WORLD HEALTH ORGANIZATION
EUROPE

TOXIC OIL SYNDROME

TEN YEARS OF PROGRESS

Instituto de Salud Carlos III

Toxic oil syndrome

Ten years of progress

Edited by: Benedetto Terracini

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ABSTRACT

This report provides an update on research on the toxic oil syndrome (TOS) since the last WHO review was published in 1992. Although it has been known since the late 1980s that the disease was caused by the consumption of illicitly refined rapeseed oil, it has been obvious for many years that a concerted scientific effort was needed to precisely identify the agent(s) responsible for the outbreak and to assess the pathogenesis and clinical evolution of the condition. Although many questions remain to be answered and no animal model for TOS has been identified, much knowledge has now been gained on the biological properties of chemicals found in the adulterated oil, on the pathogenesis of the disease and on the conditions by which the illegal refining might have produced toxic compounds. These investigations are relevant not only to protecting the health of TOS victims but also to preparedness for similar circumstances in the future.

Keywords

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  DK-2100 Copenhagen Ø, Denmark

Contents

Contributors

Dr Ignacio Abaitua
Centro de Investigación sobre el Síndrome del Aceite Tóxico y Enfermedades Raras (CISATER), Instituto de Salud Carlos III, Madrid, Spain

Dr Joaquin Abian
Structural and Biological Mass Spectrometry Unit, Department of Medical Bioanalysis, Instituto de Investigaciones Biomédicas de Barcelona (IIBB), Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Dr Benjamin C. Blount
Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA, USA

Dr Rosa Elena Calaf
Structural and Biological Mass Spectrometry Unit, Department of Medical Bioanalysis, Instituto de Investigaciones Biomédicas de Barcelona (IIBB), Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Dr Blanca Cárdaba
Department of Immunology, Clínica de la Concepción, Fundación Jiménez Díaz, Madrid, Spain

Dr Montserrat Carrascal
Structural and Biological Mass Spectrometry Unit, Department of Medical Bioanalysis, Instituto de Investigaciones Biomédicas de Barcelona (IIBB), Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Dr Yolanda Castaño
Structural and Biological Mass Spectrometry Unit, Department of Medical Bioanalysis, Instituto de Investigaciones Biomédicas de Barcelona (IIBB), Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Professor Richard Doll
Epidemiological Studies Unit, Oxford, United Kingdom

Dr Eva Estirado de Cabo
Centro de Investigación sobre el Síndrome del Aceite Tóxico y Enfermedades Raras (CISATER), Instituto de Salud Carlos III, Madrid, Spain

Dr Soledad Gallardo
Department of Immunology, Clínica de la Concepción, Fundación Jiménez Díaz, Madrid, Spain

Dr María Luisa García de Aguinaga
Centro de Investigación sobre el Síndrome del Aceite Tóxico y Enfermedades Raras (CISATER), Instituto de Salud Carlos III, Madrid, Spain

Professor Emilio Gelpí
Department of Medical Bioanalysis, Instituto de Investigaciones Biomédicas de Barcelona (IIBB), Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Mr Jordi Gibergans
Department of Biological Organic Chemistry, Institut d'Investigacions Quimiques i Ambientals de Barcelona (IIQAB), Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Dr Agustín Gómez de la Cámara
Clinical Epidemiology Unit, Research Department, Hospital Universitario 12 de Octubre, Madrid, Spain

Dr Maravillas Izquierdo Martínez
Centro de Investigación sobre el Síndrome del Aceite Tóxico y Enfermedades Raras (CISATER), Instituto de Salud Carlos III, Madrid, Spain

Dr Edwin Kilbourne
Toxic Substances and Public Health, Agency for Toxic Substances & Disease Registry, Atlanta, GA, USA

Dr Carlos Lahoz
Department of Immunology, Clínica de la Concepción, Fundación Jiménez Díaz, Madrid, Spain

Dr María del Mar Plaza Cano
Centro de Investigación sobre el Síndrome del Aceite Tóxico y Enfermedades Raras (CISATER), Instituto de Salud Carlos III, Madrid, Spain

Ms Concepción Martín-Arribas
Centro de Investigación sobre el Síndrome del Aceite Tóxico y Enfermedades Raras (CISATER), Instituto de Salud Carlos III, Madrid, Spain

Professor Angel Messeguer
Department of Biological Organic Chemistry, Institut d'Investigacions Quimiques i Ambientals de Barcelona (IIQAB) Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Dr Anna Morató
Department of Biological Organic Chemistry, Institut d'Investigacions Quimiques i Ambientals de Barcelona (IIQAB) Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Dr Benoit Nemery
Research Unit of Lung Toxicology, Department of Occupational, Environmental & Insurance Medicine and Division of Pneumology, Katholieke Universiteit Leuven, Leuven, Belgium

Dr Sonia Paytubi
Structural and Biological Mass Spectrometry Unit, Department of Medical Bioanalysis, Instituto de Investigaciones Biomédicas de Barcelona (IIBB), Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Dr Juana Peña
Structural and Biological Mass Spectrometry Unit, Department of Medical Bioanalysis, Instituto de Investigaciones Biomédicas de Barcelona (IIBB), Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Dr Rossanne M. Philen
The Epidemic Information Exchange (*Epi-X*), Office of Scientific and Health Communications, Centers for Disease Control and Prevention, Atlanta, GA, USA

Dr Manuel Posada de la Paz
Centro de Investigación sobre el Síndrome del Aceite Tóxico y Enfermedades Raras (CISATER), Instituto de Salud Carlos III, Madrid, Spain

Dr Victoria del Pozo
Immunology Department, Clínica de la Concepción, Fundación Jiménez Díaz, Madrid, Spain

Dr Francisco Pozo Rodríguez
Pneumology Department, Hospital Universitario 12 de Octubre, Madrid, Spain

Dr Rosario Prieto
Structural and Biological Mass Spectrometry Unit, Department of Medical Bioanalysis, Instituto de Investigaciones Biomédicas de Barcelona (IIBB), Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Dr Natalia Reig
Structural and Biological Mass Spectrometry Unit, Department of Medical Bioanalysis, Instituto de Investigaciones Biomédicas de Barcelona (IIBB), Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Dr Victoria Ruiz Méndez
Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Seville, Spain

Professor Stanislaw Tarkowski
Department of Environmental Health Hazards, Nofer Institute of Occupational Medicine, Łodź, Poland

Professor Benedetto Terracini
Department of Biological Sciences and Human Oncology, University of Turin, Turin, Italy

Joint WHO/CISAT Scientific Committee for the Toxic Oil Syndrome

Professor Emilio Gelpí
Department of Medical Bioanalysis, Instituto de Investigaciones Biomédicas de Barcelona (IIBB), Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain

Dr Edwin Kilbourne
Toxic Substances and Public Health, Agency for Toxic Substances & Disease Registry, Atlanta, GA, USA

Dr Carlos Lahoz
Department of Immunology, Clínica de la Concepción, Fundación Jiménez Díaz, Madrid, Spain

Dr Benoit Nemery
Research Unit of Lung Toxicology, Department of Occupational, Environmental & Insurance Medicine and Division of Pneumology, Katholieke Universiteit Leuven, Leuven, Belgium

Dr Rossanne M. Philen
The Epidemic Information Exchange (*Epi-X*), Office of Scientific and Health Communications, Centers for Disease Control and Prevention, Atlanta, GA, USA

Dr Manuel Posada de la Paz
Centro de Investigación sobre el Síndrome del Aceite Tóxico y Enfermedades Raras (CISATER), Instituto de Salud Carlos III, Madrid, Spain

Dr Arne Svejgaard
Department of Clinical Immunology, University Hospital of Copenhagen, Copenhagen, Denmark

Professor Stanislaw Tarkowski
Department of Environmental Health Hazards, Nofer Institute of Occupational Medicine, Łodź, Poland

Professor Benedetto Terracini
Department of Biological Sciences and Human Oncology, University of Turin, Turin, Italy

Foreword

From its sudden and frightening outbreak in May 1981, the toxic oil syndrome (TOS) has proven to be not only a new disease but one that continues to be a complicated, intriguing and often perplexing scientific challenge. It is also a disease that thousands of survivors must continue to live with on a daily basis.

Perhaps no other disease outbreak in medical history has been studied in such depth for so long. Hundreds of research studies have been conducted, embracing epidemiology, toxicology, clinical medicine, in vitro and in vivo studies and, most recently, immunology. As a foodborne disease with fatal consequences, TOS initially called attention to the need to strengthen food safety regulations and their enforcement. Nevertheless, despite the best procedures being in place, foodborne or other outbreaks will undoubtedly occur and the investigations into TOS have resulted in a wealth of information on how to go about studying such outbreaks in the future – from the gathering of basic information, to the collection and preservation of samples, to the follow-up of patients. Rather than starting anew, the lessons learned from the long-term study of TOS can be usefully applied. I hope that this book will provide guidance to public health authorities whose task it is to deal with such outbreaks.

The WHO Regional Office for Europe has been involved with TOS since the early days of the outbreak. At the request of the Government of Spain, WHO provided expertise in clinical medicine, epidemiology and toxicology and took part in various committees in the first years following the outbreak. Since 1987, a memorandum of understanding between the Spanish authorities and the Regional Office has formed the framework for the TOS investigations. The agreement also established a joint scientific committee of experts appointed by the Spanish authorities and WHO. Today, the WHO/CISAT Scientific Committee for the Toxic Oil Syndrome consists of nine experts from six countries in Europe and North America.

On behalf of WHO, I should like to thank the members of the WHO/CISAT Scientific Committee for their dedication and commitment to finding answers to the complex questions raised by this enigmatic disease. I should also like to acknowledge the efforts made by the authors and to thank them for their contributions.

As has been the ultimate aim of WHO throughout the years, I sincerely hope that the answers that have been found – and will continue to be found – will benefit those individuals still afflicted with this debilitating disease.

Marc Danzon
WHO Regional Director for Europe

Preface

Emilio Gelpí and Manuel Posada de la Paz

In 1992 WHO published the second book on the toxic oil syndrome (TOS) (1), gathering all available information on this disease to that date. In that book, the bases of the clinical definition and follow-up of TOS patients, as well as of the origin of the disease according to all the available epidemiological data, were clearly laid out. Since then, and as part of a carefully planned strategy reflected in the various chapters of this book, the WHO/FIS (now WHO/CISAT) Scientific Committee for the Toxic Oil Syndrome undertook a major effort on four fronts. First, various projects were supported aiming at the full chemical characterization of the oil matrix. Their purpose was to search for a component or family of components not endogenous to edible rapeseed oil, and the route by which it/they were incorporated into the fraudulently commercialized oil. Second, attempts were made to reproduce, on both a laboratory and an industrial scale, the refining process to which the suspect oil had been subjected, in an attempt to establish the conditions under which the toxin(s) were generated and to provide sufficient amounts of reconstituted oils for toxicological studies. Third, the search for an animal model in which to study the disease was undertaken. Finally, work on the possible immune origin of the intoxication was stepped up, with promising results to date.

As a result of all these efforts, we can be more confident today that we are on the right track. This is true even though the etiology of TOS is clearly elusive on account of its unique nature and the lack of a proper animal model in which to test the potentially toxic compounds. Nevertheless, it is important to recognize that TOS is not an isolated case in this respect: several other diseases that also lack an animal model have been described, such as various immune diseases that have been only partially developed in animals (2). Furthermore, if an immune origin could be confirmed it would account for the present situation.

We have indeed learned and progressed a great deal since the publication of the last TOS book, but this is a difficult problem that has taught us what needs to be done and what not to do if a case like this should ever occur again. The present volume is an account of the work carried out within the last decade of TOS research, supported and supervised by the WHO/CISAT Scientific Committee. A detailed summary of the multidisciplinary approach designed by this committee and the goals achieved to date have recently been published in Environmental Health Perspectives *(3) as well as in a comprehensive review of all epidemiological features of this outbreak (4). We trust that these two publications, together with the present book, will contribute to bringing researchers in this field up to date with the latest developments and achievements.*

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Introduction

Benedetto Terracini

This is the third book published by WHO on the outbreak of the condition that came to be called toxic oil syndrome (TOS), which struck Spain in 1981. The two previous books described, respectively, early observations (*1*) and scientific findings gathered throughout the 1980s (*2*). A crude comparison of the book published 10 years ago with the present one mirrors developments in TOS-related science in the last decade. The detailed consideration of the chemistry of TOS-related oils in Chapter 4 and Annex 4, as well as the length of Chapter 6 on the immunological features of the condition, reflect the amount of new, original investigations completed during the 1990s in these crucial areas. In contrast, there was no need for another chapter on pathology, since the early and late pathological features of TOS had been adequately described before 1990.

The complexity of the chemical approach indicates the new rationale and the encouraging perspectives for attempting to produce toxic oils in the laboratory and to mimic the "accident" postulated to have occurred in 1981. The fatty acid esters of 3-(*N*-phenylamino)-1,2-propanediol (PAP) stand out as new markers of toxicity (if not hazardous in themselves) and their toxicity and metabolism have become a major focus of research. This has implied laboratory work leading to the development of an ad hoc expertise in the use of aniline derivatives in biological assays, which merits a separate description in Annex 2.

The increasing relevance of the chemical and immunological investigations has brought about the need for repositories of oils and biological materials (tissues, sera, DNA). These are described in Chapter 2.

Also, the description of attempts to reproduce at least some clinical features of TOS in laboratory animals is dealt with in more detail than in the previous book. By and large, results have been deceptive. Although many experimental systems remain to be tested, there is room for the thought that conventional laboratory animals may be refractory to anything comparable to TOS. Before accepting this negative conclusion, however, much attention should be paid to the quality and design of the experiments that have been carried out, as set out in Chapter 5. Finally, the multiple social and human implications of an episode such as TOS – ranging from legal implications to scientific publications, and from long-term clinical follow-up to risk communication – has led us in Chapter 7 to comment on the ethical issues underlying the clinical and epidemiological investigations. These issues are common to the surveillance of victims of other environmental disasters.

In terms of causality in medicine, TOS is a paradigmatic case of circumstances in which the strength and consistency of the epidemiological evidence are not paralleled by findings in the chemical and biological laboratory. Within the classical schemes of causality in observational research (*3*), what is currently missing is the identification of the chemical agent(s) in the oil produced by illegal refining of denatured rapeseed oil, and therefore the interpretation of the toxic mechanism of such agent(s). The inability to fully or partially reproduce a condition comparable to TOS in laboratory animals might indicate a pathogenetic mechanism exclusive to the human species. This is the case for other autoimmune conditions in humans.

Thus, should the indictment of the fraudulent oil as the cause of TOS be a subject for discussion 20 years later? The answer is a definite no: the weight of the epidemiological evidence and the variety of design in the underlying studies provide strong and convincing evidence. This is not only the opinion of the contributors to this book in 2002; it was expressed, both to WHO and to the Spanish courts, by Sir Richard Doll nearly 20 years ago. The report written on that occasion is included as Annex 1. Sir Richard's analysis remains a model of causal thought, and provides guidance to other circumstances requiring the interpretation of unexpected outbreaks of previously unrecorded diseases brought about by previously unrecorded causes.

In spite of the robustness of the causal evidence, however, from time to time the media raises the fact that the chemical agent in the toxic oil has not been identified and that the disease has not been reproduced in laboratory animals. This is presented to the lay public as an alleged testimony to the inconsistency and untruth of the fraudulent denatured rapeseed oil hypothesis (*4*). These reports neither convey any constructive criticism of the investigations that have supported such a hypothesis, nor provide any evidence for any alternative hypothesis. Correspondingly, no support for any alternative hypothesis transpires from the more than 300 citations that can be found in Medline under the name of the disease.

This book is dedicated to Norman Aldridge (1919–1996), who over 15 years addressed his brainpower and expertise to the definition of a scientific strategy for investigating TOS. Norman Aldridge was not only an outstanding investigator; he was generous with his knowledge and his patience. The contributors to this book are conscious of the privilege they have had to collaborate with him.

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1

Epidemiology of toxic oil syndrome: advances since 1992

Rossanne M. Philen, Manuel Posada de la Paz, Benedetto Terracini and Edwin Kilbourne

The World Health Organization has periodically updated knowledge about the toxic oil syndrome (TOS) since the epidemic occurred in 1981. The first WHO publication on TOS appeared in 1984, and was followed by a second in 1992 (*1,2*). The latter mentioned a number of studies that were under way, planned or suggested. Many of those have since been completed or begun. In this chapter we provide an update of the epidemiological work on TOS from 1992 to the present.

Descriptive epidemiology

Although the descriptive epidemiology of the TOS epidemic has been thoroughly covered in the past, it will be briefly summarized here (*1–5*).

The epidemic began on 1 May 1981, and four months later a case definition requiring two major criteria – or one major and four minor criteria (see Chapter 3) – was developed for the new illness. This case definition was used to determine which patients to include in the patient registry, later called the

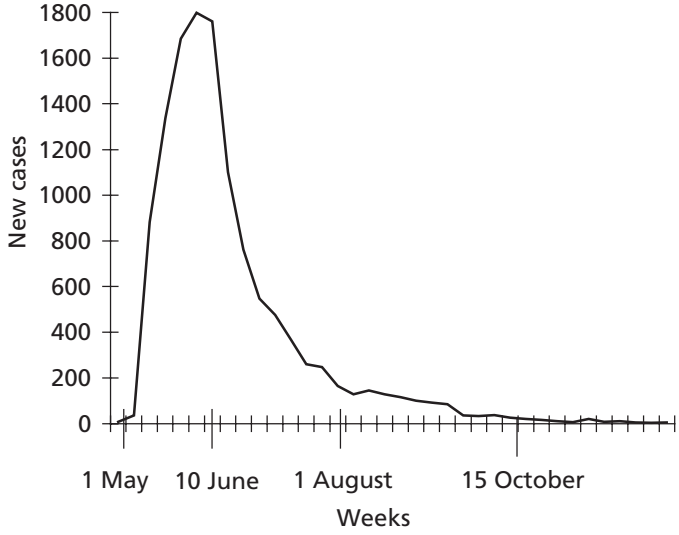
Official Census (OC) (*6*). The first OC was released in October 1981, five months after the beginning of the epidemic. In spite of the official case definition, however, the criteria were not systematically followed for months after the epidemic began. Records were often collected from hospitals and health units and entered into the OC irrespective of whether the case definition had been followed. The OC was successfully used for research and other purposes for a number of years. A review by the WHO/CISAT (formerly WHO/FIS) Scientific Committee for the Toxic Oil Syndrome closed the OC as of May 1997 with 19 904 patient records (*7*).

Women comprise 11 897 (60.8%) and men 8007 (39.2%) of the records in the census (*3*). The greatest number of TOS-affected people were aged 30–50 years and belonged to the lower socioeconomic groups, while only 38 (0.2%) of the TOS cohort were children under 1 year of age. While the greatest number of cases (14 445) occurred in the Madrid province, the highest rate of affected people (over 300 per 100 000 population) was found in the provinces of Segovia and Palencia. The vast majority of cases were reported from 14 of Spain's 52 provinces, although a few were reported from other provinces, as well as one case in a Swiss citizen and another in an American citizen. A carefully executed study determined that all these people had been exposed to the toxic oil in Spain immediately prior to becoming ill.

While the announcement of the oil–disease relationship is thought to have played a major role in the decline in new cases after 10 June, the epidemic curve began to fall about a week before this date, possibly because all of the contaminated oil had been consumed. The possibility that people still had toxic oil at home, however, led to an official effort to recall suspect oil, beginning on 30 June 30, whereby oil was exchanged for pure olive oil at government expense.

Although one case of TOS was reported as long as a year after the epidemic finished, this was clearly an exception and it was determined that the person in question had saved some oil from the epidemic period and had consumed it much later. Thus, the vast majority of cases occurred during the initial epidemic period, a pattern compatible with a point source epidemic. There is substantial evidence to support the hypothesis that TOS was a short-term, point source epidemic (*8*). First, the epidemic curve is consistent with a short-term point source exposure, with a rapid peak and decline (Fig. 1.1). Second, oil denatured with aniline had been in circulation in many areas of Spain for some time; TOS, however, occurred only in the Madrid area, where oil from the RAELCA company was distributed, and not in other areas where denatured rape-seed oil was known to be sold. Third, the contaminant OOPAP, the 1,2-dioleoyl ester of 3-(*N*-phenylamino)-1,2-propanediol (PAP), which has been established to be associated with TOS in a dose–response fashion, was found only in oil distributed by RAELCA that had been refined at the ITH refinery

Fig. 1.1. Epidemic curve for TOS, 1981



(*9*). Finally, a study of oil containers implicated in the epidemic showed that almost all of those manufactured by RAELCA contained oleic acid anilide (OAA), a chemical marker shown in both toxico-epidemiological studies (see below) to be associated with illness in a dose–response manner. Of the nine other types of similarly shaped bottle in use at the time of the epidemic, only 1.2% were found to contain OAA (*8*).

Analytical epidemiology

Overview of all new studies

Traceback studies

While the precise etiological agent in the oil responsible for TOS has still not been identified, progress has been made in determining the process that may have led to the production of a toxicant in the aniline-denatured rapeseed oil. One study, which yielded a great deal of information about possible contaminants and how the contamination might have occurred, required investigators to visit two French rapeseed oil processing companies. These companies had been identified in Spanish administrative and judicial records as those exporting aniline-denatured rapeseed oil to Spain in 1981. The oil exported to Spain was found to have been taken from stock, and the remainder sold for human consumption in France without any known adverse health effects. The important difference between the oil exported to Spain and that sold in France

was that aniline equivalent to 2% by weight was added to most of the Spanish oil but not to any of that sold in France (*10*).

This study also identified other possible sources of contamination, one of which was whether the tanker trucks used for transporting the oil to Spain had been contaminated by previously carried industrial chemicals. Investigators found no assurance that the trucks had been cleaned in a manner appropriate for the transport of a food product before the oil was loaded. Since the oil was ostensibly for industrial use, however, such cleaning would not have been a requirement. Since the clinical manifestations of TOS were not those of aniline toxicity, the investigators concluded that the etiological agent was likely to be either (*a*) a contaminant in the aniline, (*b*) a contaminant introduced during transportation, or (*c*) a product of a reaction of normal oil components or of materials used in refining with either aniline or the potential contaminants mentioned under (*a*) or (*b*) (*10*).

Mortality studies

The preliminary results of a pilot mortality study that sampled 1000 TOS patients were presented in the previous book (*2*). After three mailings of a letter and questionnaire, only 66% of the sample had responded, but eventually the investigators were able to obtain responses from virtually all remaining patients (or surrogates) when they were contacted by telephone. This preliminary mortality study found that in 1981 there was clear-cut excess mortality in the cohort (standardized mortality ratio (SMR) 6.51, 95% confidence interval (CI) 3.92–10.17). However, from January 1982 through to 7 March 1988 there was no statistically significant overall excess mortality, except during 1982–1983 among people aged <65 years (SMR 2.26, 95% CI 1.03–4.29). From 1983 onwards, this study found a consistent decrease in overall mortality. These data led the investigators to conclude that TOS altered the patterns of mortality among affected people, and recommended that an analysis of deaths by cause be conducted within the TOS cohort (*11*).

Because of the success of the pilot study, a mortality study of the entire cohort was performed, in which every living member of the TOS cohort was contacted by mail or telephone to determine overall mortality as well as mortality by cause (*12*). Virtually every living member of the cohort and family members of those who were known to have died were contacted in order to identify all deaths from 1 May 1981 to 31 December 1994. The investigators collected data on cause of death from death certificates and determined the underlying causes of death, which were then coded using the ninth revision of the International Classification of Diseases (ICD-9). The study found that 1663 deaths had occurred in that period among TOS cohort members, giving a crude mortality rate of 8.4%. Mortality was highest in 1981, with an SMR of 4.92 (95% CI 4.39–5.50) compared with the Spanish population as a whole.

The highest SMR (20.41, 95% CI 15.97–25.71) was seen among women aged 20–39 years during the period 1 May 1981 to 31 December 1982. Women under 40 years of age and who were affected by TOS were at greater risk of dying in most time periods than their unaffected peers, while older women and men were not (*12*).

Over the follow-up period, mortality of the cohort was less than expected compared with mortality of the Spanish population as a whole or with general mortality among the population of the 14 provinces where the epidemic occurred. Investigators also found that, except for deaths attributed to external causes (including TOS and pulmonary hypertension), all causes of death were lower in TOS patients compared to the Spanish population as a whole. The most frequent underlying causes of death were: TOS, 350 (21.1%); circulatory disorders, 536 (32.3%); and malignancies, 310 (18.7%). This study concluded that, while on average people affected by TOS were not at greater risk of dying over the 13-year study period than any of the comparison groups, women aged 20–39 years were at greater risk of dying (*12*).

This second mortality study, which extended observations through to 1994, found the same reduction in overall mortality for all major causes of death (*12*). This reduced mortality could result from errors such as failure to identify all deaths in the cohort, data entry errors or misclassification, despite the fact that the study controlled for these issues very carefully. In addition to contacting all TOS patients or patient proxies, the investigators checked death certificates for all but 8 of the 1663 deaths. This procedure was adequate in identifying false positives but not in identifying false negatives. Mortality in the TOS cohort was lower than in either the general Spanish population or that of the 14 provinces where most of the TOS patients lived. The investigators also studied deaths in the TOS cohort in several smaller communities and found that the cohort and the community had similar SMRs, although that of the TOS group remained slightly (though not statistically significantly) lower (*12*).

Various possible explanations exist for the decreased mortality. It could be the result of a "healthy survivor" effect, because people in the TOS cohort receive more medical care than the average citizen, or because the disabilities of many TOS patients protected them from other risks, such a motor vehicle and work-related accidents and other injuries. A reduction in other risk factors could also have played a role, for example if the TOS patients smoked or drank less than usual. Economic benefits may also have helped, by improving the socioeconomic status of the patients.

Subsequent to the study cited above, a follow-up of the cohort for mortality was carried out for the period 1995–1999. An additional 761 deaths were added to the previous number, giving a total through to the end of 1999 of 2424 deaths from all causes. In the three years for which comparison data for

the general Spanish population are available, the SMRs of the TOS cohort remained below that of the Spanish population as a whole. For 1995, 1996, 1997, 1998 and 1999, the SMRs were respectively 0.82 (95% CI 0.69–0.97), 0.84 (95% CI 0.71–0.98), 0.83 (95% CI 0.70–0.97), 0.93 (95% CI 0.80–1.08) and 0.77 (95% CI 0.66–0.91) (*13*).

The only group of TOS patients in which mortality was higher than that of the general Spanish population was that of females between the ages of 0 and 39 years. A study on cause of death in females under 40 years of age showed that the most important cause of excess mortality was TOS. The authors defined TOS as an underlying cause of death where it could be clearly documented that the persons in question had been continuously ill with TOS since first developing the disease. The study examined deaths in young women attributed to TOS from 1 May 1981 through to 1994, and found that the observed-to-expected ratios were 38/16.4 = 2.32 in 1983–1988 and 24/15.6 = 1.54 in 1989–1994. Of the 62 deaths in this age group between 1983 and 1994, 31 were attributed to TOS as the underlying cause. The authors proposed that this increase in death rates could reflect an increased exposure to the oil (*14*). Incidence, readmission and death rates were also almost twice as high in women as in men during 1981–1982.

A preliminary study to examine the sensitivity and specificity of causes of death given on death certificates compared to those that could be postulated from the clinical history was completed for 825 of the 1773 TOS deaths registered from 1981 to 1994. The sensitivity for cause of death by ICD-9-CM code was found to range from 0.37 (95% CI 0.1–0.72) for codes 580–629 (diseases of the genitourinary system) to 0.74 (95% CI 0.67–0.8) for codes 140–239 (malignant neoplasms). The specificity was more satisfactory, ranging from 0.84 (CI 0.81–0.87) for codes 390–459 (diseases of the circulatory system) to 0.99 for both codes 140–239 and codes 320–389 (diseases of the nervous system), both having 95% CI 0.98–0.99 (*15*).

Toxico-epidemiological studies

Although TOS has long been linked to the consumption of aniline-denatured rapeseed oil, the precise etiological agent remains undiscovered more than 20 years later, in spite of intense efforts on the part of many investigators. The first toxico-epidemiological study (*16*) found a strong dose–response relationship between the concentrations of OAA in the oils and the risk of developing TOS in the families that used those oils, suggesting that OAA could be used as a marker for oils that contain (or contained) the causal agent. This study, however, investigated only a relatively small geographical area in Madrid.

In the second toxico-epidemiological study (*17*), the chemical composition of oils obtained from TOS-affected (case, N = 59) and unaffected (control, N = 70) families in a larger geographical area were examined for contaminants.

Case oils had higher concentrations of fatty acids and sterols that are found in particularly high concentrations in rapeseed oil. Most importantly, not only were case oils found to have more frequent and more extensive contamination with OAA and other fatty acid anilides than control oils but, again, the levels of OAA were present in a dose–response relationship, risk increasing sharply with increasing concentrations of OAA.

Thus both studies (*16,17*) allowed for the conclusion that a high concentration of OAA was a specific marker for oils that contained the etiological agent of TOS. Both established that the consumption of specific oils containing fatty acid anilide contaminants, in particular OAA, was associated with an increased risk of developing TOS. In addition, more recent laboratory work, using chemical analytical methods developed long after the epidemic period, has led to the identification of a family of compounds, the di-fatty acid esters of phenylamino propane-diol. One specific member of this family, OOPAP, has been shown to be even more strongly associated with development of TOS than the fatty acid anilides, suggesting that oils containing OOPAP may be a more important marker for potentially toxic oils than those containing OAA. The odds ratio for exposure to OOPAP (26.4, 95% CI 6.4–76.3) was found to be much higher than the odds ratio for exposure to OAA (4.1, 95% CI 2.2–7.8) when the same oils were considered. Of particular importance is the fact that OOPAP was found in oil from the only refinery whose oil was clearly associated with TOS (*8,9*). These compounds are being evaluated as possible etiological agents and several are being tested in animal studies (see Chapter 5).

Although to date most of the research with PAP compounds has been with OOPAP, OPAP (the 3-oleoyl ester of PAP) or an oil containing a heterogeneous mixture of various PAP esters, it is important to remember that these compounds are only a few of the many possible PAP esters likely to be present in the oils. PAP has three sites where a fatty acid anilide could react, and the reaction could take place at one, two or all three sites, resulting in a number of different compounds. Additionally, there are many different fatty acid anilides in the TOS oils, so that three different fatty acid anilides could react with the PAP in different orders. Thus a large number of contaminants is possible and any one or any combination of these could be the causative agent of TOS (*9*).

Other etiological studies

One study examined the hypothesis that 5-litre plastic containers of rapeseed oil associated with TOS, and which contained OAA, had a characteristic shape. Investigators measured fatty acid, sterol, and fatty acid anilide levels in oil from containers of a variety of shapes. A total of 1673 oil containers collected during the Spanish Government's oil exchange programme were then

linked to TOS cases reported in the official government census of patients. Although rapeseed oil (identified by the presence of brassicasterol) was found in 798 (47.7%) of the bottles examined, contamination with fatty acid anilide occurred in only 329 (19.6%). Of these, 319 (97%) were oil containers of the shape sold by RAELCA, an oil distribution company in Madrid that has been strongly implicated in the epidemic.

The first aniline-denatured oil that RAELCA purchased to be refined specifically for distribution was refined at the ITH refinery in Seville, and it is this oil that has been most directly associated with the TOS epidemic. The only toxic oil linked to a specific refinery was that associated with rapeseed oil from the ITH refinery in Seville, and the epidemic began shortly after this oil was delivered to RAELCA for retail sale (*8*). On the basis of these findings, we conclude that oil refined by ITH and distributed by RAELCA was the principal, and probably the only, oil responsible for the TOS epidemic, and that it was a point source epidemic. Information on the history and treatment of this oil should continue to yield important clues towards identifying the etiological agent of TOS (*8*).

Liquid chromatography combined with atmospheric pressure ionization–tandem mass spectrometry has also been used for the analysis of oils associated with TOS (see Chapter 4). The oils used in this work were the same as those analysed in the second toxico-epidemiological study described above (*17*). These more recent analyses have focused on measuring PAP, OPAP and OOPAP. OOPAP and OPAP were found more frequently and at higher concentrations in TOS case oils than in control oils, with odds ratios of 13.7 (95% CI 5.0–38) and 21.9 (95% CI 6.1–78), respectively. Other fatty acid esters of PAP are also likely to be present in the case oils. More significantly, OOPAP and OPAP were found in aniline-denatured rapeseed oil refined at ITH, yet they were not detected in samples of unrefined aniline-denatured rapeseed oil delivered to ITH. These results show that the esters of PAP were products of the ITH refining process and were not formed spontaneously during storage, in contrast to the anilides that can form spontaneously during storage and transport of aniline-denatured rapeseed oil. PAP esters were not detected in samples of other aniline-denatured rapeseed oils that were refined elsewhere and that were not associated with illness. These findings support the hypothesis that one or more of the fatty acid esters of PAP was the etiological agent of TOS *(18)*.

Discussion and conclusions

The TOS epidemic has long been known to have resulted from the consumption of rapeseed oil denatured with aniline. Nevertheless, the recent research cited here has established the existence of numerous compounds in the denatured oil refined at ITH, any one or a combination of which could be

the direct causative agent of TOS. It appears that, in all likelihood, rapeseed oil denatured with aniline that was not refined at ITH was not associated with illness. This is a major development, for it substantially narrows down the number of oils that could potentially be used in animal or other toxicity studies.

Could OOPAP be the etiological agent of TOS? While it is the PAP compound found in the greatest concentration in the suspect oils, this fact alone is not enough to implicate it. Nevertheless, it is clear that in both published and unpublished reports, OOPAP is found only in the oil refined at ITH (*8,19*). The role of OOPAP and other related compounds in the disease process remains to be investigated. It is not yet known whether the causative agent is OOPAP alone, another contaminant in the oil, or a combination of several of the oil contaminants.

Various laboratories have attempted to reproduce the toxic oil. Recently, at least one has succeeded in producing an oil that is very similar in certain specific chemical characteristics to oils from the epidemic period that have been associated with illness, as well as to samples of the oils from the ITH factory that made up the shipment of oil implicated in the TOS epidemic (see Chapter 4). Reproduction of anything comparable to the toxic oil has been an extremely difficult and tedious task (*20,21*). This difficulty, together with the fact that the reproduction of oils containing PAP esters (and which are therefore likely candidates for the toxic oil) is apparently very sensitive to a slight variation in manufacturing conditions, suggests that the initial event that produced the toxic oil at the ITH refinery was unique and very unlikely to be replicated elsewhere. These newly produced oils will be important for future animal studies (see Chapter 5), since the etiological agent may not be stable and thus may have degraded or decomposed in the original oils.

Evidence to support a point source outbreak continues to accumulate. As mentioned, the shape of the oil containers, the finding of compounds associated both with the rapeseed oil and with oils specifically associated with the RAELCA oil distribution company, the epidemic curve, and the decrease in cases after the oil was removed from circulation all support a single source for the epidemic.

Eosinophilia–myalgia syndrome

The WHO Regional Office for Europe, in collaboration with the Fondo de Investigación Sanitaria, Spain, and the Food and Drug Administration, National Institutes of Health, National Institutes of Mental Health and Centers for Disease Control and Prevention in the United States, sponsored an international workshop in May 1991 (*4*). Researchers and clinicians from throughout the world met to share information about two very similar diseases: TOS and eosinophilia–myalgia syndrome (EMS).

The workshop concluded that TOS and EMS have many similarities. Both are related to consumer products found to contain numerous trace contaminants that remain to be identified, including the etiological agents of both disorders. Both EMS and TOS cause patients to experience intense, incapacitating myalgia and a marked peripheral eosinophilia. Both also display many rheumatic manifestations, and the long-term complications of both include pulmonary hypertension, peripheral neuropathies and joint contractures. Both disorders are highly likely to derive from the same or similar physiological pathways. EMS and TOS have their origin in an immunological response to a toxic exposure. Both have very similar vascular lesions and fibrosis in tissues, and both exhibit the same remarkable eosinophilia. The workshop reinforced WHO's recommendations that additional research was needed into the etiological agents, preferred treatments and ways of avoiding similar problems in the future.

One of the more striking parallels between EMS and TOS is that both disorders may have very similar etiological agents. It has been determined that 3-(phenylamino)-1,2-propanediol, an important TOS oil contaminant, can be biotransformed to 3-(phenylamino) alanine, a contaminant that is of substantial importance in EMS research (*22*). This work thus provides a chemical link between EMS and TOS (*23*).

A major implication of the existence of the EMS epidemic is that TOS is not an absolutely unique process: one other similar – albeit not identical – epidemic has occurred. That being the case, it is even more important to pursue TOS research, because it is likely that TOS represents only one of a group of diseases related to an environmental exposure. Other rheumatological and immunological diseases, such as drug-induced lupus, may also have a cause-and-effect relationship with environmental exposure (*24,25*). Future similar catastrophes cannot be prevented with any level of certainty without a better understanding of the etiology and pathogenesis of the TOS outbreak.

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2

Sample repositories

Ignacio Abaitua, Concepción Martín-Arribas, Victoria del Pozo and Manuel Posada de la Paz

From the beginning of the TOS epidemic, we considered collecting biological samples from TOS patients for future research. Many hospitals involved in the epidemic began collecting different types of biological specimen, such as serum and biopsy samples and organs, but this was not conducted in any systematic manner.

In June 1981, when the relationship between the consumption of some kind of oil and TOS was accepted as valid by the Spanish Government, steps were taken to withdraw the oil from sale and from households where there were still remains of the oil. A procedure was introduced whereby people could replace any suspect oil with pure oil. In this way, many thousands of suspect oil containers were exchanged. Data on the identity of people who handed in suspect oil were also collected.

With time we became more convinced of the need to establish repositories for biological samples and oil samples, so that these could be available for future research. At the same time, the need to establish a follow-up of the basic clinical aspects of the cohort was also recommended by the research team.

Oil repository

After collection, the oil samples were stored at several depots, the most significant being that at Arganda (Madrid) (*1,2*). Most of the containers

were labelled with some kind of identification. Documentation linked to the labels was also kept. For the purposes of research, thousands of stored containers were subsequently reviewed and the information on the labels was matched to the documentation from the exchange process. In this way, some 1500 containers that had very likely been handed over by TOS-affected people were identified. The shape and other characteristics of the original containers were also recorded. This led to the creation of the oil repository at the Centro de Investigación Síndrome Aceite Tóxico (CISAT) at the Instituto de Salud Carlos III in Madrid. Recently, a new selection identified around 800 samples considered of interest for research.

Repository for sera and other biological samples

When the current team at CISAT started its work in 1987 it attempted to recover, wherever possible, all the serum and other biological samples from TOS patients that had been collected from the beginning of the epidemic but that were dispersed in different laboratories and hospitals throughout the country (*3*). This task was carried out with rigour and patience over several years. Samples had to be brought from different places in a way that ensured that they remained intact and unchanged, especially in the case of frozen samples. It was also essential to obtain the relevant documents, or at least a copy, for each sample.

The materials include serum samples, frozen tissues and tissues preserved in formaldehyde and other agents. All data have now been computerized, and over the last two years tissue samples and paraffin blocks of selected organs not affected by the disease have been prepared for DNA extraction.

Table 2.1 shows the number of samples collected at CISAT according to type of organ and means of storage.

Suitable equipment for storing all the samples had to be provided at CISAT. Samples or specimens in formaldehyde are stored in cabinets. In the case of frozen materials, however, it was necessary to purchase large-capacity freezers capable of providing temperatures of –80 °C and to ensure adequate storage in the case of a power cut (in which case an alarm automatically warns those in charge). In addition, the serum repository is an active depot in that not only are samples recovered from the beginning of the epidemic kept, but new samples are continually added from patients previously not contacted.

DNA bank

Progress in experimental sciences and genetic research has made possible the development of new techniques for the analysis of biological materials. Thus the creation in the last few years of a DNA bank for TOS patients has allowed us to return to the study of TOS and to increase our knowledge on the etiology and pathogenesis of the disease.

Table 2.1. Biological samples in the CISAT collection

Item	Paraffin blocks	Formaldehyde 10%	Frozen	Total
Spleen	61	87	10	158
Brain	398	264	7	669
Heart	163	179	31	373
Digestive tissue	216	38	19	273
Liver	132	252	237	621
Bone	50	37	6	93
Muscle	125	144	42	311
Nerve	80	21	3	104
Pancreas	76	9	2	87
Skin	48	28	4	80
Lung	360	197	109	666
Kidney	251	224	51	526
Adipose tissue	37	15	58	110
Vessels	68	16	2	86
Genital tissue	107	12		119
Lymph nodes	68	3		71
Other	387	259	93	739
Total	2627	1785	674	5086

Since 1992, DNA from the lymphocytes of TOS patients who participated in two case-control studies and from cells cultured for immunological studies has been stored (*4–8*). The total numbers of cases and controls from whom DNA samples are available and their distribution by sex are shown in Table 2.2.

Advances in genetic technology, such as the polymerase chain reaction (*9*) and the work of the Human Genome Project to map and sequence the human genome (*10,11*), are accelerating our understanding of the genetic determinants of different diseases. The purpose of our bank is the same as that of other DNA banks: the performance of epidemiological research on genetic risk factors and genetic polymorphism. It could also be useful in future research to genetic level. In addition to DNA, however, living cells are very valuable for several reasons: (*a*) they can be used for detailed biochemical and molecular studies; (*b*) they may enhance the available supply of DNA;

Table 2.2. Numbers of people from whom DNA samples were taken

	Total	Males	Females
Controls	487	200 (42%)	287 (58%)
Cases	2306	747 (31%)	1559 (69%)
Total	2793	947 (34%)	1846 (66%)

and (*c*) they may represent the exclusive biological resource for investigating high-risk groups of patients from whom additional blood samples would be difficult or impossible to obtain. It is therefore very important to continue to "immortalize" cell lines.

Thus in 1997 we decided to create a DNA and cell bank in order to collect samples from TOS patients and their relatives, wherever possible. Biological samples from patients, with their informed consent, are taken during clinical check-ups. Samples are sent to the Immunology Department of Fundación Jiménez Díaz, where the DNA and cells are extracted and stored.

Extraction of DNA

To purify DNA from white blood cells we use a commercial kit (QIAamp® DNA blood kit, QIAGEN) based on spin columns, a rapid method that yields DNA of very good quality. DNA is purified from 2–4 ml of blood, and multiple aliquots of the stock DNA in TE buffer are stored at –80 °C. The concentration in each sample ranges between 0.5 µg and 65 µg. Whole blood is stored at –80 °C in two aliquots, kept in different freezers for security reasons.

Cell isolation from blood samples

Blood is shipped as soon as possible to the laboratory and maintained at 25 °C until the lymphocytes are separated. For this purpose we use the Ficoll-Hypaque technique, which separates the blood components into saline-diluted plasma and peripheral mononuclear cells. Thus, 10 ml of blood yields 10 ml of diluted plasma and cells that are separated into 8 aliquots. These are frozen in RPMI-1640 with 10% heat-activated fetal calf serum and 10% dimethyl sulfoxide. In this way, a good yield can be obtained when lymphocytes are later thawed and immortalized (Fig. 2.1).

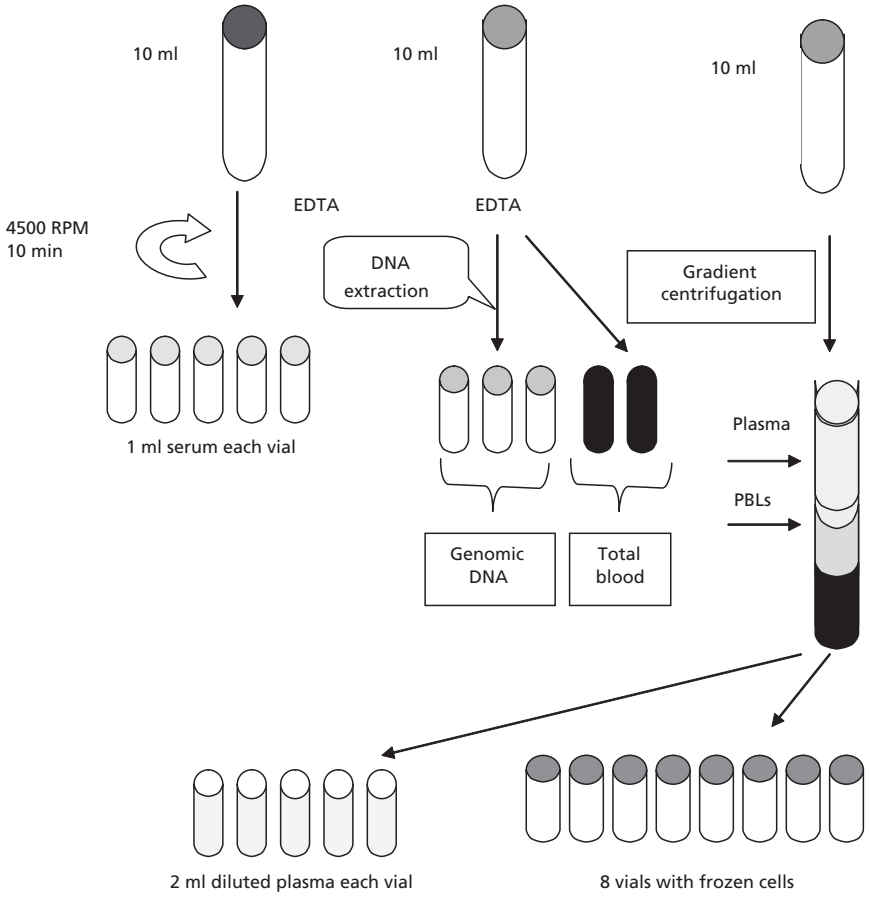
A computer program has been developed for managing the bank and controlling the sample inventory. Computer application has been designed in accordance with the Organic Law 5/1992 on regulation of automatic treatment of individual data (LORTARD). Thus files containing primary personal data (names, addresses, etc.) are separated from those containing data on the sample and its derivatives. Samples are identified by a unique bar code and this code is registered as primary data in the files and sample storage. In this way, the sample can be handled anonymously in the laboratory.

