>
<tr>
<td>2-Fr</td>
<td>700</td>
<td>AG-1</td>
<td>900</td>
</tr>
<tr>
<td>3-Fr</td>
<td>1 100</td>
<td>AG-2</td>
<td>800</td>
</tr>
<tr>
<td>4-Fr</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AG-3</td>
<td>700</td>
</tr>
<tr>
<td>2-OV&lt;sup&gt;b&lt;/sup&gt;</td>
<td>900</td>
<td>F-1</td>
<td>ND</td>
</tr>
<tr>
<td>BDR/81</td>
<td>700</td>
<td>F-2</td>
<td>ND</td>
</tr>
<tr>
<td>C.B.</td>
<td>700</td>
<td>F-5</td>
<td>ND</td>
</tr>
<tr>
<td>DMT-82</td>
<td>900</td>
<td>F-6</td>
<td>ND</td>
</tr>
<tr>
<td>F-3</td>
<td>ND</td>
<td>M3/1136</td>
<td>ND</td>
</tr>
<tr>
<td>F-4</td>
<td>ND</td>
<td>P1/1880</td>
<td>ND</td>
</tr>
<tr>
<td>JMM</td>
<td>1 200</td>
<td>P2/856</td>
<td>ND</td>
</tr>
<tr>
<td>M1/1882</td>
<td>900</td>
<td>SHG</td>
<td>ND</td>
</tr>
<tr>
<td>M3/763</td>
<td>ND</td>
<td>VLL-82</td>
<td>1 000</td>
</tr>
<tr>
<td>M3/957</td>
<td>1 300</td>
<td>X/82/15</td>
<td>900</td>
</tr>
<tr>
<td>M3/1050</td>
<td>ND</td>
<td>X/82/21</td>
<td>1 300</td>
</tr>
<tr>
<td>P1/1695C</td>
<td>ND</td>
<td>XI/82/25</td>
<td>800</td>
</tr>
<tr>
<td>P1/1919</td>
<td>800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRM</td>
<td>800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGH-1</td>
<td>600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X/82/2</td>
<td>800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X/82/6</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X/82/27</td>
<td>900</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XI/82/23</td>
<td>800</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XI/82/26</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XI/82/27</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> ND = Not detected.

<sup>b</sup> It is not clear from the source the content of anilides in sample 2-OV.

Source: Vioque & Ventura (70).

nickel or vanadium; only iron was positive (63). An initial report (56) indicated that analyses were carried out for the above-mentioned elements except calcium, plus mercury and zinc; only iron was detected at abnormal concentrations (between 6 and 15 ppm).

In an early study carried out in 1981 (57), a US Food and Drug Administration laboratory examined the elemental composition of 14 samples of TOS-associated oils. These samples were examined by instrumental neutron activation analysis and inductively coupled argon plasma emission spectroscopy, while three samples were examined by...
energy-dispersive X-ray fluorescence spectrometry. The results are summarized in Table 22. These analyses did not indicate unusual levels of any of the elements examined for, except for the high levels of chlorine and bromine in one sample. However, the significance of this study is unclear in view of the reports that indicate that only two of the 14 samples analysed contained oleoyl anilide at a level higher than 150 ppm, which was the limit of detection, while two other samples probably also contained anilides but at levels slightly lower than this limit (57). The sample containing high levels of chlorine and bromine belonged to the latter category.

The conclusions of the US Food and Drug Administration agree with those from the National Institute of Toxicology in Seville (72), carried out by atomic absorption spectrometry on 15 TOS-related samples, in that all the reported elements (aluminium, antimony, cadmium, chromium, cobalt, copper, lead, manganese, nickel, selenium and zinc) were below toxic levels.

Beaubernard et al. (65) reported that metals were not detected (except cadmium at 3 parts per billion) in two samples of oils, one TOS-related and one edible rapeseed oil.

In an early study of 19 samples mainly composed of mixtures of rapeseed oil and animal fats, 17 contained chlorine in the range of <5 to 600 ppm (33). The two samples free of chlorine did not contain aniline and were not associated with TOS cases. In contrast, 13 out of the 17

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Table 21. Concentrations of mineral oil in samples of TOS-related oils containing over 1000 ppm anilides

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mineral oil (ppm)</th>
<th>Sample</th>
<th>Mineral oil (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37 000</td>
<td>SGH-1</td>
<td>&lt; 1 000</td>
</tr>
<tr>
<td>2-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31 000</td>
<td>X/82/2</td>
<td>&lt; 1 000</td>
</tr>
<tr>
<td>1A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5 000</td>
<td>X/82/6</td>
<td>&lt; 1 000</td>
</tr>
<tr>
<td>F-3</td>
<td>&lt; 1 000</td>
<td>X/82/15</td>
<td>&lt; 1 000</td>
</tr>
<tr>
<td>F-4</td>
<td>&lt; 1 000</td>
<td>X/82/27</td>
<td>&lt; 1 000</td>
</tr>
<tr>
<td>M3/957</td>
<td>&lt; 1 000</td>
<td>XI/82/23</td>
<td>&lt; 1 000</td>
</tr>
<tr>
<td>P1/1695C</td>
<td>&lt; 1 000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Although not stated, these samples are probably pure aniline-denatured rapeseed oils.

Source: Koch, G.K., personal communication, 1983.
Table 22. Elemental composition of some samples of TOS-related oils

<table>
<thead>
<tr>
<th>Element</th>
<th>Range found (µg/g)</th>
<th>Element</th>
<th>Range found (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beryllium</td>
<td>&lt; 1</td>
<td>Cobalt</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Boron</td>
<td>&lt; 5</td>
<td>Nickel</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Fluorine</td>
<td>&lt; 100</td>
<td>Copper</td>
<td>&lt; 0.5–1</td>
</tr>
<tr>
<td>Sodium</td>
<td>18–33</td>
<td>Zinc</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.1–9</td>
<td>Germanium</td>
<td>&lt; 45</td>
</tr>
<tr>
<td>Aluminium</td>
<td>&lt; 20</td>
<td>Arsenic</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Silicon</td>
<td>&lt; 20</td>
<td>Selenium</td>
<td>&lt; 0.2</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>&lt; 4–26</td>
<td>Molybdenum</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>Chlorine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 60–446–1</td>
<td>Cadmium</td>
<td>&lt; 0.08</td>
</tr>
<tr>
<td>Potassium</td>
<td>&lt; 5–28</td>
<td>Tin</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Calcium</td>
<td>1–20</td>
<td>Antimony</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Titanium</td>
<td>&lt; 1</td>
<td>Tellurium</td>
<td>&lt; 0.08</td>
</tr>
<tr>
<td>Vanadium</td>
<td>&lt; 1</td>
<td>Thallium</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>Chromium</td>
<td>&lt; 0.2</td>
<td>Lead</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Manganese</td>
<td>&lt; 0.5</td>
<td>Bromine&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17–28–1</td>
</tr>
<tr>
<td>Iron</td>
<td>&lt; 0.5–1</td>
<td>Remaining elements</td>
<td>&lt; 10–&lt; 100</td>
</tr>
</tbody>
</table>

