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PHD

Method development for the analysis of organochlorine residues in human milk

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METHOD DEVELOPMENT FOR THE ANALYSIS OF ORGANOCHLORINE
RESIDUES IN HUMAN MILK.

Submitted by MARK P. SEYMOUR BPharm MSc MPS CChem MRSC
for the degree of PhD
of the University of Bath
1987.

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I am pessimistic about the human race because it is too ingenious for its own good. Our approach to nature is to beat it into submission. We would stand a better chance of survival if we accommodated ourselves to this planet and viewed it appreciatively instead of sceptically and dictatorially.

E.B. White

DEDICATION.

To my late father,

Capt. L.R. Seymour

(1927-1975)

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PREFACE.

Modern science and technology appears to be advancing at an unprecedented rate on so many fronts from the biological (such as molecular biology) to the physical (such as semiconductor technology). Underpinning much of the new pure and applied science are a variety of analytical techniques which rarely attract much attention from other scientists let alone members of the public. Analytical chemistry is frequently regarded by non-analysts as a "service" rather than a subject worthy of fundamental research in its own right, yet many important conclusions drawn by research workers rely heavily on analytical results. If the analytical chemistry is unreliable so too must be any conclusions drawn from these analyses. It follows, therefore, that unequivocal conclusions require unequivocal analyses and as modern research becomes increasingly more complex and fundamental, so the demands made on analytical chemistry become ever more stringent. This, in itself must surely be sufficient justification for supporting fundamental research into analytical chemistry. Existing analytical techniques must be developed and new approaches devised if analytical chemistry is to meet the challenge presented by the other scientific disciplines it serves.

The aim of this thesis is not only to describe the development of a novel approach to the analysis of human

milk for organochlorine residues, but also to show how advances in analytical techniques have enabled more meaningful research to be carried out regarding the toxicological significance of organochlorine pollution and thereby illustrate, in a small way, the important role analytical chemistry has to play in modern science and society.

SUMMARY.

The development of a novel approach to the analysis of human milk for residues of organochlorine contaminants is discussed. The synthesis and isolation of selected polychlorobiphenyl congeners is also described. The problems of environmental contamination with organochlorine pollutants is briefly reviewed with particular emphasis on the contamination of human milk. Analytical methodology is discussed broadly under the headings of extraction, clean-up and quantitation. Each section begins with a brief review of relevant analytical techniques followed by details of the experimental work undertaken in the development of a new approach to the analysis. The new method involved adsorption of 10 ml of milk onto a selective adsorbent followed by Soxhlet extraction with hexane. The extract was then reduced in volume using a Kuderna-Danish evaporator and cleaned up using an HP-GPC method. The HP-GPC clean-up procedure produced a lipid fraction and a residue fraction for each sample. The lipid fraction was removed, the solvent evaporated and the lipid weighed in order to provide a gravimetric determination of the lipid content of the milk. After the addition of a known amount of an internal standard, the residue fraction was further reduced in volume to about 1 ml and selected organochlorine residues were then determined using a GC-MS method employing 70 eV positive ion EIMS with selected ion monitoring. The

method was applied to twelve human milk samples. The results of the milk analyses and the performance of the method are discussed.

ACKNOWLEDGEMENTS.

I should like to express my gratitude to my supervisors, Drs T.M. Jefferies and L.J. Notarianni, for their advice, encouragement and patience. I should also like to thank Dr A.J. Floyd for spending so much of his time training me in the techniques of GC-MS.

My thanks are also due to Messrs. R. Sadler and C. Cryer for their extensive technical assistance and to Mr. I. Duncan for his help and his thought-provoking discussions.

Finally, I gratefully acknowledge the financial support provided by the Pharmaceutical Society of Great Britain in awarding me the Leverhulme Scholarship.

1. INTRODUCTION.

Note on the numbering of PCB congeners.

Throughout this work, the IUPAC system due to Ballschmiter and Zell (1) for numbering chlorinated biphenyls is used to identify individual congeners.

1.1. Organohalogenes in the Environment.

For many years now, the pollution of the environment with organohalogenes has been the focus of major research projects around the world and the volume of literature now covering the topic is vast. The contamination is extremely widespread and traces of organohalogenes can be found over much of the Earth's surface. The sources of this contamination are manifold and pollution has arisen both from normal usage of organohalogen products as well as from industrial accidents.

Organohalogenes, and in particular organochlorines, have been widely used in the past for many purposes. During World War II, DDT was used to control malaria and typhus and was found to be extremely effective in this role. After the war, DDT became widely used for other purposes including agricultural pest control.

Research into organochlorine pesticides (OCPs) produced

other materials for pest control such as the chlorinated dimethanonaphthalenes (the "drins"), the 1,2,3,4,5,6-hexachlorocyclohexanes (HCHs, formally known as BHCs), hexachlorobenzene (HCB) and many others. These materials were both cheap to produce and effective.

The polychlorinated biphenyls (PCBs) were another class of organochlorines with a diversity of uses. Mixtures of PCB congeners were made by direct chlorination of biphenyl and from 1930 to 1977 were marketed by the Monsanto Corporation under the trade name "Aroclor". During that time, a number of other companies also manufactured PCB mixtures and sold them under various brand names like "Clophen", "Phenoclor" and "Kanechlor". Uses of PCB mixtures included dielectric fluids in transformers and capacitors, heat exchanger fluids, printing ink additives and many others (2).

Organochlorines were known to be very stable and lipophilic. These two properties led to persistence of organochlorine residues in the environment and bioconcentration within the food chain. Instances of acute human toxicity had been observed for all these materials quite early in their histories but these were generally due to gross exposure to the compounds. As early as 1951, Laug et al. (3) had demonstrated the presence of DDT in the milk of lactating women, but the problems of toxicity (either acute or chronic) did not

really attract much attention until the early 1960s when the serious ecological impact of the widespread and largely indiscriminate use of OCPs was described by various authors including Rachel Carson in her book "Silent Spring" (4). These revelations attracted public and therefore political interest and during the 1960s the monitoring and control of environmental levels of OCPs became an important issue.

In the climate created by this upsurge in interest in environmental pollution, many OCP residue analyses were carried out, mainly using gas chromatography. It was common to find chromatographic peaks which did not correspond to any known OCPs amongst all the identifiable pesticide residues. S. Jensen, a Swedish scientist identified these peaks as being due to PCBs and published this finding in 1966 (5).

The numerous studies conducted worldwide on the chemistry, toxicology and ecology of persistent OCPs and PCBs culminated in bans or severe restrictions being imposed on their use by most developed countries during the 1970s and the manufacture of many of these materials has dropped dramatically as a result. Despite these measures, the environmental persistence of OCPs and PCBs, as well as the continued use of some OCP products and the widespread use of old equipment containing PCBs, will ensure that organochlorine residues will remain in the

environment for many years to come.

The World Health Organization (WHO) has taken considerable interest in environmental pollution and has commissioned several studies on a number of relevant topics. The results of these studies are published in various forms including the WHO Environmental Health Criteria series which includes monographs on DDT (6) and PCBs (7). The WHO, in conjunction with the Food and Agriculture Organization of the United Nations (FAO) use the data available to them to produce FAO/WHO acceptable daily intake (ADI) levels for many contaminants including several OCPs. Setting such limits is an extremely difficult task and the limits chosen are open to debate. However, they always aim to err on the side of caution and ADI levels are generally set at one or two orders of magnitude below the expected "no observable effect" level. Due to the particular problems encountered when trying to assess the toxicological properties of variable mixtures, the FAO/WHO have not set an ADI limit for PCBs although a limit of $1 \mu\text{g kg}^{-1} \text{ day}^{-1}$ total PCB has been suggested (8, 9). Residue limits for contaminants in individual foodstuffs are also set by many countries. A particularly controversial example of this is the 2 ppm limit set in 1979 by the US FDA (10) for PCBs in game fish which, in certain parts of the USA, are known to contain relatively high levels of PCBs. This limit was set after consideration of both toxicological and

commercial factors as well as the analytical capabilities of the time. This restriction has been seen as rather arbitrary by some workers, especially in view of the difficulties in interpreting pertinent toxicological data.

Another group of organohalogenes which have caused considerable concern over recent years are the remarkably toxic polychlorodibenzofurans (PCDFs) and polychlorodibenzo-p-dioxins (PCDDs). These substances are produced as impurities in the manufacture of other organochlorine materials (e.g. 2,4,5-T), by improper incineration of certain organochlorines or, in the case of PCDFs, by merely heating PCBs. Incidents of environmental contamination by these materials have occurred, such as that at Seveso in Italy in 1976. Fortunately, in most cases the levels have been quite low but contamination of the environment with PCDFs or PCDDs, when it arises, is a matter of serious concern. A striking example of this is the case of a housing estate in New York State, USA which was built on contaminated land. The soil was found to contain over 300 ppb of 2,3,7,8-TCDD and more than 200 families had to be relocated (11). Much effort is currently being directed towards controlling the release of PCDFs and PCDDs into the environment particularly by incinerators and organochlorine manufacturing plants. In this context, disposal of organochlorines can be a problem (12).

The human toxicological implications of residue levels of OCPs are difficult to evaluate and risk assessment is a contentious issue. However, a lot of work has been done and the FAO/WHO have set ADI limits for many OCPs. PCBs present a particularly awkward toxicological problem since they occur as mixtures of variable composition. Much work has been carried out on the toxicity of commercial PCB mixtures, but these studies can be misleading since due to selective degradation and metabolism, the pattern of PCB congeners in the environment and in biological tissues is usually quite different from the pattern seen in commercial mixtures. The toxicological properties of different congeners varies widely (13) and the only universally useful toxicological data are based on congener-specific studies. In the past, analyses for residues of PCBs have not usually been congener-specific and furthermore, have often been of dubious accuracy. These factors have meant that PCB residue levels could not be meaningfully interpreted in toxicological terms. It has, therefore, been a natural consequence that congener-specific toxicity studies and residue analyses have risen in popularity, fuelled by the improvements in analytical technology and the wider availability of individual PCB congeners. Hence it is to be hoped that future analyses of environmental samples will be mainly congener-specific and the toxicological implications of the results will be more readily understood.

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1.2. Organochlorines in the Environment - the Toxicological Implications.

The toxicological ramifications of organochlorine contamination of the environment are hard to judge and are a major source of research and debate. We are all exposed to organochlorines, mainly through our diets, but the nature and concentration of the contaminants varies widely from person to person and from population to population. Extrapolating animal toxicity data to humans is fraught with problems, but despite this, a good deal of progress has been made. Unfortunately, a further complication is the occurrence of the OCP residues and PCB residues in admixture rather than individually. This forces the question: what are the effects of consuming several different residues at once - are the effects additive, synergistic or antagonistic? In order to help "absorb" some of the uncertainty, ADI limits include a considerable "safety margin". ADI limits are set with the idea that the stated amount of the material in question can be consumed every day for life without appreciable risk. This is an extremely difficult assessment to make since human chronic toxicity is notoriously hard to predict from animal data, particularly at low levels of contamination.

1.3. Clinicopathology of OCPs and PCBs.

The effects of OCPs on animals and humans are well documented in the literature (14).

Studies of the effects of OCP and PCB poisoning in humans have generally been made only on people who have received high doses of the organochlorine material and long-term follow-up studies have provided very useful information. However, the majority of people are never exposed to high doses of OCPs and PCBs and it is difficult to know how the effects seen in "poisoned" people relate to the rest of the population. The well known "Yusho" incident in Japan in 1968, when over 1000 people consumed PCB contaminated rice oil, and the almost identical incident in Taiwan in 1979 (the "Yu-Cheng" incident) caused many health problems amongst the victims including marked dermatological, metabolic, neurological, immunological, gastrointestinal and hepatic effects (15, 16). Follow-up studies are continuing but it is widely accepted that the health effects incurred during these events were due largely to PCDFs and perhaps polychlorinated quaterphenyls (PCQs) which were also present in the contaminated oils. This has made toxicological evaluation even more difficult.

More specific toxicological data for PCBs have been obtained from people occupationally exposed to these

compounds. The levels of PCBs encountered in such people have been extremely high in some instances and clinical effects have been observed, particularly dermatological problems, but the symptoms appear to be mild compared to "Yusho" patients and are reversible on ceasing exposure (17, 18). This is explained by the absence of PCDFs and PCQs in people occupationally exposed to PCBs.

As an example of OCP poisoning, an incident involving HCB was recorded in Turkey in the late 1950s when grain treated with HCB as a fungicide was consumed in large quantities. Approximately 500 people died as a result. Tragically, the levels of contamination were so high that several suckling infants were fatally poisoned via their mothers' milk (19). In a follow-up study, raised HCB levels were still observed in human milk samples taken from the same area some 25 years after the event (20).

The question of cancer induction is always very topical and considerable effort has been expended in trying to assess the cancer risk associated with OCP and PCB exposure. Fortunately, it appears that the risk of carcinogenesis is low for both OCPs (6, 14, 21) and PCBs (17) and PCBs appear to act as cancer promoters rather than inducers (22).

1.4. Modes of Organochlorine Toxicity.

The mechanisms by which OCPs and PCBs exert their effects in man are not well understood. Much work is being carried out, particularly in the case of PCBs. The ability of PCBs to induce the P-450 and P-448 cytochromes, especially P-448 aryl hydrocarbon hydroxylase (AHH) has come under close scrutiny. The degree and pattern of substitution of chlorines on the biphenyl nucleus appears to determine the toxicological profile of a given PCB congener. A 3,3',4,4' pattern within the overall chlorination pattern together with an ability to readily assume a planar conformation confers on a congener strong P-448 AHH inducing properties. This P-448 inducing activity is also classified as 3-methylcholanthrene (3-MC) inducing activity. Such congeners are approximately isostereomeric with, and can interact with the same receptor site(s) as 2,3,7,8-TCDD which also exhibits these P-448 AHH inducing properties (13, 23, 24). Some other congeners exhibit P-450 (or phenobarbitone type) inducing activity. Many congeners show mixed P-450/P-448 induction properties (13, 25) or indeed no observable enzyme inducing properties. Molar polarisability seems to be a good indicator of the type of enzyme inducing properties of a given congener (17).

Fortunately, the most potent P-448 inducers, which are the most toxic PCB congeners, are not major components

(usually <1%) of Aroclor mixtures (26). Receptor models for PCB toxicity have been proposed but the relationship between observed receptor levels and biological effects are not clear (13, 27, 28, 29). Further details of OCP and PCB toxicity can be found in the literature (13, 14, 15, 16, 23, 30).

1.5. The Role of Analytical Chemistry in the Control of Organochlorines in the Environment.

Much effort is expended in developing suitable analytical methods for determining organochlorine residues in various materials. The data generated by these analyses form the basis on which practical, qualitative decisions are made, so the results produced have to be reliable. So it can be seen that analytical chemistry has a central role to play in the management of the environment. Due to the low levels commonly met in environmental samples and the complexity of the sample matrices, environmental analysis is very demanding. The requirements for accuracy, precision, specificity and sensitivity, are well known and the degree of each required will depend on the analysis in hand; methods should also be properly validated. In addition to these factors, good planning and adequate quality assurance should be incorporated into any analytical exercise including the use of blanks and the judicious use of statistical methods to evaluate the results (31). The principles of environmental

analysis have been clearly set out by Keith et al. (32).

With particular regard to the analysis of samples for residues of OCPs and PCBs, several factors must be considered. As a general "rule of thumb", recoveries of residues should be greater than 80% (33). Difficulties can be encountered if several residues are to be determined simultaneously (a multi-residue analysis) since selectivity between analytes becomes an issue; this is particularly apparent in the case of PCBs. As has been mentioned previously, the most meaningful PCB data are congener-specific data; "total PCB" analyses are of little value from the point of view of the toxicologist and this is further aggravated by the incomparability of data produced by different "total PCB" methods.

Turning specifically to human milk samples, the residue analyst is faced with a complex matrix containing several OCP and PCB residues varying over about two orders of magnitude of concentration at the low parts-per-billion (parts in 10^9) to parts-per-trillion (parts in 10^{12}) level. The analysis of human milk samples for organochlorine residues calls for efficient extraction and clean-up procedures. The selectivity between analytes (and any co-extracted interferences) may be attained by fractionation or the use of high resolution chromatography with selective detection. The method of detection must also be very sensitive. In view of these

factors, the mass spectrometer has gained in popularity as a detector on account of its selectivity, sensitivity and wide linear dynamic range. Much work has gone into improving the sensitivity and specificity of the mass spectrometer in this role and techniques such as negative ion chemical ionisation show promise. Another fairly recent means of increasing sensitivity when using capillary GC analyses is the use of a large injection volume with a retention gap.

The mass spectrometer as a selective detector together with the high efficiency offered by capillary GC columns provides a solution to the daunting task of congener-specific analysis and capillary GC-MS is entirely capable of providing a complete 209 congener-specific analysis. Ideally, this would make use of standards for all 209 PCB congeners but an effective alternative to this is to use surrogate PCB standards, one for each level of chlorination. This approach gives satisfactory quantitative results and reduces the number of standards required to ten (or less) and also reduces the amount of data to be handled.

Another unique advantage of the mass spectrometer is the opportunity for using isotopically labelled spiking standards for recovery studies.

1.6. Organochlorines in Human Milk.

The first report of organochlorine contamination of human milk was made by Laug et al. in 1951 (3). Laug and co-workers analysed the milk of 32 normal, healthy, black American women from the general population in Washington DC, USA and found their milk contained an average of 130 ppb (whole milk basis) DDT. This was the first clear evidence that organohalogenes could accumulate in human milk. In the early 1960s, the increased concern about pesticide residues prompted many studies around the world including several on human milk and it became firmly established that organochlorines bioaccumulated in human milk and was transferred to suckling infants. A further nationwide epidemiological study was conducted in the USA during 1975/6 (34) and a smaller nationwide study was conducted in the UK during 1979/80 (35). It was found that many OCP residues would often exceed FAO/WHO ADI limits, for example, the 1979/80 UK study found the mean summed concentration for p,p'-DDT + p,p'-DDE to be around 44 ng ml⁻¹ on a whole milk basis and the mean concentration for dieldrin to be around 2 ng ml⁻¹ on a whole milk basis. Assuming an infant consumes 150 ml milk/kg body weight/day, this would mean that on average, an infant would receive 6.6 µg kg⁻¹ day⁻¹ p,p'-DDT + p,p'-DDE and 0.3 µg kg⁻¹ day⁻¹ dieldrin. The FAO/WHO ADI for total DDTs (DDT + DDE + TDE) is 20 µg kg⁻¹ day⁻¹ and that for dieldrin is 0.1 µg kg⁻¹ day⁻¹. It can be seen

that dieldrin residue levels exceed the limits. Similar calculations on the UK data for PCBs and HCB reveal a total PCB consumption of about $3 \mu\text{g kg}^{-1} \text{ day}^{-1}$ and a total HCB consumption of about $0.6 \mu\text{g kg}^{-1} \text{ day}^{-1}$ by the infant. A temporary FAO/WHO ADI of $0.6 \mu\text{g kg}^{-1} \text{ day}^{-1}$ was set for HCB in 1974. No FAO/WHO limit has been set for PCBs but a limit of $1 \mu\text{g kg}^{-1} \text{ day}^{-1}$ has been suggested (8, 9). The toxicological significance of these results is not known. The consumption figures are of a similar order of magnitude as the ADIs and ADIs incorporate a considerable safety margin. Furthermore, ADIs are based on the idea that such quantities can be consumed daily for life without appreciable risk. However, the effects of consuming these quantities of several residues together at such a crucial stage in human development does mean that the possibility of chronic toxicity should not be dismissed out of hand (36). A comprehensive review of the chemical contamination of human milk has been published by A.A. Jensen (37).

It has been observed that different PCB congeners exhibit varying tendencies to bioaccumulate in human milk (38, 39) and a 2,4,4',5 substitution pattern within the overall chlorination pattern seems to be very stable within the human body. Furthermore, certain congeners containing this pattern which have been identified in human milk (PCBs 118 and 156) appear to elicit some toxic effects of the P-448 enzyme inducing type, i.e. effects

qualitatively (though not quantitatively) similar to 2,3,7,8-TCDD (39). These, and other congeners identified in human milk (like PCBs 157 and 189) (39) are mono-ortho chloro- analogues of the co-planar, non-ortho substituted PCBs which are the most toxic congeners known (13). These mono-ortho derivatives tend to show mixed enzyme induction properties (13, 25) and for these reasons, PCBs 118, 156, 157, and 189 were chosen as target analytes for this project. PCB 169 was also included on account of its high toxicity and lipophilicity, although no PCB 169 was expected (or found) in the human milk samples analysed (see Section 7).

Because toxic PCB congeners accumulate in human milk, Parkinson *et al.* (40) artificially reconstructed the pattern of PCB congeners found in Japanese human milk samples and tested the AHH inducing properties of the mixture. They found the ED_{50} to be approximately seven times lower than the ED_{50} value for Kanechlor 500. This led them to suggest that ADIs set using toxicity data produced by tests on commercial mixtures should be re-evaluated.

Concern over the contamination of human milk prompted some West German experts to suggest that breast feeding should not continue beyond the fourth month post-partum (41). The experts recognised that the benefits of breast feeding far outweigh any risks associated with the infant

ingesting milk contaminated at usual levels. However, after the fourth month, although the risks associated with consuming breast milk do not seem to increase, the benefits which accrue from breast feeding tend to decrease. Consequently, the risk/benefit ratio becomes less favourable.

The overwhelming view of most investigators is that the levels of contaminants routinely found in human milk in developed countries do not generally present a serious threat to the health of breast-fed infants and the benefits outweigh any risks (42, 43). However, it seems a common view that more toxicological investigations into the effects of organochlorine contaminants, particularly individual PCB congeners, should be conducted. The effects of co-administration of several organochlorine contaminants also needs to be considered.

The process of risk assessment in this field is very difficult because of the wide variation in the type and degree of milk contamination. The problem is compounded by the fact that no control population is available for comparison. Despite the obstacles, risk assessment is an area of active research.

1.7. Scope of this Thesis.

Research is still needed into improving analytical technology associated with environmental research with a view to enhancing reliability and ultimately closing the gap between analytical data and their toxicological interpretation. In this context, Norton Nelson of New York University has said (44): "There has been a most remarkable improvement in the sensitivity and specificity of the analytical methods for these [polyhalogenated aromatic] compounds over the last 20 years. It is unfortunate that some of these techniques were not available earlier for more refined analyses of the human exposures (especially to the dioxins); this could have made studies of those exposures more rewarding. Of all aspects in this difficult field, it is perhaps in the field of analytical techniques that the most progress has been made. There is, nevertheless, room for improvement by refinement, simplification and cost reduction of analytical procedures. Only a limited number of laboratories can now undertake these highly specialized analytical techniques. Wider availability of refined and simplified techniques would be a decided advantage."

Methods of analysis for organochlorine residues in human milk can generally be broken down into the three principal areas of extraction, clean-up and quantitative determination. After describing the synthesis and

isolation of selected PCB congeners for use as analytical standards, each of the three areas is dealt with in turn. Individual sections broadly comprise an introduction to the topic followed by experimental details of the research and development work undertaken, the results and a discussion. It is hoped that this format will enable the reader to see each part of the work in its own context and more readily follow the logical development of the methods. The performance of the new methodology was assessed by applying the new procedures to spiked samples and ultimately to actual human milk samples.

2. SYNTHESIS, ISOLATION AND CONFIRMATION OF IDENTITY OF SELECTED PCB CONGENERS.

2.1. Introduction.

An investigation of commercial sources revealed that less than half of the 209 PCB congeners were commercially available, and these were too expensive to purchase. In view of this, it was necessary to resort to in-house synthesis of the chlorobiphenyls selected for congener-specific analysis. Due to their proposed toxicity (see Section 1.6.) and their detection in human milk (except PCB 169) (39) PCBs 118, 156, 157, 169 and 189 were singled out as target analytes.

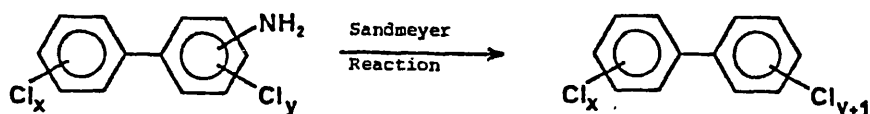
The various approaches to PCB synthesis that were considered are briefly outlined below together with the clean-up, isolation and identity confirmation procedures that were employed.

2.2. Synthetic Methodology.

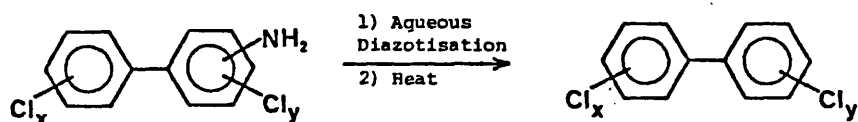
Synthetic methods for the preparation of chlorobiphenyls generally fall into two categories: i) replacement with chlorine of functional groups already attached to a biphenyl nucleus and ii) coupling of two chlorobenzenoid structures to produce chlorobiphenyls.

Replacement reactions include:

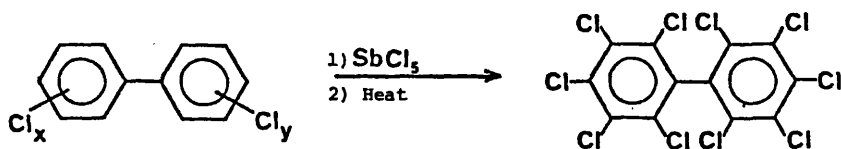
1) The Sandmeyer reaction starting with an aminochlorobiphenyl, for example:



2) Deamination reaction (i.e. replacement of an amino function with hydrogen) for example:

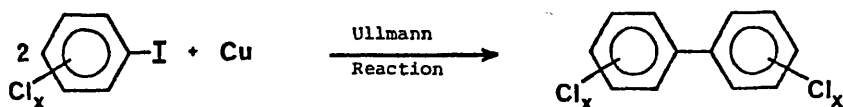


3) For decachlorobiphenyl, perchlorination using antimony pentachloride:

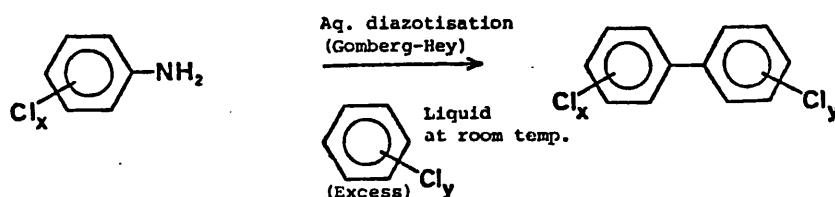


Coupling reactions include:

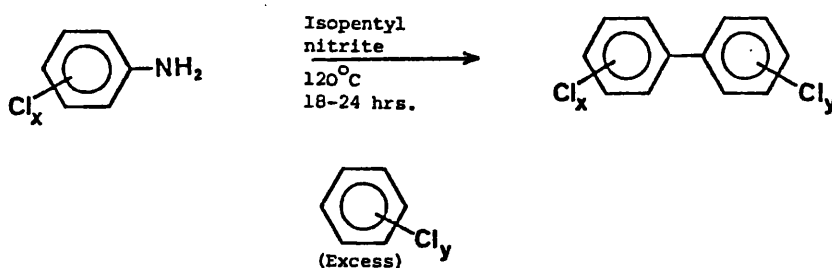
1) The Ullmann reaction, for example:



2) The Gomberg-Hey reaction, for example:



3) Cadogan coupling {45, 46}, for example:

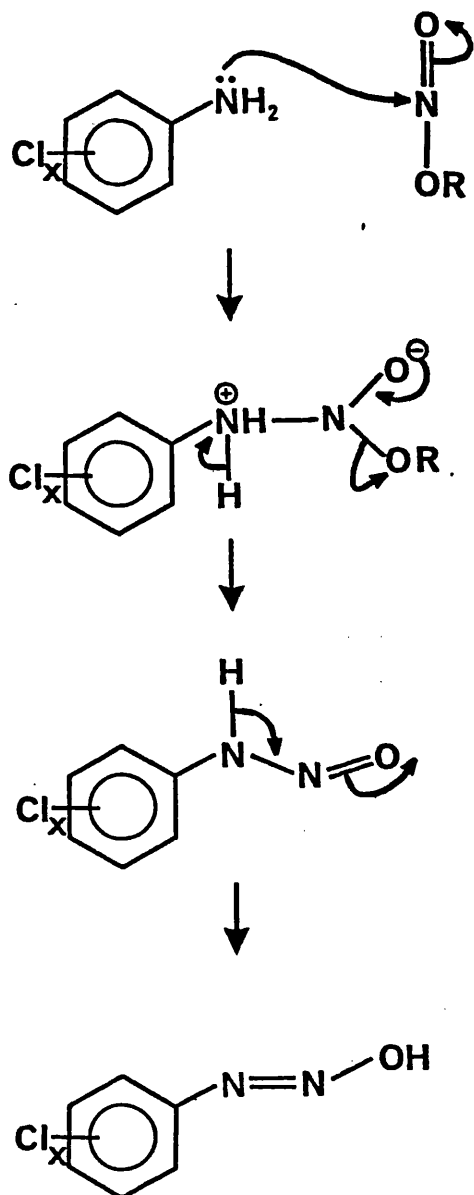


For the chlorobiphenyls required, neither the Sandmeyer reaction nor the deamination of aminochlorobiphenyls was useful due to the difficulty in obtaining appropriate starting materials. The Ullmann reaction leads to symmetrical chlorobiphenyls and since only one of the desired compounds is symmetrical (PCB 169) this route was not considered further. The Gomberg-Hey reaction, on the other hand, could have been useful, but the method requires the reaction mixture to be liquid at room temperature {47}. Since all but one of the chlorobenzenes used for the syntheses are solids at room temperature and all of them are insoluble in the aqueous diazotisation mixture, this reaction could not be carried out.

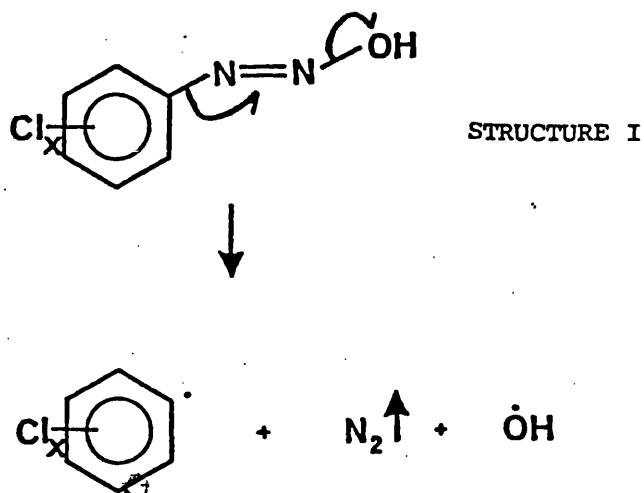
The synthetic method that was finally adopted was that of

Cadogan coupling. This is a single step non-aqueous diazotisation method which requires the use of elevated temperatures but no additional solvent medium. Under these conditions, the reaction mixture is liquid. An alkyl nitrite is used as the diazotising agent. The reaction involves free-radical intermediates and the following possible mechanism may be proposed:

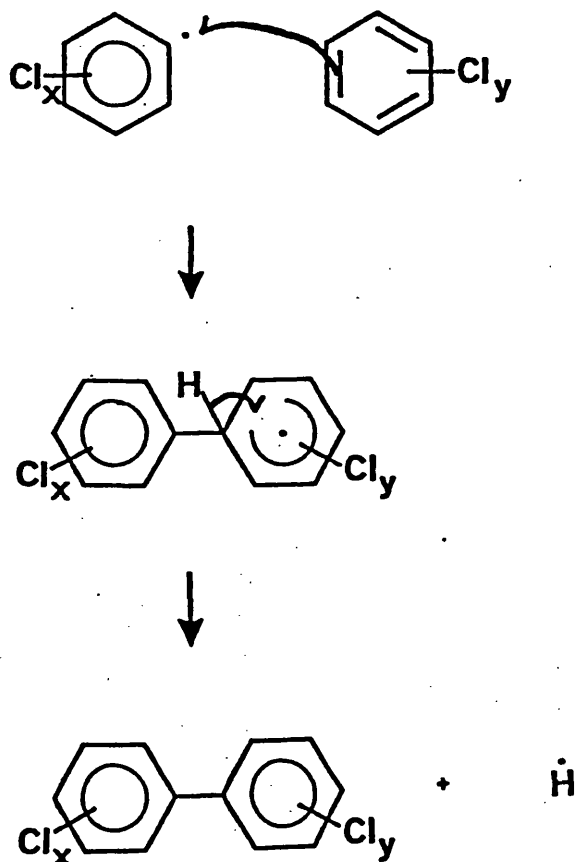
2.2.1. Cadogan coupling - a possible mechanism.



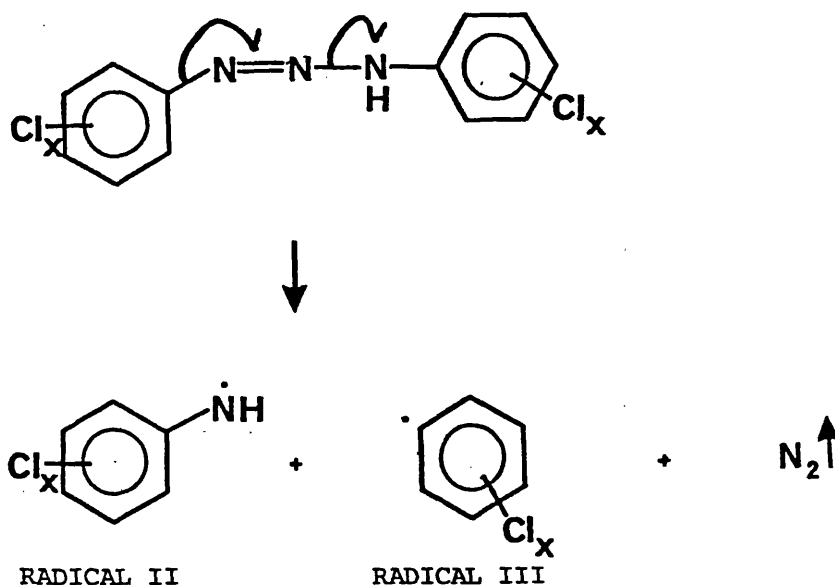
The above diazonium hydroxide may not decompose to a diazonium ion in a non-aqueous environment and it may be speculated that radicals are formed directly:



The chlorophenyl radical so formed can then couple to the chlorobenzene, which is present in excess, to form the chlorobiphenyl:



As will be seen, the reaction gives rise to many by-products and Fillipi *et al.* (48) working with aminopyridines as the aromatic amines and toluene as the substrate, examined the structures and relative proportions of the by-products formed by this type of reaction. The by-products formed in highest concentration were found to be aminobiaryls, diarylamines and diaryltriazenes. The latter would probably decompose at the elevated temperatures used for PCB synthesis and thermal decomposition of such triazenes, which are produced by self-coupling of the aromatic amine, would produce two radicals (48):



It can be seen that radical III is the same as that produced by direct decomposition of the diazonium hydroxide (structure I) and so the chloroaryl radical required for coupling to the excess chlorobenzene to form the PCB can also be produced via this triazene

intermediate. Unfortunately, decomposition of the triazene also leads to the unwanted radical II which reacts to form by-products.

As can be seen from the mechanism, regarding the chloroaniline derived ring, the biphenyl bridge forms where the amino function was originally placed and so the positions of the chlorines with regard to the biphenyl bridge can be controlled in the chloroaniline derived ring. On the other hand, the attack by the chloroaniline derived radical can take place at any unsubstituted position on the chlorobenzene and so any chlorobenzene with unequivalent hydrogens will give rise to a mixture of products. For some chlorobiphenyl congeners, this is unavoidable and as a general rule, the ring with the highest number of unequivalent hydrogens should be provided by the chloroaniline and consequently, the ring with the most equivalent hydrogens should be supplied by the chlorobenzene. This will ensure the minimum number of chlorobiphenyl products and so the yield of the desired compound(s) should be higher and the isolation procedure simpler.

2.2.2. Synthetic procedures.

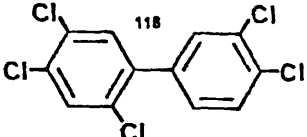
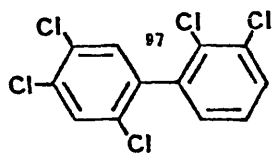
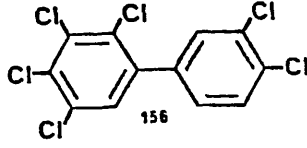
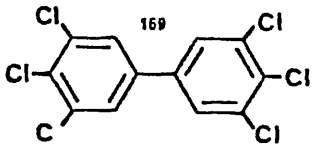
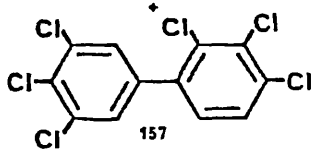
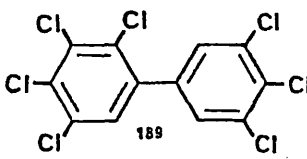
For the synthesis of PCBs 118, 156, 157, 169, 189 by Cadogan coupling it was necessary to carry out four reactions and in each case, the procedure of Mullin et

a1. (46) was followed. The three reactants in each of the reactions were i) a chloroaniline, ii) a chlorobenzene and iii) isopentyl nitrite as the diazotising agent. The identities and amounts of each component in each of the four reactions and the congeners produced is given in Table 1. Reagents were all used as received.

Experimental procedure.

For each reaction, the procedure given below was followed: The chloroaniline and the chlorobenzene were weighed out and placed in a three-necked 100 ml round-bottomed flask. A water condenser was placed in the central neck and a mercury-in-glass thermometer was placed in one of the side necks. The third neck was stoppered and was used as an access port for the subsequent introduction of the iso-pentyl nitrite. The chloroaniline and chlorobenzene were heated, with stirring, to between 120° and 130°C using a thermostatically controlled oil bath. At this temperature, the contents of the flask were liquid and the iso-pentyl nitrite was then added dropwise over a period of one hour. The reaction mixture was maintained at this temperature, with stirring, for about 20 hours. After this time, the contents of the flask were allowed to cool and solidify. In each of the four cases, the contents of the flask were a deep red-brown colour due to the production of several coloured by-products.

Table 1. Identities, quantities, sources, grades and lot numbers of reactants employed in PCB syntheses.

<u>Congeners produced</u>	<u>Chloroaniline</u>	<u>Chlorobenzene</u>	<u>Iso-pentyl nitrite</u>	<u>Product Structures</u>
97+118	2,4,5-trichloro-aniline 25 mmol=4.91g Aldrich Tech. grade Lot 72597	1,2-dichloro-benzene 250 mmol=36.75g Aldrich 98%. Lot 69877	40 mmol= 5.6 ml Aldrich 97%. Lot 60781	 
156	3,4-dichloro-aniline 12 mmol=1.94g Aldrich 98%. Lot 63324	1,2,3,4-tetra-chlorobenzene 130 mmol=28.01g Aldrich 98%. Lot 66573	20 mmol= 2.8 ml Aldrich 97%. Lot 60781	
157+169	3,4,5-trichloro-aniline 30 mmol=5.89g Aldrich 97%. Lot 38989	1,2,3-trichloro-benzene 400 mmol= 72.58g Aldrich 97%. Lot 34467	40 mmol= 5.6 ml Aldrich 97%. Lot 60781	 
189	3,4,5-trichloro-aniline 25 mmol=4.91g Aldrich 97%. Lot 38989	1,2,3,4-tetra-chlorobenzene 266 mmol=57.37g Aldrich 98%. Lot 66573	40 mmol= 5.6 ml Aldrich 97%. Lot 60781	

Note: Although iso-pentyl nitrite boils at 99°C, the reaction should still take place due to the refluxing of the vapour. However, losses may still occur on opening the flask in order to introduce the iso-pentyl nitrite. The extent of such losses is not known and may account, in part, for the low yields of PCBs obtained. Introducing the nitrite through a rubber septum may help or, alternatively, using an alkyl nitrite with a higher boiling point may be in order. For some of the reactions, it may be practicable to use a lower reaction temperature which may also affect the yields and the impurity profiles.

2.3. Clean-up and Isolation.

The chlorobiphenyls had to be separated from the reaction by-products and, in the cases of reactions producing the PCB pairs 97+118 and 157+169, the chlorobiphenyls also had to be isolated from each other (49).

2.3.1. PCB 156.

Preliminary clean-up.

The first step in the clean-up was the removal of the excess 1,2,3,4-tetrachlorobenzene. This was achieved by vacuum distillation.

The next stage was the removal of the by-products, many of which were highly coloured (and possibly carcinogenic). It was known from preliminary studies that PCBs are virtually unretained by silica/hexane (or heptane) liquid chromatographic systems (except when completely anhydrous (50)). The reaction products from the synthesis of PCB 156, were examined by TLC using a silica plate (Silica GF₂₅₄, Merck) and hexane as mobile phase. This revealed that the pigments produced by the reaction were retained by the silica. The darkest pigments (deep red-brown) were very strongly retained, but the lighter pigments displayed much greater mobility with the lightest coloured materials (very pale yellow) moving fastest. PCB 156 remained close to the solvent front.

In view of the TLC results, it was decided to effect the next stage in the clean-up using a silica/hexane liquid chromatographic column. A glass column (52 X 300 mm) was packed to a depth of about 250 mm with silica (Kieselgel 60, 70-230 mesh, Merck, wet packed in hexane). The reaction products were dissolved in a minimum of hexane and transferred to the top of the column. The column was eluted with hexane and the eluate was collected in five fractions. The first fraction was taken from the beginning of elution till just before the first pigment eluted. Four further fractions, each of about 100 ml,

were collected. The hexane was removed from each fraction using a rotary evaporator. The residue from the first fraction was white and crystalline and smelled of 1,2,3,4-tetrachlorobenzene. The residues from the remaining four fractions ranged in colour from a very pale yellow for the second fraction, to a darker yellow for the fifth fraction. Again, the residues were all crystalline and the earlier fractions smelled vaguely of the tetrachlorobenzene.

The silica column removed most of the coloured by-products but it was apparent (on account of the odour) that residual tetrachlorobenzene remained in the earlier fractions and that all fractions, save the first, still contained some pigmented material. Clearly, further clean-up was necessary but normal phase chromatography would probably not offer sufficient selectivity for separation of chlorobiphenyl isomers from chlorobenzene due to the lack of retention. In view of this, reversed-phase separations were investigated.

Stalling and Huckins (51) have shown that retention and separation of PCBs can be achieved using reversed-phase TLC with paraffin oil coated kieselguhr plates with an acetonitrile/methanol/water mobile phase. Thus the basis for reversed-phase separation had already been established.

With a view to transferring any separation to a preparative scale HPLC system with an ODS column, a variety of acetonitrile-based mobile phases were assessed using RP-HPTLC plates (RP-18, F₂₅₄, Merck). The solutes used in the RP-HPTLC experiments were:

- i) 3,4-dichloroaniline
- ii) 1,2,3,4-tetrachlorobenzene
- iii) Mixed Aroclors 1242, 1248 and 1260
- iv) Biphenyl
- v) Crude PCB 156 synthesis products (before any clean-up)
- vi) 4,4'-dibromobiphenyl.

Viewed under UV light (254 nm) it was found that 95:5% v/v acetonitrile:water gave good separation of PCBs in the Aroclor mixture and that with this system, the two main spots in the crude PCB 156 synthesis products were well separated. The lesser retained of the two spots corresponded to 1,2,3,4-tetrachlorobenzene and it was assumed that the other spot was PCB 156. The R_f values for all the solutes are given in Table 2.

Increasing water content increased retention of PCBs as expected. Adding varying amounts of methanol to acetonitrile-based mobile phases had little effect on selectivity. It was concluded that 100% acetonitrile would give adequate retention and good selectivity between PCB 156 and the tetrachlorobenzene. This was

Table 2. R_f values obtained for various solutes (see text) on RP-HPTLC plates using 95:5% v/v MeCN:water mobile phase. Spots visualised under UV light at 254 nm.

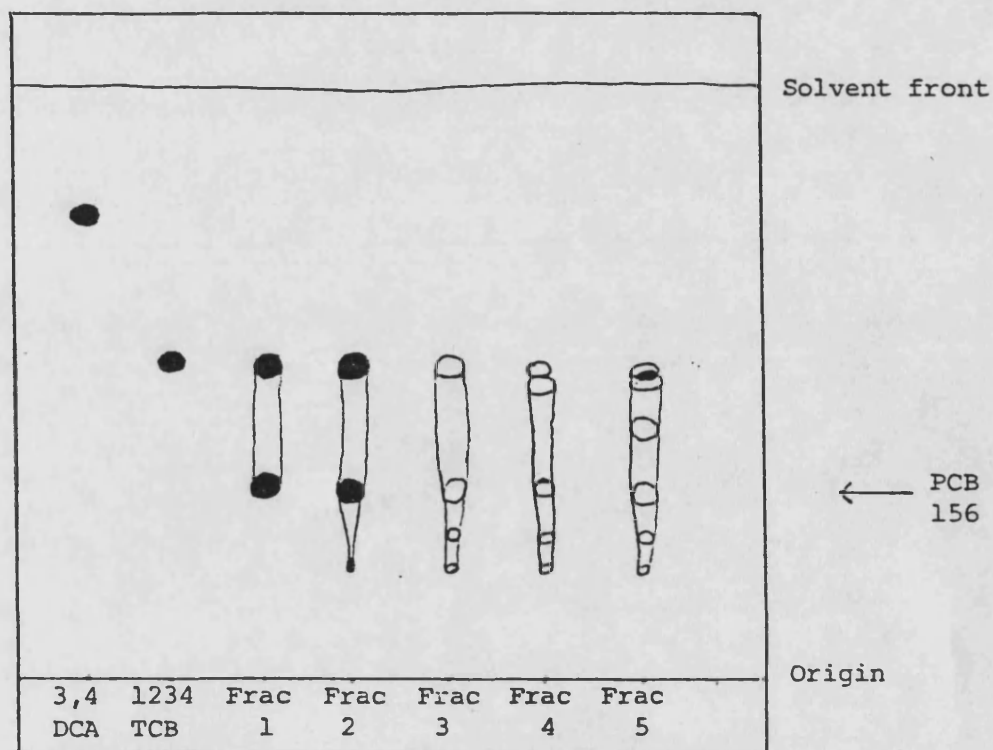
<u>Solute</u>	<u>R_f</u>
3,4-dichloroaniline	0.74
1,2,3,4-tetrachlorobenzene	0.43
Mixed Aroclors	0.21 to 0.54 (main spots at 0.51, 0.41 and 0.31)
Biphenyl	0.58
Crude PCB 156 products	0.00 to 0.69 (all coloured but faint under UV except for two very prominent spots at 0.22 (PCB 156) and 0.43 (1,2,3,4-tetrachlorobenzene))
4,4'-dibromobiphenyl	0.38

subsequently found to be the case and the following solutes were separated using an RP-18 HPTLC plate/100% acetonitrile system:

- i) 3,4-dichloroaniline
- ii) 1,2,3,4-tetrachlorobenzene
- iii), iv), v), vi), vii) First to fifth fractions respectively from silica/hexane column clean-up of PCB 156 synthesis products.

The results are shown in Figure 1.

Figure 1. TLC of solutes indicated (see text) using Merck RP-18, F₂₅₄, HPTLC plate with 100% acetonitrile mobile phase. Viewed under UV light at 254 nm.



The intensities of the spots for 1,2,3,4-tetrachlorobenzene and PCB 156 were greatest in fractions 1 and 2 and were much reduced in the subsequent fractions.

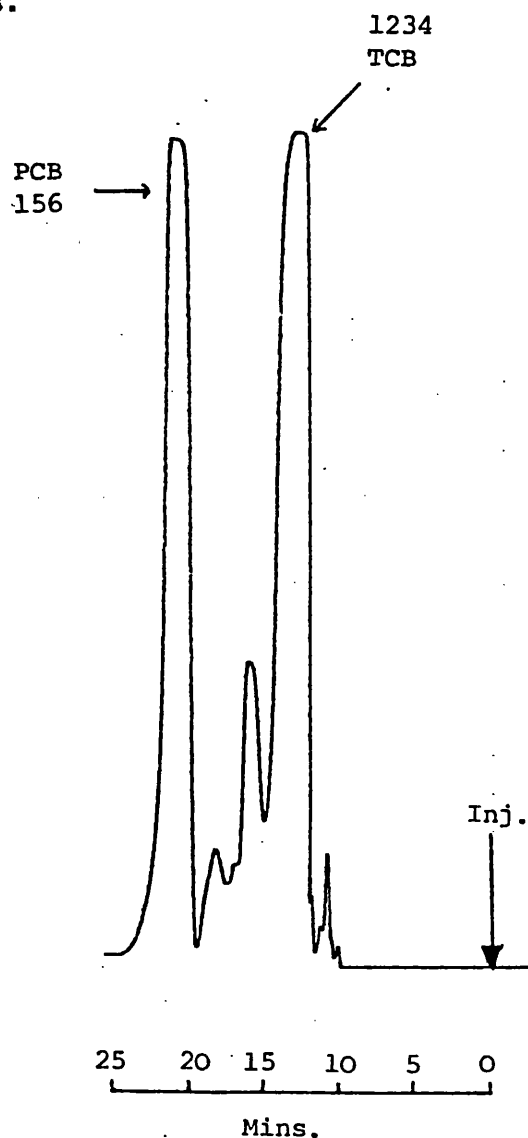
Thus it was concluded that firstly, a C₁₈ reversed phase system with 100% acetonitrile as mobile phase would probably be a useful procedure for separating PCBs from chlorobenzenes. Secondly, any residual chloroaniline (which was unlikely) would also be separated from PCBs. Thirdly, the eluate from the silica/hexane column clean-up step should be collected until about 100 ml after the first hint of colour has begun to elute (i.e. to the end of the second fraction as collected above). Collecting any more eluate would be unlikely to increase the PCB yield by very much (as witnessed by the decrease in intensity of the PCB spot in later fractions) and would increase the amount of pigmented impurity present in the sample collected.

Preparative HPLC.

In view of the TLC results, the residues from the first two fractions from the silica/hexane column clean-up were dissolved in a minimum of acetonitrile and subjected to a preparative HPLC separation using a 250 mm X 21.2 mm Zorbax ODS column with 100% acetonitrile as mobile phase. The instrument used was a Du Pont 830 preparative HPLC

unit fitted with a 2.0 ml injection loop. The flow rate was 14 ml min⁻¹ and the column effluent was passed into a Du Pont UV detector set at 254 nm, 2.56 AUFS with a preparative scale flow cell. The instrument was maintained at 35°C to increase the solubility of the crude residue in the mobile phase, to improve the peak shape and to decrease the elution time. Repeated injections of the sample solution were made and a typical chromatogram is shown in Figure 2.

Figure 2. Typical chromatogram from the preparative HPLC clean-up of PCB 156. See text for details.



The PCB 156 peak and the 1,2,3,4-tetrachlorobenzene peak both exceeded the dynamic range of the detector; this would appear to indicate that in this particular instance, the conditions of the initial vacuum distillation were not sufficiently rigorous to remove the majority of the tetrachlorobenzene.

Identification of the peaks was based on direct comparison with the RP-HPTLC results (Figure 2). The PCB fraction was collected in a round-bottomed flask directly from the outlet of the UV flow cell.

Recrystallisation of PCB 156.

The acetonitrile was evaporated off in a rotary evaporator and the PCB 156 was then recrystallised from 95% aqueous ethanol.

After the recrystallisation, the PCB still seemed to have a faint orange tinge and so was dissolved in hexane and passed through a silica Sep-pak cartridge. The cartridge retained all the pigment and on evaporation of the solvent followed by drying in a vacuum oven at 78°C for 4 hours, a white crystalline powder was left which subsequently proved to be PCB 156 (see Section 2.4.1.).