The allocation of bar code labels is also controlled by the computer application, thus avoiding duplication in assigning codes.

In addition to the need to ensure the physical safety of the samples, requirements for protecting the donor´s rights are taken into account through an Ethics Committee (see Chapter 5).

In conclusion, we are working to set up a bank of well characterized biological samples from persons included in studies performed at CISAT,

Fig. 2.1. Blood processing schedule



thus contributing to an epidemiological assessment. The samples are from volunteers who participate in the studies and who freely give their informed consent to their samples being included in the DNA bank.

Table 2.3 shows the numbers of samples stored in the DNA bank according to type.

Summary

From the beginning of the TOS epidemic, we considered collecting biological samples from TOS patients for future research. As time elapsed we became more convinced of the need to establish repositories for biological samples and oil samples, so that these could be available for future research. When the

Table 2.3. DNA bank samples

Sample type	No.
Samples containing DNA	7 601
Plasma samples	10 280
Serum samples from the same blood lots	11 795
Cell samples	16 321
Total	45 997

relationship between the consumption of some kind of oil and TOS became accepted, a procedure was introduced whereby people could replace any suspect oil with pure oil. In this way, many thousands of suspect oil containers were exchanged. This led to the creation of the CISAT oil repository. The current team at CISAT attempted to recover serum and other biological samples from TOS patients that had been collected from the beginning of the epidemic but that were dispersed in different laboratories and hospitals. The materials include serum samples, frozen tissues and tissues preserved in formaldehyde and other agents. The serum repository is also an active depot, in that new samples are continually added from patients previously not contacted. The creation in the last few years of a DNA bank for TOS patients has allowed us to increase our knowledge of the etiology and pathogenesis of the disease. Thus in 1997 we decided to create a DNA and cell bank in order to collect samples from TOS patients and their relatives.

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3

Clinical aspects

Agustín Gómez de la Cámara, Manuel Posada de la Paz, María del Mar Plaza Cano, Eva Estirado de Cabo, María Luisa García de Aguinaga, Maravillas Izquierdo Martínez, Concepción Martín-Arribas and Francisco Pozo Rodríguez

The toxic oil syndrome has been described as a multisystemic disease of unknown pathogenesis, beginning as a non-necrotizing endothelial injury with a likely autoimmune component in its inception and/or progress (*1*). In a sizable number of TOS victims, early changes evolved into long-term sequelae, but no excesses of complicating conditions (such as cancer) have been detected so far. The most prominent pathological feature is a peculiar non-necrotizing vasculitis that affects mainly the intima and involves vessels of every type and size in practically every organ (*2*).

As yet, there is no direct evidence for the pathogenetic basis of the disease. An immunological mechanism is hypothesized (*3*), based on the similarity of symptoms between TOS, graft-versus-host disease and hydantoin-induced autoimmunity. A susceptibility trait has been suggested for NAT2-defective alleles: persons with NAT2 homozygous mutant haplotypes would be at the

highest risk, whereas heterozygous and homozygous wild types would be affected only when cumulative doses exhausted a subject's metabolic capacity (*4*).

Clinical development

The disease developed in three clinically distinct phases and presented a great variety of symptoms (*3,5*). The acute phase was characterized by dyspnoea secondary to non-cardiogenic pulmonary oedema that prompted people to seek emergency hospital treatment. Chest X-rays showed alveolar-interstitial infiltration, often accompanied by pleural effusion. The criteria for case definition used for diagnostic purposes are shown in Box 3.1.

Frequent signs and symptoms included peripheral eosinophilia in 78% of patients, rash in 39% and myalgia in 80%. The onset of severe myalgia and muscle cramps was typical of the end of the acute phase. Approximately 70% of all TOS patients had pulmonary findings, combined to a greater or lesser degree with other symptoms.

About two months after the onset of symptoms, the intermediate phase began, also typically lasting two months. Sensory neuropathy was the most

Box 3.1. Diagnostic criteria for TOS

Major criteria

1. Consumption of oil presumed to be toxic before onset of illness, or occurrence of the illness in the nuclear family
2. Pulmonary pathology with radiological findings of diffuse interstitial or alveolar interstitial infiltrates, with or without pleural effusion
3. Incapacitating myalgia with functional impairment
4. Eosinophil count greater than 500 per mm^3

Minor criteria

1. Epidemic outbreak in the community
2. Severe skin itching
3. Rash or localized oedema of the skin
4. Severe and persistent dry mouth
5. Minimal or moderate myalgia
6. Neurological pathology
7. Abdominal pain
8. Clinical or analytical signs of hepatic involvement
9. Recent onset of exertional dyspnoea
10. Recent onset of hypoxaemia
11. Pulmonary hypertension
12. Cardiomyopathy
13. Vascular thrombosis

Two major or one major and four minor criteria were required for clinical case definition.

frequent clinical feature; it was characterized by intense myalgia and muscle weakness in approximately half the patients, together with a gradual onset of numbness and cutaneous hypaesthesia or painful burning dysaesthesia. The pattern of distribution was variable, but it mainly affected the distal parts of the limbs and was symmetrical. Other signs in this phase were marked weight loss and induration of the skin followed by skin infiltration. The sclerodermiform changes started with slightly indurated oedematous areas, which became progressively harder. The skin lesions were similar in some cases to a localized myxoedema or 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 (*6*). The generalized skin sclerosis was associated with neuromuscular symptoms. A number of patients exhibited hepatic cholestasis, dysphagia, pulmonary hypertension and thromboembolism of the large vessels. High levels of peripheral blood eosinophils, elevated triglycerides, elevated cholesterol and hyperglycaemia were common. The platelet count decreased during this phase and some patients developed a disseminated intravascular coagulopathy, which was the cause of death in many cases (*3*).

Later, about 59% of affected individuals developed symptoms of the chronic phase. Peripheral motor neuropathy affected 32% of all patients, and there was a similar frequency of sensory neuropathy. Involuntary muscular activity such as cramps, myoclonus and tremor were observed in 60%. Other features included scleroderma (21%), contractures (20%), pulmonary hypertension (8.2%) and hepatopathy (7.3%). These findings were combined in different proportions and with different severity.

More than 300 persons died from TOS in the first two years (*7*). The main causes of death were respiratory insufficiency in the acute phase, pulmonary hypertension and vascular thrombosis in the intermediate phase, and infectious complications, respiratory insufficiency secondary to neuromuscular weakness and pulmonary hypertension in the chronic phase.

Developments and current status

Several descriptions of the clinical development of TOS, and one on the quality of life of the survivors, have been published during the last 10 years (*8,9,* A. Gomez de la Camara, unpublished data, 1997). They focused on the natural history of the disease, the clinical picture, the prevalence of signs and symptoms and possible complications. These studies were carried out on different series of cases by different authors, with different sampling criteria and different patient participation. Nevertheless, a systematic and consistent pattern of late features was found.

Overall picture

Although many of those affected recovered from the acute and intermediate phases, some conditions – either isolated or in various combinations – are still common now, over 20 years after the onset of the disease. Over 60% of TOS victims report suffering from myalgia and paraesthesia, and contractures are present in a quarter of the affected population. Some 10% of TOS patients are recorded by the Instituto Nacional de la Seguridad Social as handicapped with permanent disability (*8*).

Respiratory system

Many organs were affected by interstitial fibrosis but, based on both clinical and histological examinations, the lungs seem to have been spared. Nevertheless, pulmonary hypertension, with clinical and pathological characteristics similar to those of primary pulmonary hypertension, has been seen in the chronic phase. Lung specimens, collected at autopsy or by biopsy during the early stages, exhibited primary endothelial injury with extensive cell proliferation and perivascular inflammatory infiltrates. The lesion evolved subsequently to intimal fibrosis up to total obliteration of the vessel lumina. These changes were the probable cause of the onset of pulmonary hypertension. On the other hand, in many patients pulmonary pressure regressed to normal values; a rational hypothesis is that this reflected a decrease in pulmonary inflammatory components and subsequent vessel permeability, an event observed in most patients with pulmonary hypertension in the initial stages (*10*). Some of these patients have exhibited a low carbon monoxide diffusion factor for some years. Throughout the intermediate and chronic phase, the cumulative incidence of pulmonary hypertension has been about 7% of the affected population in one of the largest series (A. Gomez de la Camara, unpublished data, 1997), two thirds being women. Unless a selection bias was operating, this proportion would correspond to approximately 1400 persons in the whole series. In a limited subset of patients, pulmonary hypertension showed a malignant course leading to death. Currently, active pulmonary hypertension is present in 1.5% of the study population. Complaints include palpitations, arrhythmia and dyspnoea on exertion, but the extent and importance of these symptoms are difficult to determine.

Nervous system

In the peripheral nervous system, the neuropathy gives way to cramps and myalgia, with persistent muscular atrophy and paresis in some cases. Correspondingly, involvement of the sensory nerves appears in the form of hyperaesthesia, hypaesthesia and neuritic pain. Throughout the intermediate and chronic phase, around 28% of patients exhibited clear neuropathy, both sensory and motor. Also, this finding seems to be more frequent in females and

tends to increase with age. Severe disability in the form of headaches, cramps, myalgia, myoclonia, polyneuropathy continues to affect some patients. Table 3.1 shows the distribution of neurological symptoms in a sample of patients more than 10 years after the episode (*11*). In the same sample (*11*), one tenth of those aged <50 years and one fifth of those aged ≥50 years exhibited clear neurological changes. If extrapolated to the whole TOS-affected population, this would correspond to between 2000 and 3000 persons. In another series in Colmenar Viejo (*8*), over 60% of patients complained of cramps or chronic musculoskeletal pain two years after the onset of TOS.

Comparing these rates in TOS victims with those in the general population is problematic. Population-based studies reported in the medical literature normally address specific forms of neuropathy (*12–14*) and thus in general cannot be used for comparison. Nevertheless, the high prevalence of neuropathy in the TOS series indicates that the change is largely attributable to TOS. Cramps and myalgia with incapacitation were described in clinical series of hundreds of cases shortly after the inception of the disease, at which stage muscle biopsies showed inflammatory changes of the endomysium (*15*). Thus, it seems that not only is neurological impairment a major feature of the intermediate and late phases of TOS, but that it tends to persist for many years. The discomfort caused by these symptoms has a major impact on the Nottingham Health Profile Questionnaire scores (*11*).

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. Demineralization consequent to immobilization is common. Current studies found contractures in 17% of the examined patients, most of whom were women.

The difficulty in making a differential diagnosis between contractures and osteoarthritis is well known (*16,17*). The higher prevalence of the latter in old

Table 3.1. Current prevalence of neurological symptoms in TOS patients (N = 4015)

Symptom	Percentage
Myalgia	60.8
Cramp	68.7
Numbness	62.1
Tingling	55.2
Burning	28.1
Difficulty in buttoning clothes	8.2

women compared with old men precludes an estimate of the association between contractures and TOS. Nevertheless, physicians reported their surprise at the occurrence of contractures soon after the inception of the disease (*18*).

Cutaneous system

Symptoms such as itching or numbness attributable to skin changes are mostly in remission but sclerodermiform changes, including atrophic areas, have been found in 28–38% of patients (*8,9*). The condition mainly affects distal areas of the extremities, especially the leg and foot. Skin changes are much more frequent and severe in females than in males, and they are not associated with age. The frequent finding of skin changes in young females is peculiar to TOS and is attributable to the disease.

No population prevalence estimates for scleroderma or neuropathy are available for Spain. Elsewhere the prevalence of the spectrum of scleroderma-like conditions has been found to be around two orders of magnitude lower than in the present series (*19–21*). The prevalence was higher in females and increased with age.

Cardiovascular system

According to patients' medical records checked in one of the series (A. Gomez de la Camara, unpublished data, 1997), 1.5% of the study population (60% males) have been diagnosed with myocardial infarction. Since 1981, 1% have had a thrombosis at any moment in time, the most common sites affected being the iliac artery and other arteries in the lower limbs. Another 1% (mostly females and patients with hypertension) reported diagnosis of a stroke. Interestingly enough, one third of the patients had blood cholesterol levels above 250 mg/dl and more than 40% exhibited hyperlipidaemia in general. Forty-six percent of patients had blood pressure levels above 90 mmHg (diastolic) and 140 mmHg (systolic), a higher percentage than in the general Spanish population (*9*).

Alimentary system

In one series, 55%, 16% and 6% of patients complained of pyrosis, dysphagia and disorders of intestinal rhythm, respectively, perhaps as a result of uncoordinated functioning of the alimentary tract. Neuromuscular impairments at the level of the oropharynx and upper oesophageal sphincter were detected in patients both with and without dysphagia, though more frequently in the former. During follow-up of this type of patient, significant changes in cholinergic stimulation were found, ranging from a basic neurogenic pattern to a mixed neuromyogenic one (*22*). 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.

Currently, hepatic function has improved; the most frequent findings are isolated transaminase disorders. Cholestasis and portal hypertension, cirrhosis and nodular regenerative hyperplasia are present in some patients. At any time since the beginning of the disease, some 10% have had liver changes such as chronic active hepatitis, toxic cholestatic hepatitis, nonalcoholic liver cirrhosis, nonalcoholic steatohepatitis or diffuse nodular regenerative hyperplasia; 2.2% showed signs of active hepatopathy 15 years after the outbreak. Autopsy findings in the intermediate phase suggest occasional lesions within the pancreas, while hyperglycaemia in some patients may be the result of islet cell damage. Diabetes mellitus has been diagnosed in 7.3–10.2% of samples from TOS victims (*8,9*).

Mental aspects and perceived health status

Not surprisingly, victims of this devastating disease commonly suffer mental anxiety and depression, difficulties in adapting to their predicament and, understandably in some instances, compensation neurosis. Other psychological symptoms are insomnia, drowsiness and memory disorders. These could be an expression of the incapacitating physical disease, possibly caused by organic changes in the central nervous system during the acute phase.

In one study (*11*), the Nottingham Health Profile Questionnaire, which is aimed at assessing perception of well-being, was applied to a representative sample of 840 patients. Scores were much higher than those in a sample of the general Spanish population. They show a clear pattern of very poor self-perception of state of health, which increases according the degree of clinical disease.

TOS-handicapped patients

According to the International Classification of Impairments, Disabilities, and Handicaps (*23*), the term "handicapped" implies both physical disability and social impairment. Over 10% of TOS patients are recorded by the Instituto Nacional de la Seguridad Social as handicapped with permanent disability. Of these, 101 are recognized as suffering from "great incapacity" (i.e. requiring the help of others to carry out activities of daily living) and 209 as suffering from "absolute permanent incapacity" (i.e. inability to carry out any type of work). There are 3477 recognized as suffering from "total permanent disability" (i.e. inability to carry out common activities), of which two thirds are women, with a median age of 37 years (*24*). A descriptive cross-sectional study carried out in a sample of these patients showed a severe picture. As expected, the handicapped patients showed a high prevalence of the major symptoms and clinical findings typical of TOS: joint pain (87.6%), dry mouth (84.2%), sensory loss (82.5%), cramps (81.3%), dyspnoea (78%), loss of

strength (76.8%), fatigue (75.1%) and depression or anxiety (71.8%). Skin changes typically associated with TOS were present in 57.1%. Although 65% had mobility problems, only 32.8% showed contractures. Only four patients suffered from pulmonary hypertension. Cognitive impairments were identified in 60%. As for disabilities, problems of locomotion have been observed in over 80% of the handicapped patients, with 23% needing various kinds of aids to carry out their daily activities. Prevalences higher than in the rest of the TOS patients were found for reaching activities (52%), social activities and housework (40%), gripping activities (36%) and difficulties with personal care (30%). These disabilities were commonly associated with a certain degree of sensorimotor neuropathy (*25*).

Treatment

No particular treatment (e.g. corticosteroids, azathioprine, penicillamine, plasmapheresis, vitamin E, superoxide dismutase, vasodilators, analgesics, anti-inflammatories) produced any convincing therapeutic effect on the disease. Steroids administered during the acute and intermediate phases did not appear to prevent the chronic phase, although the general thought was that they reduced eosinophil levels and produced an improvement in patients with pulmonary oedema. No controlled trial or otherwise planned clinical study was ever carried out in TOS patients. At present, most patients are given symptomatic treatment and physical rehabilitation. Those with particular disorders such as pulmonary hypertension receive therapy specific to those conditions (*1*).

Other pathologies

Other pathologies suggested as related to TOS need to be confirmed. These are thyroid dysfunction and carpal tunnel syndrome, which in one series were reported to occur in 26% and 2.8% of TOS patients, respectively (*9*).

Between 1% and 2% of patients have been diagnosed with cancer since the beginning of the epidemic, with a distribution corresponding to that to be expected in the general population (A. Gomez de la Camara, unpublished data, 1997).

Conclusion

In the medical taxonomy before 1981 there was no record of any TOS-like condition. Thus, in spite of its obvious ability to progress, the natural history of the disease is still an open question. This chapter has set out to present current knowledge on the development of the syndrome over 20 years. The clinical picture described and the course of the syndrome in patients are representative of the TOS population as a whole, as they are based the results of the largest follow-up studies published (*8,9*).

The excess of affected women, with a 2:1 female:male ratio in the TOS database as well as in the selected studies, is consistent with early descriptions of the outbreak (*1*). Over 10 years later, the prevalence of both severe scleroderma-like conditions (i.e. clear cutaneous changes or atrophic skin) and of clear-cut neurological changes was 2–3 times higher in women than in men. In those aged <50 years, the proportion free of scleroderma, neuropathy and contractures was 1.5 higher in men than in women, while in those aged ≥50 years the corresponding ratio was 3. TOS patients in the upper tenth percentile of the distribution of the Nottingham Health Profile Questionnaire scores included more women than men (*11*). The reason for TOS affecting more women than men remains unknown.

Nevertheless, a distinction between TOS patients exhibiting clear-cut conditions and those with borderline signs of the disease is acceptable only for descriptive reasons. Given the current ignorance of the clinical development of TOS, dubious or nonspecific signs or symptoms should not simply be assigned to the typical post-disaster syndrome. The overall pattern seems to be peculiar to TOS. Residual doubts require assiduous follow-up of the largest possible number of patients in order to ascertain their condition and to offer them the best available medical and social care.

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4

Chemistry

Emilio Gelpí and
Manuel Posada de la Paz

In the first 10 years after the outbreak of the TOS epidemic, chemical analyses performed on oils from oil repository samples (*1*) produced a wealth of data on the presence and levels of fatty acid anilides (*2,3*) and their correlation with the occurrence of cases in the TOS population. A detailed account of the analytical results reported by various laboratories was summarized in the previous WHO publication in 1992 (*4*). The last 10 years, however, have been marked by the confirmation of the presence of another family of aniline-derived chemical compounds (the esters of 3-(*N*-phenylamino-1,2-propanediol (PAP)) and by the definition of the possible events leading to the generation of anilides and PAP esters during oil refining (*5*). Toxico-epidemiological findings (see Chapter 1), based on the presence of these compounds in case oil samples, have recently been reviewed in the literature (*6*).

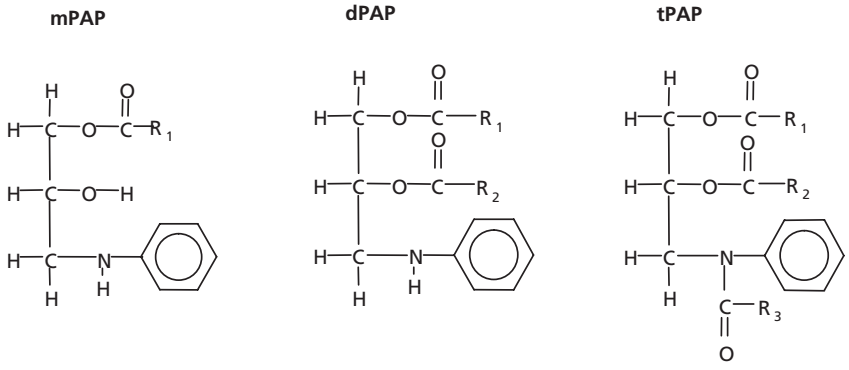
Chemical analyses and identification of new PAP derivatives

Although, soon after the epidemic, Vázquez Roncero et al. (*7*) reported on the presence of PAP and its mono-oleoyl and di-oleoyl esters (OPAP and OOPAP, respectively; see Annex 3 for compound nomenclature), these findings did not attract the full attention of the scientific community working on this issue until the last decade. Using new analytical techniques, based on the direct analysis of TOS-associated oils by liquid chromatography combined with atmospheric pressure ionization tandem mass spectrometry (LC-MS/MS), a research group

at the Centers for Disease Control and Prevention (CDC) in the United States managed to identify more than 20 new PAP-derived compounds (Fig. 4.1 and Table 4.1). These were identified as esters and ester amides of PAP (see Annex 3) that are generated by the reaction of aniline with the triglycerides in the oils (*8–10*). For this purpose, oil samples were not fractionated or extracted but were simply diluted with 1-propanol to a concentration of 50 mg oil per ml propanol, and 15 µl of the diluted samples were then injected into a reversed-phase liquid chromatographic column eluted with a gradient of water with 0.1% acetic acid (phase A) and methanol with 0.1% acetic acid (phase B). The effluent from the LC column was introduced by an atmospheric pressure chemical ionization interface into a tandem mass spectrometer that allowed extremely specific structural determinations and sensitive analyses, using the parent ion scanning technique. Identifications of most of the analytes were verified with pure standards (*11*). In this regard it is worth mentioning that a major effort to synthesize properly certified batches of standards of possible etiological agents has been supported by the WHO/CISAT Scientific Committee for the Toxic Oil Syndrome. The main aim was to certify the purity of the products used in animal toxicity experiments, in order to avoid any uncertainty in the response of the test animals to the tested compound. A more detailed account of the synthesis procedures is provided in part 3 of Annex 4.

Analysis of the three oils from the refinery with the best epidemiological links to TOS cases – Industria Trianera e Hidrogenación (ITH) in Seville – showed that the ITH refining process did produce PAP esters, supporting the hypothesis that ITH refining was responsible for the toxic agent(s) (*4*). Contrary to the anilides, these esters do not form spontaneously in the oils: unrefined oils did not contain them even though they contained anilides and were stored for years under the same conditions as the refined oils (*8*).

Fig. 4.1. General structures of compounds related to TOS



Source: Schurz et al. *(9)*.

Note: The R groups are alkyl groups associated with a specific fatty acid.

Table 4.1. Components identified in toxic oils[a]

dPAP	
R_1	R_2
palmityl	linoleoyl
palmityl	oleoyl
linolenyl	linolenyl
linolenyl	linoleoyl
linolenyl	oleoyl
linoleoyl	linoleoyl
linoleoyl	oleoyl
oleoyl	oleoyl
stearyl	oleoyl
stearyl	stearyl
linoleoyl	eicosenyl
oleoyl	eicosenyl

mPAP
R_1
linoleoyl
oleoyl

tPAP		
R_1	R_2	R_3
linoleoyl	oleoyl	linolenyl
linoleoyl	linoleoyl	linoleoyl
linoleoyl	linolenyl	oleoyl
linolenyl	oleoyl	stearyl
oleoyl	stearyl	linolenyl
oleoyl	linoleoyl	oleoyl
linoleoyl	oleoyl	oleoyl
oleoyl	oleoyl	oleoyl

[a] R_1, R_2, R_3: see note to Fig. 4.1.

As also shown by these authors, the heating of a locally purchased canola oil denatured with 1% aniline under vacuum at 300 °C for four hours produced relatively high amounts of PAP diesters (360 µg/g). This initial experimental observation was taken as a point of departure in reproducing ITH refining on a semi-industrial scale (see below).

More recently, an exhaustive search of the oil matrix for all possible aniline-related compounds initially generated and/or still present in TOS-related oils was undertaken by the WHO/CISAT Committee. This was effected by contracting the work out to specialized laboratories (at the Mayo Clinic in Rochester, Minnesota and at the Institute for Biomedical Research

in Barcelona) to carry out a comprehensive chemical characterization and quantification of extraneous components of TOS-related oil samples (see part 1 of Annex 4). The work done in Barcelona represents to date the most exhaustive screening of toxic oils ever performed. Over 2600 different samples were analysed by a new method based on automated solid phase extraction on strong cation exchange (SCX) cartridges and HPLC-APCI MS/MS quantification of all anilides and PAP derivatives present in these samples. The method has recently been published (*12,13*) and is summarized in part 3 of Annex 4.

The selective SCX extraction is effective for the screening of more basic aniline-derived compounds such as the PAP esters compared to the more polar fatty acid amides initially discovered in suspect oil samples. On the other hand, the work carried out at the Mayo Clinic focuses on the use of methanolic extracts without any further clean-up, followed by high resolution MS analyses of lower-molecular-weight polar analytes, and thus can be considered complementary to the SCX extraction method. A combined evaluation of the results obtained by both methods shows that, other than fatty acid anilides and PAP esters, no major aniline-related family of compounds is present in these oils. This is important, as it serves to focus the work of all toxicity studies on these two classes of compound.

Thus, the application of this potent methodological approach has permitted the search for and identification of all aniline-related structures produced during the refining of TOS-related oil. Although the compounds detected to date relate only to the fatty acid anilide and PAP ester families, previously identified within the oil matrixes, a large number of different and new structures have been identified. This is presented in a more detailed manner in part 2 of Annex 4.

Taking into account the structural similarities of the PAP esters with the diacylglycerol structure of phosphoglycerides, the main components of cell membranes, and specifically with PAF (platelet activating factor), a possible mechanism of toxicity was suggested and is currently under study (*14*). Thus, the new data provided by the implementation of modern analytical technology in the re-examination of TOS-associated oils provided important new knowledge. Re-analysis of the oils coming from the two sets of toxico-epidemiological case-control studies (see Chapter 1) showed that the di-oleoyl ester of PAP was a stronger specific marker of case relatedness than the oleoyl anilide previously used in these studies (*6*). This new information has been very important in planning simulated refining experiments (see below), as well as in selecting oils for animal experiments.

Simulated refining

With this data to hand, the WHO/CISAT Committee set out to search for the conditions that could have been responsible at the ITH refinery for the

generation of this new family of aniline-derived compounds. This was needed not only to explain what could have gone wrong at the industrial level, but to provide a strategy for duplicating the process at pilot plant level in order to produce enough amounts of simulated TOS oil for animal toxicity experiments. Although this type of work was contracted out to a large British organization specializing in food research (Leatherhead Food Research Association) and to a university laboratory in the United States specializing in marine oils, no clear-cut results were forthcoming despite some claims in the literature (*15*). Lately, however, the Instituto de la Grasa in Seville seems to have obtained the conditions for the generation of PAP esters during oil refining, as reported in part 4 of Annex 4.

Briefly, oil refining at ITH can now be reproduced at the Instituto de la Grasa and also at the Instituto de Investigaciones Químicas y Ambientales de Barcelona, at a pilot plant and on a laboratory scale, respectively. Thus, the production of oil following the pattern of available ITH oil (i.e. containing OOPAP and anilides in significant quantities) has been achieved. Though the variables that may influence this chemical process differ at the laboratory and industrial levels, deodorization seems to be one of the crucial variables for the formation of these compounds (*5*), and very high temperatures and short heating rates (less than 15 minutes) are two key factors. On an industrial scale, reaching high temperatures requires that the vacuum is nearly null during the first phase of the process, and changes in vacuum pressure must be modified and adapted during the course of deodorization. Nevertheless, a well standardized laboratory method now produces oil samples rich in all PAP derivatives, and these have been used to enrich refined oils for toxicological studies.

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5

Experimental toxicology

Stanislaw Tarkowski and Benoit Nemery

Why experimental studies? An important first reason for performing experimental studies in relation to TOS is to strengthen the conclusion obtained from epidemiological studies that the disease was caused by the consumption of aniline-adulterated and subsequently refined rapeseed oil. Indeed, although several of Hill's criteria (*1*) conferring causality to the epidemiological association between TOS and the consumption of refined rapeseed oil were met (*2*), one of these conditions remains frustratingly unfulfilled – namely the reproduction of the disease after the experimental administration of the putative causative agent.

Another equally, if not more important, reason for carrying out experimental studies is that the culprit(s) need to be identified in order to prevent the occurrence of future similar disasters. As long as the exact etiology of the outbreak has not been established and the mechanisms that led to the presence of highly toxic agents in the oil have not been elucidated, it will be impossible to be sure that similar outbreaks will not occur again, either with therapeutic drugs and similar agents (as occurred with the eosinophilia–myalgia syndrome (*3*)) or with other industrially processed foods or feeds.

Finally, experimental studies into the etiology and pathogenesis of TOS should shed light on the causes and biological mechanisms of possibly similar diseases such as autoimmune diseases, the etiology of which is still largely elusive. Thus, this experimental research should contribute to biomedical science in general.

In this chapter, we first summarize the state of existing knowledge regarding experimental studies up to approximately 1990, as was described

by the late Norman Aldridge in the previous monograph (*4*). We then summarize published experimental studies that have been performed in the last decade, including some unpublished experiments performed within a strategy developed by the international body whose current official name is the WHO/CISAT Scientific Committee for the Toxic Oil Syndrome.

Summary of earlier studies (1981–1990)

Experimental studies carried out during the first ten years since the outbreak of TOS were described and discussed in detail in the previous monograph (*4*).

Attempts to reproduce the condition in laboratory animals were begun very early after the outbreak of the syndrome in 1981. Initially, the oil samples were collected by individual scientists and used in experiments based on a variety of study protocols. The unknown authenticity of the oils often led to questioning of the relevance of results obtained in such studies. Later, when the hypothesis of the origin of the disease had strengthened, it was recommended at a WHO meeting in 1983 that only those case-related oils containing at least 700 µg/g of fatty acid anilides should be subjected to detailed analysis and toxicological investigation (*5*).

Acute respiratory symptoms and highly increased blood eosinophilia seemed to be the most reasonable endpoints to look for in experimental studies. However, most of the early experimental studies on animals that were administered purported case-related oils did not result in the disease or even a part of it. Other studies presented findings that could not be reasonably related to the syndrome. Therefore, it was not possible to define an experimental model of the disease or of any of its specific signs or symptoms.

Most studies used rats, which were treated in some cases with oils containing large amounts of anilides. Fewer studies have been carried out on other species, including mouse, guinea pig, hamster and rhesus monkey. In addition to the mere attempt to reproduce the disease, various hypothetical mechanisms of the development of the disease were investigated. Several of these postulated an involvement of free radicals (*6*), but conclusive evidence was lacking. The toxicity of one oil, for example, was tested using rats deficient in selenium and vitamin E, while another group received a diet supplemented with these substances (*7*). No differences were found in rats dosed with the assumed case-related oil containing anilides.

In one study, in which rats were administered a "case oil" for 200 days, an increase in the collagen content of the skin was reported (*8*). Although the authors claimed that the oil sample used in the experiment was the oil ingested by affected people, its content of fatty acid anilides was not reported. In addition, the 200-day duration of the feeding period was unrealistically long in view of the much shorter time to onset of TOS in affected people.

Another two early studies reported lung toxicity in rats. In the first study (*9*), rats were fed oil containing 2 ppm aniline and 1500 ppm anilides. In the other study (*10*), "case oil" containing 2035 ppm oleic acid anilide (OAA) and 700 ppm 3-(*N*-phenylamino)-1,2-propanediol (PAP) administered to rats caused respiratory difficulties and lung oedema, and also enlargement of the thymus and spleen. None of these early findings appears to have been fully published.

No other studies during the first ten years since the outbreak of TOS revealed any toxic effects in experimental animals treated with the assumed case-related oils. According to Aldridge (*11*) possible explanations of these negative findings, apart from the doubtful case-relatedness of the tested oils, are that TOS is a disease unique to humans, that animals are less sensitive to the postulated toxic agent, that the whole syndrome is caused by more than one substance and/or that one or more of these substances are unstable.

The epidemiological evidence for an association of fatty acid anilides with case oils is very strong (*12*), with the major anilide being OAA. There is, however, no evidence that any anilides have an etiological role in TOS, and this was already clear in the 1980s. OAA was shown in one study (*13*) to cause significant depression of the overall capacity of lipid synthesis in the lung and adipose tissue, but not in the liver. These findings were, however, not confirmed by another study (*14*).

The oleoyl esters of PAP were synthesized and tested for their acute toxicity in mice as early as the 1980s (*15–17*). In those experiments the most toxic compounds turned out to be the parent diol and the mono-oleoyl ester, which caused lung changes (congestion and infarction) after intraperitoneal but not after oral dosing (see below for a fuller description of these studies).

Altogether, the efforts of various scientific research groups during the first ten years after the outbreak of TOS were unsuccessful, either in identifying the etiological agent(s) responsible for the development of this toxic syndrome or in establishing an animal model suitable for investigating the natural history of the disease.

Discussing the state of the art of toxicological investigations during the first decade, the WHO/CISAT Committee raised a number of pertinent questions and recommended a more systematic approach to the development of new experimental studies on case-related oils.

On the basis of epidemiological knowledge available by the end of the 1980s (*12*), it became possible to select from oils kept in the repositories since 1981 those with much higher probability of an association with TOS. Case-relatedness and the presence of anilides (the only extraneous components sufficiently well characterized at that time), together with substantial amounts of rapeseed oil in selected samples, were considered reliable indicators of such an association. The anilides for which there was a strong epidemiological and

analytical evidence of their presence in case oils were the anilides of oleic, linoleic and linolenic acids. Although, according to Aldridge (*4*), one could not rigorously exclude that in certain circumstances (such as in a mixture with other substances) some of the fatty acid anilides could be involved in producing TOS in humans, the available experimental evidence led one to conclude that the fatty acid anilides should be regarded only as markers of case oils.

It was further considered by the WHO/CISAT Committee that, in order to establish the role of any of the aniline derivatives as potential etiological agent(s), it was necessary that a simulated oil refining process be carried out using labelled isotopes (*4*). This would permit the determination of a balance sheet for aniline for all the steps of the process. Such an experiment should also permit the definition of possible reactions between aniline and constituents of rapeseed oil, and the identification of hitherto unknown derivatives of aniline and their chemical structures.

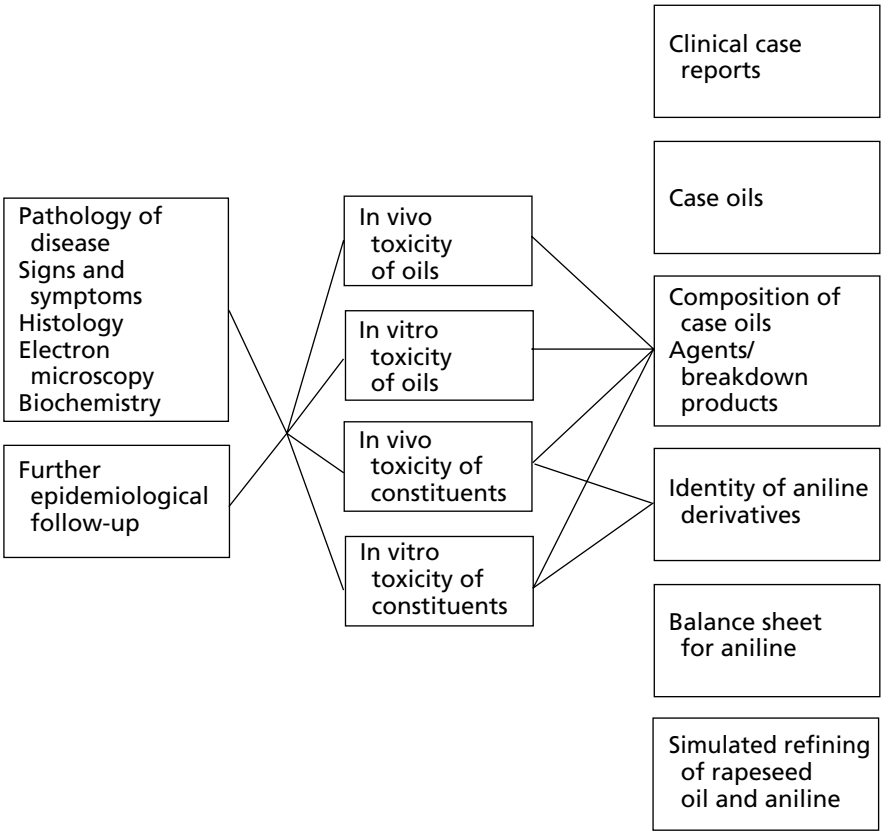
Further toxicity studies on the fatty esters of PAP were recommended by the WHO/CISAT Committee on the basis of the recent epidemiological observations and a report that claimed that these compounds caused respiratory problems and histological changes in the lungs of mice (*17*). With regard to the need for a more systematic approach to future experimental toxicology studies, it was agreed that the algorithm defined by Aldridge (*4*) (Fig. 5.1) should guide the WHO/CISAT Committee's decisions on continued toxicological research. Such an approach was found to be particularly applicable, since the identification of case-related oils had become much more reliable and since oils were becoming available that had been experimentally produced through processes intended to simulate the 1981 illegal refining of the denatured oil.

At that stage of the toxicological research and knowledge, the accepted algorithm included in vitro studies of oils and their constituents. It became clear to the WHO/CISAT Committee, however, that these types of study would have limited applications until the in vivo experiments resulted in sound effects of tested oils. They would then be useful for testing specific mechanistic hypotheses. Up to then, studies performed in in vitro systems had been used mainly for testing known constituents of case oils (*18,19*). Although these studies produced important information on the biological activity of these compounds, assessing their relevance to TOS was difficult. Consequently, during the 1990s, the focus on in vitro studies was much reduced.

Experimental studies in the period 1991–2001

In this section we attempt to cite all experimental toxicity studies, published in the generally available scientific literature between 1990 and 2001, that are relevant to the question of whether or not TOS (or some of its features) can be reproduced in some experimental system. In addition, we summarize relevant

Fig. 5.1. Approaches in experimental studies to determine the etiology of TOS



unpublished studies conducted mainly at the initiative of the WHO/CISAT Committee. Studies focusing on immunological effects are discussed in Chapter 6.

Since the early 1990s, two main types of experiment have been carried out:

- studies to investigate the fate and biological effects of selected individual agents present in case-related oils, such as fatty acid anilides and PAP and some of its esters; and
- screening studies to discover whether case-related oils and "reconstituted" oils (see Chapter 4) produce any adverse effect in experimental animals.

Studies to investigate the fate and possible toxicity of selected agents

Toxicity of fatty acid anilides

As indicated above, up to the late 1980s the possible role of fatty acid anilides as the toxic agents in TOS had been extensively studied. The possibility was finally discounted on various grounds, mainly their lack of significant toxicity in experimental animals (*4*). They thus became to be viewed only as markers of case-related oils.

Nevertheless, experimental studies were conducted by Khan et al. (*20,21*) in the rat using linoleoyl anilide, with and without heating. Male and female Sprague-Dawley rats received oral doses of 250 mg/kg test compound (in mineral oil) on alternate days for two weeks (7 doses in total) and were killed 1, 7 and 28 days following the last dose. Various alterations at some point in time were noted in organ weights (increases in the relative weights of spleen and lungs), haematology (decreases in red blood cell counts, without changes in eosinophils), serum biochemistry (decreased hepatic enzymes) and immunological parameters (decreases in IgM levels and splenic T-suppressor cells, increases in IgA and IgG levels), but these changes neither appeared consistently across groups nor were they dose-related. Consequently, these studies did not lead to any change in the previous conclusions concerning the absence of a significant role of anilides in TOS. The same authors (*22*) also investigated the in vivo formation of fatty acid anilides in rats given aniline (2 mmol/kg via gavage) and found that several fatty acid anilides were rapidly formed.

A series of studies was conducted to elucidate the metabolism of the anilide *N*-phenyllinoleamide (*19,23–28*). In an isolated perfused rat liver system it was shown that *N*-phenyllinoleamide could be hydrolysed, presumably by an amidase, to aniline and linoleic acid (*26*). Hydrolytic cleavage also occurred in human polymorphonuclear leukocytes (*27*). In addition, in these cells, *N*-phenyllinoleamide was also shown to undergo hydroperoxidative metabolism (via 15-lipoxygenase) as in human nasal polyps (*24*). In the rat, hydrolytic cleavage of OAA was shown to occur in many tissues and to be most rapid in white adipose tissue (*29*). The extent of hepatic amidase activity in humans is not known, but OAA hydrolase activity was lower and more variable in human white adipose tissue than in that of rats (*29*); this has been proposed as a possible basis for a higher susceptibility of some humans to fatty acid anilides. This research line does not appear to have been pursued.

The possible effects of fatty acid anilides on the metabolism of arachidonic acid were studied in mouse peritoneal macrophages and in human endothelial cells in culture. In mouse peritoneal macrophages, high concentrations (1 mM) of *N*-phenyllinoleamide interfered with the metabolism of arachidonate via

the cyclooxygenase and lipoxygenase pathways (*23,25*), but the authors rightly concluded that the relevance of these in vitro effects to the pathogenesis of TOS remained uncertain. Similarly, the complex alterations in prostanoid synthesis observed when cultured human endothelial cells were incubated with *N*-phenyllinoleamide or OAA are difficult to interpret (*30,31*).

Heiskanen and co-workers conducted a number of mechanistic studies into the effects of various fatty acid anilides on the activation of human polymorphonuclear leukocytes (*32–35*). Oleic and linoleic acids and their corresponding anilides moderately induced the production of reactive oxygen metabolites (from concentrations of 100 µM onwards) and they modulated the agonist-induced activation of neutrophils (*32,33*) without affecting the expression of leukocyte adhesion molecules (*35*). Palmitic and erucic acids and their respective anilides exhibited quantitatively and qualitatively different effects (*34,36*). The authors concluded from their studies and those of Bioque et al. (*27*) that the studied toxic oil contaminants were not by themselves potent activators of phagocytic cells, but that several of these contaminants possessed the potential to modify the activation of such cells (*35*). To what extent these speculations have a bearing on the pathogenesis of TOS remains to be established.

In a series of studies, Bell and co-workers investigated the immunotoxic potential of OAA in mice. In an initial publication (*37*) it was reported that B10.S mice treated for six weeks with pure OAA via osmotic pumps (delivering approximately 12 µl/day) developed increased levels of serum IgE and IgM, and then IgG1, with splenomegaly and increased cytokine gene expression (IL-6, IL-1). The changes were interpreted as suggestive for autoimmunity. In further studies (*38*), similarly treated A/J (H-2a) mice developed, within a week of exposure, an "acute lethal wasting disease" characterized by weight loss, cachexia, apathy and breathing difficulties, which had not occurred in B10.S (H-2s) (*37*) or C57BL/6 (H-2b) mice, although the latter strain did exhibit splenomegaly and polyclonal B-cell activation. However, in an experiment described in an article containing only little information about the dosing protocols and actual results (*39*), acute deaths and "sickness" with "breathing difficulties" attributed to emphysema or pulmonary oedema were reported in both A/J mice and B10.S mice given three intraperitoneal injections (25 µl) per week, over a period of 7 or 14 days, of OAA or *N*-phenyllinoleamide (both as 250 mg/ml in ethanol). In A/J mice, but not in B10.S mice, marked NF-B activation in splenic T cells and peritoneal macrophages (and in aorta and gut, but not liver, lung and kidney) was found after 1 and 4 days of intraperitoneal administration of OAA with osmotic pumps (*40*). Later, the authors (*41*) confirmed the strain-dependence of the immune response to OAA using splenocytes and T cells obtained from A/J and B10.S mice. The authors speculated that the acute wasting syndrome shown in the A/J mice was due

to slow acetylation and preferential production of toxic and immunogenic products, whereas the relative clinical resistance of the B10.S mice was related to their fast acetylation. These observations concerning the strain-dependent immunotoxicity of OAA are potentially interesting, but they require confirmation. Their significance is difficult to assess at this stage, because the relevance of the mode of administration (continuous infusion using osmotic pumps), the purity of some of the OAA used in the first experiment, and some of the inconsistencies between different publications from the same group are of concern.

It should be noted, however, that in an independent experiment in female Swiss mice (strain not reported), oral administration of 50 mg/kg of OAA (89% pure) for five consecutive days also led to progressive weight loss, increases in serum immunoglobulins and decreases in T lymphocytes in the spleen (*42*). Other immunotoxic effects of fatty acid anilides have been postulated, such as when tested in the popliteal lymph node assay, but these findings are discussed in Chapter 6.

It may be concluded that, although fatty acid anilides are not devoid of biological activity, none of the studies conducted with these agents has provided any substantial clue that they are causally involved in the pathogenesis of TOS. Consequently, the statement of 10 years ago still stands: that these anilides are probably essentially markers of toxic oil and that they are unlikely to be, by themselves, the causative toxic agents of the syndrome.

Toxicity of PAP and fatty esters of PAP

Following the discovery of mono- and diesters of PAP in case-related oils (*15,16,43*), the question arose as to the intrinsic toxicity of PAP and these esters.

Toxicity studies were initially carried out in Swiss mice at the Instituto de la Grasa in Seville. From the resulting publication (*17*) it appears that the administration of PAP led to deaths and gross and histopathological alterations in the spleen, lungs, liver and kidneys only after intraperitoneal administration (3 daily doses of 465 mg/kg bw) but not after oral administration (11 daily doses of 465 mg/kg bw). Similarly, the administration of the oleoyl monoester of PAP (OPAP), dissolved in olive oil, resulted in some deaths after intraperitoneal administration (6 daily doses of 465 mg/kg bw) but not after oral administration (8 daily doses of 465 mg/kg bw). However, with both routes of administration the authors reported macroscopic (organ weight changes) and histological evidence of damage to the lungs (including lesions described as infarctions), spleen and liver, particularly in female animals. Administration of similar doses (by weight, but not in molar terms) of the dioleoyl diester of PAP (OOPAP) did not result in death or organ damage by either route (only males tested).