<sup>a</sup>All samples except two (93 ± 28 µg/g and 446 ± 29 µg/g, respectively) contained < 60 µg chlorine per gram as determined by neutron activation analysis.

<sup>b</sup>One sample contained approx. 0.075 µg bromine per gram.

Source: Carver et al. (57).

samples were related to TOS patients, even though aniline was found in only five of the 13 samples. The Special Investigation Team (33) associated the presence of chlorine in these cases with the tetrachloroethylene (perchloroethylene) used in the production of oils obtained from animal fats (trioleins) and which was one of the fractions included in TOS-related oils.<sup>a</sup> Vioque et al. (7) and Pestaña (73) also reported high concentrations (200–400 ppm) of chlorine in some samples of TOS oils. These authors hypothesized that these high levels of

<sup>a</sup>In 1988/1989, olive oil from Spain was found to be contaminated with tetrachloroethylene. This was attributed to the use of this solvent in the Foss-Let method of measuring the oil content of olives. Instead of distilling the solvent, the olives and the solvent were added to the olive cake (J.B. Rossell, personal communication, 1990). This contamination could also have happened in 1980/1981 during the TOS episode.
chlorine could be attributed to the extraction solvent tetrachloroethylene, although no experimental evidence was given. High levels of chlorine were also reported by Yurawecz et al. (74) and Gardner et al. (75), who detected concentrations of 440, 39 and 28 ppm in three samples of TOS-related oils; they attribute the origin of these chlorine-containing compounds to chloropropanediol diesters (see page 129).

Hernández Bolaños (76) reported higher concentrations of chlorine and nitrogen compounds in 14 TOS-related oils than in edible oils; the content of both elements ranged from 100 to 200 mg/l (77). The origin of nitrogen-containing compounds was attributed to aniline and their derivatives, but no explanation was found for the chlorine-containing compounds; however, they were in the form of nonvolatile and water-insoluble products. The content of phosphorus was reported to be similar in TOS-related and edible oils (76), with a range of 4 to 20 mg/l (77).

No major effort has been made regarding sulfur-containing compounds, even though aniline-denatured rapeseed oil from RAPSA was found to contain 800 ppm total sulfur and various TOS-associated oils ranged from 350 to 700 ppm sulfur, as against 90 to 140 ppm for pure olive oils (78). Sulfur in rapeseed is known to originate mainly from sulfur-containing glucosinolates in the seed, and its final concentration in the oil depends on the method of production. Thus, the literature contains reports of 100–200 ppm isothiocyanates in expelled oil and 1600–1800 ppm in extracted oil (79). However, most of the sulfur is removed during oil processing, which normally consists of degumming, deacidification, bleaching and deodorization. According to a study by Ahmad & Ali (51), deodorization is the step that chiefly reduces the sulfur content, to under <5 ppm.

Chlorine and nickel were excluded because of their variable occurrence, mostly in concentrations too small to be of toxic significance (7).

Sánchez Sáez et al. (80, 81) determined the cadmium content in serum and the selenium content in whole blood from 20 TOS patients and compared the results with cadmium values from 20 healthy volunteers and with selenium values in 20 patients with other diseases. The cadmium and selenium values in both groups were similar, with concentrations lower than 5 parts per billion for cadmium and from <10 to 162 ng/ml for selenium.

Volatiles
The National Centre for Food and Nutrition found no detectable amounts of solvents in TOS-related oils (63). However, Carver et al. (57)
detected low amounts of trichloroethylene (<0.02 – 4.8 ppm) and tetrachloroethylene (<0.01 – 0.30 ppm) in five TOS-related oils.

Three oils were compared with an edible rapeseed oil for their content of vinyl chloride (58). The method used was a headspace-gas chromatographic technique, with a detection limit of 5 ng/ml. The results were negative in all cases.

Vioque (1) reported the detection by GC/MS of several compounds, but all of them were aniline derivatives. Olias Jiménez et al. (45) also performed a study of the volatile compounds in three different samples of TOS-associated oils. By single ion monitoring GC/MS, they identified several aniline derivatives or related compounds (see above), as well as some non-nitrogen-containing compounds; these were mainly straight chain hydrocarbons with from 8 to 12 carbon atoms, both saturated and unsaturated, several aldehydes and free fatty acids from C6 to C10. The non-nitrogenous compounds were said to be characteristic of the thermal and oxidative degradation of fats (45).

Beaubernard et al. (65) found no significant levels of volatiles in a TOS-associated oil. Guitart (82) reported the presence of several highly volatile compounds, all of them apparently products of oil degradation, in three TOS-related oils after headspace-gas chromatographic analysis. However, the differences between chromatographic profiles of TOS and edible oils were interpreted to be quantitative but not qualitative.

Artificial antioxidants
The concentration of artificial antioxidants in one TOS-related oil was analysed by the National Institute of Toxicology (58), which found low concentrations of butylated hydroxyanisole and very low concentrations of butylated hydroxytoluene and tert-butylhydroquinone in this oil.

Other compounds
The content of cyanate ion in two TOS-related oils and in one edible rapeseed oil was studied (58). The concentration of this ion was higher in the edible oil (50 µg/l) than in the TOS-related oils (40 and 30 µg/l) but both concentrations were without toxicological importance. The lack of significant concentrations of cyanate and isocyanate was also reported by Hernández Bolaños (76) in 14 TOS-associated oil samples. Using HPLC, isothiocyanates were reported to be absent in three TOS-related oils (71). An unspecified number of TOS-associated oils were analysed for isothiocyanates and toluene diisocyanate (59), also with
negative results. As discussed above, the detection of isothiocyanates in rapeseed oils could be expected. The National Institute of Toxicology (61) analysed serum, blood and/or urine from a great number of TOS patients, and the results were negative or without toxicological importance for cyanate and sulfocyanate.