Yield.

240 mg of PCB 156 were obtained.

2.3.2. PCBs 157 and 169.Vacuum distillation and column chromatography.

The crude reaction products from the synthesis of PCBs 157 and 169, which were a deep red-brown colour (as with PCB 156), were subjected to vacuum distillation to remove excess 1,2,3-trichlorobenzene and then silica/hexane column chromatography in a similar manner to PCB 156. It was assumed that PCBs 157 and 169 and residual trichlorobenzene would all be unretained by the silica/hexane system and so eluate was collected until about 100 ml of yellow pigment had been eluted as suggested by the TLC results for the PCB 156 silica/hexane column clean-up (see Section 2.3.1.).

Separation of PCB 157 and PCB 169.

At this stage it became necessary to address the problem of chromatographic selectivity between the two isomers produced in the synthesis of PCBs 157 and 169. The isomers produced by the Cadogan synthesis are inevitably very similar, containing the same number of chlorines and the same substitution pattern in one of the rings (the

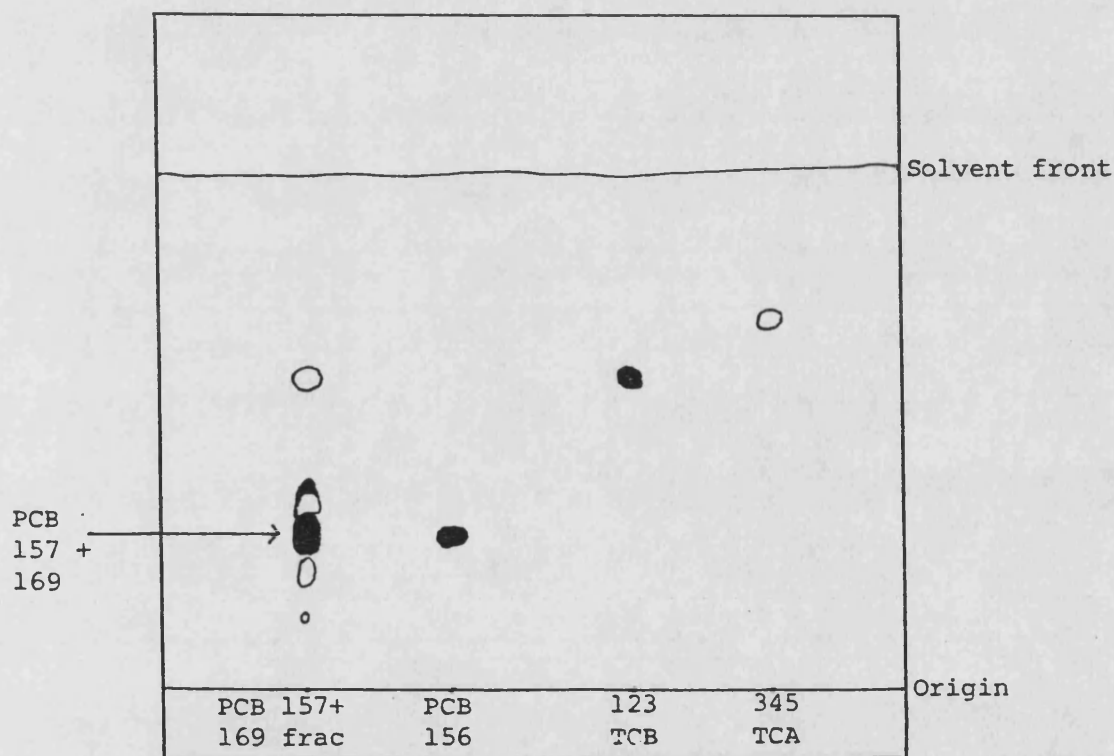
ring provided by the chloroaniline). The chlorines in the ring provided by the chlorobenzene will retain the same pattern with respect to each other in both isomers; however, the pattern of substitution with respect to the ring bridge will be different. Hence, the only difference between the two chlorobiphenyl isomers produced in a two-isomer synthesis will be the position of the ring bridge in one of the rings. This will probably give rise to differences in dipole moment and also differences in molecular geometry (such as length-to-breadth ratio and inter-ring dihedral angle). However, C₁₈ stationary phases are neither dipole moment nor geometry selective to any useful degree. Acetonitrile does possess a dipole moment and is a polarisable molecule and so may give rise to some selectivity on that basis, but acetonitrile is a linear molecule and so would not be expected to provide selectivity on the grounds of molecular geometry. Consequently, it was thought unlikely that a C₁₈ /100% acetonitrile system could effect adequate separation of isomer pairs produced by Cadogan coupling. This was borne out in practice in the case of the PCB 157/169 pair. Using RP-HPTLC plates (C₁₈, F₂₅₄, Merck) and 100% acetonitrile, the following solutes were examined for their chromatographic behaviour:

- i) PCB fraction from vacuum distillation and silica/hexane column clean-up of PCB 157/169 synthesis products.

- ii) PCB 156 (as a marker compound).
- iii) 1,2,3-trichlorobenzene.
- iv) 3,4,5-trichloroaniline.

The results are shown in Figure 3.

Figure 3. RP-HPTLC results for attempted separation of PCBs 157 and 169 using 100% acetonitrile mobile phase. (See text for details). Spots visualised under UV light at 254 nm.



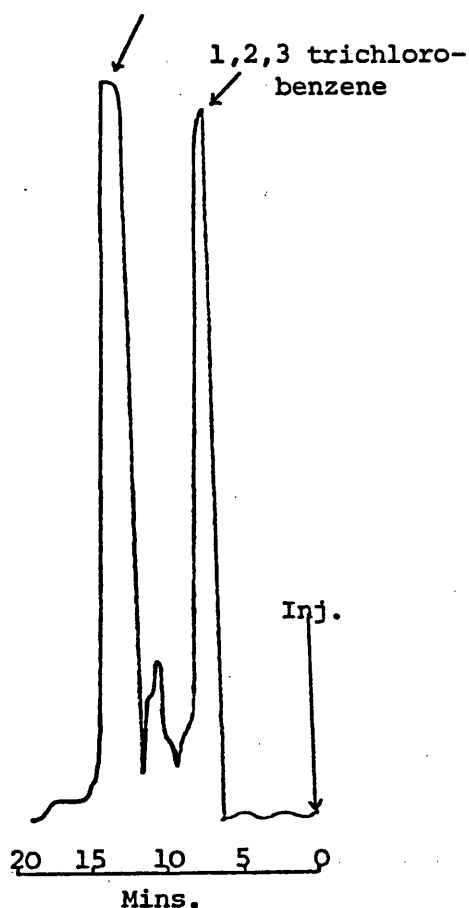
As can be seen, there was no apparent separation between PCBs 157 and 169. It also happens that PCB 156 would co-elute with PCBs 157 and 169 using this system despite being quite different in terms of substitution pattern.

Hence, this acetonitrile system was considered unsuitable for the separation of the isomers produced in a single Cadogan coupling synthesis. However, some preliminary studies were carried out using a 25 cm X 5 mm i.d. Hypersil Phenyl column (Shandon Southern, Runcorn, UK, 5 μ m particle size) with acetonitrile based mobile phases with the aim of achieving selectivity between PCBs by the use of the phenyl substituted stationary phase. 100% acetonitrile resulted in virtually no retention. With a 20 μ l injection volume, 60:40% v/v acetonitrile:water gave some separation of PCBs 157 and 169 (k' PCB 157 = 11.0, k' PCB 169 = 11.9, R_s = 1.0) but column efficiency and peak shape were very poor, possibly due to poor wetting of the stationary phase by the mobile phase, and loading capacity was very low due to the high water content of the mobile phase.

It was considered at this time that the residual 1,2,3-trichlorobenzene and pigment impurities represented a hindrance to the study of the separation of the two isomers. Consequently, the residue from the silica/hexane column clean-up was further purified by using the Zorbax ODS/100% acetonitrile preparative HPLC system described for PCB 156 (see Section 2.3.1.) but with an injection volume of 8 ml. No separation of the two PCB isomers was observed, but the residual 1,2,3-trichlorobenzene and pigments were removed. A typical chromatogram is shown in Figure 4. The PCB fraction was collected directly from

the detector flow-cell outlet. The acetonitrile was removed by rotary evaporation but no recrystallisation was performed.

Figure 4. Preliminary HPLC clean-up of PCBs 157 & 169 after synthesis. See text for details. PCBs 157 + 169



Tetrahydrofuran (THF) was considered as an alternative mobile phase component for the separation of PCB isomers. THF, like acetonitrile, possesses a marked dipole moment, but in addition it is a cyclic molecule and it was considered that this aspect of its structure would enable THF to induce selectivity between PCBs by exploiting differences in geometry. It was expected that small

differences in the pattern of chlorination in PCB isomers would give rise to differences in the orientation of THF molecules about the biphenyl nucleus and in the way the molecules stack in the solvation process, thus leading to differences in chromatographic behaviour, i.e. selectivity. Using a 55:45% v/v THF:water mobile phase with the Hypersil Phenyl column described above, baseline separation between PCBs 157 and 169 was obtained with decreased retentions.

Prompted by the increase in selectivity induced by the use of THF, the HPTLC separation of PCBs 157 and 169 was attempted using a C₁₈ RP-HPTLC/THF:water system, since a C₁₈ based separation would mean that a Preparative ODS HPLC column could be used for the separation (a preparative ODS column was already available for use whereas a preparative phenyl column was not). The solutes examined were:

- i) PCBs 157/169 after clean-up by vacuum distillation, silica/hexane column chromatography and ODS/100% acetonitrile preparative HPLC.
- ii) PCB 156.

The R_f values obtained for two different compositions of THF:water mobile phase are shown in Table 3.

Table 3. Separation of PCBs 157 and 169 using RP-HPTLC plates (RP-18, F₂₅₄, Merck), and THF:water mobile phases. Spots visualised under UV light at 254 nm.

<u>Solute</u>	<u>R_f values in</u> <u>80:20 THF:water</u>	<u>R_f values in</u> <u>70:30 THF:water</u>
PCBs 157+169	0.58 (faint) 0.64 (intense)	0.35 (faint) 0.44 (intense)
PCB 156	0.60 (intense)	0.39 (intense)

The TLC results showed that with both mobile phases there was complete separation of PCBs 157 and 169 and also, incidentally, good separation from PCB 156. The 70:30% v/v THF:water phase had about the same eluotropic strength as 100% acetonitrile for this separation, but selectivity was much improved. Hence it was expected that preparative HPLC using an ODS/THF:water system would probably give excellent selectivity between very similar PCB isomers and this was subsequently confirmed by the preparative scale separation of PCBs 157 and 169.

Preparative HPLC separation of PCBs 157 and 169.

The preparative scale HPLC separation of PCBs 157 and 169 was carried out under the following conditions:

Mobile phase: 75:25% v/v THF:water

Flow rate: 7 ml min⁻¹

Column: 250 X 21.1 mm Zorbax ODS (5-6 μm particles).

Injection vol.: 2.0 ml saturated solution in mobile phase.

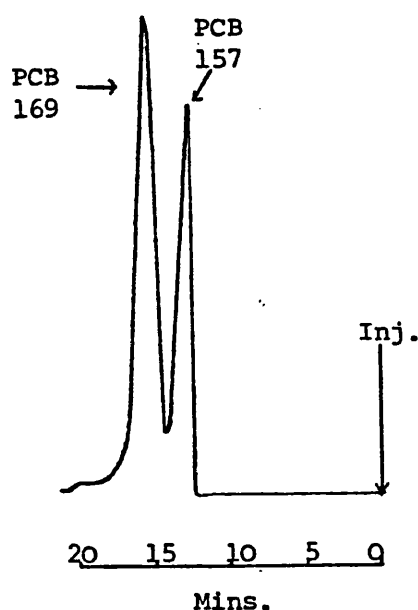
Detector: UV at 297 nm, 2.56 AUFS.
Preparative cell.

Oven temp.: 35°C

The UV detector was set at 297 nm which is off the absorption maximum. This was to prevent the absorbance exceeding the dynamic range of the detector.

The cleaned up PCB 157/169 mixture from the ODS/acetonitrile HPLC clean-up was dissolved in a minimum of mobile phase. Repeated injections were made and the individual isomer fractions were collected directly from the detector flow cell outlet. A typical chromatogram is shown in Figure 5.

Figure 5. Chromatogram from preparative scale
HPLC separation of PCBs 157 and 169.



As can be seen, the separation of the two isomers is very good. The identities of the two peaks were assigned as shown in Figure 5. on the grounds that PCB 169 has no dipole moment whereas PCB 157 does. Consequently, one would expect PCB 157 to have a greater affinity for the polar mobile phase than PCB 169, and so PCB 157 would be expected to elute first (49). Furthermore, the synthetic precursors were such that one would expect PCB 157 to be formed in greater quantities than PCB 169; the RP-HPTLC experiments using the THF:water mobile phases (Table 3.) showed that the more intense isomer spot on the plate, which was assumed to be PCB 157, had the higher R_f value and so would be expected to elute first in an elution chromatography mode. Identity assignments were subsequently confirmed (see Sections 2.4.2. and 2.4.3.). It should be noted that due to the wavelength offset of

the detector, the relative intensities of the peaks in Figure 5. are not a true indication of the relative quantities of the two isomers. The chromatogram also shows that there was slight tailing of the peaks; this could have led to contamination of the PCB 169 fraction by PCB 157 but this was obviated by taking PCB 169 as a "cut" rather than collecting it from the valley point.

When the separation was complete, the THF:water solvent was removed in a rotary evaporator by starting at a low temperature to remove the THF then using a much higher temperature to drive off the water. At this stage, PCB 169 still displayed a very faint yellow colouration. The separate isomers were then recrystallised.

Recrystallisation of PCBs 157 and 169.

Both PCBs were recrystallised from absolute ethanol and dried in a vacuum oven at 80°C. This removed the residual colour from PCB 169 and both isomers were obtained as white, crystalline materials. However, PCB 169 appeared to have a slightly waxy quality.

Yields.

935 mg of PCB 157 were obtained.

214 mg of PCB 169 were obtained.

2.3.3. PCB 189.

Vacuum distillation and column chromatography.

After synthesis, the majority of the remaining 1,2,3,4-tetrachlorobenzene was removed by vacuum distillation. The deep red-brown residue was dissolved in a minimum of hexane and transferred to a silica column for removal of the pigments as described previously (see Sections 2.3.1. and 2.3.2.). The eluate was collected until about 100 ml of the first yellow band had eluted. The hexane was then removed in a rotary evaporator to leave a slightly yellow crystalline residue. This residue smelled of 1,2,3,4-tetrachlorobenzene. The residue was then re-dissolved in a minimum of acetonitrile.

RP-HPTLC of PCB 189 synthesis products.

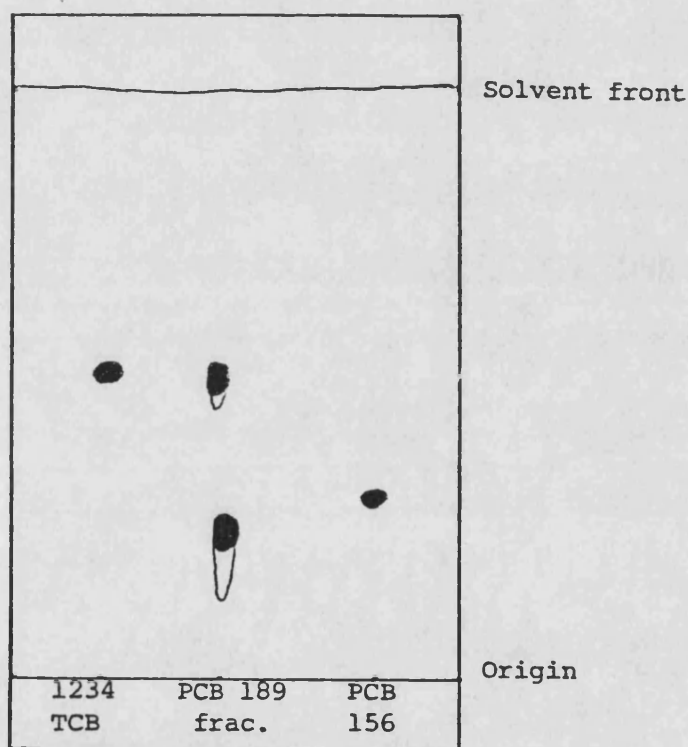
Knowing that the synthetic route used would produce only one PCB congener, it was considered that the ODS/acetonitrile HPLC system used for the clean-up of PCB 156 would probably be equally successful for PCB 189. This was confirmed using reversed-phase HPTLC. Using RP-18, F₂₅₄, HPTLC plates (Merck) and 100% acetonitrile as mobile phase, the following solutes were chromatographed:

- i) 1,2,3,4-tetrachlorobenzene.
- ii) PCB fraction from silica/hexane column clean-up.

iii) PCB 156 (as marker compound).

The results are shown in Figure 6.

Figure 6. RP-HPTLC of PCB 189 synthesis products using 100% acetonitrile as mobile phase (see text). Viewed under UV light at 254 nm.



As can be seen, PCB 189 was well separated from the tetrachlorobenzene. Slight residues of by-products were also in evidence but did not appear to pose any problem. In view of the results, preparative HPLC using the Zorbax ODS column with 100% acetonitrile as mobile phase was employed as the next step in the clean-up procedure.

Preparative HPLC of PCB 189.

The following chromatographic conditions were employed:

Mobile phase: 100% acetonitrile
Flow rate: 11.2 ml min⁻¹
Column: Zorbax ODS 250 X 21.1 mm (5-6 μm particles)
Injection vol.: 2.0 ml saturated solution in mobile phase.
Detector: UV at 254 nm. 2.56 AUFS.
Preparative cell.
Oven temp.: 55°C

Repeated injections of the acetonitrile solution were made and the PCB 189 fraction was collected directly from the outlet of the UV flow cell. A typical chromatogram is shown in Figure 7. Identities were assigned by direct comparison with the RP-HPTLC results.

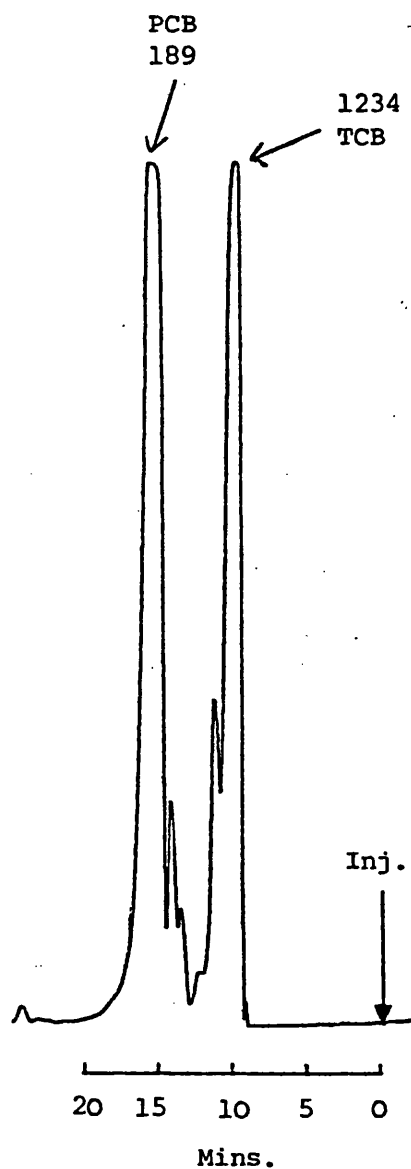
Recrystallisation.

The PCB 189 was recrystallised from methanol and dried in a vacuum oven at 80°C for 4 hours.

Yield.

749 mg of PCB 189 were obtained.

Figure 7. Chromatogram of preparative HPLC
isolation of PCB 189.



2.3.4. PCBs 97 and 118.

Vacuum distillation and column chromatography.

The crude reaction products were subjected to vacuum distillation in order to remove the excess 1,2-dichlorobenzene. The red-brown residue was then

dissolved in a minimum of heptane and passed through a silica column in a similar manner to that described previously (see Sections 2.3.1., 2.3.2. and 2.3.3.) using heptane as the mobile phase. The eluate was collected until about 100 ml of the first yellow band had eluted. The heptane was then evaporated off using a rotary evaporator.

RP-HPTLC of PCBs 97 and 118 synthesis products.

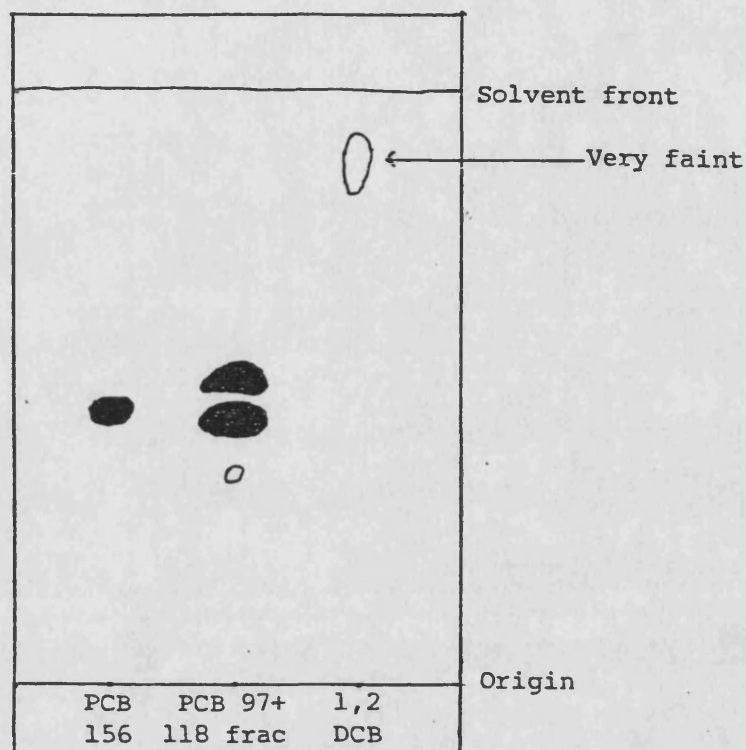
Using RP-HPTLC, F₂₅₄ plates (Merck) with 70:30% v/v THF:water as mobile phase, the following solutes were examined for their chromatographic behaviour:

- i) FCB 156 (as a marker compound).
- ii) The PCB 97/118 fraction from the silica column clean-up.
- iii) 1,2-dichlorobenzene.

The results are shown in Figure 8. From the results it can be seen that a THF:water mobile phase provided selectivity between the two isomers as well as separation from residual impurities. These results were used as a basis for the preparative scale separation of PCBs 97 and 118.

Figure 8.

RF-HFTLC of PCB 97/118 synthesis product using 70:30 THF:water mobile phase (see text). Viewed under UV light at 254 nm.



Preparative HPLC separation of PCBs 97 and 118.

In order to increase retention and therefore the separation of the two isomers, a mobile phase of 65:35% v/v THF:water was chosen. The residue from the silica column clean-up was dissolved in a minimum of mobile phase and the isomers were separated using the following chromatographic conditions:

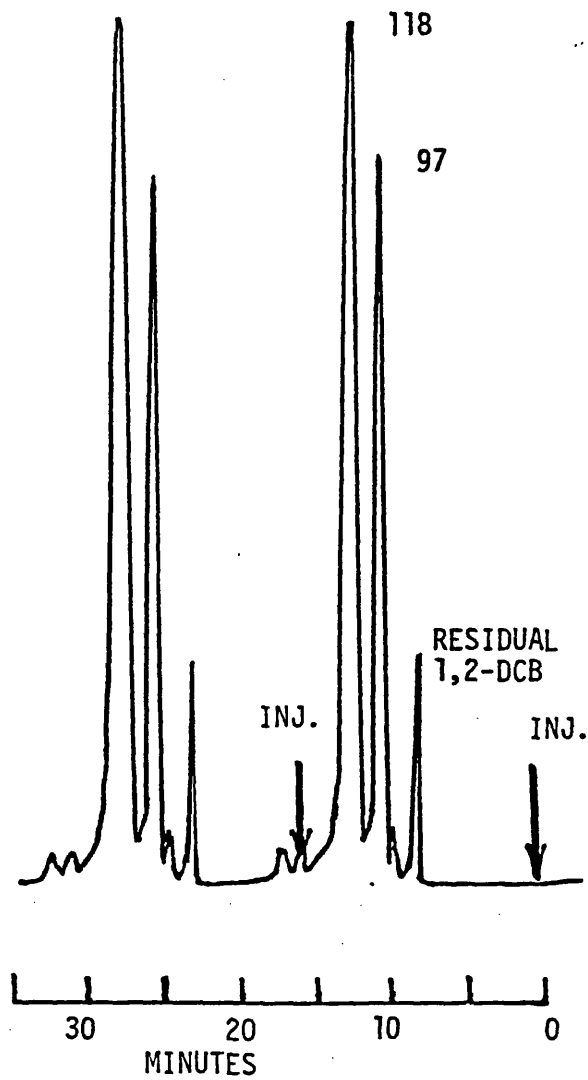
Mobile phase: 65:35% v/v THF:water
Flow rate: 11.6 ml min⁻¹
Column: Zorbax ODS 250 X 21.2 mm (5-6 μ m particles)
Injection vol.: 2.0 ml saturated solution in mobile phase.
Detector: UV at 254 nm. 2.56 AUFS.
Preparative cell.
Oven temp.: 50°C

Repeated injections of the impure PCB mixture in mobile phase were made and the two isomers were collected directly from the outlet of the detector flow cell. A typical chromatogram is shown in Figure 9. Assignments were made on the basis of dipole moment as discussed previously (49) (see Section 2.3.2.) and subsequently proved correct (see Sections 2.4.5. and 2.4.6.).

Unlike PCBs 157 and 169, the PCB 97/118 residue from the silica column clean-up was not subjected to an ODS/acetonitrile HPLC clean-up and so the sample separated on the ODS/THF:water was much dirtier as can be seen from Figure 9. On evaporation of the mobile phase, using a rotary evaporator, both PCB 97 and PCB 118 were off-white in colour. In view of this, it may have been profitable to have performed a preliminary ODS/acetonitrile HPLC clean-up but this would have been very time consuming. Instead, a second silica/heptane

column clean-up was performed for PCB 118.

Figure 9. Typical chromatogram for preparative HPLC separation of PCBs 97 and 118. See text for details.



Second silica column clean-up for PCB 118.

Due to the slight orange colouration, PCB 118 was passed through a second silica/heptane column in a similar manner to that described above except that the fraction collection was stopped just before any colour eluted. The heptane was then evaporated from the PCB fraction in a rotary evaporator. Some of the coloured band was then collected and spotted onto a fluorescent TLC plate. The plate was then viewed under UV light at 254 nm. Some quenching of fluorescence was observed and it was assumed that this was due to residual PCB 118 in the coloured band; it was thus assumed that some PCB 118 was not recovered.

PCB 97 was not subjected to a second silica/heptane clean-up since it was not required for any further studies.

Recrystallisation.

In an attempt to remove the colouration by recrystallisation, PCB 97 was recrystallised three times from 50:50% v/v methanol:acetonitrile but some colour still remained.

PCB 118 was recrystallised from methanol.

Both the impure PCB 97 and PCB 118 were dried in a vacuum oven at 78°C for 4 hours. PCB 97 was slightly off-white and PCB 118 was white. Both were crystalline.

Yields.

360 mg of PCB 118 were obtained (low yield due to some being left behind in second silica column clean-up)

720 mg of PCB 97 were obtained.

2.4. Confirmation of Identity and Assessment of Purity of PCBs 97, 118, 156, 157, 169 and 189 after Synthesis and Isolation.

Confirmation of identity was provided by examination of proton magnetic resonance spectra, mass spectra and comparison of melting points with published values where available. These data, together with knowledge of the synthetic procedure, permitted unequivocal identification of the synthesised PCB congeners.

Purity was assessed using the above techniques in conjunction with capillary gas chromatography. Impurity peaks in the spectroscopic data allowed tentative identification of the types of contaminants present.

70 eV electron impact mass spectra are reproduced in

appendix 1 as is a table of 270 MHz proton NMR data for each of the PCB congeners synthesised.

2.4.1. PCB 156.

Melting point: 124.1° to 124.9°C at 0.3°C min⁻¹.

No published value available for comparison.

Proton NMR: The proton NMR data for PCB 156 are tabulated in appendix 1. The data are entirely consistent with the assigned structure and agree well with published data {46, 52}. Impurity signals were recorded at:

δ (ppm) relative to TMS.

Approx. 0.94 (broad)	Too weak to determine multiplicity
Approx. 1.22 (triplet)	Very weak
Approx. 1.58 (broad singlet)	Very weak
Approx. 2.18	Barely discernible
Approx. 3.70	Too weak to determine multiplicity

Mass spectrometry: The 70 eV EI mass spectrum (see appendix 1) was typical of a hexachlorobiphenyl, exhibiting the correct m/z values, isotope clustering and fragmentation patterns {53}. Some very weak impurity peaks were visible, notably:

<u>m/z</u>	
29	Very weak
43	Very weak
57	Very weak
149	Very weak
394/396/398	Extremely weak

Capillary gas chromatography: The following conditions were employed:

Column: 39 m 0.25 mm id fused silica
polymethylsiloxane 0.2 μ m film.

Carrier gas: Hydrogen at 17 p.s.i.

Oven programme: 100°C for 1 min. then 4°C min⁻¹ to 250°C.
Hold.

Detector: Flame ionisation.

Inj./det. temp.: 275°C

Injection mode: 2 to 4 μ l in heptane solution, split (low ratio).

Instrument: Perkin-Elmer Sigma 3 gas chromatograph.

Some small, very early eluting peaks (t_r' all less than 3.0 mins.) were visible near the solvent front. PCB 156 eluted at $t_r' = 34.1$ minutes with a peak height of 107.5 mm. No impurity peaks were visible after $t_r' = 3.0$ minutes.

Summary and discussion:

It was clear from the spectroscopic data and the synthetic method that the compound was indeed PCB 156 and was sufficiently pure for use as an analytical standard. There were undoubtedly impurities and the main contaminants, as evidenced by the spectroscopic data, would appear to be residual ethanol from the recrystallisation, and a hydrocarbon-like material. The very weak triplet in the proton NMR at $\delta = 1.22$ may well be due to the methyl group of ethanol and the extremely weak signals at $\delta = 2.18$ and 3.70 could be due to the hydroxyl proton and the methylene protons respectively. The very weak signals at $\delta = 0.94$ and 1.58 could be the methyl and methylene protons respectively of a hydrocarbon chain. The source of this hydrocarbon-like material is not clear but may be carry-over from the silica/hexane column clean-up; alternatively, it may be that the hydrocarbon is a degradation product from the ODS phase used in the preparative HPLC work.

The mass spectrometric data exhibited some well known impurity peaks. Those at $m/z = 29, 43$ and 57 are commonly derived from hydrocarbon chains and would normally be weak if the hydrocarbon-like substance is present as an impurity since hydrocarbon chains tend to fragment heavily. The peak at $m/z = 149$ is usually indicative of a phthalate plasticiser. The peaks at $m/z = 394, 396$ and

398 were of uncertain origin but may well have been due to a very low level of a heptachlorobiphenyl produced during synthesis by higher-chlorinated chloroanilines or chlorobenzenes in the reactants. All the impurity peaks were very weak and were not of great significance but they did appear to indicate the presence of a hydrocarbon-like compound, a trace of residual ethanol, a trace of phthalate and possibly a trace of a heptachlorobiphenyl.

The capillary gas chromatogram showed the congener to be quite pure although the chromatographic conditions were such that very volatile compounds such as solvents, would not have been resolved from the solvent front. There were a number of small impurity peaks between the solvent front and $t_r = 3.0$ minutes; the identities of these materials was unknown but the retention times were undoubtedly too short to have been due to a long hydrocarbon chain type of compound. No impurities were seen eluting after $t_r = 3.0$ minutes.

Overall, the sample of PCB 156 was found to be of sufficient purity for use as an analytical standard.

2.4.2. PCB 157.

Melting point: 150.5° to 151.0°C at 0.5°C min⁻¹.

No published value was available for comparison.

Proton NMR: The proton NMR data for PCB 157 are tabulated in appendix 1. The data concur with the assigned structure of PCB 157 and agree well with published data (46, 52). Impurity signals were recorded at:

δ (ppm) relative to TMS.

Approx. 1.22	Too weak to determine multiplicity
Approx. 1.55 (singlet)	Weak
Approx. 2.18	Extremely weak - barely discernible
7.54	Extremely weak - barely discernible

Mass spectrometry: The 70 eV EI mass spectrum (see appendix 1) was typical of a hexachlorobiphenyl, exhibiting the appropriate m/z values, isotope clustering and fragmentation patterns (53). There were no notable impurity peaks.

Capillary gas chromatography: Chromatographic conditions were as for PCB 156 (see Section 2.4.1.).

Some small, very early eluting peaks were visible and appeared, on the whole, to be the same contaminants as those seen in the PCB 156 sample. The adjusted retention time, t_r' for these moderately volatile impurities were no more than 3.0 minutes for all except one very minor contaminant (peak height = 1.5 mm) which had a t_r' value of 4.7 minutes. A later eluting, very minor impurity at $t_r' = 37.2$ mins. (peak height = 1.0 mm) was identified as PCB 169. PCB 157 eluted at $t_r' = 35.4$ mins. with a peak height of 153.5 mm. No other impurities were seen.

Summary and discussion:

From the spectroscopic data and the method of synthesis, the compound was undoubtedly PCB 157. Purity appeared to be at least adequate for use as an analytical standard.

The NMR data showed extremely small amounts of ethanol in the sample as evidenced by the signals at $\delta = 1.22$ and 2.18 which could be the methyl and hydroxyl protons respectively. No signal was visible at $\delta = 3.70$ where one would have expected to find the methylene proton signals for ethanol; this was probably due to the signals being beyond the detection limit of the instrument. The weak signal at $\delta = 1.55$ could be due to methylene or methine protons in a hydrocarbon chain but no corresponding methyl proton signal could be seen. The extremely weak signal at $\delta = 7.54$ was due to the presence of very low

levels of PCB 169 (confirmed by capillary GC).

The mass spectrum showed no notable impurities.

The capillary GC results showed the presence of low levels of the same moderately volatile contaminants as seen in PCB 156. There was also a very small peak at $t_r' = 4.7$ mins.; whether this was a true contaminant or an artifact is not known but the concentration was too low to have been of significance. The presence of PCB 169 at a low concentration was witnessed by the small peak (height 1.0 mm) at $t_r' = 37.2$ mins. The adjusted retention time and peak height of PCB 157 were 35.4 mins. and 153.5 mm respectively. Assuming equal response factors and peak shapes for the two isomers, the PCB 169 represented only about 0.6% of the two PCBs present which was considered acceptable.

It was concluded that the PCB 157 was of acceptable purity. The identification confirmed the elution order of PCBs 157 and 169 as was assigned during the RP-HPTLC and preparative HPLC isolation procedures (see Section 2.3.2.). The presence of small amounts of PCB 169 was expected but did not appear to be a significant problem. Other than the presence of PCB 169 and the apparent absence of phthalate, the impurity profile appeared to be similar to that of PCB 156 with very low levels of a hydrocarbon-like contaminant and traces of ethanol. There

were also the contaminants of moderate volatility in the capillary GC.

2.4.3. PCB 169.

Melting point: 192.0° to 193.0°C at 0.4°C min⁻¹.

Literature value 201° to 202° (54).

Proton NMR: The proton NMR data for PCB 169 are given in appendix 1. The data are consistent with the structure of PCB 169 and agree with published data (46, 52). Impurity signals were observed at:

δ (ppm) relative to TMS.

Approx. 0.94 (triplet)	Strong, distorted
Approx. 1.22 (singlet)	Strong, broad
Approx. 1.55 (singlet)	Moderate, sharp
Approx. 7.10 to 7.50 (multiplet)	Very weak

Mass spectrometry: The 70 eV EI mass spectrum (see appendix 1) was typical of a hexachlorobiphenyl (53).

Several notable impurity peaks were seen including:

<u>m/z</u>	
29	Weak
43	Weak
57	Weak
149	weak

Capillary gas chromatography: Conditions were as for PCB 156 (see Section 2.4.1.).

Impurity peaks were observed at t_r' times up to 3.0 minutes and appeared to be the same materials as those seen for PCBs 156 and 157. The concentrations of these substances were also similar to those seen with the other congeners. Another impurity was seen at $t_r' = 20.7$ mins. with a peak height of 1.5 mm. No other peaks were seen until $t_r' = 35.4$ mins. and this was identified as PCB 157 (peak height = 2.0 mm). PCB 169 eluted at $t_r' = 37.2$ minutes with a peak height of 145.5 mm. No other significant peaks were observed.

Summary and discussion:

The data for PCB 169 were quite difficult to interpret unequivocally. The melting point was depressed by about 9°C and the NMR results showed the presence of high-field impurities. The NMR signal at about $\delta = 0.94$ bore some resemblance to that for a methyl group attached to a

methylene group but it was considerably distorted in that it was broadened and the central signal of the triplet was unusually large and the downfield signal was strangely shortened. The broad signal at $\delta = 1.22$ could have been due to the methylene protons of a hydrocarbon chain. The singlet at $\delta = 1.55$ may have been due to some type of long hydrocarbon chain protons but this is not certain. The very weak multiplets in the aromatic region are probably due to low levels of PCB 157.

The mass spectrometric results indicate the presence of phthalates ($m/z = 149$) and hydrocarbon chains ($m/z = 29, 43$ and 57).

The gas chromatography results showed the presence of some moderately volatile contaminants as was seen in PCBs 156 and 157. PCB 157 was a contaminant as expected and assuming equal FID response factors and peak shapes, PCB 157 comprised about 2.0% of the two PCB isomers present. This could account, in part at least, for the depression of the melting point. The small impurity at $t_r = 20.7$ mins. could be a long-chain hydrocarbon-like compound, but if this was so it is unlikely that there would be enough of this material to account for the high field signals encountered in the proton NMR spectrum.

In summary, PCB 169 would seem to contain a number of impurities at a low level. The melting point was

depressed and the sample itself had a slightly waxy consistency. It was, however, decided that the sample was pure enough to be used as an analytical standard provided the level of purity was borne in mind.

The positive identification of the substance as PCB 169 confirmed the elution order assignments made during the RP-HPTLC and preparative HPLC separations (see Section 2.3.2.).

2.4.4. PCB 189.

Melting point: 151.0° to 153.0°C at 0.5°C min⁻¹.

Literature value 162° to 163°C (55).

Proton NMR: The proton NMR data are reported in appendix 1 and are consistent with the assigned structure of PCB 189. The results also agree well with published data (46, 52). Impurity signals were seen at:

δ (ppm) relative to TMS.

1.05 (singlet)	Weak, broad
1.25 (singlet)	Weak
1.64 (singlet)	Weak, broad
Approx. 2.18 (singlet)	Very weak
3.49 (singlet)	Moderate

Mass spectrometry: The 70 eV EI mass spectrum (see appendix 1) was typical of a heptachlorobiphenyl (53) displaying the correct m/z values, isotope clustering and fragmentation patterns. Several impurity peaks were also seen including the following:

<u>m/z</u>	
29	Weak
43	Weak
57	Weak
149	Weak

Capillary gas chromatography: Conditions were as for PCB 156 (see Section 2.4.1.) except that the solvent was hexane instead of heptane.

The PCB 189 eluted at $t_r' = 38.6$ mins. and went off scale on the recorder. Twenty minor impurity peaks eluted between $t_r' = 21.5$ and 36.9 mins. but very early eluting contaminants were absent.

Summary and discussion:

The melting point was depressed by about 10°C and the NMR showed high field impurities. The singlet at $\delta = 3.49$ was due to residual methanol and it may be speculated that the broad singlet at $\delta = 1.64$ may be the hydroxyl proton

but this seems unlikely. The origin of the other signals was unknown but may have been due to some hydrocarbon-like compound.

As before, the mass spectrum indicated the presence of low levels of hydrocarbon chain and phthalate impurities.

The capillary gas chromatography revealed the presence of a number of impurities but none of the early eluting impurities seen in PCBs 156, 157 and 169 was seen in PCB 189. This may be due to the use of HPLC grade methanol as the recrystallising solvent for PCB 189 instead of ethanol, which was used for the other three congeners. The concentration of each of the impurities eluting between $t_r = 21.5$ and 36.9 mins. was low but together they may add up to a significant level. It is possible that these compounds were hydrocarbon-like but this is conjecture.

The NMR indicated moderate levels of methanol and consequently, the PCB 189 was dried in a vacuum oven for a further four hours before use. However, the depressed melting point was unchanged by this which would indicate that the level of methanol had not been as high as originally thought.

In conclusion, PCB 189 seemed to contain low levels of a hydrocarbon-like contaminant and some phthalate, but

despite this, was deemed suitable for use as an analytical standard.

2.4.5. PCB 118.

Melting point: 106.0° to 106.5°C at 1.0°C min⁻¹

Literature values 105° to 107°C (55) and 112° to 113°C (56).

Proton NMR: The proton NMR data are tabulated in appendix 1 and are consistent with the assigned structure of PCB 118. Results also agree well with published data (46, 52). Impurity signals were seen at:

δ (ppm) relative to TMS.

Approx. 0.87 (triplet)	Very weak
Approx. 1.25 (singlet)	Weak
Approx. 1.55 (singlet)	Moderate
Approx. 3.50	Too weak to determine multiplicity

Mass spectrometry: The 70 eV EI mass spectrum (see appendix 1) was typical of a pentachlorobiphenyl (53) displaying the correct m/z values, isotope clustering and fragmentation patterns. Some impurity peaks were seen, most notably:

<u>m/z</u>	
29	Very weak
43	Weak
57	Weak
149	Weak
360/362	Very weak
392/394/396/398	Very weak Cl ₇ cluster

Capillary gas chromatography: Conditions were as for PCB 156 (see Section 2.4.1.) except that the solvent was hexane rather than heptane. No early eluting contaminants were visible at all. An extremely small peak (peak height < 1 mm) eluted at $t_r' = 21.6$ mins. and four extremely small peaks (none > 1 mm) eluted between $t_r' = 25.2$ and 26.0 mins. The most notable impurity peak eluted very late at $t_r' = 44.8$ mins. (peak height 5 mm) and was broad due, at least in part, to it eluting in the isothermal region of the chromatogram; the source of this peak was not known. PCB 118 eluted at $t_r' = 32.0$ mins. with a peak height of 149.5 mm.

Summary and discussion:

The melting point of PCB 118 agreed well with one of the published values but was about 6°C lower than the other.

The proton NMR again revealed the presence of a low level

of a hydrocarbon-like material together with a very small amount of residual methanol from the recrystallisation.

The mass spectrum contained weak hydrocarbon chain peaks at $m/z = 29, 43$ and 57 together with a trace of phthalate at $m/z = 149$. Interestingly, there appeared to be a very slight trace of a heptachlorobiphenyl as indicated by the typical Cl_7 cluster between $m/z = 392$ and 398 . The peak pair at $m/z = 360$ and 362 was of unknown origin, but the two peaks were of similar intensity which may indicate that they were the central peaks of a Cl_6 cluster, accounting for the large error in intensity measurements with such weak signals, with the other peaks of the cluster being too weak for the mass spectrometer to detect; this could imply that these peaks were due to a hexachlorobiphenyl. The presence of hexachloro- and heptachlorobiphenyls could arise if the original reactants used in the synthesis contained higher chlorinated impurities, but these would not usually be expected to come through the HPLC separation. It is, however, conceivable that such low concentrations as indicated by the mass spectrum, could remain in the isolated PCB 118.

The capillary gas chromatogram seemed to indicate that the PCB 118 was quite pure. There were a few very minor peaks at $t_r' = 21.6$ mins. and at $t_r' = 25.2$ to 26.0 mins. but the main impurity peak seemed to be a broad peak at

$t_r' = 44.8$ mins. This could be the heptachlorobiphenyl seen in the mass spectrum but the chromatographic peak would appear to be much too big to result from this heptachloro- congener. The material was rather late eluting and appeared in approximately the right chromatographic region for a heptachlorobiphenyl. The peak was also rather broad which may suggest that it was some sort of artifact such as a column bleed peak.

In conclusion, it would appear that PCB 118 was quite pure and suitable for use as an analytical standard. Very small amounts of methanol remained, as shown by the the NMR results, together with the hydrocarbon-like impurity seen in the other congeners that were synthesised. The mass spectrum revealed traces of a heptachlorobiphenyl and possibly a hexachlorobiphenyl which could have arisen from impurities in the starting materials for the synthesis. The capillary gas chromatogram was relatively "clean" except for a very late eluting, broad peak which was of unknown identity. There was no cross-contamination with PCB 97 and the positive identification of PCB 118 confirmed the original assignment of the reversed phase chromatographic elution order of PCBs 97 and 118 (see Section 2.3.4.).

2.4.6. PCB 97.

Melting point: 78.0° to 79.0°C at 0.5°C min⁻¹

Literature value 81° to 82°C (55).

Proton NMR: The proton NMR data are tabulated in appendix 1 and confirm the structure as being that of PCB 97; the results agree well with published data (52). Impurity signals were seen at:

δ (ppm) relative to TMS.

Approx. 1.55 (singlet)	Weak
Approx. 2.00 (singlet)	Weak
Approx. 2.18	Too weak to determine multiplicity
Approx. 3.50	Too weak to determine multiplicity

Mass spectrometry: The 70 eV EI mass spectrum was typical of a 2,2'-chloro- substituted pentachlorobiphenyl (57). Impurity peaks were observed at:

<u>m/z</u>	
43	Extremely weak
57	Extremely weak
149	Weak

Capillary gas chromatography: Conditions were as for PCB 156 (see Section 2.4.1.) except that a concentrated

solution in hexane was used.

There were 12 impurity peaks between $t_r' = 18.8$ and 26.8 mins. though these were very minor compared to the PCB 97 peak which was well off-scale. The largest impurity peak eluted at $t_r' = 36.8$ mins. but this too was very small relative to the PCB 97. PCB 97 itself eluted at $t_r' = 28.3$ mins.

Summary and discussion:

The melting point obtained for the sample agreed quite closely with that published in the literature.

The proton NMR showed the usual weak high-field impurity signals indicative of a hydrocarbon-chain type of compound, together with a very small amount of residual methanol.

The mass spectrum also showed weak hydrocarbon peaks and a weak phthalate peak. The $[M-Cl]^+$ cluster is remarkably prominent compared to the $[M-Cl]^+$ cluster of most other congeners. This effect has been noted previously (57) and is typical of 2,2'-chloro- substitution when the remaining ortho positions are unsubstituted.

The capillary gas chromatogram revealed a number of impurities, though none of the very early eluting

compounds seen with some of the other PCB congeners synthesised was in evidence. PCB 97 was not required for any further work but overall, it would seem to be moderately pure and could possibly be used as an analytical standard provided due consideration was given to the impurities present. PCB 97 did contain a coloured contaminant carried over from the synthesis. The other contaminants appeared to be similar in nature and quantity to the contaminants in the other PCB congeners synthesised. No cross-contamination with PCB 118 was evident.

2.4.7. Identification and assessment of purity of synthesised PCB congeners: Conclusions.

One impurity was common to all the congeners synthesised, this being the high-field hydrocarbon-like impurity indicated by the NMR results. The mass spectra for all the congeners except PCB 157 showed hydrocarbon chain and phthalate impurity peaks and recrystallisation solvent was also a common contaminant.

Pigments seemed to be absent from all the congeners required as analytical standards (i.e. all except PCB 97) but PCB 97 was slightly off-white due to minor contamination with a coloured material.

Melting points were generally depressed relative to

published values.

The very early eluting impurity peaks in the capillary gas chromatograms were only present where ethanol was used as the recrystallising solvent but were absent where methanol was used. Consequently, it would appear that the ethanol was the source of these volatile impurities.

It is of interest that where the congeners were synthesised in pairs (PCBs 157/169 and 97/118), separation by preparative HPLC seemed to be very effective and cross-contamination appeared to be absent in the case of PCBs 97 and 118 and only very minor in the case of PCBs 157 and 169. Identification of these congeners confirmed the original assignment of elution orders during the RP-HPTLC and preparative HPLC work.

In summary, all the congeners required as analytical standards were deemed to be of suitable quality for that purpose despite the presence of some impurities. Increased purity would have been desirable and more specific identification of the impurities themselves would have undoubtedly helped pinpoint the sources of contamination, but these goals would have required an inordinate investment of time and consumables. Further to this, a more quantitative assessment of purity would also have been desirable and techniques such as differential thermal analysis could have been helpful in this context.

The impurities could have arisen from several sources; reaction by-products are a possible origin, both from the intended reactants and from impurities in the starting materials themselves such as higher or lower chlorinated analogues of the reactants. Other sources of impurities include the solvents used for sample processing which could contain plasticisers, such as phthalates, and perhaps hydrocarbon-like impurities. The preparative ODS HPLC column used for the clean-up and separation of congeners may have itself been a source of impurities since it was old and of unknown history and was found to be partially dried out before being used in this work. Consequently, some of the ODS phase could have been stripped from the column and found its way into the samples, but it is not likely that large quantities of ODS were removed as this would have resulted in severe degradation of column performance which was never observed.

3. EXTRACTION OF ORGANOCHLORINE RESIDUES FROM HUMAN MILK.

3.1. Introduction.

Organohalogen residues in milk occur almost entirely in the lipid globules and efficient extraction requires the disruption of these fat globules to permit dissolution of the fat and residues in a suitable solvent. There are a vast number of extraction procedures and it would probably be true to say that there are as many extraction procedures as there are laboratories performing organohalogen residue analysis on human milk. Procedures can be loosely categorised, for convenience, into three groups: i) Discrete step solvent extraction (e.g. separating funnel or mechanical blending with solvent) often followed by centrifugation to aid phase separation, ii) combined extraction/clean-up methods whereby extraction and (partial) clean-up are performed simultaneously, and iii) continuous extraction methods such as Soxhlet extraction or steam distillation. The three categories are inevitably somewhat arbitrary since extraction and clean-up steps often merge and there are often elements of clean-up in extraction and vice-versa. Simple extraction with a hydrocarbon solvent alone is usually inefficient and in order to disrupt the lipid globules, various techniques may be employed. Acetone is often used as an extracting solvent since it strips away the "membrane" surrounding the lipid allowing subsequent

extraction of the fat and residues by solvent (often hexane). Acetonitrile and ethanol have a similar effect on the "membrane". Physical methods such as sonication, freeze-drying and slurring with an adsorbent also crack the emulsion permitting extraction of the lipids. All the methods below make use of some form of membrane-disrupting technique (either a solvent or a physical method) prior to or simultaneously with solvent extraction.

3.1.1. Discrete step solvent extraction.

The official methods of the UK Laboratory of the Government Chemist (LGC) (58), the US Association of Official Analytical Chemists (AOAC) (59) and the US Environmental Protection Agency (EPA) (60) all fall into this category.

The UK LGC extraction method for OCPs and PCBs in human milk involves blending 20 ml of milk with 140 ml of a 50:50% v/v acetone:hexane mixture in an homogeniser. The mixture is then transferred to a centrifuge from which the supernatant is removed and passed through a column of anhydrous sodium sulphate into a Kuderna-Danish evaporator. After reduction in volume, the extract is cleaned-up using an alumina column. The main drawback of the method is that it is labour intensive.

The AOAC method, or a variant thereof, is widely used. It is very labour intensive and involves diluting the milk with ethanol and adding oxalate. This is then extracted with 50:50% v/v diethyl ether:petroleum ether followed by centrifugation. The extract is washed with water, dried through anhydrous sodium sulphate and lipid is removed by acetonitrile partitioning.

The US EPA describes one method for OCPs and two for PCBs. The OCP method and the "micro" method for PCBs are the same in principle and involve homogenising the milk with acetonitrile in a tissue grinder. The homogenate is then centrifuged, the supernatant is diluted with an aqueous phase and extracted with hexane. The EPA "macro" method for PCBs is similar to the method of Tessari and Savage (61) whereby extraction is performed with acetone using centrifugation, then the remaining coagulated material is further extracted with hexane followed by centrifugation. The acetone and hexane extracts are combined, washed with 2% sodium sulphate solution, dried and concentrated. Preliminary lipid removal is by acetonitrile partitioning. Again, the methods are very labour intensive.