These data led the WHO/CISAT Committee to commission a further investigation of the toxicity of PAP and OPAP at the Medical Research Council's Toxicology Unit in Leicester, United Kingdom. In this study (*44*), groups (n = 6) of Wistar-derived rats, Lewis rats and MF1 mice (all females) received intraperitoneal injections of PAP (150, 250 or 350 mg/kg bw) or OPAP (300, 375 or 450 mg/kg bw) dissolved in arachis oil for 14 consecutive days. Dosing with OPAP did not lead to mortality or toxicity, as assessed from body weight changes, autopsy findings and histology of various organs. However, the two highest doses of PAP were highly lethal for the rats of both strains, with all animals exhibiting adhesive peritonitis, and half the Wistar-derived rats (but only one Lewis rat) exhibiting pulmonary thromboemboli (considered to be secondary to vascular damage in the mesenteric tissue). Mice treated with PAP intraperitoneally also developed peritonitis and lung haemorrhage, but no thromboemboli. Oral administration of PAP (350 mg/kg bw for 14 days) to Wistar rats (no other strain or species studied) did not lead to focal or systemic lesions. Daily intravenous administration of PAP (350 mg/kg bw for up to 10 days) to Wistar rats (no other strain or species studied) caused local inflammation and intravascular fibrin deposits, but no other significant organ damage. OPAP was not administered orally. No animals exhibited an increase in blood eosinophilia. It was concluded from this investigation that it would "seem unlikely that PAP was responsible for any of the acute pathologies seen in the development of the toxic oil syndrome in man" (*44*). While this is probably a reasonable conclusion as far as PAP is concerned, the lack of toxic effects caused by intraperitoneally administered OPAP in rats and mice contradicts the initial study by Maestro Durán et al. (*17*), which suggested that similar doses of OPAP may be toxic when given intraperitoneally to mice. The discrepancies between the two studies may be due to differences in susceptibility between the mouse strains used, their background health, the vehicles used to dissolve OPAP (olive oil vs arachis oil) or histopathological diagnostic accuracy.

In an independent study (*39*) in which the linoleic diester of PAP (LLPAP) was administered intraperitoneally (25 µl of pure compound three times a week for two weeks) in mice, the authors reported weight loss, lung damage (thromboses, haemorrhage and congestion in B10.S mice; congestion and emphysema in A/J mice), and a "20-fold increase in peripheral eosinophilia". Linoleic acid was much more toxic. The oleoyl (C1), linoleoyl (C2) diester of PAP (OLPAP) was also administered and apparently led to little or no toxic effects. Overall, this study is difficult to interpret because of the paucity of experimental details. To our knowledge the findings have not been replicated.

Only a few mechanistic studies have been conducted with the PAP esters. Heiskanen & Savolainen extended their in vitro studies concerning the activation of polymorphonuclear leukocytes by fatty acid anilides (see above) to PAP,

OPAP and OOPAP (*45*). PAP and OPAP did not increase the production of reactive oxygen metabolites, whereas OOPAP did so slightly. OOPAP was also the only compound to interfere, at micromolar concentrations, with the phorbol ester-induced production of reactive oxygen metabolites. Closa et al. (*46*) studied the intestinal absorption and biotransformation of various PAP esters in the rat, and showed that these are absorbed and then distributed and stored in different organs, particularly in the liver and brown adipose tissue. There was evidence that the fatty acid composition of the PAP esters was changed, presumably as a result of de-esterification and re-esterification. Interestingly, PAP esters with a long acyl chain in the C-1 position showed an inhibitory effect on the synthesis of the (structurally similar) platelet activating factor.

In conclusion, if we assume that it is valid to test chemical agents in experimental animals to evaluate their risk to humans, then the simplest conclusion from the available information is that it does not appear that PAP or its oleoyl esters have a sufficiently high degree of acute mammalian toxicity to explain the acute manifestations of TOS. Indeed, OOPAP did not exhibit any acute toxicity (*17*), PAP was shown to be toxic only after intraperitoneal administration and not after oral dosing (*44*) and the toxicity that was produced in mice with OPAP, if any, was observed only at high doses (several daily administrations of hundreds of milligrams per kg body weight) (*17,44*). These doses should be considered in relation to the concentrations of the PAP esters that have been found in case oils, which were in the order of tens to hundreds of milligrams per kg oil (ppm) (*43*). It follows that the amount of oil that would have to be consumed to achieve the toxicity observed with the pure PAP esters would have to be in the order of at least one kg oil per kg body weight (assuming the concentrations of these chemicals remained stable between the time of the outbreak and their detection in case-related oils). Of course, such calculations are not valid if humans are much more susceptible to the toxic effects of the PAP esters. Moreover, the toxic effects produced in mice or rats also appeared to differ from those observed in TOS.

Another word of caution relates to the fact that, of the many potential fatty acid esters of PAP, only OPAP and OOPAP (and to some extent LLPAP and OLPAP (*39*)) were evaluated for their acute toxicity. It is conceivable that one or more other, perhaps "minor" fatty acid esters of PAP are orders of magnitude more toxic than the oleoyl esters. Another distinct possibility is that these compounds are relatively nontoxic when given alone, but that this is not the case when they are present together with other compounds of equally low toxicity, such as fatty acid anilides for instance, or other constituents of the oil or the diet. It is conceivable – though at this stage entirely speculative – that a particular aniline derivative of low intrinsic toxicity influences the metabolism and, hence, toxicity of another otherwise harmless food constituent. So far, no

experiments have tested the effect of the simultaneous administration of more than one agent among those found in case-related oils. Although this would be feasible, it would be a formidable undertaking to do so systematically in view of the large number of possible combinations that would have to be tested.

For the time being, the available evidence suggests that, like the fatty acid anilides, the fatty acid esters of PAP are good markers for the toxic oil but that they too are devoid of substantial toxicity by themselves (at least the oleoyl esters) in the species and strains of animals tested thus far.

Studies to investigate the effects of reconstituted oils

Rationale and methodology

This approach was the logical complement to the attempts to reconstruct the process that had led to the production of toxic oil from the refining of aniline-denatured rapeseed oil.

As described in Chapter 4, various laboratories were requested to refine aniline-denatured rapeseed oil and to modify the different steps involved in the refining process in order to achieve a composition that would resemble the toxic oil in terms of its most important markers, namely anilides and PAP esters. Thus various modifications (and their combinations) were made to factors such as the time and storage temperature before refining, the temperature (rate of increase and level reached), duration and pressure of distillation or deodorization, and the agents used for the degumming. Eventually, a refining procedure leading to an oil having the same chemical profile as case-related oils was reproduced in Seville in 1999 (*47*). The method of checking whether the resulting product resembled the toxic oil consisted of a chemical analysis (levels of marker anilides and PAP esters), but samples were also tested for their potential to cause toxicity in experimental animals. In other words, it was hoped or assumed that a "successful" refining process could be identified by a bioassay performed shortly after the production of the refined oil (in order to avoid the loss of any reactive chemicals during storage). Admittedly, the bioassay had not been validated as such, but the premise was that if a reconstituted oil produced toxicity in animals, this would be indicative of biological activity. On the one hand, this would provide the long-awaited experimental verification of the epidemiological findings and, on the other hand, it would also hopefully lead to discovering the exact etiological agent by a process of further "chemical dissection" of the implicated material.

On the basis of theoretical and practical considerations, the WHO/CISAT Committee adopted the following pragmatic approach and testing protocol.

- Mice were chosen as the animal species, mainly for reasons of convenience (low cost, easy handling, low amounts of test material required) but

also because of the availability of inbred strains with differing genetic susceptibilities to pharmacological or toxic agents, infectious organisms and immunological exposures. The following four strains were selected: BALB/c, A/J, C57BL/6 and DBA/2. BALB/c and DBA/2 mice express the same haplotype (H-2d) for the major histocompatibility complex, whereas A/J mice express the H-2a and C57BL/6 mice express the H-2b haplotype (*48*). A/J mice are slow acetylators, whereas BALB/c and C57BL/6 mice are fast acetylators (*49*). A/J and BALB/c mice tend to mount Th2-type immune responses, whereas C57BL/6 mice tend to mount Th1-type responses (*50*), even though the responses of different strains with regard to, for example, airway hyperresponsiveness, pulmonary eosinophil influx and elevations of serum antigen-specific IgE levels in response to allergen sensitization and challenge are not always associated (*51*). To reduce the size of the experiments, only female mice were studied because of the preponderance of females among the human TOS victims (*52*).

- The oral route of administration was chosen as the most relevant in view of the foodborne nature of the poisoning.
- A dosing regimen of five successive days was considered appropriate in order to mimic the human situation, and a duration of observation of five or six more days was also considered adequate to detect acute responses, in view of the estimated latent period in humans. The doses to be given were chosen to be relatively high (up to 5 ml/kg bw) in order to maximize responses and to allow for a markedly lower susceptibility of mice than humans.
- Dilutions of the oil were made with olive oil, and control animals received olive oil.
- The endpoints of study consisted mainly of body weight changes (a non-specific but relatively sensitive index of general well-being), pulmonary damage and blood eosinophilia. The latter two types of endpoint were selected on the basis of the human experience in the acute phase of TOS, where the main features had consisted of acute lung injury and blood eosinophilia. Lung damage was assessed by measuring wet and dry lung weight (a very reproducible and sensitive parameter of acute lung injury (*53*)) and by histology. Other organs were also preserved in formaldehyde for histological analysis, should this prove necessary.

In addition to the bioassays carried out with the protocol described above, two samples of reconstituted oils (RS0099O219, RSA9900125) and a case-related oil (CO-756) were also subjected to more standard toxicity protocols by a commercial facility (RCC Ltd, Itingen, Switzerland) in a number of different animal species (mice, rats, guinea-pigs, mini-pigs) according to the good laboratory practices (GLP) and international requirements. These included acute

toxicity tests (i.e. single dose) and subacute repeated dose protocols (7 days, and in some instances 28 days) with full conventional assessments of clinical, biochemical and haematological parameters, as well as histological evaluation.

None of the findings described below have been published in the scientific literature. Detailed reports were provided to the WHO/CISAT Committee, which keeps paper and electronic archives of these data.

Initial studies of 16 oil samples in Leuven and Łodź

These studies were conducted in two different laboratories (Laboratory of Pneumology, Unit of Lung Toxicology, University of Leuven, Belgium, and Department of Toxicology and Carcinogenesis, Nofer Institute of Occupational Medicine, Łodź, Poland), each studying two different strains (BALB/c and C57BL/6 in Łodź; A/J and DBA/2 in Leuven). All oil samples were coded and the investigators were blinded as to their chemical compositions. Table 5.1 shows the origin and composition of the oil samples that underwent a bioassay in the four strains of mice in Leuven and Łodź. The first 10 of these samples were tested at three different doses (1.25, 2.5 and 5 ml/kg bw) and the following six samples were tested only at the highest dose of 5 ml/kg bw. The oil was given on five consecutive days by oesophageal intubation. The animals (female, 6–8 weeks old) were not starved prior to dosing and no anaesthesia was used. A fixed volume of 5 ml/kg bw was given, with the test oil being diluted with olive oil where necessary. The treated groups contained 5–6 mice. Concurrent control animals (2–5 mice) remained untreated (Leuven) or received pure olive oil (5 ml/kg bw) (Leuven and Łodź). The data from these control animals were pooled, thus giving a total of 49–51 control animals for each strain.

During and after the dosing period the animals were weighed and observed for clinical signs every day until sacrifice 10 or 11 days after the first dose. The mice were killed by an intraperitoneal dose of pentobarbital. A differential cell count was done on blood from the abdominal vena cava or a tail vein. The gross macroscopic appearance of lungs, heart and major abdominal organs was assessed. The left bronchus and pulmonary vessels were clamped and the left lung was removed. This was weighed fresh (wet lung weight) and after 48 hours of drying at 60 °C (dry lung weight); lung weights were expressed in relation to body weight, and the ratio of wet to dry lung weight was also determined. The heart–(right) lung complex was instilled via the trachea with 10% formaldehyde, using a syringe, and placed in formaldehyde for subsequent histological analysis. Liver, kidney, thymus, nerve and muscle, spleen and any visually abnormal tissue or organ were also preserved in formaldehyde.

These experiments were not performed according to formal GLP criteria, but every effort was made to work according to standardized protocols and best practices. The reasons for choosing research-oriented institutions, rather

than contract laboratories experienced in routine toxicity testing, related to costs but also to flexibility and to the possibility of rapidly taking initiatives for further experiments should anything be found. Histological evaluation was performed by the respective institutions' pathologists, but all slides of lung tissues were reviewed by an external animal pathologist (Dr H.-G. Chevalier, Experimental Pathology Services AG, Muttenz, Switzerland), who generally confirmed both the absence and presence of lesions found in the initial assessments.

Overall, the first 10 samples gave completely negative results. No treatment-related mortality occurred, and no consistent changes were found with regard to body weight evolution, wet or dry lung weights or blood eosinophils in any of the four mouse strains. Occasional statistically significant differences compared to pooled control values could be discounted on the basis of aberrant values in concurrent control animals, or they could not be ascribed any biological significance because of the lack of consistent dose–effect relationships. (For two oils that had given somewhat doubtful results in terms of increases in lung weight, repeat tests failed to reproduce the original findings.) Histological examination of lung tissue did not reveal any significant or consistent anomalies in A/J and DBA/2 mice. In the BALB/c and C57BL/6 mice, some animals exhibited minimal lymphocyte infiltrations around the small pulmonary arteries after exposure to 5 ml/kg of four oils, but this was not a consistent or a dose-related finding (Table 5.1). The next six samples, which were only tested at the high dose of 5 ml/kg bw, also gave essentially negative results. Lung histology again revealed perivascular lymphocyte infiltration in isolated animals of the treated BALB/c and C57BL/6 mice for two oils.

As can be seen from Table 5.1, the first ten reconstituted oil samples generally contained relatively high amounts of OAA but no appreciable amounts of OOPAP. Consequently, these oils may not have met the conditions for being "toxic". It is perhaps not surprising, therefore, that the bioassays with these oils turned out to be essentially negative. Among the six samples tested subsequently, there was (unknown to the experimenters) one sample of a case-related oil (containing 53 ppm OOPAP), one sample of a control (i.e. non-case-related) oil and four samples of reconstituted oils originating from Oregon, which had turned out to contain relatively high amounts of OOPAP (20–160 ppm). However, neither the case-related oil nor the latter oils with high OOPAP produced any pulmonary toxicity or increased blood eosinophilia in the bioassay.

For some oils, the bioassay was repeated later with a longer period of observation after dosing. Thus oil "E" 024706 was tested again in the four strains, at a dose of 5 ml/kg bw, and animals were studied on days 10 and 50. This test was also negative. Similarly, oil A2147 (case-related oil) and the Oregon oil with the highest OOPAP concentration (2W1S16T210) were

Table 5.1. Refined oil samples tested in four strains of mice

No.	Origin[a]	Code	Refining process			OAA (ppm)	OOPAP (ppm)[b]	Doses tested (ml/kg bw per day) given orally for five consecutive days	Remarks[c]
				Deodorization					
			Storage (weeks)	Temperature (°C)	Duration (hours)				
1	IGS	1S/270-6	1	270	6	140	nd	1.25, 2.5, 5.0	
2	IGS	3S/270-6	3	270	6	460	0.22	1.25, 2.5, 5.0	Perivasculitis (1C+1B)
3	IGS	8S/270-6	8	270	6	910	0.45	1.25, 2.5, 5.0	Perivasculitis (2C)
4	Oregon	ADW4T270	4 (16°C)	270	4	1 700	0.28	1.25, 2.5, 5.0	
5	Oregon	W1S50T270	1 (50°C)	270	4	1 500	2.0	1.25, 2.5, 5.0	
6	Oregon	W4S50T270	4 (50°C)	270	4	5 500	nd	1.25, 2.5, 5.0	
7	LFRA	E 024706		270	4.5	1 200	3.4	1.25, 2.5, 5.0	Perivasculitis (1C+2B)
8	LFRA	H 024672		230	4.5	3 500	nd	1.25, 2.5, 5.0	
9	LFRA	O 024700		270	10	1 300	nd	1.25, 2.5, 5.0	Perivasculitis (2C+2B)
10	LFRA	R 024690		250	5.5	2 500	nd	1.25, 2.5, 5.0	Perivasculitis (1D)
11	Oregon	2W1S16T210	1 (16°C)	210	4	2 100	160	5.0	
12	Oregon	2W1S16T230	1 (16°C)	230	4	550	55	5.0	Perivasculitis (2B)
13	Oregon	2W1S16T250	1 (16°C)	250	4	570	130	5.0	
14	Oregon	2W1S16T270	1 (16°C)	270	4	420	20	5.0	
15	CISAT	A2081				nd	nd	5.0	
16	CISAT	A2147				940	53	5.0	Perivasculitis (3C+3B)

[a] IGS = Instituto de la Grasa, Seville, Spain; Oregon = Department of Food Science and Technology, Oregon State University, USA; LFRA = Leatherhead Food Research Association, Surrey, United Kingdom. Two oils were supplied by CISAT.

[b] nd = not detected.

[c] In the group given 5 ml/kg bw, one or two animals had histology showing minimal perivascular cell infiltration in the lung (B = BALB/c, C = C57BL/6, D = DBA/2).

given for five days, at 2.5 ml/kg bw, to A/J and DBA/2 mice and the animals (including appropriate controls) were killed on days 10 and 48. However, no significant findings emerged from this longer observation period.

Studies at RCC

These studies were begun in 1999 after a "successful" refining procedure had been achieved in Seville (*47*).

The following three oil samples were tested (see Table 5.2 for their characteristics): CO-756 (a case-related oil), RS-A990125 (a reconstituted oil containing a high concentration of anilides but little or no OOPAP) and RS0099#219 (a reconstituted oil supplemented with PAP esters). Rapeseed oil was used as a control. Acute (single dose) and repeated oral dose studies were performed, based on the OECD Guidelines for the Testing of Chemicals, in various species (MRL/lpr mice, Sprague-Dawley rats, Himalayan spotted guinea-pigs, Göttingen mini-pigs and domestic Lohmann Selected Leghorn laying hens). Table 5.3 presents the types of test that were performed. Haematology and histology of multiple tissues were evaluated at the indicated points in time.

In no study was there any treatment-related death and no treatment-related clinical or other signs of toxicity were recorded. In the acute rat study, one female treated with RSO099#219 exhibited a subacute interstitial pneumonia, which could have been treatment-related. The same was observed in the acute guinea-pig study with 5 ml/kg. In mice, there were inconsistent anomalies in haematology indices and extramedullary haemopoiesis (mainly granulopoiesis) was more frequently observed, mainly in mesenteric lymph nodes but to a lesser extent also in mandibular lymph nodes and in the spleen, as well as in the bone marrow (28-day study), of treated mice compared

Table 5.2. Composition of oil samples tested by RCC or subsequently in Leuven and Łodź

Code	Type[a]	Anilides (ppm)	OOPAP (ppm)	Tested by:
CO-756	Case oil	551	11	RCC
RSA990125	Refined "normal"	4 702	0.2	RCC
RSO099#219	Refined "supplemented"	5 681	412	RCC, Leuven, Łodź
RSO099#5	Refined "extreme"	1 366	1 065	Leuven, Łodź
X or No. 17	Refined "accidental"	10 701	124	Leuven, Łodź
RSO160401	Refined "supplemented"	10 562	232	Leuven, Łodź

[a] Refined "normal" = usual refining process for vegetable oils; Refined "accidental" = refining process simulating that used to produce TOS-related oil; Refined "supplemented" = refined "accidental" process supplemented with PAP esters synthesized in the laboratory; Refined "extreme" = refining process under extreme conditions to increase the content of PAP esters.

to controls. According to the authors of the RCC summary report, the proportion of control animals with extramedullary haemopoiesis was higher than the physiological range observed in their experience, but the animals treated with test oils seemed to be affected even more frequently, though without a consistent dose–response relationship. The authors attributed the increased extramedullary granulopoiesis, including that in the control mice, to a nonspecific immunomodulatory effect in the MRL/lpr mice, and they concluded that the relationship of this finding remains unclear. It should be noted that this type of mouse was selected on the basis of its genetic propensity

Table 5.3. Toxicity studies performed by RCC

Study	Species	Oil	RCC project	Dose (ml/kg)	No. per group	Observation
Acute toxicity	Mouse	CO-765 RS-A990125 RSO099#219	721710 721721 721732	5	3 M, 3 F	14 days
	Rat	CO-765 RS-A990125 RSO099#219	721743 721754 721765	5	3 M, 3 F	14 days
	Guinea-pig	CO-765 RS-A990125 RSO099#219	721776 721787 721798	5	3 M, 3 F	14 days
Repeated 7 days	Mouse	CO-765 RS-A990125 RSO099#219	721800 721811 721822	0, 1, 2.5, 5	6 M, 6 F	7 + 1 or 14 days
	Mouse	CO-765 RS-A990125 RSO099#219	721343 771208 771221	0, 1, 2.5, 5	10 M, 10 F	7 + 1 days
	Rat	CO-765 RS-A990125 RSO099#219	721833 721844 721855	0, 1, 2.5, 5	3 M, 3 F	7 + 14 days
	Guinea-pig	CO-765 RS-A990125 RSO099#219	721866 721877 721888	0, 1, 2.5, 5	3 M, 3 F	7 (8) + 14 (13) days
	Mini-pig	RS-A990125 RSO099#219	721890	5	1M, 1 F	7 + 14 days
Repeated 28 days	Mouse	CO-765 RS-A990125 RSO099#219	771254 771210 771232	0, 1, 2.5, 5	10 M, 10 F	28 days
	Laying hen (neurotoxicity)	RS-A990125 RSO099#219	806106	0, 10	6 F	28/29 days

to develop autoimmune disease (*54,55*). It is not known whether the latter has any bearing on the possible induction of extramedullary haemopoiesis, but no other features of autoimmunity were apparent in these studies.

In conclusion, no substantial toxicity was elicited when a case-related oil or two reconstituted oils with chemical compositions apparently similar to case-related oils were tested according to prevailing international guidelines for toxicity testing, either by giving a single dose or repeated oral doses in several species. The occurrence of extramedullary haemopoiesis in mice is of unclear significance, but it does not appear to be of major relevance for human TOS.

Further studies in mice with reconstituted oils

Further studies were performed, in Łodź and in Leuven, with other reconstituted oils enriched with PAP esters (see Table 5.2) following the rationale described above and according to similar or slightly modified experimental protocols. The detailed results of these experiments have been reported to the WHO/CISAT Committee.

In none of these experiments was any major pulmonary toxicity observed. The following observations are nevertheless of possible relevance. In Łodź, mice (BALB/c and C57BL/6) from the groups treated with test oils occasionally exhibited alterations in lung histology, consisting of minimal perivascular, peribronchial or interstitial infiltration with lymphocytes, and these were sometimes accompanied by increased thickness of the arterial wall and swelling or proliferation of the endothelium. The single most significant finding was that oil RSO160401 caused increased thickness of the small pulmonary arteries with swelling of endothelial cells in three out of four C57BL/6 mice given five daily doses of 5 ml/kg bw and killed on day 10, while this was not observed in the other groups (controls and mice given 1.25 or 2.5 ml/kg bw). In Leuven, no pulmonary or other abnormalities were found in A/J mice. In contrast, DBA/2 mice exhibited significant increases in lung weights and histological abnormalities after dosing with oil X or oil RSO099#219. However, the histopathological changes consisted mainly of centrilobular inflammation, which is a nonspecific feature that could also be caused by infection, and the findings of two successive experiments were not internally consistent. Dosing with oil RSO160401 (1.25, 2.5 or 5 ml/kg bw for seven consecutive days) gave no significant lung changes when the animals (A/J, DBA/2, BALB/c) were killed on day 8, but there was a moderate increase in blood eosinophils (counted by flow cytometry) in BALB/c mice given 5 ml/kg bw (6.2% vs 4.0% in controls). No significant changes were found in total serum IgE as measured by ELISA.

The conclusion of these recent additional experiments is that no major acute disease could be produced in mice by the administration of reconstituted oils with a chemical composition resembling that of case-related oils.

Nevertheless, some apparently treatment-related alterations in lung weight and pulmonary histology, as well as in blood eosinophilia (see Chapter 6) occurred sometimes in specific mouse strains (BALB/c, C57BL/6, DBA/2), whereas this did not happen in A/J mice. These alterations have not always been consistent, and the generally small numbers of animals in the groups have often precluded obtaining meaningful statistical analyses. Consequently, the findings need to be interpreted with great caution and they must be repeated by independent experiments.

General conclusion

This chapter on the experimental toxicology of TOS may appear disappointing at first sight. Even in the second decade after the accident, nobody appears yet to have been able to reproduce the syndrome in experimental animals. Yet considerable resources and tremendous efforts have been devoted to this issue, and a large number of experimental animals of different species and strains have been subjected to various testing protocols.

How can one attempt to explain this frustrating state of affairs?

A possibility that will undoubtedly come to mind is that TOS was simply not caused by the ingestion of toxic oil. The failure to reproduce the human syndrome in experimental animals has indeed been used by opponents of the toxic oil etiological theory to negate its validity altogether. However, the epidemiological evidence in favour of the toxic oil hypothesis is overwhelming, and no alternative hypothesis, speculation or conspiracy theory has withstood the test of scientific scrutiny. Consequently, the failure, so far, to reproduce the syndrome in animals requires a different type of explanation.

One explanation could be that (some) humans were exquisitely and uniquely susceptible to the deleterious effects of the toxic oil. This susceptibility could be due to genetic factors, as suggested by the fact that not all those who consumed the oil were equally afflicted. Although genetic susceptibility factors have been identified (*56*), they did not confer very highly increased risks of becoming affected by TOS. Also, these genetic factors were not unique to the human species. Moreover, such extreme species sensitivity to the effects of foreign chemicals, whereby only one species is severely poisoned while all others remain virtually unaffected, would be highly unusual. In fact, although species differences in toxic responses do exist, we know of no other instances where a substance exhibits severe toxicity in one species and no toxicity at all in all the other species tested. Nevertheless, TOS could be the first such example. In this case, the use of "humanized" SCID (severe combined immunodeficiency disease) mice should be helpful, as should the in vitro use of cells or tissues of human origin. However, in vitro studies have hitherto not been particularly rewarding, and it is not easy to design suitable experiments with the available candidate materials.

Failure to cause toxicity in experimental animals may also be due to the fact that the most appropriate experiments have not yet been performed and that not all possible experimental approaches have been utilized.

The specific markers of the toxic oil (either anilides or PAP esters) have consistently proven to be devoid of serious toxicity when given orally to experimental animals. This may mean that these agents are indeed nothing more than markers of toxic oil, but alternative possibilities should also be considered.

One possibility is that in the tested animals the gastrointestinal absorption (with de-esterification and re-esterification) of these substances differs substantially from that which takes place in humans, where a substantial fraction of ingested lipids may reach the pulmonary circulation directly via the thoracic duct. In other words, oral administration, which appeared to be the most relevant route of administration, may well be the least suitable for testing such fatty ester derivatives in animals. Hence, seemingly less physiological modes of administration, including intratracheal instillation, should not be dismissed.

Another possibility is that only the most abundant fatty acid derivatives have been studied, i.e. mainly those derived from oleic acid, and to a lesser extent also those derived from linoleic and linolenic acids. It is conceivable that derivatives of other fatty acids exhibit a more pronounced toxicity. In this respect, a recent paper by Parke & Parke (*57*) pointed out that involvement of erucic acid anilide was never tested and they speculated, without any serious backing, that rapeseed oil of low content of erucic acid (or its derivative) is likely responsible for TOS. This line should perhaps be investigated further.

Still another possibility is that neither the anilides nor the PAP esters are very toxic in themselves, but that they become toxic when given together in particular combinations, or with other (e.g. dietary) lipids or components. As alluded to previously, this type of hypothesis is difficult to investigate without any serious hints as to which combinations are likely to occur or to be hazardous.

The possibility that TOS was caused by a combination of the ingestion of toxic oil and another still unidentified environmental or other factor (e.g. a latent infection) would not only explain why the markers of toxic oil have not proven to be particularly toxic when given to animals, but also why case-related oils and reconstituted oils have remained negative. Experimental toxicology is always conducted with the healthiest possible animals and with the least possible interference from other chemicals, but this might explain paradoxically why the "best" studies have been generally unrewarding, whereas those that were considered of lower standard sometimes yielded positive (and nonreproducible) findings. A solution would be to design suitable experiments involving infected animals or animals that have been immunologically "primed" (e.g. by treatment with adjuvants).

Finally, it must also be recognized that it has been difficult, if not impossible, to reproduce many human diseases in experimental animals. This is particularly the case with immunologically mediated diseases, such as autoimmune disorders, although some models are available that partially reproduce these conditions (*55,58,59*). There is, in fact, only limited evidence for environmentally induced autoimmune disease in humans (*60*). Consequently, the findings, particularly in the more recent experimental studies, of pulmonary alterations or changes in blood eosinophils (even if only minor and not always consistent) should be regarded as potentially important leads for further investigation. The etiology of many human diseases is complex and multifactorial. In many instances it is still largely elusive so that several disorders, such as the autoimmune diseases, are often still considered to be idiopathic because the external causative agents have not been properly investigated. In this way, some of the experimental studies that have been conducted in relation to TOS may well not have achieved their stated purpose of advancing our knowledge regarding its etiology. Nevertheless, it may be hoped that they will contribute usefully to biomedical research in general.

Summary

Up to now, experimental studies performed in a variety of laboratory animals, using case-related oils or laboratory-produced oils apparently having compositions similar to those of case-related oils, have failed to reproduce the symptoms of human TOS. None of the in vivo or in vitro studies performed with toxic-oil-specific components, such as fatty acid anilides and esters of PAP, have provided evidence that these markers are causally involved in the pathogenesis of TOS. This failure, however, must not be used to argue against the toxic oil etiology; the epidemiological evidence in favour of this etiology is overwhelming and none of alternative hypotheses have withstood the test of scientific scrutiny. Recent experimental studies using laboratory-produced oils having apparently similar compositions as case-related oils have produced mild but possibly significant effects (such as eosinophilia and pulmonary alterations) in some strains of mice. Further research is warranted to obtain a suitable animal model of TOS.

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6

Immunology

Soledad Gallardo, Blanca Cárdaba, Victoria del Pozo and Carlos Lahoz

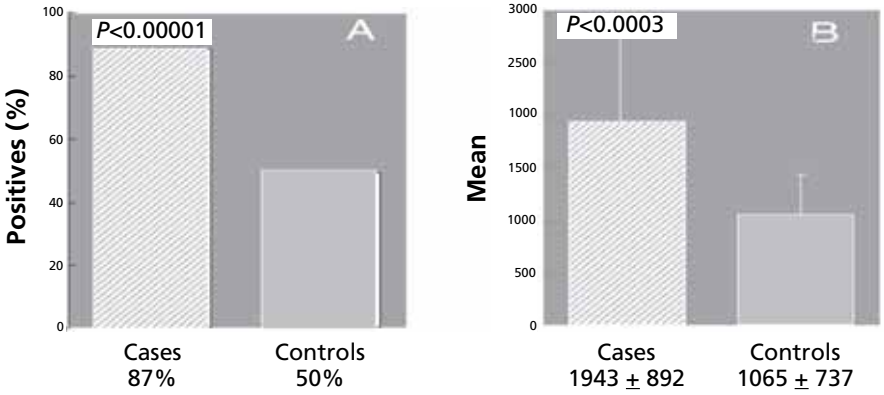
There are many reasons for presuming that immunological mechanisms operated in the pathogenesis and continuation of TOS. The condition has been suggested as being similar to graft-vs-host disease in many respects. The first immunological features described were high levels of IgE, eosinophilia and reduced numbers of CD8 T cells and basophils (*1*). Subsequently, some reviews reported immunological findings in TOS (*2–4*). This chapter brings together most of the immunological studies performed after the previous WHO publication in 1991 (*5*). The findings are dealt with under six main headings:

- serological findings
- immunopathological studies
- genetic studies
- in vitro studies
- animal studies
- work in progress and future perspectives.

Serological findings

In the acute phase, the most important serological finding was the high level of soluble interleukin-2 receptor (sIL-2R) detected in sera of TOS patients: an average of 1943 U/ml vs 1065 U/ml in controls ($P<0.0003$). This study was performed in 126 individuals (98 cases and 28 controls) using a case-control design (Fig. 6.1). In a subsequent study, IgE levels in TOS patients were 2.44 times

Fig. 6.1. Results of sIL-2R in sera from the acute phase of TOS patients and controls



A: percentage of positive sera; B: mean ± standard deviation.

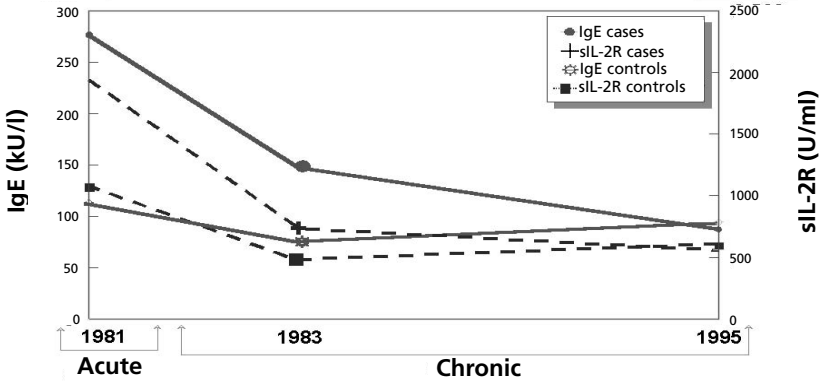
higher than those of controls (276 ± 847 vs 113 ± 177 U/ml). Levels of soluble CD23 antibodies (sCD23) in TOS patients were also increased compared to controls (322 ± 321 vs 209 ± 207 U/ml) (*6*). These results are in agreement with those of other authors that describe, in both TOS and eosinophilia–myalgia syndrome (EMS), elevated sIL-2R and IL-4 in serum and a high number of blood eosinophils (*7,8*).

Several groups have investigated the possibility that autoimmune mechanisms were involved in the pathogenesis of TOS. Some reported, in the acute phase, low titres of autoantibodies against smooth muscle, mitochondria, nuclei, lymphocytes, cardiolipin, DNA and collagen, which were not related to the progress of the disease (*9,10*). IgG antibodies against human collagen were found in 28 out of 44 serum samples obtained from patients during the acute phase (*11*). This study also detected antiphospholipid antibodies in TOS patients. All of these antibodies decreased as the disease progressed.

In a case-control study performed under blind conditions with sera from acute-phase patients, we did not find autoantibodies against smooth muscle, mitochondria, nuclei, gastric parietal cells, lymphocytes, extractable nuclear antigens or collagen type I-IV (*3*).

In the chronic phase, sIL-2R, sCD23 and IgE tended to recover to normal levels, but two years after the outbreak levels of sIL-2R and IgE were still high ($P<0.05$) (*12*). The development of these serological parameters is represented in Fig. 6.2. Clinically, the chronic phase is characterized by the presence of autoimmune-like conditions such as scleroderma, sicca syndrome and myositis. Antibodies against C-reactive protein were detected in patients during the chronic phase of TOS, but only when the protein was

Fig. 6.2. Development of sIL-2R and IgE in sera from TOS patients in different phases



denatured (*13*). The explanation given by the authors was that absorption of toxic products may have caused liver damage, causing abnormal biosynthesis and/or alterations of acute-phase proteins. Antiphospholipid antibodies with different specificity have been found in a high percentage of TOS patients, although their significance remains unclear because there was no significant association between these antibodies and the clinical disease (*14*).

Immunopathological studies

The data obtained from patients during the acute phase of TOS, such as elevated sIL-2R and IgE, eosinophilia and lymphocytic infiltration in lung tissues, lead to speculation of T cell activation (*15*).

Advances in molecular biological techniques over the last few years now make it possible to use methods that were not available when the disease occurred (*16*). Thus, preserved lung tissues from patients who died from TOS were used to study the mRNA specific to several cytokines and markers of activity. For this purpose, an mRNA extraction procedure was developed and used on paraffin-embedded lung tissue obtained during autopsies on 26 TOS patients with pulmonary involvement (*17*). Tissues from 15 non-TOS-affected patients, autopsied at the Pathology Department of Fundación Jiménez Díaz in Madrid, were used a controls. Cytokines tested were IL-2 and γ-IFN produced by the Th1 subset of T lymphocytes, and IL-4 and IL-5 produced by Th2 cells; mRNA specific for GM-CSF, CD25 (IL-2 receptor) and CD23 (low-affinity receptor for IgE) were also analysed**.**

A statistically significant increase in cytokines corresponding to Th2 responses with respect to Th1 were found in lung specimens from patients

who died from TOS (P = 0.03) (Fig. 6.3). This did not occur with specimens from non-TOS controls (*18*).

In summary, high levels of IL-5, IL-4 and CD23 antibody messenger RNA were detected in lung specimens from TOS patients. These data are the first clear evidence that an immunological mechanism was directly implicated in this illness. Thus, T cells could play an important role in the induction and maintenance of inflammation: (*a*) specific Th2 cytokines such as IL-5 could explain eosinophilia found in TOS patients, owing to the fact that IL-5 is an eosinophilopoietic factor that induces selective eosinophil recruitment in different tissues; and (*b*) IL-4 facilitates the persistent IgE antibody production by B cells also found in TOS patients.

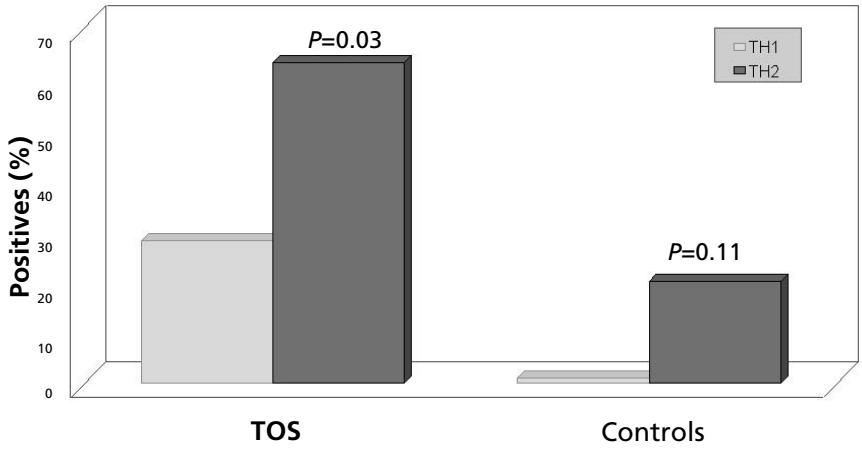
In addition, positive immunohistochemical localization of IL-4 has been demonstrated in the skin of patients with EMS and TOS (*19*).

Furthermore, Ten et al. (*20*) found eosinophil infiltration and degranulation in tissues of 52 TOS patients, especially during the acute phase. Tissues were examined by specific immunofluorescence for the eosinophil granule major basic protein (MBP), and deposits of this eosinophil inflammatory mediator were found in the lungs. Serum MBP was significantly elevated during all phases of the disease. Thus, tissue damage caused by eosinophil-derived protein may at least partially explain many of the pathogenic features of TOS.

Genetic studies

The human leukocyte antigen (HLA) plays a pivotal role in the binding and presentation of antigenic peptides to T cells. A highly polymorphic

Fig. 6.3. Results of Th1 and Th2 cytokines in lung of TOS patients who died because of TOS



genetic region located on chromosome 6 codifies for these elements. Different haplotypes have been associated with several immune diseases, especially in the autoimmune and allergic processes.

Genetic susceptibility appears to be involved in TOS. After the outbreak, HLA-DR2 antigen was associated with patients in the acute phase of the disease, but not with those in the chronic phase (*21*). Several reports have described associations between the chronic stage of the disease and HLA, with HLA-DR3 and HLA-DR4 antigens being postulated as risk factors (*22*). Recently, an increase of frequencies of HLA-A24 and HLA-DR4-DQ8 and a decreased in HLA-B blank frequencies have been associated with this stage (*23*).

The HLA class II antigens were re-analysed in a well designed case-control study. Triplets of subjects (n = 265) composed of chronic patients (n = 117), non-affected family members (n = 71) and non-related controls (n = 77) were studied. Also, HLA class II antigens were analysed in patients who had died from TOS (n = 34) and in TOS patients (controls) who died from non-TOS-related causes (n = 13). No significant association was found between HLA and the disease in surviving patients. In contrast, an increase in phenotypic frequency of DR2 antigen was found in patients who died from TOS: 73.5% compared with 25.6% in TOS patients ($P<0.001$), 28.5% in non-affected family members ($P<0.001$), 23.9% in non-related controls ($P<0.001$) and 38.4% in dead controls ($P = 0.03$) (*24*). Table 6.1 shows the results obtained when DR2 antigen frequencies were compared among groups. These results showed that some DR2 patients were prone to die from the disease before reaching the chronic stage, suggesting that DR2 is in fact an aggravating factor for this disease.

In vitro studies

As a result of immunotoxicological findings, the in vitro effect of some TOS-related products was studied in lymphocyte proliferation tests. The aim was to evaluate not only the possible immunological memory owing to previous contact between lymphocytes with toxic agents, but also their direct toxic effects.

For this purpose, a design-matched case-control was used. Cases (n = 25) and controls (n = 50) were selected through a simple random sample. Patients were selected according to the clinical criteria used by Kilbourne et al. (*25*). None of controls had either clinical criteria or symptoms of TOS. The products tested were the oleoyl monoester of PAP (OPAP), the dioleoyl diester of PAP (OOPAP), oleoyl anilide, linoleoyl anilide and erucyl anilide. The parameters studied were incorporation of thymidine ^{3}H, cellular cycles, IL-2 membrane receptor and sIL-2R in supernatant of cell cultures. No differences were found between cases and controls. It therefore appears that TOS patients do not maintain immunological memory, at least within the period studied

Table 6.1. Results for DR2 antigen among all groups tested

Comparison	Marker	Patients and controls[a]				*P* uncorrected	*P* corrected	RR, EF
		a	b	c	d			
Dead from TOS vs dead from other causes	DR2	25	9	5	8	0.03	ns[b]	
Dead from TOS vs non-related controls	DR2	25	9	17	54	<0.001	<0.001	RR = 8.82 EF = 0.64
Dead from TOS vs non-affected family members	DR2	25	9	22	55	<0.001	<0.001	RR = 6.9 EF = 0.62
Dead from TOS vs surviving TOS patients	DR2	25	9	30	87	<0.001	<0.001	RR = 8.05 EF = 0.63
Dead from non-TOS-related causes vs non-related controls	DR2	5	8	17	54	ns	ns	
Dead from non-TOS-related causes vs non-affected family members	DR2	5	8	22	55	ns	ns	
Dead from non-TOS-related causes vs surviving TOS patients	DR2	5	8	30	87	ns	ns	

[a] a and c: numbers of patients and controls, respectively, with the marker; b and d: numbers of patients and controls, respectively, without the marker.

[b] ns = not significant.

(14 years after the epidemic). Negative results obtained in cultures from the chronic phase are also in accordance with those obtained in serological studies (*12*).

Based on the hypothesis that the initial target is endothelial cells, several studies focused on detecting the effect of TOS-related products in cultured human endothelial cells. It was found that that oleic and linoleic acid anilides impair prostanoid synthesis (*26*) and release of arachidonic acid (*27*).

Several in vitro studies have demonstrated that TOS-related products cause cell death (lymphocytes and endothelial cells) and, owing to the relationship between apoptosis and autoimmunity, the possibility of cell death by apoptosis was studied. The DNA degradation, hypodiploid peak and differences in morphology found in linolenyl anilide (LNA)-treated lymphocytes led to the conclusion that LNA causes apoptosis. This was not found with other tested products. Fig. 6.4 shows acridine orange (AO) and propidium iodide (PI) stained cells cultured with LNA.

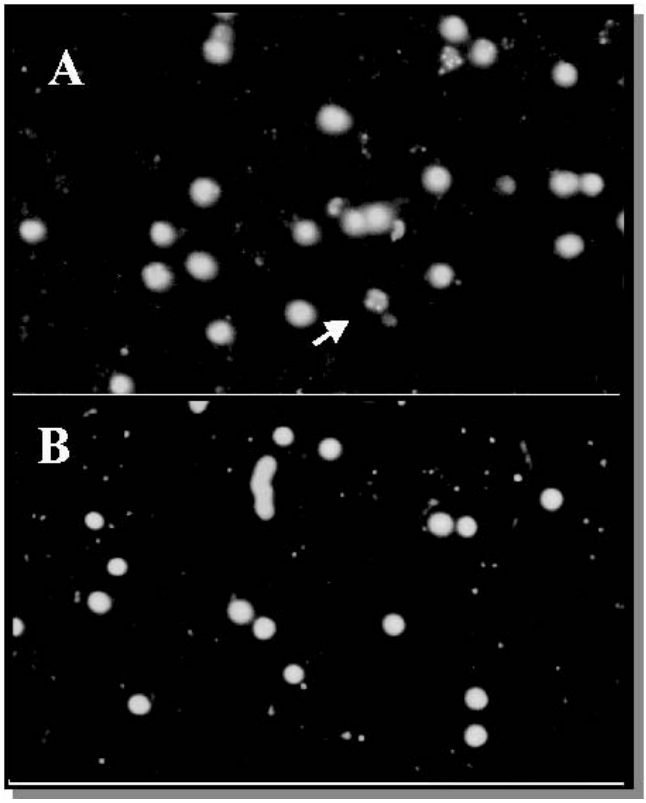
Animal studies

The epidemiological, clinical and pathological descriptions of TOS are well established, but much remains unclear concerning the etiopathogenic mechanisms and the etiological agents involved. This is in part due to the absence of an animal model.

In the last few years, several groups have studied various animal strains (with different haplotypes), not only with synthesized TOS products but also with "reconstituted" oils enriched with different concentrations of OOPAP. A strain-dependent susceptibility was demonstrated in a murine model of TOS after treatment with oleic acid anilide (one of the possible agents implicated) (*28*). C57BL/6 mice (fast acetylators) showed a Th2-like response and A/J mice (slow acetylators) a Th1-like response, suggesting that these results resemble the human conditions in TOS with development of either acute lethal disease or a chronic autoimmune-like disease. These studies, however, despite the interesting results, did not use controlled toxic samples.