Vioque (1) reported that detergents (anionic tensioactives, sulfate or sulfonate type) were not present in an unspecified number of oil samples. Yurawecz et al. (74) and Gardner et al. (75) reported the detection by MS techniques of mixed palmitic, stearic, oleic and linoleic acid diesters of 3-chloro-1,2-propanediol (the chloropropanediol diesters can be produced by the reaction of hydrochloric acid with triglycerides at higher temperatures). These compounds were detected in three TOS-related oil samples (total chlorine content of 440, 39 and 28 ppm), but not in a sample of oil from an unaffected Spanish household. The sample containing 440 ppm of total chlorine could have had as much as 2500 ppm of chloropropanediol diesters. Gardner et al. (75) reported the isolation and mass spectrometric characterization of monoesters of chloropropanediol. According to the authors, these findings could be accounted for by hydrolysis of triglycerides with hydrogen chloride at 110 °C. This would indicate that these oils had been exposed to hydrogen chloride during the process of refining, possibly to remove aniline.

Artificial colorants (no other specifications) were not present in TOS-associated oils (66). However, Martínez-Conde (83) reported the purification from TOS-related oil samples of an unidentified dye that showed high toxicity in vitro and in vivo when tested under certain conditions. This substance was not chemically characterized, but some evidence led to the conclusion that it was a polar compound, probably having a phenol-type structure with an acidic radical in the molecule, and with an approximate boiling point of 300 °C. It turned green when reduced and red when oxidized. The process of oxidation–reduction did not seem to cause degrading structural changes, although this substance tended to polymerize in its oxidized form. The salt of the oxidized dye showed a great capacity for emulsifying, and destroyed rat adipocytes and hepatocytes in culture in only one minute. In in vivo experiments with mice, the fraction of the purified oil containing the oxidized form of the dye killed all the animals after 12–48 hours when injected intraperitoneally, whereas none of the mice treated with other oil fractions died (83). Despite these promising findings in 1983, no more information on this subject seems to have been reported.
Artigas et al. (25, 71) reported the detection by GC/MS of phthalate in two oil samples; they also detected chlorobenzene and nonylphenol in one of them. No attempt was made to quantify them. The detection of an unsaturated acid of molecular mass 308 in some of the samples was also reported; however, this compound was also detected in pure rapeseed oil.

Maleic anhydride was not detected in TOS-related oils (9).

No experimental evidence of the presence of other types of product known to be associated with rapeseed, such as aflatoxins, oxazolidinethiones, thioglycosides, isothiocyanates and nitriles, has been found in suspect oils other than in trace amounts, as is the case for two thioureas and a phenylisothiocyanate reported by Viayna Roca (78) as well as the thiourea derivative reported by Kammüller et al. (52).

**Microorganisms**

No studies or reports have been published on contamination of TOS-related oils by microorganisms. However, Koch (34, 63, 66) reported that microbiological analyses were carried out (no other specifications indicated) with apparently negative results.

The microbiological results of the studies carried out with samples obtained from biopsies or necropsies of the TOS patients were negative or not different from the control population for several species of viruses, bacteria, chlamydiae, mycoplasmas, fungi and eukaryotic parasites such as rickettsiae (84). Despite this data, Kimbrough (59) claimed that “the pattern of the illness is inconsistent with the usual course of events observed in poisoning cases or exposure to naturally occurring toxins that elicit allergic responses. It would seem more likely that the illness was caused by an as yet unidentified microorganism”.

**Conclusions**

TOS-related oils were a mixture of aniline-denatured rapeseed oil and various oils and animals fats of diverse origin containing a high proportion of saturated fatty acids in the 2-position of triglycerides. Their acidity index was higher than that of refined seed oils, and the peroxide index was abnormally high concomitant with a reduced stability to spontaneous oxidation in air.

Fatty acid profiles in TOS-related oils are characterized by a high content of oleic acid (18:1) and linoleic acid (18:2) and low amounts of erucic acid (22:1).
The presence of fatty acid anilides was the only differential parameter consistently appearing in profiling studies carried out by HPLC or capillary GC procedures.

The peroxidative hypothesis, based on the high peroxide values and low oxidation stability, is supported by experiments showing increased malondialdehyde production in rat liver homogenates and by the identification of various oxidized products arising from fatty acids and aniline.

After refining, the original 20 000 ppm aniline content of TOS-related oils is reduced to traces (<0.5–1.1 ppm) while anilides are found in the range of 300 to 2000 ppm. Aniline is essentially removed by refining and by reactions with different oil constituents. Most of the reaction products, except for the fatty acid anilides, are still unidentified so that the fate of the whole of the added aniline remains unaccounted for. Presumably, part of the aniline not in the form of fatty acid anilides could have been present as oxidation derivatives, a few of which have been identified. These derivatives or oxidized products could have formed spontaneously during storage, transport or refining. Oxidized anilides with leukotoxin-related structures have been definitely identified, but direct toxicity studies are lacking.

Experimental data prove that not only can anilides be formed spontaneously during the transport and storage of aniline-denatured oils (both before and after refining) but that during refining a balance between removal and de novo synthesis is established. Nevertheless, the final concentration of anilides is drastically reduced after deodorization. The fatty acid anilides arise mainly by reaction of aniline with triglycerides. Reaction with free fatty acids seems to be a minor process.

A strong epidemiological correlation has been found between the presence of anilide and case-related TOS oils, which is also true for aniline. The anilide content varies widely in different samples and, due to interlaboratory discrepancies, assays should be properly validated. This is also true for the methods of synthesizing authentic anilides.

The content of sulfur compounds is very low (only about 10% of the aniline nitrogen would be chemically combined with sulfur) and most of it is removed during oil processing.

High levels of chlorine-containing compounds in some TOS-related samples have been reported. These levels have been attributed to various sources: to the use of perchloroethylene in oil processing from animal fats; to the presence of chloropropanediol diesters found to be toxic to rats; to the presence of batches contaminated with PCBs or
chlorophenols; and/or to the incorporation of waste solvent as solvent-impregnated olives from Foss-Let oil determinations.