Variants of the AOAC method include those of Yakushiji *et al.* (38) who employed a KOH saponification step as part of the extraction, and that of McKinney *et al.* (62) who used hexane instead of petroleum ether in the AOAC

method. Suzuki *et al.* (63) investigated different extracting solvents with the AOAC method and found hexane/acetonitrile/ethanol gave the best organohalogen recoveries with the least lipid extraction.

Some other interesting solvent extraction techniques include: i) extraction with acetonitrile followed by addition of water, adsorption of the residue onto charcoal and elution with 50:50% v/v acetone:hexane (64). Luke and Doose (65) adsorbed the milk onto alumina, extracted with acetonitrile in a blender, filtered, and back-extracted with petroleum ether. Rogirst *et al.* (66) employed an official Belgian method which is similar to that of the UK LGC. In this, 2:1 v/v acetone:hexane was used to extract 50 g of milk in an homogeniser then the phases were allowed to separate after which the extract was washed with water, dried with anhydrous sodium sulphate and concentrated in a rotary evaporator. Clean-up was by alumina column chromatography.

Procedures of the above type probably account for the majority of extraction methods used worldwide. They are generally well tried and tested and give good, reproducible results. They are, however, complex and labour intensive and often consume large amounts of laboratory materials.

3.1.2. Combined extraction/clean-up procedures.

These methods may be grouped into three main types: i) saponification followed by solvent extraction, ii) acid digestion followed by solvent extraction and iii) Adsorption of the sample and chromatographic elution of the residues. Saponification (38, 67) and acid hydrolysis (68), like other chemical degradation procedures, are often used for the analysis of PCBs alone in the presence of OCPs since some OCPs are destroyed by the severe conditions. Triglycerides are hydrolysed and subsequently removed by solvent extraction or column chromatography. A quick and simple acid digestion method of particular note is that of Veierov and Aharonson (68). In their method, concentrated sulphuric acid was slowly added to the milk which was then extracted with petroleum ether. The aqueous layer was then discarded and the extract further cleaned by the addition of more sulphuric acid. The extract was then reduced in volume for analysis by GC-ECD. PCBs and several OCPs were analysed by this method. Recoveries were generally very good and reproducible. The two main disadvantages of the method are the loss of dieldrin and endrin which are degraded by the acid (69), and the loss of the lipid necessitating a separate determination of lipid content. The method is, however, remarkably straightforward.

The third technique has been used successfully for PCBs

{70} and OCPs {71} in milk. The milk is slurried with a chromatographic adsorbent (silica or Florisil) which is then added to the top of an already packed adsorption chromatography column. Organohalogenes are typically eluted with 20:80% v/v dichloromethane:petroleum ether. The method is very simple to use but a compromise between the volume of milk and the size of the column has to be found; low levels of OCPs and PCBs would require a large volume of milk to be extracted which in turn would necessitate the use of a large adsorption column. Hence, low limits of detection could be expensive in terms of consumables, but the method is potentially very useful.

3.1.3. Continuous extraction methods.

Continuous extraction methods appear to be an underused form of organohalogen extraction. They can be very effective and are generally much less labour intensive and cheaper to perform than other methods. Continuous extraction methods include sweep co-distillation {72}, Soxhlet extraction and exhaustive steam distillation. Musial *et al.* {73} used Soxhlet extraction to extract OCPs and PCBs from human milk. In their method, the milk was simply dispersed in anhydrous sodium sulphate prior to extraction. More recently, Bush *et al.* {74} freeze-dried human milk samples and then Soxhlet extracted the remaining milk solids. Soxhlet extraction is a very thorough extraction method and is not labour intensive.

The effectiveness of the extraction technique can give rise to "dirty" samples, but this may be offset, to some extent, by adsorbing the milk onto a selective adsorbent prior to Soxhlet extraction (see Section 3.2.2.).

A very simple and elegant method of extraction for OCPs and PCBs, is exhaustive steam distillation using a modified Nielson-Kryger apparatus (see Section 3.2.1.). Veith and Kiwus, who developed a modified Nielson-Kryger apparatus (75) investigated its use in the extraction of organohalogenes from several matrices (but not milk). During operation of the equipment, the matrix, which must be aqueous, is boiled and the condensed steam falls through a layer of solvent. The solvent extracts the steam volatiles (assuming the steam volatile materials exhibit favourable partitioning behaviour) after which the condensate returns to the boiling sample via a siphon tube. The solvent is usually of low volume and quite clean thus obviating further concentration or clean-up before GC analysis. For these reasons, and the exceptional speed and simplicity of the technique, steam distillation should be the method of choice for organohalogen residue extraction whenever possible. Veith and Kiwus included in their work a comparison of their steam extraction with a Soxhlet extraction plus Florisil clean-up method for extracting PCBs from catfish. On inspecting the resulting gas chromatograms, they noted an increase in size of the early eluting PCB peaks relative

to the later peaks in the steam extract compared to the Soxhlet extract. The authors attributed this to the greater recovery of early congeners using steam extraction. Unfortunately, this may not be the case and as will be seen in Section 3.2.1., the difference is probably due to poorer recovery of the later eluting peaks when using steam extraction. Cooke *et al.* (76) found that steam extraction of PCBs from fat was very inefficient and it would seem reasonable to assume that the most lipophilic congeners, which are generally the later eluting congeners on a gas chromatogram, would exhibit the poorest recoveries. The work carried out on steam extraction methods for milk samples described in Section 3.2.1. would seem to confirm this view. Further drawbacks of the method include the breakdown of hexachlorocyclohexane isomers during extraction (see Section 3.2.1.1.) and the need to determine lipid content by a separate method.

3.2. Experimental Investigation of Organochlorine Residue Extraction Techniques.

A number of different milk extraction procedures were investigated but the majority were found to be time-consuming, labour-intensive and expensive in terms of materials; such methods will not be considered here. Some procedures do not permit the recovery of lipids which means that a sub-sample of milk has to be analysed for

fat content. For these reasons, attention was focussed on two extraction methods: exhaustive steam distillation and Soxhlet extraction.

Exhaustive steam distillation does not permit the recovery of lipids, but the simplicity and cost effectiveness of the method made it very attractive. The method was found to be extremely effective for the extraction of many OCPs and PCBs from water, but as will be seen, it was not effective for the extraction of highly chlorinated PCBs from milk and caused the breakdown of hexachlorocyclohexane isomers and so, unfortunately, the method had to be abandoned.

Soxhlet extraction is a very well established technique and gives very thorough solvent/matrix contact. Furthermore, the extracting solvent is hot which aids extraction but may cause breakdown of thermally labile materials. Soxhlet extraction was finally adopted as the extraction method due to the advantages of lipid recovery, simplicity and the partial clean-up provided by the adsorption of the milk onto a selective substrate before extraction.

3.2.1. Exhaustive steam distillation.

The equipment used was described by Veith and Kiwus (75) (see Figure 10) but constructed with an extracting

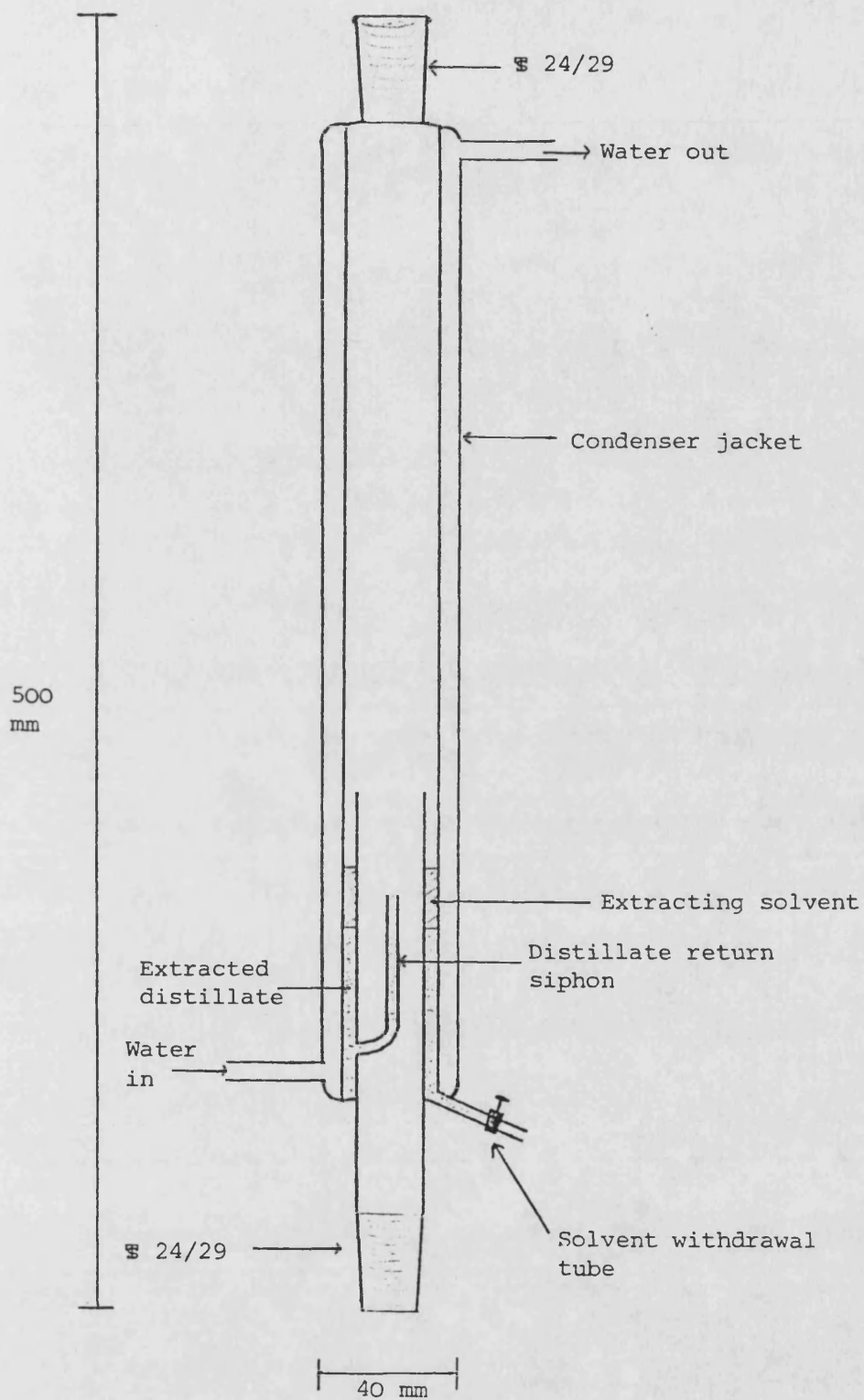


Figure 10. Steam Extraction Apparatus

NOT TO SCALE

solvent capacity of about 4 ml. During operation of an extractor, the aqueous liquid to be extracted is placed in a round bottomed flask (RBF). The extractor is placed on top of the flask using ground glass fittings. The drain stopcock is closed and clean glass-distilled water is poured into the trap until the level is just past the bottom of the return siphon. The condenser water is then turned on. The extracting solvent may be placed either in the RBF and allowed to distil over and condense into the trap, or it may be placed directly in the trap by running it down the inner condenser wall of the extractor. This latter method is very useful for quantitative work since if an internal standard is incorporated in the extracting solvent added in this way, any losses of the extract on removal from the extractor are compensated by the internal standard. This avoids the need to wash the extractor walls down into the extract. If the internal standard solution were added to the RBF instead of the trap, account would have to be taken of the recovery of the internal standard itself which could be a source of considerable error.

The sample in the RBF is then boiled and the steam containing the volatiles condenses and falls through the extracting solvent. The distillate, after extraction by the solvent layer, returns via the siphon to the RBF.

Assessment of extractor function was carried out with

both spiked water and spiked milk (cow's and human) samples. All solvents used were of HPLC grade.

Experiment 1.

The first experiment was conducted as described below using only spiked milk in order to obtain a preliminary qualitative assessment of the technique. PCBs were used as the test analytes.

Five samples were evaluated as follows:

1) n-Heptane (4.0 ml) containing approximately 150 $\mu\text{g ml}^{-1}$ of Aroclor 1242 was pipetted into a clean 250 ml RBF and the heptane was gently removed on a rotary evaporator. Cow's milk (15 ml) was added, swirled in the flask and then sonicated for 10 minutes to incorporate the PCBs into the milk. Glass-distilled water (120 ml) was then added plus a few anti-bumping granules. Clean heptane (4.0 ml) was pipetted into the flask and the sample was boiled vigorously for two hours. The heptane layer was then collected from the extractor and analysed using capillary GC-FID.

2) As for sample 1 but using n-heptane (4.0 ml) containing approximately 150 $\mu\text{g ml}^{-1}$ Aroclor 1248 as the spike.

3) As for sample 1 but using n-heptane (4.0 ml) containing approximately $150 \mu\text{g ml}^{-1}$ Aroclor 1260 as the spike.

4) As for sample 1 but using n-heptane (4.0 ml) containing approximately $50 \mu\text{g ml}^{-1}$ of each of Aroclor 1242, Aroclor 1248 and Aroclor 1260 (i.e. a total concentration of about $150 \mu\text{g ml}^{-1}$) as the spike.

5) Using blank n-heptane as the spike.

The GC-FID conditions were as follows:

Column: 25m CP Sil 5 0.35 mm id 0.16 μm film.

Program: 85°C for 1 min. $5^{\circ}\text{C min}^{-1}$ to 200°C . Hold.

Inj./Det. temp.: 250°C .

Carrier: Helium at 8 p.s.i.

Injection: $1\mu\text{l}$ split approx. 1:5.

Instrument: Perkin-Elmer Sigma 3 gas chromatograph.

The chromatograms produced by the samples were compared directly to those produced by the original spiking solutions.

Note. Cow's milk was used instead of human milk firstly because it is more readily available and secondly, using the above method, cow's milk would not normally contain detectable levels of OCPs and PCBs.

Results and discussion.

The heptane distilled over and floated in the trap as expected. Steam containing the volatile materials then condensed and fell through the heptane layer permitting extraction. A number of conclusions were drawn from the above experiment. Firstly, the milk tended to froth very badly and even boil over into the extractors; this was probably due to the proteins in the milk. Further work revealed that simmering the milk gently for 45 minutes prior to boiling prevented excessive frothing, probably by denaturing the proteins.

It was clear from a comparison of the capillary GC chromatograms produced by the samples and the spiking solutions that recoveries became progressively poorer for those PCB congeners having longer retention times. The later eluting, higher chlorinated and therefore more lipophilic PCB congeners were not effectively extracted from the milk. This can be seen in Figure 11 which shows the chromatogram for the extract of the milk spiked with the triple Aroclor mix (sample 4 above) together with that for the original spiking solution. No internal standard was used but estimates of recoveries were approximately 60% for the lower chlorinated congeners and around 0% for the higher chlorinated PCBs. These values are merely estimates and difficulties with the milks boiling over into the extractors may have adversely

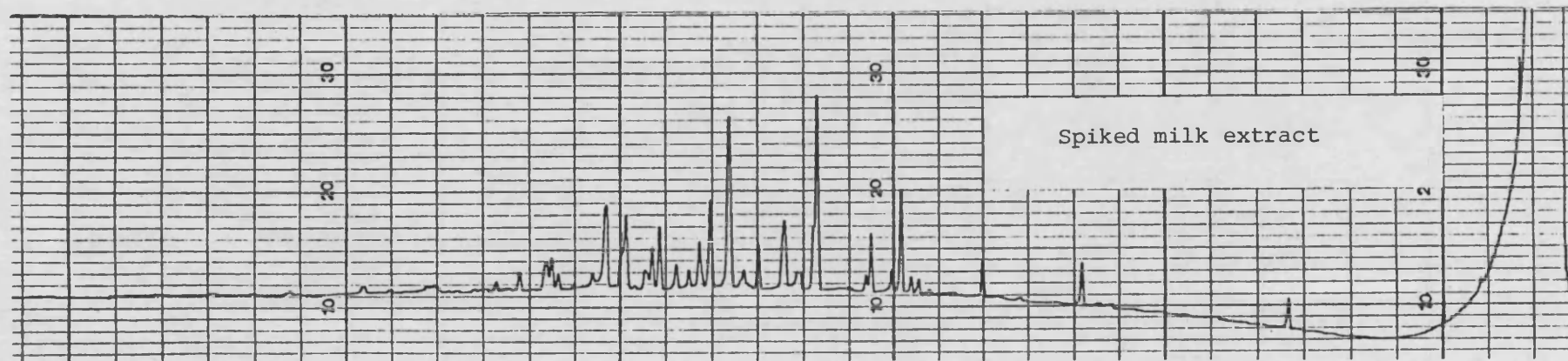
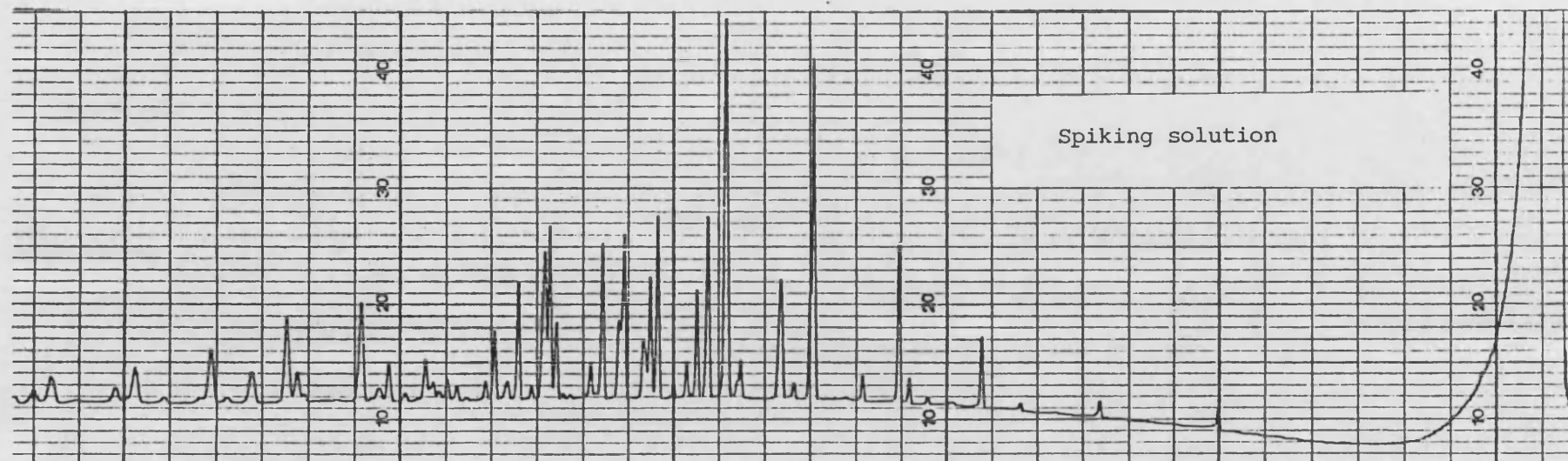


Figure 11.

30

20

10

Minutes after injection.

affected the recoveries.

There were a few small peaks in the blank milk but these were of minor significance. All the extracts smelt musty.

The reasons for the loss of the higher chlorinated congeners are not clear at this stage but it was thought that the most likely explanation was that the milk constituents, particularly the lipid, retained the higher chlorinated materials effectively reducing their vapour pressures and thus hindering steam extraction. It was, however, considered that the higher chlorinated PCBs could have been lost during rotary evaporation of the spiking solution or retained by binding to the flask walls but such explanations were thought unlikely. In order to examine the cause of the losses, further experiments were conducted.

Experiment 2.

The aim of this experiment was to confirm that PCBs are steam volatile and could be extracted using this apparatus under suitable conditions. The opportunity was also taken to repeat the extraction of spiked milk in order to reassess recoveries. Six samples were extracted as follows:

- 1) n-Heptane (4.0 ml) containing approximately $50 \mu\text{g ml}^{-1}$

of each of Aroclor 1242, Aroclor 1248 and Aroclor 1260 was pipetted into a 250 ml RBF. The solvent was gently removed in a rotary evaporator. Cow's milk (15 ml) was added and the flask was sonicated for 10 minutes. Glass-distilled water (85 ml) was added together with heptane (4.0 ml) and a few anti-bumping granules.

2) A duplicate of sample 1 was prepared.

3) A blank milk sample was prepared as for sample 1 but using heptane (4.0 ml) in place of the Aroclor solution.

4) The triple Aroclor mix (4.0 ml) was pipetted into a 250 ml RBF. Glass-distilled water (100 ml) was added together with a few anti-bumping granules.

5) A duplicate of sample 4 was prepared.

6) A blank water was prepared by placing glass-distilled water (100 ml) in a 250 ml RBF.

The samples were simmered gently for 45 minutes to prevent excessive frothing of the milk then all samples were boiled more vigorously for two hours. The heptane extracts were then examined by capillary GC as in experiment 1.

Results and discussion.

The absolute recoveries for the extracts could not be determined accurately since no internal standard was used but it was clear from the replicates that the recoveries from the milks for the lower chlorinated congeners was about 85%, dropping to about 40% for moderately chlorinated PCBs and finally falling to nearly 0% for the most highly chlorinated compounds. These recoveries represented a significant improvement over those obtained in experiment 1 and the increase was almost certainly due to the care taken in not allowing the samples to boil over.

The milk blank contained a few small peaks.

The water spikes were virtually indistinguishable from the original spiking solution with essentially 100% recovery for all peaks. The water blank displayed no significant peaks.

Hence, it was seen that the PCBs were all steam volatile and steam-extracted well from water. However, it was clear that something in the milk held the higher chlorinated congeners back or, alternatively, something in the preparation of the milk spikes caused loss of the higher chlorinated components.

Gas chromatograms of a milk spike, a water spike and the original spiking solution can be seen in Figure 12.

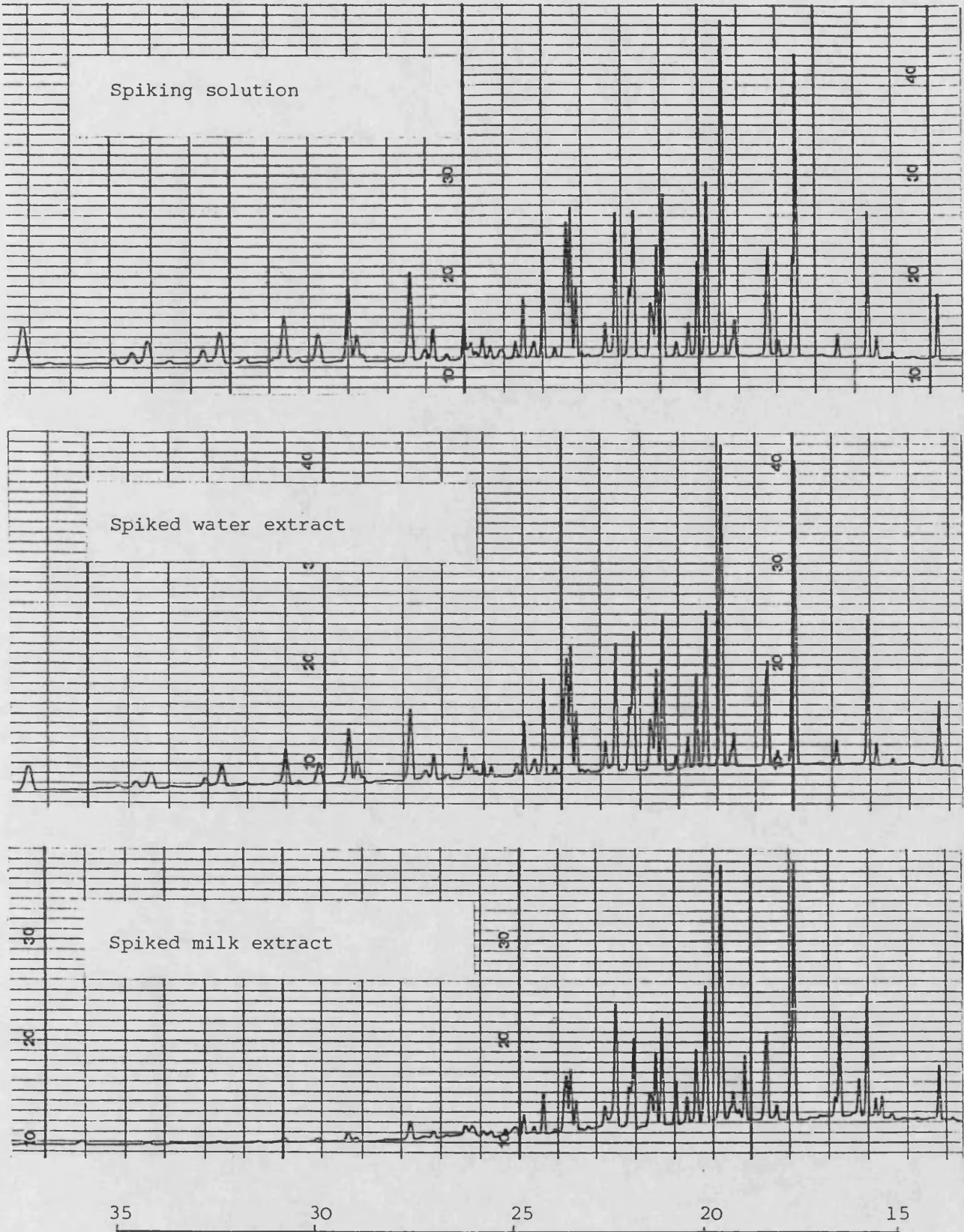
Experiment 3.

In order to determine if the evaporation of the n-heptane spiking solution using a rotary evaporator caused the loss of the higher chlorinated congeners, the 50 + 50 + 50 $\mu\text{g ml}^{-1}$ mix of the three Aroclors (2.0 ml) was added to a 250 ml RBF. The solvent was evaporated thoroughly and heptane (2.0 ml) was added to re-dissolve the residue. The sample was then analysed by GC as in experiment 1 together with the original spiking solution.

Results and discussion.

The evaporated sample showed no significant distortion of the PCB pattern and in fact, any losses that did occur, however slight, appeared to be of the lower chlorinated, more volatile congeners as may be expected.

So it appeared that the poor recovery of the higher chlorinated PCBs in milk spikes was indeed due to retention by milk components. Subsequent experiments were therefore aimed at attempting to release the bound PCBs.



Minutes after injection

Figure 12.

Experiment 4.

The first attempt to release the bound PCBs made use of sulphuric acid to try to degrade whichever component of the milk was responsible for the binding. Triglycerides and possibly proteins were considered to be the most likely candidates for causing the retention and both of these would be hydrolysed by the action of strong acid. It was appreciated, however, that this treatment would probably degrade certain OCPs should they be included as analytes at a later stage.

Six samples were prepared as follows:

1) The 50 + 50 + 50 $\mu\text{g ml}^{-1}$ Aroclor 1242 + 1248 + 1260 solution in n-heptane (4.0 ml) was pipetted into a 250 ml RBF and the solvent was gently removed using a rotary evaporator. Cow's milk (15 ml) was added and the flask was sonicated for 10 minutes. Concentrated sulphuric acid (2 ml) was added slowly and the sample was swirled for 1 minute. Distilled water (85 ml) was then added together with heptane (4.0 ml) and some anti-bumping granules.

2) A duplicate of sample 1 was prepared.

3) A water spike was prepared as in 1 above by substituting glass-distilled water for the milk.

4) A duplicate of 3 above was prepared.

5) A blank milk was prepared as in 1 above by substituting blank heptane for the Aroclor solution.

6) A water blank was prepared as in 3 above by substituting blank heptane for the Aroclor solution.

All samples were simmered gently for 45 minutes in order to avoid excessive frothing of the milk (although the acid reduced the frothing of the milk considerably). The samples were then boiled more vigorously for a further two hours. The heptane extract was then collected and analysed by GC together with the original spiking solution as in experiment 1.

Results and discussion.

The acid caused browning of the milk but reduced frothing on boiling.

The milk blank solution was clear on visual inspection but smelled musty and gave an extremely "dirty" chromatogram. The water blank, on the other hand, was very clean. Both blank chromatograms can be seen in Figure 13.

Both water spikes showed no distortion of the PCB pattern

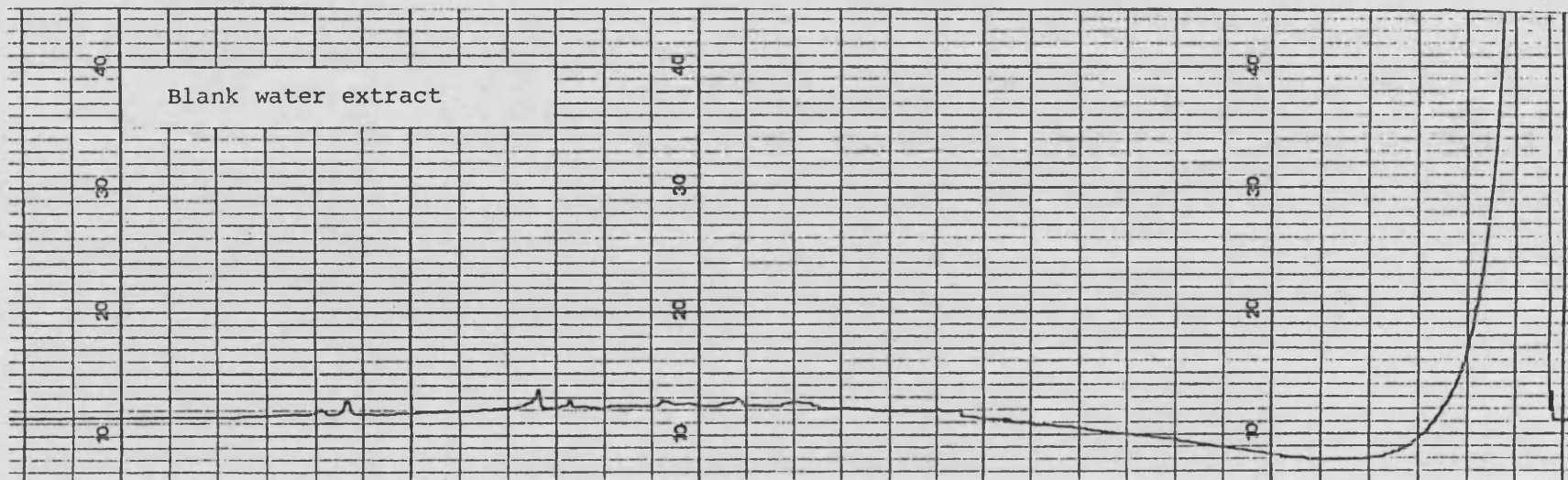
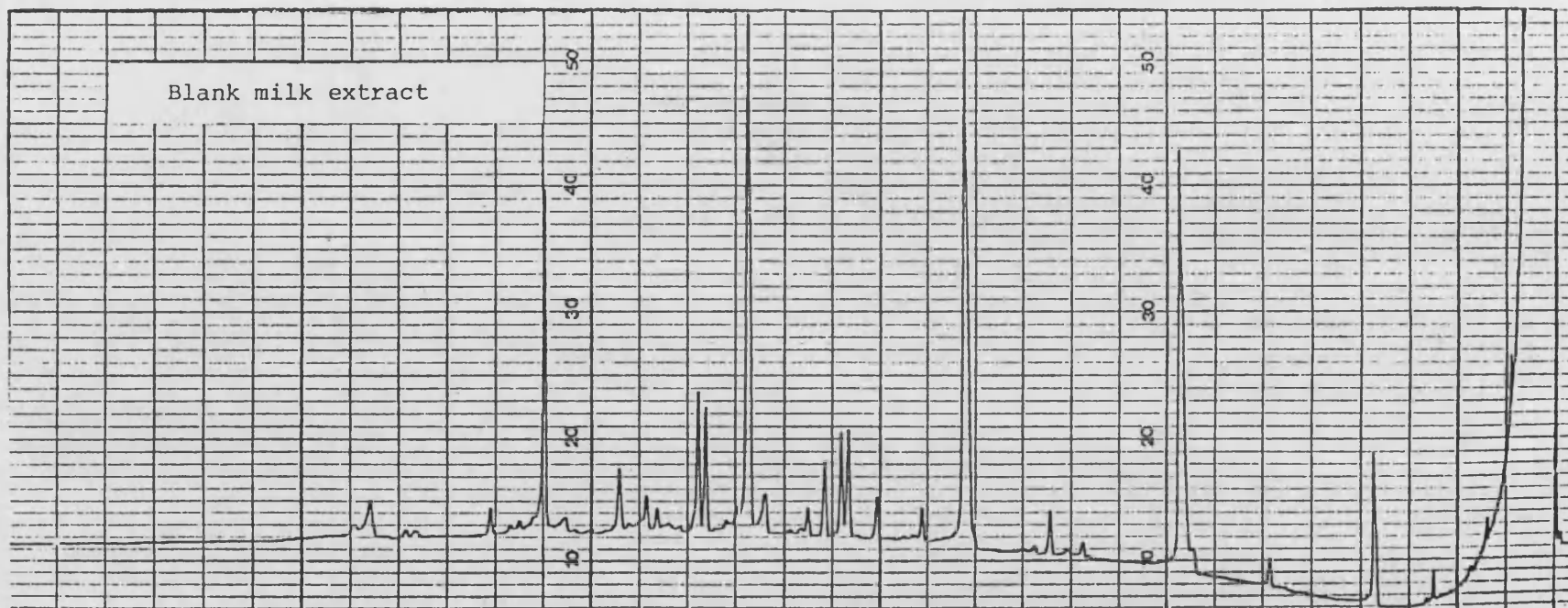


Figure 13.

25 20 15 10 5
Minutes after injection.

on GC examination but in contrast, the milk extracts showed no improvement in recovery of PCBs with the same marked cut-off of the higher chlorinated congeners in evidence. Furthermore, the acid degradation of the milk gave rise to several interfering peaks. The chromatograms for a water extract, a milk extract and the original spiking solution are shown in Figure 14.

In conclusion, the acid treatment did not release the bound PCBs from the milk and the breakdown of the milk gave rise to several interfering peaks in the milk extract chromatograms. In view of this, a different approach was taken using alkaline degradation of the milk.

Experiment 5.

In this experiment, sodium hydroxide was used to degrade the milk with the hope of releasing the bound PCBs. Experiment 4 was repeated for the cow's milk samples only and replacing the 2 ml of the concentrated acid with 10 ml of 0.1 M sodium hydroxide solution. Again, this treatment would be expected to degrade a number of OCPs.

Results and discussion.

The blank was cleaner on chromatographic examination than that produced by the concentrated sulphuric acid

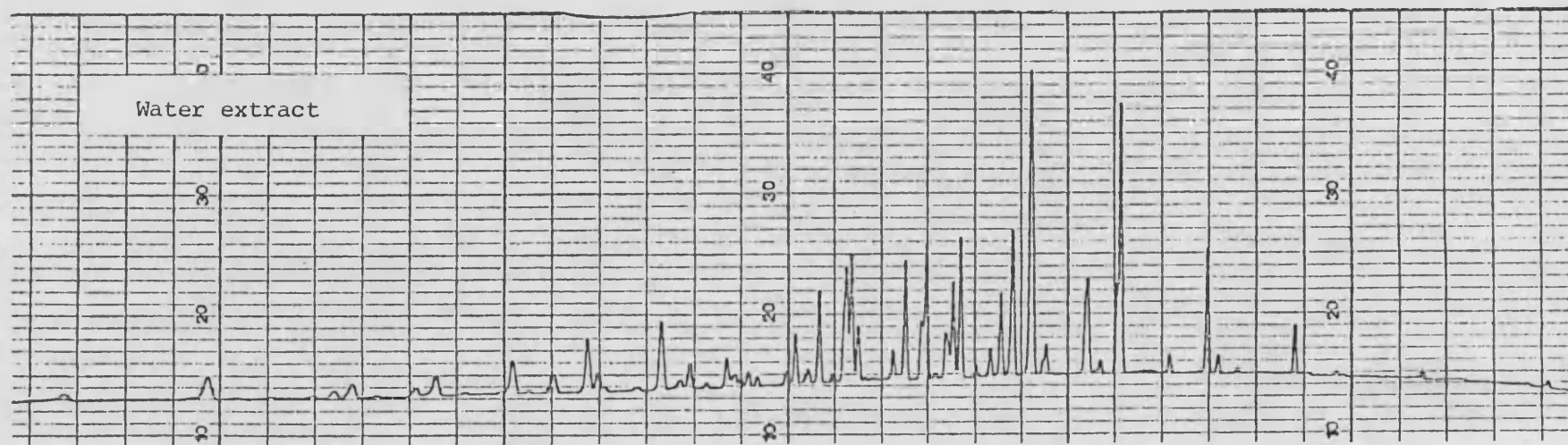
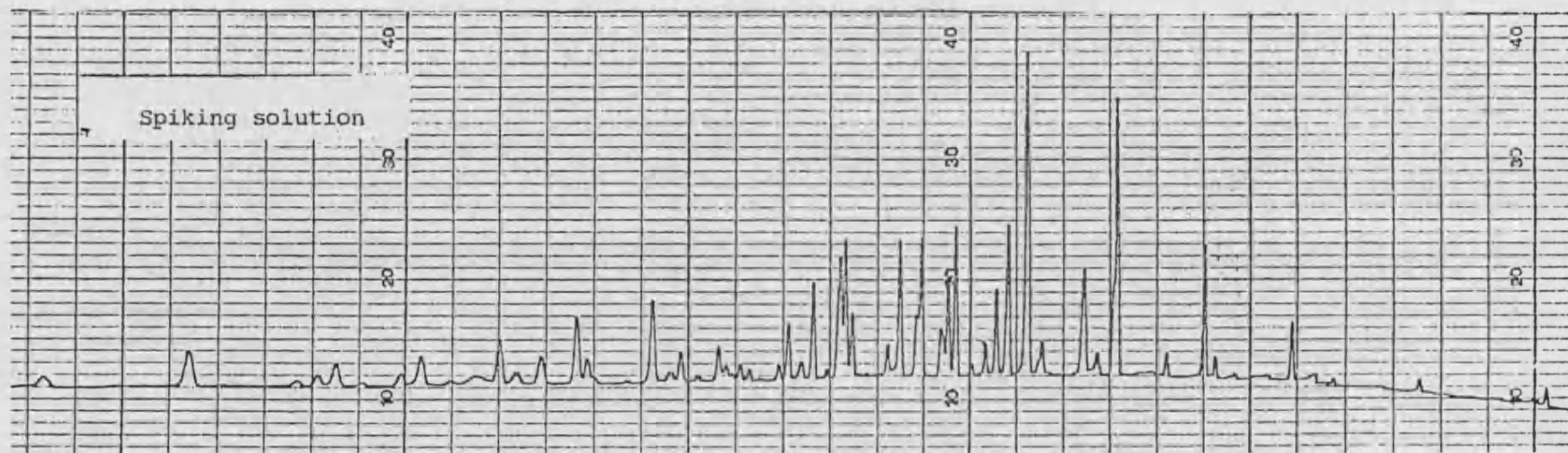


Figure 14.

30 25 20 15 10

Mins. after injection

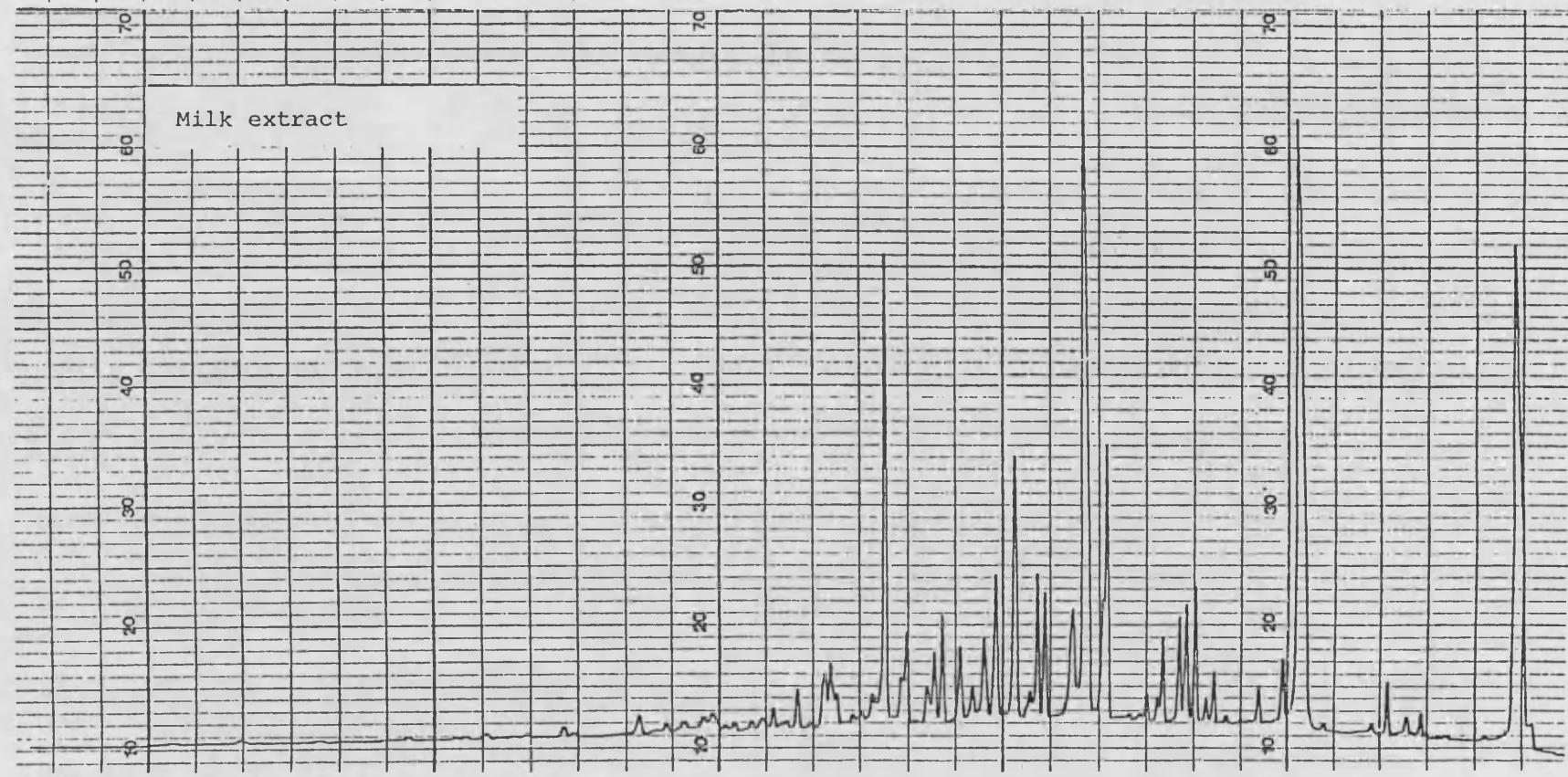
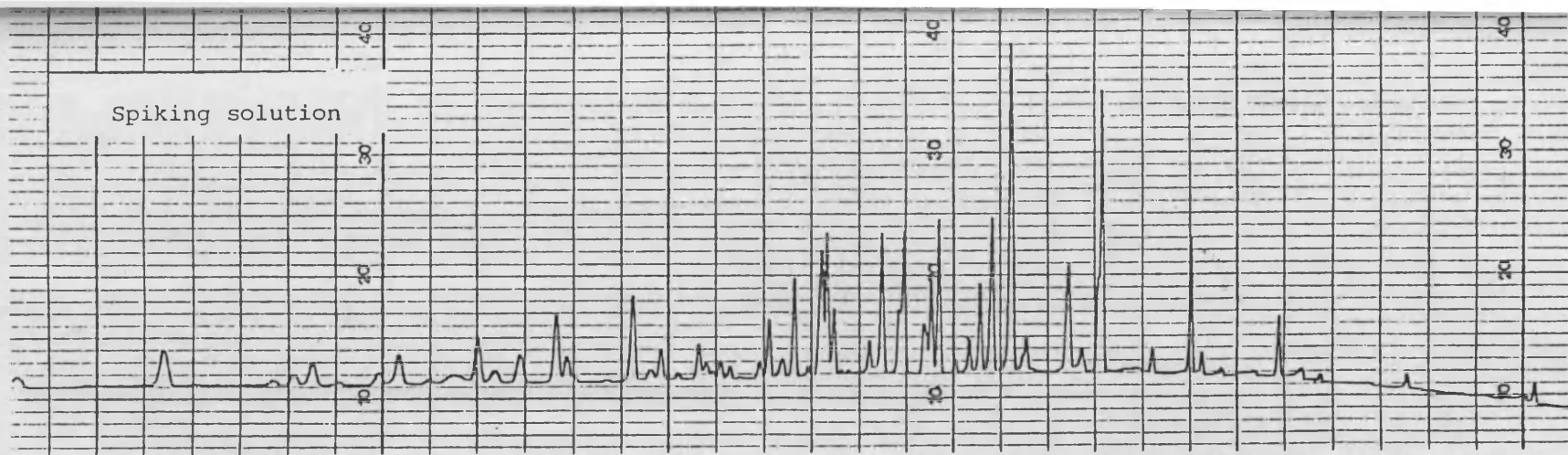


Figure 14 (contd.)

30 25 20 15 10
Mins. after injection

degradation, but there were still a number of interfering peaks.

The recoveries of higher chlorinated PCBs remained unchanged by the alkaline hydrolysis. Chromatograms for the blank, a milk spike and the spiking solution can be seen in Figure 15.

Experiment 6.

Saponification of milk triglycerides is commonly carried out using potassium hydroxide rather than sodium hydroxide. It was decided to use a more strongly alkaline medium than had been provided by the 0.1 M sodium hydroxide in experiment 5. To this end, 4 M potassium hydroxide (15 ml) was used but before conducting a spiking experiment, it was realised that the formation of soft soap may well cause excessive frothing so a preliminary experiment to assess this problem was carried out:

Cow's milk (15 ml) was placed in a 250 ml RBF. 4 M KOH (15 ml) was added with swirling and the sample was allowed to stand for 30 minutes after which time it had become very soapy. Glass-distilled water (70 ml) was added together with anti-bumping granules and heptane (4.0 ml). An extractor was placed on top of the RBF and the liquid was boiled. Immediately, the mixture frothed

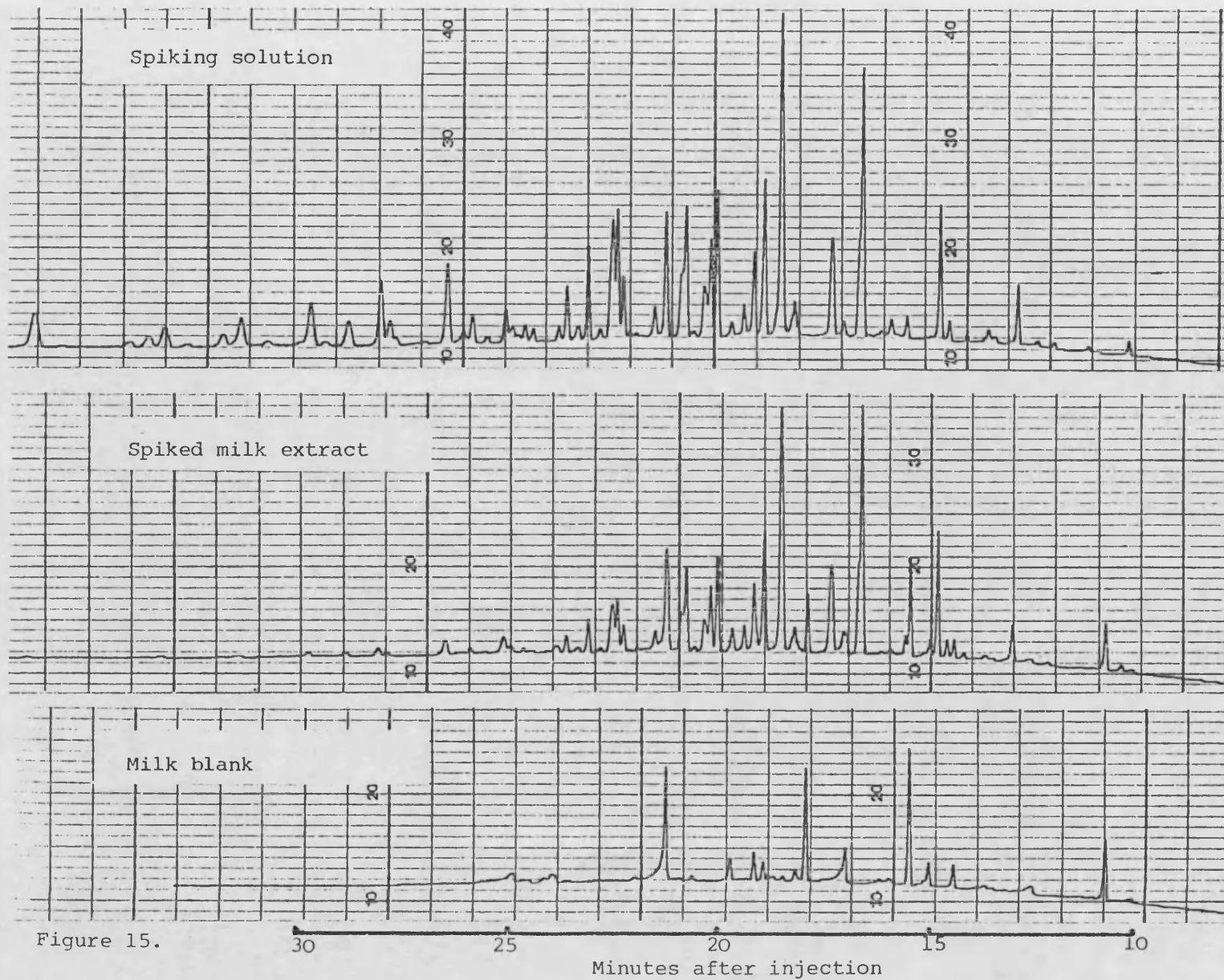


Figure 15.

violently but on adding 1.5 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ frothing was much reduced and extraction could be carried out unimpeded.

The spiking experiment was then conducted using cow's milk only. Three samples were prepared:

1) The approximately $50 + 50 + 50 \mu\text{g ml}^{-1}$ Aroclor 1242 + 1248 + 1260 solution in heptane (4.0 ml) was pipetted into a 250 ml RBF and the solvent was gently removed using a rotary evaporator. Cow's milk (15 ml) was added and the flask was sonicated for 10 minutes. 4 M KOH (15 ml) was added with swirling and the sample was allowed to stand for 30 minutes. Glass-distilled water (70 ml) was added followed by 1.5 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to precipitate the soap and crack the emulsion. A few anti-bumping granules were added together with 4.0 ml of heptane.

2) A duplicate of sample 1 was prepared.

3) A blank milk was prepared by substituting heptane (4.0 ml) for the Aroclor mixture in sample 1.

The samples were extracted for two hours after which time the heptane extracts were analysed together with the spiking solution using GC as in experiment 1.

Results and discussion.

Frothing was minimal and did not hinder the extraction process.

No improvement in recovery was observed and the extracts exhibited the same cut-off of the higher chlorinated PCBs as before.

Surprisingly, the blank was very clean with only a few small peaks in evidence; this may have been due to the precipitation of some of the milk breakdown products by the calcium chloride.

Experiment 7.

Chang-Yen and Sampath (77) claimed that the addition of oxalic acid to estuarine sediments prior to extraction by steam distillation enhanced the recoveries of certain organochlorine materials by virtue of its slight reducing properties and its acidity acting on various components of the matrix to release the analytes. By this stage, it seemed most likely that the higher chlorinated PCBs were being held back by the lipids and free fatty acids in the milk and the degraded milk respectively. In view of previous experiments it was considered unlikely that oxalic acid would improve the recoveries of PCBs from milk and unfortunately, this was found to be the case.