An increase in blood eosinophilia in mice treated with the linoleic diester of PAP has also been described (*29*). Recent experiments performed in our laboratory showed that administration by gavage of synthesized TOS oils with different concentrations of OOPAP caused a slight increase of peripheral blood eosinophils in BALB/c mice (Fig. 6.5) and an increase in serum IgE levels in DBA/2 mice, which share the same haplotype (H-2d) (Fig. 6.6). In the data shown in Fig. 6.5, the population of eosinophils was detected by flow cytometry by counting the double positive cells to VLA-4 and Gr-1 (the only population of granulocytes that express VLA-4). The basal and final percentages of eosinophils were recorded individually for each mouse. These responses were not observed in other strains tested with different haplotypes:

Fig. 6.4. Apoptosis induced by 20 µg/ml of linolenyl anilide on human lymphocytes after two days of culture.



A: Arrow points to an apoptotic cell with a still intact membrane.
B: Normal cells were cultured with 0.2% ethanol.

C57BL/6 (H-2b) or A/J mice (H-2a). It would therefore appear that the response depends on the strain of mice tested, which may be indicative of a strain-specific response to TOS oils.

Discussion

Many immunological goals have been achieved since the publication of the previous WHO book in 1992 (*5*), in which immunological mechanisms were suspected but not yet demonstrated.

A high level of sIL-2R in sera from patients at the acute phase of TOS is one of the parameters that indicates a process in which T cell activation may play an important role. Interleukin-2 receptor (IL-2R) is the membrane receptor for IL-2, a lymphokine that regulates the proliferation of activated

Fig. 6.5. Flow cytometry analysis of peripheral blood eosinophils from BALB/c mice (n = 61) treated with oil RSO160401 and control mice (n = 36) treated with olive oil

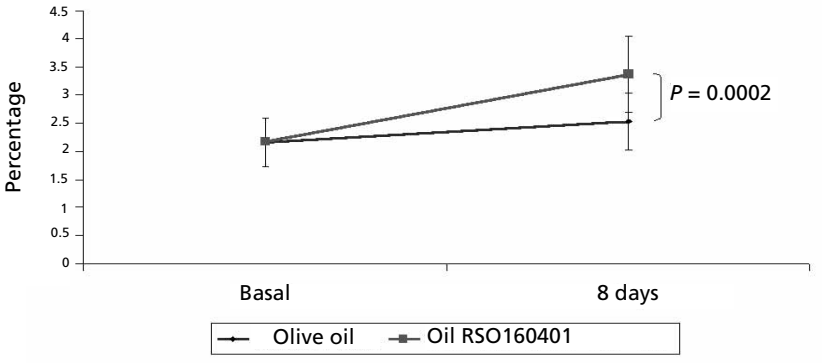
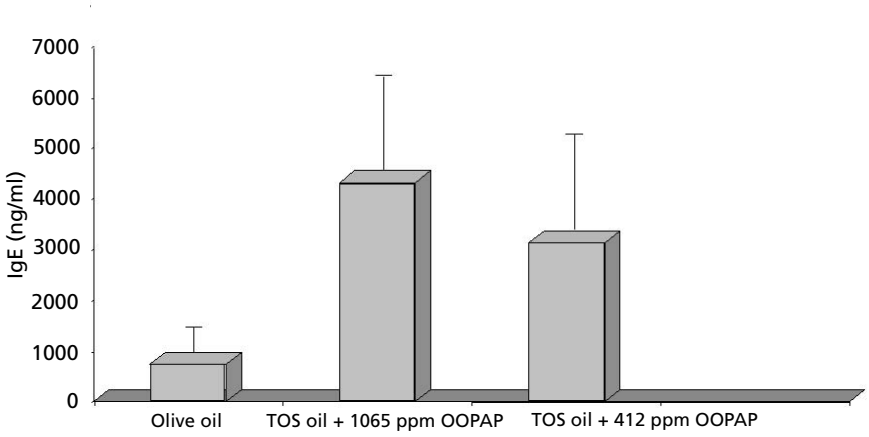


Fig. 6.6. Serum IgE levels (mean ± SD) obtained in DBA/2 mice treated with 10 ml/kg oil enriched with two different OOPAP concentrations and using olive oil as control



T lymphocytes. Its soluble counterpart, sIL-2R, is elevated in several autoimmune processes: multiple sclerosis (*30*), systemic lupus erythematosus (*31*), rheumatoid arthritis (*32*) and primary Sjögren's syndrome (*33*). It has also been reported that sIL-2R levels are correlated with blood eosinophilia and lung function (*34*). These data suggest a possible link between T cell activation, eosinophils and impairment of lung function in inflammatory processes.

This cascade of events possibly occurred in TOS patients. Also, high levels of sIL-2R were reported in the sera of seven EMS patients (*35*). In conclusion, high levels of sIL-2R, IgE and sCD23 detected in TOS patients comprise the first indirect evidence of a T cell activation in TOS, and may explain some characteristic features of the acute phase of the disease.

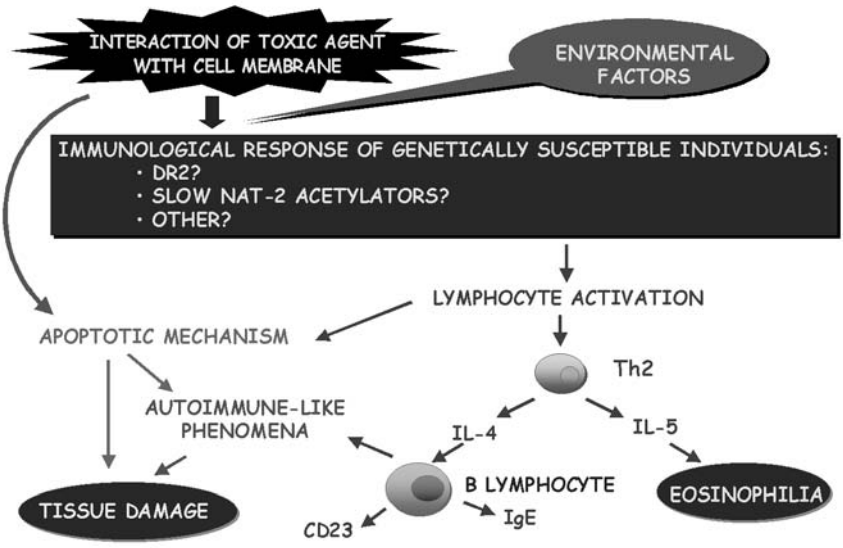
HLA studies have demonstrated that DR2 antigens are associated with severity of disease in TOS (*24*). As far as we know, this is the first time that deceased patients from an epidemic have been HLA typed, emphasizing the importance of storing genomic DNA where emergency situations may occur and opening the possibility for re-analysis afterwards. This kind of study will help to understand the correct pathogenic implications of the disease process.

The peripheral eosinophilia, the rise in serum IgE, sCD23 and sIL-2R (*6*), the increase in CD23, Th1 and Th2 cytokine profile in the lungs of TOS patients (*18*) and the similarities to graft-vs-host (*36,37*) and autoimmune diseases are strong evidence for an immune mechanism in TOS. We postulate an activation of Th2 cells, giving rise to the secretion of the Th2-dependent cytokines. The secretion of these Th2 cytokines could explain the eosinophilia (IL-5) and the increased levels of IgE and sCD23 (IL-4) found in the acute phase. Fatty acid anilides of oleic, linoleic and linolenic acids were considered reliable indicators of toxic oils (*38*). However, it is possible that these products by themselves do not promote immune response but, once absorbed and transported to the liver, undergo transformation into other metabolites capable to eliciting the immune activation of T cells, not only in the liver but also in other organs and tissues. This may be one reason why memory T cells are not able to recognize the original products (*12*).

Thus, there is further evidence that both immunotoxic and immune reactions were involved in the pathogenesis of TOS. A hypothetical mechanism is shown schematically in Fig. 6.7.

TOS is a multifactorial disease and probably depends on the interaction, in genetically susceptible individuals, between the time and amount of oil ingested and the presence of non-specific "adjuvant" factors (differences in lifestyles and exposure rates and other environmental factors) (*39*). The unsolved question is whether this genetic susceptibility is due to DR2 or to other genetic polymorphisms associated in linkage disequilibrium with this antigen. It is possible that the product of other genetic loci related to DR2 could be implicated in this response. Analysis of chromosome 6 has revealed that *N*-acetylglucosaminyltransferase (an enzyme implicated in the acetylation of fatty acids) is located on this chromosome (*40*). There are different polymorphisms of this enzyme, implicated in slow or quick acetylation, that could be involved in the metabolization of different fatty acids. It might be interesting to investigate whether there is a relationship between both loci in relation to the development of TOS.

Fig. 6.7. Hypothetical mechanism operating in the development of TOS



Work in progress and future perspectives

A bank of samples from TOS patients and controls has existed at the Fundación Jiménez Díaz in Madrid for several years. At present the collection has approximately 46 000 samples (sera, plasma, cells and DNA) from about 3000 individuals. The bank continues to store samples from case and control subjects. In addition, it has samples from patients who participated in a feasibility study on the cohort of TOS patients. Individual clinical records are collected in a database.

TOS has been described as a disease in search of an animal model (*4*). Some results obtained in animals partially reproduce the human disease and the response depends on the strain of mouse tested, which is indicative of a strain-specific response. It also appears that oils tested are more toxic when the OOPAP concentration is higher. Thus, the search for an animal model should not be abandoned, and other strains of mice with different haplotypes that could be of potential interest should be tested. On the other hand, knockout or HLA-transgenic mice could be a good model for elucidating the role of HLA in TOS. These mice do not express their endogenous Class II molecules but instead express the human Class II antigens DR and/or DQ (*41*).

Contamination of food by chemicals or metals has become more common in recent years. The origins of these foodborne epidemics are not understood and the diseases that occur are often new and misdiagnosed (*42*). In fact,

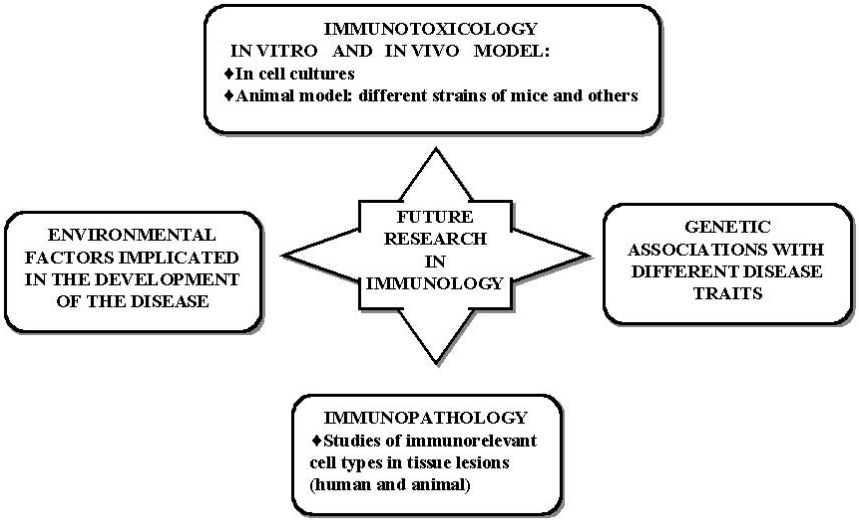
In fact, some mechanisms appear to be common to a variety of agents, while different mechanisms appear to produce similar diseases (*43*). We believe that the molecular pathology approach presented here could be applicable to a broad range of other poorly understood disorders, and could greatly promote the understanding of the immunological mechanisms associated with these disorders. Many clinical aspects of TOS resemble those of other illnesses whose causes are still unknown, such as scleroderma (*44,45*), hypereosinophilic syndrome (*8*) and EMS. In fact, EMS presents many features in common with TOS: severe myalgia, multisystem involvement, high levels of peripheral blood eosinophilia and a relationship to the use of consumer products (*1,46*). The indirect evidence for the T cell activation found in TOS has also been reported in EMS, associated with high levels of sIL-2R. The close similarity between TOS and EMS emphasizes the importance of updating knowledge of new diseases. In fact, scleroderma and related disorders may be triggered by exposure to some environmental agents in people with a pre-existing susceptibility (*47*), and certain host factors (such as genetic background) may have played a role in determining their susceptibility to the illness. Thus, assessment of the effect of genetic polymorphism on xenobiotic metabolism would indicate the potential metabolic capacity of the victims at the time of the event. An increase in NAT2-defective alleles in TOS patients compared with unrelated controls, adjusted by age and sex, has been recently described *(48)*. Thus, impaired metabolic pathways may have contributed to the clearance of the toxicant(s), leading to a low detoxification or accumulation of toxic metabolites. This may suggest a possible role for impaired acetylation mediating susceptibility in TOS.

The discovery of the toxic agents could shed light not only on the cytotoxic mechanisms operating in TOS but also on those of other diseases. The further elucidation of mechanisms of apoptosis could also shed light on the mechanism of toxic damage to cells and tissues of TOS patients.

Despite these findings, however, knowledge of the possible genetic basis of TOS is still poor. On the other hand, TOS is a good model for studying genetic mechanisms, since it has well characterized causative environmental factors, a well known clinical picture and a defined immune response. Thus studies could be made of chromosomes with genes potentially relevant to the development of TOS (study of candidate genes) or by using a genome-wide search for new relevant genetic elements related to this kind of disorder, after which the genetic studies could be completed by analysing the other chromosomes.

The search for the cause of TOS should not be abandoned. In fact, although many aspects of TOS have been clarified in the last few years, other immunological goals still remain to be attained in term of immunotoxicology, immunopathology, animal models and genetic studies. Fig. 6.8 shows points to be developed in immunological studies.

Fig. 6.8. Areas to be developed in immunological studies



Summary

The first immunological features described in TOS were eosinophilia and reduced numbers of CD8 T cells and basophils. Subsequently, case-control studies found high levels of soluble interleukin-2 receptor (sIL-2R) and high total IgE in serum samples from TOS patients. Immunopathological studies revealed an increase in Th2 cytokine (IL-4, IL-5) in lung specimens from TOS patients. Also, eosinophils and their derivates (MBP) were demonstrated in pulmonary tissues. Genetic studies indicated HLA-DR2 antigen to be an aggravating factor for the disease. Results obtained in mice partially reproduce some signs of the human disease (slight increase in blood eosinophils and RNA from pulmonary tissues with a Th2 cytokine profile). It is very important that the search for an animal model should not be abandoned, and other strains of mice with different haplotypes that could be of potential interest should be tested. On the other hand, knockout or HLA-transgenic mice could be a good model for elucidating the role of HLA in TOS. Individual clinical records are being collected in a database in order to further investigate mechanisms in the light of new developments in knowledge.

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7

Ethical and social aspects

Benedetto Terracini and Concepción Martín-Arribas

The epidemic that occurred in 1981 in Spain was unique, because the condition that became known as TOS had not been previously been recorded and because its etiology turned out to be most unusual. In addition, the TOS episode took place in the complex social milieu typical of western societies at the end of the twentieth century. This was characterized by a complex of motivating forces, such as people's expectations from medical practice and from research, governments' responsibilities in health care, limited resources for science, and access to information and the freedom of the press. These and other basic values favour the appearance of circumstances that demand that a choice be made between options that are equally valid on moral grounds.

As yet, the ethical problems created by environmental accidents and disasters (to which, to a certain extent, TOS can be equated) have received limited attention. Medline provides only a handful of entries for the key words "environmental disasters + ethics". Some guidance was given in a previous WHO publication (*1*) and in guidelines on the interaction between scientists and the community when local excesses of diseases of environmental origin are suspected or identified (*2*). An epidemiological focus on the consequences of environmental disasters implies consideration of such issues as informed consent, the guarding of privacy, peer review and dissemination of results. Individual interest is involved in so far as people may either expect a return (such as compensation) or be disturbed by the idea of being labelled as

contaminated or diseased (*1*). The need for consistency between research and community interests has also been stressed (*3*).

This chapter focuses first on those aspects of the TOS episode that have implied or may imply an ethical consideration. In doing this, we refer to the classical framework provided by the principles of beneficence, nonmaleficence, autonomy and justice (*4*). We then describe the work of the Ethical Committee of CISAT, which is currently dealing with the epidemiological and clinical investigations on TOS victims. Finally, we report on the role of victims' associations in the development of knowledge about TOS.

Soundness of the scientific evidence, knowledge and decision-making

A major preventive measure was the public announcement, on 10 June 1981, that oils sold by street vendors in unlabelled containers of a certain shape were responsible for the "obscure disease" whose lethal consequences had been reported by the media during the previous weeks. The announcement had probably only a limited role in causing the epidemic to subside (the decline in the epidemic curve started before the announcement), but it represented a landmark in terms of risk communication and reinstatement of public confidence in the Government's ability to keep an unexpected event under control, as well as in the potential of epidemiological research.

The events of late May and early June that led to the public announcement included the following.

- A rudimental case-control study was carried out, prompted by the exceptional occurrence of the disease in an infant aged less than six months who was routinely fed a food oil. It compared the dietary habits (reported by their mothers) of 62 children admitted to hospital with TOS and as many children admitted for other conditions. Of these children, those who had consumed cooking oil sold by itinerant vendors in 5-litre bottles were respectively 100% and 6% (*5,6*).
- Informal surveys by physicians found that all those who were interviewed reported previously consuming cooking oil illegally sold by street vendors.
- Chemical analyses of oils collected from households and local outdoor markets were carried out at the Central Customs Laboratory: the oils were determined to be rapeseed oil denatured with 2% aniline.

Admittedly, professional epidemiologists would have hesitated to endorse the Government's statement; the evidence relied on only one epidemiological study carried out in only one hospital. This study investigated many dietary variables, so that the likelihood of spurious positive associations was high. In addition, possible bias from limited comparability between cases and controls

had not been given sufficient attention. The study in Navas del Marques, which later provided firmer evidence of the association (*7*), as well as all the other "early" case-control studies (see Annex 1), had not yet begun at the time of the announcement. In terms of causal inference, with the exception of the statistical strength of the association (with an unusually high relative risk), most classical criteria of Bradford Hill (*8*) were not met, including consistency of findings in independent studies of dose–response relationship, biological plausibility and reproducibility of findings under experimental conditions. The extent to which the Government's officials issuing the announcement on 10 June were aware of these limitations, and the weight they gave to the underlying uncertainties, is not known. The need for action prevailed over the need for a more adequate background for causal inference. This was fortunate and, in a way, represents a good example of the application of the precautionary principle *avant la lettre* (*9*).

Respect for individual autonomy in prevalence studies

In any clinical follow-up study, limited participation brings about a selection bias leading to loss of precision in estimating the occurrence of outcomes of any sort. This is even more so when outcomes cover a spectrum of conditions ranging from (almost) well-being to severe handicap. When follow-up extends for years or decades, it is difficult to quantify the extent to which prevalence estimates are flawed by limited representativeness. Those who refuse to participate may do so because they are in good health or because they feel that they do not need any attention in addition to that already provided, because their condition is so poor that they cannot move from home, or for other reasons.

In clinical studies on TOS survivors, an invitation to visit a clinical unit usually stated that acceptance would benefit both the person's health and scientific knowledge, but it also emphasized the freedom of the person not to participate. It is also acknowledged that research aimed at understanding the biology of a new disease such as TOS is potentially beneficial, though not necessarily to those entering the study.

Protecting privacy and maintaining confidentiality, as well as respecting the cultural setting in which the research is conducted, have been stressed as obligations and responsibilities of epidemiologists (*10*). Occasionally, TOS patients with few or no current symptoms or signs of the disease expressed the desire not to be contacted for follow-up studies. This was based on understandable psychological grounds, including the fact that such patients did not want their friends and neighbours (and possibly spouses) to know that they were TOS survivors. There have also been rumours of people being discriminated against when seeking work. To reduce these negative aspects of follow-up, letters of invitation and any other request for information from CISAT are mailed in plain envelopes.

Health care and research

Over the years, decisions on caring for TOS patients went through different phases. Shortly after the outbreak, ad hoc units were created for the care and follow-up of TOS victims, the underlying idea being that the care of TOS patients needed specialized staff. Centralization of the care of TOS patients would have also prevented fragmentation of therapeutic approaches and would have helped the collection of data for clinical studies. Later, such units were dismantled except for five, which were preserved expressly for clinical studies. A new unit has recently been opened by CISAT, where clinical investigations offered to TOS patients are relatively complex and where the mechanism for clinical follow-up through active recall is likely to be more efficient than elsewhere.

The debate continues as to whether or not the attention needed by TOS patients in the chronic phase requires "TOS specialists". The decision to close a number of ad hoc follow-up units was taken on the grounds that the state should not discriminate among citizens. Since the late 1980s, the Spanish National Health Service has been proven to work relatively well and to be able to take care of any severe condition with the same degree of effectiveness. Most conditions exhibited by TOS survivors in the late phases are not specific to TOS and occur also in patients suffering from other diseases, such as pulmonary hypertension and scleroderma. On the other hand, TOS victims report feeling more comfortable in sharing their anxieties concerning the uncertainties of their condition with TOS "experts" (admittedly, there is a certain degree of TOS specificity in such anxieties and uncertainties). In addition, TOS patients have some privileges (which they understandably wish to retain) such as access to all medicines at no extra charge, including those that are not considered to be "essential".

Thus, within the TOS episode, there was room for several sources of inequality (*a*) between TOS victims and patients suffering from other conditions requiring as much attention as TOS and (*b*) between TOS survivors having access to the ad hoc follow-up units and other TOS survivors. People living in and around Madrid have easier access to follow-up units than those living in other areas hit by the epidemic. Logistically, they are also the most suitable patients for inclusion in trials intended to assess the diagnostic ability of relatively sophisticated procedures (such as instrumental diagnosis of neurological impairment) or the occurrence of indirect consequences of TOS (such as psychological impairment in the offspring of TOS patients). This, however, leads to inequality associated with place of residence. On the other hand, any positive results from these studies may also benefit those not included in the corresponding databases.

These inequalities are far from being unique to the TOS episode. They reflect typical conflicts between research and care delivery. Inequalities in

access to health care have also been reported following other environmental accidents, such as that at Bhopal (*11*).

The Ethical Committee of CISAT

The Ethical Committee was created by the Director of the Instituto de Salud Carlos III in 1997, at the request of the Centers for Disease Control and Prevention (CDC) in the United States, within the framework of an agreement between the two institutions on research on TOS involving human subjects and biological materials stored in Madrid. Over the years, the Committee has evaluated all clinical and epidemiological studies on TOS initiated at CISAT that require observations on TOS victims and/or the use of biological materials stored at CISAT. The Committee has stated that it is also willing to involve itself in TOS projects carried out elsewhere, but as yet this has not happened. The Government of Spain has produced comprehensive norms for clinical trials, whereas as yet no legal restrictions on observational studies have been issued.

The Committee comprises nine persons chosen by the Director of the Instituto de Salud Carlos III. Five of these (two epidemiologists, one nurse, one technician and one secretary) are from CISAT, two are TOS victims, and one is a physician with a masters degree in bioethics. The ninth member, the Chairman, is a senior non-Spanish epidemiologist.

Since both TOS victims and epidemiologists investigating the disease sit on the Committee, it is not an independent body. On the other hand, in the absence of a legal framework for ethical committees dealing with observational studies, and indeed in the absence of experience in this type of activity in Mediterranean countries, it is preferable for the Committee to work through consensual procedures whose terms of reference are defined on a case-by-case basis.

On average, the Committee meets twice a year. It has set its own rules of procedure and has examined about a dozen new projects, the scientific soundness of which had previously been approved by the WHO/CISAT Scientific Committee for the Toxic Oil Syndrome.

Communication of findings

The Ethical Committee considers the communication of findings of clinical studies to have three levels, each having a different interlocutor, and has established the following criteria.

- An individual report on instrumental or laboratory findings is to be forwarded to each participant. It should provide guidance on the clinical significance of findings, if any, and should be written in understandable terms. For instance, it was agreed with the investigators that reports of individual data on HLA antigens should state that "the purpose of these tests is not to show the presence or absence of any disease. On the contrary,

similarly to blood groups, each person has different values that only indicate personal characteristics". Investigators are also asked to provide participants with an address and/or phone number where additional information can be obtained, if desired.

- A report on the overall findings of any study is to be sent to the health authorities as well as to the whole group of participants (i.e. the victims' associations).
- There is to be a conventional scientific publication addressed to the scientific community.

Biological samples

A common problem in molecular epidemiology is created by the potential use of stored biological material that had originally been collected and processed for clinical purposes (such as histological specimens in paraffin wax or tissues preserved in formaldehyde). The Ethical Committee has established that the use of these tissues does not require a posteriori consent of the donors, provided that they are processed under strict anonymity. However, informed consent is required for the prospective collection (mainly from TOS patients undergoing clinical follow-up) of blood and other biological fluids to be stored in the biological bank that has been created. This strategy conforms to international guidelines (*12*).

The Ethical Committee and investigators at CISAT have been aware of the risks involved in generic, unconditional informed consent to any "use for research" of biological materials. The forms on informed consent offered to individuals to be included in a study specify in great detail the purpose of the study. The following is a case in point.

In 1997, before the creation of the Ethical Committee, a study (*13*) was carried out on the distribution of genotypes governing enzyme polymorphisms in a number of TOS survivors and in two sets of individually matched controls (one unaffected sibling and one friend in each case). All participants were asked to give their informed consent in writing, after having been informed in great detail of the purpose of the study. At the end of the study, given the possibility of additional studies being conducted *on the same subject*, it was decided that the unused materials should be preserved. Three years later, this material turned out to be suitable for use in a new study designed to compare a number of genomic features between cases and controls. Given the restricted nature of the original informed consent, it was decided to contact all participants to obtain their informed consent again for the new set of analyses. Of the approximately 400 persons involved (cases and controls), 80% were traced; none refused to give their informed consent for the new project. The exclusion from the database of the other 20%, who could not be traced because of changes of address and other logistical reasons, would have greatly reduced the number of full triplets expected to be included in the analysis. The Committee decided that the case was characterized by some unusual circumstances:

- the restriction of the original informed consent had been due to excess zeal on the part of the investigator, and had not been requested by the individual participants;
- none of the contacted people had refused the second request for informed consent and it was reasonable to assume that there would have been no refusals from these people, given that they were untraceable for reasons unrelated to their attitude towards the study; and
- given the specific circumstances, the validity of the study could be considered as a "superior" objective in terms of its benefit to the community.

The current attitude of the Committee is that the forms should not specify in excessive detail the design and scope of the relevant studies, provided that the consent is granted *exclusively* for studies aimed at unravelling the natural history of TOS, thus excluding the use of the biological materials in studies unrelated to TOS.

Donors are informed that "it is most unlikely that the results will either have a bearing on the treatment you are receiving or predict the evolution of your disease. However, if you wish, you will receive them" (followed by a clear request to inform CISAT of any future change of address). Participants are informed of their right to provide their biological materials anonymously and to withdraw them from the bank at any time.

The associations of TOS victims

Spain has a tradition of neighbourly activities, and the return of democracy in 1975 saw an upsurge of "neighbours' associations" throughout the country. These associations were the natural place for the TOS victims to gather, exchange experiences and decide on common action. Formal associations of TOS victims started to be created in late 1981; these came together in provincial federations early in 1982 and later evolved into three major groups.

One of the first problems to be tackled by the associations was the discrimination between TOS victims who were employed in 1981 and those who were not. According to the social security regulations, only the former were entitled to sickness benefit for 18 months, after which they had the right to go before a medical tribunal. If recognized as unfit to work, they could then receive a pension. This lack of equity was the major reason for a number of "sit-ins" in churches, lasting for up to two months, in late 1982 and early 1983. In addition to social security coverage for all, requests were made for registration of all victims and clinical follow-up.

The first meeting on TOS organized jointly by the Spanish Government and WHO took place in Madrid in 1983. On that occasion, the victims' associations defined four goals: research into the causes and natural history of TOS, clinical follow-up, social reintegration of the victims (a novel idea

in those days) and recognition of legal responsibilities. Marches to WHO headquarters in Geneva and to the WHO Regional Office for Europe in Copenhagen took place in 1985 with the aim of stressing to WHO the importance of these goals.

On the international front, a major breakthrough was reached in 1987 when, on the basis of a request by the victims' associations, the European Parliament approved a resolution that included specific recommendations to the Spanish Government and to the European Commission. The former aimed at pursuing the objective of scientific research, health care and justice mentioned above (point 19 requested the Spanish authorities to "study and evaluate the failure of the current programme for social reintegration of the TOS victims"). Requests addressed to the European Commission related to actions that the Commission could take to prevent episodes similar to TOS occurring in the future. These included the need to investigate social inequalities in dietary habits, which may bring about differential risks in foodborne intoxication. They also included the need to develop mechanisms to ensure that, in the case of environmental disasters, action should include social and occupational as well as health-related aspects.

In terms of research, the TOS episode illustrates the potential of victims' associations in understanding environmental disasters and estimating their impact. They have helped in providing rosters of patients for inclusion in ad hoc studies. They have helped explain the reasons for studies being undertaken, and they have helped clarify the difference between activities aimed at providing treatment and studies on specific issues related to biological mechanisms. In the case of clinical–epidemiological studies on samples of patients, the associations have helped avoiding misunderstandings that often appear in this type of investigation (inclusion in or exclusion from the sample may be perceived as a kind of discrimination). Above all, they have contributed to creating an atmosphere of reciprocal trust between victims and investigators.

Conclusion

The management of the TOS outbreak has implied a variety of problems that cannot be faced without ethical and social considerations. Hopefully, this experience may help in planning measures to prevent similar episodes in the future.

Summary

The specific social and ethical problems raised by the TOS epidemic were due to the fact that it consisted of an outbreak of a previously unknown disease whose cause, early in 1981, was totally unknown. Progress made in understanding the etiology of the disease created an unusual level of communication involving scientists, public health authorities, TOS victims and the public. An early event (in June1981) was the assessment of the soundness of the scientific

evidence of causality, which was relevant to public health decisions. The possibility of confusion between research and intervention has also merited attention. Other ethical issues raised by the episode included individuals' autonomy with regard to their participation in follow-up clinical studies and in investigations requiring the collection of biological samples. These aspects are reviewed in the present chapter, which also describes the role of victims' association in the implementation of the results of clinical studies.

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8

Future research strategies

Manuel Posada de la Paz and Emilio Gelpí

Since May 1981, when the TOS epidemic appeared, much effort has been directed by individual researchers, research groups and organizations worldwide towards identifying the toxic agent(s) and elucidating the mechanisms of toxicity and the pathogenesis of the disease. In this regard, the WHO/CISAT Scientific Committee for the Toxic Oil Syndrome has played a major role in defining an adequate research strategy and in coordinating efforts on various fronts. All of the steps taken in fields such as etiological research, mechanisms of production of toxic chemical compounds and their identification, aspects related to individual susceptibility and implication of the immune system in the pathogenesis, constitute a sound basis on which to move forward in an attempt to accomplish the final objectives in TOS research.

In parallel, all aspects related to clinical research and follow-up of the TOS-affected cohort have always been one of the main objectives that have been diligently pursued, bearing in mind that this was a unique disease in which autoimmune phenomena seem to have played an important role.

As illustrated in the previous chapters of this book, substantial progress has been made to date in TOS research, especially in the fields of epidemiology, clinical outcome, immunology and chemistry, both of the oil matrix and of the chemical changes arising from the refining process. Nevertheless, there are still some unknown aspects of the intoxication that will not be completely understood until a suitable animal model is found that will permit a full explanation of the pathogenesis and the identification of the toxic agent(s).

This book summarizes most of the latest achievements reached in TOS research. Initially, this research was divided rather artificially into traditional fields such as epidemiology, clinical research, chemistry, toxicology and pathogenesis. With time, however, the WHO/CISAT Committee has led the way to an integration of all the available data into a common body of knowledge around this unique event, adding in the process novel aspects related to ethical topics. Thus, considering all we know for certain at this time, are we in a position to define the priority fields for future research? The answer is yes, we are. Our accumulated unique and profitable experience over the past 10 years shows that, besides concentrating on those areas where the most important questions remain to be answered, we need an in-depth and systematic monitoring of all possible subjects of interest in TOS, focusing at the same time on the proper convergence of different types of expertise within these areas. Some of them have been the object of much work since the beginning of the syndrome (epidemiology, clinical aspects, chemistry, in vivo and in vitro toxicology and immunology), others (immunology, toxicology and pathogenesis) are being revisited in the light of the latest results obtained, while new ones (lipid absorption, distribution and metabolism) need to be considered. Thus, with all available knowledge in mind we can take a forward look at short- and medium-term strategies, as summarized below.

Epidemiology

This has been the field in which all the important findings have been made, which in turn have opened the door to progress in other areas. Continued research in this field has shown that the newly identified compounds derived from 3-(*N*-phenylamino)-1,2-propanediol (PAP) could be better markers of TOS than the fatty acid anilides, since patients who had consumed oils with a high PAP content were at higher relative risk. Nevertheless, the role of epidemiological research in the future should focus on studying both the pathogenesis and toxicology of TOS in collaboration with experts in these areas and on new research compatible with current knowledge of the disease.

The biological and DNA sample repositories are a valuable development, not only in terms of clarifying the pathogenesis but also for specific clinical studies. Current data appear to indicate that TOS-affected people manifest signs and symptoms that are due to an interaction between toxic and individual genetic factors. This line of approach could be useful in understanding some aspects of the disease that have been impossible to determine up to now.

Clinical aspects

Clinical research is playing a major role at the present time. Psychological care, self-perceived health status and the search for health outcomes in susceptible subgroups of the cohort are some of the issues to be addressed in the near future.

The great challenge in the future will be to describe the natural history of the disease, including long-term mortality studies and the possible appearance of late outcomes in the whole cohort. Thus epidemiology has an important role to play in long-term follow-up studies.

Chemistry

Data on new families of chemical compound based on PAP ester derivatives, specific factors in the refining of the oil that influenced the production of these compounds (high temperatures and vacuum conditions) and the production/conversion kinetics of these substances cover a wide and complex field. Nevertheless, detailed chemical characterization of foreign components has permitted the performance of studies using both "simulated" oils (obtained through proper refining procedures) and/or "reconstituted" oils (oils supplemented with known amounts of compounds previously identified in case and simulated oils). Further progress in the full chemical characterization of toxic oils will be helped by the development of new analytical procedures, such as the determination and unequivocal identification of new aniline compounds at very low levels of concentration.

Toxicology

The latest studies performed on animal models, following standard intoxication guidelines and using refined oils tailored to the purpose, have produced different results. Nevertheless, although scarce, some results are promising. In particular, the fact that some strains of mice have shown eosinophilia in response to some oils has opened up new possibilities for research.

The definition of the toxicological pathway is one of the greatest challenges in the identification of the agent. The finding of an animal model would open the door to many basic research studies related to TOS and also to other autoimmune conditions of toxic origin. Systematic tracking of specimens and strains following standardized protocols will be carried out, in order to study oil toxicity combined with specific experimental studies from pathogenic hypotheses.

One of the possibilities is that TOS may have been caused by various toxic compounds. It has been discovered that many of the aniline derivatives found in the oil do not show the same kinetic behaviour in animals or act similarly in vitro. Thus we should not discount the possibility that several compounds, acting at the same time in different systems, may have caused this systemic disease.

Immunology

The possible involvement of an autoimmune mechanism was under consideration from the beginning of the epidemic, but interest in more extensive

immunotoxicological work has recently been strengthened by direct studies of immunological parameters in TOS patients. These studies show activation of immune mechanisms, demonstrated by a predominant Th2 response in lung tissues and a higher frequency of HLA-DR2 in those patients with more severe disease. Some of these observations leave the door open for research on other immunological biomarkers. In addressing the issue of a possible immune etiology of TOS, work may be carried out along the following lines.

Animal model

- Susceptible mice may be immunized with an appropriate antigen prepared from one or more of the aniline derivatives associated with TOS. Animals should be primed towards an immune response by sensitizing them with a suitable adjuvant and then dosing them with reconstituted oil.
- The intrinsic adjuvant effect of toxic oil may be tested when administered together with an antigen, such as ovalbumin.
- Work may be carried out on a model of a spontaneously developing human disease, such as scleroderma, in tight-skinned mice.
- Human HLA transgenic mice may be used to demonstrate genetic restriction.

Human studies

- Immunohistochemical studies may be carried out on TOS tissues with special interest for immune and inflammatory cells.
- Genetic studies should continue on candidate genes implicated in TOS

Lipid metabolism

Since the TOS syndrome was related to the presence of abnormal lipid-like molecules (anilides and esters of PAP), it seems logical to investigate in detail how such molecules could interfere with the absorption, metabolism and/or distribution of components of normal lipid metabolic pathways in humans. In this connection, the search for biochemical markers of lipid metabolism disruption at different stages should be pursued.

Pathogenesis

It is clear that pathogenic research would be strengthened by the development of an animal model to test the various hypotheses. In its absence, analysis of the expression of certain genes, the use of in vitro studies on human cells, and the search for a protein expression pattern are the best ways forward. Greater efforts should be made to find determinant susceptibility factors, in order to identify the most suitable animal model for this disease.

There is still a long way to go, and only a comprehensive and systematic approach will yield optimum final results.

Annex 1

The etiology of the Spanish toxic syndrome: interpretation of the epidemiological evidence[1]

Sir Richard Doll

The disease that caused some 20 000 people to be ill in central and north-western Spain in the summer of 1981 had not previously been known to medical science. Even in retrospect, it has not been possible to identify any similar outbreak before 1981 and no similar cases have been detected since, either in Spain or elsewhere. Research into the cause of the disease has led many people to conclude that there was a toxic substance in some batches of oil that were sold for human consumption by street vendors. Laboratory studies have, however, failed to demonstrate toxicity in any of the samples that were recovered, no specific chemical that might have caused the disease has been identified, and the conclusion that the oil was responsible rests primarily on the epidemiological evidence. The purpose of this report is, therefore, to review the epidemiological evidence to see whether the conclusion is justified or whether the possibility of some other cause needs to be considered. In presenting the report, I have assumed that the clinical, pathological and

[1] First issued by the Regional Office in October 1985 as document SPA/CEH 502, and including the Addendum of June 1987.

toxicological features of the disease are not open to question, and have referred to them briefly only in so far as they help to interpret the epidemiological data.

In preparing the report, I have read in whole or in part the papers referred to in the list of references and in Appendix 1. Many of these are unpublished and were provided by the Spanish Ministry of Health, the World Health Organization (WHO) Regional Office for Europe, the Plan Nacional para el Síndrome Tóxico in Madrid, and by individual scientists in response to personal requests. I have also visited Madrid and had the opportunity of discussion with Dr M.J. Clavera Ortiz and Dr J. Martinez Ruiz, who were known to be critical of the conclusion that the toxic syndrome was attributable to the consumption of toxic oil.

In summarizing the facts as I believe them to have occurred, I have not given references if the facts can be found in the report of the WHO Working Group on the Toxic Oil Syndrome, which met in Madrid in March 1983 (*1*). For others I have given a reference. Interpretation of the meaning of the facts (for example, the last paragraph of the following section on Clinical and pathological features) is, however, purely personal.

Clinical and pathological features

Most of the affected subjects presented with an acute episode of fever, cough and dyspnoea, which was often accompanied by myalgia, skin rashes and changes in the chest X-ray suggesting noncardiogenic pulmonary oedema. Eosinophilia was present in over 90% of patients by the third week and sometimes persisted for many months. Most patients recovered spontaneously within a few weeks. Myalgia, however, frequently persisted for many months and in some 15–20% of cases progressed into a chronic phase that was characterized by symptoms and signs in many systems, including peripheral neuropathy, sclerodermatous changes and severe salivary and lachrymal hyposecretion. A few patients presented only in the chronic phase, particularly in the later stages of the epidemic, and this complicates the analysis of the descending limb of the epidemic curve.

Some 2% of patients altogether and 3.5% of those admitted to hospital had died from the disease by 30 October 1982. At autopsy, the lungs in the acute stage showed diffuse septal oedema and changes predominantly affecting the capillary endothelial cells with minimal evidence of inflammation; many other organs showed a non-necrotizing vasculitis. In the chronic stage, the most prominent feature was vasculitis of the small arteries with widespread fibrosis and atrophy of affected organs.

No evidence of any specific infection was found in life or at autopsy and immunological changes were few, apart from an early transient increase in serum IgE.

These clinical and laboratory features exclude the possibility of a psychological origin and of a helminth infection, and are reminiscent of some cases of periarteritis. The eosinophilia, which had suggested a helminth infection, weighs strongly against an infection of any other sort and suggests a toxic origin.

Epidemiological evidence

Characteristics of the outbreak

The epidemic was first recognized early in May 1981, but cases are known to have occurred from early in April, if not sooner. From early May, the increase in incidence was explosive and the peak of the epidemic occurred early in June.

The decline began about a week before there was any public suspicion that the disease might be due to the consumption of toxic oil, and the disassociation between the beginning of the decline and the beginning of public awareness that oil might be responsible for the disease is made more marked if an induction period of a few days to a week is required before the appearance of symptoms. Many of the new cases reported during the decline of the epidemic presented in transitional or chronic stages of the disease, and it is not possible to tell from reported data how rapidly exposure to the causal agent diminished.

Geographically, the epidemic was almost confined to 14 provinces in central and north-west Spain. No cases occurred outside Spain, but a few (fewer than 200) occurred in other provinces. Within the affected region, the epidemic spread progressively north-west from Madrid to León.

Other features of the epidemic include:

- the occurrence of clusters of cases close together in time within families;
- the occasional recurrence of symptoms in patients who returned to their homes after discharge from hospital in the early stages of the epidemic;
- the absence of secondary cases outside family clusters;
- a slightly higher rate in women than in men;
- a fairly uniform distribution by age, except that no cases occurred in children under six months;
- an absence of clusters associated with institutions that are characteristically affected in epidemics of infectious diseases (e.g. schools, military camps), although a few outbreaks did occur in convents; and
- a concentration of cases in the industrial suburbs of Madrid, with a tendency to avoid both the most, and the least, wealthy areas.

Many of these features are consistent with either an infectious or a toxic etiology; some, however, weigh against infection, particularly the absence of

secondary cases, the rarity of cases in infancy despite a high incidence in women of reproductive age, and the social distribution. Recurrence on returning home during the early stages of the epidemic also tends to favour a toxic origin from exposure at home.

The experience of Legionnaire's disease, which does not give rise to secondary cases and which was initially thought to be toxic in origin when an epidemic was first recognized in the United States, must, however, make one hesitate to exclude infection solely on these grounds.

Case-control studies

Evidence of association

The idea that the consumption of a particular type of oil was the responsible factor arose from the results of enquiries about the background to the admission of paediatric cases to the Niño Jesús Hospital in Madrid. Around the middle of May, Dr Tabuenca Oliver (*2*) became convinced that the disease was toxic in origin and he sought the collaboration of the Institute of Hygiene and Safety at Work to look for heavy metals in biological specimens from affected children. By 1 June, he had come to believe that the disease was due to food poisoning, and in the first days of June, a questionnaire was administered to the parents of 62 affected children and 62 children with other conditions about the food that these children had consumed shortly before admission. The results showed a striking difference in the proportions who had consumed daily oil that was marketed and sold as olive oil in 5-litre plastic containers bearing no trademark or seal (100% against 6.4%) (*3*). This led to a public announcement on 10 June that oil of this type was responsible for the outbreak and to an offer, on 26 June, to exchange all similar oil for pure olive oil at government expense. It is clear, however, that the oil to which cases of the disease have subsequently been linked has not always had precisely the characteristics described by Tabuenca Oliver (some samples, for example, were sold in other containers with brand names) and the term "street oil", which will be used in the rest of this report, will imply only oil that was sold by street vendors in street markets, or in small shops that had bought the oil from street sources.

All the other case-control studies that were carried out were conducted after 10 June, when the concept that street oil was responsible for the disease was imprinted on the public mind, and this has to be borne in mind when assessing the results. Most, however, were conducted before the offer was made to exchange the oil (Table A1.1).

Unfortunately, the first study has not been described in detail anywhere and some of its features are described differently in different accounts. It is therefore difficult to assess its validity. A brief summary of the principal account, including some of the conflicting information, is given in Appendix 2.

It is clear, however, that detailed questions were asked about a wide variety of foods, that the replies recorded were in general appropriate, that the results were internally consistent in that the control children who consumed very little street oil consumed more oil of other types, and that few other differences of possible importance were observed (*3*).

The results of 12 studies undertaken to check the conclusion of the first are summarized, together with those of the Niño Jesús Hospital study, in Table A1.1. The results have been extracted from the original reports, whenever possible, but for four sets not included in the 1984 WHO report (*1*) (d, e, f, and g) I have had available only Kilbourne's (1985) report to the WHO Steering Committee (*12*). Different figures have been given in some instances elsewhere, usually because of the choice of different controls for comparison, but occasionally for reasons that are unclear. Table A1.1 includes data from all control series, combining, when available, the separate data for controls selected at random and those matched for selected characteristics.

All 12 studies confirm that street oil had been consumed in nearly every instance by the affected individuals or families. In each instance, the proportion that had consumed street oil was higher for the affected families or individuals than for the controls. In 11 studies the difference was statistically significant and in 7 it was highly significant ($P<0.001$). In sum, the proportion that had consumed street oil was 94% (232/248), while the proportion in the controls (weighted by the number of affected families or individuals in each study) was 40%, suggesting that the risk with a positive history relative to that with a negative history was increased approximately 25 times and unlikely to be increased less than about 17 times (95% lower confidence limit).

In one study (b) detailed enquiries were made about the source of the oil, and the association was found to be much stronger when oil was considered that had been purchased from particular vendors. Of 32 affected families who had bought street oil, 24 had purchased it from a vendor who was identified by his appearance, vehicle and street cry, while only 5 out of 23 similar control families had done so. These figures give a relative risk for oil purchased from this vendor compared to that for oils purchased from other vendors of 10.8.

Similar associations with particular vendors were found in studies f (5 out of 8 who had purchased from street vendors against 17 out of 72) and h (9 out of 19 who had purchased from street vendors against 3 out of 15). The original description of the results of study h is, however, unclear and the results have been presented differently in different reports (*13*).