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Nearly a decade after the appearance of TOS, no direct evidence for the pathogenic basis of the disease has been found. However, immunological mechanisms are presumed to have been involved in the pathogenesis, based on the similarity of the array of symptoms in TOS, in graft-versus-host disease (GVHD) and in hydantoin-induced autoimmunity. This array includes the following symptoms seen in the acute phase of TOS: maculopapular exanthema, pruritus, fever, interstitial pneumonitis, dyspnoea, eosinophilia, vasculitis, polyarthritis, high serum IgE levels, lymphadenopathy, decreases in both the total T cell population and the T suppressor/cytotoxic subpopulation, and the appearance of autoantibodies. Likewise, the chronic phase of TOS has many parallels with chronic GVHD in terms of symptoms, such as scleroderma-like lesions, sicca syndrome, peripheral neuropathy and inflammatory myopathy.

Another fact that implicates immunological mechanisms in the pathogenesis of TOS is that the illness progressed to the chronic phase in only a small fraction of acutely ill individuals. This sensitivity of only a fraction of a population to the adverse effects of exposure to an agent is characteristic of immunologically mediated toxicities.

This chapter summarizes the findings related to changes in immunological parameters, and discusses them in the light of present knowledge.
of the syndrome. In addition, guidelines for future research are set out, based on the facts described here.

The immunological findings on TOS are difficult to interpret, as the studies show several problems: lack of proper coordination and definition of cases for immunological studies; insufficient data due to small numbers of individuals studied for any one immunological factor; investigations not made under "blind" conditions and in most cases without adequate experimental design for the hypothesis to be tested; and use of serum from different clinical phases, stored under different conditions.

The Acute Phase

Serological findings
The most comprehensive report of the immunological studies carried out from the initial phase of the outbreak until a year later was made by the Spanish Ministry of Health and Consumer Affairs (1). Data included the results obtained in three immunology laboratories from different hospitals in Madrid: Fundación Jiménez Díaz, Clínica Puerta de Hierro and Centro Ramón y Cajal. The study included more than 500 patients, but not all determinations were carried out for every patient. For those tests performed by all three laboratories, the results were in agreement with each other.

Serum IgG. In most cases the IgG levels were within the normal range (mean, 1010 mg/dl). In 16% of cases, values below the mean were found; normal levels were reached after 2–3 weeks.

Serum IgA and IgM. Mean values of 198 and 184 mg/dl, respectively, were within the normal range.

Serum IgE. Levels were increased in 52% of patients: 210 ± 34 IU/ml (mean ± SE) for one group studied (normal values, 35 ± 30 IU/ml) (2). Even higher values have been reported for other groups of patients: a mean of 403 IU/ml, with more than 1000 IU/ml occurring in 5% of the cases (1).

Complement. At the beginning of the disease, 35.4% of patients had a moderate increase in C3 level (mean, 140 mg/dl). After several weeks C3 levels returned to the normal range. The same initial increase was seen for C4 levels in 42.8% of patients with acute TOS (mean, 42 mg/dl).

Haemolytic activity. In 96 of 100 patients in which CH50 levels were measured during the acute phase, normal values were found. The four exceptions had low levels.
Immune complexes. High concentrations of circulating immune complexes occurred in only 4.5% of the 44 patients tested for their presence.

Regarding reactivity against exogenous antigens and autoantigens, one report (3) describes the presence of specific IgE antibodies against oleoyl anilide in the serum of patients in the acute phase (17 out of 25 samples of serum, 68%). Specific IgE was determined by solid phase radioimmunoassay. The antibody activity was stopped by heating for 30 minutes at 56 °C and by previous incubation of the serum with unlabelled antigen. No association was found between IgE serum levels and the presence of antibodies. Negative results were found using atopic serum and normal controls. However, the presence of these specific IgE antibodies could not be demonstrated by a different group of investigators (4, 5).

Antistreptolysin O antibodies occurred at a level of more than 150 Todd U/ml in 44% of 530 patient serum samples, versus 16% in normal control serum samples.

Precipitins against Aspergillus fumigatus were found in 39.5% of 162 cases analysed, compared with a 5% incidence in a normal population.

One out of 50 samples of serum was positive for cold agglutinins at a titre of 128.

Antinuclear antibodies were found by indirect immunofluorescence in 41.3% of 126 serum samples from patients in the acute phase. However, the titres were generally low, ranging between 10 and 40. Antibodies against extractable nuclear antigen, mitochondria, gastric parietal cells, thyroid cells and immunoglobulin (rheumatoid factor) were consistently negative.

In 6.9% of 116 cases investigated, antilymphocyte antibodies were detected.

Cellular findings
Eosinophils were increased in more than 50% of patients, with a mean count of 2521 ± 335 cells per mm\(^3\) (mean ± SE) versus 217 ± 21 in normal individuals (1). Some reports (see reference 5 for a review) describe an increase in eosinophil levels in 85% of 74 patients studied.

Deposition of major basic protein (MBP) in several tissues and high serum MBP levels have been demonstrated (6).

Basophils were decreased in 100% of the 93 patients studied: 9 ± 1.4 basophils per mm\(^3\) versus 31 ± 1.6 basophils per mm\(^3\) in normal controls (2).
A study (3) of the peripheral blood lymphocyte surface antigens of 52 patients determined that 87% of these patients had a diminished number of CD3 lymphocytes as compared to a control population. (The mean percentage of CD3 peripheral blood lymphocytes was 47.4% for the patient population as compared to 62.8% for the normal population.)

The CD8 cell subpopulation was decreased in 78.5% of these patients (mean value: 13.1% versus 27.5% in the normal population) (1, 2). In contrast, the percentage of CD4 peripheral blood lymphocytes remained within normal limits (40.2%).

Findings in the Chronic Phase

IgE levels decreased gradually, reaching a mean value of 76 IU/ml after one year. At that time, only 19.6% of the chronic phase patients had elevated levels of serum IgE and 18.1% had increased IgG values. C3 levels were decreased in 2.9% of the chronic TOS patients one year after their initial symptoms appeared. Immune complexes containing IgG and/or IgM occurred in over 50% of the individuals in a study on 52 patients (IgG complexes in 55.7% and IgM complexes in 40.3%). In most cases the levels of immune complexes were low. High levels were found in only 5.8 to 10% of chronic TOS patients, depending on the laboratory (1, 5).