Six samples were prepared:

1) The approximately 50 + 50 + 50 $\mu\text{g ml}^{-1}$ Aroclor 1242 + 1248 + 1260 solution in heptane (4.0 ml) was pipetted into a 250 ml RBF and the solvent was gently removed in a rotary evaporator. Cow's milk (15 ml) was added and the flask was sonicated for 10 minutes. 0.1 M oxalic acid (85 ml) was added together with a few anti-bumping granules and 4.0 ml of heptane.

2) A duplicate of sample 1 was prepared.

3) A blank milk was prepared as in sample 1 but substituting heptane (4.0 ml) for the Aroclor spike.

4) A water spike was prepared by substituting glass-distilled water (15 ml) for the milk in sample 1.

5) A duplicate of sample 4 was prepared.

6) A blank water was prepared by substituting heptane (4.0 ml) for the Aroclor solution in sample 4.

The extractors were put in place and the samples were simmered for 45 minutes followed by two hours of more vigorous boiling. The heptane extracts were then examined by GC as in experiment 1.

Results and discussion.

The milk blank was very dirty and the peaks in the chromatogram were the same as those seen for the sulphuric acid treated milks in experiment 1. As anticipated, the milk spikes still showed no improvement in the recovery pattern.

As before, water spikes gave essentially 100% recovery for all congeners and the water blank was clean by GC.

Experiment 8.

The final experiment in the series was the extraction of a spiked milk sample for 24 hours in order to find out whether extending the extraction time would improve the recoveries of the PCBs. Three samples were prepared:

1) The approximately 50 + 50 + 50 $\mu\text{g ml}^{-1}$ solution of Aroclors 1242 + 1248 + 1260 in n-heptane (4.0 ml) was pipetted into a 250 ml RBF. The solvent was gently removed in a rotary evaporator and cow's milk (15 ml) was added. The flask was sonicated for 10 minutes and glass-distilled water (120 ml), a few anti-bumping granules and n-heptane (4.0 ml) were added.

2) A second spiked sample of milk was prepared as above but using a solution containing 170 $\mu\text{g ml}^{-1}$ Aroclor 1260

in n-heptane (4.0 ml) in place of the Aroclor triple mixture.

3) A blank milk was prepared as in sample 1 but substituting clean heptane (4.0 ml) for the PCB solution.

The extractors were put in place and the samples were extracted for 24 hours taking care not to allow the milk to boil over during the initial stages of the extraction. After 24 hours, the heptane extracts were removed and examined by GC as in experiment 1.

Results and discussion.

The recoveries of higher chlorinated PCBs were much improved and some of the early Aroclor 1260 peaks seemed to show very good recoveries (say >90%). However, a cut-off effect was still in evidence and the later eluting Aroclor 1260 peaks only showed recoveries of about 40%. As expected, all the lower chlorinated PCBs in the Aroclor triple mixture spike were all extracted with high recoveries.

An added complication was that the milk blank was quite dirty and extracts prepared in this way may need further clean-up or analysis using a selective GC detector.

Chromatograms for the Aroclor 1260 milk spike, the milk

blank and the original Aroclor 1260 spiking solution are shown in Figure 16.

Despite the improved recoveries, the remaining cut-off effect was regarded as sufficient cause to reject this method of extraction, particularly as it is the higher chlorinated congeners which tend to accumulate in human milk.

Conclusions on Steam Extraction.

The unavoidable conclusion from the preceding eight experiments is that certain milk components, most probably the lipids, retain the more lipophilic PCB congeners thereby reducing their vapour pressure and so inhibiting steam extraction. Even 24 hour extraction or degradation of the milk matrix (including hydrolysis of triglycerides) failed to release the higher chlorinated PCBs and it may be concluded that these more lipophilic PCBs remained bound to the free fatty acid residues produced by the degradation. In addition, such drastic conditions would probably degrade many OCPs had these been investigated. It was decided, therefore, to abandon this approach to milk extraction.

In contrast to milk, water spikes gave very clean extracts and essentially 100% recovery of all PCBs. As a point of interest, further studies were carried out on

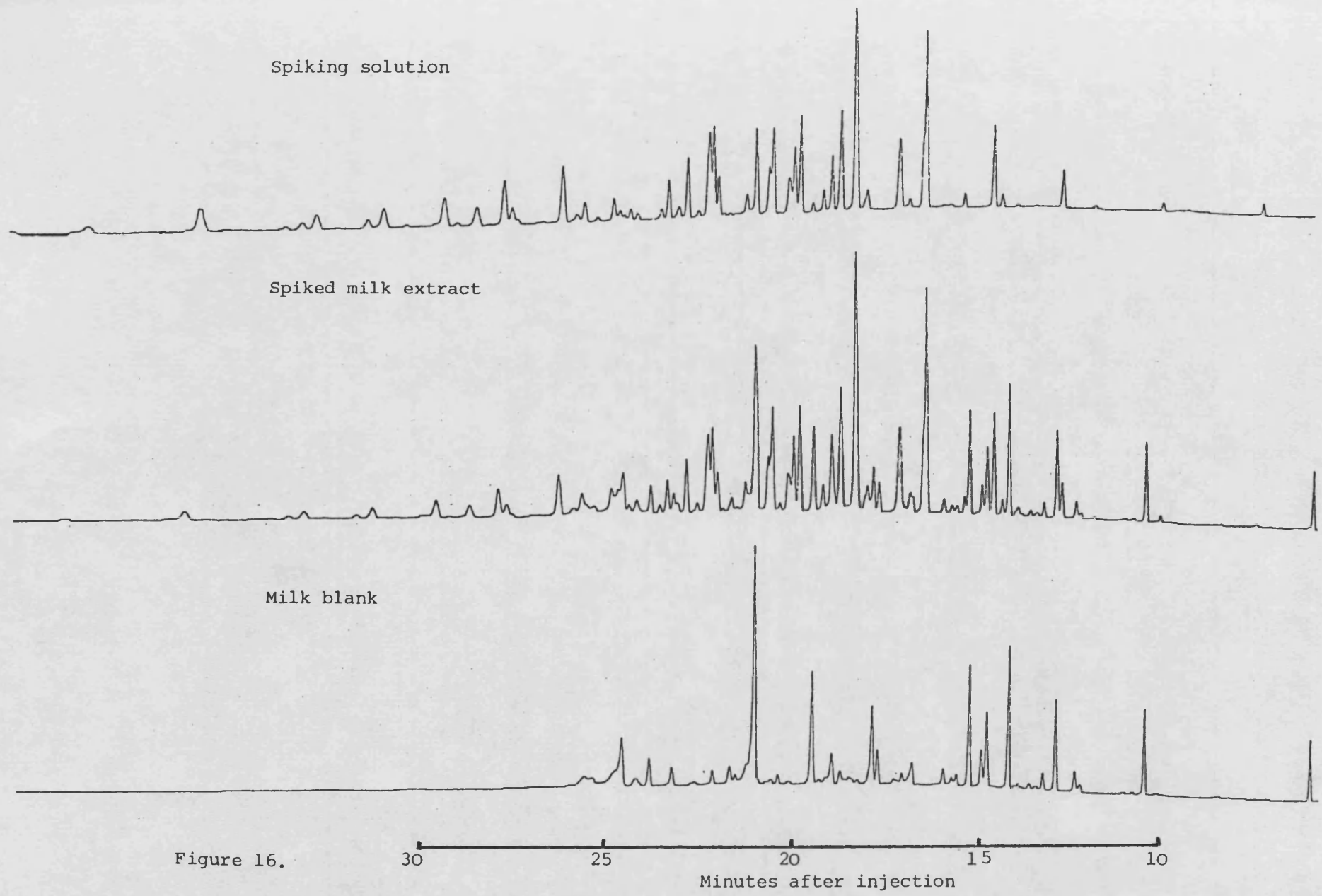


Figure 16.

30 25 20 15 10
Minutes after injection

the steam extraction of water samples.

3.2.1.1. Steam extraction of OCPs and PCBs from water (78).

Three litres of tap water in a 5L RBF were spiked with an acetone solution (1.0 ml) containing approximately 40 ng ml⁻¹ of each of the following organochlorine compounds:

Hexachlorobenzene, alpha-HCH, beta-HCH, gamma-HCH, delta-HCH, o,p'-DDE, p,p'-DDE, o,p'-DDT, p,p'-DDT, Aldrin, Dieldrin, Endrin, PCBs 118, 156, 157, 169, 189.

A modified extractor with an additional condensing coil in the top (see Figure 17) was used since the rate of steam production from 3L of water was considered sufficiently high to merit an increase in condensing power in order to prevent vapour loss.

The modified extractor was placed on top of the 5L flask and distilled water was run into the extractor trap until the level was just above the bottom of the return siphon. n-Heptane (1.0 ml) containing 54 ng ml⁻¹ of 4,4'-dibromobiphenyl was run down the inner wall of the extractor into the trap; this served both as the extraction solvent and as an internal standard for quantitative GC analysis.

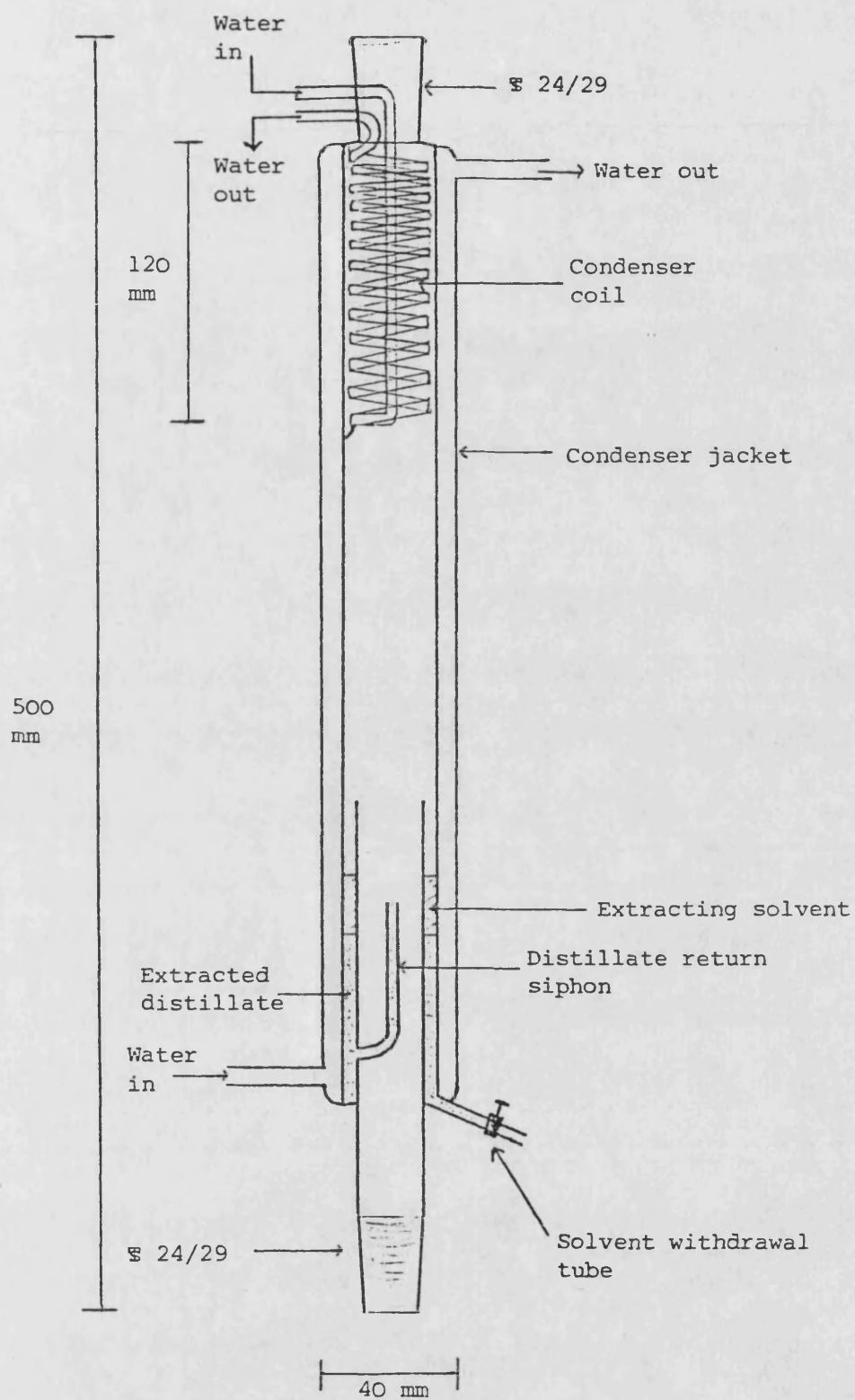


Figure 17. Modified Steam Extraction Apparatus

NOT TO SCALE

The water was steam extracted for 2 hours and recoveries of the analytes were determined by capillary GC-ECD as follows:

Column: 25m OV-17 0.25 mm id 0.2 μ m film.
Program: 100°C for 2 mins. Ballistic rise to 120°C. 1.3°C min⁻¹ to 250°C.
Inj. Temp.: 270°C
ECD Temp.: 350°C
Injection: 2.5 μ l. Splitless for 2 mins.
Instrument: Perkin-Elmer 8320B capillary GC.

Quantitation was performed by measuring the peak area ratio (analyte/internal standard) and comparing this directly to the corresponding peak area ratio for a standard containing the same amount of each analyte as the original spiking solution plus the internal standard.

Results and discussion.

The recoveries are shown in Table 4.

The spiking experiment represents the recovery of approximately 13 pg ml⁻¹ of each analyte from the water and in view of this, the recoveries were very encouraging. The low recoveries of the HCH isomers has subsequently been traced to the breakdown of these materials to two different trichlorobenzene isomers

Table 4. Recoveries of selected OCPs and PCBs for steam extract of tap water spiked at approx. 13 pg ml^{-1} per analyte.

<u>Analyte.</u>	<u>Recovery.</u>
HCB	62.5%
α -HCH	32.0%
β -HCH	10.1%
γ -HCH	10.9%
δ -HCH	Not detected.
o,p' -DDE	94.3%
p,p' -DDE	94.0%
o,p' -DDT	93.0%
p,p' -DDT	88.4%
Aldrin	75.8%
Dieldrin	94.6%
Endrin	93.8%
PCB 118	83.5%
PCB 156	82.4%
PCB 157	89.9%
PCB 169	95.2%
PCB 189	85.7%

during extraction.

Further work has shown that the recovery of Aroclors from water spikes is routinely over 90% using this technique and the method really merits further investigation.

It was realised that environmental water samples would not produce "clean" organohalogen extracts since such samples contain many other steam volatile materials. Using a non-selective or semi-selective GC detector for the analysis of these types of extract would give rise to extremely complex chromatograms (and experience has borne this out) but GC-MS provides an elegant solution to this problem. Despite the complexity of environmental samples extracted in this way, sample clean-up is not necessary since all the materials in the extract are inherently volatile and so would not be expected to damage a capillary GC column.

3.2.2. Soxhlet extraction.

Experiment 1.

The first experiment with Soxhlet extraction involved spiking milk with a mixed Aroclor solution in acetone, followed by adsorption onto a substrate prior to Soxhlet extraction. The extract was then reduced in volume and cleaned up using the HPLC clean-up method described in

Section 4.2.2.. Both the lipid and the organochlorine fractions were evaporated to dryness. The organochlorine fraction was re-dissolved in n-heptane with an internal standard and analysed by GC-FID. The lipid from each milk was weighed. The adsorbent chosen was a 50:50% mix by volume of Florisil:anhydrous sodium sulphate. The sodium sulphate was used to absorb the water and the Florisil was chosen for its polar adsorptive properties which it was hoped would retain some of the unwanted components of the milk matrix such as protein and electrolytes, but not the lipid or the organochlorine residues.

All solvents were of HPLC grade.

The spiking solution was $120 \mu\text{g ml}^{-1}$ Aroclor 1242 + $124 \mu\text{g ml}^{-1}$ Aroclor 1248 + $218 \mu\text{g ml}^{-1}$ Aroclor 1260 in acetone.

The recovery standard for GC analysis was made by taking 50.0 ml of the above spiking solution plus 3.0 ml of a $347.2 \mu\text{g ml}^{-1}$ solution of 4-chloro-m-cresol in n-heptane (as internal standard) and making this up to 100.0 ml with heptane to give a solution containing $60 \mu\text{g ml}^{-1}$ Aroclor 1242 + $62 \mu\text{g ml}^{-1}$ Aroclor 1248 + $109 \mu\text{g ml}^{-1}$ Aroclor 1260 + $10.42 \mu\text{g ml}^{-1}$ 4-chloro-m-cresol in 50:50% v/v acetone:heptane.

Six extracts were prepared from a single sample of pooled

human milk as follows:

1) The spiking solution (1.0 ml) was mixed with 10g of human milk in a 100 ml beaker. Florisil (10 g of 60-100 mesh ASTM) and anhydrous sodium sulphate (10 g) were mixed together and added slowly, with mixing, to the spiked milk. The slurry was then transferred quantitatively to a 30 x 80 mm cellulose Soxhlet thimble with 3 x 2 ml heptane washings. The adsorbed milk was then Soxhlet extracted with heptane for three hours at a rate of about 60 cycles per hour. The extract was gently evaporated just to dryness in a rotary evaporator and re-dissolved in 1 ml of heptane. This was taken up into a 2 ml syringe. The extract flask was washed with 0.8 ml of heptane and this too was taken up into the syringe with the extract. The sample was cleaned up using HP-GPC:

Column: Polymer Laboratories PLRP-S 300 x 7.5 mm.
10 μ m particles. 100 nm pores.
Mobile phase: 20:80% v/v Propan-2-ol:n-heptane.
Flow rate: 2.3 ml min⁻¹.
System temp.: 35°C.
Detection: Refractive index at 10⁻³ RI units f.s.d.
Instrument: DuPont 830 HPLC unit.

Lipid and organochlorine fractions were collected as described in Section 4.2.2. into 25 ml RBFs.

The solvent for each fraction was gently removed in a rotary evaporator. The lipid fraction was weighed and the organohalogen fraction was re-dissolved in heptane (2.0 ml) containing $10.42 \mu\text{g ml}^{-1}$ 4-chloro-m-cresol.

The organochlorine fraction was then analysed by GC-FID as follows:

Column: 25m CP Sil 5. 0.35 mm id. 0.16 μm film.
Carrier: Helium at 8 p.s.i.
Program: 85°C for 1 minute. 5°C min^{-1} to 250°C.
Hold.
Inj/Det temp.: 280°C.
Injection: 1 μl split approximately 1:5.
Instrument: Perkin-Elmer sigma 3 gas chromatograph.

Peak areas were measured using a Hewlett-Packard HP3390A integrator.

2) & 3) Two other replicates of sample 1 were prepared.

4) & 5) Two blank milks from the same milk sample as samples 1, 2 and 3 were prepared as above but omitting the spiking solution.

6) A blank sample of Florisil (10 g) plus anhydrous sodium sulphate (10 g) was extracted and analysed together with the milk samples.

Results and discussion.

The milk samples became brown during the extraction. Much of the brown colour remained on the adsorbent but some passed into the extract giving the extract a straw colour.

After extraction, the used adsorbent from the milk samples was examined and found to be slightly caked which may have occluded the passage of the solvent during extraction.

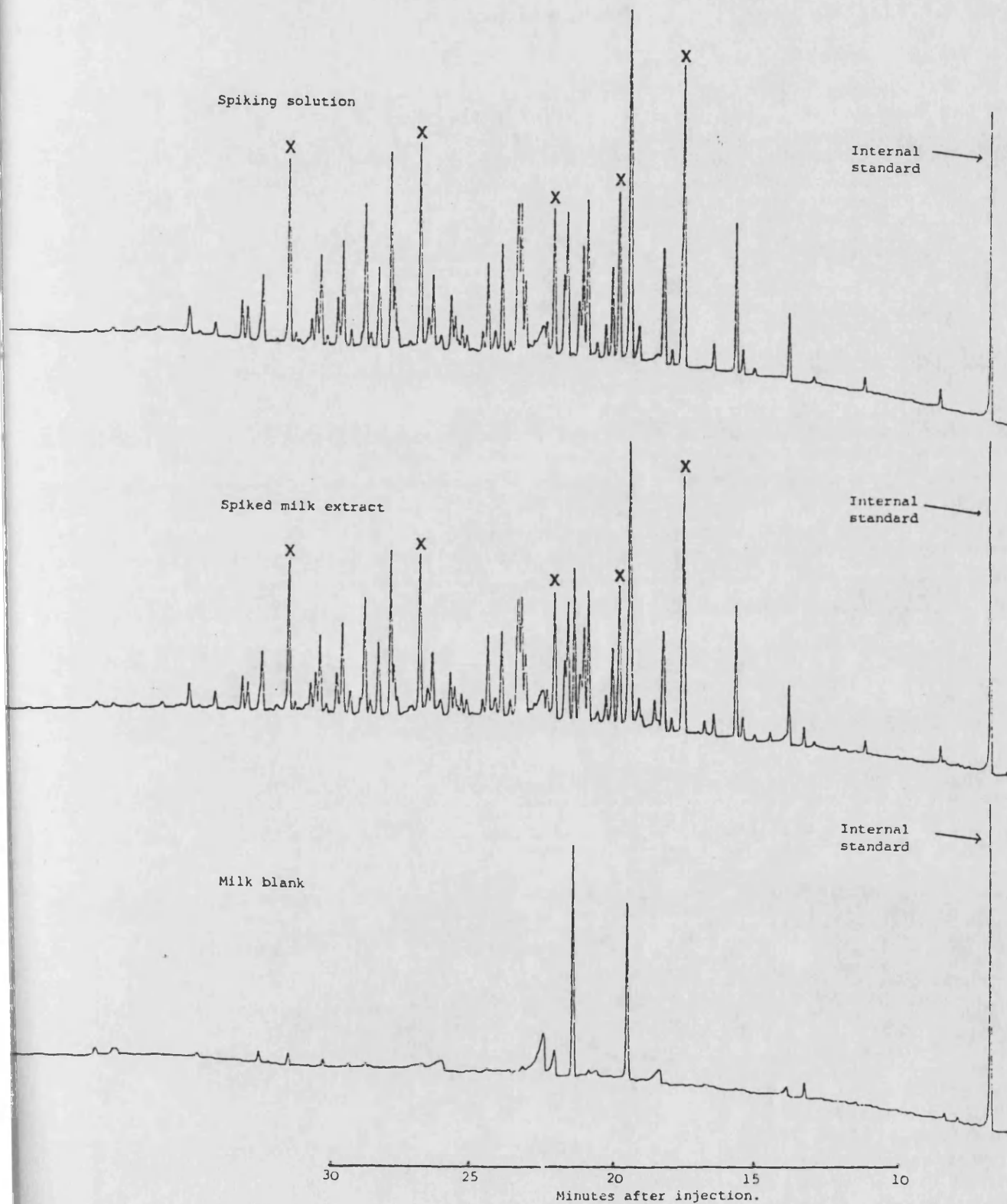
Quantitative analysis showed that recoveries for five selected PCB peaks across the chromatographic range (from 18 minutes to 32 minutes retention time) varied from about 62% to 77% with no significant distortion of the overall PCB pattern. A typical chromatogram for a PCB fraction from a spiked milk together with a milk blank and a recovery standard are shown in Figure 18.

As can be seen, the blank contains a few interfering peaks but on the whole is quite clean. The interfering peaks may have been due to other OCP residues or perhaps phthalates from the solvents as well as milk components.

Lipid determinations ranged from 1.51% to 1.54% m/m in the milk which seemed low and subsequently proved to be about 60% of the accepted value. This recovery value for

Figure 18.

Peaks marked X were used for recovery calculations.



the lipids is about the same magnitude as that for the PCB residues.

In summary, the method gave a fairly clean extract after HF-GPC clean-up with no significant discrimination between PCB congeners. The lipid was also recoverable for gravimetric determination of fat content. The recoveries were poor (62% to 77% for the PCBs and 1.51% to 1.54% m/m for the lipid) but showed promise; occlusion due to caking of the wet adsorbent seemed the most likely cause for this.

Experiment 2.

A similar experiment to that above was attempted using 5g acidic alumina plus 5g basic alumina plus 10g anhydrous sodium sulphate as the adsorbent. The aim was to make use of the different adsorptive properties of the aluminas. Unfortunately, the mixture tended to set hard on contact with milk and so the experiment was abandoned. Attention was then turned back to Florisil as an adsorbent.

Experiment 3.

It was decided to dry the adsorbent after the addition of the milk. Because of this, sodium sulphate was not used and was replaced by keiselguhr as a filtering aid. The adsorbent used was 70:30% v/v Florisil (60-100

mesh):kéiselguhr which was itself Soxhlet extracted with heptane before use.

A spiking solution containing approximately 300 ng ml^{-1} Aroclor 1254 and approximately 30 ng ml^{-1} of selected OCPs was prepared in n-heptane. The OCPs chosen were hexachlorobenzene, alpha-HCH, beta-HCH, gamma-HCH, delta-HCH, o,p'-DDE, p,p'-DDE, o,p'-DDT, p,p'-DDT, dieldrin and endrin.

An identical solution containing all the analytes plus 32 ng ml^{-1} of 4,4'-dibromobiphenyl was prepared in heptane as a recovery standard for the GC analysis.

The following six extracts were prepared:

- 1) About $2/3$ of a $30 \times 80 \text{ mm}$ Soxhlet thimbleful of adsorbent was placed in a glass mortar. Human milk (10 ml) was pipetted into a beaker and weighed. The milk was poured onto the adsorbent in the mortar and the beaker was re-weighed to determine the amount of milk transferred. The adsorbent and milk were mixed with a pestle. The sample was then dried in a fan oven at 60°C for 30 minutes with occasional mixing. The sample was then transferred quantitatively with hexane washings to a $30 \times 80 \text{ mm}$ cellulose Soxhlet thimble. The sample was extracted overnight into a 250 ml RBF using about 120 ml hexane at a cycling rate of about 40 cycles per hour. The

extract was filtered through an anhydrous sodium sulphate bed (previously washed with hexane), into a Kuderna-Danish evaporator fitted with a 10 ml graduated receiver tube. The extract was evaporated to about 1.5 ml and cleaned up using the HPLC method described in experiment 1. Lipid and organochlorine fractions were collected. The lipid fraction was evaporated to dryness over air for a gravimetric determination of fat and the organohalogen fraction was evaporated over clean, dry air to about 5 ml when 1.0 ml of a 32 ng ml⁻¹ solution of 4,4'-dibromobiphenyl in heptane was added as internal standard for the GC analysis. Evaporation was then continued to a volume of about 1.0 ml. This was then analysed by capillary GC-ECD as follows:

Column: 25m OV-17 0.25 mm id 0.2 µm film.
Carrier: Hydrogen at 65 cm s⁻¹ (10 psi).
Program: 100°C for 2 mins. 1.3°C min⁻¹ to 230°C.
Injector temp.: 270°C
Detector temp.: 350°C
Injection: Splitless for 2 minutes. 2.5 µl injection with 1 µl "chaser".
Instrument: Perkin-Elmer 8320B capillary GC.

Quantitation was carried out by direct proportional comparison of peak area ratios (analyte/internal standard) for the samples to the respective ratios produced by the recovery standard.

- 2) A duplicate of sample 1 was prepared.
- 3) A sample of cow's milk was prepared in the same manner as the human milk in sample 1.
- 4) A sample of cow's milk was prepared in the same way as the human milk in sample 1 but the adsorbent was spiked with 1.0 ml of spiking solution prior to drying in the oven.
- 5) A blank adsorbent was spiked with 1.0 ml of the spiking solution prior to extraction. This spiked adsorbent was subsequently processed as a milk sample.
- 6) Blank adsorbent was extracted, cleaned up and analysed as a milk sample.

Results and discussion.

Lipid determinations for the milks were:

Human milk sample 1	2.46% m/m
Human milk sample 2	2.54% m/m
Cow's milk sample 3	3.07% m/m
Cow's milk sample 4	3.06% m/m

These values for lipid content are much more realistic than those seen in experiment 1. The chromatogram for the

blank adsorbent was quite clean but did show a few small interfering peaks two of which co-eluted with delta-HCH and dieldrin.

Recoveries of OCPs from the spiked adsorbent ranged from between 74.3% for o,p'-DDT to 115.3% for p,p'-DDT except for delta-HCH which was recovered at a level of 50.9%. Five PCB peaks from the Aroclor 1254 pattern were selected and showed recoveries between 75.2% and 104.3%.

Recoveries from the cow's milk spike could not be accurately determined due to interfering peaks co-eluting with many of the analytes but on the whole, recoveries appeared to be similar to those observed in the spiked adsorbent.

The human milk duplicates showed the presence of several OCPs and PCBs and reproducibility appeared to be quite good although interferences made accurate assessment of this impossible.

Extraction was carried out overnight both to ensure good extraction and for convenience; such a long extraction time may not, however, be necessary.

Overall, the recoveries of lipids and OCPs/PCBs seemed quite good. The drying process seemed to help and the adsorbent was much more porous during extraction. The

drying was not complete and the adsorbent was still slightly damp when transferred to the Soxhlet thimbles, but this did not seem to be of any major significance and more severe drying may have had an adverse effect on recoveries.

It was clear that despite the clean-up, not all the target analytes could be determined using GC-ECD because of interferences and GC-MS would, ultimately, have to be used.

Experiment 4.

Lipid recoveries were still unknown and so this experiment was designed to determine the recovery of triglycerides using the Florisil/keiselguhr adsorbent with overnight Soxhlet extraction.

Three replicate samples were prepared according to the following method:

Approximately 2/3 of a 30 x 80 mm Soxhlet thimbleful of of the 70:30% v/v Florisil:keiselguhr adsorbent was placed in each of three glass mortars. A solution containing 15.00 g/100 ml mixed triglycerides (Witepsol H15) in heptane (2.0 ml) was pipetted onto each lot of adsorbent. Glass-distilled water (10 ml) was added to each and each sample was placed in the fan oven at 60°C

for 30 minutes. After the drying, each sample was transferred quantitatively with hexane washings to a 30 x 80 mm cellulose Soxhlet thimble and extracted overnight into a 250 ml RBF using hexane at about 30 cycles per hour. The extracts were evaporated to near dryness using a rotary evaporator, transferred with hexane washings to a tared, clean glass vial and the remaining solvent was evaporated over air at about 40°C. The lipid residues were weighed and the recoveries calculated.

Results and discussion.

Recoveries for the three replicates were recorded as 54.5%, 68.8% and 70.0%. These were disappointingly low and appeared to be due, at least in part, to poor permeability of the adsorbent and poor draining of the thimble during siphoning.

Experiment 5.

Experiment 3 was repeated with the following modifications using the same human and cow's milk samples:

i) The spiked adsorbent was spiked with the OCP/PCB spiking solution as before but was also spiked with 300 mg mixed triglycerides and glass-distilled water (10 ml). This spiked adsorbent was then dried in the fan oven with

the milk samples.

ii) Glass spacers were placed under the Soxhlet thimbles in the extractors to promote proper draining of the thimbles.

Results and discussion.

Lipid content for the milks were determined to be:

Human milk sample 1	2.47% m/m
Human milk sample 2	2.83% m/m
Cow's milk sample 3	4.68% m/m
Cow's milk sample 4	4.27% m/m

The human milk lipid values were similar to those obtained in experiment 3 but the cow's milk values were markedly increased. Reproducibility was not particularly good and probably not acceptable.

The lipid recovery from the spiked adsorbent was only 77.5%.

Lipid recoveries seem to have been improved by using the glass spacers in the extractors and during the extraction it was noted that draining of the thimbles was much improved by this modification. However, it was clear that the adsorbent was unsuitable for the extraction of

lipids.

OCP/PCB recoveries were similar to those obtained in experiment 3 but a disturbing development was the apparent total loss of endrin which exhibited almost 0% recovery from the spiked adsorbent. No explanation could be found for this (the spiking solution itself was analysed and found to contain endrin in the correct amount). As will be seen, this problem arose again in subsequent experiments and is discussed in Section 4.2.3.. No cause was ever established and the problem remains unresolved.

A repeat of this experiment revealed no improvement in the lipid recovery or the reproducibility of lipid determination but endrin was recovered at a level of 109.3% from the spiked adsorbent. Other organochlorines were recovered from the spiked adsorbent at levels between 88.3% for alpha-HCH to 124.7% for one of the PCB peaks. Recoveries from the spiked cow's milk ranged from 74.7% for HCB and 123.6% for p,p'-DDT. High recovery values were due to the presence of interferences.

Reproducibility for the determination of selected OCPs in the human milk samples was variable with the worst relative standard deviation being 21.0% for HCB and the best being 6.2% for p,p'-DDT but these values may be misleading due to interferences in the GC-ECD

determination.

Thus, OCP/PCB recoveries seemed to be very promising but lipid recoveries were disappointingly low and so efforts were directed towards improving lipid extraction.

Experiment 6.

Two other adsorbents were assessed for lipid extraction.

Four samples were prepared:

1) Approximately 3/4 of a 30 x 80 mm Soxhlet thimbleful of fibrous cellulose for column chromatography (Whatman) was placed in a glass mortar. Fresh human milk (10.0 ml) was pipetted on top of the adsorbent and mixed in. The mixture was dried in a fan oven at 60°C for about 40 minutes. The sample was then transferred quantitatively with hexane washings to a 30 x 80 mm cellulose Soxhlet thimble and extracted overnight with hexane into a 250 ml RBF. The extract was evaporated to near dryness using a rotary evaporator, was transferred quantitatively with hexane washings to a tared, clean glass vial and the remaining solvent was evaporated off over air at about 40°C. The lipid residue was then weighed.

2) The above procedure was repeated substituting the milk with distilled water (10.0 ml) and a 15.00 g/100 ml solution in heptane of mixed triglycerides (2.0 ml).

3) Sample 1 was repeated but substituting the cellulose with 50:50% v/v silica (70-230 mesh ASTM):Florisol (60-100 mesh ASTM) with a few anti-bumping granules mixed in to increase porosity.

4) Sample 2 was duplicated using the Silica:Florisol adsorbent described for sample 3.

Results and discussion.

On wetting, the cellulose became "mushy" and did not dry well in the fan oven despite occasional re-mixing. However, the cellulose-adsorbed samples seemed to extract very well in the Soxhlets. The milk adsorbed onto the cellulose turned straw yellow in colour, but the cellulose did not retain any significant amount of the colour.

The silica/Florisol mixture dried rather better in the oven but did not appear to be quite as porous as the cellulose. Much of the yellow colouration in the extracted milk was retained by the silica/Florisol.

Lipid content for the human milk samples were determined as:

Cellulose adsorbent:	3.87% m/v
Silica/Florisol adsorbent:	3.47% m/v

The triglyceride recoveries were determined to be:

Cellulose adsorbent:	96.0%
Silica/Florisil adsorbent:	80.1%

It was quite apparent that the cellulose permitted far more efficient extraction of the lipids, but the mushy consistency of the damp cellulose made quantitative transfer from the mortar to the thimble awkward. Furthermore, the cellulose adsorbent milk extract was much dirtier on visual inspection compared to the silica/Florisil adsorbent extract.

In view of these results, it was decided to mix Florisil and cellulose in a ratio of 50:50% by volume, and try this as an adsorbent.

Experiment 7.

Experiment 5 was repeated using a fresh human milk sample together with cow's milk samples, a spiked adsorbent and a blank, but using 50:50% v/v fibrous cellulose:Florisil (60-100 mesh).

Results and discussion.

Lipid determinations for the human milk duplicates were 2.87% m/v and 2.70% m/v and for the cow's milk were

3.30% m/v and 3.23% m/v. These values are in much better agreement than those produced using the Florisil/keiselguhr adsorbent. The triglyceride recovery from the spiked adsorbent was 92.1% which was deemed acceptable.

The adsorbent seemed to be very permeable to the solvent during extraction, yet the Florisil retained a lot of the colour produced and also retained any residual water. As a consequence of this latter observation, it was considered unnecessary to filter the extracts through an anhydrous sodium sulphate bed and so they were merely filtered through Whatman No.1 filter papers.

It seemed that the fibrous nature of the cellulose particles prevented ordered packing of the Florisil thus increasing permeability.

The blank was found to be quite dirty on GC analysis and appeared to contain certain OCPs, particularly gamma-HCH and dieldrin. This was attributed to the cellulose which was manufactured during the 1960's and was of plant origin. Consequently, OCP residues were to be expected. As a result, in future extractions, the adsorbent was Soxhlet extracted with heptane before use.

Recoveries for OCPs and PCBs were not calculated but on inspection appeared to be very good except for endrin

which was again lost. Delta-HCH recovery, as before, was lower than desired. Typical chromatograms for human milk and the blank adsorbent are shown in Figure 19.

Further experimentation.

Repeats of experiment 7 revealed several points:

i) Washing the adsorbent by Soxhlet extraction prior to use gave rise to a much cleaner blank.

ii) Delta-HCH recoveries were routinely lower than other HCH isomers and was typically around 65%.

iii) Both OCP and PCB recoveries were acceptable and adequately reproducible (see Section 6.2.2.) except for delta-HCH which routinely exhibited an unusably low recovery.

iv) Lipid recoveries were typically around 94% and reproducible (see Section 6.2.2.).

v) Endrin still appeared to be "lost" occasionally. It was noticed that endrin recovery was either very good or virtually zero and never in between the two extremes. No explanation can be offered for this strange effect.

vi) The chromatographic background on ECD tended to be

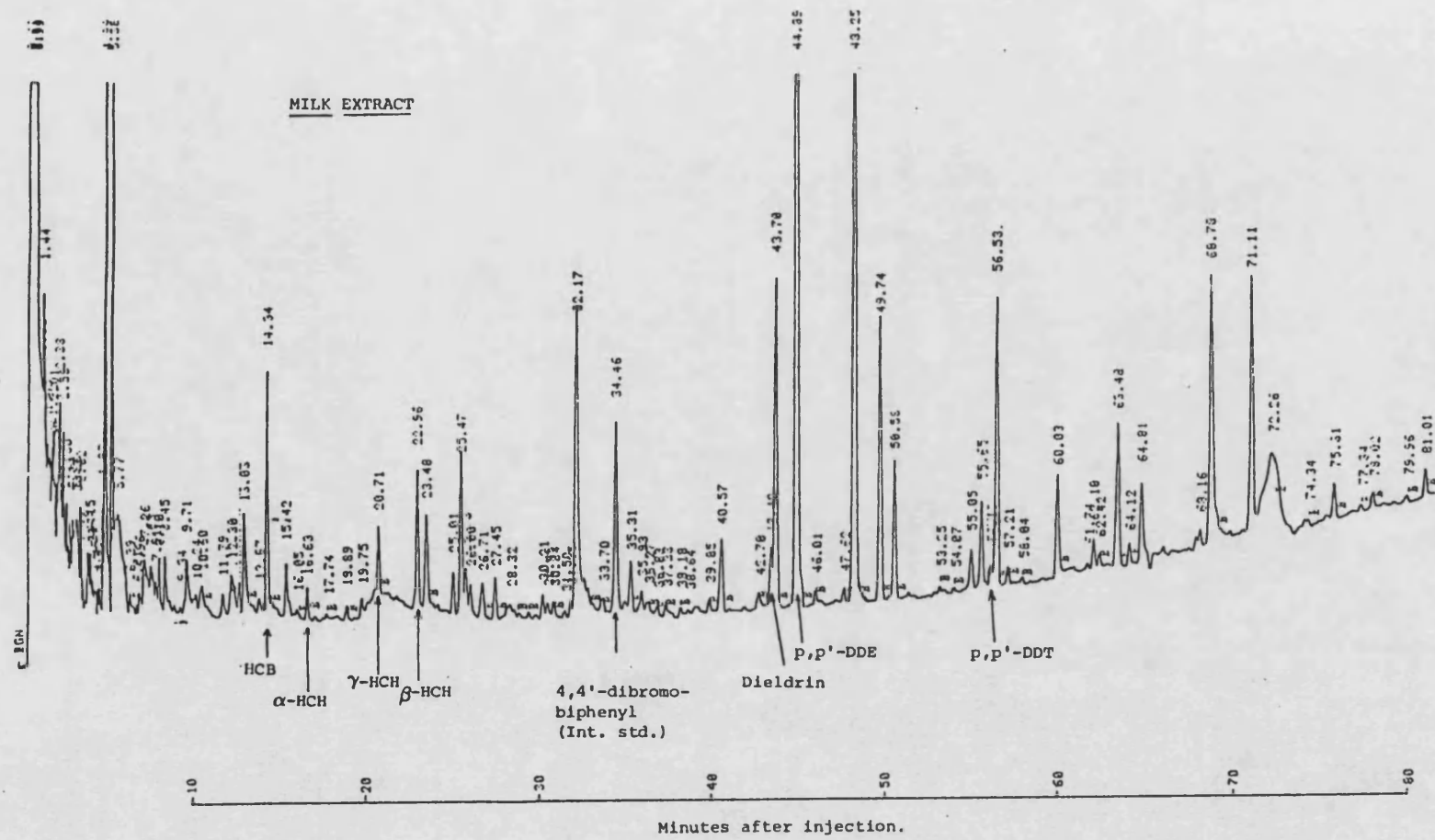


Figure 19.

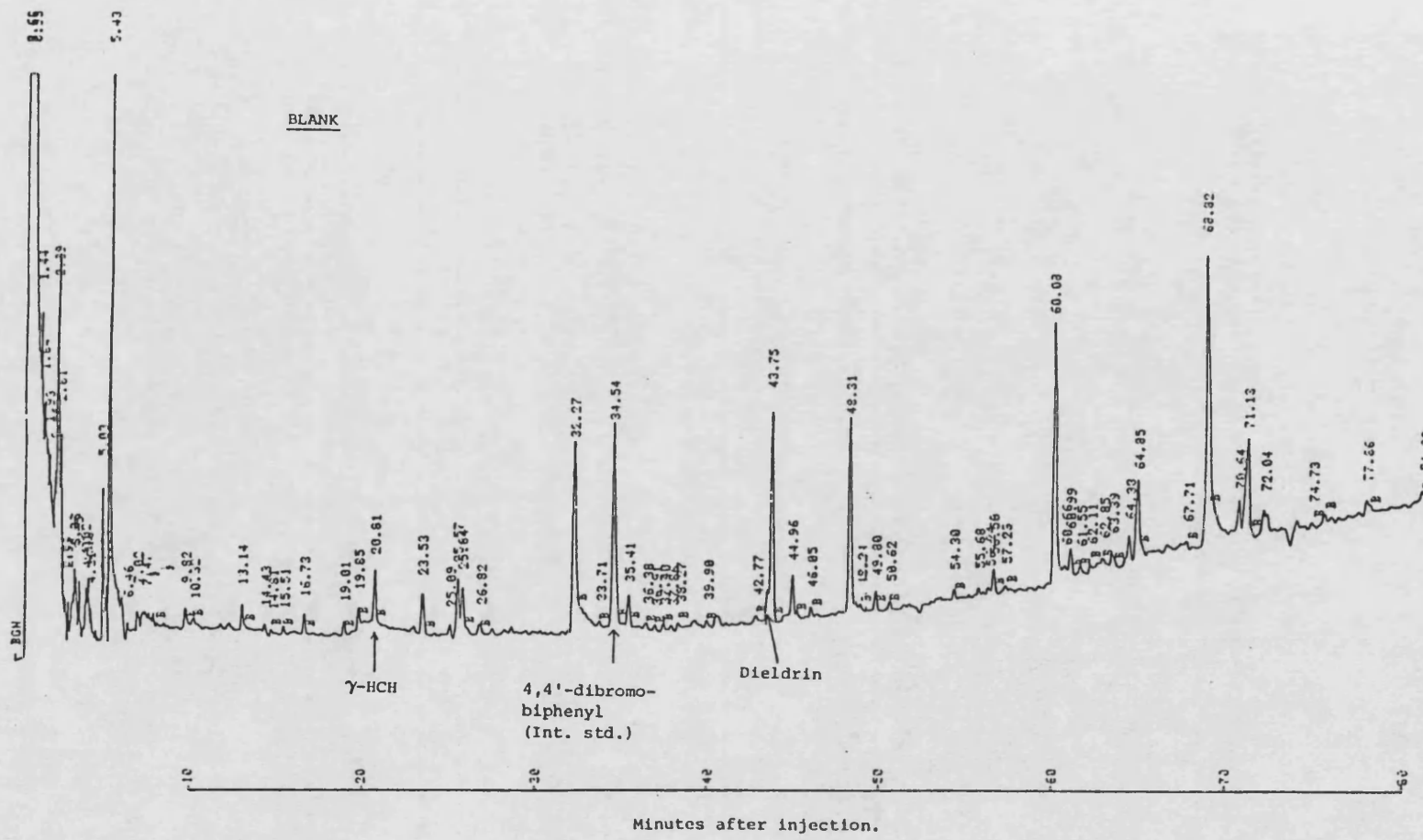


Figure 19 (contd.)

too "dirty" for direct determination of analytes and GC-MS would have to be used.

vii) Although lipid recoveries were determined using an artificial mixture of triglycerides (Witepsol H15 is a mixture of saturated triglycerides designed to melt at body temperature) it was considered that recoveries based on this mixture would adequately reflect milk fat recoveries despite the fact that triglycerides in milk are bound in a protein sheath. The mixing and drying of the milk with the adsorbent almost certainly destroys the integrity of the milk fat globules and so releases the milk lipids. Consequently, recoveries of the Witepsol were considered an adequate indicator of milk lipid extraction.

viii) The Florisil/fibrous cellulose provides a porous adsorbent substrate which permits good recovery of both lipid and organohalogen residues, but appears to retain a lot of unwanted components and breakdown products of the milk matrix. Residual water does not seem to pass into the hexane thus obviating the need to dry the extract.

Recoveries, reproducibility data and other method performance characteristics for the entire extraction, clean-up and quantitation procedure were further assessed in an extraction experiment involving five replicate adsorbent spikes. Details of this are to be found in Section 6.2. *et seq.*

4. TECHNIQUES USED FOR THE CLEAN-UP OF MILK EXTRACTS.

4.1. Introduction

The concentrations of OCPs and PCBs relative to the natural components of human milk are very low. For example, the lipid content of human milk is routinely in the region of 10^6 times greater than that of an individual organohalogen contaminant. Some methods of analysis combine the extraction and clean-up whereas others produce relatively "dirty" extracts which require extensive clean-up. All methods of extraction eliminate the water component of the milk, but other milk constituents, particularly lipids, often remain in the extract and must be removed prior to quantitation. Clean-up may be regarded as an enrichment step whereby the proportion of organohalogenes in the sample relative to the interferences is increased. With approximately a 10^6 fold excess of interfering compounds over the analytes, an efficient clean-up is technically difficult especially for multi-residue samples where discrimination between the analytes must be minimised.

The ultimate aim of a clean-up procedure is to provide a sample of at least adequate quality and concentration for the method of determination to be employed. In practical terms, this usually means that all the lipids, macromolecules and ionic species must be removed. The

degree of tolerance of other materials, such as steroidal compounds, vitamins etc. again depends on the method of determination, but major contamination with these substances should generally be avoided.

Clean-up methods rely on physico-chemical differences between the analytes and the interferences. This exploitation of differences can also be used to fractionate the analytes into groups and thereby result in a combined clean-up/fractionation of the sample which is often required prior to, say, GC-ECD determination of organohalogenes.

The discussion that follows outlines the main approaches to the clean-up of human milk extracts and includes references to combined extraction/clean-up type methods. A general theme throughout the literature is that removal of the lipids poses the major challenge to the analyst since extraction methods tend to eliminate the majority of the more polar milk constituents (such as proteins, carbohydrates, electrolytes etc.) and this is reflected in the nature of the clean-up procedures.

The clean-up procedures may be summarised as: i) solvent partitioning, ii) chemical degradation, iii) cryogenic precipitation of lipid, iv) adsorption column chromatography including combined clean-up/fractionation, v) gel permeation chromatography and vi) HPLC. Combined

extraction and clean-up procedures are described in Section 3.1.2.. Extract concentration procedures are also discussed.

4.1.1. Solvent partitioning.

In this type of method, the lipid-containing extract, in a hydrocarbon solvent, is partitioned with a more polar organic solvent such as acetonitrile (59) or dimethylformamide (DMF) (79). The more polar phase, containing the organohalogens, is then removed, diluted with an aqueous solution, and back-extracted into a hydrocarbon solvent. The lipids remain largely in the original hydrocarbon phase and the organohalogens should end up in the second hydrocarbon phase. De Faubert Maunder *et al.* (79) reported adequate clean-up and recoveries using this approach with DMF as the polar solvent. Where PCBs are concerned however, particularly with acetonitrile as the polar solvent, the recoveries of some congeners may be poor due to unfavourable partitioning properties (80). The method is time-consuming, expensive in terms of consumables and the extract may need further clean-up.

4.1.2. Chemical degradation.

These methods of clean-up are generally used for the determination of PCBs alone in the presence of OCPs and

almost certainly result in the degradation of many of the pesticides. Consequently, they will not be considered in detail but include oxidation of DDT and its derivatives to dichlorobenzophenones for subsequent removal prior to the determination of PCBs (81). However, this may cause loss of lower-chlorinated PCBs as well (82).

Saponification and acid hydrolysis techniques essentially form parts of combined extraction/clean-up methods and are described below. However, Brevik (83) used acid and alkali treatment separately, post-extraction, as a clean-up procedure for a few selected DCPs.

4.1.3. Cryogenic precipitation of lipids.

This simple method of removing lipids involves cooling the extract containing the organohalogenes to about -78°C (84) using, for example, methanol/solid CO_2 mixture. At this temperature, lipids and other substances precipitate out of solution and the supernatant liquid can then be removed. The method has been used successfully but the possibility exists for trapping residues in the solidified lipid. Again, extracts may need further clean-up.

4.1.4. Adsorption chromatography.

Further to the combined extraction/clean-up slurry

techniques, adsorption chromatography clean-ups for lipid-containing samples are very widely used (85). A variety of stationary phase materials (often topped with a layer of anhydrous sodium sulphate as a drying agent) has been used including Florisil (61, 63, 74, 86, 87, 88) and alumina (89) for lipid removal and Florisil, alumina (90), silica gel (73, 90, 91), magnesium oxide (92) and carbon (93) for further clean-up and fractionation. Recoveries of different compounds vary considerably.

Retention of PCBs and OCPs is controlled by the activity and composition of the adsorbent and the composition of the mobile phase. With so many variables, the volume of literature covering this topic is vast. Adsorption chromatography is possibly the most widely used technique and allows fractionation which is frequently required prior to quantitation by methods of low selectivity. Alumina and silica are particularly common materials for separation of OCPs from each other and from PCBs. Carbon too has been used for this purpose, but also to separate PCB congeners from each other on the basis of different degrees of ortho substitution.

Mobile phases vary very widely, though they are always of low to intermediate polarity.

The main problems with adsorption column clean-ups are batch-to-batch variation of chromatographic properties

and difficulty in controlling the activity of the adsorbent. These problems can be controlled by careful standardisation of each batch of adsorbent and perhaps using elution markers such as azulene. Methods can also be quite labour intensive and expensive in terms of consumables, and each fraction has to be analysed separately.

Evidently, removal of lipids by adsorption chromatography is not difficult, but effective and reproducible fractionation can be a problem and adds to the complexity of the analytical procedure. With the advent of very selective quantitation techniques, it would seem more efficient to expend less effort on extensive adsorption chromatography clean-ups and fractionations, and make use of the considerable selectivity offered by techniques such as GC-MS to differentiate between analytes.