Reality or artefact

To interpret these results we have first to decide whether the association that has been observed with the consumption of street oil was real or whether it was an artefact of the method of enquiry. In principle, the results could have been

Table A1.1. Case-control studies

Study (reference)	Location	Date	Study unit	Selection of subjects		Consumption of "street oil"		
				Cases	Controls	Cases	Controls	*P* value
a (*3*)	Madrid	(?)1–8 June	Individual	See Appendix 2	See Appendix 2	62/62 (100%)	4/62 (6%)	<0.001
b (*4*)	Navas del Marqués	11 June	Family	27/30 affected families in town	108 families: 54 selected randomly 54 randomly after matching for size	27/27 (100%)	30/108 (28%)	<0.001
c [a]	Pozuelo de Alarcón	17–18 June	Family	Families of patients from Pozuelo district admitted to Clinica Puerta de Hierro	Neighbourhood families approached in defined order	42/48 (88%)	32/96 (33%)	<0.001
d	Madrid	–	Individual	–	–	7/7 (100%)	28/84 (33%)	<0.001
e	Madrid	–	Individual	–	–	9/9 (100%)	34/104 (33%)	<0.001
f	Madrid	–	Family	–	–	8/8 (100%)	72/204 (35%)	<0.001
g	Madrid	–	Family	–	–	52/58 (90%)	615/1725 (36%)	<0.001
h (*5*)	Chozas de Abajo	15–22 June	Family	All affected families	Randomly selected families	19/19 (100%)	15/19 (79%)	>0.05
i (*6*)	Cerezo de Arriba	19 June	Family	All affected families in village	Other families [b]	13/13 (100%)	25/44 (57%)	0.002

j (*7*)	San Cristobal de Entreviñas	17–25 June	Family	(?)All affected families	Two sets: selected at random and matched	10/10 (100%)	8/19 [c] (42%)	0.002
k (*8*)	Bocigas de Perales	11–17 June	Family	All affected families	All other families	11/11 (100%)	22/33 (67%)	0.03
l (*9*)	Arconada [d]	End July	Family	All affected Individuals	All unaffected families	18/18 (100%)	9/21 (57%)	<0.001
m (*10*)	Colmenar Viejo	26 June	Family	Patients from Colmenar admitted to Ramón y Cajal Centre	Neighbourhood families as (c)	16/20 (80%)	6/20 (30%)	0.002

[a] J. Andres et al., unpublished data, 1981.

[b] Unclear how chosen: must have been most available as altogether 173 persons were included in 57 families out of 266 inhabitants.

[c] Only 9 replies to this question out of 10 matched controls.

[d] Comparison is made between affected individuals and unaffected families. Different figures are given by Rigau-Pérez (*11*): 18 case families, all consumed street oil; 21 control families, 12 consumed street oil. The original WHO report (*1*) also states that all the affected individuals except one (number not stated) at the locality of Lantadilla had consumed street oil, as had the 12 people in the 5 unaffected families.

produced artificially by bias in the selection of cases or controls or, since the information was subjective, by bias in the way the interviews were conducted or recorded, or in the way the subjects responded to the enquiry.

Selection bias is effectively ruled out by the design of some of the studies, which included all (or practically all) the cases that had occurred in a particular area or had been admitted to a particular hospital (studies b, c, i, j, k, l) and by the selection of controls that consisted of all the unaffected families in an area (studies i, k, l), a random sample of them (studies b, j) or neighbouring families matched according to stringent criteria (studies b, c, j, m). As each set of studies led to results that were practically identical with most of those obtained by other means, we can conclude that selection bias cannot have been responsible for the results, unless a positive history of the consumption of street oil had been made a criterion for the diagnosis of the disease. This, however, cannot have been the case as many of the affected patients were diagnosed before 10 June, and even after 10 June the concept that street oil was the responsible factor continued to have the status of an unproved hypothesis for a considerable period.

Interviewer bias is more difficult to exclude, as blind interviewing was not done and would not have been practicable. One way of testing for the possibility of bias in interviewing affected families or affected patients is to examine separately the results obtained for patients who were interviewed in the belief that they suffered from the syndrome, but were eventually shown to have had some other condition. It is not known, however, whether any such patients were interviewed. Subject bias leading control patients to underestimate consumption can be tested for by comparing the results obtained from control histories with estimates of the frequency with which street oil was purchased that have been obtained in other ways. No objective figures for the consumption of street oil are, understandably, available, but the purchase of street oil by 30–40% of families in the affected strata of Spanish society seems not to have been unrealistically low (*14*). The one figure for the proportion of individuals who had consumed street oil that is out of line with the rest is the 6% for control children reported in the initial enquiry (*3*). This low figure is unlikely to be due to chance. It could have arisen if the hospital had drawn its patients from a relatively low consumption area; but it could reflect a lower intensity of questioning of the control patients.

The short intervals between the presumed period of exposure and the onset of symptoms (approximately one week) and before the interviews were conducted (seldom more than a few weeks) should have prevented the introduction of any major bias owing to differential recall. Respondent bias could, however, have been introduced if the purchase of street oil was regarded by controls as being discreditable or was regarded by affected patients as being advantageous. The former may have been the case to some small extent, but

seems unlikely to have affected the control histories materially. The latter is unlikely to have been influential in early June 1981, as the offer to exchange street oil for pure olive oil was not made until 26 June, while special economic aid was not provided until September.

I conclude that bias is unlikely to have influenced the results to any important extent. It follows that, as the differences in the consumption of street oil between affected families and patients on the one hand and control families and patients on the other are large, the association between exposure to street oil and the development of the disease must be largely real.

Confounding or causality

It does not, of course, necessarily follow that the consumption of street oil, which was invariably said to precede rather than to follow the onset of the disease, necessarily caused the disease. It could have caused the disease, or the purchase and consumption of oil could have been confounded with some other factor that was the direct cause, such as the consumption of other food or the use of another commercial product purchased in the same way. The distinction between these two explanations for an observed association is the central problem of many epidemiological studies. The distinction is seldom easy to make, but experience has gradually accumulated that often enables it to be made with a fair degree of confidence. Case-control studies commonly provide much of the relevant evidence; but the distinction can never be made on the results of such studies alone.

The evidence from case-control studies includes the consistency of the association in different studies, the strength of the association, the quantitative relationship between the dose of the suspected agent and the estimated risk of developing the disease, the temporal relationship between exposure and the onset of the disease, and the existence of other associations and the inter-relationships between the different factors.

Failure to find a consistent association in different studies would weigh against a causal relationship. Consistency, however, accords with both a causal relationship and confounding, and weighs in favour of cause only when it extends over different circumstances and different cultures. As all the present studies were undertaken in similar circumstances in one country, the fact of consistency contributes little apart from helping to exclude chance and bias, as discussed above.

The strength of the relationship is another matter, as cause becomes progressively more likely as the strength of the relationship increases. No specific limit can be set to the size of the relative risk that excludes confounding, but past experience suggests that confounding is seldom likely to be the explanation if the lower 95% confidence limit of the estimated relative risk is greater than 3. As, in the present case, the estimated relative risk is of the order

of 25 : 1, with a 95% lower limit greater than 17 : 1, this certainly weighs in favour of the hypothesis that the oil caused the disease.

Moreover, this estimate of the relative risk is in all probability too low, for two reasons: (*a*) because it has not taken account of the greater risk associated with purchase from specific salesmen recorded in three studies; and (*b*) because control families were sometimes chosen for comparison with affected families that were matched precisely for place of residence. It is understandable that controls were matched in this way, as the demonstration of a substantial difference between cases and controls matched for place of residence helps to reduce the possibility of confounding between the purchase of street oil and some special feature of the town or village such as the prevalence of infection. It may, however, have had the effect of grossly reducing the estimate of the relative risk if individual salesmen visited different villages and high-incidence villages were chosen for study (as in studies i and l).

A progressive increase in risk with the amount consumed would also help to support a causal relationship. Quantitative estimates of food consumption are, however, difficult to make and the difficulty is enhanced with an item of food such as oil, which is not consumed by itself but is used for many purposes in association with other foods. To obtain any worthwhile estimates, detailed attention would have to be paid to the design of the questionnaire and pilot studies would need to be conducted to test the validity of the questions. It is not surprising, therefore, that few of the studies that were carried out during the heat of the epidemic obtained any quantitative data at all. In one study (c), an attempt was made to relate the proportion of the members affected in each of 48 families to the average amount of suspect oil consumed per month by each member of the family and to various indirect measures of oil consumption, such as the amount of salads, mayonnaise, etc. or the amount of fried food. All the correlations proved to be negative (i.e. the higher the proportion of members affected, the less the amount of oil consumed), but few details are given in the paper and it is not clear what was actually done. In four other studies, estimates were made of the numbers of families or persons consuming different amounts of oil per unit of time. In three (studies h, i and j), based respectively on 32 affected and 60 unaffected members of the same families, 13 affected and 25 unaffected families all of which were exposed, and 10 affected and 20 unaffected families, no material differences of any sort were observed between the affected and the unaffected. In the fourth (b), which has been reported in the greatest detail, a comparison was made between the oil consumption of 56 patients and 58 unaffected members of affected families, and the proportion of patients was found to increase progressively from 26% (9/35) when the consumption was less than a quarter litre per week, through 53% (27/51) when it was a quarter to a half litre per week, to 71% (20/28) when it was more than a half litre per week. One month later, however (9 July),

when further enquiries were made of a subgroup of families (32 affected and 23 control) who had used street oil, no association could be found between the risk of illness and the estimated weekly consumption. In sum, the lack of a consistent dose–response relationship weighs against a causal role for the oil, but in view of the difficulties of obtaining accurate information about personal consumption, particularly in the circumstances in which the enquiries had to be made, the weight to be attached to the finding can be only small.

Individual histories have indicated a latent period of a few days to two weeks between the first consumption of street oil and the onset of symptoms, but no evidence to denote a specific temporal relationship appears to have been sought in the case-control studies. Two (c and m) enquired about purchase "after Easter", two (b and j) enquired about purchase "after 1 April", while one (h) (which did not apparently specify a date for the purchase of street oil in general) asked a subgroup about purchase from specific vendors in the last two weeks of April. The responses to these questions are compatible with a causal relationship, but do not strengthen belief in it.

In several of the studies, information was sought about other possible factors, including other items sold by travelling salesmen (b, c and m), the consumption of other foods (a, b, c, h and j), exposure to household materials and domestic animals (b, h and j) and housing and general social conditions (b). Several associations were observed with potential sources of toxic material. In the initial study at the Niño Jesús Hospital (a), associations were found with spices, processed cheese, canned fruit and canned vegetables (see Appendix 2). At Navas del Marqués (b), a greater proportion of affected families than of control families were found to have purchased a particular shampoo also commonly sold by itinerant vendors (29.6% against 1.7% in the first study and 11.0% against 0.0% in the second) and a greater proportion of affected members than of healthy members of the same families were found to have consumed salads (89% against 60%). Multivariate analysis, however, failed to show a significant association between illness in the family and the consumption of salads, even when street oil was omitted from the analysis. At Pozuelo de Alarcón (h), an association was found with the purchase of wine from street vendors (20/48 families against 22/96) but this difference, unlike that for the purchase of oil, ceased to be statistically significant when it was limited to families who had bought from street vendors since 1 April. In brief, no factor was found that was as closely or consistently associated with the syndrome as the purchase of street oil, or that differentiated as well between affected and unaffected families, and it is not possible to attribute the association with street oil to confounding with any of the many other factors that were examined.

Dr Clavera Ortiz and Dr Martínez Ruíz (personal communication) believe that the late Dr Muro had observed a strong association with the consumption

of tomatoes that had been grown in one part of the country, but data to support this hypothesis were not available for assessment.

Other epidemiological evidence

Hypotheses derived from case-control studies can be tested epidemiologically in two ways: (*a*) by seeing whether the results can be used to predict successfully the subsequent risk of developing the disease in people with different degrees of exposure to the suspect agent; and (*b*) by seeing how well the postulated relationship fits the observed incidence of the disease in place and time. In the present case, the first method is not available, as new cases of the toxic syndrome ceased to occur in 1981, and we have to depend on the second method alone. This method can, however, provide crucial evidence, particularly when we are dealing with a disease like the toxic syndrome, which is not known to have occurred in any other place or at any other time, when it is unreasonable to suggest that the disease should have had more than one cause. In these circumstances, outlying observations that do not appear to fit in with the geographical and temporal distribution of the disease may serve to destroy the hypothesis or, if they can be explained, to provide strong evidence that it is, in fact, correct. Several outlying observations of this type that have been reported are, therefore, examined in detail below.

Geographical location

One of the most striking pieces of evidence is the localization of the epidemic to one part of Spain, and the question arises how the oil sold in that part of the country during the spring and early summer of 1981 differed from that sold elsewhere. The histories obtained from affected families and from street vendors operating in the areas indicate that most of the suspect oil was distributed by three suppliers (RAELCA, Aguardo El Prado and JAP); but many other sources have also been suspected, either because the oils supplied were associated with the occurrence of individual cases of the disease or because they were found to contain unusually large amounts of anilide, indicating that some components had been imported for industrial use rather than for human consumption. Neither of the studies that were undertaken by the Special Investigation Team of the Interministerial Commission (*15*) nor that by Clavera Ortiz (*16*) succeeded in delineating a clear network of supplies that corresponded to the affected areas, and this must weigh against the idea that a few batches of oil were responsible for the production of the disease. It cannot, however, exculpate street oil altogether. The many samples that were surrendered at the end of June in exchange for pure olive oil had many different characteristics and it is impossible, at this stage, to define precisely the characteristics of those that were and those that were not associated with disease. Moreover, the conduct of legal proceedings has clouded the issue,

as it has been against the interests of both vendors and suppliers to allow themselves to be associated with the sale of any batches of oil that might be accused of being toxic. The intricacies of the trade in oil, some of which had been imported for industrial use and refined and sold improperly for human consumption, may eventually be sorted out by judicial enquiry; but at present it is possible to conclude only that, if the oil was responsible, it must have been because of the special toxicity of a relatively small number of batches that were imported, processed and widely distributed in the first half of 1981, some of which were imported by RAPSA at San Sebastían, handled by RAELCA and refined by ITH in Seville or DANESA-BAU in Madrid. The identification of a few pathways by which identified batches reached all affected families would be strong evidence that street oil was responsible. This, however, has not been achieved. The failure to identify common pathways certainly weakens the case against street oil; but, in the peculiar circumstances of the trade, it does not prove that the oil was not responsible.

Temporal distribution

Cheap brands of so-called olive oil have been sold by street vendors throughout Spain for many years and it follows that, if the epidemic was due to such oil, it must have been due to oil produced in a new way or supplied from a new source. This is compatible with the vast majority of the information obtained from affected families or individuals, as practically all of those interrogated had bought and consumed new supplies of oil shortly before the symptoms of illness appeared. Specific examples include: (*a*) the 11 affected families in study k, all of which had purchased street oil during the last days of April, while the 22 unaffected families that had also purchased it had purchased the oil before the beginning of the month, and (*b*) the outbreaks in three of the four convents that are described in outline in Table A1.2.

Some of this "new" oil that was consumed by people who subsequently developed the disease can be traced back to five batches of denatured rapeseed oil that were imported as industrial oil between March and May 1981 and were subsequently refined and distributed during the epidemic in April, May and June 1981, or shortly before it began (*15,17*). The significance of this temporal association is, however, diminished by the inability to demonstrate that the distribution of the batches related specifically to the geographical area in which the disease occurred.

What was initially thought to be stronger evidence was the decline of the epidemic following the announcement that the disease was due to adulterated oil and the subsequent exchange of samples for pure olive oil at government expense. In fact, however, the decline in incidence had begun a week or more before the announcement was made (*16*), when the idea that street oil might be responsible for the disease was limited to a small group of research workers.

Table A1.2. Consumption of suspect "olive oil" and occurrence of symptoms in four convents

Convent	Population	Date	Purchase of oil	Use of oil	Occurrence of illness
1		Mid-February	100 litres from a street vendor mixed with 20 litres previously purchased		
	23 nuns	February to beginning of May		Dressing for salads and vegetables	Unspecified number complained of general weakness, several of slight fever and chest pain, treated symptomatically
	23 nuns	Early May to end June		Used for cooking as well because "more nutritive". All but 12 litres used	
	23 nuns	End May			Typical cases of toxic syndrome. 20 affected, 8 of whom developed chronic symptoms with one death
	Chaplain	February to end May		1 meal a day	Unaffected
	Relative of Superior	February to end May		Ate very little oil, because on special diet for medical reasons	Unaffected
2	35 nuns	Early May	10 litres JAP oil from local shop		
	26 nuns and chaplain in retreat	15–25 May		5 litres consumed mainly for salads	13 nuns developed mild symptoms: cough, dyspnoea, myalgia; 6 progressed to chronic phase
	9 nuns	15–25 May		Nil	Unaffected
	35 nuns and chaplain	25 May to end June		5 litres consumed mainly for salads	1 of additional 9 nuns developed acute symptoms progressing to chronic phase
	56 laywomen	Early May to end June		Same meals as nuns but used soybean oil for dressings	Unaffected

3	43 nuns	May	20 litres JAP oil from same shop as used by Convent 2		
	42 nuns	Mid-May to end June		18 litres consumed as dressings for salads and vegetables	42 developed dyspnoea, myalgia; 9 progressed to chronic phase
	1 nun	Absent for 16 days of period			Unaffected
	70 laywomen	Mid-May to end June		Same meals as nuns, but used soybean oil for dressings	Unaffected
4		End April	Gift of 20 litres oil bought from street vendor		
	13 nuns	Early May to 31 May		Oil used for all purposes (salads, cooking, etc.). Taste poor, mixed with other oils. 2.5. litres to 1 litre soybean and 1 litre sunflower seed oil. 12.5 litres original oil consumed [a]	3 developed acute symptoms diagnosed as toxic syndrome
	Physician, gardener, chaplain	Early May to 31 May	Visited convent. No meals	Unaffected	

[a] Use of oil ceased because physician suspected a possible connection between illness and the ingestion of oil.

It must therefore be concluded that the temporal distribution of the epidemic adds little or nothing to the weight of evidence in favour of the hypothesis. It is, however, generally compatible with the hypothesis, with the possible exception of the one outlying observation described below.

Outlying cases

According to the WHO report (*1*) fewer than 200 cases have been registered as occurring in people who lived outside the affected region, the great majority of whom have been found to have had meals in the affected region before the onset of their illness (*14*). Several such cases were referred to in the report of the WHO Regional Office for Europe (*1*) and some of them have been described in detail by Posada et al. (*18*).

According to the latter authors, the toxic syndrome is recorded in the Government's census of affected persons as having occurred in four families in Seville, which is approximately 300 km away from the affected region. Representatives of each family were interviewed personally. Two families had visited the epidemic area at the time of the epidemic, when they had consumed oil of the suspect type. Two families had not.

One of the latter families consisted of a man, his wife and daughter, and a niece who was living with them temporarily. Food was bought from local stores, not from travelling salesmen, and all food products purchased had brand names that implied sanitary control. Meals were prepared by the wife and eaten at home. Food oil was acquired from the ITH oil refinery in which the man worked, his last allotment having been obtained some time in May 1981. This oil was used until the middle of June, when the family learnt through the news media that the epidemic might be due to street oil and that the refinery was thought to be implicated in its distribution. In June, both the wife and the daughter developed acute respiratory symptoms. The wife's illness was accompanied by a rash and dry mouth and was followed by temporary alopecia and weight loss, but no definitive diagnosis was made. The daughter's illness, which persisted for two weeks, was followed a month later by characteristic skin, joint and muscular lesions accompanied by marked eosinophilia (2400 cells/ml), and was diagnosed as the toxic syndrome.

The other family had five members (a man and his wife, a son, a daughter and a grandmother). Food products were again bought only from local stores and only with formal brand names. Meals were prepared by the grandmother and were eaten at home. The husband was employed as principal administrator of the same company that employed the head of the first family and he also brought oil home directly from his workplace. The last allotment was obtained in April or May, but the dates when it was first and last consumed are unknown. Only the wife became ill. In July, she developed a rash, followed a week later by a dry cough and dyspnoea on exertion associated with a small

pleural effusion. Three weeks later she developed skin, muscle and joint symptoms accompanied by an eosinophilia of 2280 cells/ml, and later still she developed severe neuropathy and loss of weight. The illness, which was characteristic of the toxic syndrome, has been registered as such in the official census.

The ITH refinery, for which the two heads of families worked, refined denatured rapeseed oil for RAELCA, the oil distributor with whose products the epidemic has been most strongly linked. Supplies of oil were allocated only to the two men referred to above and no cases occurred in the families of any of the other 22 employees.

The evidence provided by these four Seville families is extremely persuasive. Its importance is not diminished by the fact that several members of the two affected families of ITH staff did not become ill, despite having consumed the same oil, as attack rates appreciably less than 100% were characteristic of the epidemic. This was observed, for example, in two of the convents listed in Table A1.2 and can be attributed to individual differences in dosage and susceptibility. Its importance would be diminished if many sporadic cases had occurred outside the affected region that could not be related to the oil sold in the affected region, but this appears not to have been so. The possibility exists, however, that the history of oil consumption was taken into account in registering sporadic cases, and the weight of the evidence that they provide would be greatly increased if it were possible to show that no case had been excluded, simply because it had not been possible to link it with the suspect oil. One way in which this might be done is suggested later (Appendix 3). Meanwhile, it would be helpful if a register could be published of all the sporadic cases with an indication of how each was connected with the suspected cause.

One observation that does not easily fit the oil hypothesis is the outbreak of disease in the convent at Casarrubios del Monte (referred to as convent No.1 in Table A1.2). This differed materially from the outbreaks in the three other convents that were referred to earlier. The facts, which are discussed below, are described differently, in some minor respects, in the reports by the WHO Regional Office for Europe (*1*) and by Diaz de Rojas et al. (*19*). Where they differ I have preferred the account in the latter report, even though it dates from a later period, because the information was obtained in a special study that made use of convent records and individual clinical files, as well as the results of personal interviewing.

In convents 2, 3 and 4, the illness was confined to individuals who consumed the suspect oil (bought for two convents from a local store at an especially low price and not directly from a street vendor) and the first symptoms appeared a few days after the oil began to be used. In convent 1, the situation differed in two ways: the oil was purchased in February, well before the outbreak of the epidemic, and only very mild and nonspecific symptoms were reported for two months after the oil began to be used.

The occurrence of such mild symptoms during March and April can perhaps be attributed to the fact that the oil was initially used only for salads and vegetables, so that the amount consumed was quite small. On this basis, the development of typical signs and symptoms of the disease later in May is explicable by the fact that the amount consumed was increased early in May, when the oil began to be used also for cooking, on the grounds that it would be better for the nuns who had been unwell than the cheaper sunflower oil that had been used for cooking previously.

Whether the February purchase is consistent with the timing of the import and refining of the suspect oils is more difficult to decide. The village in which the convent was situated had a particularly close connection with the owners of the firm (RAELCA) which processed and distributed some of the suspect oil and, according to Diaz de Rojas et al. (*19*), there is no problem as the records show that "the first batch of refined and denatured rapeseed oil arrived in Spain in the first days of February 1981, and it was supplied to the convent by the importing factory by the middle of the same month". There is a problem, however, if other reports are correct. In the initial report of the WHO Working Group (*1*) the oil is said to have been purchased by the convent "in the first 10 days of February" and all records agree that the first shipment of suspect oil to RAELCA was received on 11 February (*15,16*). The discrepancy would diminish if the Spanish word *decena*, which was used in the original report and was translated in the WHO report as "10 days" is translated more correctly by the phrase "10 days or so". Even so, it is difficult to attribute symptoms to oil purchased in this period. Most of the shipment of oil received by RAELCA on 11 February is said to have been returned to the importers, as the colour and smell were bad and only 500 kg were retained. No record exists of what happened to this small batch, but it is not easy to see how it could have been refined so successfully and distributed so quickly that 100 litres of it could have been regarded as good oil and sold to the convent within a few days. And it is even more difficult to believe that toxic oil could have come from any other source at this time, when no other cases occurred in Spain until April. Repeated enquiries have failed to shake the belief of the nuns that the oil was purchased "en la primera decena de febrero" and that no further oil was purchased until July (*20*), and the only suggestion that it might have been later is contained in a statement by Sr López (*21*) under legal examination that he had sold approximately 120 litres of oil received from RAELCA to the convent three or four times between the end of 1980 and the months of February or March 1981.

There remains the evidence of the few subjects who, while resident in the affected area, developed the disease but could not be shown to have consumed any street oil. It is unreasonable to suggest that a unique disease that has never been known to occur at any other time or in any other place could have had

more than one cause. If it could be shown that even one person who developed the disease could not have had any exposure to the suspected agent (either the oil or the toxic chemical that is supposed to have been in the oil) that would provide good grounds for exculpating the oil altogether. It is possible that some similar chemical may, in other circumstances, produce similar reactions elsewhere on another occasion (presuming, that is, that a chemical caused the disease) but we should not accept more than one cause for the cases that occurred in Spain in the summer of 1981.

According to the data in Table A1.1, no evidence could be obtained that street oil had been consumed by 16 individuals or affected families out of a total of 310 (5.2%). This, however, is not the same as saying that they cannot have consumed it. Some individuals may have consumed it without their knowledge when away from home, some may not have remembered accurately what they bought or ate, and some may have had reasons for suppressing the truth.

Kilbourne (*12*), for example, reported to a meeting of the Scientific Steering Committee that representatives of two of the affected families in study f initially denied purchasing street oil, but that on re-interview a daughter in one family intervened to say that such oil had been purchased, while a neighbour reported that the second family had certainly bought the oil, but did not wish to admit it as they had resold the oil in their own shop. In both cases the positive histories were subsequently confirmed.

I have no information about the six affected families who are reported not to have purchased street oil in study g, but further information has been given about the six similar families reported in study c. Of the ten affected individuals in the six families, six may have unwittingly consumed the suspect oil at home, as oil of unknown brand had been purchased in street markets, and three may have consumed the oil elsewhere. One man remains, in whom the diagnosis has been confirmed, who states that he did not consume any oil at all. A register of affected subjects that are reported not to have consumed the oil, comparable to the register of sporadic cases outside north-west Spain referred to previously, could provide a further useful resource for research.

Toxicological evidence

None of the samples of street oil that were collected in Spain in the summer of 1981 has been shown to contain chemicals that are toxic to animals in laboratory tests, other than aniline, which was present in small amounts and produces, in larger amounts, effects in humans that are quite different from those observed in the epidemic. Nor has it been possible to produce samples that have toxic effects analagous to those observed in humans by reproducing the processes by which it is thought that oils imported as industrial oils were refined and blended to provide cheap substitutes for olive oil. This could be because street oil was not the cause of the epidemic.

Alternatively, the experiments may have failed for technical reasons. First, species differences in susceptibility may make it extremely difficult to demonstrate toxicity in laboratory animals, even when they are known to be toxic to humans. This has been true of several pharmaceutical products that have caused serious side effects in a substantial proportion of treated patients (for example, the appetite suppressant aminorex fumarate that was introduced in Switzerland in 1967 and the beta-blocker practolol that was introduced in the United Kingdom in the mid-1970s) and, more pertinently perhaps, it was true of the substance added to margarine, which is believed to have caused the outbreak of "margarine disease" in Holland in 1960 (*22*).

Second, the long time that elapsed between the peak of the epidemic and intensive laboratory testing of the oil, and the probable dilution of many noxious samples with other samples unrelated to the production of the disease, may have so reduced the concentration of the toxic element that its effects could no longer be shown.

Third, the agent may have been introduced into the toxic batches incidentally in, for example, the treatment of the oil before it was imported, so that the attempts to reproduce a toxic oil experimentally have necessarily failed.

Conclusion

Proof that a particular agent causes disease in humans can seldom be obtained conclusively, unless the disease is so mild that it is justifiable to attempt to reproduce it experimentally in humans. Occasionally it may be possible to test the idea by a properly designed experiment in prevention. More often, however, our conclusion has to be based on evidence that falls short of logical proof, and the correctness (or incorrectness) of the conclusion is demonstrated subsequently by our ability to control or to predict the future incidence of the disease. In these circumstances, we are commonly guided by the results of experiments in laboratory animals. Positive evidence that the suspected agent produces an analagous disease in animals provides strong support for the idea that it is the cause of disease in humans; but negative evidence does not necessarily rule out the idea and should not be regarded as outweighing epidemiological evidence, if the epidemiological evidence is strong.

In the present case, toxicity tests in animals have not demonstrated the existence of any hitherto unknown toxic material in the suspect oil and the question is, therefore, whether the epidemiological evidence is sufficiently strong to implicate the oil on its own. Review of this evidence provides no reason to suppose that the association observed between the development of the disease and exposure to street oil bought between April and June is due either to bias or to confounding with any other factor with which the purchase and consumption of street oil was associated. This leaves causality as the most natural explanation. The lack of evidence for an alternative explanation is not,

however, sufficient to justify concluding that the observed association reflects cause and effect.

Against the idea of causality is the fact that no clear relationship has been observed between the dose of oil consumed and the risk of developing the disease, the failure to obtain evidence of exposure for all affected subjects, the time relationships between the import of suspect oil and the purchase of refined oil and the development of typical symptoms by the nuns at the convent of Sta. Cruz de Casarrubios del Monte, and the failure to demonstrate geographical limits to the sale of specific batches of suspect oil corresponding to the region in which the epidemic occurred.

In favour of causality is the strength of the association deduced from interviews with affected and unaffected individuals and families, the generally close temporal relationship between the purchase and consumption of oil and the occurrence of disease, and the fact that so many (if not all) of the sporadic cases occurring outside the affected region were found to have been exposed to the suspect oil by peculiarities of their personal behaviour. In this respect, the four cases that occurred in Seville, about 300 km from the affected region, are particularly notable.

In my opinion the evidence against causality is inconclusive for the following reasons:

- reliable estimates of the amount of an item of food that has been consumed, when it is not consumed by itself but is used in the preparation of other foods, are always difficult to obtain and estimates are likely to be particularly unreliable when questionnaires are designed hurriedly without an opportunity for pre-testing, as was necessarily the case in the emergency in which the Spanish case-control studies were undertaken;
- not all personal histories can be expected to be accurate and some truly positive histories are nearly always recorded as negative;
- the long latent period observed in one convent, during which only some minor malaise occurred, can be attributed to an increase in dose following prolonged exposure to an unusually low dose, while confusion in the reported dates can be postulated to explain the apparent incompatibility between the time the suspect oil was first imported and its purchase by the convent; and
- the failure to define geographical limits to the distribution of toxic batches of oil corresponding to the distribution of the disease can be attributed to the confusion caused by the anxiety of distributors and vendors not to be implicated as responsible for the epidemic.

It is, in contrast, difficult to see how the evidence in favour of causality could have been produced artificially; unless, perhaps, the evidence that so many

of the sporadic cases had been exposed to the oil (such as the four cases that occurred in Seville) was due to bias against registering individuals as suffering from the toxic syndrome, if they were not known to have had any such exposure.

In the absence of laboratory evidence for a contaminant of oil that could have caused the characteristic pathology of the toxic syndrome, a conclusion has to be based on the pathological and epidemiological evidence alone. The former strongly suggests a toxic origin for the disease. The latter strongly suggests that the disease was due to the consumption of oil that was sold as olive oil but was actually made from other sources, in the course of which a toxic substance of unknown character was either introduced or formed.

There are, however, too many gaps in the evidence to allow the conclusion that oil was definitely the cause. Such a conclusion could, however, be reached, even in the absence of toxicological evidence, if some of the gaps were filled. In view of the very high relative risks estimated from the case-control studies it would, in my opinion, be a proper conclusion if it were possible to define the sources of supply that explained both the temporal and geographical distribution of the disease; or if it were possible to provide a satisfactory explanation of the events at the Casarrubios del Monte convent and to show that practically all the sporadic cases outside the affected region had had the opportunity of consuming the suspect oil and that there had been no bias in their diagnoses, produced by knowledge that the subjects had been exposed to the oil. Two steps that might help to achieve this aim would be the publication of a list of sporadic cases with a note of the extent to which they were known to be exposed, and an investigation along the lines of that described in Appendix 3.

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Appendix 2. Niño Jesús Hospital case-control study

The most detailed account of the Niño Jesús hospital study is that given by Casado-Flores et al. (*3*), in which it is stated that a dietary investigation was carried out among the parents of the children admitted to the hospital during the first days of June. The affected children were those among the first 46 affected families, totalling 62 in all, while the 62 controls were selected without any defined procedure from other inpatients or surgical outpatient clinics. At least 42 items of food and drink were enquired about, and the proportions consuming them during the days prior to the children's first admission to hospital are shown in histogram form. Very few numbers are given in the text, and attention is drawn solely to the difference in the proportions consuming unbranded oil sold in 5-litre containers (100% against 6.4%) and other oils (Cases: all other types 0%. Controls: branded olive oil 22.5%; branded olive oil and sunflower oil 19.4%; sunflower oil 19.4%; oil "home-produced or straight from an oil press" 16.1%; seed oil with a registered mark bought loose 14.5%)[2] and to the similarity in the proportions consuming running mains water, fresh fruit, biscuits and pastas, various types of pulse vegetables, eggs, green vegetables, fish, sweets and cakes, yoghurt, chocolates and cocoa.

Examination of the histogram shows, however, several other differences between the groups, though all are smaller than the difference in the consumption of unbranded olive oil in 5-litre plastic containers. The largest differences were: various spices (cases 80%, controls 43%); processed cheese (54% and 0%); canned fruit (35% and 20%); canned vegetables (22% and 0%); pastas (22% and 42%); fresh cheese (20% and 42%); lamb (18% and 0%); and branded custard (17% and 56%).

Finally it is noted that the investigation continued to be carried out on successive children admitted for the same syndrome, and that the findings did not vary when 124 with the syndrome were compared with 121 without.

[2] Oil consumption is specified only for 61 out of 62 controls; yet those who used seed oil with a registered trade mark bought loose are described as "the remaining nine".

In other accounts, some of the details differ. Tabuenca Oliver (*2*), who was one of those responsible for the Niño Jesús Hospital study, lists many other questions and implies that patients were selected from (for example) those who relapsed quickly after discharge from hospital and households with a large proportion of sick members. According to him, 97% (not 100%) of patients had consumed the oil compared with 6.4% of the controls.

Rigau-Pérez (*11*), in his summarized account of the study, states that food consumption histories were obtained from approximately 30 patients with the toxic syndrome between 1 and 4 June and from additional patients between 4 and 8 June. Altogether 124 cases and 124 controls were interviewed and all the affected patients had consumed the oil.

Appendix 3. Investigation of cases of toxic syndrome in Seville

The evidence relating the occurrence of sporadic cases of toxic syndrome outside the main affected region carries great weight in helping to decide whether the consumption of oil from certain suspect sources caused the disease. It does so, however, only if it can be demonstrated that the diagnosis of such cases was not biased by knowledge of the history of exposure. One objective sign of the disease was the occurrence of substantial eosinophilia (2000 or more eosinophils per ml). The possibility of the existence of such bias could be investigated by examining the records of the pathological departments of all Seville hospitals and listing all the patients who were found to have such blood counts between (say) April and August 1981. An independent medical review of the final diagnosis of the conditions from which the subjects were suffering, and a comparison of the diagnoses after review with those in the register of cases of the toxic syndrome and of the histories given by any patients included in one list, but not in both, would indicate whether the possibility of diagnostic bias existed.

The etiology of the Spanish toxic syndrome: interpretation of the epidemiological evidence

Addendum, 8 June 1987

New epidemiological evidence

In the 20 months that have passed since the submission of my report on the Spanish toxic syndrome, further epidemiological evidence has become available: namely, that provided by four papers reported to the Liaison Group of the WHO Steering Committee, which met in Madrid on 27 and 28 January 1987.

Orcasur case-control study

The first paper gives the detailed results of a case-control study that was carried out in Orcasur, a working class neighbourhood in south Madrid, from 29 October to 6 November 1981 (*1*). The preliminary results had been reported previously in outline by Dr Kilbourne and were included in Table A1.1 of my 1985 report (reference f), when "street oil" was recorded as having been used in all 8 of the affected households in comparison with 72 out of 204 unaffected households. In this study, 277 houses were visited systematically and information about household oil consumption was obtained from 212. No-one was available for questioning in 59 houses, residents in 4 houses bought no oil for household consumption, and residents in 2 "were apparently unwilling to provide data the interviewer considered reliable".

In the preliminary report, which had been available in the summer of 1985, eight households were recorded as having reported cases of the toxic syndrome in at least one household member, but review of the reported illnesses subsequently showed that they met a strict definition of the syndrome in only five. The sources from which oil had been obtained by these five households and by 207 households in which no definite disease was reported are shown in Table A1.3. All five had used oil from travelling salesmen, as against 71 of the 207 (34.3%) unaffected households, and all five had obtained oil from the *mercadillo* (a Saturday open-air market), as against 27 of the unaffected households (13%). The three households that had previously been classed as

having affected members on the basis of inclusion in the official census of cases, but whose diseases have not met the strict diagnostic criteria now used, are classed as unaffected in Table A1.3. In all three, oil had been purchased from travelling salesmen, but it had been purchased from the *mercadillo* in only one.

Late cases

The second paper is a preliminary report of cases that occurred several months after the epidemic had ended. The authors (*2*), who had worked for the Plan Nacional para el Síndrome Tóxico and who subsequently reviewed data collected during the legal investigation of the epidemic, came to learn of a family in which cases occurred approximately 7 months after the epidemic began, and a single case in a man whose symptoms began about 12 months after it began.

The present paper is limited to the description of the latter case. The man complained of "diffuse myalgia, cramps, decreased motility of the elbows and weight loss" of increasing severity from June 1982, which led to hospital admission on 22 October when he was found to have an eosinophil count of 16 200 cells/ml and an IgE of 202 i.u. Typical signs of the chronic toxic syndrome were recorded, with muscular atrophy, contractures of the upper extremities and scleroderma, and a skin biopsy showed increased collagen deposition and perivascular infiltration by mononuclear cells and eosinophils. He improved gradually on treatment with corticosteroids and was discharged

Table A1.3. Sources of oil in affected and unaffected households

Sources of oil	Number of:			
	affected households (5)		unaffected households (207)	
Grocery store or supermarket	4		164	
Molino [a]	1		31	
Almacenes or *a granel* [a]	0		15	
Travelling salesmen				
Mercadillo [a]	5	5	27	71
Door to door	1		45	
Outside Orcasur	0		3	

[a] *Molino*, a small oil-processing facility associated with an olive farm; *almacenes*, an establishment selling wholesale; *a granel*, obtained from bulk supply in own container; *mercadillo*, Saturday open-air market.

Source: Cañas & Kilbourne (*1*).

on 23 January 1983. During the previous summer he had travelled constantly and, when in Madrid, had normally eaten in restaurants in the centre of the city. In April 1982 his mother was taken ill, and died on 5 October 1982 after being diagnosed as having cirrhosis of the liver. From April to July 1982 the man lived in his mother's house, using oil from a container that is said to have been typical of the sort associated with the outbreak except that the container was labelled. There is reason to think that his mother had used very little oil from this container, having previously consumed pure olive oil that had been provided by her daughters. The oil, unfortunately, is not available for examination as it is being retained for use in a case for damages, which the man is trying to obtain from the oil merchant who supplied it.

Peripheral cases

The third paper provides a preliminary account of the results of detailed investigation of the cases recorded as occurring outside the 14 provinces that were principally affected (*3*), which, it had been suggested, might help to fill the gaps in the epidemiological evidence. All such cases have been included that are referred to in the official census of cases, in the list of cases maintained by the Ministry of Justice, and in the list of cases reported from hospitals outside the area that was compiled by the Ministry of Health in 1981.

Cases have been included only if they met the following criteria: for acute cases, the presence of a "typical radiographic pattern" (presumably in a chest X-ray) with an eosinophilia of 500 cells/ml or more or the finding of non-cardiogenic pulmonary oedema and vascular endothelial damage at autopsy; for chronic cases, one of the above accompanied by neuropathy, scleroderma, pulmonary hypertension, substantial weight loss or sicca syndrome.

Altogether, 268 cases that meet these criteria have been traced and 241 have been reviewed and classified in one of the following four categories.

1.	Became ill outside the epidemic area and exposed only to oil obtained outside the area	41
2.	Became ill outside the epidemic area but exposed to oil obtained from inside the area	39
3.	Became ill inside the epidemic area and exposed to oil obtained from inside the area, but moved outside the area before the diagnosis was made	158
4.	Incorrectly included in the list of cases because of a coding error	3

A further 3 cases have proved impossible to classify and 24 cases are awaiting review. Twenty-three of the latter have province codes in the official census (implying that the illness began in the epidemic area) and are likely to be classed in category 3.

The clinical records of 29 of those assigned to category 1 have been obtained. These show that 10 satisfy the clinical definition of a case but that 19 do not. Records of the remaining 12 are still being sought.

Of the ten confirmed peripheral cases, five occurred in Vizcaya, which is located on the north border of the epidemic area, three occurred in Seville, one occurred in Badajoz and one in Alicante. The three that occurred in Seville have been reported previously (*4*, see also original report above) and have all been associated with the consumption of suspect oil that was refined in the town. The remaining two are still being investigated, but as yet no links with the suspect oil have been obtained.

Characteristics of oils related to the toxic syndrome

The fourth paper (*5*) provides a detailed account of a new attempt to see if it is possible to associate the occurrence of the disease with a particular type of oil in an objective and unbiased way. Samples of oil were obtained from two warehouses in which oils were stored that had been obtained from households in two contiguous towns in Madrid province (Alcorcón and Leganés) during the Spanish Government's oil exchange programme in June and July 1981. Contact was made with the families concerned and the clinical records were reviewed of all the cases that were reported to have occurred. Of the 195 specimens originally selected, 14 were excluded because they were duplicates or because the family from which the oil came could not be located. Eighty-eight of the remainder were rejected because the oil did not come from a typical container or because there was doubt about its association (or lack of association) with a typical case of the disease, and the remaining 93 (29 from affected and 64 from unaffected families) were coded and sent to the laboratories of the Centers for Disease Control in Atlanta, United States, where they were analysed in ignorance of the class from which they had come. The results are striking. Oils from affected families were characterized to some extent by differences in the content of at least four fatty acids and two sterols, but notably by the presence and the amount of aniline and three fatty acid anilides. For each of these four last chemicals, the probability that the differences could be produced by chance was less than 1 in 10 000. The results for oleic acid anilide (which was slightly, but not significantly, more closely associated with the disease than the three others) are summarized in Table A1.4, from which it is seen that the risk was increased approximately 19-fold (13/3 ÷ 11/48) when the sample contained more than 600 µg of oleic acid anilide per gram of oil compared with that when the chemical was not present.

Discussion

The new evidence is of variable quality. That provided by the Orcasur case-control study strengthens the association with the consumption of "street oil"

Table A1.4. Concentration of oleic acid anilide in oils from affected and unaffected families

Household	No. with samples containing oleic acid anilide (μg/g)					No. of households
	0	1–100	101–600	601–1200	1201 or more	
Affected	11	2	3	6	7	29
Unaffected	48	6	7	3	0	64
Total	59	8	10	9	7	93

Source: Kilbourne et al. (*5*).

in so far as it provides a complete and publicly available account of one of the 14 case-control studies that had hitherto been described only in outline, and pinpoints a particular source for the oil (namely a Saturday *mercadillo*) rather than one associated with travelling salesmen in general; but it is not qualitatively different from that previously available. The evidence from enquiring into the background of the few cases that appear to have occurred after the general epidemic was over could be of crucial importance, but that now presented is too incomplete to be of any material help.

There remains the evidence from enquiring into the background of people recorded as having developed the disease outside the affected provinces and that from a new examination of samples of the available oils. The first is still incomplete, but the results already obtained were enlightening. The fact that 95.6% of the cases that have been adequately investigated (219 out of 229) have been shown either to have occurred in people who had eaten in the epidemic area or not to meet the criteria needed for a positive diagnosis, and that it has been possible to demonstrate a link with the consumption of the suspect oil in 8 of the remaining 10 cases (3 definitely and 5 because they occurred in a province bordering on the epidemic area), provides strong support for the idea that the disease was due to the consumption of certain specific oils.

The second piece of evidence strengthens it even more. Not only does it confirm the existence of a general association between adulterated edible oil and the development of the disease, but it provides this in an objective manner that cannot have been biased by knowledge of the presence of disease. It also greatly strengthens the epidemiological evidence by providing clear evidence of a dose–response relationship, which had been lacking from the previous case-control studies, and, moreover, evidence of such a gross risk with high concentrations of anilide (nearly 20 times that observed in the absence of anilide) that the association is extremely unlikely to be due to confounding between the use of adulterated oil and some other hypothetical agent.

Conclusion

In my report, I concluded that the epidemiological evidence led most naturally to the conclusion that the consumption of oil that was sold as olive oil, but was actually made from other sources, was responsible for the disease, and that the evidence against causality was inconclusive. I added, however, that there were too many gaps in the evidence in favour of causality to allow the conclusion that oil was definitely the cause.

The new evidence has filled some of the gaps. First, it has provided evidence that the number of exceptional cases outside the affected area is extremely small and that, in a high proportion of the few that did occur, some special exposure to adulterated oil either did exist or can be presumed to have existed. Second, it has provided objective and unbiased evidence of a dose–response relationship between the risk of developing the disease and the concentration of certain chemicals (anilides) in oil that are not found in any natural oil. Moreover, the increase in risk with high concentrations is so great that it is most unlikely to be an artefact due to association with any factor extrinsic to the oil.

With the addition of this new evidence, I conclude that adulterated oil was the cause of the toxic syndrome.

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Annex 2

Guidelines for the use of aniline derivatives related to toxic oil syndrome in biological and toxicological assays

Jordi Gibergans, Anna Morató and Angel Messeguer

Aniline derivatives related to TOS, that is fatty acid anilides and PAP derivatives, are highly lipophilic compounds. Consequently, their use in biological and toxicological assays could be troublesome owing to their poor aqueous buffer solubility. This has caused numerous problems for the research groups involved in these investigations, and we have frequently been asked about the handling of these substances in aqueous media and how to improve their solubility.