With respect to cellular parameters in the chronic phase, the eosinophil and basophil counts in most cases returned to normal levels within a few months. In the spring of 1982, a decreased CD3 cell population was found in only 10%, and a decreased CD8 cell population in only 5% of the chronic cases investigated. The most frequent finding at that time was an increase in the percentage of the CD8 cells to a mean of 31.6%, which is within the normal range.

One study (7) reports the detection of autoantibodies in the chronic phase. Autoantibodies having titres above 80 were found with specificities against nuclear antigens (27% of symptomatic versus 8% of nonsymptomatic individuals tested), against smooth muscle (12 vs. 8%, respectively) and against mitochondrial antigens (8% of patients with symptoms tested). In this study, no autoantibodies with titres $\geq 80$ were demonstrated in normal controls.

Other authors (8) did not find autoantibodies in serum from patients in the chronic phase. As described in a study of 27 patients made one year after the onset of the disease, cell proliferation against T and B cell
mitogens was reduced in patients with severe illness. All these data seem to indicate some alteration in the immune system leading to graft-versus-host or autoimmune responses (9, 10).

A relationship has been suggested between major histocompatibility (HLA) haplotype and the development of certain clinical features in the chronic phase of TOS (11). When chronically ill patients were grouped by clinical features exhibited, far more patients with pneumonia had the HLA haplotype DR2 than did those without pneumonia. In the same group of patients, the presence of DR2 was also correlated with the presence of serum glomerular basement membrane antibodies as detected by both enzyme-linked immunosorbent assay (ELISA) and immunofluorescence.

In addition, an association with haplotypes DR3 and DR4 has been described in several chronic patients (1).

Other investigators (12) found IgG antibodies against human collagen in 28 out of 44 serum samples from patients during the acute phase but, curiously, the titres of these antibodies decreased during the chronic phase. In addition, the antibodies to glomerular basement membrane may react with type IV collagen. (Anticollagen antibodies have been described in scleroderma.)

From an immunopathological point of view, two reports support immunological mechanisms of damage in certain tissues. Antiprocollagen and antifibronectin antibodies have been found in the sclerodermoid-like lesions (13). In addition, Oliva et al. (14), in biopsies of the minor salivary glands from TOS patients, found lymphoepithelial infiltrates of memory T cells (CD45RO+) exhibiting digital projections towards the junction of the ductal and myoepithelial cells. Both these and other authors (15) found no deposits of immunoglobulins in the damaged tissues. The finding of T cells infiltrating the salivary gland suggests a possible cytotoxic role for these cells in TOS.

Kammüller et al. (16, 17) pointed to the similarity of the constellations of symptoms seen in GVHD, in autoimmune reactions induced by hydantoins and in TOS. Since some clinical and laboratory findings (eosinophilia, elevated IgE and T lymphocytopenia) occur in common in chemically induced GVHD and in TOS (18), one can hypothesize that the toxic agent in TOS induced abnormal antigens in cellular membranes of the patient. If this novel antigen were presented in association with an appropriate HLA class II product (e.g. DR3, DR4) it would lead to the activation of autologous CD4 helper T cells, thus giving rise to an autoimmune-like disease.
A recent study used the popliteal lymph node assay in an attempt to identify an inbred strain of mice in which the assay could discriminate between TOS-associated and case-control oils. Eight strains differing in their H-2 (murine major histocompatibility complex) haplotypes gave various patterns of response to a mix of case-associated oils, a mix of case-control oils and edible rapeseed oil. None of the response differences proved to be statistically significant for the small group sizes used, though one strain (C57BL/10J) showed a trend towards significance. The assay was next used to measure the responses to fractionated samples of the oils in the strain C57BL/10J that discriminated best between the the three types of unfractionated oil. The ability of the assay to discriminate among the samples was no longer evident when the responses to oil fractions were analysed (N.R. Rose et al., unpublished observations, 1989).

Discussion

Fig. 18 shows a scheme of the possible immunopathogenic pathways during both the acute and the chronic phases of TOS.

The most prominent immunological features in the acute phase were the high levels of eosinophils found in 85% of patients tested, the low basophil count in 94% and the decrease in the CD8 peripheral blood lymphocytes in 78.5%. A related finding is the increase of serum IgE in 52% of the patients.

The decrease in basophil count, together with the presence of specific IgE antibodies against oleoyl anilide, seems to indicate that immediate hypersensitivity reactions had developed, although the soluble mediators of type I hypersensitivity reactions have not been assayed.

Another possibility is that the toxic agent reacted directly with eosinophil membranes, eliciting in some way the release of immediate hypersensitivity mediators. Such a hypothesis is lent credence by the work of Garcia Gil et al. (19). Alternatively, a direct toxic effect could have resulted in a decrease in CD8 lymphocytes, leading to an immunological hyperreactivity, with elevated IgE possibly resulting from a release of interleukin 4. The presence of antibodies against ubiquitous antigens and the high eosinophil counts could be attributed to the release of interleukin 5 induced by the toxic agent.

Deposition of major basic protein from eosinophils has been demonstrated, pointing to a possible pathogenic role of eosinophils in the tissue damage of TOS patients.
Fig. 18. Possible immunopathogenic pathways of TOS

Toxic agent from denatured oil → Altered autoantigen

Directed interaction of toxic antigen with lymphocytes and/or eosinophils

**Acute phase**

Type 1 hypersensitivity?

- Eosinophils
- IgE
- Activation of inflammatory cells

<table>
<thead>
<tr>
<th>IL-5</th>
<th>IL-4</th>
<th>IL-6</th>
<th>GMCSF</th>
</tr>
</thead>
</table>

Activated helper CD4 T cell

IL-1

Resting CD4 T cell

IL-2 (clonal expansion)

B cell → Plasma cell

Autoantibody → Autoimmune disease

Chronic phase

**Key**: IL = interleukin; GMCSF = granulocyte macrophage colony stimulating factor; CD = cluster of differentiation; GVH = graft-versus-host.
Similar pathogenic mechanisms are involved in other diseases, such as systemic lupus erythematosus, the acute phase of GVHD and hyper-IgE syndrome. A decrease in CD8 cells, eosinophilia and high IgE levels have occasionally been described for these diseases.