4.1.5. Gel permeation chromatography (GPC).

Gel permeation has gained wide acceptance as a primary clean-up technique for the removal of lipids from fatty samples. Retention characteristics of a GPC column are governed largely by a mixture of size exclusion and adsorption type mechanisms; the retention volumes of materials chromatographed using GPC can, therefore, be varied by using different mobile phase compositions. This is particularly true for smaller molecules which are

exposed to a greater surface area in the GPC column however, in general, less subtle control may be exercised over large molecules since they are excluded from much of the stationary phase surface. This type of selectivity tuning is probably due almost entirely to shifts in the adsorption kinetics and equilibria since modest changes in mobile phase composition are unlikely to have a large effect on the size exclusion properties of the system. Different solvents will give rise to different degrees of swelling of the polymer gel and differences in the diffusion properties of the analytes within the mobile phase, however, it is unlikely that these variations would have as much effect on the selectivity as would changes in adsorptive properties. The theory and practice of GPC has been extensively reviewed elsewhere (94).

Bio-Beads SX-2 (95, 96) and, more recently, Bio-Beads SX-3 (97, 98) have been widely investigated and used for the primary clean-up of fat-containing samples. Lipid capacity is generally high (depending on the size of the column) but solvent consumption is quite large and there is only very limited scope for fractionation of PCBs and OCPs should this be required. Depending on the nature of the samples and the subsequent method of determination, samples may need further clean-up after GPC. Recoveries are generally very good (97, 98) and the lipid is recoverable. The column is also re-usable which permits automation.

An interesting advance in this field is the use of high performance GPC materials and Majors and Johnson (99) demonstrated the separation of DDT from lipids using a high performance GPC method. As will be seen later (see Section 4.2.) the advantages of using high performance GPC include high throughput, low solvent consumption, high lipid capacity, low analyte fraction volume, recovery of the lipid and column re-usability (100).

4.1.6. High performance liquid chromatography.

The advantages of high resolution and low solvent consumption offered by HPLC are an attractive contrast to traditional low resolution adsorption column chromatography. Very little work has been carried out using HPLC as a primary clean-up step and much of this work has been performed using preparative scale HPLC (with concomitant high solvent consumption) or the technique was employed only as a secondary clean-up/fractionation step (101). Both normal and reversed-phase methods have been used.

The use of HPLC as a primary clean-up technique was, however, ably demonstrated by Gillespie and Walters (102) using silica as the stationary phase and various hexane/dichloromethane mobile phases. With such systems, lipids are strongly retained and organohalogens tend to

elute before the fats. Using only an analytical scale column, OCPs could be separated from 100 mg of lipid and with a semi-preparative scale column, resolution of 500 mg of fat from OCPs and PCBs was achieved. However, the latter separation required large volumes (>400 ml) of mobile phase due to long retention times. Increasing the dichloromethane content of the mobile phase reduced elution times of both organochlorines and lipids and hence decreased the solvent consumption, but this was at the expense of lipid capacity. Lipid was more effectively removed by back-flushing with 100% dichloromethane. Repeated injections with or without back-flushing did not appear to be detrimental to the column performance. Recoveries appeared to be very good.

This impressive example of an HPLC clean-up as devised by Gillespie and Walters suffered from the major drawback that lipid was very strongly retained by their normal phase system and despite being able to cope with remarkably large amounts of lipid, the price of high lipid capacity was high solvent consumption or the inconvenience of back-flushing with a change of solvent composition. A means of avoiding this would be to elute the lipids before the organochlorines and provided the total elution volume for the most retained solute was fairly low, solvent consumption would be low and back-flushing could be avoided. This goal was attained as described later (see Section 4.2.).

4.1.7. Concentration techniques.

Concentration of residue extracts is an area of major concern. Nearly all residue methods require a concentration step which involves evaporation of solvent and this presents the analyst with two problems. Firstly, are there any undesirable residues in the solvent itself? Secondly, how can evaporation of the residues themselves be prevented? The required solvent purity depends on the analytes being determined and should not contribute significantly to the concentration of analyte in the sample. It also depends on the degree of concentration and the selectivity of the method of determination. Overall, the solvent should not interfere with the determination of the analytes. For residue analysis, the solvents generally have to be of very high grade in view of the very low concentrations of analytes and the large amount of solvent evaporation encountered in many methods. Great care must be taken to avoid contamination of the solvent with any potential interferences (103) and some form of solvent quality control is desirable.

The problem of analyte loss by volatilisation is particularly acute in residue analysis. The majority of PCBs and DCPs are not very volatile but the smaller, lower chlorinated species can be lost, particularly when

the levels encountered are in the part-per-billion range or lower. Rinne and Groh (104) demonstrated that rotary evaporation under vacuum was unreliable for trace analysis unless a vacuum controller was used.

A more common means of solvent evaporation in residue analysis is the use of the Kuderna-Danish apparatus. The evaporation is more gentle than that obtained with rotary evaporators and the Snyder column permits partial reflux which helps wash any residues on the glass walls down into the receiver tube. The reflux action also helps to prevent analyte losses by vapour entrainment. It is still good practice, however, to wash down the walls of the evaporator. Recoveries are generally better than those obtained by rotary evaporation.

Solvent evaporation using the Kuderna-Danish apparatus is slow but a more serious drawback is the possibility of sudden, violent eruptions of the solution which can result in gross losses (103). This is particularly true as the solvent volume becomes small and it may be wiser to perform the final stages of evaporation to a low volume over a stream of clean, dry air or nitrogen. Despite the relative gentleness of evaporation using the Kuderna-Danish concentrator, Gillespie and Walters (102) reported significant losses of organochlorines using this equipment. They also found that addition of a lipophilic substance (paraffin oil) as a "keeper solution" was of

little benefit.

A general rule in trace analysis is that clean extracts should not be evaporated to dryness since significant losses of analyte can occur. Even evaporation to a low volume (0.5 ml or less) can give rise to large, irreproducible losses {103}. This is particularly true for the more volatile residues such as HCB and HCH isomers. However, less volatile materials such as PCBs seem to be able to tolerate gentle evaporation to dryness as was demonstrated in Section 3.2.1..

Another common, and very gentle solvent evaporation technique is that of evaporation over a stream of gas. This can be very time consuming and so is generally only used for the removal of small amounts of solvent, but its main advantage is its gentleness. Residues may, however, be left on the vessel walls and so the vessel should be rinsed down.

4.2. Experimental Investigation of Sample Clean-up Techniques.

The clean-up methodology to be used in the analytical procedure was, of necessity, developed simultaneously with the extraction and quantitation procedures. However, very early on it became apparent that the crude extract would contain interfering materials of which the most

abundant would be lipids. It was appreciated that the fat content of the milk would have to be determined and so the recovery of the lipids would be a desirable property of the clean-up procedure, but also speed, efficiency, robustness and low running costs would have to be incorporated. The only methods which fulfilled these criteria to any degree were adsorption chromatography and gel permeation chromatography. Adsorption chromatography, despite being well established, was rejected on the grounds of having high running costs and the difficulty in recovering the lipid. This left gel permeation chromatography. Conventional GPC has become quite popular for this kind of clean-up but has the two major drawbacks of high initial costs and high solvent consumption. It was quickly realised that the latter disadvantage could be ameliorated by the use of modern high performance polymeric columns used in a GPC mode and in view of this, all investigations into clean-up methodology were directed towards developing a high performance GPC (HP-GPC) type procedure.

4.2.1. HP-GPC - initial analytical scale investigations.

Initial investigations into the use of HP-GPC centred around the use of a Hamilton PRP-1, 150 x 4.1 mm column with 10 μ m particles of the styrene/divinylbenzene copolymer. Knowing that the sample would be in a lipophilic solvent such as a hydrocarbon after extraction,

n-heptane was chosen as a starting point for the selection of a mobile phase. Heptane was chosen in preference to hexane on account of its lower volatility.

Experiment 1.

The following chromatographic conditions were employed:

All solvents were of HPLC grade.

Column:	Hamilton PRP-1 150 x 4.1 mm. 10 µm particles.
Mobile phase:	100% heptane.
Flow rate:	Approximately 1.0 ml min ⁻¹
Detection:	Refractive index at 10 ⁻⁴ RI units fsd.
Injecton vol.:	20 µl
Column temp.:	40°C
Instrument:	DuPont 830 preparative HPLC unit.

The following solutes were investigated (all solutions in n-heptane):

Witepsol H15*	Approx. 0.5% m/v
Cholesterol	Approx. 0.5% m/v
4,4'-dibromobiphenyl	Saturated.
Aroclor 1242	Approx. 0.5% m/v
Aroclor 1260	Approx. 0.5% m/v

Acetone

Approx. 5% v/v

*Witepsol H15 is mixture of C₁₂ to C₁₈ saturated triglycerides used as a suppository base.

Witepsol and cholesterol were chosen as typical lipid materials and 4,4'-dibromobiphenyl was chosen as a marker compound. The Aroclors were used as typical organochlorine mixtures and acetone was examined since at this stage it was thought that acetone may be incorporated in the extracting solvent.

For these solutes, under the above conditions, the following results were obtained:

<u>Solute</u>	<u>t_r (mins)</u>	<u>Comments</u>
Witepsol H15	1.7	Unretained - eluted in void volume.
Cholesterol	2.6	Some retention.
4,4'-diBrbiph.	3.4	Most retained solute. k' = approx. 1
Aroclor 1242	2.8	Broad with tailing shoulder.
Aroclor 1260	2.5	Broad with marked tailing shoulder.
Acetone	2.7	Some retention. Negative RI peak.

The retention mechanisms involved were probably a mixture of gel permeation and adsorption. All the solutes are lipophilic and would be attracted by both the mobile and the stationary phases. The stationary phase would probably be able to retain alicyclic and aromatic materials more effectively than aliphatic compounds and this, together with the large size of the triglyceride molecules in the Witepsol and the high affinity for the mobile phase, probably accounts for the almost total absence of retention for the Witepsol.

Cholesterol is a smaller molecule than the Witepsol triglycerides, is polycyclic and possesses polar groups (a hydroxyl function and a double bond). Hence cholesterol would not be expected to be totally excluded from the channels in the stationary phase and would probably have more adsorptive affinity for the stationary phase compared to Witepsol. These factors would be expected to give rise to some retention and this appears to be the case.

The 4,4'-dibromobiphenyl and the PCBs are all much smaller molecules and so would pass freely into the stationary phase channels. They are also aromatic and these two factors would explain the retention of these materials. It will be noted that the higher chlorinated Aroclor 1260 is slightly less retained than the lower chlorinated Aroclor 1242 with the 4,4'-dibromobiphenyl

being the most strongly retained of the halogenated biphenyls. This may be explained by an increasing affinity for the mobile phase with increasing halogenation and a decrease in the co-planarity of the biphenyl nucleus with increasing ortho substitution which may lead to a less favourable conformation for adsorptive retention by the stationary phase.

Acetone too was retained to some degree by virtue of its small molecular size and its unsaturation.

Peak shapes were generally quite broad but fairly Gaussian with some tailing in evidence. The Witepsol peak was very sharp, however, since it was unretained and so did not suffer band-broadening effects due to mass transfer and non-equilibrium. This is a very useful advantage since in effect, the triglycerides never "see" the stationary phase and so capacity is limited only by mobile phase solubility and the band broadening due to eddy and longitudinal diffusion. Both these diffusion effects are probably small for triglycerides. Thus, lipid capacity is high and encroachment of the lipid band on the retained solutes is minimised.

Of course, all the solutes displayed very low capacity factors compared to more usual HPLC modes and this was expected in view of the nature of the solutes and the mobile phase. All the solutes, apart from the

triglycerides, eluted in quite broad bands due to classical band broadening mechanisms, including mass transfer effects, operating on the retained materials. Polymeric columns are particularly prone to band broadening due to slow mass transfer kinetics. The Aroclor peaks were further broadened (both Aroclor peaks had tailing shoulders) by virtue of the fact that they are mixtures and each component has slightly different retention properties.

The system was maintained at 40°C to reduce the viscosity of the lipid solutions, to increase solubility and to maintain acceptable peak shape.

Overall, the results for this experiment were very encouraging since separation of the organohalogenes from the triglycerides (but not the cholesterol) was achieved in a short time and with a low elution volume.

Experiment 2.

The aim of this experiment was to decrease the retention of the cholesterol in order to separate it from the organohalogenes, but at the same time, avoid decreasing the retention of the organohalogenes and maintain high lipid capacity. In an attempt to achieve this, 20% tetrahydrofuran (THF) was added to the heptane as a modifier. It was expected that this cyclic, slightly

polar molecule would decrease the retention of all solutes, but it was hoped that the greater porosity of the polymer gel to the halogenated biphenyls would maintain some retention for these materials while the much larger and partially excluded cholesterol would be more susceptible to the greater solvating power of the mobile phase and would exhibit a relatively marked loss of retention. As will be seen, these hypotheses appear to be, at least in part, successful.

The chromatographic conditions were as for experiment 1 except:

Mobile Phase:	20:80% v/v THF:n-heptane
Flow rate:	Approx. 0.8 ml min ⁻¹

Solutes were as for experiment 1 except that the 5% acetone was omitted and 100% n-heptane was injected in its place. Witepsol, cholesterol and 4,4'-dibromobiphenyl were injected in both heptane and mobile phase solutions to see if the solvent had any significant effect on retention. The Aroclors were injected only in heptane solution.

Results.

<u>Solute</u>	<u>t_r (mins)</u>
Witepsol H15	1.5
Cholesterol	1.7
4,4'-diBrbiph.	2.3
Aroclor 1242	2.0
Aroclor 1260	1.9
n-Heptane	1.7

Retention times were found to be unaffected by whether the injection solvent was heptane or mobile phase. As can be seen, the cholesterol was virtually unretained whereas the organohalogens maintained at least some retention. Interestingly, the Witepsol appeared to be less retained than when 100% heptane was used as the mobile phase. The most likely explanation for this is that the flow rate was inaccurately controlled but it may be that the stationary phase channels contracted under the influence of the new mobile phase and reduced what little permeability there was available to the triglycerides with heptane as the mobile phase; this could also reduce the retention of the cholesterol. However, this mechanism is merely conjecture. Peaks were all much sharper than with the 100% mobile phase.

So, the addition of a slightly polar modifier was found

to provide superior separation of the organohalogens from the lipids. The main drawback was the very low elution volume which would make the timing of fraction collection critical and probably too exacting for routine use.

Experiment 3.

The purpose of this experiment was to see if Aroclors could be separated from the lipids in an Aroclor+cholesterol+triglyceride mixture using the system developed in experiment 2, and to collect the PCB fraction for subsequent examination by capillary GC-FID.

The chromatographic conditions were as for experiment 2 except that the separation was carried out at ambient temperature in an attempt to increase retention.

The solutions used were:

i) Approx. 0.16% m/v Aroclor 1242 + approx. 0.16% m/v cholesterol + approx. 0.16% m/v Witepsol H15 in 14:86% v/v THF:n-heptane.

ii) Approx. 0.16% m/v Aroclor 1260 + approx. 0.16% m/v cholesterol + approx. 0.16% m/v Witepsol H15 in 14:86% v/v THF:n-heptane.

iii) Approx. 0.25% m/v cholesterol + approx. 0.25% m/v

Witepsol H15 in 20:80% v/v THF:n-heptane.

With an injection volume of 20 μ l, the mass of Aroclor injected for solutions (i) and (ii) was approximately 32 μ g.

Duplicate injections were made for each solution and the PCB fraction was collected as shown in Figure 20 and adjusted to 1 ml. The "PCB fractions" for the solution of cholesterol + triglycerides were used as blanks. The PCBs and blanks were examined by capillary GC under the following conditions:

Column:	25 m 0.35 mm id CP Sil 5
Carrier gas:	Helium at 8 psi
Oven prog.:	85°C for 1 minute 5°C min ⁻¹ to 200°C. Hold.
Injection:	Split approximately 1:5
Instrument:	Perkin-Elmer Sigma 3 GC.

Standard solutions of Aroclor 1242 (144 μ g ml⁻¹) and Aroclor 1260 (170 μ g ml⁻¹) were made up in n-heptane.

The injection volume for the samples was 5 μ l and for the standards was 1 μ l.

The capillary GC chromatograms for the fractions collected in Figure 20 are shown in Figure 21. As can be seen, the blank appears to be quite clean with only minor

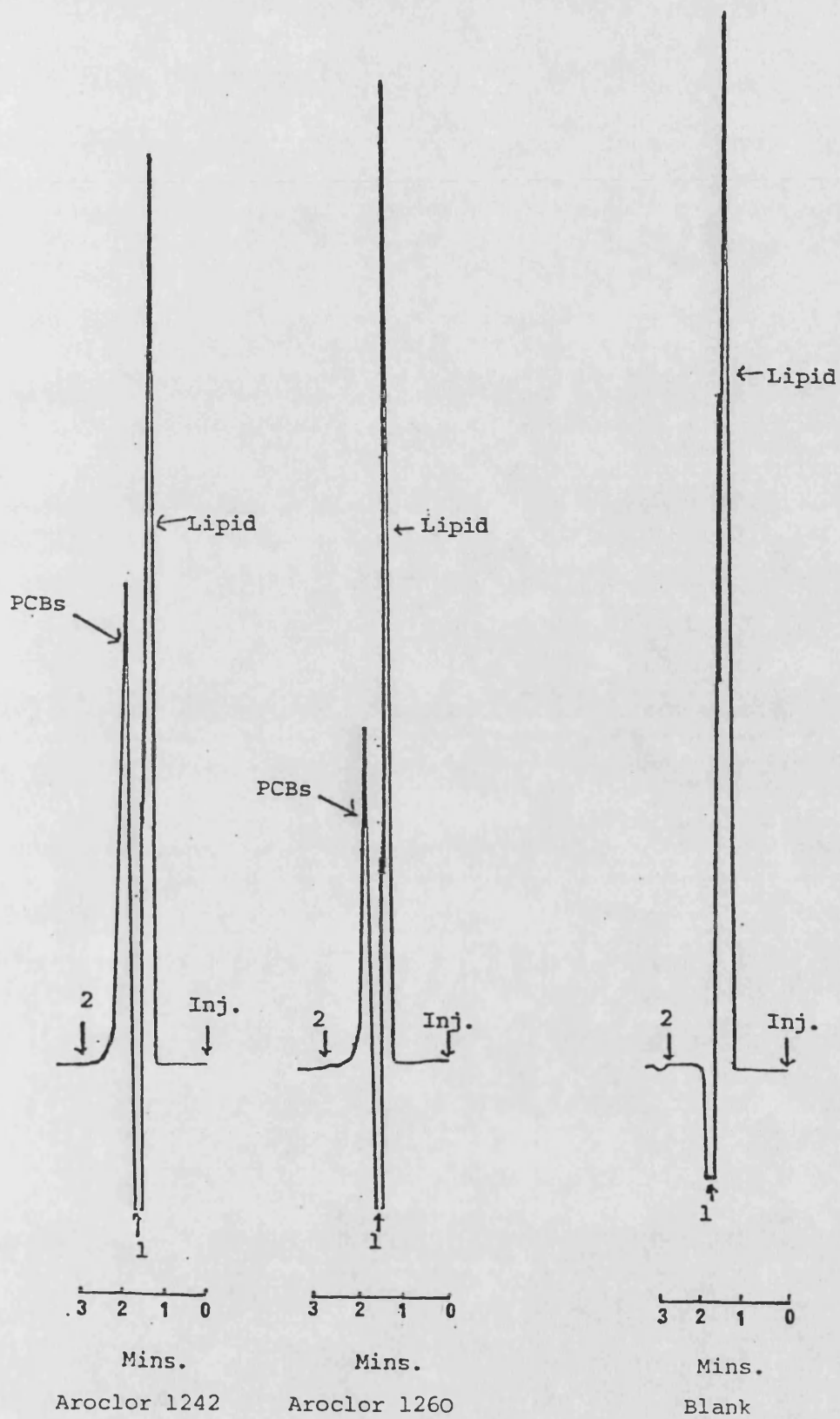
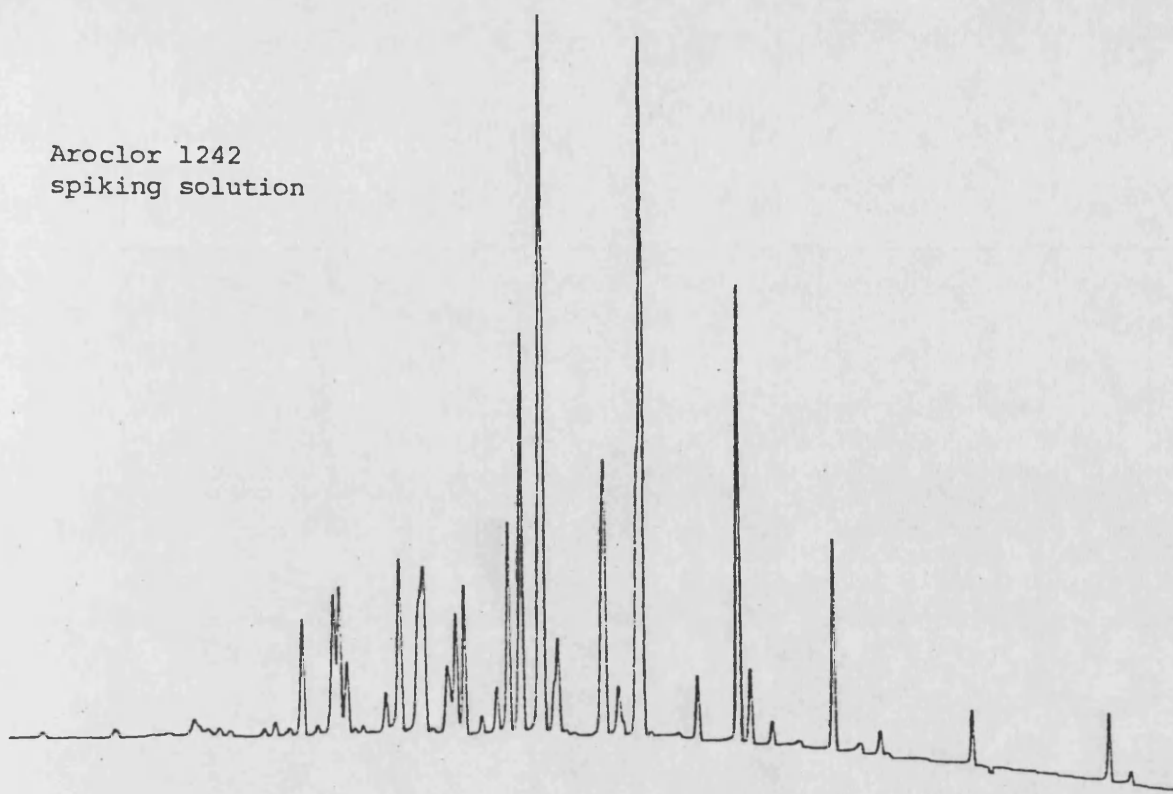


Figure 20.

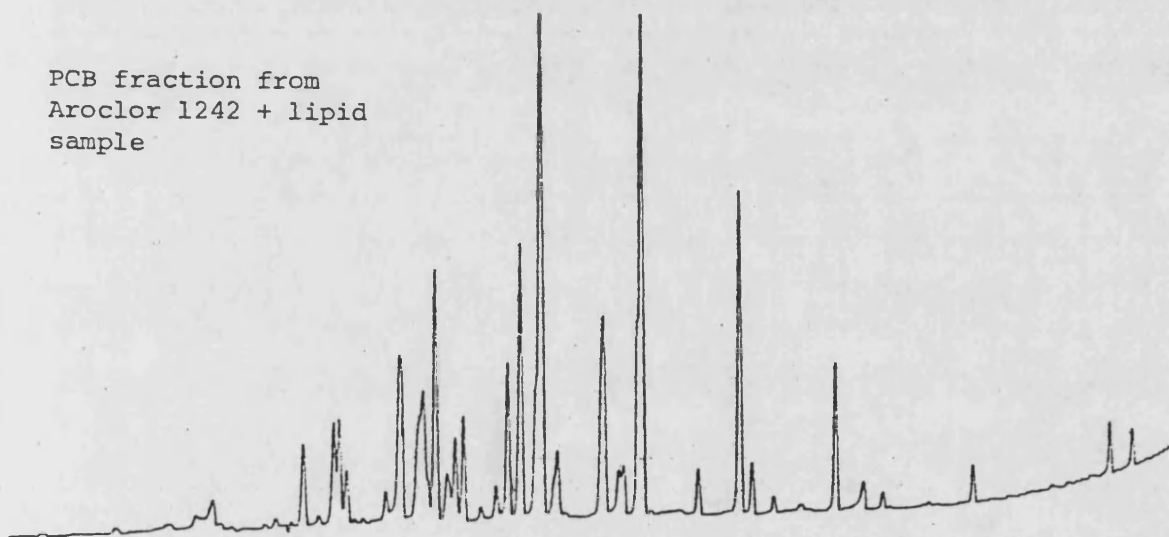
1=Start fraction collection.

2=Stop fraction collection.

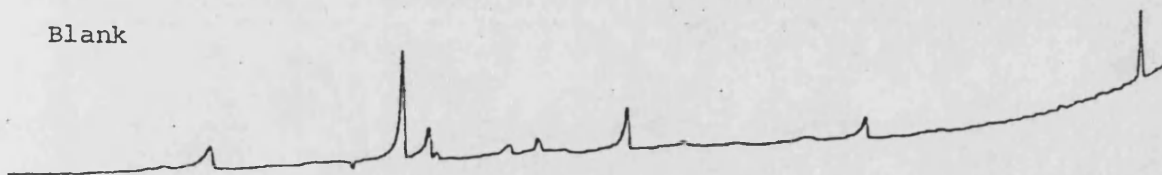
Aroclor 1242
spiking solution



PCB fraction from
Aroclor 1242 + lipid
sample



Blank

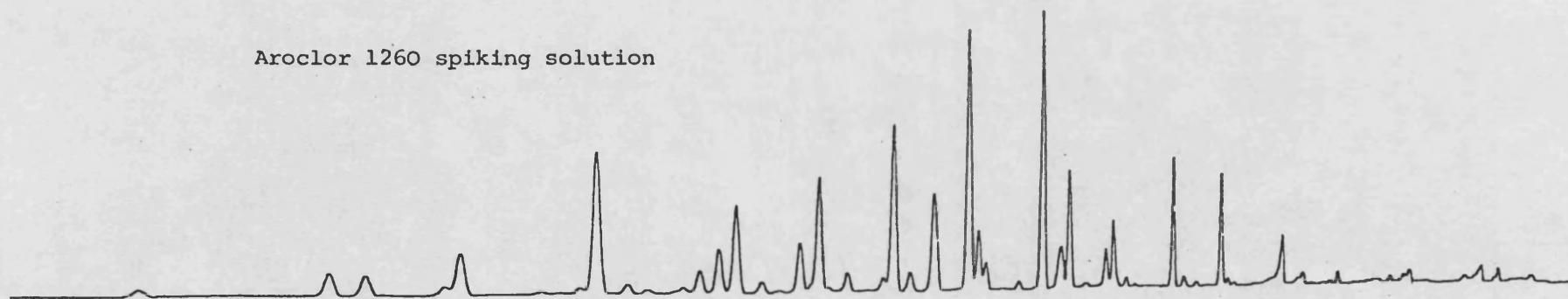


25 20 15 10

Minutes after injection

Figure 21.

Aroclor 1260 spiking solution



PCB fraction from Aroclor 1260
+ lipid sample

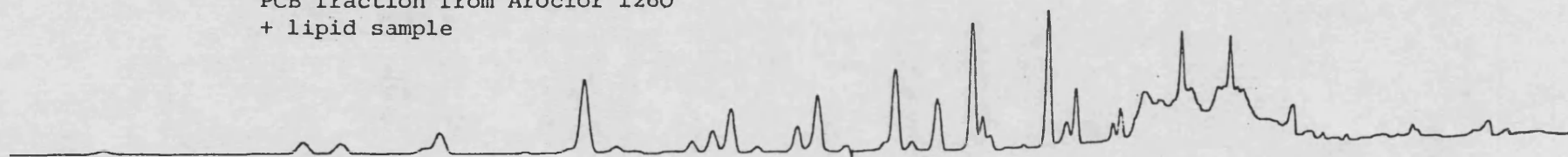


Figure 21
(contd.)

45

40

35

30

25

20

Minutes after injection

interference, and the Aroclor patterns for both the Aroclor 1242 and 1260 fractions are essentially identical to those given by the respective standards.

So, it was clear that HP-GPC was effective and did not give rise to any conspicuous losses of PCB congeners.

It was found that reducing the temperature of the HPLC column to ambient did not increase retention significantly and it was considered that a higher lipid content would produce a lipid band that would overlap with the organohalogen fraction. Consequently, attempts were made to increase the separation between the lipids and organohalogens. Decreasing the THF content of the mobile phase to 15% produced no significant improvement with the only notable effect being a slight increase in the retention of the cholesterol. As a result, a different modifier for the heptane was sought.

Experiment 4.

Propan-2-ol was chosen as the new modifier since it was hoped that cholesterol would have a high affinity for the alcohol, but the PCBs would be less soluble in a propan-2-ol/heptane mobile phase than in the THF/heptane phase. On these grounds, increased retention of the Aroclors was expected while the cholesterol was expected to elute very early and this was indeed found to be the case.

The chromatographic conditions were as for experiment 3 except:

Mobile phase: 15:85% v/v propan-2-ol:n-heptane
Flow rate: 0.9 ml min⁻¹

The solutes were:

- i) Witepsol H15 approx. 0.5% m/v in mobile phase.
- ii) Cholesterol approx. 0.5% m/v in mobile phase.
- iii) Aroclor 1242 approx. 0.5% m/v in n-heptane.
- iv) Aroclor 1260 approx. 0.5% m/v in n-heptane.
- v) Witepsol H15 approx 0.25% m/v + cholesterol approx. 0.25% m/v in mobile phase.
- vi) Aroclor 1242 approx. 0.16% m/v + cholesterol approx. 0.16% m/v + Witepsol H15 approx. 0.16% m/v in 14:86% v/v THF:n-heptane.
- vii) Aroclor 1260 approx. 0.16% m/v + cholesterol approx. 0.16% m/v + Witepsol H15 approx. 0.16% m/v in 14:86% v/v THF:n-heptane.
- viii) 100% n-heptane.

20 µl of each solution was injected and the retention times were recorded. For solutions (v), (vi) and (vii), injections were made in duplicate and the PCB fractions from each were collected and adjusted to about 1 ml for subsequent examination by capillary GC. The "PCB fraction" for solution (v) was used as a blank.

Results.

<u>Solute</u>	<u>t_r (mins)</u>
Witepsol H15	1.7
Cholesterol	1.8
Aroclor 1242	3.4
Aroclor 1260	2.9
n-Heptane	1.7

Typical chromatograms for solutions (vi) and (vii) with the fraction collection times can be seen in Figure 22.

As can be seen from the retention times, Witepsol and cholesterol were both virtually unretained as expected, but the PCBs were very well removed from the lipids. PCB peaks were quite broad (about 1.5 ml). As before, the higher chlorinated Aroclor 1260 was slightly less retained than the Aroclor 1242. The PCB fractions were examined by capillary GC under the same conditions as described under experiment 3. The GC chromatograms from the fractions shown in Figure 22 are shown in Figure 23 together with a blank and standard Aroclor solutions. As can be seen, there is no discernible distortion of the PCB pattern and the blank was very clean.

Thus, a useful HPLC system was devised whereby the PCBs were completely separated from lipids and the PCB fraction volume was still very low. Lipids were virtually

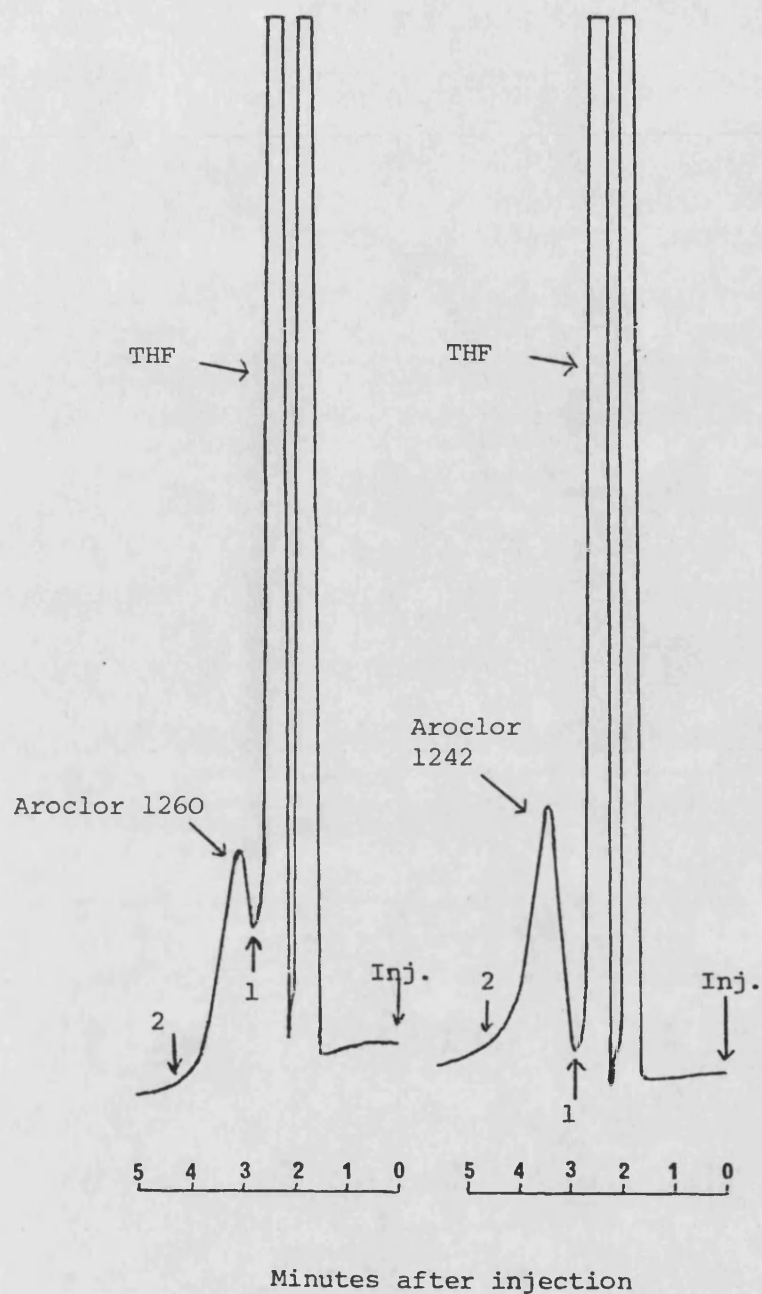
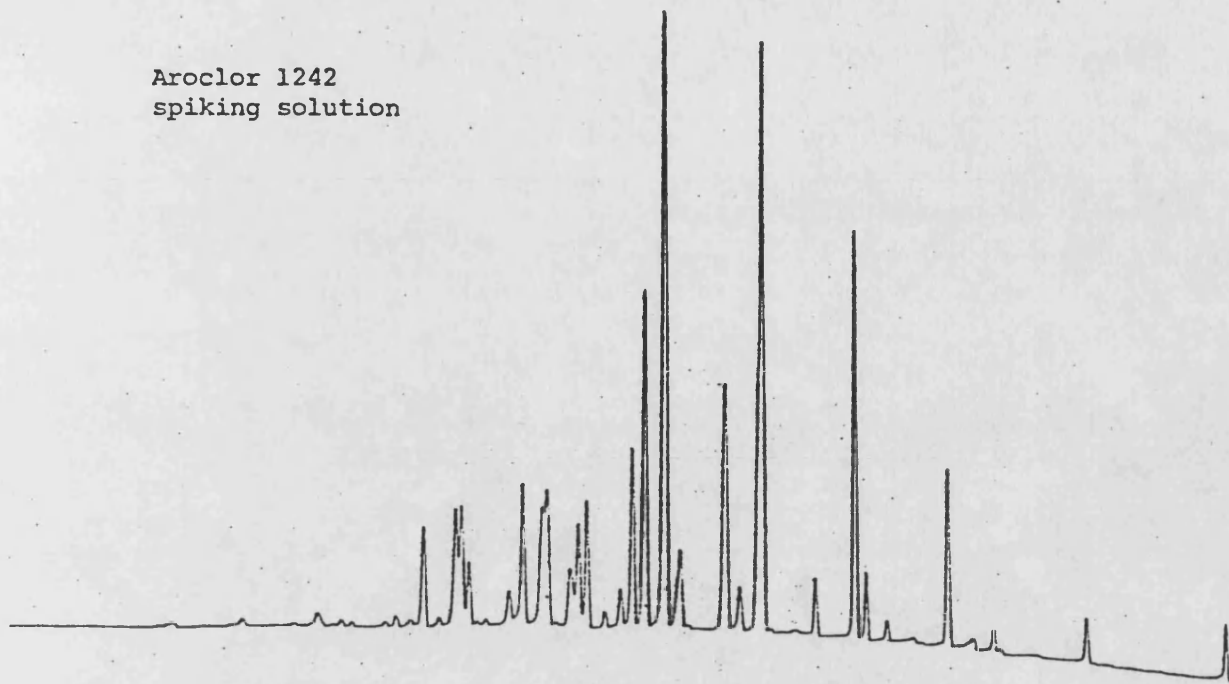
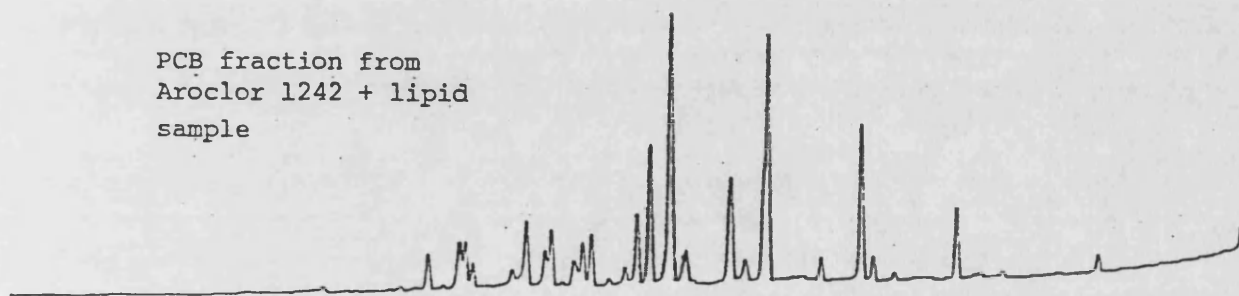


Figure 22. 1=Start fraction collection
2=Stop fraction collection

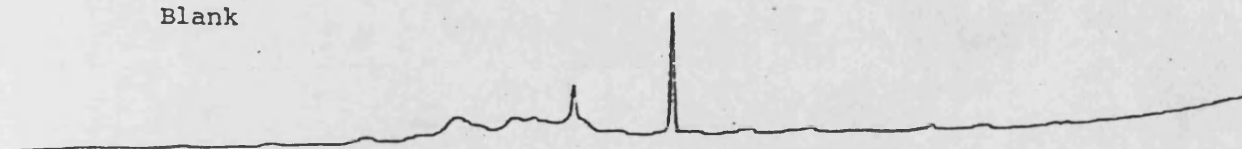
Aroclor 1242
spiking solution



PCB fraction from
Aroclor 1242 + lipid
sample



Blank



30 25 20 15 10
Minutes after injection

Figure 23.

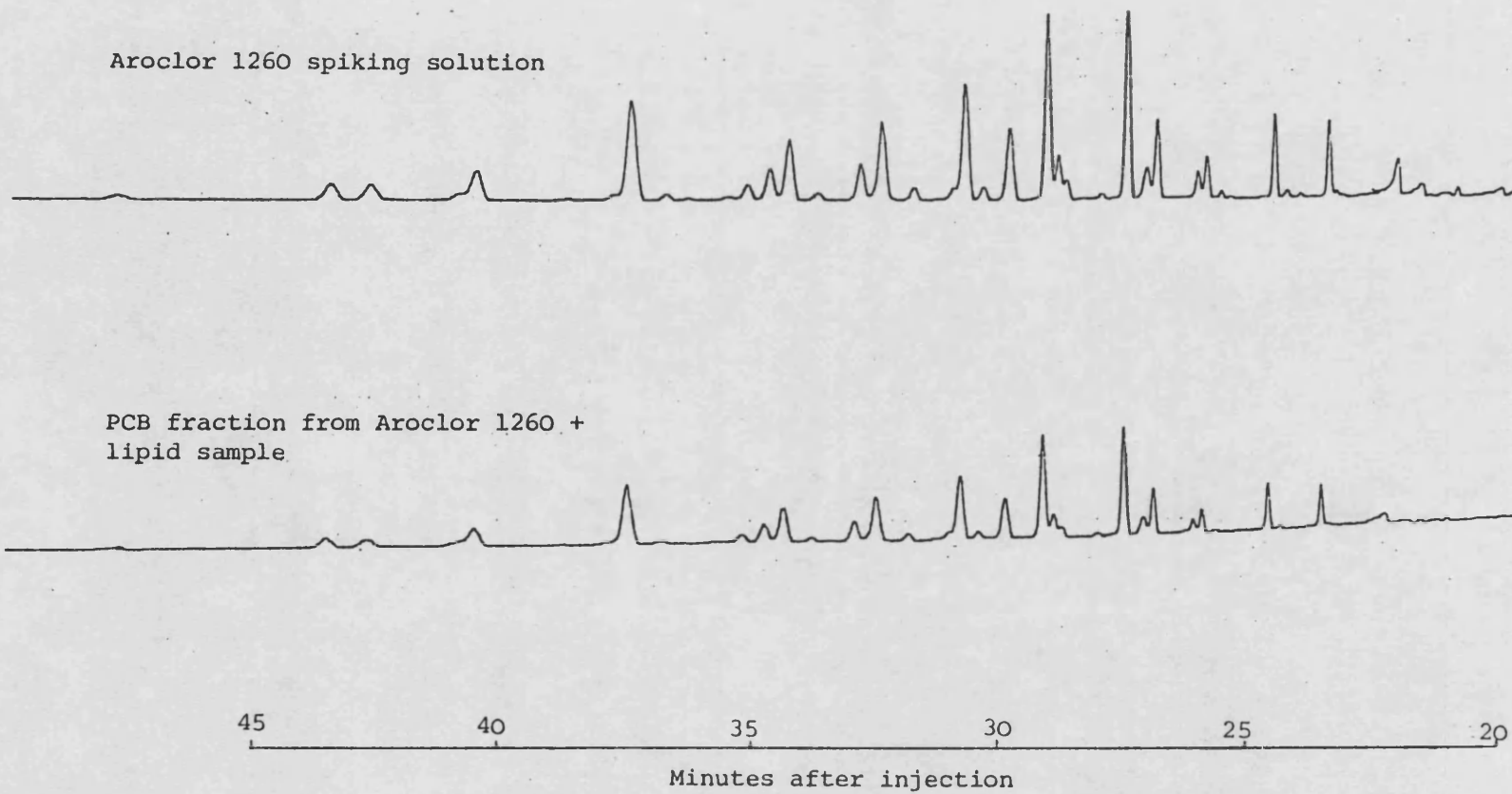


Figure 23 (contd.)

unretained thus maintaining a high lipid capacity but a narrow chromatographic band width. The next step was to scale the separation up to a method suitable for the clean-up of larger samples and also to assess the ruggedness of the system.

4.2.2. Semi-preparative HP-GPC clean-up.

Experiment 5.

The column chosen for the larger scale work was a Polymer Laboratories PLRP-S 300 x 7.5 mm column with 10 μm particles and 100 nm pores. This is a semi-preparative column and like the Hamilton PRP-1 phase, is a styrene/divinylbenzene co-polymer.

The following chromatographic conditions were employed:

Column: Polymer Laboratories PLRP-S 300 x 7.5 mm column with 10 μm particles and 100 nm pores.

Mobile phase: 15:85% v/v and 20:80% v/v propan-2-ol:n-heptane.

Flow rate: 2 to 4 ml min^{-1} .

Detection: Refractive index at 5×10^{-4} to 2×10^{-3} RI units fsd. Ambient temperature.

Column temp.: 35°C.

Injection vol.: 0.5 to 2 ml

Instrument: DuPont 830 preparative HPLC unit.

The flow rate is given above as a range from 2 to 4 ml min⁻¹. The reason for this is that the pump used was a constant pressure system and the injection of large amounts of lipid (up to 300 mg) caused marked, but transient, drops in flow rate. Initial flow rates were kept at about 4 ml min⁻¹ but this dropped to about 2 ml min⁻¹ on injection of large amount of viscous lipid solution. Heating the system to 35°C helped to alleviate this effect and also maintained acceptable peak shapes.

Two different mobile phase compositions were investigated in order to assess the sensitivity of the system to propan-2-ol content.

The range of the RI detector was changed as required in order to keep the PCB and cholesterol peaks on-scale as the injection volume was increased.

Solutes.

- i) Approx. 15% m/v Witepsol H15 in mobile phase.
- ii) Approx. 0.5% m/v cholesterol in mobile phase.
- iii) Approx. 0.5% m/v Aroclor 1242 in mobile phase.
- iv) Approx. 0.5% m/v Aroclor 1260 in mobile phase.
- v) Approx. 0.5% m/v Aroclor 1242 + approx. 0.5% m/v cholesterol + approx. 0.5% m/v Witepsol H15 in mobile

phase.

vi) Approx. 0.5% m/v Aroclor 1260 + approx. 0.5% m/v cholesterol + approx. 0.5% m/v Witepsol H15 in mobile phase.

Injections were made as shown in Tables 5 and 6.

Results.

The results are set out in Tables 5 and 6.

The separations of 0.5% m/v Aroclor 1242 and 0.5% m/v Aroclor 1260 from 0.5% m/v cholesterol + 15% m/v triglycerides using a 2.0 ml injection and 20:80% v/v propan-2-ol:n-heptane mobile phase can be seen in Figure 24.

It can be seen from the results that the injection of large amounts of lipid in the 2.0 ml injections produces a considerable drop in flow rate compared to the 0.5 ml injections. However, an important feature of the results is that neither the drop in flow rate nor the large amount of lipid affects the adjusted capacity factors for the PCBs i.e. the k' values remain constant despite the changes in injection volume, flow rate and lipid mass. This is quite remarkable since an injection of 2.0 ml of 15% triglycerides represents an injection of 300 mg of lipid which may well be expected to disturb the

Table 5. Retention of solutes using 15:85 propan-2-ol: n-heptane mobile phase.

<u>Solution</u>	t_r (mins) 0.5 ml inj.	k' of peak crest.	t_r (mins) 2.0 ml inj.	k' of peak crest.
i) Witepsol H15	2.3 to 3.3	Unretained	3.1 to 4.8	Unretained
ii) Cholesterol	2.6 to 3.0	Approx. 0.2	3.1 to 4.2	Approx 0.2
iii) Aroclor 1242	3.8 to 5.4	Approx. 0.9	Not done	Not done
iv) Aroclor 1260	3.4 to 5.0	Approx. 0.8	Not done	Not done
v) Aro. 1242+lipid	3.9 to 5.5	Approx. 0.9	5.4 to 8.0	Approx. 0.9
vi) Aro. 1260+lipid	3.6 to 5.2	Approx. 0.8	4.9 to 7.7	Approx. 0.8

Table 6. Retention of solutes using 20:80 propan-2-ol: n-heptane mobile phase.

<u>Solution</u>	t_r (mins) 2.0 ml inj.	k' of peak crest.
i) Witepsol H15	3.2 to 5.0	Unretained
ii) Cholesterol	3.2 to 4.3	Approx. 0.2
iii) Aroclor 1242	Not done	Not done
iv) Aroclor 1260	Not done	Not done
v) Aro. 1242+lipid	5.7 to 8.3	Approx. 0.9
vi) Aro. 1260+lipid	5.1 to 7.9	Approx. 0.8

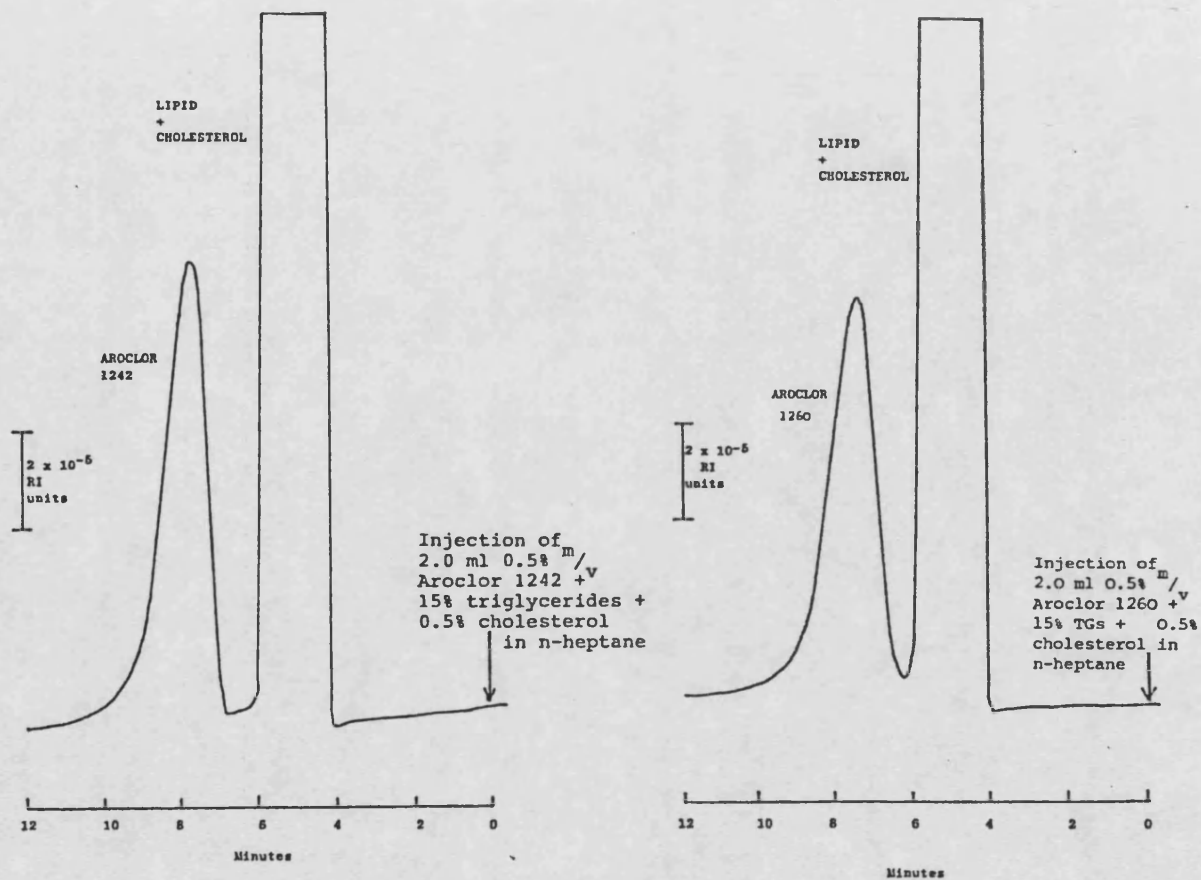


Figure 24.

Column: Polymer Laboratories PLRP-S 300 x 7.5 mm
 10 μ m particle size 100 nm pores
 Mobile phase: 20% v/v propan-2-ol in n-heptane
 Flow rate: 2.3 ml /min

equilibrium between mobile and stationary phases with marked effects on retention, but this does not appear to be the case. On reflection, this is perhaps not so surprising with a GPC type system since the retention of the organohalogenes takes place largely within the channels of the polymer which are not accessible to the lipids. Thus, the mobile phase composition probably remains largely unchanged within the channels where the organohalogenes spend much of their time on column. Consequently, the capacity factors of the PCBs are not affected by the triglycerides. Absolute retention times are, of course, affected by variations in flow rate.

With the 2.0 ml injection, the PCB peak is much broader because the injected sample band is that much wider on introduction, but despite this effect, both Aroclor 1242 and Aroclor 1260 remained completely separated from the cholesterol and the triglycerides using this system.