For these reasons, the WHO/CISAT Scientific Committee for the Toxic Oil Syndrome suggested the preparation of general guidelines for the manipulation of these aniline derivatives. This study comprised the determination of the solubility of selected aniline derivatives in organic solvents, in different aqueous buffers, and in aqueous buffer containing minimum amounts of more strictly selected organic solvents (those usually compatible with cell culture assays, i.e. dimethyl sulfoxide and ethanol). In the case of negative results,

a strategy involving the preparation of liposomes encapsulating the aniline derivatives was also contemplated.

Solubility studies

The aniline derivatives selected for the study were oleic acid anilide, PAP, LPAP (mixture of regioisomers L(1)PAP and L(2)PAP) and LLPAP. These compounds, in addition to being among the most frequently used and assayed, cover a wide spectrum of solubility of all the aniline derivatives synthesized so far within the TOS research projects.

The organic solvents selected were olive oil, hexane, chloroform, acetone, dimethyl sulfoxide (DMSO) and ethanol. The solubility of the different aniline derivatives in 10 mM solution in these solvents was studied at 23 °C (Table A2.1). The aqueous media assayed were water, phosphate buffer (0.1 M, pH 7.4), phosphate buffer (0.1 M, pH 6.5), TRIS-HCl buffer (0.1 M, pH 7.4) and TRIS-HCl buffer (0.1 M, pH 8.0). The solubility of the different aniline derivatives in 1 mM solution in these buffered media was studied at 23 °C (Table A2.2). Finally, the solubility of test compounds in 10–100 μM concentration in aqueous media containing 0.5% or 1% (v/v) ethanol or DMSO at 23 °C was also determined (Table A2.3). In all these cases, the solubility of compounds was assessed by using a magnifying glass, which permitted the observation of micelles or vesicles in those cases where they were formed.

As shown in Table A2.1, all aniline derivatives tested are soluble in the organic solvents assayed at 10 mM concentration. Only in the case of oleic acid anilide and PAP is sonication recommended to achieve a homogeneous solution.

As shown in Table A2.2, only PAP is soluble at 1 mM in the different buffer solutions assayed. The rest of the compounds are insoluble.

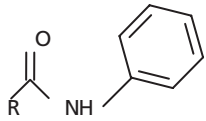
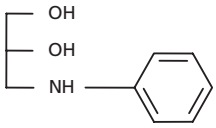
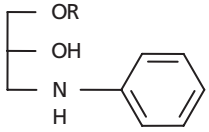
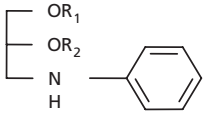
As shown in Table A2.3, addition of 0.5–1% (v/v) ethanol or DMSO to the above buffer solutions did not significantly improve the solubility of the different aniline derivatives. Thus, oleic acid anilide remained essentially insoluble, as did LLPAP. In the case of LPAP, homogeneous solutions were obtained only with solutions containing 1% DMSO at the lowest concentration, that is 10 μM.

In summary, at 23 °C:

- all compounds are soluble in organic solvents at 10 mM;
- PAP is soluble in buffered aqueous media (1 mM);
- LPAP is only soluble at 10 μM in solutions containing 1% DMSO; and
- oleic acid anilide and LLPAP are insoluble in buffered aqueous media.

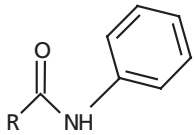
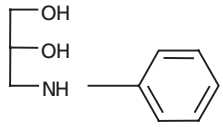
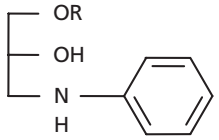
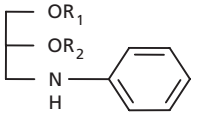
Thus, for the in vivo administration, olive oil can be a suitable vehicle for intraperitoneal dosages. On the other hand, these results indicate that the manipulation of fatty acid anilides or diesters of PAP (and in a significant extension monoesters as well) for in vitro assays should be carried out using an alternative administration procedure.

Table A2.1. Solubility of different aniline derivatives (10 mM) in organic solvents at 23 °C

Substance	Solvent	Solubility[a]
OLEIC ACID ANILIDE  R = oleic acid	Olive oil	✓✷
	Hexane	✓
	Chloroform	✓
	Acetone	✓
	DMSO	✓
	Ethanol	✓
PAP 	Olive oil	✓✷
	Hexane	✖
	Chloroform	✓
	Acetone	✓
	DMSO	✓
	Ethanol	✓
LPAP  R = acyl residue from linoleic acid	Olive oil	✓
	Hexane	✓
	Chloroform	✓
	Acetone	✓
	DMSO	✓
	Ethanol	✓
LLP AP  $R_1 = R_2$ = acyl residue from linoleic acid	Olive oil	✓
	Hexane	✓
	Chloroform	✓
	Acetone	✓
	DMSO	✓
	Ethanol	✓

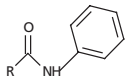
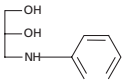
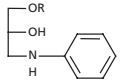
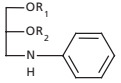
[a] ✓ = soluble; ✖ = insoluble; ✷ = sonication needed.

Table A2.2. Solubility of different aniline derivatives (1 mM) in buffer solutions at 23 °C

Substance	Aqueous solution	Solubility[a]
OLEIC ACID ANILIDE  R = oleic acid	Water	—
	Phosphate buffer, 0.1 M, pH 7.4	✖
	Phosphate buffer, 0.1 M, pH 6.5	✖
	TRIS-HCl buffer, 0.1 M, pH 7.4	✖
	TRIS-HCl buffer, 0.1 M, pH 8	✖
PAP 	Water	—
	Phosphate buffer, 0.1 M, pH 7.4	✓
	Phosphate buffer, 0.1 M, pH 6.5	✓
	TRIS-HCl buffer, 0.1 M, pH 7.4	✓
	TRIS-HCl buffer, 0.1 M, pH 8	✓
LPAP  R = acyl residue from linoleic acid	Water	—
	Phosphate buffer, 0.1 M, pH 7.4	✖
	Phosphate buffer, 0.1 M, pH 6.5	✖
	TRIS-HCl buffer, 0.1 M, pH 7.4	✖
	TRIS-HCl buffer, 0.1 M, pH 8	✖
LLPAP  $R_1 = R_2$ = acyl residue from linoleic acid	Water	—
	Phosphate buffer, 0.1 M, pH 7.4	✖
	Phosphate buffer, 0.1 M, pH 6.5	✖
	TRIS-HCl buffer, 0.1 M, pH 7.4	✖
	TRIS-HCl buffer, 0.1 M, pH 8	✖

[a] ✓ = soluble; ✖ = insoluble; — = not assayed.

Table A2.3. Solubility of different aniline derivatives (10–100 μM) in aqueous media containing 0.5% or 1% (v/v) of ethanol or dimethylsulfoxide at 23 °C

Substance	Concentration of compounds (μM)	Phosphate buffer 0.1 M, pH 7.5				Phosphate buffer 0.1 M, pH 6.5				Water		
		Ethanol 1% (v/v)	DMSO 1% (v/v)	Ethanol 0.5% (v/v)	DMSO 0.5% (v/v)	Ethanol 1% (v/v)	DMSO 1% (v/v)	Ethanol 0.5% (v/v)	DMSO 0.5% (v/v)	0%	Ethanol 1% (v/v)	DMSO 1% (v/v)
OLEIC ACID ANILIDE	100	insoluble	slightly soluble	—[b]	—	—	—	—	—	insoluble	—	—
	50	—	—	slightly soluble	slightly soluble	—	—	—	—	—	—	—
	20	—	—	—	—	—	insoluble	—	—	insoluble	—	—
R = oleic acid	10	slightly soluble	—	—	—	slightly soluble	insoluble	—	—	insoluble	—	insoluble
PAP	100	soluble	soluble	—	—	—	—	—	—	slightly soluble	—	—
	50	—	—	soluble	soluble	—	—	—	—	—	—	—
	20	—	—	—	—	—	—	—	—	slightly soluble	—	—
	10	soluble	—	—	—	soluble	soluble	—	—	slightly soluble	—	soluble
LPAP	100	insoluble[a]	slightly soluble	—	—	—	—	—	—	slightly soluble	—	—
	50	—	—	insoluble	slightly soluble	—	—	—	—	—	—	—
	20	—	—	—	—	—	—	—	—	slightly soluble	—	—
R = acyl residue from linoleic acid	10	slightly soluble	—	—	—	slightly soluble	soluble	—	—	slightly soluble	—	soluble
LLPAP	100	insoluble[a]	insoluble	—	—	—	—	—	—	insoluble	—	—
	50	—	—	insoluble	slightly soluble	—	—	—	—	—	—	—
	20	—	—	—	—	—	—	—	—	insoluble	—	—
R_1 = R_2 = acyl residue from linoleic acid	10	insoluble	—	—	—	insoluble	insoluble	—	—	insoluble	—	slightly soluble

[a] Forms micelles or vesicles easily visible with magnifying glass.

[b] — = not assayed.

Stability of the solutions

The stability of the above solutions was monitored by HPLC under the following conditions. A Nucleosil 100-5 cyano (15 × 0.4-cm, 5-µM) column was used. The mobile phase consisted of hexane and isopropyl alcohol containing 4% water at 1 ml/minute. The elution conditions were: from minute 0 to minute 2 isocratic at 5% iPrOH; from minute 2 to minute 5 a gradient up to 8% iPrOH; from minute 5 to minute 11 a second gradient up to 20% iPrOH. The DAD detector was set at 245 nm. Under these conditions, retention times for LLPAP, L(2)PAP, L(1)PAP, PAP and oleic acid anilide were 2.01, 3.97, 4.89, 10.63 and 4.13 min, respectively (Fig. A2.1). Thus, the same HPLC column permitted the analysis of all PAP derivatives by using a common elution gradient programme. As can be seen, all four compounds appear cleanly separated. In this respect, the use of the cyano column makes it also possible to separate the small amount of PAP regioisomer that is formed during the preparation of the parent compound.

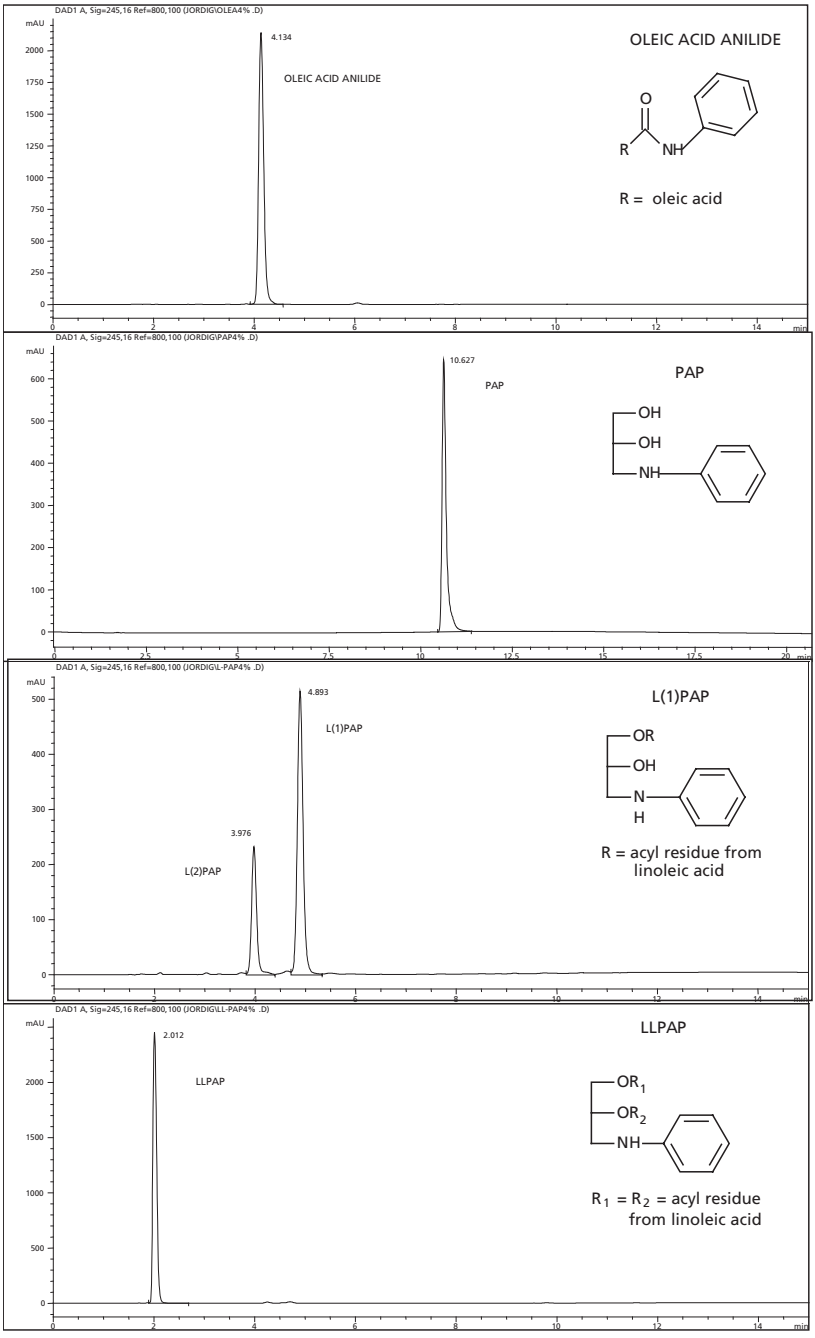
The stability of their solutions in the different solvents used was tested after 60 days at –20 °C. From the results obtained it can be concluded that these PAP derivatives and oleic acid anilide solutions are stable under the indicated storage conditions. Only minor changes in the composition of L(1)PAP and L(2)PAP mixtures were observed and these were due to the acyl migration process, which depends on the solvent used. Therefore, although the storage of pure compounds at low temperatures is always recommended, solutions of PAP derivatives or fatty acid anilides in organic solvents (preferentially hexane or halogenated solvents) are stable for several weeks if maintained at –20 °C and in the absence of light and oxygen. This recommendation is particularly important for those derivatives containing polyunsaturated fatty acyl moieties (inter alia linoleoyl and linolenyl). In our experience, linolenyl anilide and PAP acylated derivatives containing linolenyl residues are highly unstable even at low temperatures.

Preparation of liposomes

Use of liposomes is a well established procedure for increasing the bioavailability and stability of bioactive compounds. Depending on the hydrophilic/lipophilic character of the compound to be encapsulated, it will remain in the inner (aqueous) part of the liposome or be integrated into the lipid cover. In the case of aniline derivatives, the latter will be prevalent. Thus, it will be expected that either fatty acid anilides or PAP mono- and diesters will remain in the external, lipid layer of the liposome and will be released from this layer into the assay medium.

Thus, small liposomes of several compositions loaded with the substrates LPAP, LLPAP and oleic acid anilide, and containing dipalmitoylphosphatidylcholine (DPPC) and cholesterol (CHOL), were prepared as follows:

Fig. A2.1. HPLC profiles for oleic acid anilide, PAP, LPAP (mixture of regioisomers) and LLPAP



(1) DPPC : CHOL (0.6 : 0.4% molar) blank
(2) DPPC : CHOL : SUBSTRATE (0.6 : 0.35 : 0.05% molar)
(3) DPPC : CHOL : SUBSTRATE (0.6 : 0.3 : 0.1% molar)
(4) DPPC : CHOL : SUBSTRATE (0.6 : 0.25 : 0.15% molar)

The appropriate amounts of lipids were mixed in chloroform and dried by rotation under reduced pressure at 60 °C to obtain a thin film. The lipid film was maintained connected to a vacuum pump for two hours. The lipids were then hydrated with 2 ml of 10% (by weight) sterile saccharose solution. Liposomes were sized before and after a process of sonication with a 13 mm diameter ultrasound probe for 2 minutes. After sonication the liposomes were subjected to centrifugation to eliminate titanium probe residues. The liposomes were lyophilized and rehydrated with 10% (by weight) sterile saccharose solution to re-analyse their size. The initial, sonicated and final liposomes encapsulating LPAP, LLPAP or oleic acid anilide were analysed by HPLC.

In this case the HPLC analyses were performed by reverse-phase on a RP-18 LiChrospher 100, 5 µm particle size, 12.5 × 0.4 cm column (Merck), using 10 mM triethyl ammonium acetate buffer, pH 6.8 (solvent A) and methanol (solvent B) as mobile phases. The DAD detector was set at 235 nm. For the analysis of LPAP liposomes, the time gradient elution consisted of a linear ramp from 80% B to 100% B (15 minutes) and these conditions were maintained for up to 20 minutes (t_R = 11.4 minutes for L(1)PAP and 11.7 minutes for L(2)PAP). For LLPAP and oleic acid anilide liposomes, the time gradient elution used was a linear ramp from 90% B to 100% B (10 minutes) and these conditions were maintained for up to 20 minutes (t_R LLPAP = 16.1 minutes; t_R OLA = 6.2 minutes).

Liposome characterization

Quantitative determination of phospholipid was performed by McClare's colorimetric method. In this method, phospholipid phosphorus in the sample is first acid-hydrolysed to inorganic phosphate. This is converted to phosphomolybdic acid by the addition of ammonium molybdate, and the phosphomolybdic acid is quantitatively reduced to a coloured compound by ascorbic acid. The intensity of the colour is measured spectrophotometrically, and is compared with calibration standards to give the content of phosphorus and hence phospholipid.

Microscopic analysis

Observation of liposomes under the microscope permits the detection of test compounds on the surface of the liposome that would indicate deficiencies in the encapsulation.

Different molar ratios of the lipid components of liposomes and the appropriate substrate were assayed to ascertain the liposome composition for achieving an optimum encapsulation of the aniline derivatives. According to the results obtained, composition number 3 appears to be that of choice. Once the liposomes were formed, different analyses were carried out to determine the efficiency of encapsulation. Liposomes were assayed by HPLC to confirm the presence of the corresponding aniline derivative.

In this context it is worth mentioning that PAP monoesters undergo acyl rearrangement, particularly under acid conditions. For this reason, use of chloroform for the preparation of the liposome might favour interconversion between m(1)PAP and m(2)PAP derivatives (L(1)PAP and L(2)PAP in the case of our model compounds). This fact should not affect the encapsulation ability of these compounds, but can alter the isomer composition. The use of dichloromethane instead of chloroform may help to freeze the above interconversion, and this practice can be recommended when highly pure samples of m(1)PAP derivatives are required. In the case of m(2)PAP, it is very difficult to avoid its partial conversion into the corresponding m(1)PAP during any sort of manipulation procedure.

To calculate the composition ratio, phospholipid contents were also estimated. Finally, analysis under the microscope revealed that aniline derivatives were encapsulated into the liposome since no occurrence of these derivatives, in particular those that are solid at ambient temperature (i.e. the anilide), was observed on the surface of the lipid particles. It was also observed that these liposomes can be lyophilized and further rehydrated without detectable degradation or release of the encapsulated substrates.

From the above studies it can be concluded that encapsulation into liposomes should be the method recommended for performing in vitro assays with fatty acid anilides and PAP monoesters (mPAP) and diesters (dPAP). Although a detailed description of the preparation of these liposomes has been provided we recommend, as in the case of standards and test compounds, that there should be a unique source of these liposomes for potential users, thus ensuring the reproducibility and reliability of liposome preparations. Should our laboratories be chosen as that source, liposomes containing known amounts of the above aniline derivatives would be provided as lyophilized samples, and the user would need only to resuspend the appropriate aliquot as described above.

Acknowledgements

We thank Núria Almiñana and Francesca Reig (Department of Peptides and Proteins, IIQAB) for their advice on the preparation and manipulation of liposomes.

Annex 3

Proposal for the updating and rationalization of the nomenclature on abbreviations of PAP compounds

Joaquin Abian, Emilio Gelpí and Angel Messeguer

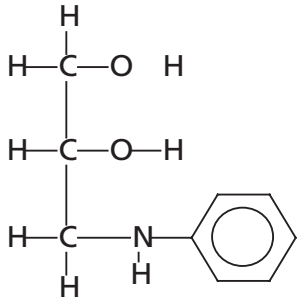
Generic names for basic structures

Two main groups of phenylaminopropanols are addressed. The basic molecules will be named as follows.

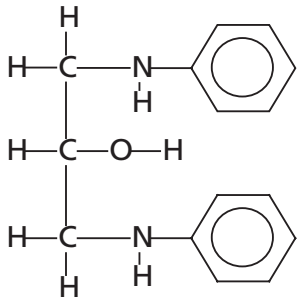
- 3-(*N*-phenylamino)-1,2-propanediol will be indicated by the well known denomination PAP.
- Diphenylaminopropanols will be generically named bisPAP. There are two possible positional isomers for bisPAP. To specify a particular isomer, two numbers in parentheses will be used: bis(1,3)PAP and bis(2,3)PAP.

Generic names for PAP and bisPAP derivatives

PAP and bisPAP generate an array of compounds bearing different acyl substituents in positions 1 and/or 2 of the molecule, as well as on the nitrogen



PAP

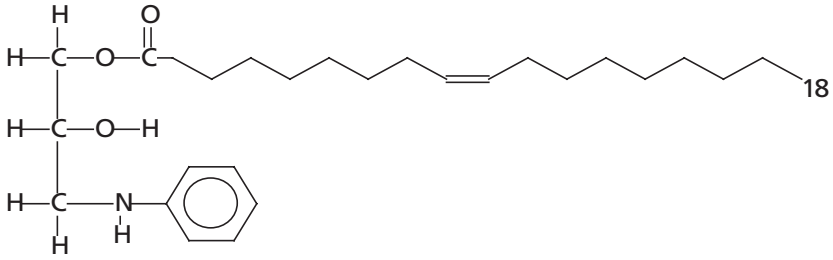


bisPAP
bis(1,3)PAP

of the phenylamino groups. To generically cover all possibilities the following nomenclature is proposed.

Monoacylated derivatives

- mPAP and mbisPAP to generically denominate a monoacylated PAP and monoacylated bisPAP, respectively.
- m(i)PAP to specify the acyl group is in position i (that is, 1 or 2) of PAP.
- m(N)PAP to specify the acyl group is in the nitrogen of the phenylamino group of PAP.
- m(N)bisPAP when the acyl group is on the nitrogen in position 3.
- m(N')bisPAP when the acyl group is on the nitrogen of the phenylamino group in position 1 or 2.



Generic names	*Specific name*[1]
mPAP	O(1)PAP (preferred to OPAP)
m(1)PAP	

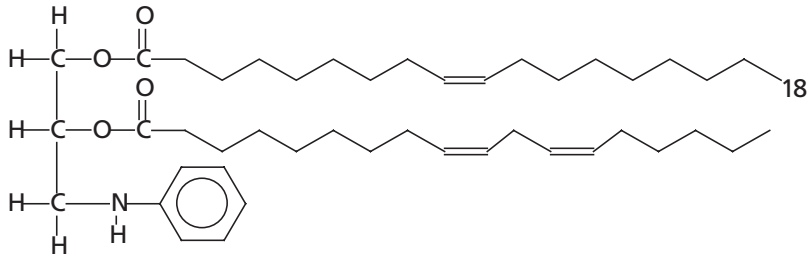
[1] The specific name as indicated throughout this proposal denotes the "preferred" chemically correct terminology indicating the position of the acyl substituent. For practical purposes, however, the WHO/CISAT Scientific Committee for the Toxic Oil Syndrome accepts the simplified name on the understanding that, unless otherwise specified, the acyl group is always in position 1.

Diacylated derivatives

- dPAP and dbisPAP to generically denominate a diacylated PAP and diacylated bisPAP, respectively.
- d(i,j)PAP when the two acyl groups are in positions i and j.
- d(i,N)PAP when one acyl is in position i and the other is on the nitrogen of the phenylamino group.

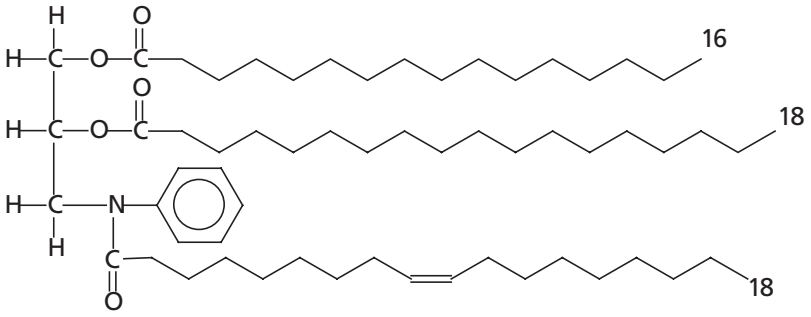
Triacilated derivatives

- tPAP for compounds with three acyl groups at 1, 2 and N.



Generic names
dPAP
d(1,2)PAP

Specific name
OL(1,2)PAP referred to OLPAP)



Generic name
tPAP

Specific name
PSO(1,2,N)PAP (preferred to PSOPAP)

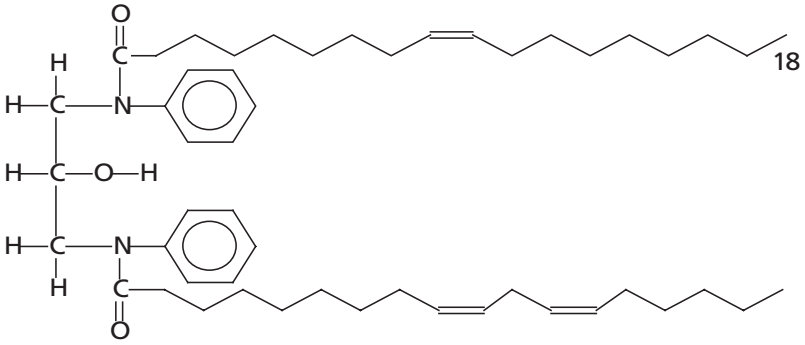
Specific names for PAP and bisPAP derivatives

It is suggested that the nature of the acyl residues be identified as follows.

- Capital first letter of the different fatty acids of the compound followed by the positions where they occur (1, 2, N or N') in parentheses, and then the name PAP or bisPAP.

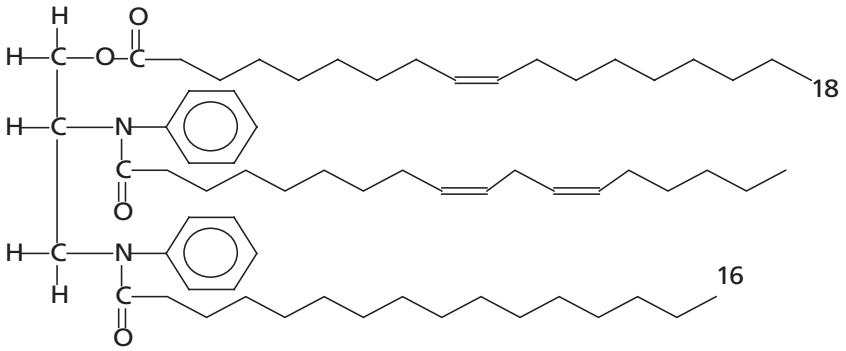
- When the first letter is the same for two fatty acids, a second letter in lower case will be added to one (e.g. linoleic acid (L) and linolenic acid (Ln)).
- The presence of acyl residues at different nitrogen atoms should be denoted by N (for 3-amino substitution) and N'.

Thus, a dioleoyl ester of PAP will be abbreviated OO(1,2)PAP. OLn(N,N')bis(1,3)PAP would indicate that the oleoyl residue is at one nitrogen atom whereas the linolenyl residue is at the other nitrogen atom of bis(1,3)PAP.



Generic names
dbisPAP
d(N',N)bisPAP

Specific name
OL(N',N)bis(1,3)PAP



Generic names
tbisPAP
t(1,N',N)bisPAP
t(1,N',N)bis(2,3)PAP

Specific name
OLP(1,N',N)bis(2,3)PAP

Annex 4

Part 1. Chemical analyses of TOS oils

Rosa Elena Calaf, Juana Peña, Montserrat Carrascal, Sonia Paytubi, Natalia Reig, Rosario Prieto, Yolanda Castaño, Benjamin C. Blount, Manuel Posada de la Paz, Emilio Gelpí and Joaquin Abian

Over the last several years great efforts have been made to achieve a thorough chemical characterization of TOS-related oil samples. It is now accepted that the particular conditions under which the aniline-containing oils were refined led to the formation of the toxic compounds responsible for the disease. The chemical characterization of the oils is thus very important in order to understand the processes that took place in the refineries and to elucidate the etiological agent(s). After the outbreak of the disease, exhaustive analyses carried out mainly by high-performance liquid chromatography with UV detection (HPLC-UV) demonstrated that several aniline derivatives were present in these oils (*1*). Two different toxico-epidemiological studies targeted two families of compounds as chemical markers of the toxic oils: fatty acid anilides and acylated derivatives of 3-(*N*-phenylamino)-1,2-propanediol (PAP) (*2*–*5*). The quantification of these compounds in TOS-related oils is essential to achieving further breakthroughs on epidemiological traits and

refinery conditions related to these oils. Table A4.1 gives the terminology of PAP and its derivatives.

Our laboratory optimized and validated analytical methods for the routine analysis of PAP derivatives and anilides in oils (*6–8*). Using these methods, more than 2600 oil samples (approximately 7000 analyses) were quantified over a two-year period. In general, two aliquots were taken from each sample of oil delivered for analysis, one for PAP analysis and the other for anilide analysis (Fig. A4.1).

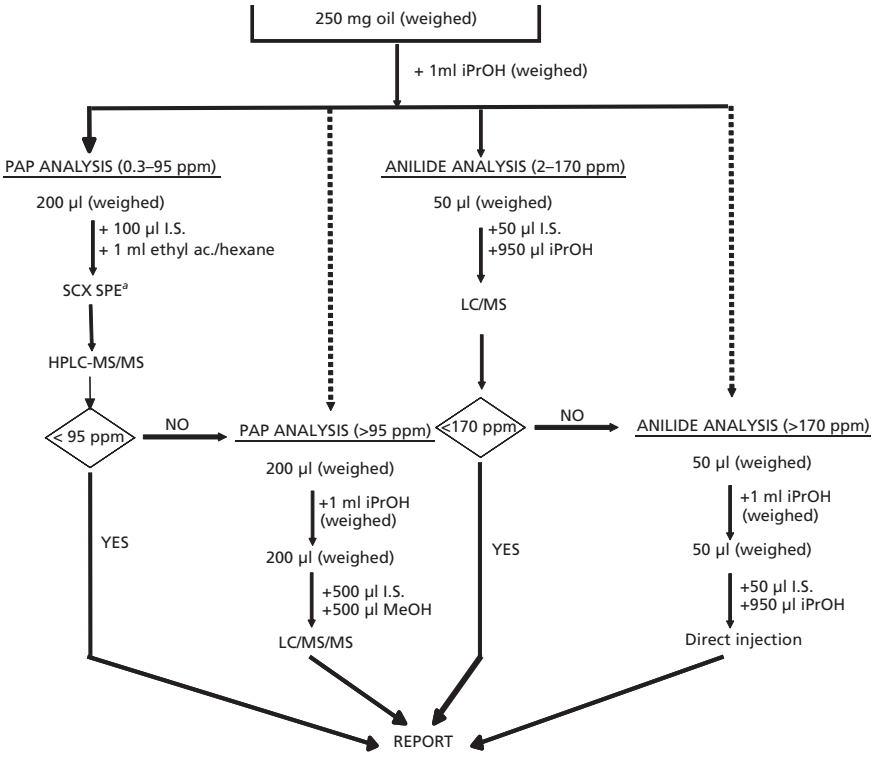
The oil samples analysed came from two sources. The main group of samples came from the CISAT storage collection in Madrid and correspond to oils recovered by the Spanish Government shortly after the outbreak of the illness in 1981. The other group corresponds to newly treated oil samples from refining studies attempting to reproduce the original processing conditions. The total amount delivered from CISAT was 10 ml. For high sensitivity quantitation of PAP derivatives, the corresponding aliquot was preconcentrated with a strong cation exchange (SCX) column prior to its analysis by liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). The complete procedure is shown in Fig. A4.2. In other cases the oil was analysed directly after appropriate dilution (Fig. A4.1). The aliquot taken for the analysis of anilides was directly injected and analysed by liquid chromatography coupled with mass spectrometry (HPLC-MS).

Typical chromatograms from PAP and anilide analyses are shown in Fig. A4.3. Chromatography was carried out using a Hewlett Packard model 1100 HPLC with a binary pump and a UV detector. Samples were injected

Table A4.1. Nomenclature of PAP and its derivatives

Abbreviation	Name	Molecular weight
PAP	3-(*N*-phenylamino)-1,2-propanediol	167
LPAP	2-hydroxy-3-(*N*-phenylamino)propyl linoleate	429
HPAP	2-hydroxy-3-(*N*-phenylamino)propyl heptadecanoate	421
OPAP	2-hydroxy-3-(*N*-phenylamino)propyl oleate	431
HHPAP	2-heptadecanoyloxy-3-(*N*-phenylamino)propyl heptadecanoate	671
LLPAP	2-linoleoyloxy-3-(*N*-phenylamino)propyl linoleate	691
HLPAP	2-linoleoyloxy-3-(*N*-phenylamino)propyl heptadecanoate	681
HOPAP	2-oleoyloxy-3-(*N*-phenylamino)propyl heptadecanoate	683
LOPAP	2-oleoyloxy-3-(*N*-phenylamino)propyl linoleate	693
OLPAP	2-linoleoyloxy-3-(*N*-phenylamino)propyl oleate	693
OOPAP	2-oleoyloxy-3-(*N*-phenylamino)propyl oleate	695

Fig. A4.1. Scheme of the complete procedure for oil analysis

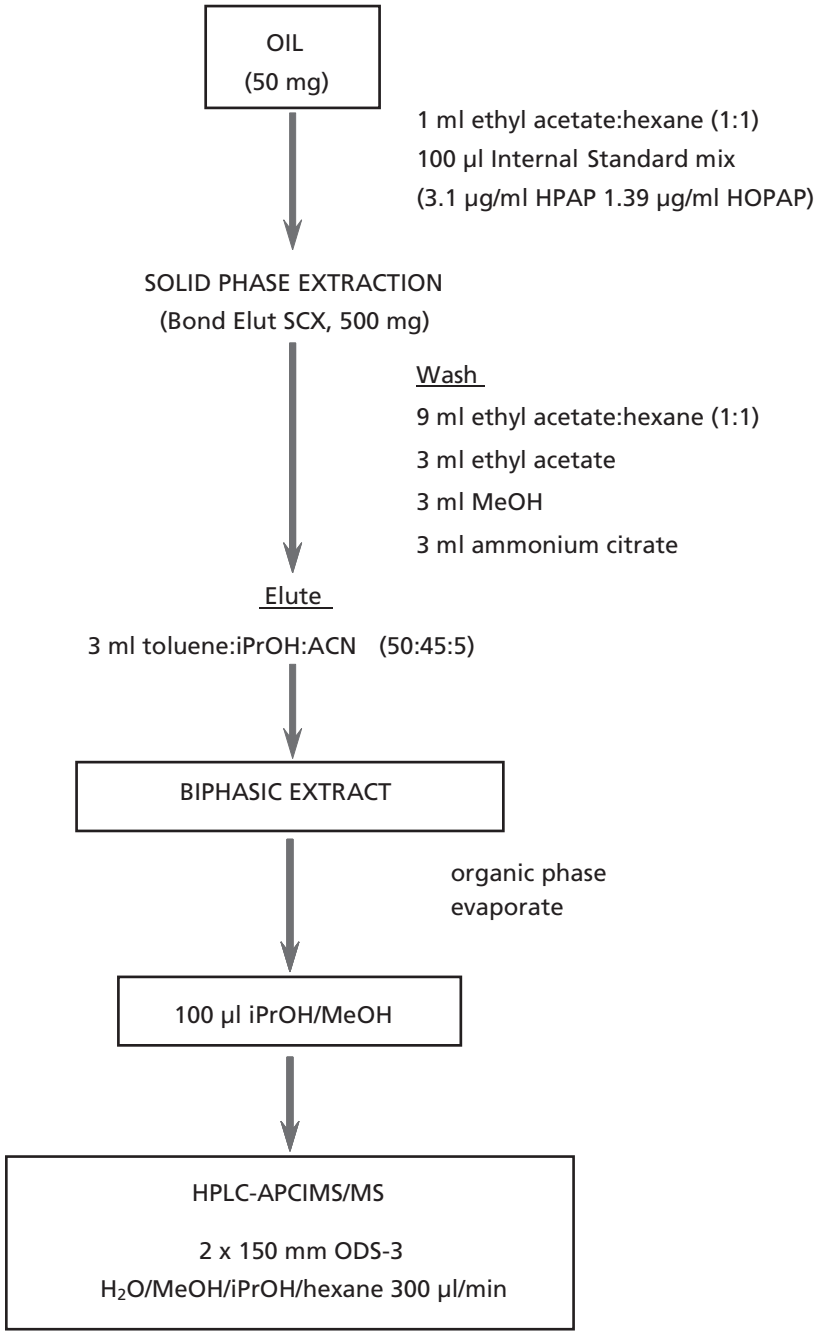


[a] Strong cation exchange solid phase extraction.

with a Triathlon automatic injector (Spark Holland B.V., Emmen, Netherlands) provided with a 50-µl loop.

The analytical column was a reversed-phase Partisil ODS-3 (5-µm particle size, 2 × 150 mm) from Tecnokroma (Barcelona, Spain) preceded by a 2 × 10-mm ODS precolumn (Upchurch Scientific, Oak Harbor, WA, United States). The injection volume was 10 µl and the flow rate 300 µl/min. Target compounds were separated under gradient conditions. Solvent A was composed of water/methanol 20/80 (0.1% acetic acid) and solvent B was isopropanol/methanol 20/80 (0.1% acetic acid, 0.5% hexane). The gradient started at 20% of B, increased to 80% in 0.1 minute, then increased again to 100% of B in 3.5 minutes, where it was held for 5 minutes. Quantitative measurements were achieved using two different internal standards: HPAP for quantitation of monoacyl PAP derivatives and HOPAP for quantitation of diacyl PAP derivatives. Analyses were carried out in the precursor-ion scan mode. $[M+H]^+$ ions at m/z 420, 432, 684.5, 692.5, 694.5 and 696.5 (HPAP, OPAP, HOPAP, LLPAP, LOPAP and OOPAP, respectively) were monitored in the first quadrupole and the common product ion at m/z 132 in the

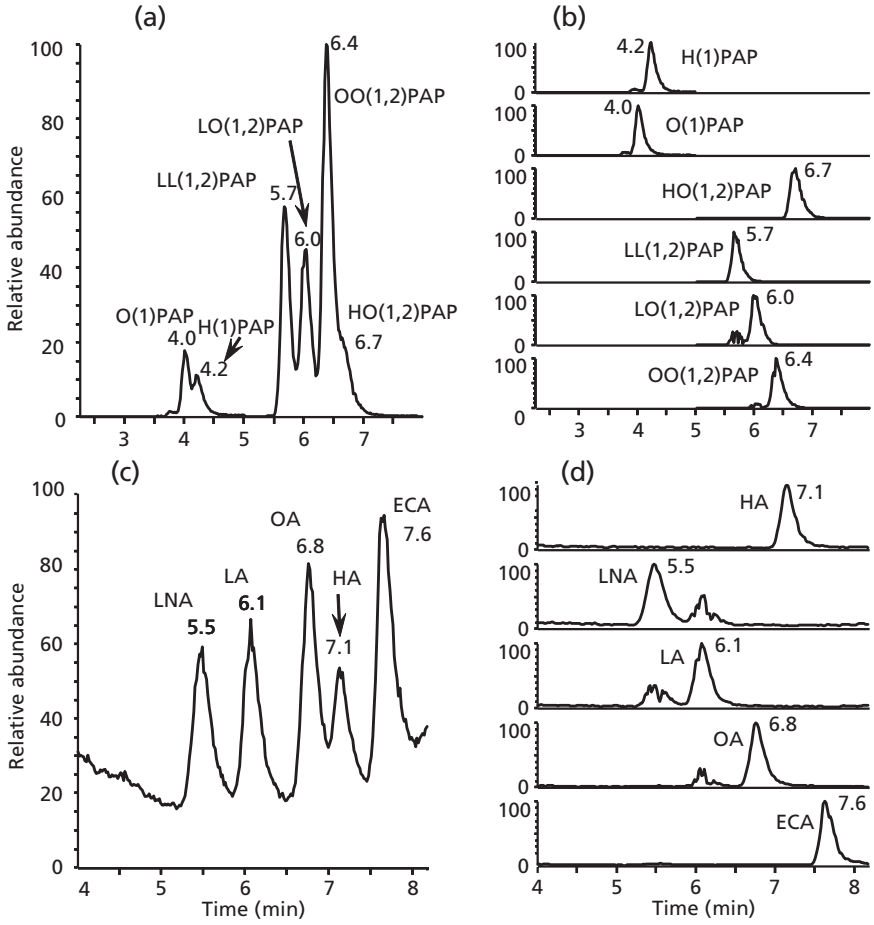
Fig. A4.2. Detailed procedure for PAP analysis in oils by solid phase extraction and HPLC-MS/MS



second analyser (third quadrupole). The collision energy was set at –25 eV for monoacyl PAP derivatives and –35 for diacyl PAP derivatives. The collision gas (argon) pressure was 1.5 mTorr and the multiplier voltage was set at 1800 V.

The methods reported here provided a highly selective, sensitive and reliable way of quantifying PAP derivatives and anilides in oil samples. In this respect, owing to their different original sources, TOS-related oils do not show a homogeneous composition. They can often be classified by odour, colour and viscosity. Despite this heterogeneity, these methods produced total (Fig. A4.3a,c) and selected (Fig. A4.3b,d) ion chromatograms with

Fig. A4.3. Analysis of PAP derivatives (a,b) and anilides (c,d) in two different rapeseed oil samples spiked with 3–26 ppm PAP derivatives and 40–100 ppm anilides, respectively[a]



[a] LA = *N*-phenyl linoleamide; LNA = *N*-phenyl linolenamide; OA = *N*-phenyl oleamide; ECA = *N*-phenyl eicosanamide; HA = *N*-phenyl heptadecanamide.

no detectable interference from other oil components. In the case of PAP derivatives this is the combined result of the high selectivity of the extraction procedure and the MS/MS analysis.

Fig. A4.4 shows the distribution of TOS-related samples in terms of their content for the most abundant PAP and anilide derivatives, OOPAP and *N*-phenyl oleamide, respectively. More than 50% of the oils analysed did not contain detectable amounts of OOPAP; when detected, it was quantified in most samples at levels below 5 ppm. On the other hand, 25% of the oil samples contained anilides in the 8–100 ppm range and only 35% of the samples did not show detectable amounts of these compounds. The lack of detectable amounts of PAP derivatives and anilides in some of these oils is due either to the high dilution of the original aniline-containing oil with other edible oils or to its lack of relation to the toxic oil batches. As PAP derivatives need more astringent conditions for synthesis than anilides, it could be possible that some oils containing anilides do not contain PAP derivatives. The relative distribution of fatty acids in PAP derivatives and anilides in TOS-related oils is shown in Fig. A4.5. The relative abundance of the different components follows a similar pattern in all of the oils analysed, and seems to reflect the average distribution of fatty acids in these oils. It is still not clear whether these results imply a nonspecific chemistry for the formation of PAP derivatives in oils, or that they are the consequence of transesterification processes after the initial synthesis. Some comments on the method development and validation are indicated below. A more exhaustive description of the methods can be found elsewhere (*6*–*8*).

Fig. A4.4. Summary of the quantification results obtained for OOPAP and *N*-phenyl oleamide in the analysis of TOS-related oil samples (n = 2600 oil samples)

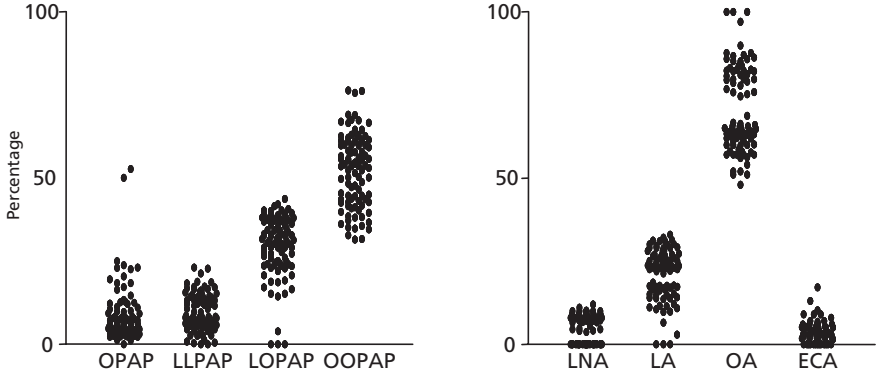
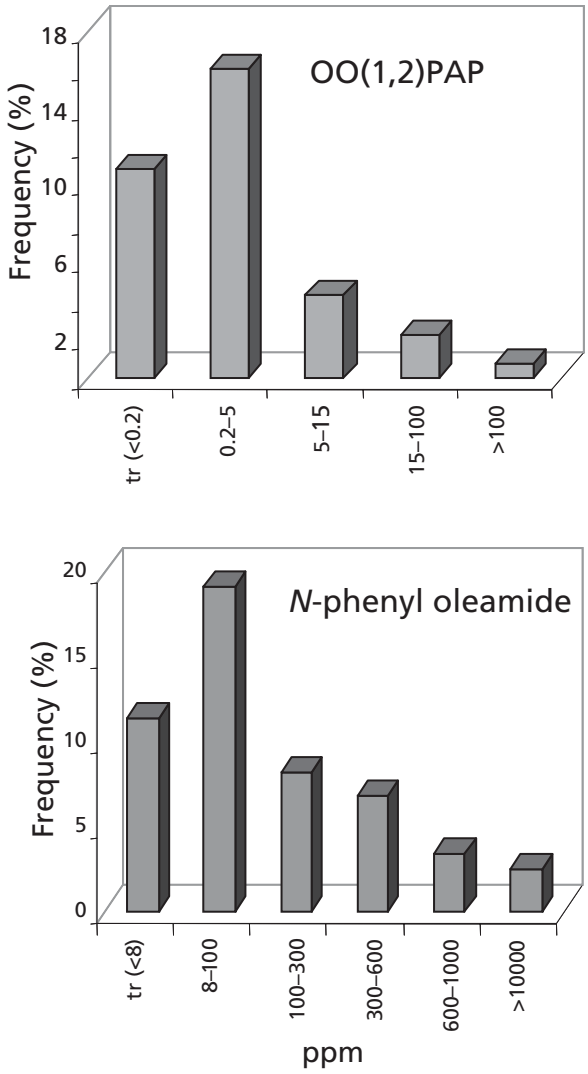


Fig. A4.5. Relative percentages of the major PAP and anilide compounds detected in TOS-related oil samples (94 and 90 randomly selected samples from PAP derivatives or anilide-containing oils, respectively)



Development of a quantitative analytical method for PAP derivatives

Some methods for the analysis of PAP derivatives based on the direct analysis of oil samples by HPLC-MS/MS have been reported by groups involved in TOS research. These methods used synthetic anilides as internal standards (*9*) or semi-quantitative estimations (*10*). The main drawback of these methods

was the poor sensitivity owing to the direct analysis of diluted oil samples. In our experience, direct injection of diluted samples led to contamination of the system, columns and ionization source and to a steady loss in sensitivity. Despite the excellent selectivity of the MS/MS method (typical chromatograms contain no apparent interfering peaks), co-eluting matrix compounds can affect analyte ionization and thus reduce sensitivity and reproducibility. Moreover, the need to keep the total amount of material injected at a reasonable level (in terms of sample solubility, column overloading or system contamination problems) determines the minimum detection level that can be reached in routine analyses.