One problem is the lack of correlation between eosinophilia, basophilopenia and the decrease in the CD8 lymphocyte population. However, these parameters are undoubtedly regulated by T cells. In addition, changes induced in the T cell population may be qualitative rather than quantitative in nature. Support for this hypothesis comes from the work of Leung et al. (20), in which the in vitro production of IgE by lymphocytes from patients with hyper-IgE syndrome but normal CD8 cell counts could be regulated down to normal levels by the addition of CD8 cells from normal individuals.

The existence of immunological mechanisms in the chronic phase is very likely, though not yet demonstrated. The suggested association of the disease with particular DR haplotypes such as DR3 and DR4, the presence of autoantibodies, and certain of the clinical and immunopathological findings may suggest autoimmune-like mechanisms such as those found in scleroderma or Sjögren’s syndrome.

References


17. Kammüller, M.E. et al. Chemically induced autoimmune reactions and Spanish toxic oil syndrome. Focus on hydantoins and related


Future research

Epidemiology

The studies discussed here have either already begun or are planned for the future. They can be classified roughly into two types: etiological studies designed to elucidate the origins of the TOS epidemic; and follow-up studies designed to determine the ultimate consequences of the epidemic among affected people.

Etiological studies

Peripheral cases study
The purpose of this study is to collect information on the small proportion of cases that occurred outside the 14-province area of central and northwestern Spain where the epidemic was centred. Of particular interest is the source of the oil to which these cases were exposed, its prior treatment, and its type and extent of contamination. This study would attempt to explain why so few cases occurred outside the principally affected area, despite the apparently widespread distribution of rapeseed oil denatured with aniline.

Study of oils by container type
After the first toxico-epidemiological study, investigators found that the apparently uniform group of “typical” containers could be further
distinguished into a number of types. The 3000 oils stored with FIS are being analysed for anilide content, and any association with container type will be described.

"Amplified" toxico-epidemiological studies
The toxico-epidemiological method can be used to test etiological hypotheses other than the association of fatty acid anilides with illness. In principle, any chemically stable suspect etiological agent (or correlate of the etiological agent) ought to appear more frequently and/or in greater quantity in case than in control oils. Candidate compounds for such studies include chlorpropanediol, 3-aminophenyl-1,2-propanediol (and its mono- and di- fatty acid derivatives), pentachlorophenol, dioxins and furans.

Follow-up studies

Clinical register study
Approximately 25 000 records of TOS patients have been reviewed, and the data should be ready for analysis shortly. The question has recently arisen regarding the best way to validate data collected by the several people (chart abstractors) involved in the chart reviews. A validation study is to be conducted involving estimation of the degree of intra-abstractor agreement (comparison of repeat reviews done by a specific abstractor with the initial reviews done by him/her on a sample of clinical histories) for all abstractors, as well as estimation of the degree of inter-abstractor agreement for the five principal abstractors.

Systematic collection of detailed clinical data
The long-term clinical follow-up of the sample of 500 patients (100 in each of five institutions) is being pursued (see Chapter 1). In some locations, logistical problems have delayed implementation of the study. In others (e.g. Salamanca) data collection is being accomplished quite satisfactorily and is effectively population-based.

Collection of data on large numbers of patients
Recently, the possibility has arisen of an ongoing study of a substantially larger group of patients, using the system of medical care run by the Spanish National Institute of Health. While important theoretical difficulties accompany any long-term clinical follow-up study (e.g. lack of comparison information for most indicators of morbidity, and selection bias arising from less than complete participation by the persons being studied) such a study would yield important clinical information.
Moreover, a large clinical follow-up study offers the possibility of far greater statistical power than that available from the sample of 500 study. Discussions are to be held with National Institute of Health personnel regarding the manner in which data on a large number of patients might be accumulated. In any case, any large clinical study would not replace the detailed sample of 500 study.

Mortality studies
A system for ascertaining deaths in the TOS cohort is urgently needed. However, access to death certificate data at the national level is stringently protected by Spanish law, presenting a difficulty that investigators in FIS have so far been unable to resolve. Some mechanism for systematic ascertainment of deaths (with causes) for all (or at least a major population-based portion) of the cohort of affected persons is absolutely necessary. The problem of access to death certificate data is being actively pursued.

Cancer registry in the Autonomous Community of Madrid
This project would provide important data on cancer incidence in one of the most populated areas of Spain. As some three quarters of affected people live in the Autonomous Community of Madrid, registration of all tumours in this area would be helpful in approaching the problem of cancer follow-up in the TOS cohort as well.

The project is regarded as very desirable, but very difficult to implement. Its implementation may be pursued by the Government of the Autonomous Community of Madrid, and FIS is considering supporting this effort. However, appropriate investigators would have to be identified, and a pilot study to determine feasibility may have to be done. Methods other than registering all Madrid cancer cases for follow-up of cancer among affected persons are also being considered.

While the question of excess cancer in TOS patients is an important one, the appropriate method of approaching this problem still needs to be considered. Experts in cancer registries, particularly those involved in the network of cancer registries in Spain, will have to be consulted.

Follow-up of patients with specific clinical pictures
Such studies could involve follow-up of patients with specific chronic manifestations of TOS, such as neuropathy, hepatopathy, sclerodermiform skin changes and pulmonary hypertension. Such patients can be identified for study as soon as the TOS clinical register is compiled. Samples identifying those TOS patients who presented specific clinical
characteristics would then be made available to investigators to follow these patient subsets.

**Malformations in offspring of TOS patients**

FIS has information on offspring born to parents, one or both of whom were affected by TOS, in Madrid Province over a period of five years. These data have not yet been subjected to quality control. Although exhaustiveness (and representativeness) can hardly be verified, analysis of these data may provide useful information both on the occurrence of birth defects and on reproductive trends in the affected population.

**Toxicology**

Case oils have been identified by toxico-epidemiological studies (J; Posada, M. & Abaitua, A., unpublished data, 1989). These oils are now almost nine years old and, with the exception of their use in one animal study described below, they should be stored at low temperature and retained for further analytical studies as the need arises.

**Toxicity of case oils**

Three cats should be administered the maximum tolerated dose of a case oil for several weeks. The same dose of control (non-case) oil should be administered to another group of three cats.