Changing the composition of the mobile phase from 15% to 20% propan-2-ol in n-heptane had no discernible effect on the retention or peak shape of any of the solutes.

In conclusion, this semi-preparative system enabled complete separation of PCBs from 300 mg of triglycerides plus 10 mg of cholesterol injected over 2.0 ml. Elution times and fraction volumes were both very much lower than with conventional GPC systems and both the PCBs and

lipids could be recovered. The separation was unaffected by changing the mobile phase composition from 15% to 20% propan-2-ol in n-heptane.

These results were very encouraging and demonstrated that separation on a large scale such as would be required for milk samples was feasible and the next step was to assess recoveries of PCBs and lipids.

Experiment 6.

The recoveries of PCBs and triglycerides were determined using the following HPLC system:

Column: Polymer Laboratories PLRP-S 300 x 7.5 mm
column with 10 μ m particles and 100 nm
pores.

Mobile phase: 20:80% v/v propan-2-ol:n-heptane.

Flow rate: 2.3 ml min⁻¹.

Detection: Refractive index at 10⁻³ RI units fsd.
Ambient temperature.

Column temp.: 35°C.

Injection vol.: 2.0 ml

Instrument: DuPont 830 preparative HPLC unit.

The following solutions were made up:

i) 45 μ g ml⁻¹ Aroclor 1242 + 48 μ g ml⁻¹ Aroclor 1248 +

102 $\mu\text{g ml}^{-1}$ Aroclor 1260 + 15.00% m/v Witepsol H15 in n-heptane.

ii) 15.00% m/v Witepsol H15 in n-heptane.

iii) 45 $\mu\text{g ml}^{-1}$ Aroclor 1242 + 48 $\mu\text{g ml}^{-1}$ Aroclor 1248 + 102 $\mu\text{g ml}^{-1}$ Aroclor 1260 + 10.4 $\mu\text{g ml}^{-1}$ 4-chloro-m-cresol in n-heptane.

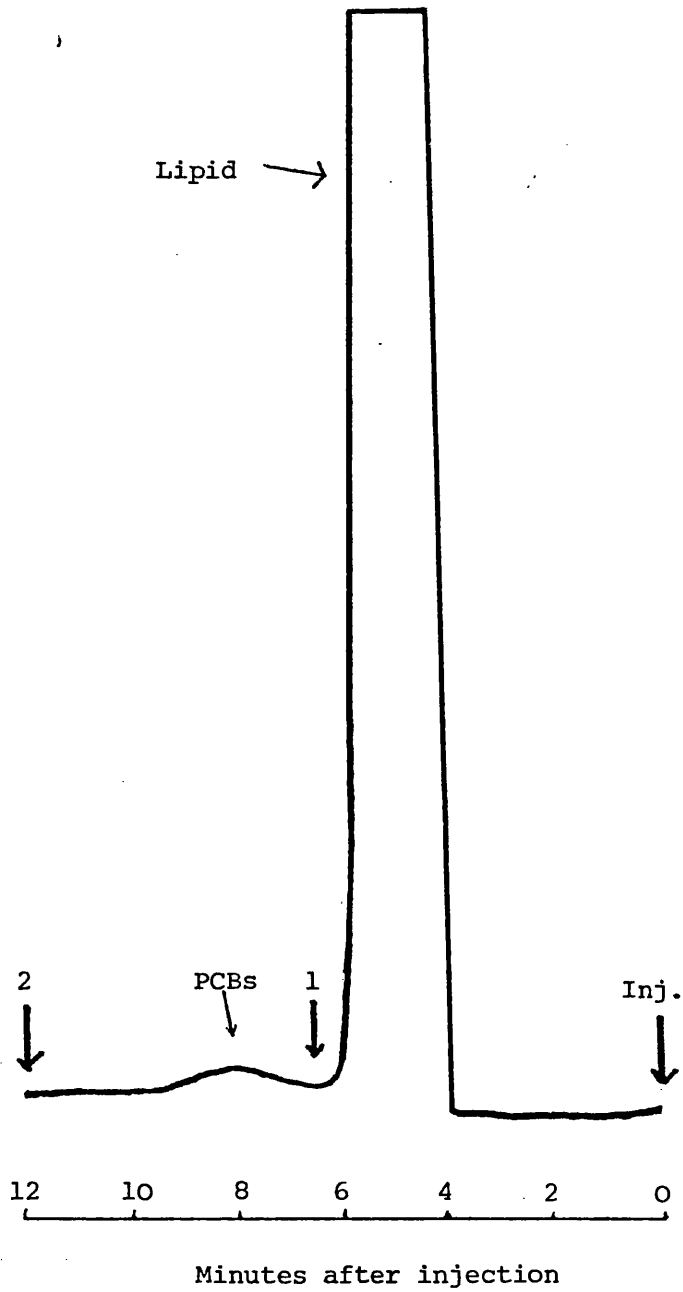
iv) 10.4 $\mu\text{g ml}^{-1}$ 4-chloro-m-cresol in n-heptane.

The chlorocresol was used as an internal standard for subsequent GC analysis.

The recovery experiment assumed that the injection loop volume was exactly 2.0 ml. The flow rate was maintained at 2.3 ml min^{-1} during sample elution.

The following injections were made: One injection of solution (ii) was made and the lipid fraction collected. Five replicate injections of solution (i) were made and the lipid fractions from the first three were collected whereas the PCB fractions were collected for all five replicates. Fraction collection times were dictated by the position of the lipid band as seen on the chart recorder; the fractions were typically collected as shown in Figure 25.

The lipid fractions were evaporated to dryness in a rotary evaporator and the residues were weighed in order to determine recoveries.



1=Start fraction collection
2=Stop fraction collection

Figure 25.

The PCB fractions were gently evaporated almost to dryness in a rotary evaporator and 2.0 ml of the internal standard solution (iv) were added to each to redissolve the residues.

Results.

The recoveries for the four lipid fractions were determined to be:

98.0%, 98.9%, 96.3% and 96.6% (mean = 97.5%).

After addition of the internal standard solution (iv), the PCB fraction was examined by capillary GC using the conditions described under experiment 3 and using an integrator to measure peak areas. Solution (iii) was used as a recovery standard. Five peaks across the chromatographic range of PCBs were chosen to be representative of PCB recovery and the peak area ratios (relative to the internal standard) for the five peaks in each of the five replicate PCB fractions were then compared directly to the respective peak area ratios in the standard. Assuming a linear calibration curve with zero intercept for each peak, the recovery for each of the five peaks in the five replicates was calculated. The standard values used for the recovery calculations were obtained by taking the mean peak area ratio for three replicate injections of the standard solution (iii) for

each of the five selected PCB peaks and assigning 100% recovery to these mean values.

In addition, the area ratio of the peaks eluting at $t_r=19.80$ and 37.80 was calculated for each of the five replicates as a crude measure of pattern distortion.

Results.

Results are shown in Table 7.

This approach to determining PCB recovery relies on several assumptions including: a) there are no losses of PCBs during the evaporation step, b) the chosen peaks are representative of all PCBs and c) the variation of GC peak areas, particularly the internal standard peak, are not so great as to be misleading with regard to the reproducibility of the HPLC recoveries.

These assumptions are probably acceptable although the internal standard peak is very early-eluting and significant variation in the measurement of its area is quite possible; this may account for some of the variation seen in the results. This is borne out by the observation that the rank order of recoveries is virtually the same for each peak (for example, replicate 2 gives the highest recovery for all five peaks and replicate 5 gives the lowest for all save peak 3). This

Table 7. %Recoveries for selected PCB peaks from five replicate injections of PCB + triglyceride solution.

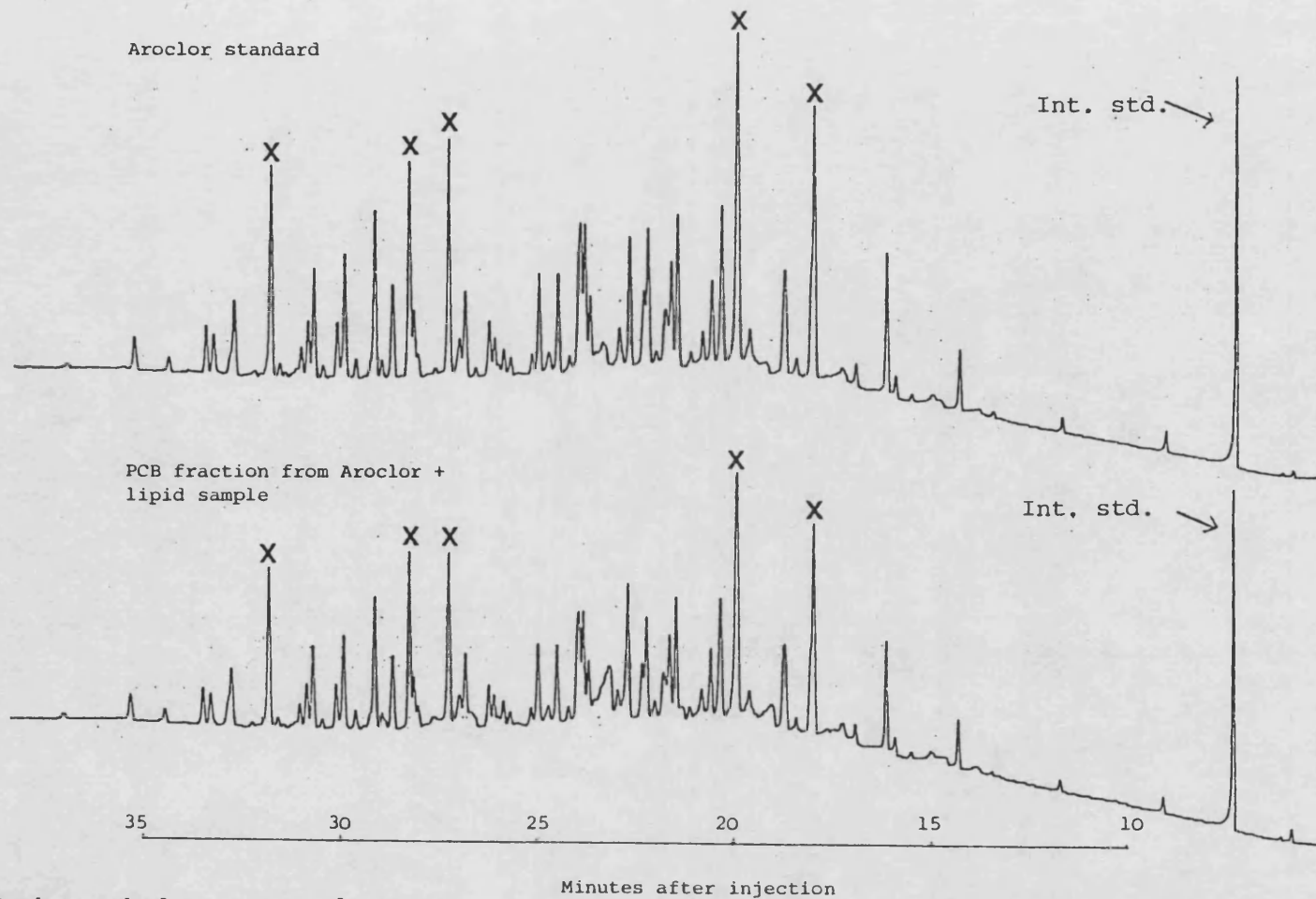
<u>Replicate number</u>	<u>t_r (min) peak 1</u> 18.00	<u>t_r (min) peak 2</u> 19.80	<u>t_r (min) peak3</u> 28.00	<u>t_r (min) peak4</u> 29.40	<u>t_r (min) peak 5</u> 37.80	<u>Area ratio peak @ 37.80 mins</u> <u>peak @ 19.80 mins</u>
1	92.7	97.9	86.8	89.9	94.8	0.3906
2	108.0	113.9	106.3	102.9	100.9	0.3605
3	102.7	107.6	100.6	101.7	99.7	0.3738
4	94.4	99.9	95.6	95.6	100.5	0.4058
5	86.5	91.6	88.1	87.6	93.0	0.4094

effect would be expected if there was variation in the area of the internal standard (either a measurement error or an error in the addition of the internal standard). It could, of course, be due to genuine variation in the recoveries, but it is possibly a little unlikely that all peak recoveries would vary together quite so concertedly, despite the fact that all the congeners co-elute from the HPLC.

In contrast, the very low split ratio used for the GC injection may have given rise to some variable discrimination amongst the congeners, especially the higher boiling point components and this may account for the variation in the peak area ratio for the peaks at 19.80 and 37.80 minutes.

A typical capillary GC chromatogram for a cleaned-up PCB fraction is shown together with a standard injection in Figure 26. GC conditions were as described under experiment 3 except that the temperature was programmed to 250°C instead of 200°C. As can be seen, pattern distortion appears to be minimal and the clean-up of the sample seems to be excellent.

In conclusion, recoveries for both lipid (300 mg) and PCBs (approximately 400 µg) were very good. This assumes that the HPLC injection loop was exactly 2.00 ml in volume but the error should not be large. The PCB



Peaks marked X were used for quantitation.

Figure 26.

fraction was collected directly after the elution of the lipid band and was about 10 ml in volume. The final step in the development of this clean-up technique was the investigation of OCP elution profiles.

Experiment 7.

Using the HPLC system as described in experiment 6, the retention of a number of organochlorine pesticides was investigated. Individual solutions of the following OCPs were made up in n-heptane at a concentration of approximately 0.03% m/v (except for beta-HCH which was made up as a saturated solution):

alpha-HCH, beta-HCH, gamma-HCH, delta-HCH, p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, p,p'-TDE, o,p'-TDE, p,p'-TDE olefin, chlordane (technical), aldrin, endrin, dieldrin, hexachlorobenzene.

For 2.0 ml injections and a flow rate of 2.3 ml min⁻¹, the elution windows (the times after injection after which elution begins and ends) for each OCP together with the elution windows for PCBs, biphenyl, lipid and n-heptane, are given in Table 8.

Peak shapes were generally quite broad, but all the organohalogenes tested except technical chlordane and aldrin would be completely separated from up to about

Table 8. Elution windows for selected compounds using HP-GPC clean up with PLRP-S column (300 x 7.5 mm), 20:80 propan-2-ol:heptane at 2.3 ml min⁻¹. 2.0 ml injection. System temp. 35°C.

<u>Solute</u>	<u>Elution window</u> (mins. after injection.)
α -HCH	7.3-9.2
β -HCH	7.3-9.0
γ -HCH	8.0-10.2
δ -HCH	6.7-7.7
p,p'-DDT	6.9-8.5
o,p'-DDT	6.4-8.2
p,p'-DDE	6.6-8.6
o,p'-DDE	6.6-8.1
p,p'-TDE	7.2-9.2
o,p'-TDE	6.8-9.1
p,p'-TDE olefin	7.0-9.2
Chlordane (technical)	In lipid-7.8
Aldrin	In lipid-7.4
Endrin	6.6-8.1
Dieldrin	6.5-8.2
Hexachlorobenzene	6.4-8.3
PCBs (mixed Aroclors)	7.0-9.7 (200 ppm level)
	6.4-10.4 (0.5% level)
Lipid (15% triglycerides + cholesterol)	4.0-6.4
n-heptane	3.8-5.8

300 mg of lipid material.

The total elution volume from the point of injection to the point where all the tested materials had completely eluted was less than 24 ml and the OCP/PCB fraction was less than 12 ml. The chromatogram for a 2.0 ml injection of the 0.03% m/v p,p'-DDT solution in n-heptane is shown in Figure 27.

In conclusion, PCBs and a wide variety of OCPs were separated from triglycerides using this HP-GPC system and the method showed considerable promise as a clean-up technique for milk extracts.

4.2.3. Comments on the use of the HP-GPC clean-up method.

The advantage of the HPLC clean-up described above are quite evident and include speed of separation, low solvent consumption and a re-usable column. The visual picture of the separation provided by the RI detector was found to be very useful for indicating when the lipid fraction had ended which is particularly important for samples where lipid content varies as with human milk.

Experience with the system has also proved it to be very durable. After approximately 18 months of use, having been exposed to a large number of crude extracts and other solutions, the column test mixture was re-run and

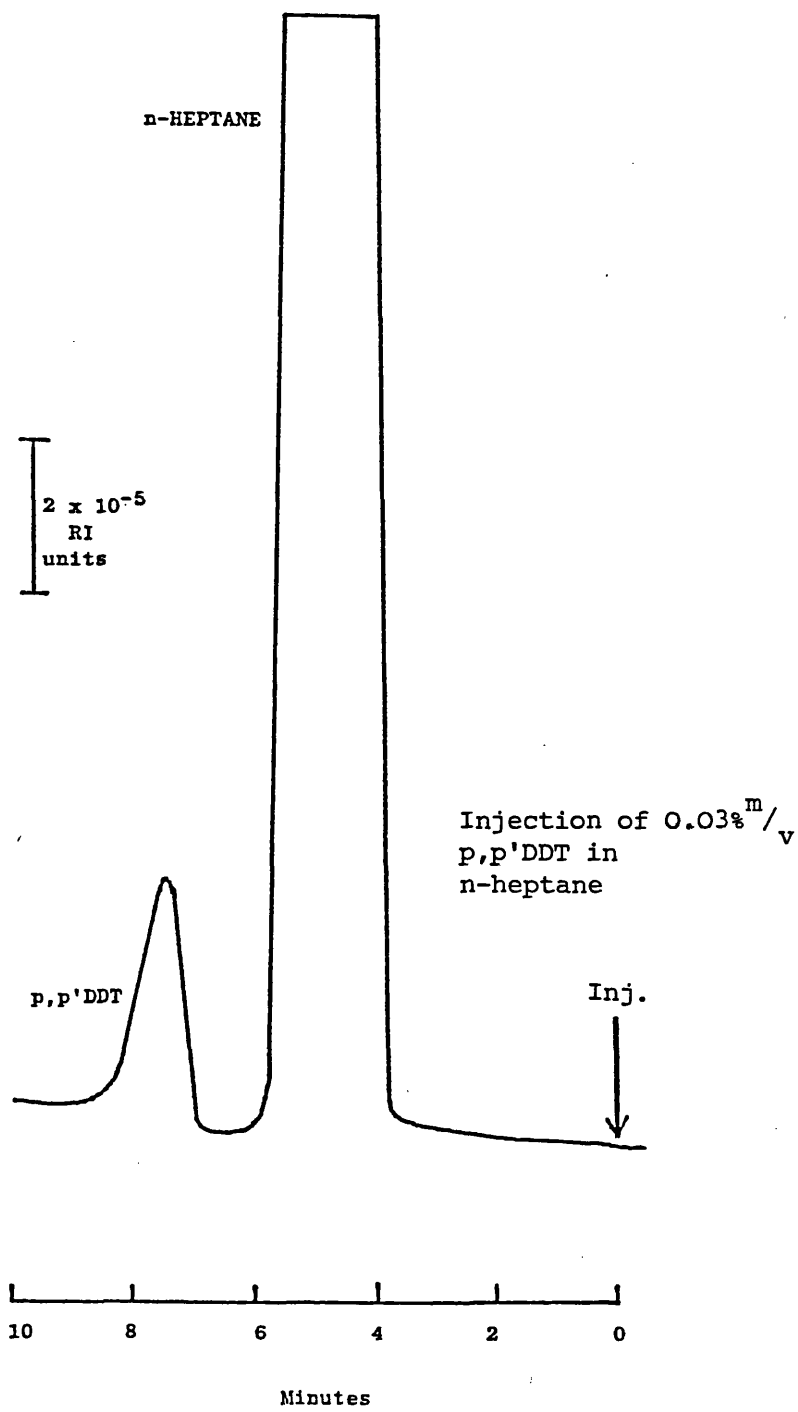


Figure 27.

the result compared with the original test chromatogram supplied by the manufacturer. The two chromatograms are reproduced in appendix 2. Inspection of the results show no discernible deterioration of column performance despite the harsh use.

In contrast to the advantages, however, there are a number of minor problems which have come to light as a result of experience gained in using the system for the clean-up of milk extracts:

The milk extracts were always straw-yellow in colour. Most of this pigmentation elutes within the lipid fraction but some does frequently pass into the OCP/PCB fraction. This colouration does not, fortunately, appear to hinder the GC determination of the residues.

On reducing the volume of the OCP/PCB fraction to a volume suitable for HP-GPC injection, a white precipitate is thrown down (about 1 mg per 10 ml mobile phase). Investigation of the source of this precipitate showed it to stem from the mobile phase itself. The identity of this precipitate was not determined but may have been due to plasticiser or molecular sieve resins. Again, the presence of this precipitate did not appear to affect the determination of the residues.

Extracts of higher fat content (say >300 mg lipid in

2 ml) can give lipid bands which overlap with the OCP/PCB fraction. As a result, milk samples were routinely introduced over two injections.

The use of this approach to sample clean-up requires complete transfer of the sample to the HPLC column. This quantitative transfer forces the operator to wash the sample vessel and syringe with solvent and inject these washings as part of the sample. This implies the use of very low volumes of solvent for washing (about 1 ml in total) with the possibility of leaving traces of the sample in the vessel or syringe. Fortunately, experience has proved this problem to be negligible and a sample plus washings can be introduced over two injections without difficulty. A further potential problem with this method is the introduction of air with the sample since air cannot be totally expelled from the syringe without the risk of sample loss. Again, experience has shown the system to be highly tolerant of air and any air bubbles pass straight through the column and detector.

A more trying practical problem is the blockage of the system by particles in the injected samples. Attempts to filter the samples through a zero dead volume membrane filter prior to injection proved to be impracticable due to the high viscosity of the extract. Thus, an in-line filter had to be incorporated in the system after the injection loop. This filter frequently became blocked

(after about every third or fourth injection) and had to be back-flushed. This is a major inconvenience and causes the couplings to become worn which in turn can lead to leakage. Incorporating a valve switching back-flushig system would prevent this difficulty but would be expensive.

A more serious and perplexing problem which may or may not be the result of the HP-GPC clean-up was the occasional "loss" of endrin residues observed in recovery studies conducted to assess the recoveries of the whole extraction, clean-up and quantitation procedure. When this loss occured, it appeared to be almost total with the endrin recovery dropping to around zero. At all other times, recoveries were routinely very good. It may be that this loss occurs during the HPLC although it could arise during extraction or during volume reduction in the Kuderna-Danish evaporator. If the loss does occur during the HPLC, it could possibly be due to different amounts of lipid causing shifts in retention with the endrin moving into the lipid. However, if this were the case, one may expect to see a variability in endrin recoveries rather than the "all-or-nothing" type of effect observed. This particular problem remains unsolved but has not been observed with any other analyte.

A final disappointment, rather than problem, is the early

elution of aldrin and technical chlordane preventing their quantitative recovery. In connection with this, it would be desirable to slightly increase the retention of the OCP/PCB fraction without increasing its total volume too much, in order to make the timing of fraction collection less critical and move residues, including chlordane and aldrin, further from the lipid. This is a difficult task since solvents which increase the retention of the organohalogenes tend to be polar. Introducing polar solvents to the system would severely limit the solubility of lipids in the mobile phase, but an interesting possibility exists by virtue of the size exclusion mechanism of the separation. Sample extracts are routinely introduced in 100% hexane solution. The lipid and the hexane are both unretained and elute together, i.e. the lipid band remains largely in the hexane slug. As a result, the constitution of the mobile phase may be less important in terms of maintaining lipid solubility on injection. Furthermore, at the temperature of the separation (35°C) the triglycerides in human milk would be fluid and would not cause total blockage even if they were to come out of solution. Consequently, it may be possible to further modify the mobile phase, perhaps by increasing the proportion of propan-2-ol, to prolong the retention of the organohalogenes without severely limiting the lipid capacity of the system. The organohalogenes should enter the channels within the polymer gel and would "see" the more polar mobile phase

therein whereas the lipid would be excluded and "see" mainly the hydrocarbon slug in which it was injected, thus maintaining its solubility. Such a hypothesis would have to be investigated by thorough experimentation. Of course, using a longer column would increase resolution but would also increase elution times and volumes.

5. QUANTITATION OF ORGANOCHLORINE RESIDUES.

5.1. Introduction.

The method of determination of organohalogens to be employed depends on several factors; the type and number of analytes, the range of analyte concentrations, the "cleanness" of the processed sample and the type of information required. A vast number of methods of detection and quantitation of organohalogens have been devised. In this particular analysis of human milk samples, it was necessary to develop a multi-residue method capable of determining several organochlorine pesticides and the PCBs simultaneously with a low limit of detection and a high degree of specificity whilst also minimising the total time and cost for the analysis.

5.1.1. Methods of determination - an overview.

A wide range of techniques have been brought to bear on the problems of OCP and PCB analysis. To a large extent, the choice of technique depends on the type of information required and the amount of qualitative and quantitative detail sought. The determination of OCP and PCB residues is made very demanding not only by the large number of potential analytes, but also by the very wide range of concentrations of analytes that may occur within a sample and any successful analytical method must

address and deal adequately with these problems.

Some of the less common techniques that have been used, particularly for PCBs, include IR, NMR, radioimmunoassay (RIA), TLC (105) and isotope dilution analysis (IDA) (106). These can all be used for screening and, to a greater or lesser extent, quantitation. HPLC has also been used for a number of applications with regard to organochlorine analysis (107). All of these methods except perhaps RIA and IDA, however, generally lack sufficiently low limits of detection for residue work. By far the most common method for qualitative and quantitative determination of OCPs and PCBs is gas chromatography and the discussions that follow will be concerned primarily with this technique.

5.1.2. Methods of determination using gas chromatography.

Before deciding which form of gas chromatography is to be used, the overall objectives of the analysis must be identified, that is, how much qualitative and how much quantitative information is required. OCPs are relatively simple to identify and quantify by gas chromatography since they tend to occur as single compounds or as simple mixtures of compounds with each component displaying easily exploitable differences in chromatographic behaviour. There are exceptions to this generalisation, however, and some OCPs, such as technical chlordane or

camphechlor, occur in the environment as mixtures of many congeners in a similar fashion to PCBs, and when such mixtures arise in a sample, the analytical methodology can become very involved. Equally, if a large number of OCPs is to be determined or interferences are present, steps must be taken to simplify the gas chromatographic data and it may become necessary to fractionate the sample using liquid chromatography or to use a "two-dimensional" technique such as GC-MS or perhaps GC-FTIR.

The PCBs, on the other hand, are more difficult to determine. The large number of congeners cover a very wide chromatographic range and complete resolution of all 209 congeners is hard to achieve. Although all 209 congeners are unlikely to be present in a human milk sample, or indeed any other environmental material, the requirement for resolution is still very demanding.

In order to avoid resolving all 209 congeners, various means of quantifying the "total PCB" content of a sample have been devised. Such methods were developed in the past when total resolution was beyond the technical ability of most laboratories but many, if not the majority of laboratories persist in using "total PCB" methods despite the fact that congener-specific analyses can now be carried out without great difficulty. Of course, congener-specific data may not be required if, for example, the analysis is designed merely as a screen

for PCBs or for monitoring variations in total PCB contamination, but as will be seen, the inherent variability and practical problems of the "total PCB" methods may render them unreliable, even for these applications. But it is evident that because of the widely differing toxicological profiles of different PCB congeners, any analysis designed for generating data for toxicological evaluation must, *de rigueur*, be congener-specific.

5.1.2.1. Perchlorination.

The principle of perchlorination is that all chlorobiphenyl congeners are exhaustively chlorinated up to decachlorobiphenyl and so may be determined as a single substance (108, 109). The total concentration may be expressed either on a molar basis or on a weight basis. The latter could be accomplished by perchlorination of a known amount of a standard PCB mixture (such as an Aroclor) and comparing the sample peak with that obtained for the standard; the sample may then be said to contain PCBs equivalent to a given amount of the standard mixture.

The perchlorination is usually carried out using antimony pentachloride with or without a halogen carrier. Perchlorination without a halogen carrier has been found to be very temperature dependent and care must be taken

to avoid incomplete perchlorination (110). Even if perchlorination is quantitative, the sample may have to be cleaned up prior to the reaction in order to remove potential interferences such as biphenyl (111), although biphenyl itself is unlikely to occur in human milk. If GC-ECD is to be used for the determination, the sample has to be cleaned up again after perchlorination, using adsorption chromatography, so as to remove residual electron capturing impurities which would otherwise impair the proper functioning of the detector (110). The method does decrease the limit of detection since all the PCBs are determined in one very electron capturing peak, but all information about the distribution of individual congeners is lost and so the result is of very limited value for toxicological purposes. In addition, the result may not be comparable to the answer produced using another method (111). Problems with contamination of the antimony pentachloride itself with chlorobiphenyls or brominated compounds have been reported (112). This is potentially a major source of error and blanks must be run. The method has been shown to be susceptible to wide variations in accuracy (113) and although it appears to be quite straightforward to perform, in reality it is quite labour intensive and overall, perchlorination is probably best avoided.

5.1.2.2. Dechlorination.

In contrast to perchlorination, dechlorination to biphenyl has been investigated as an alternative. Again, the aim is to determine PCBs as one chromatographic peak (biphenyl). The dechlorination has been carried out by heterogeneous catalytic dechlorination using nickel boride generated *in situ* (114). The method suffers from many of the drawbacks of perchlorination including interference from biphenyl itself if present in the sample, loss of all congener-specific information and lack of comparability with other methods. Furthermore, the biphenyl generated is more difficult to detect at low levels than chlorinated biphenyls since biphenyl has poor electron capturing properties and no chlorines; consequently, biphenyl is not amenable to detection by ECD or by Hall electrolytic conductivity detector (HECD). Finally, the nickel boride reduction has been found to be unreliable and not necessarily quantitative (115, 116). In conclusion, this method should not be employed.

5.1.2.3. Carbon skeleton gas chromatography.

This ingenious method again involves catalytic dechlorination of the PCBs with the aim of determining "total PCBs" as one peak (biphenyl) (117). Hydrogen gas is used both for the reduction and as the GC carrier gas. The catalyst, which may be palladium or platinum, can be

incorporated into the injector port of a gas chromatograph and dechlorination takes place within the injector port (76, 118, 119). This method suffers from some of the problems of nickel boride reduction such as difficulty in detecting low levels, loss of congener information and problems with interferences. However, it is more convenient and less labour intensive than the nickel boride method.

5.1.2.4. Pattern/peak matching and chemometrics in PCB analysis.

With pattern matching approaches, quantitation of a PCB sample is carried out by comparing the chromatographic pattern of the sample to that produced by a known amount of a standard mixture. Such methods probably account for the majority of "total PCB" analyses. One of the original peak-matching types of analysis was devised by Webb and McCall (120). In their method, the PCB standard was chromatographed on a polymethylsiloxane phase using packed column GC. By carrying out extensive analysis of each peak, a form of mixed response factor was calculated for all the congeners eluting in each peak. The peaks in a sample are then quantified using these response factors and the "total PCB" concentration can be calculated by summing the results for each peak. This type of procedure has several drawbacks: Firstly, each peak in the sample may contain a different distribution of congeners

compared to the standard, and so the mass of PCB per unit of peak area will be different in sample and standard. Secondly, the pattern of peaks present in the sample and standard may be different and some sample peaks may, unknowingly, be due to interferences that have not been removed by the clean-up. Thirdly, when used with ECD, the poorly electron capturing, lower chlorinated congeners, may go undetected. Fourthly, no congener-specific information is obtained and finally, the result obtained is probably not comparable with those obtained by other methods.

A similar approach, using capillary columns, was proposed by Bush et al. (121). This method is essentially a congener specific method. A response factor for each peak is used for quantitation and since most peaks are due to only one congener, the results are almost completely specific for each component. However, where congeners co-elute, a mixed response factor is used as with the Webb and McCall method.

An unusual method for PCB determination employs capillary GC but uses selected peaks to represent the total level of PCB contamination. Essentially, the method is congener-specific for a few characteristic components and ignores the rest, yet information regarding the total PCB burden is inferred from this limited amount of data. This approach has been adopted for determining PCB residues in

fish {122}. Provided the pattern of congeners is fairly constant in all samples, this method could be useful for detecting variations in levels of PCBs, but should the distribution of congeners vary from sample to sample, as may well be the case in biological matrices, the results from this type of analysis could be very misleading. A comparison of this type of peak matching method with a full congener-specific method has shown the method to be only semi-quantitative {123}.

Chemometric techniques have also been applied to PCB analysis and have been used to analyse mixtures of PCBs by level of chlorination, having already established the elution windows for each isomer group {124}. In a similar vein, pattern recognition by a partial least squares regression has also been used to quantify PCBs {125}. In both cases, the sample pattern is fitted mathematically to a combination of known patterns and the final result is expressed as an amount of a mixture of known components which may themselves be mixtures. The techniques are useful for classifying the component mixtures (e.g. mixed Aroclors) in the sample as a form of "fingerprint", although it must be appreciated that the combination of mixtures as assessed by the data reduction does not necessarily imply that this was the profile of the mixture when the sample was originally contaminated, particularly if the matrix has been subjected to environmental modification. Fingerprinting may be useful

for identifying the source of contamination and as well as this chemometrics can, of course, be used for quantitative analysis. Due to the extensive calculations that have to be carried out, these methods have to be computerised. Various methods have been devised and depending on the approach used and the conditions of the data collection and reduction, congener-specific information may or may not be provided.

In conclusion, pattern matching types of analysis can be useful in some instances. The simple types are prone to inaccuracy, particularly when the sample pattern does not match the standard pattern very well. The more complex chemometric methods can be more informative and may be helpful in identifying a source of pollution, but complex methods may still not provide the congener-specific information required for toxicological studies.

5.1.2.5. OCP and congener-specific PCB analysis. Theory and practice.

Different PCB congeners can have widely differing toxicological profiles (13) and in view of this, any study of the toxicological effects of PCB contamination must be based on a congener-by-congener analysis. Furthermore, the only true picture of total PCB contamination is a complete 209 congener analysis. For these reasons, the trend in PCB analysis over the past

few years has been towards high resolution, congener-specific analyses. The principle of congener-specific analysis is simple - each of the 209 possible congeners is treated as an individual analyte and is quantified as such; the practice of total congener-specific analysis, on the other hand, is quite formidable.

Evidently, congener-specific analysis demands the use of capillary GC. Packed GC columns are unable to deliver sufficient resolution for congener-specific work. Packed columns have their place in pattern matching, perchlorination or dechlorination methods but as has already been pointed out, these methods possess major drawbacks and are of limited use. With multi-residue OCP analysis, packed column GC analysis often necessitates the fractionation of the sample, usually by adsorption chromatography, so the sample has to be analysed over many injections. A final limitation to packed column work is the increase in the limit of detection due to the relative broadness of the peaks. The only real advantage of packed columns is their robustness.

The choice of detector determines the way a congener-specific, multi-residue analysis will be implemented. With very low levels of organochlorine analytes, only three detectors offer the sensitivity required for routine use; the electron capture detector (ECD), the Hall electrolytic conductivity detector (HECD) and the

mass spectrometer (MS).

The ECD is very widely used for organohalogen residue work and is simple and robust. It is generally regarded as being only semi-selective in that it detects some types of compounds (electron capturing substances) better than others and as a result, it will not tolerate "dirty" samples as are often encountered in environmental work. Another problem is the very wide variation in response factors between similar compounds and amongst the PCBs, for example with a modern ECD the response factors for PCBs vary over two orders of magnitude (46). Consequently, wide differences in limits of detection are observed for different PCB congeners.

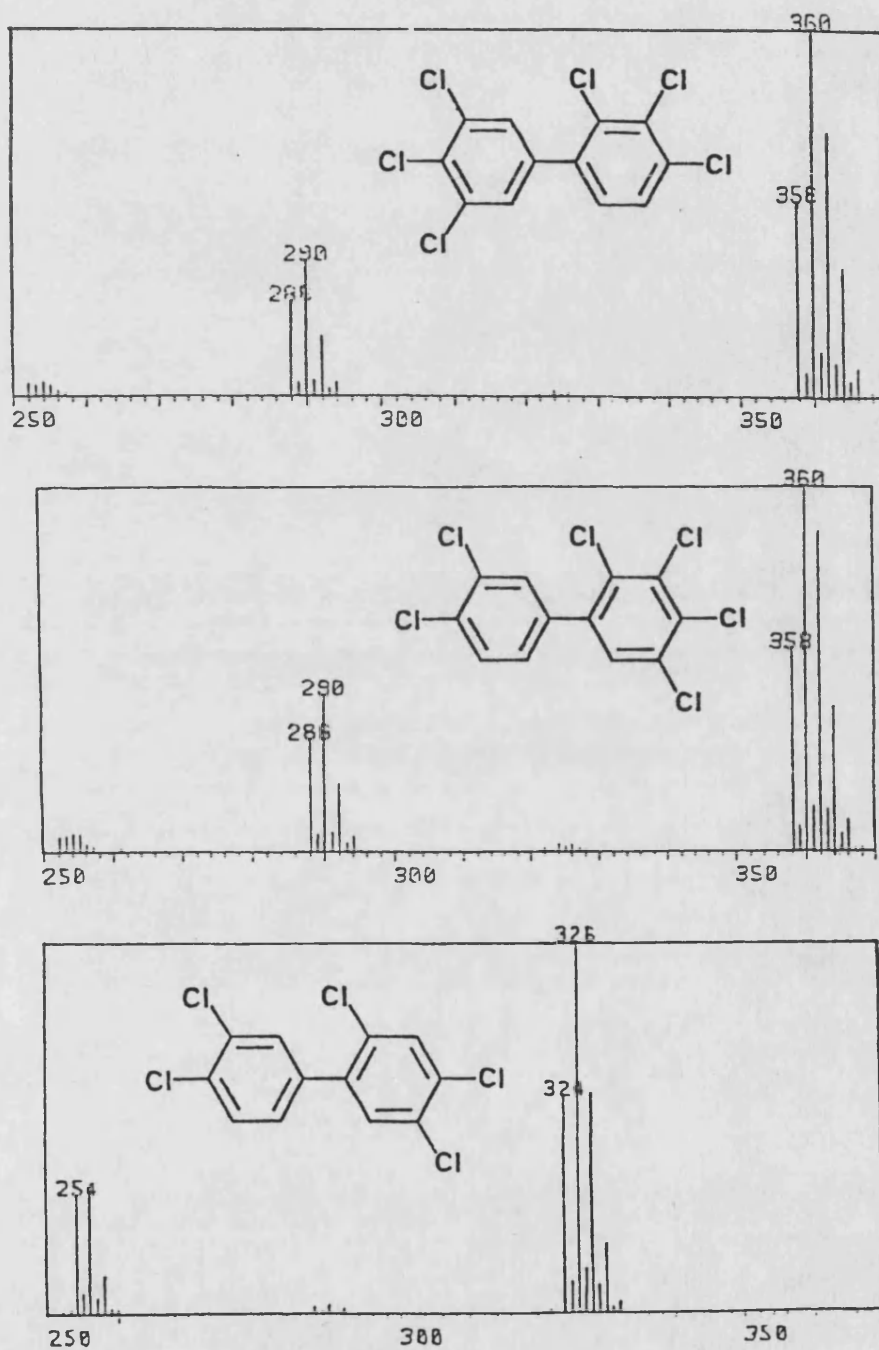
The HECD is a more appropriate detector since it can be made very selective for chlorinated compounds and response varies linearly with chlorine content (126). Consequently, the HECD is able to tolerate less clean samples and gives a predictable range of response factors which vary over only one order of magnitude.

With both the ECD and the HECD, all compounds are detected on one chromatographic trace. This means that either the GC separation has to be extremely efficient, or the sample has to be fractionated. When detecting OCPs and PCBs using these detectors, some fractionation is inevitable since the efficiency required for separating

all the OCPs and the PCBs in one run is not routinely available. However, it is possible to completely separate virtually all 209 PCBs from each other (39, 46) and this is achieved by using a long, non-polar column such as a 60 m SE-54, and hydrogen as carrier gas. The aim is to develop as much column efficiency as possible. Stationary phase selectivity is of no use with such a large number of analytes since a selective column would merely alter the elution order but would not simplify the chromatogram.

The mass spectrometer has several advantages over the ECD and HECD as a detector by virtue of its ability to distinguish between compounds with different mass spectra. GC-MS can be regarded as a two-dimensional technique with a chromatographic dimension and a mass spectrometric dimension running at right angles such that mass spectrometric "slices" through the chromatogram are obtained. Each m/z value in the mass spectrometric dimension can be regarded as an individual detector channel and so compounds which give ions at different m/z values can be distinguished by the MS even if they co-elute chromatographically. This immediately allows the OCPs to be distinguished from the PCBs so that OCPs and PCBs do not have to be separated by fractionation prior to GC-MS. Using 70 eV electron impact MS (EIMS), a problem does, however, arise with the PCBs. EIMS cannot easily distinguish between PCBs with the same number of

chlorines (i.e. isomers) or between PCBs differing by two chlorines (127); in the latter case, the higher chlorinated congener can be quantified independently of the lower but not vice-versa. There is also a problem of interference when the analyte contains, say, n chlorines and a co-eluting congener contains (n+1) chlorines with a 2,2'-chloro- substitution pattern; this arises because of the increased abundance of the $[M-Cl]^+$ fragment in 2,2'-chloro- PCBs. Inspection of the 70eV EI mass spectrum of PCB 97 (see Appendix 1) illustrates this. Other inter-congener interferences can occur such as between congeners differing by one chlorine or between a lower chlorinated congener and a low intensity fragment ion from a much higher chlorinated compound, but these interferences are of a low order and the interfering compound would have to be present in considerable excess over the analyte. The potential for inter-congener interference can be assessed by inspection of the relevant mass spectra. As an example, the 70eV EI mass spectra of two hexachloro- and one pentachlorobiphenyl can be seen in Figure 28. The three primary results of this increased selectivity are firstly that the OCPs do not have to be separated from the PCBs. Secondly, only PCBs which interfere in the mass spectrometric dimension have to be resolved chromatographically. Thirdly, MS is very selective against other interferences and so can tolerate relatively dirty samples. Furthermore, GC-EIMS response factors for PCBs range over only about one order



70 eV EI mass spectra for two hexachlorobiphenyls and a pentachlorobiphenyl.

Figure 28.

of magnitude (128) giving a more even spread of limits of detection.

Due to the selectivity of the mass spectrometer as a detector, its ability to distinguish OCPs from PCBs and to differentiate between groups of PCB congeners, the capillary column used for the GC does not have to separate all the analytes but merely has to separate analytes which do not have distinguishable ions. On non-polar phases, PCBs tend to elute in tight clusters according to the level of chlorination with the lower chlorinated isomers eluting earliest (129). This clustering of compounds with virtually identical mass spectra means that interference is very likely. For this reason, there is scope for employing selective phases when using EIMS as the detection mode in order to scatter isomers and reduce the likelihood of serious interference. The reduced requirement for pure efficiency also means that shorter columns can be used, hence reducing total analysis time. Of course, the right sort of selectivity has to be employed. It will be seen that polytrifluoropropylsiloxanes are useful in this context and mixing phases can be a very powerful means of tuning selectivity (130). The technique of window diagramming (131, 132) provides an excellent guide as to the proportions of stationary phases to be combined. Indeed, phases need not be physically mixed and coated into one column, but columns of the different phases can be

coupled in series to achieve the same result (133). Other forms of selectivity tuning such as differential pressure or temperature control in sequentially coupled columns (130) offer very fine control over selectivity but are probably too specialised for routine application and in many instances, may not be necessary.

An exciting development in stationary phase technology over the past few years has been the emergence of liquid crystalline phases (134). The phases are very selective for positional isomers and molecular geometry, particularly length-to-breadth ratio, exerts a major influence over the retention of PCBs by these types of phases (135). This is especially useful for GC-MS methods since it is positional isomers which pose the most serious interference problems.

A unique advantage of employing MS as the detection mode is offered by the use of isotopically labelled recovery standards. Isotopically labelled analogues of the analytes represent perfect recovery standards since the physical and chemical properties are essentially identical to those of the analyte.

It will be appreciated that there are several modes of operation of MS as a GC detector (136) such as positive (137) or negative (138) chemical ionization, pulsed positive ion negative ion chemical ionization (PPINICI)

{139} as well as the more common electron impact (EI) mode. Any of these modes may be used with different scanning techniques such as full scan {140}, limited scan {141} or selected ion monitoring {142}. The diversity of GC-MS techniques including discussions of their advantages and disadvantages, are very well documented in the literature {136}. A notable advantage of chemical ionization techniques is the increase in sensitivity over EIMS for many compounds. For example, the dimethanonaphthalene OCPs fragment heavily under EIMS but using CIMS, fragmentation is markedly reduced {143} thus effectively concentrating the ion current in fewer ions which in turn results in increased sensitivity.

A major drawback of attempting to quantify 209 PCB congeners plus a variety of OCPs is the apparent need for a pure sample of each analyte for use as a standard. The situation is further complicated by the difficulty in obtaining pure samples of the majority of PCB congeners and it may well become necessary to synthesise standards in-house. A useful way of avoiding this problem is to use surrogate standards. The idea behind this approach is that PCB congeners are grouped together into sets such that the response factors for each congener within a set are as similar as possible. The congener in each set which possesses a response factor nearest to the average response factor is then chosen as being representative of every congener within that set. This representative

congener is the surrogate or secondary standard for the set. Each individual PCB congener is then quantified using the surrogate standard from its set. This approach has been used for PCB quantitation using both ECD (144) and MS detectors (145). With ECD detection, the situation is complicated by the wide range of response factors seen amongst the different congeners and this means that there will be many sets containing only a few congeners if the surrogate is to be acceptably representative. In the work of Cooper et al. (144), an ECD method with 31 surrogate standards was employed for congener-specific analysis.

Using mass spectrometry as the detection mode, the situation is simplified considerably since the range of response factors is much narrower and congeners fall naturally into sets of congeners with different numbers of chlorines within which response factors are very similar. Hence, Slivon et al. (145) made use of only nine surrogate standards to quantify individual PCB congeners by GC-MS.

5.1.2.6. Chromatographic conditions for OCP and congener-specific PCB analysis.

The choice of stationary phase and detector have already been discussed. The three remaining factors involved in the construction of a capillary GC method are the choice of carrier gas, the injection mode and the column size

and film thickness.

For high resolution multi-analyte work, hydrogen is the carrier gas of choice (146). Hydrogen exhibits a shallow van Deemter curve and this means that efficient separation is maintained throughout a greater length of the column and also over a wider span of temperature during a programmed run than with any other gas. Hydrogen also develops more plates per unit time than any other carrier gas which means that analysis times are minimised. Helium represents an acceptable alternative but should be rejected in favour of hydrogen whenever possible.

With regard to injection mode, there are a number of techniques available for use with capillary columns including split, splitless, on-column and programmable temperature vaporization (PTV).

Split injection involves discarding a large proportion of the injected material which inevitably raises the limit of detection. This alone is sufficient reason for rejecting this technique since low limits of detection are a primary requirement for residue analysis. Discrimination between analytes may also be a problem.

Splitless injection is commonly used in residue analysis since most of the injected material reaches the column.

Choice of temperature, solvent and injection volume are critical, however, since the ability of the solvent to wet the stationary phase and the length of column over which the sample spreads (band broadening in space (147)) play a central role in establishing good peak shape and hence resolution. Large injection volumes are desirable from the point of view of sensitivity but until recently, large injection volumes were not generally used with splitless injection because of the difficulty in avoiding band broadening and also the possibility of damaging or contaminating the stationary phase. Cold trapping of solutes is one way of achieving large injection volumes, but a simple and very elegant alternative is to use a retention gap as proposed by Grob (147). This not only serves as a guard column but also permits re-concentration of the solutes at the inlet of the coated part of the column while the solvent evaporates. These advantages should make the retention gap a routine device for splitless injections. Discrimination and thermal decomposition can be a problem with splitless injections.

On-column injection is a widely used technique and may be implemented in a variety of ways. Peak shape is generally excellent and discrimination is not usually a problem providing the injector design is satisfactory. Interestingly, Tuinstra *et al.* (148) found no significant difference between the results obtained using splitless and on-column injection for the analysis of PCBs.

The programmable temperature vaporizer (PTV) injection system offers an effective form of injecting a sample by evaporating the solvent before volatilising the solutes. Discrimination is virtually eliminated and the technique exposes thermally labile substances to the minimum of thermal shock. PTV injection is a powerful technique but is probably of little advantage for OCP and PCB work.

The final consideration is that of column dimensions. Essentially, this decision requires a compromise between efficiency, analysis time and robustness. Narrow bore, thin film columns offer high efficiency and shorter analysis times but are less robust. Long columns increase efficiency although very long columns cannot be effectively optimised over their whole length, even when using hydrogen carrier gas, and analysis time is prolonged. The final choice of column depends very much on the application.

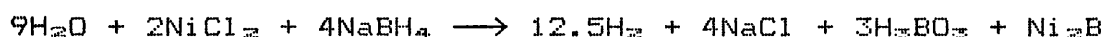
5.2. Experimental Investigations into the Quantitative Determination of Organochlorine Residues.

Initial studies were directed at the investigation of the catalytic reduction of PCBs with a view to quantifying "total PCBs" as a single substance (biphenyl). This line of experimentation was quickly abandoned and all subsequent work was aimed at using capillary GC-ECD and GC-MS for congener-specific PCB analysis with

simultaneous determination of selected OCPs.

5.2.1. Catalytic hydrodechlorination of PCBs using nickel boride.

The hydrodechlorination method of Dennis and Cooper (114) was employed whereby a precipitate of nickel boride is produced *in situ* according to the reaction:



All experiments were conducted using PCBs; OCPs were not investigated. However, Dennis and Cooper looked at OCPs (114, 149, 150, 151) and found that a mixture of partially dechlorinated products was obtained for most OCPs. The same workers also claimed that Aroclor 1254 underwent 97% conversion to biphenyl using this technique (152) but Kennedy *et al.* (116) found that the conversion of PCBs using this approach was somewhat less efficient than had been expected.

A series of fifteen experiments on the nickel boride reduction of PCBs was carried out and these are described in detail in reference (115). The results showed that conversion of PCBs to biphenyl was not quantitative and the degree of conversion became worse the higher the level of chlorination of the original PCBs. This in itself was sufficient cause to reject the method, but

other problems were also noted:

- i) Biphenyl is a volatile material and is easily lost.
- ii) Biphenyl has poor electron-capturing properties and has a high limit of detection using GC-ECD. As a result, other less sensitive detectors have to be used and the limit of detection is, therefore, poor despite "concentrating" all the PCBs into one peak.
- iii) Biphenyl itself is an environmental contaminant and may interfere with the determination of PCBs (although one would not expect to detect biphenyl in human milk).
- iv) The reduction reaction is sensitive to changes in reaction conditions.

For these reasons, the method was not considered further.

5.2.2. Investigations into the WCOT capillary gas chromatography of PCBs and OCPs.

A number of choices have to be made regarding the operation of a capillary GC method including internal standard selection, injection mode, detector choice, carrier gas, column type and temperature program.

5.2.2.1. Internal standard choice.

The internal standard choice for all GC-ECD and GC-MS analyses was 4,4'-dibromobiphenyl. This was selected for

three reasons: (i) it elutes in a convenient position (approximately mid-way) in the chromatogram for PCB/DPC analyses, (ii) it is electron-capturing and (iii) it has a high, even mass base peak under 70 eV EIMS and a characteristic mass spectrum. Bromobiphenyls have not been used extensively in the U.K. and so 4,4'-dibromobiphenyl would not be expected to appear in human milk samples and indeed, no bromobiphenyls were ever detected during this project. In the U.S.A., however, polybrominated biphenyls were used as fire retardants and are widely distributed in the environment. Consequently, 4,4'-dibromobiphenyl would probably not be a suitable internal standard for North American human milk samples.