Thus, for maximum sensitivity and reproducibility a selective preconcentration of PAP derivatives was desirable. We used a modification of the solid phase extraction method described by Blount & Schruz (*11*) for this purpose. This method used SCX cartridges to fractionate PAP derivatives from the rest of the neutral, apolar oil matrix. An SCX extraction was expected to be highly selective for PAP derivatives, because not many other basic compounds would be present in edible oil. The SCX method takes advantage of the different interactions that take place between the PAP derivatives and the SCX stationary phase to produce a highly selective extraction. Ionic interactions between the amino group from PAP derivatives and the SO_3^- from the sorbent determine the retention of PAP derivatives. Additionally, apolar interactions between the phenyl groups on the PAP derivatives and the sorbent also intervene in the extraction process. A careful solvent selection was therefore important to maintain the equilibrium between both interactions necessary for a high level of recovery.

The original SCX method (*11*) was developed for LC-UV analysis and used 500 mg of oil for extraction. To adapt the procedure for LC/MS analysis and to optimize recovery and reproducibility, only a tenth of the original amount of oil was extracted in our procedure. In addition, we suspected that a fraction of PAP derivatives could be lost as a citrate salt in the aqueous layer of the final eluate. These losses could be responsible for the high variability in the absolute peak areas observed in the analysis of samples spiked at the same concentration. To prevent this, an additional liquid–liquid extraction of the biphasic eluate was performed, adding 200 µl of a methylamine solution (1% in water) (Fig. A4.2). After vortexing and centrifugation, the aqueous phase was eliminated. With this treatment, reproducibility was greatly enhanced. The coefficients of variation of the absolute peak area of the internal standards HPAP and HOPAP in the LC/MS chromatograms were 25% and 15% (n = 54), respectively. Before the treatment these values were as high as 65% and 35% (n = 100) for the same standards.

Liquid chromatography

During the early stages of establishing the method, we developed an HPLC method with a cycle time of 19 minutes. This gave baseline separation of all

PAP derivatives analysed, except for co-eluting LOPAP isomers, and is still used in our laboratory for compound identification. The analysis of PAP derivatives without prefractionation required long chromatographic runs to obtain maximum separation of oil components as well as to wash out the less polar oil components from the column after elution of PAP derivatives. As indicated above, chromatographic separation is important in preventing ionization interference.

The use of solid phase extraction (SPE) sample cleanup allowed the run cycle time to be shortened to 11 minutes, a more reasonable time because of the large number of samples to be analysed. System robustness against contamination was improved using programmable switching valves to route the column eluate to the mass spectrometer only during the period where PAP derivatives elute. Under these conditions, more than 100 oil samples could be injected without compromising analytical performance.

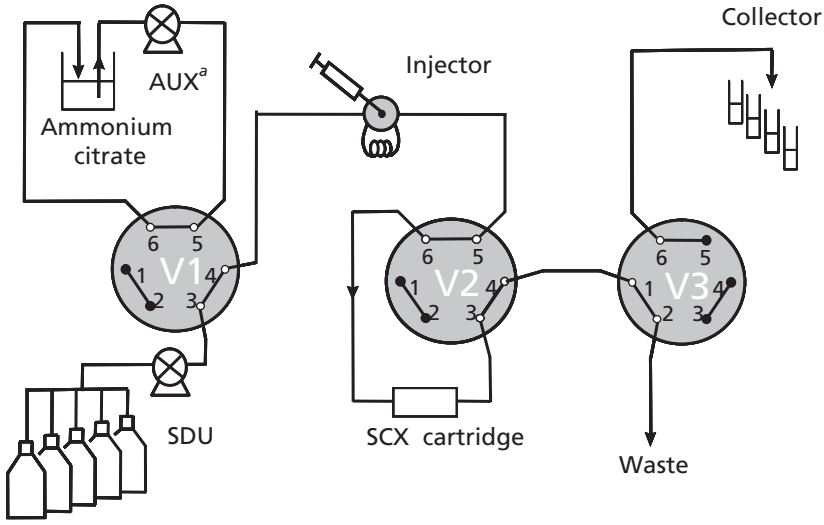
Automation

The main drawbacks of the analytical procedure were found in the sample preparation steps. These manual SPE steps were labour- and time-intensive and required skilled and attentive personnel to assure constant recovery. To circumvent these problems, we adapted the SPE protocol for automatic extraction and solved the problems derived from the use of the immiscible solvents required for the SCX extraction. Automatic extraction was carried out using a Prospekt system (Spark Holland, Netherlands) (Fig. A4.6).

Initial protocols used for automatic extraction were designed to mimic the solvent order and flow rates used for manual extraction. These procedures soon proved to be inappropriate. Ammonium citrate precipitated when ammonium citrate solution was pumped after methanol. This caused the solvent delivery unit (SDU) solvent valves to become blocked after a few cycles. This problem was solved by installing an additional, independent high-pressure pump. Thus, while the main SDU pump supplied organic solvents such as methanol, isopropanol, ethyl acetate : hexane and the toluene : acetonitrile : isopropanol elution mixture, this second pump was responsible for pumping ammonium citrate (Fig. A4.6 and Table A4.2).

Major losses in the manual procedure were observed when methanol was used as an intermediate solvent between the immiscible ethyl acetate and ammonium citrate solutions. Losses were observed both in the methanol fraction (mixed with some ethyl acetate) and in the citrate fraction (mixed with some methanol), and these were probably due to the elution effect produced by these mixtures of intermediate polarity. To prevent sample losses and, at the same time, to allow switching between immiscible solvents, the Prospekt system was programmed to pump methanol and ammonium citrate with the SPE cartridge disconnected from the flow path (V2 in bypass position). After

Fig. A4.6. Scheme of the setup used in the automatic extraction procedure



[a] The AUX pump was an independent HPLC pump continuously flushing an ammonium citrate solution.

Note: V1, V2 and V3 correspond to the switching valves provided in the Prospekt system.

Table A4.2. Solvent and valve programmes for automatic extraction of PAP derivatives

Time (min)	Solvent[a]	SDU flow rate (ml/min)	Injector	V1 citrate	V2 cartridge	V3 collector	Comments
0	1	5	Load	Recycle	Cartridge	Waste	Conditioning
1:00	2						
2:00	2	0.3	Inject				Sample load
6:00	3	5			Bypass		Tubing wash
6:30	3	1			Cartridge		Cartridge wash
7:00	4	5			Bypass		Tubing wash
/:30	4	0		Input			Tubing wash (citrate)
8:30					Cartridge		Citrate
9:30	4	5		Recycle	Bypass		Tubing wash
10:00	5						
10:30	5	1			Cartridge	Collect	
12:30	4	5				Waste	System wash
13:30	0	0					

[a] 1 = isopropanol; 2 = ethyl acetate : hexane; 3 = ethyl acetate; 4 = methanol; 5 = toluene : isopropanol : acetonitrile.

the tubing had been filled with ammonium citrate, this solution was pumped for 1 minute through the cartridge. The V2 was then switched to the bypass position and the system flushed with the apolar elution solvent before elution. The sequence of solvents pumped through the cartridge was then ethyl acetate-citrate-toluene : isopropanol : acetonitrile. This unusual procedure produced a high recovery of the material in the column with minimal sample losses in the ammonium citrate fraction. A remarkable characteristic of the automatic procedure is its high reproducibility: coefficients of variation were between 2 and 8 times lower than those obtained in the manual procedures.

The initial oil dilution was also optimized for the automatic method. Using this method, only a fraction of the total sample volume could be injected into the extraction system. For practical purposes, the maximum injection volume was 200 µl. Oil dilution was calculated accordingly, in order to inject the same sample weight compared with the manual method. Linearity of the analytical method was found to be in the same range as for the manual method. Extraction recoveries were 87% and 75% for LPAP and LLPAP, respectively, and the corresponding coefficients of variation were approximately 1%, thus greatly improving reproducibility compared with manual procedures.

HPLC-MS/MS analysis and quantitation of PAP derivatives

For routine quantitation, calibration curves were performed daily using an oil matrix spiked with different levels of PAP standards (oil No. 4078). This oil was a TOS-related sample lacking any measurable level of monoacyl or diacyl PAP derivatives. Oil No. 4078 came from the same sources as oils containing aniline by-products and was collected during the same period. It was therefore considered an appropriate and representative matrix for calibration purposes.

Different heptadecanoic acid derivatives were tested as internal standards. Heptadecanoic acid is very similar to oleic acid in terms of polarity, but is absent in vegetable oils. The difference of 10 or more mass units between the heptanoyl esters of PAP and the corresponding PAP derivatives found in oils assured no isotopic interferences between mass channels. This is especially important because, for maximum sensitivity in the analysis of PAP derivatives, mass resolution can then be adjusted at a low value in both analytical quadrupoles.

Calibration curves for OPAP (0.05–2.5 µg PAP added) were calculated using HPAP, HLPAP, HOPAP or HHPAP as the internal standard. The coefficients of regression (R^2) for the lines obtained were 0.9995, 0.9915, 0.9909 and 0.9915, respectively. In the case of OOPAP these values were 0.9769, 0.998, 0.9993 and 0.9987. Thus, the best line for OPAP was produced when using the HPAP signal for calibration. In contrast, HPAP was not adequate as the internal standard for OOPAP, which was best represented by the diacyl

candidates. Two internal standards were thus selected for quantification, one for each PAP derivatives group: HPAP and HOPAP.

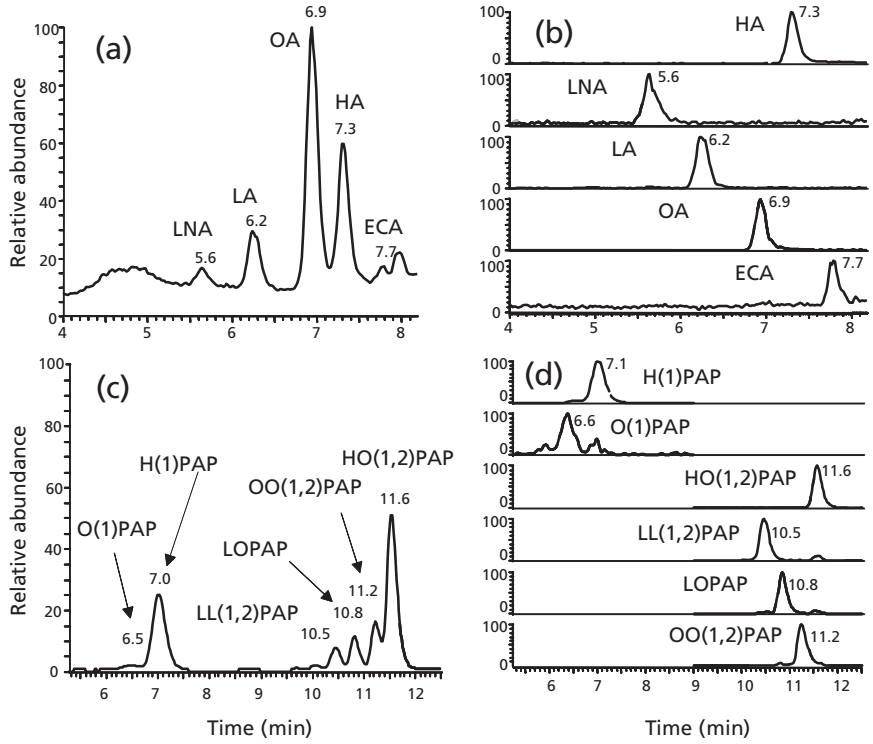
The quantitation limits of the analysis, based on a signal-to-noise ratio of 10, were set to 200 ppb for diacyl PAP derivatives and 500 ppb for monoacyl PAP derivatives by using manual extraction. Interassay coefficients of variation at different concentrations ranged from 5% (25 ppm OOPAP, n = 13) to 12% (1 ppm OOPAP, n = 18). Typical chromatograms obtained from a TOS oil sample are shown in Fig. A4.7. Calculated concentration values for oleoyl derivatives in these samples were 862 ppm (*N*-phenyl oleamide) and 1 ppm (OOPAP), respectively. Similar quantitation limits (200 ppb) were determined for monoacyl and diacyl PAP derivatives with automatic extraction. Response was linear throughout a calibration range of 0.1–100 ppb, using either manual or automatic extraction. The coefficients of determination (r^2) were greater than 0.999 in all cases. The relatively higher extraction efficiency of the automated method resulted in improved detection of monoacyl PAP derivatives (eluting around 4.5 minutes) compared to the manual method. Recovery of diacyl PAP derivatives (eluting between 5.5 and 8 minutes) was similar with the two methods. The complete HPLC-MS/MS analysis was carried out in less than 10 minutes. Samples were injected every 11 minutes, approximately the same throughput as the automatic extraction step.

Quantitative method for fatty acid anilides

Early methods described for the analysis of fatty acid anilides were based on the production of aniline from anilide using thermal or alkaline decomposition (*12–14*). These methods were not able to distinguish between the different anilides present in oils. More specific analyses were carried out using HPLC-UV with a previous purification of the analyte (*15–17*). These methods allowed the characterization of oleic, linoleic, linolenic and stearic anilides. Meanwhile, Bailey et al., using gas chromatography, demonstrated that oleoyl anilide was the major anilide component of the toxic oils (*18*). Since the anilides of oleic, linoleic and linolenic acids co-eluted on the capillary column, a mass spectrometry detector was needed for their identification. More recently, Guitart et al. described a gas chromatographic method using cross-bonded polar phases capable of separating these compounds (*19*).

Chromatography and analysis of anilides did not involve the same challenges as the analysis of PAP derivatives. These compounds are found in toxic oils at levels 20–1000 times higher than those of PAP derivatives. Moreover, this family of compounds is less heterogeneous than PAP derivatives, making co-elution problems less severe. The quantity of anilides found in TOS-related oils is highly dependent on the nature of the oil. Crude oils contain 11 000–66 000 ppm of anilides, while these values drop to about 2000 ppm after refining (*13,20*). In an exhaustive study of 195 samples of oils implicated

Fig. A4.7. Analysis of anilides (a,b) and PAP derivatives (c,d) on TOS-related oil samples[a]



[a] LA = *N*-phenyl linoleamide; LNA = *N*-phenyl linolenamide; OA = *N*-phenyl oleamide; ECA = *N*-phenyl eicosanamide; HA = *N*-phenyl heptadecanamide.

in TOS, anilides were detected in 62% of the samples at concentrations ranging from 30 to 2000 ppm (*19*).

For routine quantification, calibration curves were performed every day using a reference oil matrix (oil No. V-40) spiked with the corresponding anilide standards. This oil, which lacked any measurable level of anilides, was obtained from the same sources as oils containing aniline by-products and was collected during the same period.

Adequate chromatograms were obtained with runs of 11 minutes. Under these conditions no interferences were detected by HPLC-MS, so that the greater selectivity of MS/MS was not required (Fig. A4.7c,d). Quantification of anilides was carried out using the MS mode and monitoring the $[M+H]^+$ ions at m/z 346.5, 354, 356, 358 and 386 (*N*-phenyl heptadecanamide, *N*-phenyl linolenamide, *N*-phenyl linoleamide, *N*-phenyl oleamide and *N*-phenyl eicosanamide, respectively).

The quantification limits of the analysis, based on a signal-to-noise ratio of 10, were found to be 8 ppm for anilides. Interassay coefficients of variation were always close to 10% (16–1600 ppm *N*-phenyl oleamide, n >11).

Conclusions

The procedures described above have allowed the quantitative characterization of chemical markers of TOS in oils related to the syndrome. In marker-positive samples, anilides and PAP derivatives were found at levels up to 50 000 and 330 ppm, respectively. The relative abundance of the different fatty acid anilides and PAP derivatives correlates with the fatty acid composition of the oils. More than 2600 different samples were analysed by this method in the most exhaustive screening of suspected toxic oils carried out so far.

Acknowledgement

We should like to thank Dr Angel Messeguer (CID, CSIC, Barcelona) for providing the synthesized standards.

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Part 2. Identification of new PAP derivatives

Natalia Reig, Yolanda Castaño, Rosario Prieto, Rosa Elena Calaf, Angel Messeguer, Anna Morató and Joaquin Abian

Aniline derivatives are suspected of playing a role in the appearance of TOS. Compounds such as fatty acid anilides and monoesters and diesters of 3-(*N*-phenylamino)-1,2-propanediol (PAP) were identified soon after the syndrome appeared in oils related to TOS. Two different toxico-epidemiological studies targeted these families of compounds as chemical markers of the toxic oil, although no specific toxicity of these compounds could be demonstrated. It is now clear, however, that specific conditions during the refining of the aniline-containing oils yielded toxic compounds related to the disease. We attempt here to describe the maximum possible number of aniline derivatives present in refined oils in an effort to search for the causative agent(s). Several aniline derivatives of PAP had already been detected in these oils (*1,2*, N. Reig et al., unpublished data, 2003) and the structures of other possible, as yet undetected, compounds had been suggested. The work reported here describes an exhaustive chemical analysis of laboratory-processed oils. These oils were submitted, in the presence of aniline, to laboratory conditions under which PAP derivatives were known to be produced. Oil samples were then submitted to a series of extraction procedures to yield extracts enriched in aniline derivatives. These were analysed by on-line HPLC-UV-APCI/MS. In addition, the extracts were fractionated by HPLC and the fractions analysed by HPLC-APCI/MS/MS to

obtain structural information. The components of several large families of derivatives were easily identified and characterized by MS/MS, including anilides, mPAPs, d(1,2)PAPs and t(1,2,N)PAPs (Fig. A4.8 and Table A4.3). A new PAP positional isomeric family (d(N)PAP) was detected and characterized, as well as the bis(1,3)PAP families, the m(2)bis(1,3)PAPs and d(N,2)bis(1,3)PAPs. Two new bis(1,3)PAP families were identified: the t(N,2,N')bis(1,3)PAPs and the positional isomeric family, the m(N)bis(1,3)PAPs.

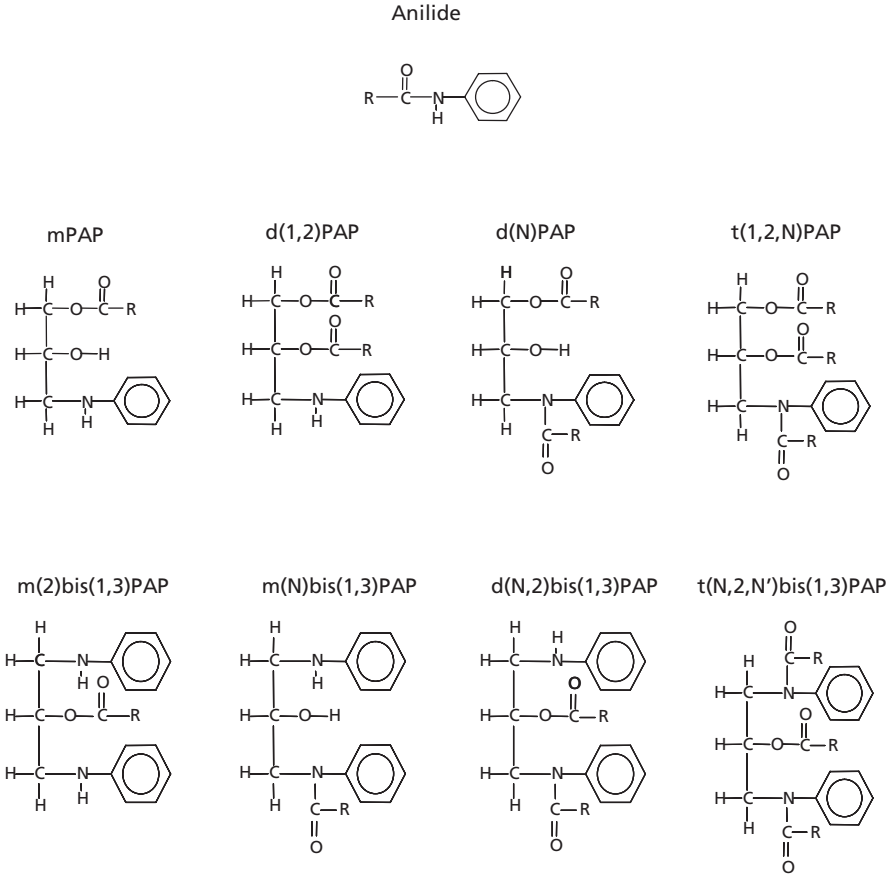
Oil synthesis and fractionation

Oil samples were laboratory-refined under conditions in which PAPs were found to be formed in large quantities. Briefly, 40 g of rapeseed oil was denatured with 2% of a mixture of aniline and ^{14}C-aniline. The radioactive label was used to trace the aniline moiety during the synthetic and analytical steps. The mixture was shaken and added to a flask preheated to 200–220 °C (addition of the mixture reduced the temperature to 120–130 °C). The mixture was then heated to 290 °C for 20 minutes. The temperature was then reduced to 200 °C and maintained until the end of the experiment two hours later. In addition to the synthetic oil, the volatile material recovered in the refrigeration coil of the reactor was also collected.

In order to remove unreacted aniline and to cover a wider range of compounds, oil samples (200 mg) were extracted with 1 : 1 mixtures of ethyl acetate : hexane and methanol : water to yield two fractions, the aqueous phase and the organic phase. The radiochromatographic profiles obtained from those fractions and from the volatile fraction showed broad radioactivity signals in the areas corresponding to aniline (aqueous phase and volatile fraction) and to anilides, mPAPs and d(1,2)PAPs (organic phase). No information about individual components could be obtained from these samples owing to the low resolution obtained from the on-line radioactivity detector, and no other signals could be detected on the chromatogram. These results suggested that, if present, unknown aniline-derived compounds should be either at low concentration in the oils or unresolved in the elution zone corresponding to the already known anilides, mPAPs or d(1,2)PAPs.

To search for new derivatives, the aqueous and organic phases were fractionated by HPLC, and the fractions were individually studied by HPLC-APCI/MS. Characteristic HPLC-UV and HPLC-radioactivity profiles are shown in Fig. A4.9. The analysis of the organic phase showed three main areas (A, B and C in Fig. A4.9). Mass spectrometric analysis of the HPLC fractions corresponding to these areas showed only the major compounds expected in oil. Compounds with one (anilides and mPAPs), two (diglycerides and d(1,2)PAPs) or three (triglycerides) fatty acids were found in zones A, B and C, respectively. Analysis of other aniline-derived compounds in the low-radioactivity fractions was, however, impeded owing to the interference produced

Fig. A4.8. Abbreviations for families of aniline derivatives and their molecular structures



Note: R corresponds to any possible fatty acid.

by major oil components such as diglycerides and triglycerides. As shown in Fig. A4.9, these components were clearly seen in the UV trace despite their low absorbance at 245 nm.

Preconcentration using SCX-SPE columns

To circumvent the problem originating from the interference of major oil components in the analysis of low-abundance aniline derivatives, a new strategy was introduced. This included SCX extraction to selectively purify amine-containing compounds from the oil matrix, as described by Calaf et al. (*3*). The procedure was applied to the oil after washing the sample with methanol/water to eliminate aniline and very polar compounds.

Table A4.3. Aniline derivatives detected in refined oils

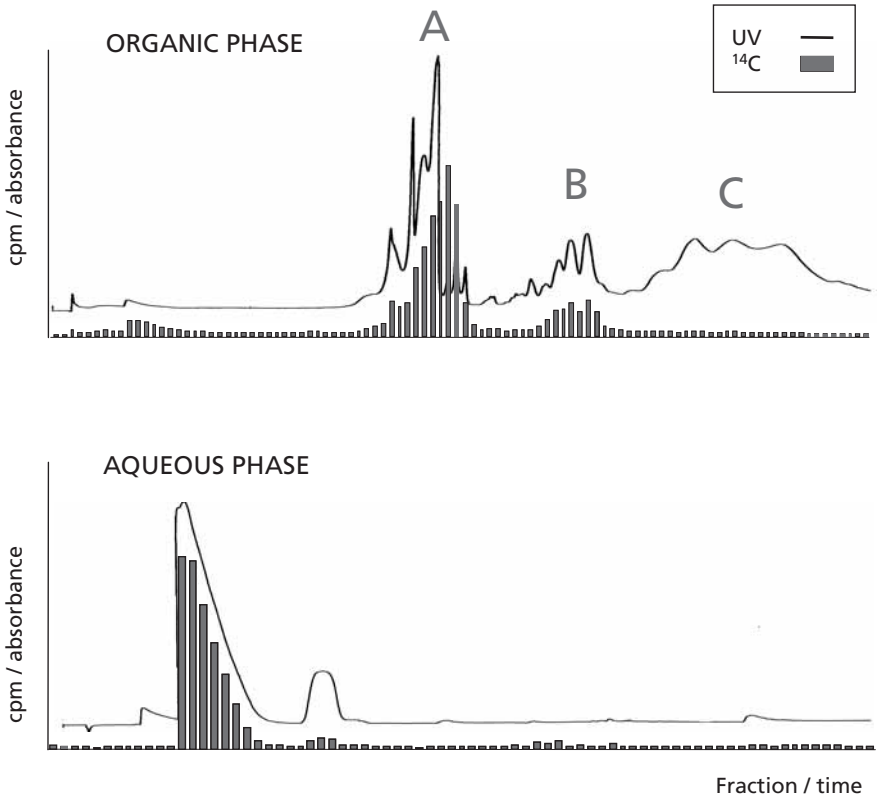
	Compound	MW of protonated molecular ion (MH+)	Oil fraction	Retention time (minutes)
Anilides	C16:2 A	328.3	SPNR1	10.60
	PoA	330.3	SPNR1	12.16
	PA	332.3	SPNR1	14.22
	LnA	354.3	SPNR1	11.75
	LA	356.3	SPNR1	13.08
	OA	358.3	SPNR1	14.64
	SA	360.3	SPNR1	16.82
	EiA	386.3	SPNR1	17.02
	AA	388.3	SPNR1	19.49
	EA	414.3	SPNR1	19.47
	BA	416.3	SPNR1	22.42
	C24:1 A	442.3	SPNR1	22.23
	LgA	444.3	SPNR1	25.32
mPAPs	C16:2 PAP	402.3	SPP2	11.58
	PoPAP	404.3	SPP2	12.98
	PPAP	406.3	SPP2	14.88
	LnPAP	428.3	SPP2	12.50
	LPAP	430.3	SPP2	13.64
	OPAP	432.3	SPP2	15.08
	SPAP	434.3	SPP2	17.61
	EiPAP	460.3	SPP2	17.52
	APAP	462.3	SPP2	20.60
	EPAP	488.3	SPP2	20.18
	BPAP	490.3	SPP2	23.55
	C24:1 PAP	516.3	SPP2	22.91
	LgPAP	518.3	SPP2	26.24

	Compound	MW of protonated molecular ion (MH+)	Oil fraction	Retention time (minutes)
D(1,2)PAPs	LnMPAP	638.9	SPP2	26.73
	LMPAP	640.6	SPP2	28.17
	OMPAP	642.3	SPP2	29.79
	C16:2LnPAP	662.6	SPP2	25.58
	C16:2LPAP	664.6	SPP2	27.17
	C16:2OPAP	666.6	SPP2	28.43
	LnPoPAP	664.6	SPP2	27.17
	LPoPAP	666.6	SPP2	28.42
	OPoPAP	668.6	SPP2	29.88
	LnPPAP	666.6	SPP2	29.19
	LPPAP	668.5	SPP2	30.46
	OPPAP	670.6	SPP2	32.00
	LnLnPAP	688.6	SPP2	26.41
	LnLPAP	690.6	SPP2	27.73
	OLnPAP	692.6	SPP2	29.27
	LLPAP	692.6	SPP2	28.89
	OLPAP	694.6	SPP2	30.48
	OOPAP	696.6	SPP2	32.00
	LnSPAP	694.6	SPP2	31.20
	LSPAP	696.6	SPP2	32.67
	OSPAP	698.6	SPP2	34.18
	LnEiPAP	720.6	SPP2	31.32
	LEiPAP	722.6	SPP2	32.37
	OEiPAP	724.6	SPP2	33.84
	LnAPAP	722.6	SPP2	33.41
	LAPAP	724.6	SPP2	34.59
	OAPAP	726.6	SPP2	36.30
	LnEPAP	748.6	SPP2	33.18

D(1,2)PAPs (contd)	LEPAP	750.6	SPP2	34.30
	OEPAP	752.6	SPP2	35.74
	LnBPAP	750.6	SPP2	35.43
	LBPAP	752.6	SPP2	36.76
	OBPAP	754.6	SPP2	38.67
	LnLgPAP	778.6	SPP2	37.57
	LLgPAP	780.6	SPP2	39.09
	OLgPAP	782.6	SPP2	41.29
	C24:1LnPAP	776.6	SPP2	35.09
	C24:1LPAP	778.6	SPP2	36.32
	C24:1OPAP	780.6	SPP2	38.00
d(N,2)bis(1,3)PAPs	LnPbis(1,3)PAP	741.6	SPP2	29.30
	LPbis(1,3)PAP	743.6	SPP2	30.47
	OPbis(1,3)PAP	745.6	SPP2	31.97
	LnLnbis(1,3)PAP	763.6	SPP2	26.66
	LLnbis(1,3)PAP	765.6	SPP2	27.92
	OLnbis(1,3)PAP	767.6	SPP2	29.50
	LLbis(1,3)PAP	767.6	SPP2	29.22
	OLbis(1,3)PAP	769.6	SPP2	30.65
	OObis(1,3)PAP	771.6	SPP2	32.13
	LSbis(1,3)PAP	771.6	SPP2	32.75
	OSbis(1,3)PAP	773.6	SPP2	34.05
	LnEibis(1,3)PAP	795.6	SPP2	31.35
	LEibis(1,3)PAP	797.6	SPP2	32.50
	OEibis(1,3)PAP	799.6	SPP2	33.88
	LAbis(1,3)PAP	799.6	SPP2	34.84
	OAbis(1,3)PAP	801.6	SPP2	36.32
t(N,2,N')bis (1,3)PAPs	LOLnbis(1,3)PAP	1029.8	SPNR1 grad II	20.83
	OOLnbis(1,3)PAP	1031.8	SPNR1 grad II	22.29
	LLLbis(1,3)PAP	1029.8	SPNR1 grad II	20.83
	LLObis(1,3)PAP	1031.8	SPNR1 grad II	22.29
	LOObis(1,3)PAP	1033.8	SPNR1 grad II	23.72
	OOObis(1,3)PAP	1035.8	SPNR1 grad II	25.70

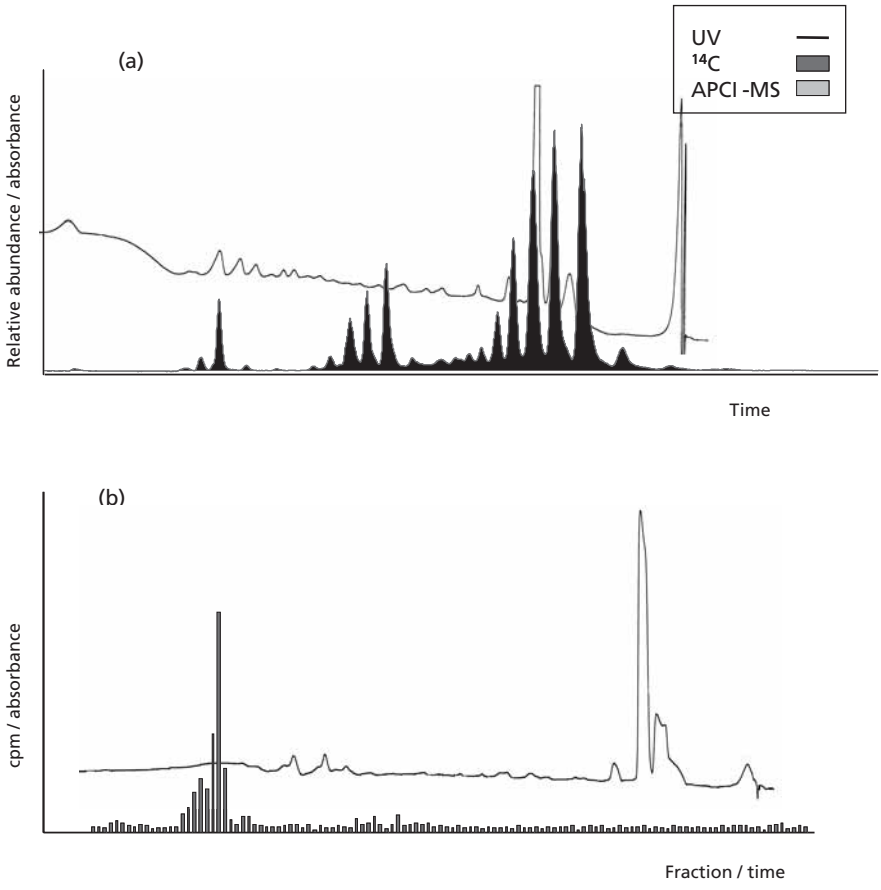
m(2)bis (1,3)PAPs	P(2)bis(1,3)PAP	481.3	SPP2	15.94
	Ln(2)bis(1,3)PAP	503.3	SPP2	13.43
	L(2)bis(1,3)PAP	505.3	SPP2	14.60
	O(2)bis(1,3)PAP	507.3	SPP2	16.14
m(N)bis (1,3)PAPs	P(N)bis(1,3)PAP	481.3	SPP2	18.09
	Ln(N)bis(1,3)PAP	503.3	SPP2	15.36
	L(N)bis(1,3)PAP	505.3	SPP2	16.62
	O(N)bis(1,3)PAP	507.3	SPP2	18.32
d(N)PAPs	LnP(N)PAP	666.6	SPNR1	26.06
	LP(N)PAP	668.5	SPNR1	27.41
	OP(N)PAP	670.6	SPNR1	28.99
	LnLn(N)PAP	688.6	SPNR1	23.26
	LnL(N)PAP	690.6	SPNR1	24.70
	Oln(N)PAP	692.6	SPNR1	26.36
	LL(N)PAP	692.6	SPNR1	26.15
	OL(N)PAP	694.6	SPNR1	27.70
	OO(N)PAP	696.6	SPNR1	29.31
	OS(N)PAP	698.6	SPNR1	31.30
	LEi(N)PAP	722.6	SPNR1	29.83
	OEi(N)PAP	724.6	SPNR1	31.36
	LA(N)PAP	724.6	SPNR1	32.00
	OA(N)PAP	726.6	SPNR1	33.48
t(1,2,N)PAPs	LLnLn(N)PAP	950.8	SPNR1 grad II	18.46
	LLLn(N)PAP	952.8	SPNR1 grad II	19.72
	LLL(N)PAP	954.8	SPNR1 grad II	21.10
	LOLn(N)PAP	954.8	SPNR1 grad II	21.10
	LLO(N)PAP	956.8	SPNR1 grad II	22.52
	OOLn(N)PAP	956.8	SPNR1 grad II	22.52
	LOO(N)PAP	958.8	SPNR1 grad II	24.18

Fig. A4.9. HPLC-UV and HPLC-radioactivity profiles from the organic and aqueous phases showing the three main areas (A, B and C) from the organic phase



This extraction method produced a very selective purification of molecules containing aniline groups, while other apolar compounds were not retained in the column. Two fractions were obtained from the SCX extraction: the non-retained (SPNR, solid phase non-retained) and the absorbed (SPP, solid phase purified) fractions. To eliminate residual triglycerides and other interferences in the SPP fraction, a second SCX extraction of the first SPP extract (SPP1) was performed to produce a highly purified extract, SPP2. The corresponding non-retained fractions were labelled SPNR1 and SPNR2, respectively. Further analyses were performed on the SPP2 and SPNR1 fractions. These fractions were analysed by on-line HPLC-UV-APCI/MS and also refractionated by HPLC in order to perform MS/MS experiments on the purified compounds (radioactivity was also monitored on the collected 30-second HPLC fractions) (Fig. A4.10 and A4.11).

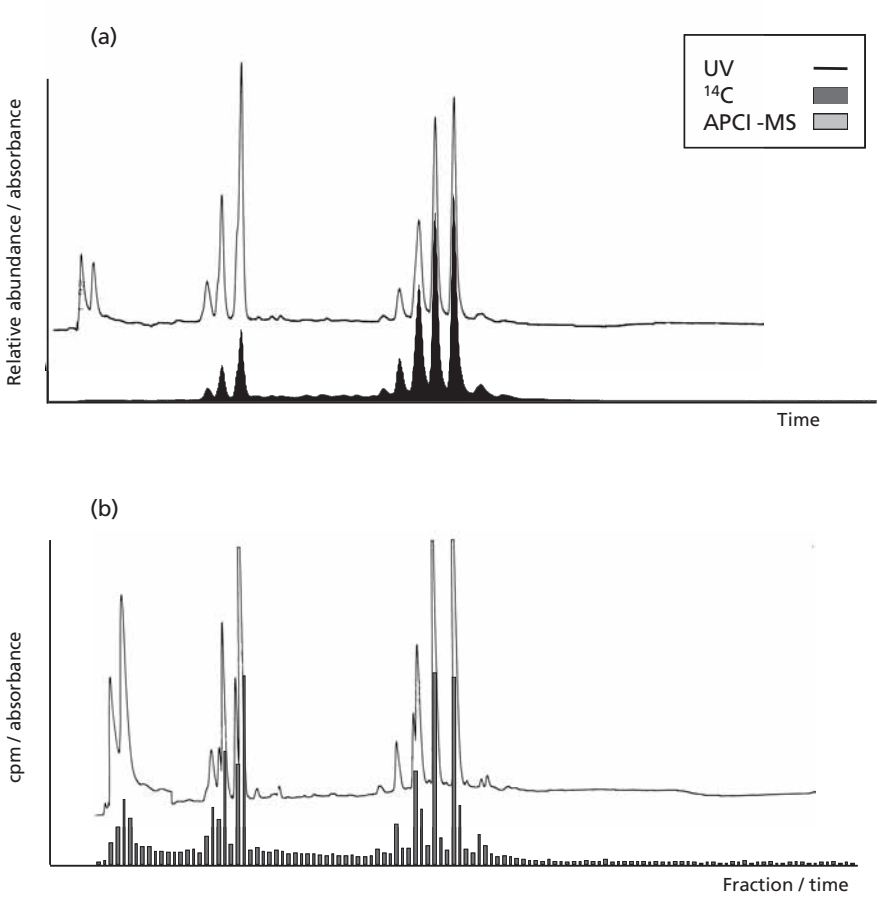
Fig. A4.10. HPLC-UV-APCI/MS (a) and HPLC-UV radioactivity (b) profiles from the SPNR1 fraction



HPLC-APCI/MS of SPP2 and SPNR1 and MS/MS analysis of HPLC fractions

The HPLC-APCI/MS data from the analysis of the SPP2 and SPNR1 fractions were studied using 3-D ion map representations of the corresponding chromatograms. These ion maps provided a rapid method of identifying families of compounds from their characteristic patterns. The relative abundance of each component of a family and their relative separation in mass and time was used as a clue to deduce where and with what intensity another component of this or another family should be found. In addition, the APCI ionization produces some confirmative fragments from these compounds derived from processes including the loss of aniline, water or one or more of the fatty acids in the structure. These confirmative fragments could be used for compound characterization without the need for MS/MS analysis, as every

Fig. A4.11. HPLC-UV-APCI/MS (a) and HPLC-UV radioactivity (b) profiles from the SPP2 fraction



family of aniline derivatives produced different and characteristic confirmative fragments.

In addition, the extracts were fractionated by HPLC-UV and the fractions analysed by HPLC-APCI/MS/MS to obtain structural information. Combining the information obtained from the 3-D ion maps and from the comparison of the MS/MS spectra of the family members with those from synthesized standards, a confident identification of the components in a family could be obtained.

The radioactivity profiles of the SPP2 and SPNR1 fractions (Fig. A4.10 and A4.11) showed a pattern similar to that shown before for the organic phase and with the three main elution areas (A, B and C) previously described (Fig. A4.9). A better separation was obtained in this case owing to the use of an optimized solvent gradient. The compounds identified from the study of

the SPP2 and SPNR1 fractions are indicated below, grouped according to the corresponding elution area. A complete list of the identified compounds is presented in Table A4.3.

Compounds in area A

The HPLC-APCI/MS analysis of the SPP2 fraction produced 3-D ion maps whereby mPAPs (retention times 11–27 minutes) were the most abundant components in the area. Twelve of these compounds were identified (PoPAP, PPAP, LnPAP, LPAP, OPAP, SPAP, EiPAP, APAP, EPAP, BPAP, C24:1PAP and LgPAP at m/z 404, 406, 428, 430, 432, 434, 460, 462, 488, 490, 516 and 518, respectively).

In addition, SPP2 displayed four major m(2)bis(1,3)PAPs at retention times of between 15 and 18.5 minutes: P(2)bis(1,3)PAP at m/z 481, Ln(2)bis(1,3)PAP at m/z 503, L(2)bis(1,3)PAP at m/z 505 and O(2)bis(1,3)PAP at m/z 507. A family of positional isomers of these compounds (m(N)bis(1,3)PAPs) was also found eluting two minutes before.

The chemical characterization of the m(2)bis(1,3)PAPs was confirmed using the synthetic standard O(2)bis(1,3)PAP that showed identical chromatographic and mass spectrometric behaviour to the corresponding oleoyl derivative in the oil. Although there was no available standard for the corresponding m(N)bis(1,3)PAP isomers, several complementary MS and MS/MS data permitted us to identify with confidence this family of compounds in oils.

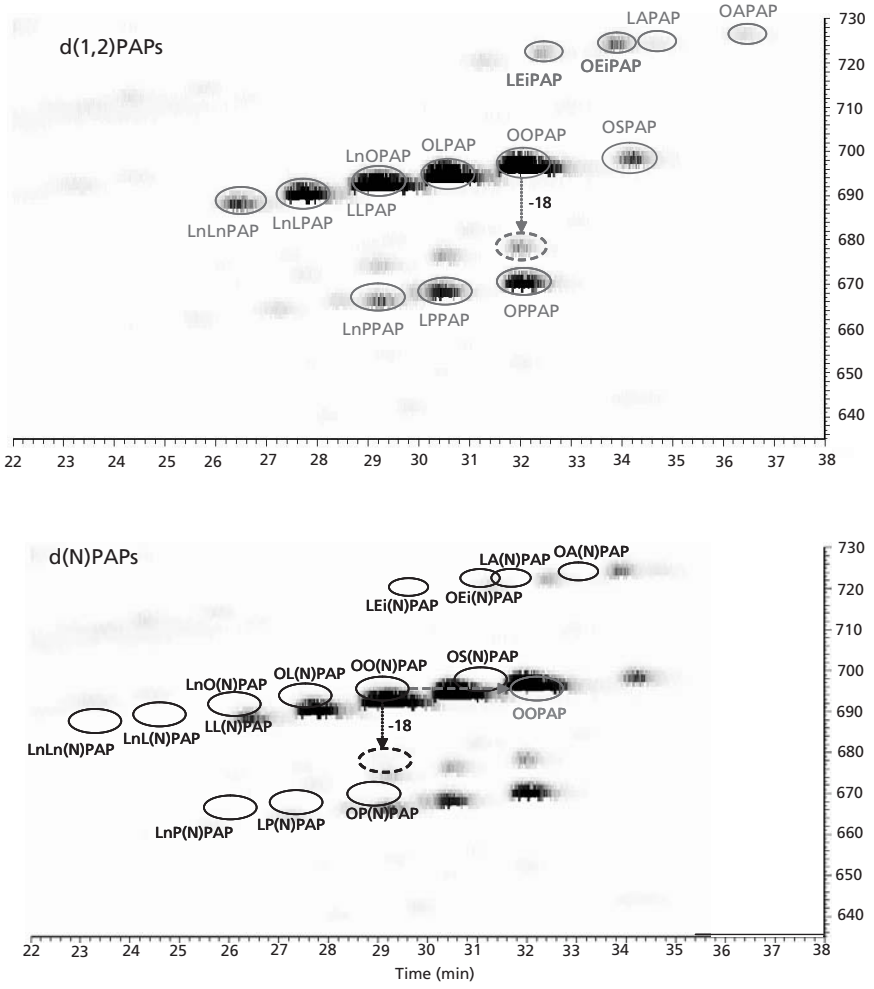
In the case of the SPNR1 fraction, fatty acid anilides were the only components detected in area A. As a whole, anilides were the more abundant aniline derivatives detected in these oils.