**Simulated refining of aniline-denatured rapeseed oil**

A major oil refining study is under way. It is a joint project involving the Leatherhead Food Research Association, the British Industrial Biological Research Association, the London Hospital Medical School, all in the United Kingdom, and the Department of Neurochemistry in Barcelona. Extensions of this study, or new studies, are described below.

Simulated refining of aniline-denatured rapeseed oil using labelled (3H and N15) aniline should be undertaken. A balance sheet for aniline and its derivatives during the various stages of refining should be constructed. The oils so obtained will be fractionated, and the various derivatives in the fractions identified and synthesized. The simulated process will be scaled up to produce sufficient oil samples for biological testing.

A processing system for oils and aniline derivatives in oil fractions should be developed so that fractions may be added to cell cultures without causing nonspecific effects.
Biological testing of oils and oil fractions in a variety of systems is needed. The range of test systems should be kept under continuous review, but those already envisaged are various cell cultures, popliteal lymph node assay, whole animals, and perfused blood vessel and liver systems.

**Biological properties of known constituents of oils**

The extensive research showing a high biological activity of anilides on the lipid biosynthetic pathway in the rat (2) and polymorphonuclear leucocytes (3) has not been confirmed in another laboratory (4). Two studies would help resolve this difference. First, anilides should be synthesized by the methods used in the respective papers and the products compared for their composition. All substances found in addition to anilides should be identified. Second, if compounds other than anilides are found, relevant experiments should be designed to test for their activity against lipid biosynthetic systems.

**Fatty acid esters of 3-aminophenyl-1,2-propanediol**

One published paper (5) has claimed that the administration of these compounds to mice causes respiratory problems and histopathological changes in the lung. This experiment should be repeated. The 3-aminophenyl-1,2-propanediol and its mono-oleoyl ester should be synthesized in pure form in sufficient amount for animal studies. A toxicity study should then be carried out in rats and mice with three dose levels in three groups of 10 animals with daily oral dosing for one week. Histopathological studies on tissues should be done on any animals showing symptoms, or after 10 weeks.

**Testing oils in in vitro systems**

A search should be made for a laboratory capable of developing procedures suitable for testing unfractionated oils, using in vitro cell cultures and other biological systems.

**Further examination of case oils identified by the toxicoc-epidemiological studies**

Two studies are recommended. First, as biologically active compounds are found in the simulated refining project, their presence or their breakdown products in case oils should be investigated. Their absence (or low concentration) in non-case control oils should also be checked. Second, case or control oils should be examined for the presence or absence of 3-amino-1,2-propanediol and its mono- and di- fatty acid esters.
Immunology

Fundamental considerations
Immunological studies contribute to a greater understanding of TOS on at least three different levels: immunodiagnosis, immunopathogenesis and immunoepidemiology.

Immunodiagnosis depends on finding immunological markers of TOS or of exposure to toxic oil. Commonly used immunological markers include particular autoantibodies to phospholipids, nuclear antigens or collagen. Levels of one or more of these autoantibodies may be higher in TOS victims than in unexposed controls. Alternatively, one particular autoantibody may be disease-specific. Immunodiagnosis is also carried out with carefully selected antibodies, including monoclonal antibodies, capable of detecting antigenic biomarkers of diseases or of exposure to the toxic agents. This application is exemplified by the use of antibody to the eosinophil major basic protein.

Immunodiagnosis would aid epidemiological studies of TOS by better defining exposures and cases, especially when the symptom complex is variable or unclear. It may also relate TOS to other examples of eosinophilic myalgia. Therefore, a number of potential immunodiagnostic tests are proposed.

Immunopathology connotes active involvement of immune responses in manifestations of disease, but does not necessarily imply immunoeptiology. A number of forms of initial tissue injury can instigate subsequent immunopathogenetic responses. For example, glomerulonephritis resulting from immune complex deposition may be caused by various infectious microorganisms and toxic agents, representing essentially the same pathogenesis with different etiologies. In the case of TOS, immunological mechanisms could quite conceivably have perpetuated but not initiated the tissue injury. Demonstration of immunopathogenesis, then, would clarify the disease process without necessarily revealing its cause.

Recent advances in technology now permit reinvestigation of fixed pathological specimens for evidence of immunological activity. A great deal of such material is available from autopsies performed on TOS victims. It is essential that the pathological picture be related to the stage of the disease and to the symptomatology of the patient. Thus, close collaboration with further pathology research is needed.

Experiments should be performed only with valid reagents, including documented and well preserved serum specimens and verified case-associated oils. Should these precautions not be observed, misleading
results will impede future investigations. Therefore, many of the suggested immunological studies need to be deferred until appropriate reagents are available.

The immunological studies should proceed in a flexible sequence from the most straightforward and inexpensive to the more intricate and speculative procedures. Therefore, unless the initial investigations implicate immunological mechanisms in TOS, more expensive studies are not justifiable.

**Guidelines for immunological investigations**

The immunological mechanisms implicated in both the acute phase and chronic phase of TOS can be investigated by *in vitro* studies on fresh lymphocytes, and by studies on samples of historical serum samples obtained from individuals with TOS. This seems a more fruitful approach than extensive work in experimental animals, as efforts to develop a suitable animal model have so far failed.

**Selection of authentic case-associated oils**

As shown by Kilbourne et al. (1), a good correlation exists between the amount of anilide found in an oil collected during the Spanish Government's oil exchange programme in 1981, and the likelihood that that particular oil was turned in by a household in which at least one person developed TOS. Thus, those case oils with chemical profiles consistent with rapeseed oil content and with the highest concentrations of anilide are the most likely to have been authentic case-associated oils and to have contained the toxic agents. Therefore, the case-associated oils to be used in these studies should be selected on the basis both of their historic designation as such and of their high anilide content. For some of the studies, pooled case-associated oils may increase the likelihood that the authentic oils are contained in the samples. Controls in these studies should include both case-control oils (historically designated as such and with low anilide content) and edible rapeseed oil.

**Selection of historical serum samples from TOS patients**

Concerns exist regarding the adequacy of storage conditions over the past nine years for the serum samples obtained from affected individuals during the TOS epidemic. Therefore, the serum to be used in the recommended studies should have been stored under adequate conditions. This can be verified by determining the IgE and complement levels of serum from TOS patients in which the IgE levels were measured eight
or nine years ago. If the IgE concentration in a serum sample has not decreased more than 50–75%, and if complement can still be measured, the serum sample can be used in these studies. If samples are used in which prior IgE levels are unknown, the present IgE levels should still be normal, and complement levels should be measurable. If possible, control serum should include both historic and nonhistoric samples from healthy individuals.