5.2.2.2. Injection mode.

Choice of injection mode has already been discussed and splitless mode was selected for its simplicity and the need to transfer as much of the injected sample as possible to the column. An extension of this was the use of a 2 m retention gap joined to the separation column with a zero dead volume butt connector. This served not only as a guard column but also permitted the use of large injection volumes and 10 μ l of heptane solutions were routinely injected (splitless for 2 minutes with an injection duration of 15 seconds).

5.2.2.3. Detectors.

Choice of the detector for residue work was limited by the need for extreme sensitivity and selectivity and most of the method development work was carried out using ECD and EIMS as detection mode. Due to the large number of analytes and the presence of many other materials in the extracts, quantitative analysis was always carried out using GC-EIMS.

5.2.2.4. Quantitative measurements on chromatographic peaks.

Quantitative measurements on chromatographic peaks were usually performed by measuring peak areas by electronic integration. Peak height measurements, when needed, were made manually for GC-ECD traces and electronically for GC-MS. Quantitation by peak areas generally shows improved precision over peak heights but peak height measurements are less prone to errors due to partial overlap of peaks. This latter advantage of peak height measurements was considered to be of minor importance in view of the high resolution offered by WCOT capillary columns and the selectivity provided by EIMS (in selected ion monitoring mode).

It was noted that the area measurement algorithm employed by the GC-MS system was unusual in that instead of

measuring the whole area of a peak, it performed quantitative calculations by dropping perpendiculars from the half-heights of a peak and then measured the area between the two perpendiculars. This was found to lead to considerable errors in quantitation if the peak in question was distorted to any degree. As will be seen later, some milk samples gave rise to distorted peaks for the 4,4'-dibromobiphenyl internal standard and also for beta-HCH. This distortion was probably due to some unidentified substance(s) (possibly residual lipids) in the extract giving rise to retention of these two compounds in the injector port or the retention gap. However, because only some of the samples but not the standards gave rise to distorted peaks, this unusual method of measurement gave rise to errors of quantitation due to the difference in the proportion of the peak area measured in the sample and the standard; this was a particularly worrying problem in samples where the internal standard peak was affected. If total peak areas had been measured, the problem would not have arisen. This matter is discussed further in Sections 6.2.1.3. subsection 14, 7.2. and 8..

5.2.2.5. Carrier gas selection.

With capillary gas chromatography, the choice of carrier gas is essentially limited to helium or hydrogen; all other gases tend to be too inefficient in terms of plate

height and the number of plates generated per unit analysis time. Analysis times are greatly prolonged when other carrier gases are used.

All of the ECD work was carried out using hydrogen as carrier gas and all the development work for GC-MS (using a 15 m DB-210 column) was carried out using helium. Later GC-MS work using a 25 m OV-17 column was conducted using hydrogen in order to reduce the analysis time and also to increase the overall efficiency of the separation. Use of hydrogen as a carrier gas implies the use of relatively high flow rates. In GC-MS this gave rise to high source pressures. The possibility of hydrogen chemical ionisation effects arising was investigated; fortunately no CI effects were seen despite the high source pressure and full-scan mass spectra obtained using hydrogen as carrier gas for GC-EIMS were identical to those obtained using helium carrier for all the OCPs and PCBs investigated.

5.2.2.6. Column selection.

With a large number of analytes and a selective or semi-selective detector, the selectivity of the stationary phase can be very important. With a semi-selective detector such as an ECD, all analytes are detected simultaneously and so each peak must be separated chromatographically from all the others. With a very

large number of analytes, as in the case of congener-specific PCB with simultaneous OCP analysis, this separation has to be achieved by sheer efficiency; selectivity would merely change the elution order and would be of little or no use in increasing resolution. For this reason, congener-specific PCB analysis using GC-ECD employs very long, non-polar (non-selective) columns such as a 60 m SE-54.

Using MS as the detection mode adds another dimension to the analysis in that only analytes that interfere mass spectrometrically (see Section 5.1.2.5.) have to be resolved chromatographically. Consequently, shorter, more polar columns were investigated (OV-17, DB-210) and compared to OV-1 for selectivity between interfering analytes, particularly PCBs with interfering ions. The aim of the work was to scatter interfering analytes chromatographically by introducing chromatographic selectivity, and thereby relax the requirement for pure efficiency thus enabling the analysis to be carried out using a shorter column with a concomitant reduction in analysis time. Further advantages of shorter columns are their decreased cost and the fact that they are optimised over a greater proportion of their length since the difference between the inlet and outlet linear gas velocities is less than in a longer column.

The internal diameter and stationary phase film thickness

were also parameters which had to be considered. Wide bore, thick film columns are more robust and have a greater loading capacity than small bore, thin film types. Unfortunately, the former are less efficient and give rise to long analysis times. High loading capacity and robustness are desirable properties when dealing with large injection volumes, but the requirement for efficiency (despite chromatographic and spectrometric selectivity) made it necessary to find a compromise and it was decided that a 0.25 mm i.d. column with a film thickness of about 0.2 μm would be suitable.

Three WCOT columns were evaluated: (i) a 39 m OV-1, 0.25 mm i.d., 0.2 μm film, (ii) a 25 m OV-17, 0.25 mm i.d., 0.2 μm film and (iii) a 15 m DB-210, 0.25 mm i.d., 0.25 μm film. All phases were chemically bonded onto the fused silica. The selectivity between OCPs, as a guide to the overall selectivity for similar types of organochlorines, was investigated for all three columns using a Perkin-Elmer 8320B capillary GC. The conditions used with each column were as follows:

Carrier gas:	Hydrogen at about 65 cm s ⁻¹ .
Injector temp.:	270°C.
Detector temp.:	350°C
Injection:	2.5 μl heptane solution with 1 μl heptane "chaser". Splitless.

i) OV-1:

Carrier pressure: 22.0 p.s.i.
Splitless time: 1.75 minutes.
Oven programme: 100°C for 2 mins. 30°C min⁻¹ to
120°C. 1.3°C min⁻¹ to 230°C.

ii) OV-17:

Carrier pressure: 10.0 p.s.i.
Splitless time: 2.00 minutes.
Oven programme: 100°C for 2 mins. 30°C min⁻¹ to
120°C. 1.3°C min⁻¹ to 250°C.

iii) DB-210:

Carrier pressure: 5.2 p.s.i.
Splitless time: 1.75 minutes.
Oven programme: 100°C for 1.7 mins. 1.2°C min⁻¹
to 190°C.

Chromatograms for each column with the selected organohalogenes labelled can be seen in Figure 29. As can be seen, the OV-1 gives poor selectivity between compounds of similar structure, for example, the HCHs and HCB tend to cluster together.

The OV-17 and DB-210 generate far greater selectivity and a more even distribution of the OCPs over the chromatographic range. The shorter DB-210 column also resulted in a shorter analysis time. For these reasons,

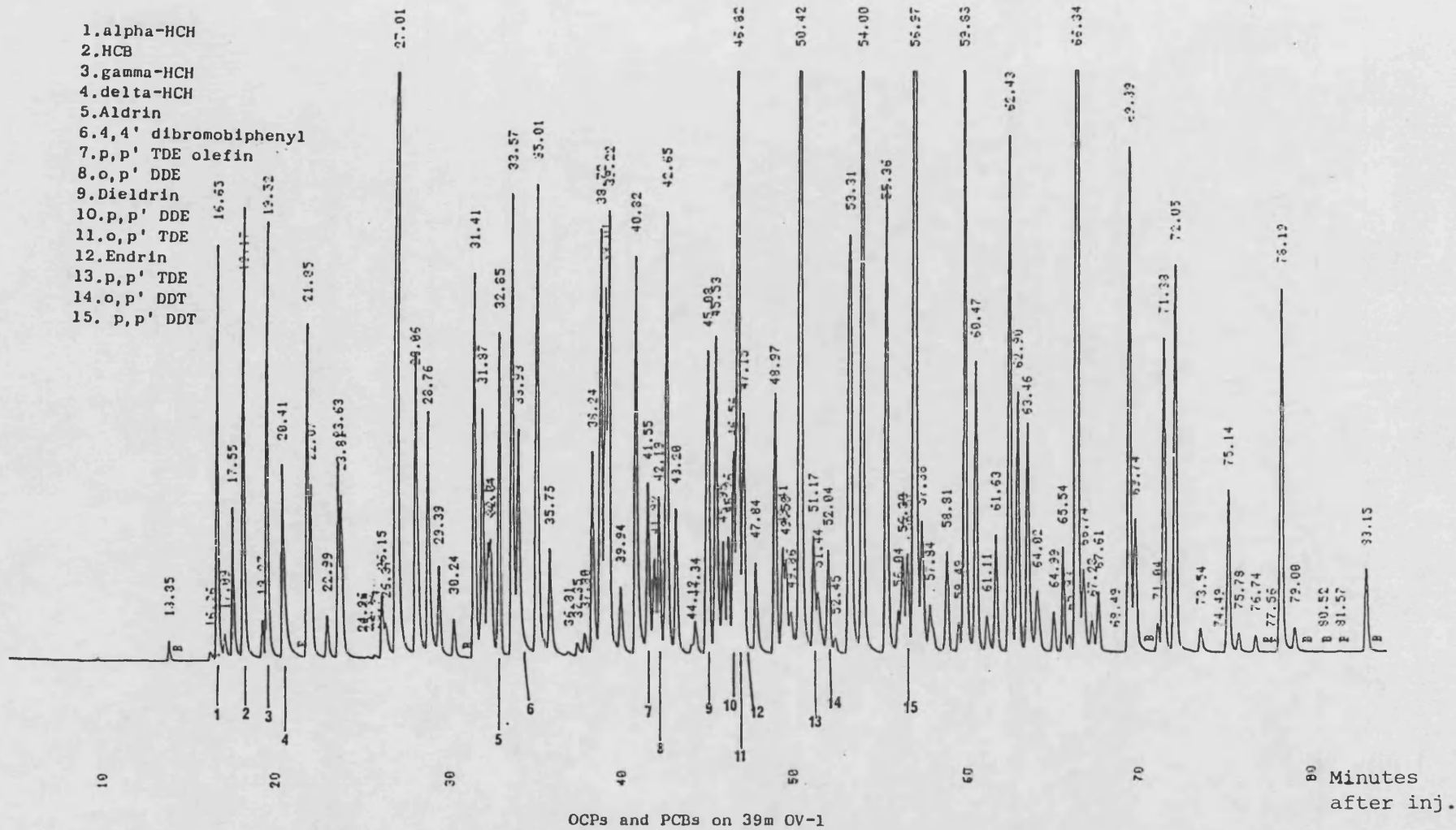
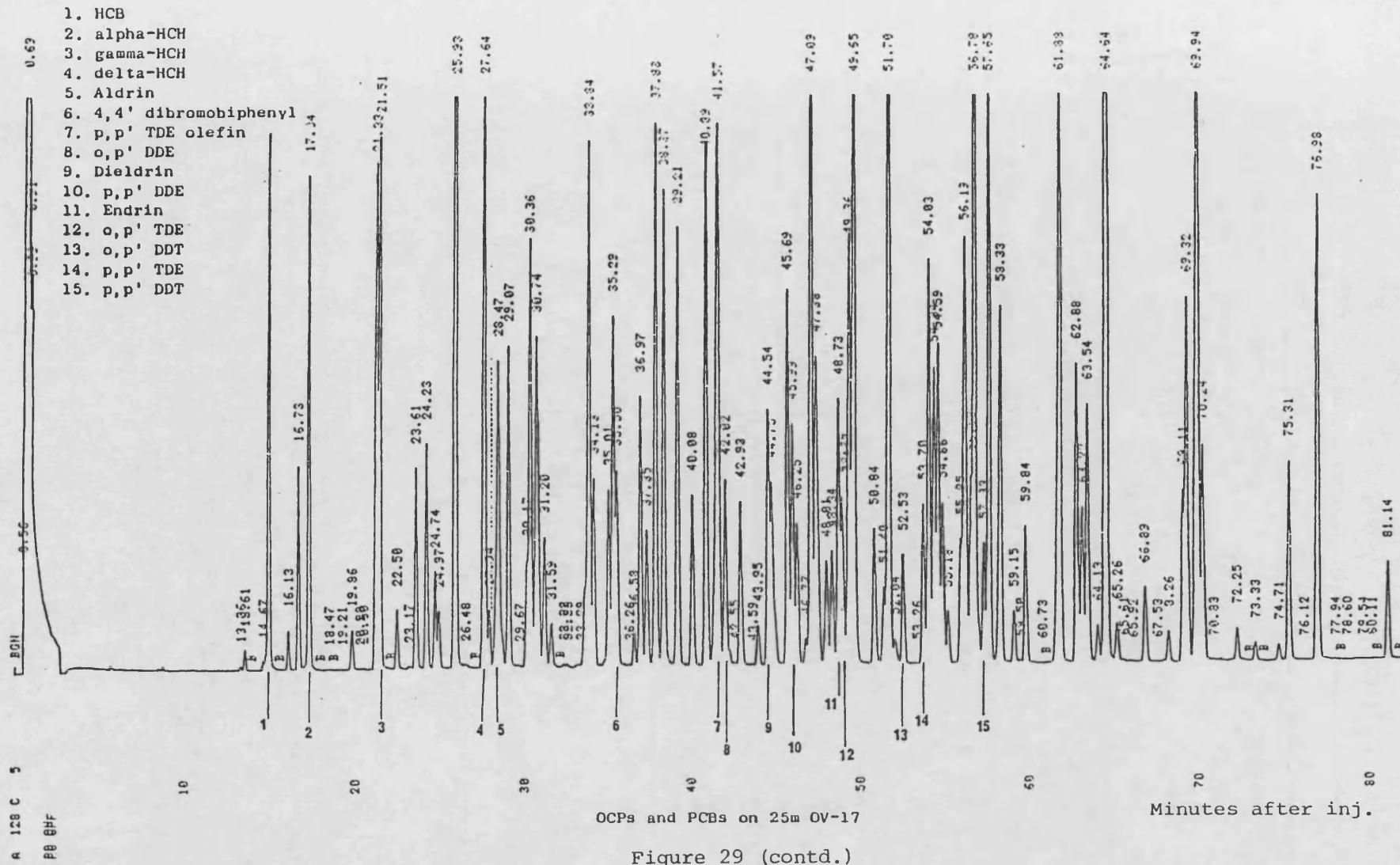
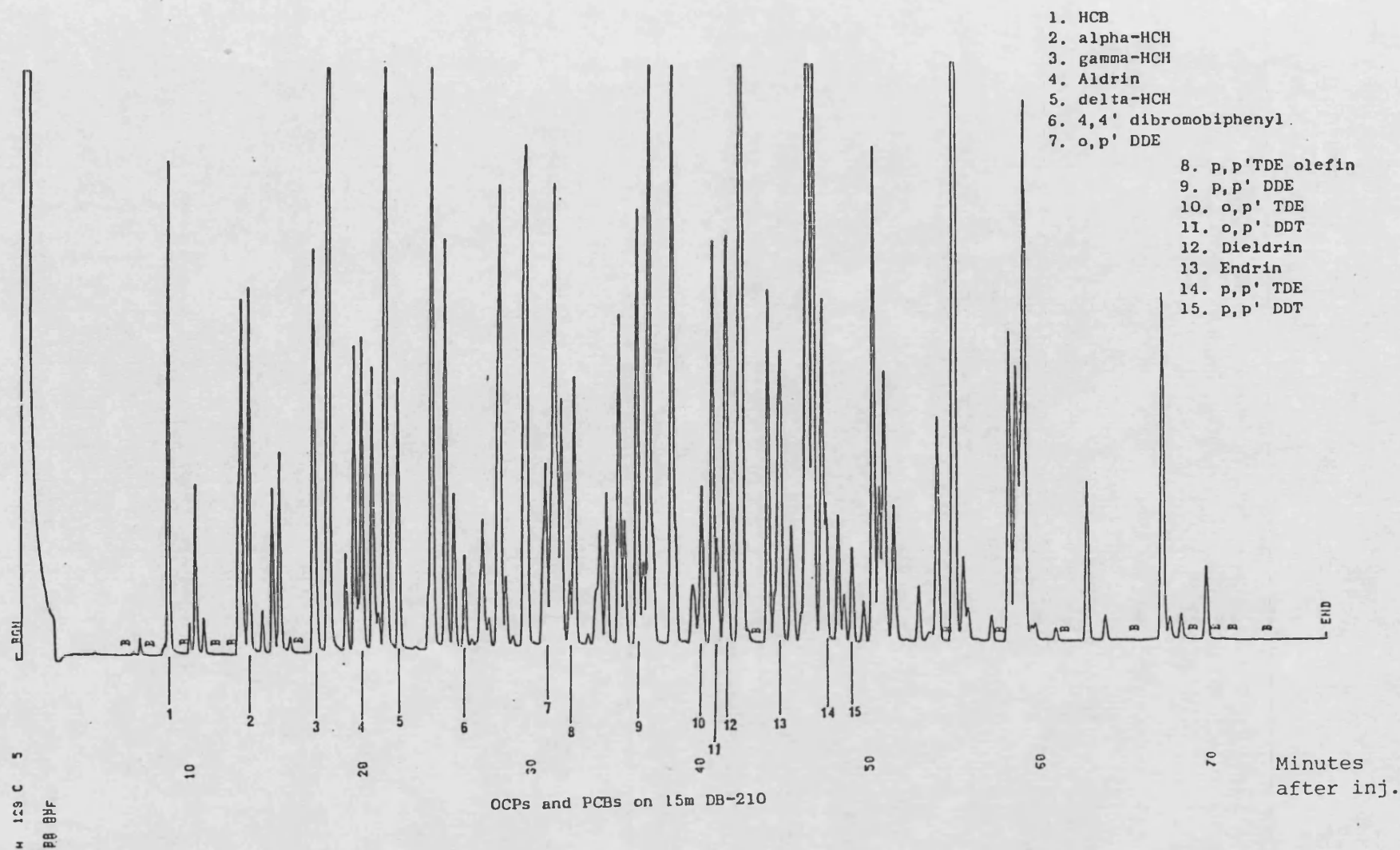


Figure 29.





OCPs and PCBs on 15m DB-210

Figure 29 (contd.)

the DB-210 was selected for further evaluation and its performance was examined using GC-MS under the following conditions:

Instrumentation: Dani 3800 capillary GC interfaced to a VG 7070E mass spectrometer.

Carrier gas: Helium at 0.25 bar (approx. 70 cm s⁻¹).

Injector temp.: 270°C.

Injection: 10 µl heptane solution. Splitless for 2 mins.

Oven programme: 86°C for 2.5 mins. 1.5°Cmin⁻¹ to 200°C.

Transfer line temp.: 180°C.

Ionisation mode: 70 eV EI. Selected ion monitoring.

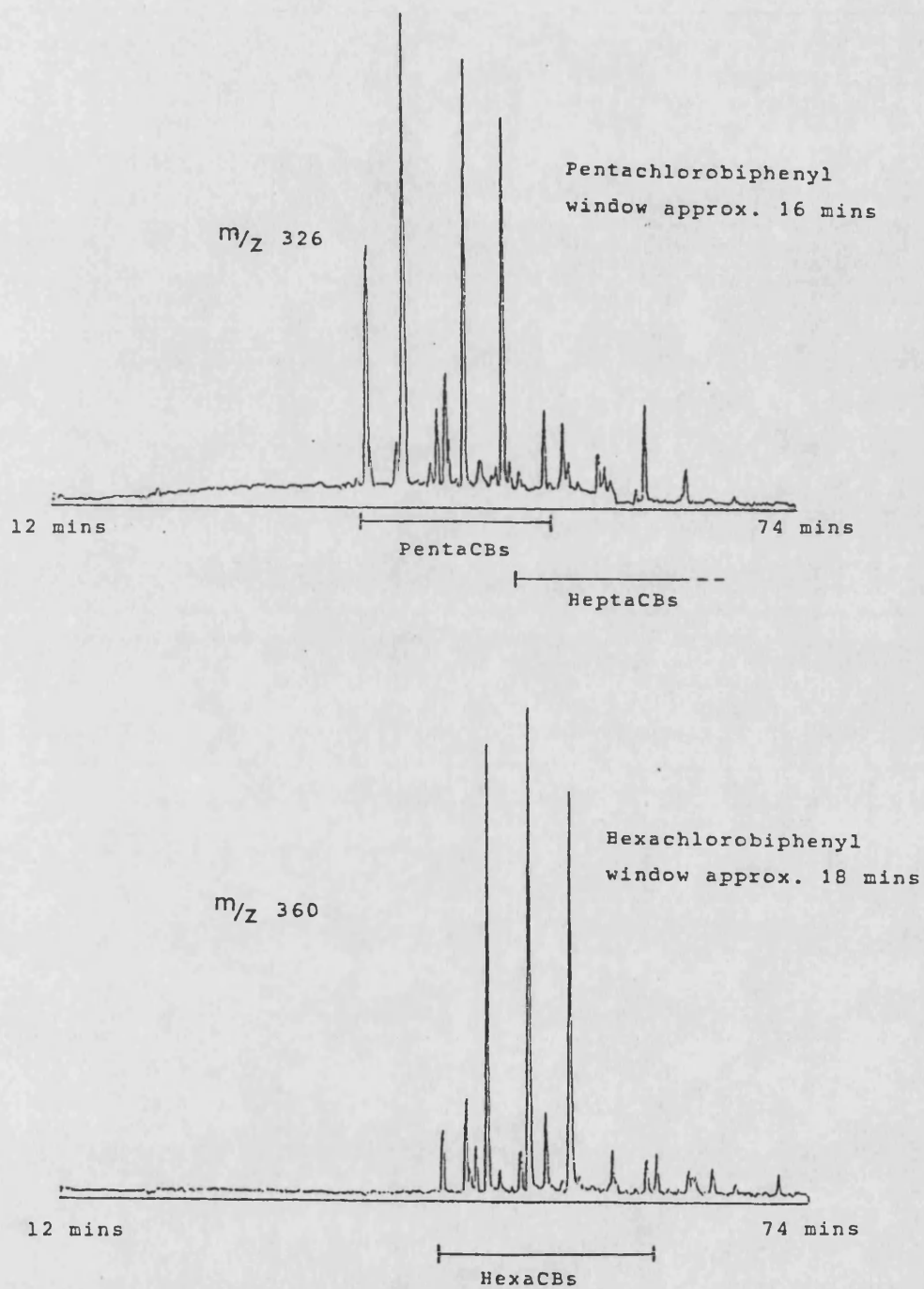
The ions monitored under SIM were the base peak ions in the molecular ion clusters for Cl₁ through to Cl₉ PCBs. With this system, a 1:1:1:1 mix of Aroclors 1242, 1248, 1254 and 1260 in heptane (approximately 100 ng ml⁻¹ of each) was injected. The elution windows for each isomer group was estimated and are listed in Table 9.

Table 9. Elution windows for PCB isomer groups.

See text for experimental details.

<u>Isomer group.</u>	<u>Elution window (mins after injection).</u>
Cl ₁	0 to 26
Cl ₂	0 to 30
Cl ₃	18 to 32
Cl ₄	27 to 43
Cl ₅	37 to 59
Cl ₆	43 to 62
Cl ₇	49 to 70
Cl ₈	63 to 80
Cl ₉	63 to 80

The estimates for octa- and nonachlorobiphenyls are not precise since only a few of these congeners were detected. The windows for penta- and hexachlorobiphenyls can be seen in Figure 30. The major pentachlorobiphenyl peaks in the Aroclor mixture were found to elute over a window about 16 minutes wide and the major hexachlorobiphenyls were found to elute over a window about 18 minutes wide. There are 46 pentachlorobiphenyls and 42 hexachlorobiphenyls (pentachlorobiphenyls comprise the largest isomer group) and the elution windows were considered sufficiently wide to permit adequate resolution of the majority of congeners within each



GC-MS elution windows for penta- and hexachlorobiphenyls

Figure 30.

isomer group. There was considerable overlap of elution windows for groups differing by one chlorine, but overlap of windows for groups differing by two chlorines (the most important source of inter-group interference) appeared to be relatively minor. Overall, it was considered that the DB-210 would probably separate the majority of spectroscopically interfering PCB congeners and separate either chromatographically or permit spectroscopic separation of all the congeners encountered in human milk samples. It was appreciated that the only way to test whether there is interference between PCBs is to inject a mixture of all 209 congeners. Unfortunately, this was not possible and it was considered unlikely that a 15 m DB-210 would, in practice, be sufficiently efficient or selective to separate out all possible interferences; for the purposes of this project, it was considered enough to separate the target analyte PCB congeners and fortunately, no interference with these was ever encountered. A longer DB-210 (say, 30 m) would give a much better chance of separating all interfering congeners when using GC-MS, especially if hydrogen carrier gas was used.

All target OCPs were separated chromatographically from each other using the 15 m DB-210 and spectrometrically from the PCBs using SIM, so that no interference problems were encountered in analysing OCPs.

Some of the milk samples (see Section 7.2.) were analysed using the OV-17 column with GC-MS and hydrogen carrier gas. All target OCP and specific PCB congener analytes were separated either chromatographically or spectrometrically from potential interferences and it was felt that OV-17 was an acceptable, though inferior, alternative to DB-210 in terms of selectivity.

5.2.2.7. Routine GC operating conditions.

The GC operating conditions were devised largely by trial and error. Long splitless times (up to 2 minutes) were used in order to maximise the solute transfer to the column. The initial oven temperature used for ECD work with the 25 m OV-17 without retention gap was set at 100°C for n-heptane (b.pt. 98.5°C) solutions so no solvent effect was obtained. The maximum injection volume used for injection without retention gap was 2.5 µl with a 1 µl heptane "chaser". The program ramp(s) and final temperature depend on the analytes, but using the 25 m OV-17 without retention gap, the following conditions were routinely used in order to elute all target OCPs and PCBs:

Instrumentation: Perkin-Elmer 8320B capillary GC.
Column: 25 m OV-17, 0.25 mm i.d., 0.2 μ m film.
Carrier gas: Hydrogen at 10 p.s.i. (nominal).
Linear gas velocity at 100°C approx. 65 cm s⁻¹.
Detector: ECD at 350°C. Nitrogen make-up gas.
Injector temp.: 270°C.
Injection: Maximum of 2.5 μ l heptane solution with 1 μ l heptane "chaser". Splitless for 2 mins.
Oven programme: 100°C for 2 mins. 30°C min⁻¹ to 120°C. 1.3°C min⁻¹ to 240°C.
Total analysis time: Up to 90 minutes.

The GC-MS work was carried out using two different chromatographic systems. The first system made use of a 15 m DB-210 column with 2 m retention gap and helium carrier. The second system comprised a 25 m OV-17 column with 2 m retention gap and hydrogen carrier. Both systems made use of a heptane solvent effect. In order to elute all target OCPs and PCBs, the following conditions were used:

DB-210.

Instrumentation: Dani 3800 capillary GC interfaced to a VG 7070E mass spectrometer.

Column: 15 m DB-210, 0.25 mm i.d., 0.25 μm film with 2 m retention gap.

Carrier gas: Helium at 0.25 bar (nominal).
Linear gas velocity at 86°C approx. 70 cm s^{-1} .

Transfer line temp.: 180°C.

Injector temp.: 270°C.

Injection: Maximum of 10 μl heptane solution with 1 μl "chaser" injected over 15 seconds. Splitless for 2 mins.

Oven programme: 86°C for 2.5 mins. 1.5°C min^{-1} to 200°C.

Total analysis time: Up to 70 minutes.

OV-17.

Instrumentation: Dani 3800 capillary GC interfaced to a VG 7070E mass spectrometer.

Column: 25 m OV-17, 0.25 mm i.d., 0.2 μm film with 2 m retention gap.

Carrier gas: Hydrogen at 0.42 bar (nominal).
Linear gas velocity at 96°C approx. 95 cm s^{-1} .

Transfer line temp.: 180°C.
Injector temp.: 270°C.
Injection: Maximum of 10 µl heptane solution
with 1 µl "chaser" injected over
15 seconds. Splitless for 100
secs.
Oven programme: 96°C for 100 secs. Ballistic rise
to 120°C. 1.5°C min⁻¹ to 230°C.
Total analysis time: Up to 70 minutes.

It will be noted that the linear gas velocities required for efficient GC-MS work, where the column outlet pressure is essentially vacuum, are much higher than those encountered in atmospheric pressure GC; this is because the density of the carrier is much lower under GC-MS conditions.

5.2.2.8. Mass spectrometry operating conditions for GC-MS.

The mass spectrometer used throughout was a VG 7070E double focussing, extended geometry instrument with a PDP 8A based operating system.

Ionisation mode.

Electron impact (EI) was chosen as the ionisation mode on account of its simplicity and reproducibility.

Furthermore, most OCPs and PCBs give a strong base peak under EI conditions. A softer chemical ionisation method would probably have provided increased sensitivity, particularly for the dimethanonaphthalenes (the "drins"), but CI conditions are more complex to operate and are less reproducible and wide differences in response factors between similar compounds are often observed.

Scanning mode.

Full scan modes offer increased scope for identification but are very much less sensitive than selected ion monitoring (SIM) modes. For ion trap detectors, however, the difference in sensitivity is less marked and full scan modes are very sensitive. Nine ion masses were selected for all the residue analyses on account of the specificity of these masses for the target analytes and the strength of the ions produced at the selected m/z values. The analytes with their corresponding m/z values are listed in Table 10.

Table 10. Selected ion masses for GC-MS SIM determination OCPs and PCBs.

<u>Analyte.</u>	<u>m/z.</u>
HCHs	183
DDTs	235
DDEs	246
"Drins"	263
HCB	284
4,4'-dibromobiphenyl (internal std.)	312
Pentachlorobiphenyls	326
Hexachlorobiphenyls	360
Heptachlorobiphenyls	394

During the chromatographic run, the mass spectrometer repeatedly scanned through all nine masses in sequence with a complete mass cycle taking approximately 1.1 seconds. A cycle time of 1.1 seconds permitted adequate sampling of chromatographic peaks to permit faithful recording of the peak shape and size. Data were stored for each of the mass channels over the entire run. High mass, even value m/z values were preferred since generally less interfering ions are produced at such values.

Mass calibration and tuning.

The mass spectrometer was mass calibrated using perfluorokerosene at a resolution of 1000 (10% valley method). Prior to a SIM run, the spectrometer was de-tuned to a mass resolution of about 500 by opening the collector slit after the analyser tube. This resulted in increased sensitivity due to an increase in ion flux, and gave rise to a "flat top" tuning effect which considerably reduces changes in analyte response factors should any drift in the mass calibration arise. Technical details of mass spectrometer operation are summarised in Table 11.

Table 11. Mass spectrometer operating conditions for SIM.

Electron multiplier potential: 2.0 to 2.2 KV.

Mass calibration: Using PFK at 1000 resolution.

Mass resolution and tuning: 500 (10% valley), "flat top".

Amplifier gain: Automatic gain control.

Voltage controlled mass scanning.

<u>Mass</u> <u>monitored.</u>	<u>Dwell time(ms).</u>	<u>Inter-channel</u> <u>delay.</u>
182.9347	100	30
235.0080	100	20
246.0001	100	20
262.8568	100	20
283.8100	100	20
311.8970	100	20
325.8801	100	20
359.8413	100	20
393.8024	100	30

The inter-channel delays for the initial and final masses in the scanning cycle were extended to allow the mass spectrometer time to settle at the beginning and end of each 1.1 second scanning sequence.

GC-MS quantitation.

The target analytes in the milk samples were: hexachlorobenzene, alpha-HCH, beta-HCH, gamma-HCH, delta-HCH, o,p'-DDE, p,p'-DDE, o,p'-DDT, p,p'-DDT, dieldrin, endrin, PCB 118, PCB 156, PCB 157, PCB 169, PCB 189. The five selected PCB congeners were chosen because of their expected toxicity and/or their reported detection in human milk.

In order to test the linearity of response of the mass spectrometer, nine solutions containing each analyte plus 56.54 ng ml⁻¹ of 4,4'-dibromobiphenyl as internal standard were analysed. The nine solutions covered three orders of magnitude of concentration for each analyte. The nominal concentrations for each analyte were 1, 3, 7, 10, 30, 70, 100, 300, 700 ng ml⁻¹. An aliquot of 10µl of each solution was analysed using GC-MS SIM with the 15 m DB-210 column and a 2 m retention gap. Chromatographic conditions were as described for this column in Section 5.2.2.7. and mass spectrometric conditions were as described in this Section except that "flat top" tuning was not used and resolution was maintained at 1000. The peak area ratios, peak height ratios and accurate concentrations for each analyte are shown in Table 12.

Plotting peak area ratios against concentration and performing a least squares linear regression for each of the sixteen analytes gave the equations and correlation coefficients shown in Table 13.

The results show that the calibrations are linear. The analyte deviating most from linearity is HCB with a peak area correlation coefficient of 0.97707 and a peak height correlation coefficient of 0.96587. This deviation is due almost entirely to curvature at the high concentration end of the calibration. This can be seen for the area regression line in Figure 31. Ignoring the 700 ng ml⁻¹

Table 12. Peak height ratios, peak area ratios (analyte/int.std.) and concentrations for nine point calibration for target analytes.
All concentrations in ng ml⁻¹.

Analyte.

HCB									
Concn.	0.099	2.96	6.92	9.88	29.65	69.18	98.83	296.5	691.8
Area	0.0356	0.0878	0.2212	0.2927	0.8555	2.0143	3.0572	9.3725	14.0471
Height	0.0317	0.1039	0.2067	0.4492	1.1309	2.2761	3.0924	13.7730	18.8461
α -HCH									
Concn.	1.00	2.99	6.97	9.96	29.88	69.72	99.60	298.8	697.2
Area	0.0102	0.0322	0.0979	0.1143	0.3351	0.8474	1.1228	4.3742	10.9233
Height	0.0123	0.0337	0.1142	0.1199	0.4017	0.8951	1.2995	5.1687	10.6486
β -HCH									
Concn.	1.00	3.01	7.02	10.03	30.08	70.18	100.26	300.8	701.8
Area	0.0082	0.0208	0.0680	0.0785	0.2314	0.5752	0.8886	3.0998	7.5177
Height	0.0088	0.0189	0.0628	0.1115	0.3425	0.4296	0.7414	4.1547	10.4120
γ -HCH									
Concn.	1.00	3.00	7.01	10.02	30.05	70.12	100.18	300.5	701.2
Area	0.0133	0.0311	0.0944	0.0982	0.2771	0.7026	1.0207	3.6809	8.7948
Height	0.0155	0.0310	0.1016	0.1283	0.3747	0.7315	1.1523	4.9781	9.3701
δ -HCH									
Concn.	1.05	3.15	7.36	10.52	31.55	73.62	105.18	315.5	736.2
Area	0.0082	0.0186	0.0595	0.0687	0.2014	0.5123	0.7956	2.8436	7.0121
Height	0.0081	0.0189	0.0655	0.0768	0.2312	0.5298	0.8763	3.8860	8.1791
op'DDE									
Concn.	0.84	2.53	5.90	8.43	25.29	59.01	84.30	252.9	590.1
Area	0.0348	0.0913	0.2419	0.2950	0.8624	2.0237	2.4416	9.6013	16.4173
Height	0.0364	0.0654	0.2275	0.4145	1.2721	1.6035	2.4467	13.4537	17.9908
pp'DDE									
Concn.	0.96	2.89	6.75	9.64	28.92	67.49	96.41	289.2	674.9
Area	0.0271	0.0705	0.1947	0.2338	0.6918	1.5810	1.9875	7.7254	15.7162
Height	0.0229	0.0721	0.1654	0.2188	0.8833	1.4960	1.7534	8.3856	19.9970
op'DDT									
Concn.	1.12	3.35	7.82	11.17	33.50	78.17	111.68	335.0	781.7
Area	0.0288	0.0632	0.1595	0.1934	0.5552	1.4278	1.8250	8.1004	15.5443
Height	0.0232	0.0607	0.1281	0.2160	0.7558	1.0299	1.4113	10.7545	18.9200
pp'DDT									
Concn.	0.87	2.62	6.10	8.72	26.16	61.04	87.20	261.6	610.40
Area	0.0121	0.0357	0.0721	0.1166	0.3083	0.8645	1.0462	4.8878	12.6687
Height	0.0149	0.0296	0.0682	0.1588	0.4327	0.7535	1.0665	6.6245	14.4360

Table 12 (contd.)

Dieldrin									
Concn.	1.11	3.34	7.79	11.14	33.41	77.95	111.35	334.1	779.5
Area	0.0047	0.0114	0.0332	0.0374	0.1262	0.2763	0.3479	1.2682	3.0678
Height	0.0054	0.0107	0.0286	0.0427	0.1590	0.2373	0.2733	1.5494	4.1805
Endrin									
Concn.	1.00	3.01	7.03	10.04	30.11	70.26	100.38	301.1	702.6
Area	0.0013	0.0050	0.0140	0.0150	0.0533	0.1182	0.1526	0.5604	1.4492
Height	0.0027	0.0050	0.0109	0.0168	0.0592	0.1190	0.1563	0.6004	1.5075
PCB 118									
Concn.	0.87	2.61	6.09	8.69	26.08	60.86	86.94	260.8	608.6
Area	0.1323	0.1721	0.3023	0.2366	0.5866	1.1685	1.5939	4.6420	10.2335
Height	0.1469	0.1158	0.3049	0.3067	0.8116	0.9785	1.5052	5.9056	9.8791
PCB 156									
Concn.	0.95	2.84	6.64	9.48	28.44	66.36	94.80	284.4	663.6
Area	0.0099	0.0273	0.0542	0.0831	0.2556	0.5682	0.9527	2.6829	6.1594
Height	0.0108	0.0259	0.0504	0.0905	0.3233	0.3782	0.6998	3.2481	7.8142
PCB 157									
Concn.	1.03	3.08	7.18	10.25	30.76	71.78	102.54	307.6	717.8
Area	0.0352	0.0662	0.1143	0.1184	0.3122	0.6733	1.0769	2.9253	6.4841
Height	0.0364	0.0595	0.1024	0.1315	0.4010	0.8304	0.8169	3.6829	7.7690
PCB 169									
Concn.	0.80	2.40	5.60	8.01	24.02	56.04	80.05	240.2	560.4
Area	0.0335	0.0528	0.0950	0.0820	0.1878	0.4501	0.6458	1.7433	3.6282
Height	0.0222	0.0354	0.0798	0.0884	0.2023	0.3846	0.6001	1.7919	3.7064
PCB 189									
Concn.	1.12	3.36	7.85	11.21	33.65	78.51	112.15	336.5	785.1
Area	0.0116	0.0238	0.0525	0.0618	0.1796	0.4592	0.7150	1.8976	3.6607
Height	0.0115	0.0212	0.0437	0.0652	0.2211	0.2932	0.5815	2.2758	4.5625

Table 13.

Regression of peak area ratios and peak height ratios on concentration for organochlorine analytes using GC-MS SIM. $y = \text{analyte/int.std. ratio}$, $x = \text{concentration (ng ml}^{-1}\text{)}$.

<u>Analyte.</u>	<u>Regression line.</u>	<u>Correlation Coefficient.</u>
HCB		
Area	$y=2.1290 \times 10^{-2}x + 0.47698$	0.97707
Height	$y=2.9202 \times 10^{-2}x + 0.51915$	0.96587
α -HCH		
Area	$y=1.5678 \times 10^{-2}x - 0.13436$	0.99902
Height	$y=1.5575 \times 10^{-2}x - 0.02749$	0.99804
β -HCH		
Area	$y=1.0745 \times 10^{-2}x - 0.07392$	0.99954
Height	$y=1.4932 \times 10^{-2}x - 0.22192$	0.99668
γ -HCH		
Area	$y=1.2588 \times 10^{-2}x - 0.07548$	0.99956
Height	$y=1.3785 \times 10^{-2}x + 0.00261$	0.99459
δ -HCH		
Area	$y=9.5485 \times 10^{-3}x - 0.08240$	0.99932
Height	$y=1.1362 \times 10^{-2}x - 0.07990$	0.99771
<i>o,p'</i> -DDE		
Area	$y=2.8798 \times 10^{-2}x + 0.26305$	0.98987
Height	$y=3.2920 \times 10^{-2}x + 0.40290$	0.96115
<i>p,p'</i> -DDE		
Area	$y=2.3678 \times 10^{-2}x + 0.03946$	0.99797
Height	$y=2.9762 \times 10^{-2}x - 0.22667$	0.99844
<i>o,p'</i> -DDT		
Area	$y=2.0454 \times 10^{-2}x + 0.00094$	0.99554
Height	$y=2.5372 \times 10^{-2}x - 0.14389$	0.98766
<i>p,p'</i> -DDT		
Area	$y=2.0776 \times 10^{-2}x - 0.23422$	0.99782
Height	$y=2.4165 \times 10^{-2}x - 0.23820$	0.99653
Dieldrin		
Area	$y=3.9331 \times 10^{-3}x - 0.01940$	0.99953
Height	$y=5.3434 \times 10^{-3}x - 0.08650$	0.99592
Endrin		
Area	$y=2.0525 \times 10^{-3}x - 0.01629$	0.99874
Height	$y=2.1422 \times 10^{-3}x - 0.01639$	0.99897

Table 13 (contd.)

PCB 118		
Area	$y=1.6661 \times 10^{-2}x + 0.15353$	0.99982
Height	$y=1.6650 \times 10^{-2}x + 0.25334$	0.98819
PCB 156		
Area	$y=9.3059 \times 10^{-3}x + 0.00241$	0.99986
Height	$y=1.1857 \times 10^{-2}x - 0.12038$	0.99783
PCB 157		
Area	$y=9.0226 \times 10^{-3}x + 0.05660$	0.99968
Height	$y=1.0226 \times 10^{-2}x + 0.01674$	0.99809
PCB 169		
Area	$y=6.4660 \times 10^{-3}x + 0.06643$	0.99878
Height	$y=6.6445 \times 10^{-3}x + 0.04622$	0.99878
PCB 189		
Area	$y=4.7320 \times 10^{-3}x + 0.06461$	0.99586
Height	$y=5.9409 \times 10^{-3}x - 0.00667$	0.99677

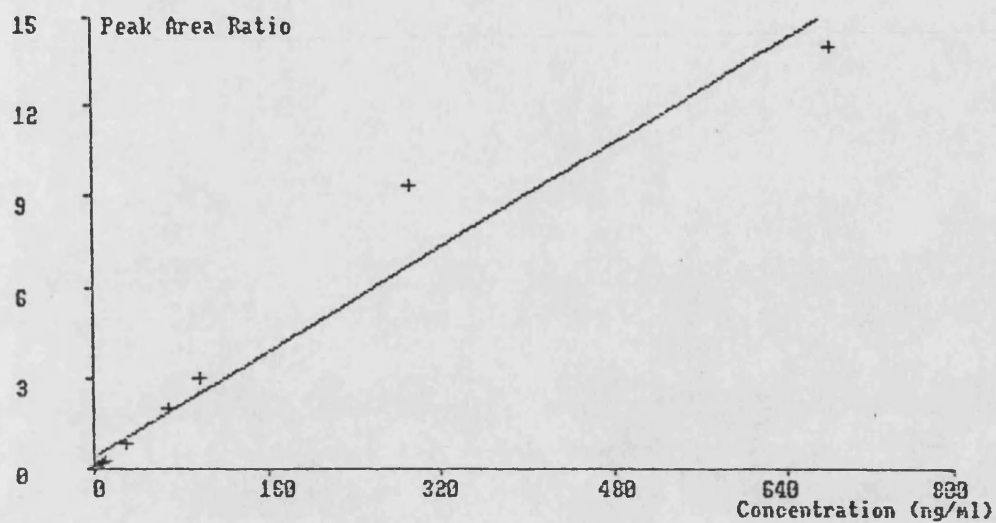


Figure 31. Regression line for GC-MS calibration with hexachlorobenzene including the 700 ng/ml standard.

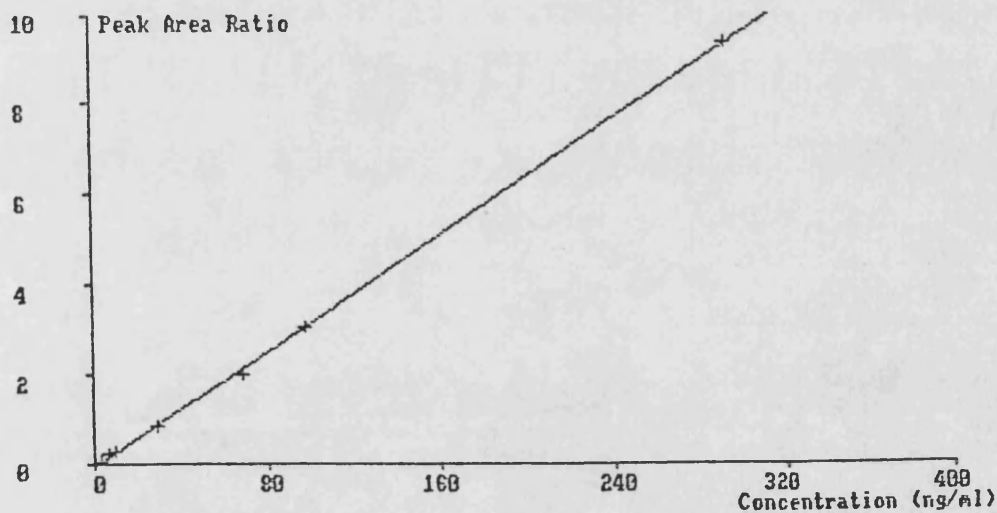


Figure 32. Regression line for GC-MS calibration with hexachlorobenzene excluding the 700 ng/ml standard.

point for HCB and re-calculating the regression line with the remaining eight points gives a much better linear fit and an area regression line of:

$$y=3.16206 \times 10^{-2}x - 0.043118 \quad r=0.99981$$

A plot of this new area regression line can be seen in Figure 32.

There was some evidence of this slight "top end" curvature for other analytes and this had reduced the corresponding correlation coefficients. These deviations from linearity were not considered to be a major drawback since the deviations were small and furthermore, it was not expected that such high analyte concentrations would be encountered in milk extracts.

An additional factor in producing scatter in the calibration lines was the lack of flat-top tuning and the subsequent discovery that there was a slight leak of the accelerating voltage high tension from the mass spectrometer resulting in drift in the mass calibration. Despite these problems, it was considered that the results provided sufficient evidence of linearity of response with concentration over at least two orders of magnitude covering the expected range of analyte concentrations.

Some analytes tended to display significant non-zero intercepts which still persisted when only the lower concentration point (1 to 10 ng ml⁻¹) were regressed. Consequently, it was decided that milk analyses should be carried out using a two point bracketing calibration technique. All analyses were subsequently carried out using the 70 ng ml⁻¹ standard and the 7 ng ml⁻¹ standard. The 7 ng ml⁻¹ standard served to anchor the lower end of the calibration. Occasionally, analyte concentrations in the samples fell outside this range and quantitation had to be carried out by extrapolation of the calibration; this was particularly true for p,p'-DDE which routinely gave analyte concentrations well above 70 ng ml⁻¹ (taking into account the approximately 10-fold concentration step in the sample work-up). Ideally, many more than two standards would be analysed with the samples, but limited access to the GC-MS system prevented this.

The regression lines for peak height ratio against concentration generally gave similar results to the peak area data. Peak height ratio precision (as evidenced by the correlation coefficients) tended to be poorer than those for the area data as expected, although there were a few exceptions to this.

Limits of quantitation were not rigorously determined but were estimated to be around 0.1 ng ml⁻¹ or better in whole milk (i.e. about 1 ng ml⁻¹ in the final extract)

for all analytes assuming a 10 μ l injection volume. Dieldrin and endrin, however would be quite difficult to quantify at this level due to the weakness of the $m/z=263$ ion used for their detection.

The problem of quantifying "total PCBs" was difficult to address. An effective method is to quantify each isomer group with a surrogate standard for each level of chlorination (see Section 5.1.2.5.) but the SIM control software provided with the mass spectrometer system only allowed ten ions to be monitored simultaneously under SIM, and nine of these (including three PCB ion masses) were already in use. Furthermore, suitable surrogate standards were not readily available. It was, therefore, decided to quantify "total PCBs" using a modification of the surrogate standard technique. It was known that the majority of PCB congeners in human milk tended to be quite highly chlorinated and Aroclor 1260 is commonly used as a "pattern matching" standard for quantitation of PCBs in milk. The ions already monitored in the SIM method were characteristic of pentachloro-, hexachloro- and heptachlorobiphenyls and these three isomer groups account for about 92% by mass (153) of the total PCBs in Aroclor 1260. Consequently, it was decided to use only these three isomer groups for the "total PCB" quantitation. Hence quantitation was effected by analysing a standard solution of Aroclor 1260 (250 ng ml^{-1} with 56.54 ng ml^{-1} 4,4'-dibromobiphenyl as internal standard)

using the SIM method already described. The total areas for penta-, hexa- and heptachlorobiphenyl peaks were measured and all three totals were summed to give a grand total area. This grand total area was divided by the internal standard area to give a grand total area ratio for the standard. A grand total area ratio was calculated in the same manner for the samples and "total PCB" quantitation was effected by direct comparison of the sample ratio to the standard ratio assuming a zero intercept and perfect linearity in the Aroclor calibration. Thus "total PCB" concentration was expressed in ng ml^{-1} Aroclor 1260.

This approach to PCB quantitation does have drawbacks: Firstly, it ignores many isomer groups, although other isomer groups probably do not contribute very much to the total PCB burden in human milk. Secondly, it assumes zero intercept and perfect linearity in the calibration; this is probably not too unrealistic but the use of only one standard during a run of samples is bound to introduce some error in quantitation. Thirdly, the "total PCB" figure produced is of little toxicological value and is not directly comparable with figures produced by other methods. Overall, the method is probably best regarded as a semi-quantitative method for the "total PCB" analysis of human milk. It should be borne in mind, however, that the target PCB congeners (PCBs 118, 156, 157, 169 and 189) were all quantified as individual analytes

independent of the "total PCB" determination. Of course, the ultimate "total PCB" analysis is a congener-specific determination of all 209 congeners.

5.2.2.9. The selectivity of GC-MS compared to GC-ECD.

In order to confirm the need for the selectivity offered by GC-MS SIM, a milk extract was prepared using the Soxhlet extraction and HPLC clean-up already described. The extract was analysed using GC-ECD with a 25m OV-17 column under the conditions described in Section 5.2.2.7. and also by GC-MS SIM with a 15 m DB-210 column as described in Section 5.2.2.7.. The ECD chromatogram is shown in Figure 33. It can be seen that although some analytes could be quantified from this, on the whole there is too much interference and the extract would have to be subjected to further clean-up and fractionation before GC-ECD determination. Figure 34 shows the selected ion chromatograms produced by the same milk sample analysed by GC-MS SIM monitoring ions of $m/z = 326$, 284 and 183 which are characteristic of pentachlorobiphenyls, HCB and HCHs respectively. These chromatograms are normalised to the largest peak in each. It can be seen that the GC-MS SIM method provides much enhanced selectivity and quantitation of all target analytes can be carried out without further clean-up or fractionation. The $m/z = 326$ mass fragmentogram shows the presence of some small interfering hexachlorobiphenyl fragment ions.