Compounds in area B

Major components of fraction SPP2 belonged to the d(1,2)PAP family of derivatives (the more abundant ones are shown in Fig. A4.12). Characteristic confirmative fragment ions were observed, corresponding to the loss of the fatty acid chain. These confirmative fragments allowed in some cases to discern whether a co-eluting compound was present or not. For example, at retention time 29 minutes, a signal that could correspond to LLPAP or to OLnPAP (m/z 692.6) was observed. Accompanying signals at m/z 410, 412 and 414 were also observed at the same retention time in the 3-D maps, producing a characteristic fork-like system. These fragments could be explained by the loss of linoleic, linolenic and oleic acids from the parent signal, and thus confirmed the presence of both PAPs. The shape of the fork-like group of signals also indicates that LLPAP eluted slightly before LnOPAP.

The d(N,2)bis(1,3)PAP family was also detected in this fraction. The APCI/MS/MS spectrum of the signal tentatively identified as

Fig. A4.12. The most abundant d(1,2)PAPs and d(N)PAPs detected in the HPLC-APCI/MS analysis of the SPP and SPNR extracts, respectively



Note: The small signal from non-retained OO(1,2)PAP present in the SPNR extracts can be used for comparison.

OO(N,2)bis(1,3)PAP was shown to be identical to that obtained from a standard. Major characteristic fragments for this family are derived from the loss of the aniline group and the fatty acid in the ester.

As expected, major compounds in area B of the SPNR1 fraction corresponded to diglycerides. d(N)PAPs were also found in this fraction (Fig. A4.12). The tentative structure of this family of compounds agrees with the fact that they were not retained in the SCX column. N-acylation probably

prevents the interaction of the amino group with the phenylsulfonate groups from the sorbent and, consequently these compounds were recovered in the SPNR fraction.

Additionally, and with a very low signal intensity, some d(1,2)PAPs were observed close to the d(N)PAP family (Fig. A4.12). d(N)PAPs and d(1,2)PAPs could be differentiated on the basis of the different extension, in which every compound loses structural fatty acids as seen in the corresponding product ion spectra. As indicated above for m(2)bis(1,3)PAPs and m(N)bis(1,3)PAPs, d(1,2)PAPS show losses of the two fatty acid chains while d(N)PAPs lose mainly the esterified fatty acid. The assignments were further confirmed after the synthesis and study of the chromatographic and mass spectrometric behaviour of standard OO(N)PAP and OL(N)PAP. This information was compared with that obtained from the analysis of the sample components and from a OO(1,2)PAP standard.

Compounds in area C

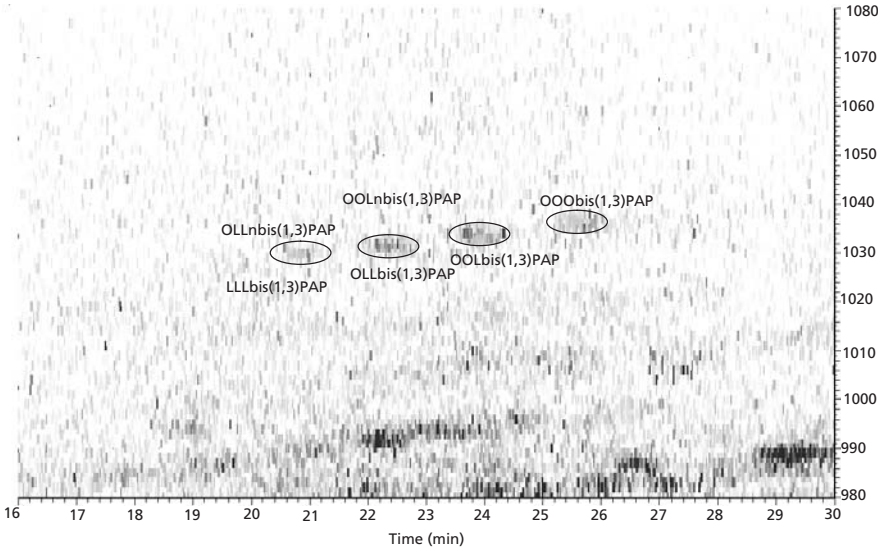
No aniline-derived compound could be identified in area C from the SPP2 fraction. In contrast, a family of t(1,2,N)PAPs was detected in the SPNR1 fraction in addition to triglycerides, which were, as expected, the most abundant components in this area. T(1,2,N)PAPs were found in very low abundance, so that sample concentration and optimized HPLC gradients were needed for its characterization. APCI/MS/MS analysis of the corresponding fractions displayed the expected fragmentation pattern for these new compounds (major losses corresponding to the loss of one or two fatty acids). The assignment was confirmed using a synthetic OOO(1,2,N)PAP standard.

The analytical conditions used for the characterization of t(1,2,N)PAPs also allowed for the identification of a new family of compounds tentatively assigned to the t(N,2,N')bis(1,3)PAP family (Fig. A4.13). Six of these compounds were initially identified on the basis of their relative retention times and molecular mass. The most intense signal corresponded to the $[M+H]^+$ ion from the trioleoyl derivative (OOO(N,2,N')bis(1,3)PAP, m/z 1036). Less intense signals were observed for the OOL (m/z 1034), OLL (m/z 1032), OOLn (m/z 1032), LLL (m/z 1030) and OLLn (m/z 1030) derivatives. To confirm these assignments, a standard of OLO(N,2,N')bis(1,3)PAP was synthesized and analysed by APCI/MS/MS (Fig. A4.14). The fragmentation pattern was identical to that obtained from the OOL derivative from the oil sample, thus allowing the assignment of the other components of the t(N,2,N')bis(1,3)PAP family.

Conclusions

A total of 117 aniline derivatives from the 9 families shown in Fig. A4.8 were identified in these samples, including anilides, mPAPs, d(1,2)PAPs, d(N)PAPs,

Fig. A4.13. Ion map of APCI/MS from SPNR1 of the most abundant t(N,2,N')bis(1,3)PAPs



t(1,2,N)PAPs, m(2)bis(1,3)PAPs, m(N)bis(1,3)PAPs, d(N,2)bis(1,3)PAPs and t(N,2,N')bis(1,3)PAPs. Only four of these families had been detected and studied previously. The anilide, mPAP and d(1,2)PAP families were the major components of our study oil. These families were composed of at least 13, 13 and 39 compounds, respectively.

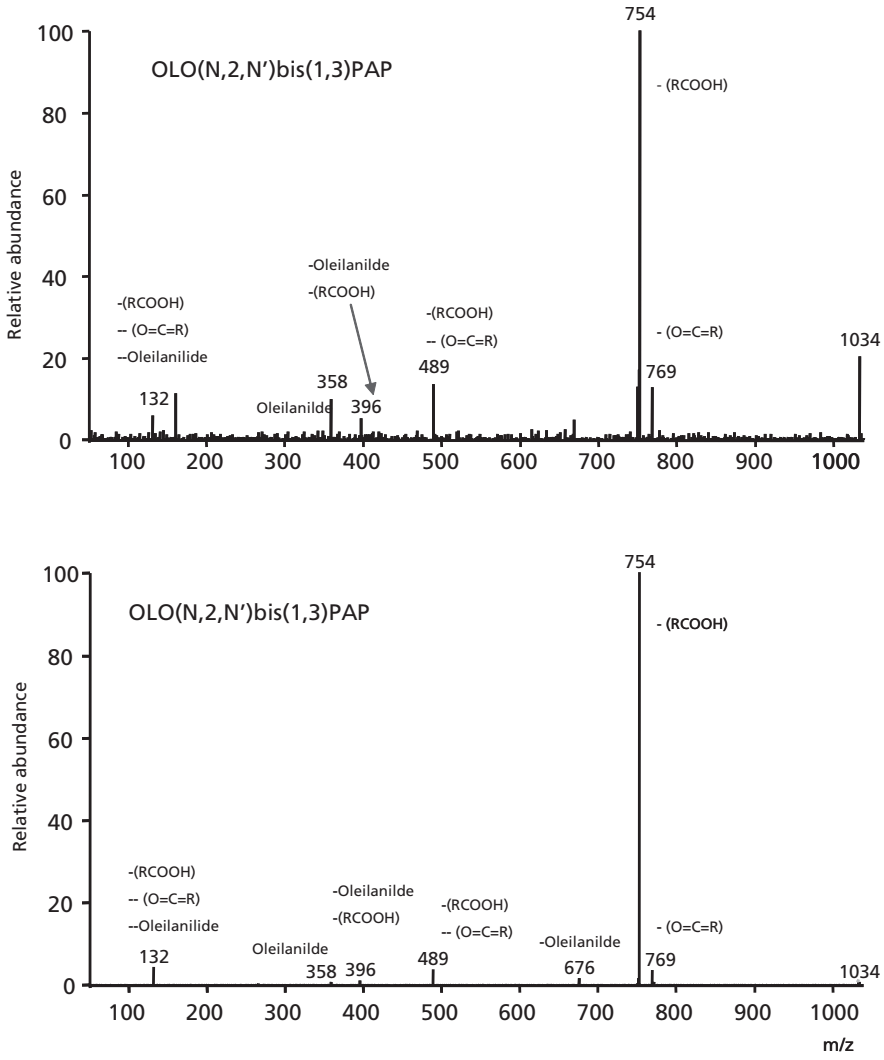
The two t(1,2,N)PAPs (OOO(1,2,N)PAP and LLL(1,2,N)PAP) previously detected by other authors in oils related to TOS (*1*) were also identified in these oils, in addition to six other t(1,2,N)PAPs containing different combinations of oleic, linoleic and linolenic acids.

Several families of compounds bearing the bis(1,3)PAP central core were detected, including 4 m(2)bis(1,3)PAP compounds and 16 d(N,2)bis(1,3)PAP compounds. Some of these compounds (all m(2)bis(1,3)PAPs, LLnbis(1,3)PAP, OLnbis(1,3)PAP, LL(N,2)bis(1,3)PAP, OLbis(1,3)PAP and OO(N,2)bis(1,3)PAP) were previously reported by Blount (*2*). Two new families with this central core were also detected: four m(N)bis(1,3)PAPs and at least six different t(N,2,N')bis(1,3)PAPs. In total, at least 30 different compounds containing the bis(1,3)PAP structure were detected in these samples by HPLC-MS. Finally, the d(N)PAP family, consisting of at least 14 components, was described for the first time in this work.

Acknowledgements

The authors thank Montse Carrascal and Juana Peña (EMEB Unit, IIBB-IDIBAPS, CSIC, Barcelona) for their technical support. Thanks are also

Fig. A4.14. MSMS spectra of OLO(N,2,N')bis(1,3)PAP from the SPNR extract and from the OLO(N,2,N')bis(1,3)PAP standard



due to the World Health Organization for financial support through Project EU/017025676.

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2. Blount, B, Schruz H. *Laboratory investigations of the TOS – progress report. Presented at the 23rd Meeting of the Joint WHO/FIS Scientific Committee for the Toxic Oil Syndrome, Copenhagen, 10–11 June 1998*. Copenhagen, WHO Regional Office for Europe, 1998 (document 98A23).
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Part 3. Synthesis of PAP derivatives

Anna Morató and Angel Messeguer

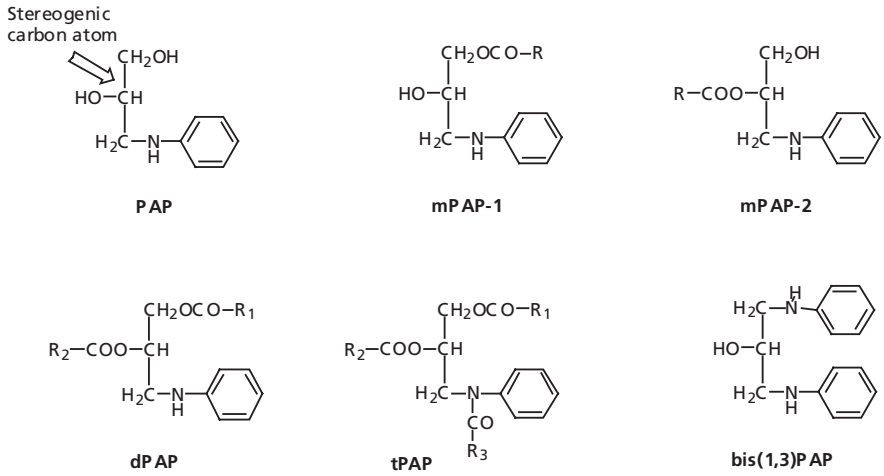
The interest in 3-(*N*-phenylamino)-1,2-propanediol (PAP) and its acyl derivatives (Fig. A4.15) in investigations related to TOS raised the question of the need to synthesize authentic standards for use in toxicological studies. As in the case of fatty acid anilides (*1*), our laboratory was commissioned by the WHO/CISAT Scientific Committee to provide high purity batches of these compounds. The anticipation that isotopically labelled samples of these PAP derivatives could also be needed led us to develop procedures versatile enough to fulfil these demands. In addition, it should be noted that PAP and its acyl derivatives are chiral molecules owing to the presence of a stereogenic centre at C-2. Thus the synthetic methodology devised would also need to cover the eventual provision of the appropriate chiral pool of PAP derivatives.

Synthesis of PAP and bis(1,3)PAP

Of all the compounds depicted in Fig. A4.15, PAP and bis(1,3)PAP could be considered as those from which the desired acyl derivatives can be obtained. We therefore devised a general method for the preparation of these hydroxylic compounds.

The treatment of glycidol, a readily available substrate, with a slight excess of aniline in the presence of methanol as solvent leads to the formation of PAP as the main component of the crude reaction mixture (Fig. A4.16a) (*1*). Under these conditions the epoxide opening is highly regioselective, and only minor amounts of the diol where aniline has been inserted at C-2 have been observed. Nevertheless, the fact that the secondary amino group present in

Fig. A4.15. Structures of PAP, its acyl derivatives mPAP, dPAP and tPAP, and bis(1,3)PAP



PAP is reactive enough to promote the opening of glycidol compromises the isolation of the pure compound in high conversion yields, and those obtained are usually moderate (50–60%). In any case, the simplicity of the experimental protocol and the ease of purification (either by distillation under reduced pressure when working in the multigram scale or by chromatographic methods, particularly TLC, for the milligram scale) makes this the procedure of choice for the preparation of this aniline derivative. Once isolated, PAP is a colourless solid, soluble in a wide variety of polar solvents and in water, and highly stable if stored at –20 °C in an inert atmosphere. Its purity can be easily monitored by HPLC, using either reverse or direct phase (A. Morató et al., unpublished data, 2003). PAP can also be analysed by GC if conveniently derived (e.g. by silylation).

The synthesis of bis(1,3)PAP involves a two-step procedure (Fig. A4.16b). Thus, reaction of epichlorhydrin with aniline under the conditions mentioned above leads to a chloro derivative intermediate, which is converted into the desired compound by treatment with a second molecular excess of aniline at high temperature. This compound is also a solid, and it should be noted that it is the only anilino derivative related to PAP that is not a chiral molecule.

Synthesis of PAP acyl derivatives

Fig. A4.17 shows the synthetic pathways for obtaining the different acyl derivatives derived from PAP. Thus, monoacylation can be carried out by allowing PAP to react with the appropriate fatty acid in the presence of *N*,*N*'-dicyclohexylcarbodiimide and 4-dimethylaminopyridine. The corresponding

Fig. A4.16. Synthesis of PAP and of bis(1,3)PAP

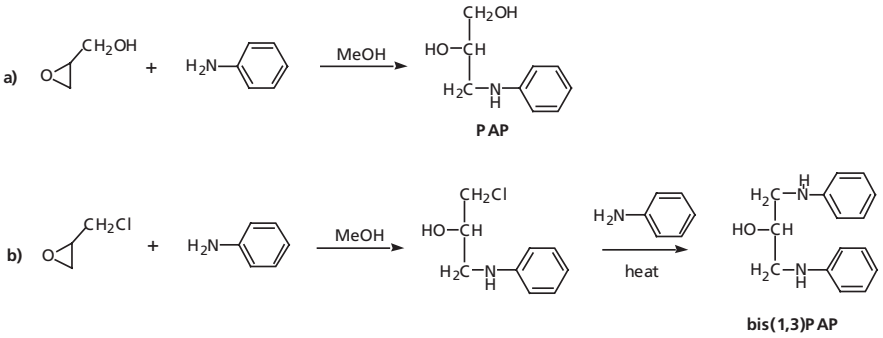
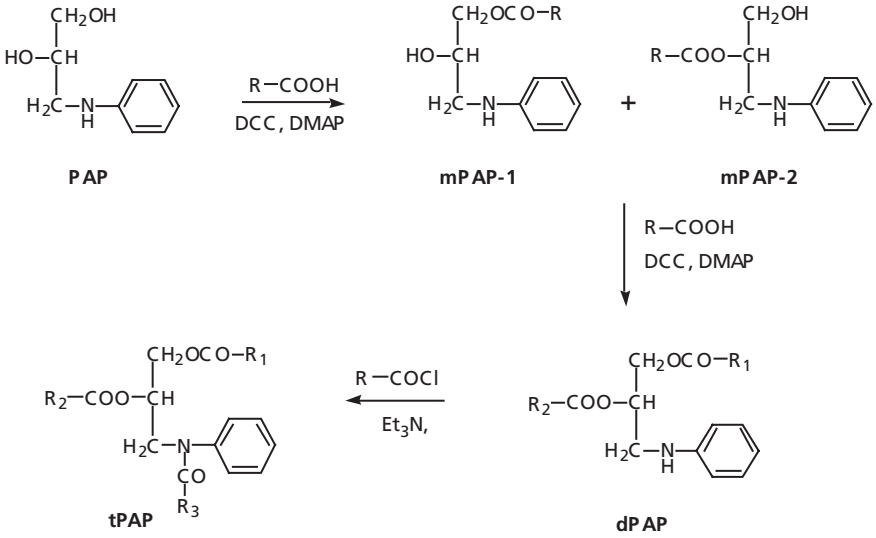


Fig. A4.17. Synthesis of PAP acylated derivatives



mPAP-1 derivative is the major component of the crude reaction mixture and can be isolated in approximately 60% conversion yield. In addition, m-PAP-2 is formed in a 10–20% conversion yield. Finally, a small amount of the dPAP derivative is also observed.

The purification of these monoester derivatives is troublesome. Actually, mPAP-1 derivatives can be isolated in high purity by chromatographic

procedures, in particular reverse phase HPLC. The use of TLC on silica gel supports leads to samples of this monoester contaminated with variable amounts of its respective mPAP-2 regioisomer. In addition to the detection of both compounds by HPLC (A. Morató et al., unpublished data, 2003), these compounds can be easily identified by NMR owing to the difference in chemical shifts of the CH_2 and CH groups, depending on whether their hydroxyl groups are acylated or not (*1*). The contamination is due to the tendency of these monoesters to interconvert to each other by acyl rearrangement. Acyl rearrangement is well known in the chemistry of glycerolipids and, in this respect, PAP derivatives exhibit similar behaviour.

The interconversion equilibrium is commonly shifted towards the mPAP-1 regioisomer, a fact that renders almost impossible the isolation and storage of pure samples of mPAP-2 derivatives. In our experience, only purification by reverse phase HPLC led to the isolation of highly enriched samples of mPAP-2 esters. Nevertheless, further manipulation of the collected eluates under carefully controlled conditions resulted in partial rearrangement to give the mPAP-1 regioisomer. (A. Morató et al., unpublished data, 2003). Moreover, this acyl migration leads to the formation of minor quantities of PAP itself.

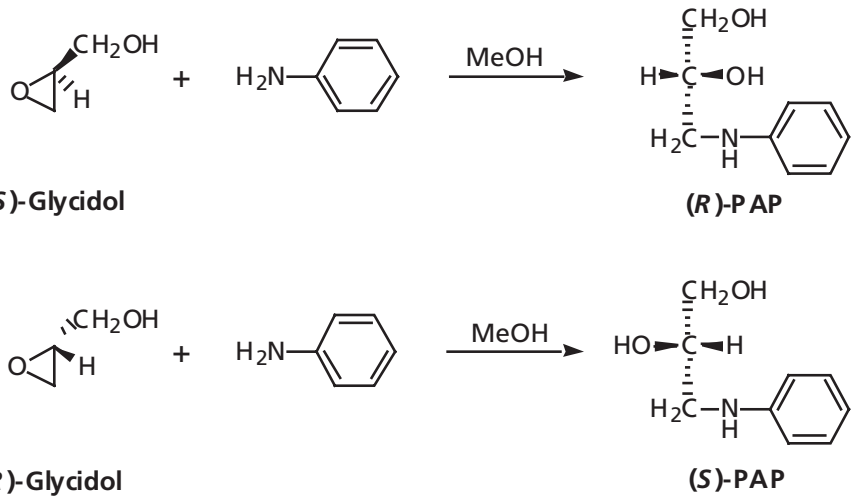
On the other hand, when the diacylated derivative is the target synthetic compound, treatment of PAP with two molecular equivalents of the fatty acid under the above conditions leads to the isolation of the corresponding dPAP diester in good conversion yields. In this case, purification of the compound is easily accomplished by flash chromatography or preparative TLC. In general, dPAP derivatives are highly lipophilic, and their stability depends on the nature of the fatty acyl residues present in the molecule. It should be noted that double acylation can be conducted with different fatty acids in order to give mixed dPAP derivatives. However, acyl rearrangement makes the synthesis of mixed dPAP derivatives with a predefined composition at C-1 and C-2 rather difficult.

The triacyl derivatives of PAP (tPAP) are obtained in good yields by treatment of the corresponding dPAP with the appropriate fatty acid acyl chloride in the presence of a tertiary amine (Fig. A4.17). In this case, acylation with an acid different from those present in the starting diester is more feasible owing to the higher stability of dPAP derivatives.

Synthesis of chiral PAP derivatives

The preparation of the chiral versions of PAP and its acylated derivatives can be carried out using the same procedure described above for the racemates, but starting from the commercial chiral pool of (*R*)- and (*S*)-glycidol (Fig. A4.18). Thus, reaction of aniline on (*S*)-glycidol leads to the formation of (*R*)-PAP, whereas the same reaction on (*R*)-glycidol gives rise to (*S*)-PAP. Likewise, acylation of the latter diol leads to (*S*)-mPAP-1 or (*S*)-dPAP, whereas the respective stereoisomers can be obtained starting from (*R*)-PAP. Both reactions

Fig. A4.18. Synthesis of PAP stereoisomers



take place with a high degree of enantioselectivity. The stereochemical purity of these compounds can be assessed by chiral HPLC, specifically by using a CHIRALCEL® column, and the results obtained are over 90% (*2*, A. Morató et al., unpublished data, 2003).

Preparation of isotopically labelled PAP derivatives

Finally, the same general procedure described above can be used to synthesize isotopically labelled PAP derivatives (*3,4*). In addition to the commercial availability of the most common fatty acids labelled with either radioactive (^{3}H and ^{14}C) or nonradioactive (^{2}H and ^{13}C) isotopes, aniline isotopomers can be obtained from commercial sources with both radioactive (^{3}H and ^{14}C) and nonradioactive (^{2}H, ^{13}C and ^{15}N) labelling. The moderate yields obtained in the formation of PAP and the high cost of radioactive aniline makes this procedure particularly delicate, and only the possibility of recovering the unreacted aniline in the final TLC purification is of some help in counterbalancing this drawback.

In conclusion, a general route has been developed for the synthesis of PAP and its diverse acyl derivatives. This route can also be applied satisfactorily to the preparation of the corresponding stereoisomers and to a wide variety of radioactive and nonradioactive isotopomers of these compounds. In general, reactions take place with satisfactory or good conversion yields, and compounds can be isolated by conventional purification procedures. Finally,

the use of chromatographic techniques, in particular different HPLC versions (direct, reverse and chiral phase), allows easy monitoring of the processes and a confident determination of the purity of the final compounds.

Summary

The interest in 3-(*N*-phenylamino)-1,2-propanediol (PAP) and its acyl derivatives in investigations related to TOS led to the need to synthesize authentic standards for use in toxicological studies. Accordingly, a general route has been developed for the synthesis of PAP and its different *O*- and *N*-acyl derivatives. This route can also be applied satisfactorily to the preparation of the corresponding stereoisomers and to a wide variety of radioactive and nonradioactive isotopomers of these compounds. Likewise, a similar general procedure has been set up for the synthesis of bis(1,3)PAP and its *O*- and *N*-acyl derivatives. Reactions take place with satisfactory to good conversion yields, and compounds can be isolated by conventional purification procedures. Finally, the use of chromatographic techniques, in particular different HPLC versions (direct, reverse and chiral phase), allows the easy monitoring of the synthetic processes and a confident determination of the chemical and stereochemical purity of final products.

Acknowledgements

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Part 4. Reproducing the refining process

Victoria Ruiz Méndez and Manuel Posada de la Paz

During the first few years after the development of the TOS outbreak, many Spanish and foreign laboratories tried to develop a method to reproduce the chemical characteristics of the oil that supposedly caused TOS. All of these trials were based on standard refining processes, without taking into consideration what could have specifically occurred with this oil (*1*). Although some visits took place in 1981 to industries involved (*2*), there was a lack of real communication between the scientists and the people working for companies involved in the refining process. A frank exchange of information on the actual conditions in use at the various refineries that fraudulently handled aniline-denatured oil at the time of the outbreak was prevented by the fear of legal consequences.

On the other hand, attempts to reproduce the refining process in the laboratory did not follow a specific chemical pattern. The presence of oleoyl anilide seemed to be the only clue to be pursued, but this had the serious drawback that this compound is spontaneously formed in aniline-denatured oils after a few days (*3*).

At the end of the 1980s a new stage began owing to: (*a*) a better characterization of case oils provided by the first toxico-epidemiological study (*4*); and (*b*) a deeper knowledge both of what was likely to have actually happened in the refineries involved and of the means of distributing the denatured oil (*5*). At the beginning of the 1990s, some chemical–epidemiological studies were published (*6,7*) and at the same time personal interviews were held

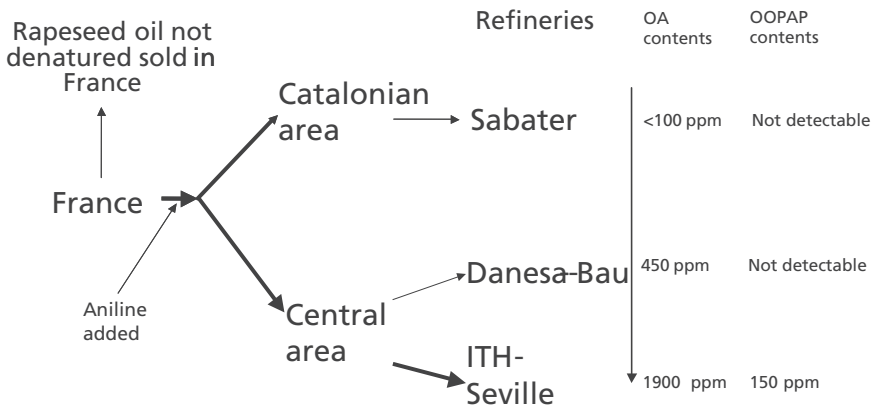
with workers at the two refineries suspected of being most involved: ITH and Danesa Bau (*8*).

Fig. A4.19 depicts the two main routes by which the denatured rapeseed oil was distributed for processing and the factories involved. Cases were not registered in the Catalonian area of delivery. The vast majority (99%) of the TOS cases occurred in the central and north-western provinces of Spain. Three cases, however, were identified in the south and were the result of ingestion of oil from the ITH refinery in Seville (*9*). The oil refined by ITH and distributed by a company in Madrid had been identified as the principal, and probably the only, oil responsible for the TOS epidemic (*10*).

Aniline derivatives were identified among several contaminants in this oil (*11,12*) but the precise etiological agent remains unknown. Nevertheless, research has established that increasing concentrations of fatty acid anilides in the oils are associated with an increased risk of developing TOS (*4,6*). Moreover, it was demonstrated that some derivatives that had been described years before (PAP derivatives) (*3*) showed a higher risk than the fatty acid anilides (*10*). Some of these substances, including PAP, OPAP and OOPAP, were found in aniline-denatured rapeseed oil refined at ITH but not in stocks of denatured rapeseed oil refined elsewhere. Moreover, these PAP esters were not detected in unrefined aniline-denatured samples of rapeseed oil delivered to ITH (*13*). Hence it is likely that the causal toxic agent(s) were formed in the denatured rapeseed oil during the refining process, and that the process varied from one factory to another.

Systematic investigation of these facts encouraged the WHO/FIS (now WHO/CISAT) Scientific Committee for the Toxic Oil Syndrome to approach the problem. OOPAP was used as a chemical marker of the refining process, much in the same way as it was used as a chemical risk marker of the disease.

Fig. A4.19. Main routes by which denatured rapeseed oil was distributed, factories involved in the refining process, and aniline derivatives detected in their oil samples



Refining process

Refining is a process of removing unwanted minor components that make oils unappealing to consumers. The two main refining routes are alkaline refining and physical refining (steam stripping, distillative neutralization), which are used for removing the free fatty acids. Some physical losses are highly desirable, for example, the removal of off-flavours, pesticides and polycyclic aromatic hydrocarbons, if present. Other losses of nutritionally valuable components, such as tocopherols and sterols, are potentially undesirable (*14*).

The alkaline neutralization process has major drawbacks: the yield is relatively low and oil losses occur owing to emulsification and saponification of neutral oil. Also, a considerable amount of liquid emuent is generated. The soaps are generally split with sulfuric acid to recover free fatty acids along with sodium sulfate and some fat-containing acid water steam.

Deodorization is essentially a steam distillation process carried out at low pressure (2–6 mbar) and high temperatures (180–220 °C). In physical refining, the fatty acids are removed by a steam distillation (stripping) process similar to deodorization. The low volatility of fatty acids (depending on chain length) requires higher temperatures in physical refining than those required for deodorization only. In practice, a maximum temperature of 240–260 °C is sufficient to reduce the free fatty acid content to about 0.05–0.1%. A prerequisite for physical refining is that phosphatides be removed to a level below 5 mg phosphorus/kg oil. In the classic refining process, this level is easily achieved during the neutralization stage, but special degumming processes may be required for physical refining of high-phosphatide seed oils.

Both alkali and physical refining are now the standard techniques used. The classical alkali refining procedure includes caustic treatment to neutralize the oil and, following physical refining, free fatty acids are eliminated by distillation during deodorization.

The processing conditions used by ITH (*8*) consisted of three steps: neutralization without excess of lye; bleaching without specified conditions (although it was stated that owing to the colour of the oil this step had been repeated); and finally deodorization in a discontinuous device of 10-tonne capacity. These differ from the standard conditions for rapeseed oil processing described by Kochhar (*1*). In fact, the latter were those recommended for caustic refining of rapeseed oil, and should be similar to those used for soybean oil, including the use of a degumming step with phosphoric acid prior to neutralization, bleaching with 2–6% of activated clays and very soft conditions of temperature during deodorization (220 °C).

Under the supervision of the WHO/CISAT Committee, three laboratories began to develop methods to reproduce, on a laboratory scale, the refining process that took place at the ITH refinery in 1981. The conditions used are shown in Table A4.4. The aim was to produce denatured, refined rapeseed oils

Table A4.4 Conditions used in the refining processes developed in three laboratories supervised by WHO/CISAT in order to reproduce an oil with the specific pattern of compounds found in toxic oil from ITH

Condition	Laboratory			
	Leatherhead	**Oregon**	**Seville 1**	**Seville 2**
Waiting time	1 week	0–4 weeks	3 days to 8 weeks	8 weeks
Storage temperature	50 °C	16 °C and 50 °C	Room temperature	Room temperature
Degumming	0.15% phosphoric acid	0.15% phosphoric acid	None	None
Neutralization	NaOH 20 °C; Be 80 °C	NaOH 20 °C; Be 80 °C	NaOH 20 °C; Be 80 °C	NaOH 20 °C; Be 80 °C
Washing	Water and brine	None	Water and brine	Water and brine
Bleaching				
– Temperature	110 °C	110 °C	110 °C	110 °C
– Percentage	1% Gador earth	2% bleaching earth	1% Gador earth	2.5% Gador earth
– Pressure	20 Torr	20 Torr	60 Torr	60 Torr
Deodorization				
– Temperature	210–270 °C (20 °C)	210–270 °C (20 °C)	200–270 °C (15 °C)	260 °C
– Time	4.5–10 hours	4.5 hours	4–6 hours	5 hours
– Pressure	7–10 Torr	7–10 Torr	3 Torr	3 Torr

with a specific pattern of compounds similar to those of known toxic oils, as observed in the ITH oils. Two laboratories worked on the project for several years (*15,16*), but on only one occasion did the levels of OOPAP approach those seen in the original ITH oil. Unfortunately, the results could not be replicated and were not validated by the Committee (*15*).

Laboratory assays

A systematic work plan was established between the Instituto de la Grasa in Seville (IGS) and CISAT for determining on both the laboratory and the industrial scale those variables that led to the production of the toxic oil. The contents of both oleoyl anilide and OOPAP in the ITH oil were used as the gold standard for these experiments (Fig. A4.19). Complete chemical characterization was performed at the Centers for Disease Control and Prevention

(CDC) in the United States and at the Instituto de Investigaciones Biomédicas de Barcelona (IIBB). In the first part of the work, after denaturing but prior to refining, each sample was stored at room temperature for 1, 2, 3, 4 or 8 weeks. Neither OOPAP nor other diesters of PAP appeared in the stored oil and only fatty acid anilides were detected in these samples (*17*).

The process stated by ITH workers as having been used at the time of the epidemic (*8*) was used initially. However, these conditions were similar to those used in the refining of olive oil (partial neutralization followed by removal of residual free fatty acids at the deodorization stage) and did not result in oils with the same characteristics as those obtained at ITH. After storage, neutralization and bleaching, the effects of deodorization temperatures and deodorization operation times on the oil were studied in order to identify the refining conditions necessary for toxin formation. The following samples were analysed: denatured crude oil and oils deodorized at 270 °C for 6 hours, 260 °C for 6 hours, 260 °C for 5½ hours, 245 °C for 6 hours, 245 °C for 4½ hours, 230 °C for 5 hours, 215 °C for 4½ hours and 200 °C for 4 hours.

The deodorization step appeared to lead to a reduction of total free fatty acids in all of the samples (*17*). This reduction was most pronounced when the deodorization temperature was above 260 °C. PAP esters were not detected in these oil samples and the quality of the bleached oils was very poor, with significant amounts of phosphorus and soaps, indicating that the refining process used was not appropriate for that type of oil.

The oil was green in colour owing to the presence of large quantities of chlorophyll or chlorophyll derivatives. Yellow and red carotenoid pigments normally present in the oil usually mask the green colour. Once the yellow and red pigments are removed in the refining process, the green colour becomes apparent, often as late in the process as deodorization (*18*). As the denatured oil was to be sold as olive oil, the green colour should have been an advantage from the point of view of fraudulent distribution. Assuming that ITH followed the typical olive oil refining process, PAP esters should not have been produced.

It is likely, therefore, that other conditions existed at the time of refining, which, owing to the presence of aniline, led to the formation of PAP esters and other PAP-related compounds. Further investigations were carried out to find the conditions under which OOPAP was produced. Accordingly, a study was undertaken to establish the importance of the different refining steps on OOPAP formation. This included degumming conditions, the effects of varying acidity and the percentage of bleaching earth, the addition procedure and operation time. It also included the possible influence of the oxidation grade of the aniline used to denature the oil. Apart from the generally poor oil quality caused by the inappropriate refining procedures, the samples had no other notable characteristics. Intermediate and final samples checked by IIBB (*19*) had no trace of OOPAP.

OOPAP was also added as a marker to a rapeseed oil in a complete refining process in order to determine the important steps in the elimination of these compounds. In this process, 1 kg of OOPAP-enriched denatured oil was neutralized with 20 °Baumé lye, bleached with 5% Gador C Earth at 110 °C for 180 minutes and deodorized under vacuum at 230 °C for 4 hours. These assays confirmed that bleaching negatively affects the presence of OOPAP. However, deodorization seemed to be positive in this assay, demonstrating that once formed, OOPAP was stable under standard deodorization conditions.

Distillation assays

In 1995, Hill et al. (*13*) performed experiments using two different samples of oil: a locally purchased canola oil (a variety of rapeseed oil produced in Canada) with 1% aniline added and a Catalonian rapeseed oil manufactured during the time of the epidemic, which contained anilides but not PAP esters or additional aniline. Both samples were heated at 300 °C for 4 hours, and PAP esters were formed in both cases. This suggests that a high temperature might be necessary to form the PAP compounds.

The main differences between the distillation assays performed by Hill and normal deodorization were that extremely high temperatures were used and that stripping steam was not introduced. At that point, the deodorization process carried out at ITH was taken into account. Discontinuous deodorizers such as those used at ITH are vessels capable of holding 10 tonnes of oil while withstanding a vacuum of 10 Torr. The deodorization process begins at around 160 °C as steam is blown in through perforated pipes fixed to the bottom of the vessel (*14*). The oil is heated to a temperature between of 180–260 °C by circulating hot mineral oil through coils inside the deodorization vessel. Mineral oils and mineral oil derivatives with a working range of between –10 °C and 330 °C were the most commonly used heating fluids in the edible oil industry at the time of the TOS epidemic (*20*).

A situation analogous to a distillation without stripping gas could occur in an industrial-scale apparatus if the agitation produced by the stripping steam was insufficient to frequently renew the layer of oil near the heating coil. In this way, the stagnant layer of oil could reach temperatures of over 300 °C.

Further systematic assays were carried out to evaluate the influence of an elevated refining temperature, such as might occur in close proximity to a deodorizer coil, and to account for storage time prior to refining (*21*). Two samples of rapeseed oil were initially prepared. The first sample was denatured with 2% w/w aniline (99.5% purity) and stored for one week prior to neutralizing and bleaching. The second sample was denatured with 2% w/w aniline (99.5% purity) and stored for three weeks before neutralizing and bleaching, which involved refining 1 kg oil from each storage time by neutralizing with 20 °Baumé (4.18M) lye and then bleaching with 5% (w/w) Gador C Earth at 110 °C for 20 minutes.

The results related to PAP derivatives obtained in these assays were very promising. Fig. A4.20 shows the results obtained for the quantification of the PAP esters. The amount of PAP esters in aniline-denatured oil increased dramatically when the temperature of the oil was raised from 250 °C to 300 °C. Those formed at 300 °C, however, were lost during processing at that temperature. The level maintained during operation at 300 °C was higher in the samples stored for three weeks before refining than in those stored for only one week. This suggests that the length of time the denatured oil is stored before refining is also a factor in the quantity of contaminants formed.

The total quantities of LLPAP and OLPAP formed during the period of heating to 300 °C were slightly lower than that of OOPAP, which is to be expected because of the relative proportions of these fatty acids in the oil. It is interesting to note that the amount of OPAP seemed to increase more during the heating period than the other related compounds. This might suggest that OPAP is an intermediate compound in the formation/decomposition of diacyl esters.

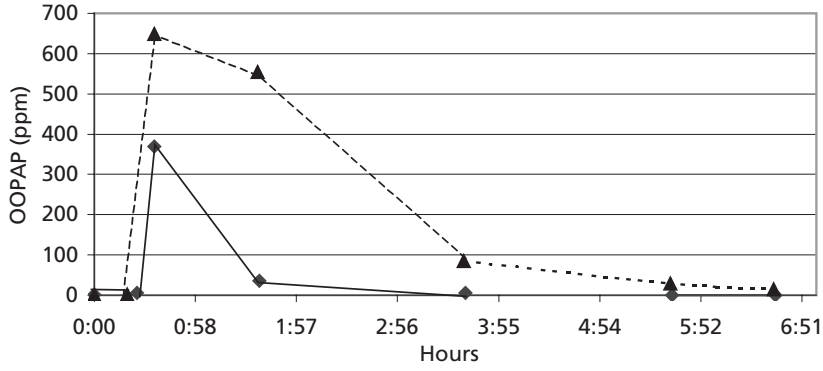
Two assays were carried out to evaluate the influence of the different refining steps on PAP ester formation during the refining of an oil supplemented with radioactive markers, confirming the results of previous experiments. Refined samples contained only approximately 3% of the initial radioactivity. This suggests that, in agreement with the results of other studies, the main compound eliminated is aniline. Although practically all the mass was recovered, radioactivity was lost in those steps where vacuum was applied, in spite of efforts to trap the labelled compounds before and after the vacuum pump.

Although there could be more than one pathway for the generation of these compounds, the heating temperature was not enough to break the acylglycerol–ester linkages; hence, a very low proportion of these compounds would have been contaminated by aniline. The greater quantities of free fatty acids and monoacyl and diacyl glycerols found at temperatures above 280 °C suggests that formation of aniline esters might be facilitated; however, high levels of PAP decreasing with time suggests that they may decompose at such a high temperature. The esters formed might be degraded more rapidly during deodorization at 300 °C than at lower temperatures.

Assays carried out at the IGS pilot plant

Three lots of 25 kg of rapeseed oil with <1% erucic acid, obtained from the Andalusia region, were denatured with 0.5 kg (2% w/w) aniline and, before refining, stored at room temperature for five weeks. Two thermocouples measured the temperature, one in the centre of the vessel and the other at the nearest point to the thermal fluid coil. Deodorization was conducted under a programmed vacuum and the steam flow was 2%/hour in the first and second assays. In the third assay, steam was not introduced.

Fig. A4.20. Amount of OOPAP before, during and after distillation at 300 °C in rapeseed oil denatured and stored for 1 week (◆) and 3 weeks (▲) before neutralizing and bleaching



In the first assay, an intermediate sample was taken when the oil reached 230 °C. After 4 hours at that temperature, the oil was allowed to cool and the vacuum was released with nitrogen.

In the second assay, after 2½ hours at 290–300 °C the oil was allowed to cool and the vacuum was released with nitrogen. Two small aliquots were taken out after 107 minutes of treatment, when the temperature reached 290 °C. There were difficulties in reaching 300 °C in such a short time.

The last assay was a distillation, under similar conditions to those used in the laboratory. After 20 minutes at 290–300 °C the oil was allowed to cool and the vacuum was released with nitrogen. Small aliquots were taken out after 47 minutes, when the temperature was 290 °C. Steam was not introduced in this assay.

Table A4.5 shows the results obtained in the quantification of PAP esters and anilides in the initial, intermediate and final samples. OOPAP was found only in this latter assay, when high temperature was applied for a shorter time and steam was not introduced. These results were consistent with those found in the laboratory assays.

Conclusions

In conclusion, the profile of aniline-containing compounds found in the oils refined by ITH was reproduced under these extreme conditions.

The hypothesis is that the ITH refinery most likely followed the normal procedure for deodorizing poor quality oil. The process ITH followed conceivably involved enormous difficulties in the bleaching step, and the strong undesirable flavour may have required an increase in the temperature used in

Table A4.5. PAP esters and anilides before, during and after deodorization and distillation of denatured, neutralized and bleached rapeseed oil

Procedure	PAP esters[a]				Anilides[b]			
	OPAP (ppm)	LLPAP (ppm)	OOPAP (ppm)	OLPAP (ppm)	LNA (ppm)	LA (ppm)	OA (ppm)	ECA (ppm)
Deodorization at 230 °C								
Bleached oil	<0.1	<0.1	<0.1	<0.2	675	2 151	5 707	146
Intermediate sample	3.3	11	21	18	834	1 934	5 128	276
Final cooled sample	<0.1	<0.1	<0.1	<0.2	742	1 743	4 702	257
Deodorization at 295 °C								
Bleached oil	<0.1	<0.1	<0.1	<0.2	675	2 151	5 707	146
Intermediate sample at 290 °C	1.7	7.4	14	16	328	975	2 755	124
Final cooled sample	0.8	3.9	8.2	9.4	119	364	996	79
Distillation at 300 °C								
Intermediate sample at 290 °C	6.6	128	361	282	1 442	6 022	11 211	219
Final cooled sample	9	40	146	131	1 439	5 946	11 357	216

[a] OPAP = oleoyl monoester of PAP; LLPAP = linoleic diester of PAP; OOPAP = dioleoyl diester of PAP; OLPAP = oleoyl-linoleoyl diester of PAP.

[b] LNA = *N*-phenyl linolenamide; LA = *N*-phenyl linoleamide; OA = *N*-phenyl oleamide; ECA = *N*-phenyl eicosanamide.

deodorization and consequently an increase in the temperature of the thermal heating fluid. These circumstances, applied to such unusual oil, could have given rise to the formation of anilides and PAP esters in the area near the heating coil, followed by the dilution of these compounds and other derivatives in the oil caused by the action of the stripping gas.

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This is the third book published by WHO on the outbreak of the condition that came to be called the toxic oil syndrome (TOS), which struck Spain in 1981. It killed several hundred people and affected more than 20 000, many of whom remain ill today. The two previous books described, respectively, early observations and scientific findings gathered throughout the 1980s.

This volume reflects the progress made in the last ten years under a carefully planned strategy undertaken on four main fronts by the WHO/CISAT Scientific Committee for the Toxic Oil Syndrome. First, various projects were supported aiming at the full chemical characterization of the oil matrix. Second, attempts were made to reproduce, on both a laboratory and an industrial scale, the refining process to which the suspect oil had been subjected in an attempt to establish the conditions under which the toxin(s) were generated and to provide sufficient amounts of reconstituted oils for toxicological studies. Third, a search was undertaken for an animal model in which to study the disease. Finally, work on the possible immune origin of the intoxication was stepped up, with promising results to date.

World Health Organization
Regional Office for Europe

Scherfigsvej 8
DK-2100 Copenhagen Ø
Denmark

Tel.: +45 39 17 17 17
Fax: +45 39 17 18 18
E-mail: postmaster@euro.who.int
Web site: www.euro.who.int

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The World Health Organization (WHO) is a specialized agency of the United Nations created in 1948 with the primary responsibility for international health matters and public health. The WHO Regional Office for Europe is one of six regional offices throughout the world, each with its own programme geared to the particular health conditions of the countries it serves.

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