**Recommended studies to ascertain whether immunological mechanisms were operative in the etiology of TOS**

A scheme for these studies is given in Table 23. It distinguishes the experiments designed to implicate immunological mechanisms in the early, acute phase from those in the late, chronic phase.

1. **Studies related to the acute phase of TOS**

*Specific IgG and IgE antibodies against the TOS agent(s).* One laboratory has reported finding IgE antibodies specific for oleoyl anilide in the serum of patients at the beginning of the disease. This finding has not been confirmed by other investigators. Therefore, further investigation on the possible existence of these antibodies would be of interest. The main difficulty that may be encountered is in obtaining a sufficient number of TOS serum samples that have been stored under the proper conditions for retention of IgE antibodies, which are more labile than IgG antibodies. Equally, the absence of a clearly defined agent in case-associated oils may explain discordant results.

*In vitro assays for release of cytokines.* Peripheral blood mononuclear cells (PBMCs) from individuals diagnosed with TOS may still be prone to release inflammatory mediators in the presence of the TOS agent(s). In addition, PBMCs from normal individuals should be tested for cytokine release as the result of direct contact with the toxic agents.

*Intradermal skin tests.* Skin tests for immediate or delayed hypersensitivity reactions may be performed on patients and controls if one or more well characterized antigens become available. However, the passage of time diminishes the value of these procedures.

*Prausnitz-Kustner (PK) test in monkeys.* As human homocytotropic antibodies can fix to primate skin, a modified PK test is recommended in which monkeys will be sensitized by intradermal injection of selected case-associated and control serum. The animals will be fed authentic oil samples and then injected with Evans' blue dye.
Table 23. Recommended studies on the possible role of immunological mechanisms in the etiology of TOS

<table>
<thead>
<tr>
<th>Acute phase&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Chronic phase&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Assay for IgE and IgG antibodies directed against TOS agent(s) in historic TOS serum</td>
<td>1. Immunocytochemical identification of T cell markers on cells in lymphocytic infiltrates in historic pathology slides of specimens from TOS patients</td>
</tr>
</tbody>
</table>

2. In vitro assays for cytokines and immediate hypersensitivity mediators, released by peripheral blood mononuclear cells from TOS patients and from normal individuals in response to TOS agent(s) in culture (e.g. IL-1, IL-2, IL-4, IL-5, tumour-necrosing factor, eosinophilic chemotactic factors) |

3. Intradermal tests for immediate or delayed response to TOS agent(s) in recovered patients and controls |

4. Prausnitz-Kustner test in primates with TOS serum samples injected intradermally and pooled case-associated oils administered orally |

1. Immunocytochemical identification of T cell markers in pathology specimens. The infiltrating cells in histological specimens can be typed by immunocytochemical reagents specific for T cell antigens and for activation antigens. Such specimens can also be stained for the expression of the human leukocyte antigen HLA-DR on tissue cells.

<sup>a</sup> Direct toxic effect $\rightarrow$ mediators release or type I hypersensitivity $\rightarrow$ increased IgE $\rightarrow$ mediators release.

<sup>b</sup> Induction of GVH-like autoimmunity or immune complex disease.
In situ hybridization with probes for mRNAs in selected cytokines. Fixed histological specimens can be subjected to in situ hybridization with appropriate probes to determine whether infiltrating mononuclear cells were synthesizing various cytokines, as predicted by the presence of mRNAs.

Immunological survey of selected patients. It would be interesting to determine whether those who developed the chronic phase of TOS developed autoantibodies during the initial phase of chronicity, or whether any of these individuals have a distinctive pattern of serum autoantibodies at present. Therefore, both historical TOS serum samples and contemporary serum samples (matched, where possible) from individuals diagnosed with late phase TOS should be screened for multiple autoantibodies.

HLA haplotyping of former TOS patients. An expanded study of HLA haplotypes of documented TOS patients and documented case controls might help to determine whether susceptibility to the toxic agent(s) was linked to the HLA haplotype. This would require that a sufficient number of these individuals could be located and would be willing to participate. The studies must relate HLA haplotype to the particular symptom complex and should include Class II determinants (DR, DP and DQ). For further studies, amplification of determinant exons from Class II genes may be achieved by polymerase chain reaction and subsequently detected by means of radiolabelled sequence-specific oligonucleotides (having sequences corresponding to the polymorphic regions of different HLA-DR, -DQ and -DP alleles.

Priority scheme
The investigations related to both the early and late phases should be carried out in the order listed in Table 23. Tasks 1 and 2 from the late phase will be performed on histological specimens already available, whereas tasks 3 and 4 in the late phase and 2 and 3 in the early phase require recontacting a sample of the original TOS victims. Tasks 3 in the late phase and 1 and 4 in the early phase will take advantage of stored serum samples.

References


Foodborne diseases, especially those with fatal consequences, cut deep into one of the basic tenets of human health and wellbeing – the need for safe uncontaminated food. The right of the consumer for healthy and safe food is one of the strategic elements of the health for all policy endorsed by the Member States of the WHO European Region.

The toxic oil syndrome that erupted suddenly in Spain in 1981 highlights clearly – and tragically – the crucial importance of food safety in daily life. It points out once again the need for food surveillance and control at all stages of production, storage, distribution, sale and use. In 1987 the Spanish Government and the Regional Office formalized their collaboration on an intensive international research effort that has led, among other things, to the strengthening of scientific capability in Spain.

This volume brings together the results of many of the studies on the syndrome, and outlines the directions that future research on the disease will follow. It also provides an update on what has been learned since the publication in 1984 of the first WHO book entitled Toxic oil syndrome: mass food poisoning in Spain. In six concisely written chapters, the authors deal with the various areas of scientific interest, namely epidemiology, clinical findings, pathology, experimental studies, the chemical composition of the oils associated with the syndrome, and immunology. A seventh chapter addresses clearly and comprehensively the areas mapped out for future research – hopefully leading to the eventual solving of one of the most puzzling episodes of mass food poisoning in history.