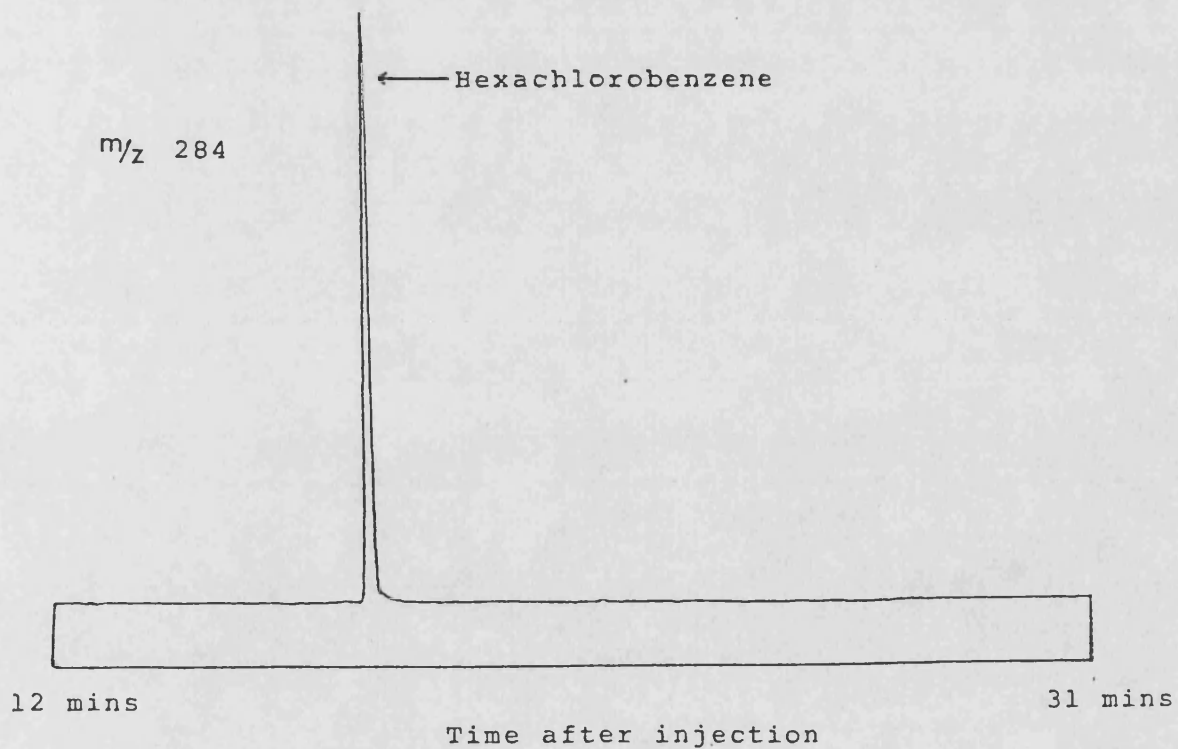
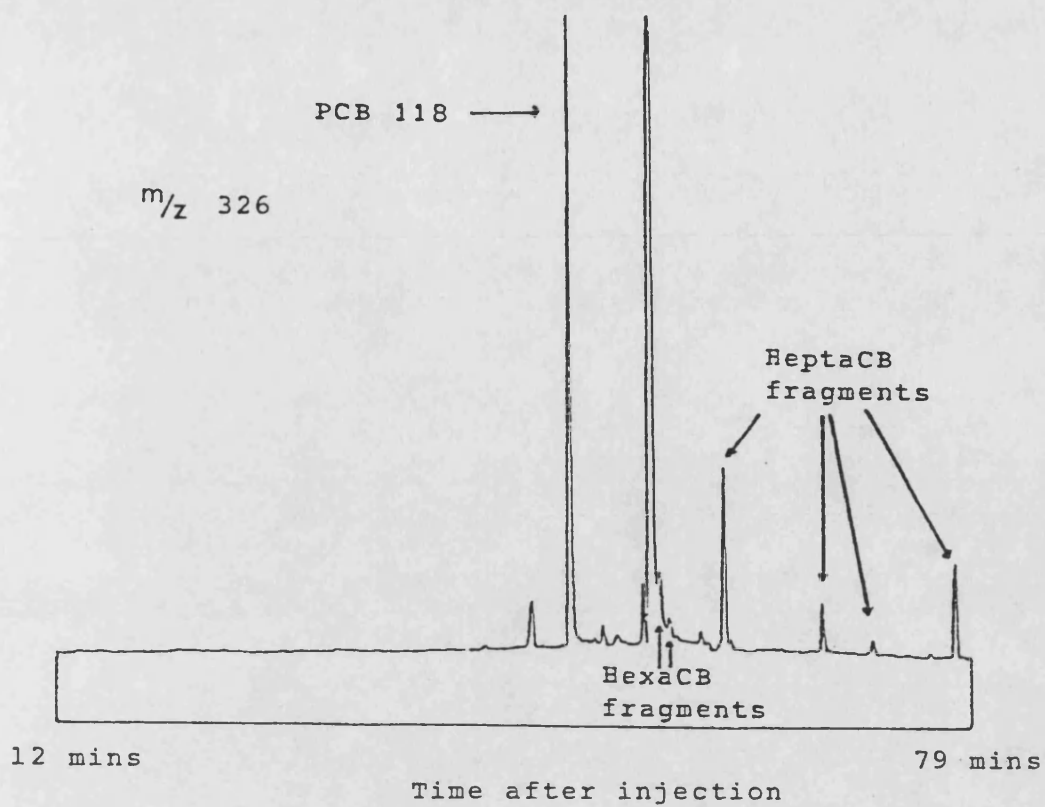


Figure 34. Human milk sample analysed by GC-MS SIM

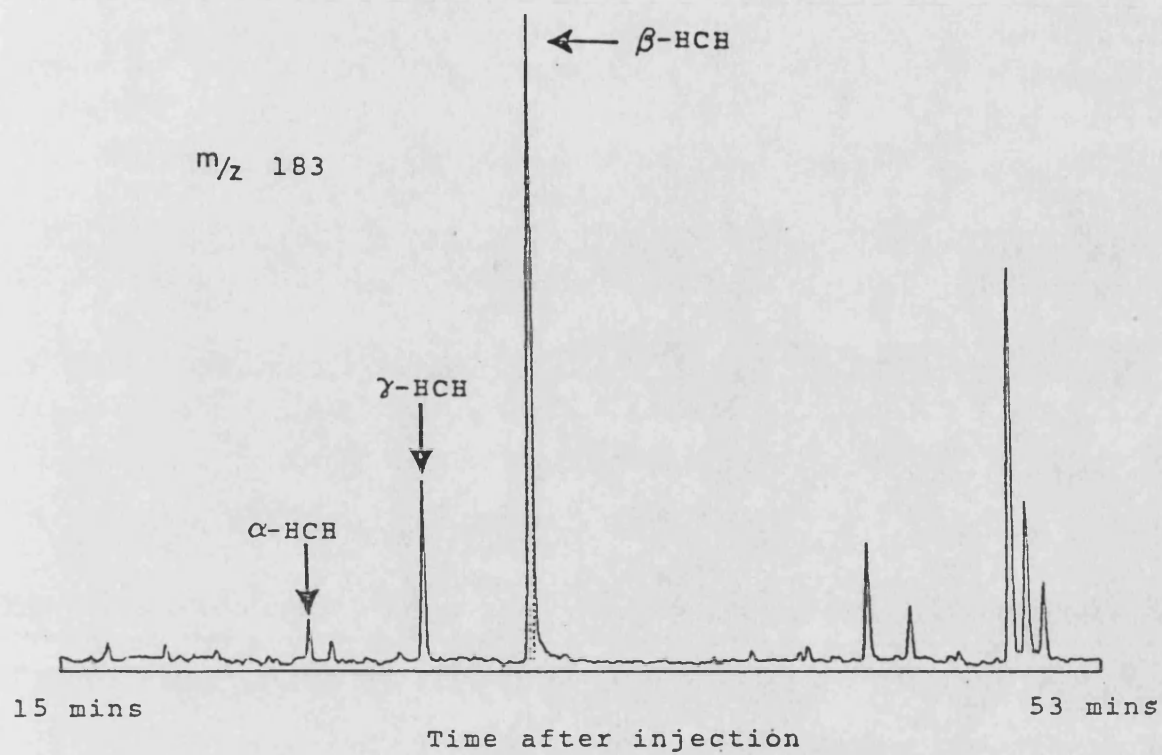


Figure 34 (contd.)

These fragment ions appear in the chromatogram because the hexachlorobiphenyls from which they are derived are present at much higher concentrations than the pentachlorobiphenyls and so the $[M-Cl]^{+}$ ions produced at $m/z = 326$ show up, but this interference is of little practical significance. The expected heptachlorobiphenyl ions at $m/z = 326$ are also visible.

In the mass fragmentogram for $m/z = 183$, there are some unidentified materials which elute after the HCHs. This was not entirely unexpected when monitoring a low, odd mass ion and since the HCHs are resolved chromatographically from these unknowns, the presence of these unidentified materials in the extracts is of no consequence.

6. ANALYTICAL PROCEDURES AND RECOVERY STUDIES.

6.1. Introduction.

In this section, a formal analytical procedure for the analysis of organochlorine residues in human milk is presented. This procedure was then employed in the analysis of five organohalogen/lipid samples in order to assess the performance of the method. Milk spikes were not used because the final extracts were to be analysed by both GC-ECD and GC-MS and milk extracts prepared using the method described are not directly amenable to analysis by GC-ECD on account of interferences in the extract. Since the addition of a further clean-up step was not desirable, the extractions were performed on adsorbents spiked with the target analytes together with a known amount of triglyceride and distilled water to mimic the lipid and aqueous content of milk. From these extracts, recovery data for lipid and organohalogen residues were obtained using both ECD and MS detection. All solvents were of HPLC grade.

6.2. The Analysis of Human Milk Samples for Residues of Organochlorine Pesticides and Polychlorinated Biphenyls.

6.2.1. Analytical procedure.

6.2.1.1. Preparation of adsorbent.

The adsorbent substrate for the extraction was prepared by mixing 60-100 mesh (ASTM) Florisil with fibrous cellulose (chromatography grade) in a 1:1 ratio by volume. Mixing was carried out in a glass mortar. The adsorbent was Soxhlet extracted overnight with heptane and dried in a vacuum oven at 80°C until a free-flowing powder was obtained. This was then stored in an airtight brown glass jar.

Note: The cellulose used was manufactured from a plant source (as determined by microscopical examination) and was approximately 25 years old. As a consequence, prior to extraction the cellulose was found to contain considerable residues of lindane (gamma-HCH) and dieldrin. After extraction, residues of these materials were much reduced and blank determinations were used to correct for any remaining contamination. It is to be hoped that newer batches of fibrous cellulose would not present such a problem.

6.2.1.2. Collection and storage of human milk samples.

Samples were collected from women attending post-natal clinics in the Bath area. Milk was expressed into sterilised glass jars with airtight caps. Samples were deep frozen at the earliest possible opportunity.

Donors were also asked to complete a brief questionnaire giving a short history of where they have lived since birth, types of occupation undertaken and any exposure to pesticides together with other details. A copy of the questionnaire is given in appendix 4.

6.2.1.3. Extraction of milk.

For each milk run, six extracts were prepared; two different milk samples, both in duplicate plus a blank adsorbent and a spiked adsorbent.

1) During storage, milk samples were deep frozen in sterilised, airtight glass jars. Before analysis, the samples were thawed after which they were shaken thoroughly to ensure sample homogeneity.

2) A clean, dry glass pestle and mortar was set out for each sample and into each was placed 2/3 of a 30 x 80 mm Soxhlet thimbleful of the adsorbent (q.v.).

3) An aliquot of 10.0 ml for each milk sample was pipetted, using a bulb pipette, into a clean, dry, tared 10 ml beaker and the weight of milk was recorded. From this, the density of the milk was calculated.

4) The weighed milk samples were poured from their beakers onto the adsorbent in the mortars and the beakers were re-weighed to determine the amount of milk on the adsorbent.

5) After adding milk to the adsorbent, the contents of the mortar were mixed thoroughly to ensure an even distribution of milk throughout the substrate.

6) Another glass mortar was prepared and adsorbent was added as for the milk samples. To this adsorbent was added 10 ml of glass-distilled water followed by 2.0 ml (bulb pipette) of a 150 mg ml⁻¹ solution of triglycerides (Witepsol H15) in heptane and 1.0 ml (bulb pipette) of an organohalogen analyte spiking solution in heptane. This spiking solution contained approximately 50 ng ml⁻¹ of each of the target analytes (the amount of each solute in the spiking solution was known accurately). This sample served as the recovery standard extract.

7) After mixing, the milk samples and spike were placed in a fan oven at 60°C in order to drive off the majority of the water. The samples were left in the oven for about

30 minutes and were re-mixed occasionally to promote drying. Drying was not usually complete.

8) After drying, the adsorbent samples were transferred quantitatively to 30 x 80 mm cellulose Soxhlet thimbles with 3 x 2 ml hexane washings. Care was taken to ensure that as much of the adsorbent as possible was transferred. The washings from the mortars were poured directly into the thimbles.

9) A blank sample of adsorbent was placed in a Soxhlet thimble and was thereafter treated as a milk sample.

10) The samples were Soxhlet extracted overnight with about 120 ml of hexane at a cycling rate of about 30 to 40 cycles h^{-1} . Small glass spacers were placed beneath the thimbles to promote efficient draining. During extraction, the milk samples tended to turn straw-brown. Much of this colour was retained by the adsorbent but some colouration always passed into the extract. Residual water tended to creep into the extractor siphon, but no water was ever seen in the extract at this stage.

11) The extracts were filtered through a hexane-washed Whatman No.1 filter into 250 ml Kuderna-Danish evaporators with 10 ml graduated receiver tubes. Extraction flasks were washed with 3 x 5 ml of hexane and these washings were added to the extracts through the

filter. The filter was then washed with about 5 ml hexane and this was combined with the extract.

12) Two-ball Snyder columns (180 mm) were placed on top of the evaporators and the hexane was evaporated off using a steam chest at about 80°C.

The first few samples processed were evaporated to about 1.3 ml, but it was found that the lipid concentration in some milk extracts became too high for the HPLC clean-up when such a low volume extract was prepared. Consequently, later milk samples were evaporated to about 3 ml and were cleaned up in two parts on the HPLC.

A further problem encountered during the concentration of the samples was spattering of the extract within the evaporator, particularly when the extract volume was low and contained entirely within the receiver tube. High lipid content also seemed to be a factor in promoting spattering. This was a serious problem since sample losses could be high despite washing down the evaporator walls. Putting a single anti-bumping granule in the receiver tube seemed to reduce spattering but did not prevent it. The only satisfactory solution was to remove the receiver tube once the extract was contained entirely within the latter and then reduce the extract to the desired volume by evaporation over clean, dry air.

Occasionally, very small amounts of water were found in the receiver tube. When this occurred, the water was left in the tube but was not injected with the extract during HPLC clean-up.

13) The low volume extracts were cleaned up using the HP-GPC system described in Section 4.2.2.. With 20:80% v/v propan-2-ol:n-heptane as the mobile phase, samples were latterly cleaned up over two injections to accommodate any samples of high lipid content. The first injection would be of about 2.0 ml and the fractions would be collected as usual. The second injection would consist of the remainder of the extract (about 1 ml) which would be loaded into the injector loop. The receiver tube would then be washed with 0.4 ml of mobile phase and this would be drawn into the injection syringe leaving any water behind, swirled within the barrel of the syringe, the majority of the air expelled (being careful not to lose any of the washing) and then added to the sample in the loop. A further 0.4 ml washing would then be carried out and added to the sample in the loop. Inevitably, some air was always injected but this did not seem to cause any problems. Fractions would then be collected as usual. The corresponding fractions from the first and second injections of a sample were combined.

14) To the organohalogen fraction was added 1.0 ml (bulb

pipette) of a solution of 50 ng ml⁻¹ (nominal) 4,4'-dibromobiphenyl in heptane as internal standard. The solution was then evaporated to about 1.0 ml over clean, dry air at about 40°C. The extract was then ready for GC-MS analysis as described in Sections 5.2.2.7. and 5.2.2.8.. Milk extracts were frequently yellow in colour at this stage but this did not seem to adversely affect the GC-MS determination.

Extracts always produced traces of a white precipitate on reducing the volume of the HPLC fraction to about 1.0 ml. This precipitate was found to derive from the HPLC grade solvents themselves. This slight precipitate did not seem to adversely affect the quantitation, but higher grade solvents would be a desirable, though costly, modification of the method.

Blockage of the HPLC system was frequently encountered. Samples were too viscous to be filtered through membrane filters before injection and pre-filtering would probably have given rise to sample losses anyway. An in-line filter was always placed immediately after the injector loop and this tended to become blocked after only a few injections. Back-flushing of the filter always cleared the obstruction but this was time consuming and caused wear of the couplings; a back-flushing valve system would have been a useful addition.

It was found that the timing of the switch from the lipid fraction to the organohalogen fraction was critical despite the use of the RI detector and chart recorder as an aid to fraction timing. During GC-MS analysis, band broadening or, rarely, splitting of the beta-HCH and the internal standard 4,4'-dibromobiphenyl peaks was observed with some milk samples. It was assumed that this was due to slight residues of lipid in the extract although it was considered strange that only these two components were affected. A longer HPLC column would give greater resolution but would also increase solvent consumption slightly.

It was realised that after HPLC clean-up the extracts were dissolved in 20:80% v/v propan-2-ol:n-heptane. After evaporation to about 1 ml the composition of the solvent would be quite different and would probably contain a much higher proportion of propan-2-ol. This could conceivably affect the quality of the GC injection with a GC method designed for the injection of heptane solutions, especially when a large injection volume is used. However, if the solvent was the source of the band broadening problem, it would be expected in all samples and this was not the case.

15) The lipid HPLC fractions were evaporated to dryness over clean, dry air at about 50°C. The lipids were then weighed and the lipid content of the milks and the lipid

spike recoveries were then calculated.

6.2.2. Recoveries of organochlorines and lipids from spiked adsorbent samples.

6.2.2.1. Experimental.

Five replicate adsorbents were spiked with lipid and organohalogens and processed as described in step 6. *et seq.* of Section 6.2.1.3.. The extracts were evaporated to approximately 1.3 ml in the Kuderna-Danish evaporators and no spattering was encountered. The samples were each cleaned up in one injection (with washings) using the HPLC system. Extracts were analysed using GC-ECD (see Section 5.2.2.7.) using peak heights, and GC-MS with the 15 m DB-210 column (see Sections 5.2.2.7. and 5.2.2.8.). The adsorbent was spiked with the following solutions:

- i) A solution containing 150.72 mg ml⁻¹ triglycerides (Witepsol H15) in heptane (2.0 ml by bulb pipette).

- ii) A solution containing the following organochlorines in n-heptane (1.0 ml by bulb pipette).:

<u>Solute.</u>	<u>Concentration (ng ml⁻¹).</u>
HCB	49.41
alpha-HCH	49.80
beta-HCH	50.13
gamma-HCH	50.09
delta-HCH	52.59
o,p'-DDE	42.15
p,p'-DDE	48.21
o,p'-DDT	55.84
p,p'-DDT	43.60
Dieldrin	55.68
Endrin	50.19
PCB 118	43.47
PCB 156	47.40
PCB 157	51.27
PCB 169	40.03
PCB 189	56.08

Results and discussion.

The recoveries and relative standard deviations are shown in Table 14. ECD peak measurements were carried out using peak heights measured manually in order to help reject interference due to peak overlap. The quantitation was made by direct comparison of the sample peak heights with those produced by a standard containing exactly the same levels of analytes as the original spiking solution plus

Table 14. Recoveries and relative standard deviations of lipids and organochlorines from spiked adsorbents.

Analyte	Mean Recovery (GC-ECD) (n=5)	Relative Std. Dev. (GC-ECD) (n=5)	Mean Recovery (GC-MS) (n=5)	Relative Std. Dev. (GC-MS) (n=5)
HCB	83.2% (n=4)	2.54% (n=4)	99.81%	13.86%
α -HCH	87.2%	4.30%	106.92%	6.83%
β -HCH	88.4%	5.56%	90.89%	9.39%
γ -HCH	90.9%	3.53%	106.51% (n=4)	4.99% (n=4)
δ -HCH	63.2%	9.38%	67.30%	8.41%
o,p'-DDE	91.5%	2.89%	86.69%	6.28%
p,p'-DDE	100.1% (n=3)	2.84% (n=3)	92.68% (n=4)	9.16% (n=4)
o,p'-DDT	94.6%	5.11%	91.66%	10.55%
p,p'-DDT	97.8%	6.01%	98.88%	10.12%
Dieldrin	87.8%	4.64%	94.09%	10.36%
Endrin	98.9%	5.49%	96.03%	8.51%
PCB 118	205.6% (n=4)	5.75% (n=4)	243.78% (n=5)	23.65% (n=5)
PCB 156	92.0%	3.20%	99.62%	8.31%
PCB 157	91.2%	2.03%	95.36%	8.74%
PCB 169	79.7%	7.56%	77.52%	11.79%
PCB 189	89.8%	2.94%	80.71%	13.38%

Lipid: Mean recovery (n=5) 94.2%
Relative Std. Dev. 2.22%

the internal standard. Linear calibration and zero intercept at the concentration level being considered had previously been established and were assumed for the purposes of the ECD recovery calculations.

GC-MS quantitation was carried out using 30 ng ml⁻¹ and 70 ng ml⁻¹ bracketing standards. Due to the constraints of GC-MS time, the standards were only analysed once during the GC-MS analysis of the samples and this probably contributed to the variability of the results.

The recoveries for all analytes except delta-HCH were acceptable. Delta-HCH recoveries were considered too poor for quantitative use and PCB 169 gave low but probably useable recoveries.

It can be seen that the ECD results tended to be less variable than the GC-MS results, probably due in part, to the problem of drift in the mass calibration which was being encountered at the time. Another factor contributing to the variability of the GC-MS results was undoubtedly the use of only single injections for each sample and standard.

The recovery for PCB 118 was extremely high. This was eventually traced to contamination of the adsorbent. The adsorbent was not homogeneously contaminated with the PCB 118 which gave rise to variable results for PCB 118 and

also made the investigation of the source of the high recoveries difficult since blank determinations were not always contaminated to the same degree. The reason for the contamination was never fully discovered but was probably a consequence of carrying out preliminary clean-up work on the PCB 118 after synthesis in the same laboratory as was subsequently used for the preparation of the adsorbent.

The reason for the low recovery of delta-HCH is not known but the compound may partially degrade during the extraction or sample concentration steps when the residues are exposed to heat.

It was appreciated that this recovery study did not constitute proper validation of the milk extraction method since the only ways to do this are to use properly spiked milk samples and also to compare the results obtained using this method to those obtained using an established method. It may be argued that milk samples cannot be spiked in such a way that the added organochlorines would be as fully incorporated into the milk matrix as the environmentally derived contaminants. Consequently, even milk spiking experiments may not reflect true recoveries.

Due to the time and materials involved, validation of the method by comparison to an established method was not

undertaken.

The extraction of milk samples served as a form of validation as well as a means of discovering methodological problems and the results of these extractions are discussed in Section 7..

7. THE ANALYSIS OF TWELVE HUMAN MILK SAMPLES.

7.1. Introduction.

The object of analysing the dozen human milk samples was not so much to generate data on the levels of contamination present but to gain some insight into the performance of the method as described in Section 6.2.1. and to go some way towards validating this approach to human milk analysis. Many aspects of the method are quite novel in the context of milk analysis and it was not expected that the results obtained from the first few runs would be reliable. Practical problems were anticipated and encountered; some of these problems have been described in previous sections. The problems, as they arose during each milk run, will be discussed below and suggestions for their correction will be proposed in Section 8.

The twelve milk samples were analysed in duplicate except for the last sample for which there was insufficient milk to permit a duplicate analysis. Runs were performed on two samples at a time in duplicate and the results for each run are given in Tables 15 to 20 together with spiked adsorbent recovery data. The milk results are NOT corrected for recoveries.

In view of the experimental nature of the analyses, it

was considered inappropriate and indeed misleading to attempt any form of statistical or toxicological analysis of the results, especially as only twelve samples were processed. Spike recoveries, agreement between duplicates and the degree of conformity with expected values (the latter being a rather subjective assessment but taken as being a broad agreement with published data, particularly reference (35)), were adopted as the main indicators of the quality of the results and the performance of the procedure.

The limits of quantitation were somewhat arbitrarily set at 0.5 ng ml^{-1} in whole milk for all the analytes and the limits of detection were set at 0.1 ng ml^{-1} in whole milk. With the modifications suggested in Section 8., the LOQs and LODs should be rather lower. These values for the LOQs and LODs were adopted for all analytes despite considerable variation in the sensitivity of the method for different compounds. Even as the method stands, the LOQ of 0.5 ng ml^{-1} in whole milk is quite conservative for some materials like HCB, to which the GC-MS system is very sensitive. In contrast, the dimethanonaphthalenes (the "drins") fragment heavily under the GC-MS conditions used and this LOQ was difficult to achieve.

The data in the tables are reported to 4 decimal places. This was done merely for completeness and to allow any subsequent calculations to be performed on the raw data.

The use of 4 places of decimals is in no way intended to imply such a high degree of precision or accuracy.

7.2. Individual Milk Analyses.

7.2.1. Run 1: Human milk samples 1 and 2.

The results for Run 1 are shown in Table 15.

The recoveries from the spiked adsorbent were very variable. HCB recovery was very low, probably due to volatilisation in the Kuderna-Danish evaporator. The blank results were high and this was indicative of contamination of the adsorbent. The remarkably high recoveries for the DDTs and PCB 118 were due to inhomogeneous contamination of the adsorbent which was not corrected by the blank. This inhomogeneous contamination probably accounts, to some extent, for the poor agreement between duplicates for some analytes, but another source of variation was the HPLC clean-up. The samples were each cleaned up over one injection and not two. The lipid content of the milk was moderately high in sample 1 and the lipid bands tended to encroach slightly on the residue bands in both the duplicates. This meant that the residue band was collected a little late and the beginning part of some early eluting residues, such as p,p'-DDE, may have been lost to the lipid fraction and resulted in low recoveries from the milk samples and poor

Table 15.

Results of the analysis of human milk samples 1 and 2 for OCP and PCB residues.

T=trace (= < 0.5 ng ml⁻¹ in whole milk.)ND=not detected (= < 0.1 ng ml⁻¹ in whole milk.)

NOTE: Although data are reported with up to four decimal places, this is not intended to imply such a high degree of accuracy or precision.

Analyte	Sample 1		Sample 2		Spike recovery
	A	B	A	B	
HCB whole milk (ng ml ⁻¹)	0.8574	0.4942	0.5768	0.7477	35.16%
HCB fat basis (µg g ⁻¹)	0.0279	0.0174	0.0242	0.0320	
α-HCH whole milk (ng ml ⁻¹)	T	T	T	T	46.68%
α-HCH fat basis (µg g ⁻¹)	T	T	T	T	
β-HCH whole milk (ng ml ⁻¹)	2.6832	2.9902	2.9228	3.1169	80.77%
β-HCH fat basis (µg g ⁻¹)	0.0874	0.1053	0.1226	0.1336	
γ-HCH whole milk (ng ml ⁻¹)	T	T	T	0.6474	70.80%
γ-HCH fat basis (µg g ⁻¹)	T	T	T	0.0277	
δ-HCH whole milk (ng ml ⁻¹)	ND	ND	ND	ND	46.96%
δ-HCH fat basis (µg g ⁻¹)	ND	ND	ND	ND	
o,p'-DDE whole milk (ng ml ⁻¹)	ND	ND	ND	ND	70.16%
o,p'-DDE fat basis (µg g ⁻¹)	ND	ND	ND	ND	
p,p'-DDE whole milk (ng ml ⁻¹)	8.8141	15.1464	19.3843	23.2669	77.97%
p,p'-DDE fat basis (µg g ⁻¹)	0.2869	0.5335	0.8128	0.9972	
o,p'-DDT whole milk (ng ml ⁻¹)	T	T	T	T	175.53%
o,p'-DDT fat basis (µg g ⁻¹)	T	T	T	T	
p,p'-DDT whole milk (ng ml ⁻¹)	7.2635	6.9952	2.5832	3.2936	209.26%
p,p'-DDT fat basis (µg g ⁻¹)	0.2364	0.2464	0.1083	0.1412	
Dieldrin whole milk (ng ml ⁻¹)	T	T	3.0472	6.0191	103.25%
Dieldrin fat basis (µg g ⁻¹)	T	T	0.1278	0.2580	
Endrin whole milk (ng ml ⁻¹)	ND	ND	ND	ND	0.00%
Endrin fat basis (µg g ⁻¹)	ND	ND	ND	ND	
PCB 118 whole milk (ng ml ⁻¹)	8.7283	3.5726	5.2038	7.2639	302.41%
PCB 118 fat basis (µg g ⁻¹)	0.2841	0.1258	0.2436	0.3113	
PCB 156 whole milk (ng ml ⁻¹)	0.5110	0.4326	T	T	128.48%
PCB 156 fat basis (µg g ⁻¹)	0.0166	0.0152	ND	ND	
PCB 157 whole milk (ng ml ⁻¹)	ND	ND	ND	ND	95.81%
PCB 157 fat basis (µg g ⁻¹)	ND	ND	ND	ND	
PCB 169 whole milk (ng ml ⁻¹)	ND	ND	ND	ND	57.73%
PCB 169 fat basis (µg g ⁻¹)	ND	ND	ND	ND	
PCB 189 whole milk (ng ml ⁻¹)	ND	ND	ND	ND	58.32%
PCB 189 fat basis (µg g ⁻¹)	ND	ND	ND	ND	
Total PCB (as Aroclor 1260)					
Whole milk (ng ml ⁻¹)	42.06	24.42	26.90	39.56	
Fat basis (µg g ⁻¹)	1.37	0.86	1.15	1.70	
Lipid result (%m/v)	3.0720	2.8392	2.3850	2.3334	
(%m/m)	3.0070	2.7789	2.3312	2.2791	
Lipid recovery from spike	96.53%				

agreement between replicates.

Spattering in the Kuderna-Danish apparatus was not a problem during this run since a fairly low temperature was used, but the evaporation took place over a longer period of time which may have allowed the more volatile residues to evaporate resulting in low recoveries and poor agreement between duplicates.

Endrin was not recovered; this has been discussed in previous sections.

The low recoveries of the higher chlorinated PCBs was probably due, at least in part, to the late collection of the residue fraction from the HPLC (higher chlorinated PCBs elute earlier from the HPLC system). This may also account for the poor agreement between "total PCB" results.

On evaporating the extract down to the low volume for HPLC clean-up and again on evaporating the cleaned up sample down to its final volume for GC-MS determination, a slight white precipitate was observed. This was found to derive from the HPLC grade solvents themselves. This precipitate did not seem to adversely affect the determination of the residues.

GC-MS analysis was carried out using the 15 m DB-210

column. Some band broadening was seen in the GC-MS results for beta-HCH and, more importantly, the internal standard, which probably affected the quantitation on account of the unusual peak measuring algorithm used by the data handling software. Some marked drift in the mass calibration of the mass spectrometer was also observed which may have resulted in slight changes in response factors despite the flat-top tuning.

Lipid recovery from the spike was good (96.53%). The lipid content of the milk samples were reasonable and exhibited fair agreement.

Overall, results were reasonable and compared fairly well with the expected values, but the practical problems encountered made the results unreliable.

7.2.2. Run 2: Human milk samples 3 and 4.

The results for this analysis are given in Table 16.

Recoveries from the spiked adsorbent were quite low on the whole on account of some spattering of the spike sample in the Kuderna-Danish apparatus. This is particularly noticeable in the lipid recovery, but on later examination of the lipid spiking solution (which was a separate solution from the OCP/PCB spiking solution), lipid was found to have precipitated out and

Table 16.

Results of the analysis of human milk samples 3 and 4 for OCP and PCB residues.

T=trace (= < 0.5 ng ml⁻¹ in whole milk.)ND=not detected (= < 0.1 ng ml⁻¹ in whole milk.)NOTE: Although data are reported with up to four decimal places, this is not intended to imply such a high degree of accuracy or precision.

Analyte	Sample 3		Sample 4		Spike recovery
	A	B	A	B	
HCB whole milk (ng ml ⁻¹)	T	T	0.6906	1.3968	38.92%
HCB fat basis (µg g ⁻¹)	T	T	0.0124	0.0243	
α-HCH whole milk (ng ml ⁻¹)	ND	ND	ND	ND	41.14%
α-HCH fat basis (µg g ⁻¹)	ND	ND	ND	ND	
β-HCH whole milk (ng ml ⁻¹)	0.6074	0.6775	1.1904	2.6944	52.48%
β-HCH fat basis (µg g ⁻¹)	0.0486	0.0540	0.0213	0.0468	
γ-HCH whole milk (ng ml ⁻¹)	ND	ND	ND	T	39.31%
γ-HCH fat basis (µg g ⁻¹)	ND	ND	ND	T	
δ-HCH whole milk (ng ml ⁻¹)	ND	ND	ND	ND	63.43%
δ-HCH fat basis (µg g ⁻¹)	ND	ND	ND	ND	
o,p'-DDE whole milk (ng ml ⁻¹)	T	T	T	T	86.88%
o,p'-DDE fat basis (µg g ⁻¹)	T	T	T	T	
p,p'-DDE whole milk (ng ml ⁻¹)	4.3030	3.2409	12.4369	17.5165	79.44%
p,p'-DDE fat basis (µg g ⁻¹)	0.3440	0.2583	0.2225	0.3042	
o,p'-DDT whole milk (ng ml ⁻¹)	T	T	0.5414	0.5557	106.91%
o,p'-DDT fat basis (µg g ⁻¹)	T	T	0.0097	0.0097	
p,p'-DDT whole milk (ng ml ⁻¹)	T	T	0.6839	0.9580	73.60%
p,p'-DDT fat basis (µg g ⁻¹)	T	T	0.0122	0.0166	
Dieldrin whole milk (ng ml ⁻¹)	0.7459	0.6692	1.1827	1.9214	95.06%
Dieldrin fat basis (µg g ⁻¹)	0.0596	0.0533	0.0212	0.0334	
Endrin whole milk (ng ml ⁻¹)	ND	ND	ND	ND	48.87%
Endrin fat basis (µg g ⁻¹)	ND	ND	ND	ND	
PCB 118 whole milk (ng ml ⁻¹)	2.1209	1.4977	1.7057	2.1233	164.57%
PCB 118 fat basis (µg g ⁻¹)	0.1695	0.1194	0.0305	0.0369	
PCB 156 whole milk (ng ml ⁻¹)	ND	ND	T	T	48.16%
PCB 156 fat basis (µg g ⁻¹)	ND	ND	T	T	
PCB 157 whole milk (ng ml ⁻¹)	ND	ND	ND	ND	52.35%
PCB 157 fat basis (µg g ⁻¹)	ND	ND	ND	ND	
PCB 169 whole milk (ng ml ⁻¹)	ND	ND	ND	ND	39.37%
PCB 169 fat basis (µg g ⁻¹)	ND	ND	ND	ND	
PCB 189 whole milk (ng ml ⁻¹)	ND	ND	ND	ND	34.91%
PCB 189 fat basis (µg g ⁻¹)	ND	ND	ND	ND	
Total PCB (as Aroclor 1260)					
Whole milk (ng ml ⁻¹)	12.47	8.26	14.10	18.07	
Fat basis (µg g ⁻¹)	0.9970	0.6583	0.2523	0.3137	
Lipid result (%m/v)	1.25	1.25	5.59	5.76	
(%m/m)	1.22	1.22	5.47	5.63	
Lipid recovery from spike	16.1%				

so the low lipid recovery was probably due mainly to improper spiking with the Witepsol solution.

A further point to notice is the low values obtained for the residue levels in the milk. The values for many analytes are markedly lower than would normally be expected especially in the case of sample 4 which was high in fat content. This may imply a poor extraction but the lipid content results for both samples 3 and 4 are reasonable and are in close agreement so poor extraction seems very unlikely and it may well be that the level of contamination for both the individuals concerned was indeed low. However, as will be seen below, technical problems with the mass spectrometer probably account for the low results.

The adsorbent was washed again prior to use and the blank results were correspondingly lower than previously obtained. The relatively high recovery for PCB 118 was again due to contamination of the adsorbent despite the further washing of the adsorbent.

Spattering in the Kuderna-Danish evaporators was not a major problem but it was observed, to a small degree, in all the samples. Volatilisation of the more volatile residues may have occurred.

Samples were cleaned up over two injections on the HPLC

system but the high lipid content of sample 4 meant that the lipid fraction was only just clear of the residue fraction. However, it was expected that all residues were fully collected for both samples 3 and 4 unless the high lipid content of sample 4 caused some residues to shift into the lipid band by causing a transient change in the eluotropic strength of the mobile phase.

A white precipitate was again seen in the samples just before HPLC clean-up and on evaporating the cleaned up samples down to their final volume.

GC-MS analysis was carried out using the 15 m DB-210 column. Chromatographic band broadening in the GC-MS analysis was not observed, but marked drift in the mass calibration was again seen and the drift was particularly bad at the high mass end resulting in considerable loss of sensitivity for high mass components. The MS was re-calibrated prior to analysing the standards which meant that the milk and the spike results could not realistically be compared to the standards. This was a major problem and probably accounts for the low results obtained, particularly for high mass components. The source of the drift was ultimately traced to a leakage of the accelerating voltage high tension to earth. This problem was rectified before any further runs were undertaken.

Endrin was recovered but its true recovery could not be determined in the light of the GC-MS problems.

Lipid results for the milks were reasonable and in close agreement.

In summary, the residue results were very unreliable, largely due to the technical problems encountered with the GC-MS.

7.2.3. Run 3: Human milk samples 5 and 6.

Recoveries could not be determined on account of the reasons described below:

Because of the possibility of losing OCP/PCB residues by taking the organochlorine fraction from the HPLC too late, it was decided to clean up each sample over two injections as before, but take the residue fraction very early. Since the samples were introduced into the HPLC in hexane solution, samples of fairly low lipid content (as would be encountered in samples introduced over two injections) first give a positive lipid peak on the RI detector followed by a negative peak due to the hexane. The residue fractions were collected starting about half way through the negative peak, but there was probably still some lipid eluting in this negative band, with the RI detector being swamped into giving a negative response

by the hexane. As a result, many samples including sample 6B and the spike were too dirty to be analysed by GC and gave hopelessly distorted bands. The GC-MS analysis was carried out using the DB-210 column which by this stage was about 18 months old and had been subjected to considerable use. These dirty samples seemed to damage the already ageing column beyond repair. Samples 5A, 5B and 6A as well as the blank and the individual analyte standards were analysed before the column finally broke down, but the spike, sample 6B and the Aroclor standard could not be analysed. The available results are given in Table 17. Broadening of the beta-HCH and the internal standard peaks was observed even in the measurable samples but was not so severe as to cause extreme distortion of the peaks although the reliability of the results must be brought into question.

Minor spattering was observed in the Kuderna-Danish evaporators but was not considered to be a significant problem. A further problem was created when heptane instead of hexane was used to wash the extracts from the Soxhlet equipment into the Kuderna-Danish apparatus. This meant that the evaporation was unusually prolonged and had to be carried out at a higher temperature. This would undoubtedly have caused residue losses by volatilisation.

A white precipitate was again seen in the samples just prior to HPLC clean-up and in the final solutions.

Table 17.

Results of the analysis of human milk samples 5 and 6 for OCP and PCB residues.

T=trace (= < 0.5 ng ml⁻¹ in whole milk.)ND=not detected (= < 0.1 ng ml⁻¹ in whole milk.)

NOTE: Although data are reported with up to four decimal places, this is not intended to imply such a high degree of accuracy or precision.

Analyte	Sample 5		Sample 6		Spike recovery
	A	B	A	B	
HCB whole milk (ng ml ⁻¹)	1.8078	1.5334	1.94		Sample 6B the spike and the Aroclor standard were unmeasur- able due to dirty samples and/or a damaged GC column
HCB fat basis (µg g ⁻¹)	0.0619	0.0536	0.0769		
α-HCH whole milk (ng ml ⁻¹)	T	T	T		
α-HCH fat basis (µg g ⁻¹)	T	T	T		
β-HCH whole milk (ng ml ⁻¹)	1.2668	1.2958	Unmeasurable		
β-HCH fat basis (µg g ⁻¹)	0.0433	0.0453	Unmeasurable		
γ-HCH whole milk (ng ml ⁻¹)	0.9273	0.4737	0.7383		
γ-HCH fat basis (µg g ⁻¹)	0.0317	0.0166	0.0293		
δ-HCH whole milk (ng ml ⁻¹)	ND	ND	ND		
δ-HCH fat basis (µg g ⁻¹)	ND	ND	ND		
o,p'-DDE whole milk (ng ml ⁻¹)	T	T	ND		
o,p'-DDE fat basis (µg g ⁻¹)	T	T	ND		
p,p'-DDE whole milk (ng ml ⁻¹)	6.8100	3.3963	16.36		
p,p'-DDE fat basis (µg g ⁻¹)	0.2329	0.2236	0.6493		
o,p'-DDT whole milk (ng ml ⁻¹)	T	T	ND		
o,p'-DDT fat basis (µg g ⁻¹)	T	T	ND		
p,p'-DDT whole milk (ng ml ⁻¹)	0.8663	0.7967	1.48		
p,p'-DDT fat basis (µg g ⁻¹)	0.0296	0.0278	0.0587		
Dieldrin whole milk (ng ml ⁻¹)	1.7624	1.6613	2.61		
Dieldrin fat basis (µg g ⁻¹)	0.0603	0.0581	0.1035		
Endrin whole milk (ng ml ⁻¹)	ND	ND	ND		
Endrin fat basis (µg g ⁻¹)	ND	ND	ND		
FCB 118 whole milk (ng ml ⁻¹)	1.7451	1.9932	1.45		
PCB 118 fat basis (µg g ⁻¹)	0.0597	0.0697	0.0577		
FCB 156 whole milk (ng ml ⁻¹)	T	ND	ND		
FCB 156 fat basis (µg g ⁻¹)	T	ND	ND		
FCB 157 whole milk (ng ml ⁻¹)	T	ND	ND		
FCB 157 fat basis (µg g ⁻¹)	T	ND	ND		
FCB 169 whole milk (ng ml ⁻¹)	ND	ND	ND		
FCB 169 fat basis (µg g ⁻¹)	ND	ND	ND		
FCB 189 whole milk (ng ml ⁻¹)	T	T	T		
FCB 189 fat basis (µg g ⁻¹)	T	T	T		
Total PCB (as Aroclor 1260)					
Whole milk (ng ml ⁻¹)					Unmeasurable due to peak distortion
Fat basis (µg g ⁻¹)					in the Aroclor standard.
Lipid result (%m/v)	2.92	2.86	2.52	2.32	
(%m/m)	2.85	2.79	2.45	2.26	
Lipid recovery from spike		92.5%			

Despite the considerable problems, the results that were obtained seemed reasonable and the duplicates for sample 5 exhibited quite good agreement. Milk lipid results seemed to be realistic and duplicates agreed quite well. Lipid recovery was also quite good.

The duplicate values in sample 5 for gamma-HCH and p,p'-DDE did not agree very well. Both peaks were broadened in both duplicates and the gamma-HCH peak suffered from minor interference in the mass fragmentogram. The apparently low values for p,p'-DDE may have been due, in part, to losses in the Kuderna-Danish apparatus as described above.

7.2.4. Run 4: Human milk samples 7 and 8.

The results for Run 4 are given in Table 18.

Recoveries obtained were generally improved but were still inferior to Run 1. After extraction, volume reduction in the Kuderna-Danish concentrators was carried out at a higher temperature (about 90°C) in order to reduce the amount of time spent in the apparatus with the aim of reducing losses by volatilisation. Unfortunately, this resulted in severe spattering for all samples. This would have reduced recoveries considerably and is reflected in the low lipid recovery. Such spattering also gave rise to poor agreement between duplicates on a whole

Table 18.

Results of the analysis of human milk samples 7 and 8 for OCP and PCB residues.

T=trace (= < 0.5 ng ml⁻¹ in whole milk.)ND=not detected (= < 0.1 ng ml⁻¹ in whole milk.)

NOTE: Although data are reported with up to four decimal places, this is not intended to imply such a high degree of accuracy or precision.

Analyte	Sample 7		Sample 8		Spike recovery
	A	B	A	B	
HCB whole milk (ng ml ⁻¹)	2.5136	1.9076	1.3654	1.7984	53.27%
HCB fat basis (µg g ⁻¹)	0.1133	0.1176	0.0776	0.0785	
α-HCH whole milk (ng ml ⁻¹)	ND	T	T	T	69.88%
α-HCH fat basis (µg g ⁻¹)	ND	T	T	T	
β-HCH whole milk (ng ml ⁻¹)	6.5350	3.8142	3.8322	4.0108	75.14%
β-HCH fat basis (µg g ⁻¹)	0.2945	0.2352	0.2179	0.1750	
γ-HCH whole milk (ng ml ⁻¹)	ND	ND	ND	T	77.05%
γ-HCH fat basis (µg g ⁻¹)	ND	ND	ND	T	
δ-HCH whole milk (ng ml ⁻¹)	ND	ND	ND	ND	58.91%
δ-HCH fat basis (µg g ⁻¹)	ND	ND	ND	ND	
o,p'-DDE whole milk (ng ml ⁻¹)	ND	ND	ND	ND	80.54%
o,p'-DDE fat basis (µg g ⁻¹)	ND	ND	ND	ND	
p,p'-DDE whole milk (ng ml ⁻¹)	62.2346	49.5248	12.9364	15.6848	91.46%
p,p'-DDE fat basis (µg g ⁻¹)	2.8046	3.0541	0.7355	0.6842	
o,p'-DDT whole milk (ng ml ⁻¹)	T	ND	ND	ND	82.60%
o,p'-DDT fat basis (µg g ⁻¹)	T	ND	ND	ND	
p,p'-DDT whole milk (ng ml ⁻¹)	4.1826	3.2753	1.3529	1.6980	76.76%
p,p'-DDT fat basis (µg g ⁻¹)	0.1885	0.2020	0.0769	0.0741	
Dieldrin whole milk (ng ml ⁻¹)	T	T	0.7170	0.9762	80.07%
Dieldrin fat basis (µg g ⁻¹)	T	T	0.0408	0.0426	
Endrin whole milk (ng ml ⁻¹)	ND	ND	ND	ND	122.22%
Endrin fat basis (µg g ⁻¹)	ND	ND	ND	ND	
PCB 118 whole milk (ng ml ⁻¹)	1.6438	1.0979	1.2109	1.6433	103.72%
PCB 118 fat basis (µg g ⁻¹)	0.0741	0.0677	0.0688	0.0717	
PCB 156 whole milk (ng ml ⁻¹)	ND	ND	ND	ND	48.92%
PCB 156 fat basis (µg g ⁻¹)	ND	ND	ND	ND	
PCB 157 whole milk (ng ml ⁻¹)	ND	ND	ND	ND	43.23%
PCB 157 fat basis (µg g ⁻¹)	ND	ND	ND	ND	
PCB 169 whole milk (ng ml ⁻¹)	ND	ND	ND	ND	20.65%
PCB 169 fat basis (µg g ⁻¹)	ND	ND	ND	ND	
PCB 189 whole milk (ng ml ⁻¹)	ND	ND	ND	ND	34.65%
PCB 189 fat basis (µg g ⁻¹)	ND	ND	ND	ND	
Total PCB (as Aroclor 1260)					
Whole milk (ng ml ⁻¹)	15.32	12.79	12.92	17.58	
Fat basis (µg g ⁻¹)	0.6905	0.7885	0.7347	0.7671	
Lipid result (%m/v)	2.2190	1.6217	1.7590	2.2923	
(%m/m)	2.1628	1.5792	1.7114	2.2290	
Lipid recovery from spike		81.25%			

milk basis, but because spattering causes a proportionate loss of lipid, the results of the analysis on a lipid basis agree much more closely and would be expected to be much more accurate (although the lipid determination itself would be low).

A white precipitate was again seen in the samples on reducing to low volumes.

With regard to the HPLC clean-up, samples were cleaned up over two injections and fractions were again taken quite early, but not as early as in Run 3.

The GC-MS analysis was carried out using the 25 m OV-17 column. Band broadening was observed for beta-HCH and the internal standard for all samples and in the case of sample 7A, actual peak splitting was seen for these two materials. This adversely affected the quantitative results for all analytes.

Endrin was recovered with high efficiency, but it was noted that the absolute peak area was markedly smaller in the spike and the standards than was normally seen and it may be that degradation or, more likely, adsorption onto glass had occurred.

Absolute values for residue levels in the milk seemed reasonable compared to published data (35) and in the

case of sample 7, the values for some analytes, especially p,p'-DDE, were quite high. On reading the completed questionnaire provided by the subject, it was discovered that she had experienced above average contact with pesticides and, having been born in 1950, many of the pesticides to which she was exposed would have been organochlorines.

The recoveries for the higher chlorinated PCB congeners were very poor, especially for PCB 169. No explanation could be found for this.

On account of the spattering, milk lipid values were a little low and agreement between replicates was poor. Lipid recovery was also low (81.25%).

7.2.5. Run 5: Human milk samples 9 and 10.

The results for this run are shown in Table 19.

During this run, a high evaporation temperature was again used with the Kuderna-Danish apparatus, but a single anti-bump granule was placed in each receiver tube. This reduced but did not eliminate spattering. Consequently, agreement between duplicates on a whole milk basis were adversely affected, but generally less so on a lipid basis.

Table 19. Results of the analysis of human milk samples 9 and 10 for OCP and PCB residues.

T=trace (= < 0.5 ng ml⁻¹ in whole milk.)
 ND=not detected (= < 0.1 ng ml⁻¹ in whole milk.)

NOTE: Although data are reported with up to four decimal places, this is not intended to imply such a high degree of accuracy or precision.

Analyte	Sample 9		Sample 10		Spike recovery
	A	B	A	B	
HCB whole milk (ng ml ⁻¹)	0.6974	0.6257	2.7482	3.2592	89.45%
HCB fat basis (µg g ⁻¹)	0.0563	0.0511	0.0952	0.0859	
α-HCH whole milk (ng ml ⁻¹)	ND	ND	ND	T	90.48%
α-HCH fat basis (µg g ⁻¹)	ND	ND	ND	T	
β-HCH whole milk (ng ml ⁻¹)	0.6112	0.6342	4.9729	5.2923	73.22%
β-HCH fat basis (µg g ⁻¹)	0.0494	0.0518	0.1722	0.1395	
γ-HCH whole milk (ng ml ⁻¹)	T	T	0.9196	1.0754	105.84%
γ-HCH fat basis (µg g ⁻¹)	T	T	0.0318	0.0283	
δ-HCH whole milk (ng ml ⁻¹)	ND	ND	ND	ND	71.67%
δ-HCH fat basis (µg g ⁻¹)	ND	ND	ND	ND	
o,p'-DDE whole milk (ng ml ⁻¹)	ND	ND	ND	ND	88.34%
o,p'-DDE fat basis (µg g ⁻¹)	ND	ND	ND	ND	
p,p'-DDE whole milk (ng ml ⁻¹)	3.9712	4.7835	14.5401	19.1904	154.15%
p,p'-DDE fat basis (µg g ⁻¹)	0.3207	0.3905	0.5034	0.5058	
o,p'-DDT whole milk (ng ml ⁻¹)	T	T	T	T	96.07%
o,p'-DDT fat basis (µg g ⁻¹)	T	T	T	T	
p,p'-DDT whole milk (ng ml ⁻¹)	0.4504	0.5611	1.0223	1.1944	101.12%
p,p'-DDT fat basis (µg g ⁻¹)	0.0364	0.0458	0.0354	0.0315	
Dieldrin whole milk (ng ml ⁻¹)	2.2468	2.8142	2.2837	4.7473	143.82%
Dieldrin fat basis (µg g ⁻¹)	0.1814	0.2297	0.0791	0.1250	
Endrin whole milk (ng ml ⁻¹)	ND	ND	ND	ND	158.94%
Endrin fat basis (µg g ⁻¹)	ND	ND	ND	ND	
PCB 118 whole milk (ng ml ⁻¹)	1.0005	T	0.8902	1.3910	116.61%
PCB 118 fat basis (µg g ⁻¹)	0.0808	T	0.0308	0.0367	
PCB 156 whole milk (ng ml ⁻¹)	ND	ND	T	T	83.10%
PCB 156 fat basis (µg g ⁻¹)	ND	ND	T	T	
PCB 157 whole milk (ng ml ⁻¹)	ND	ND	ND	ND	68.58%
PCB 157 fat basis (µg g ⁻¹)	ND	ND	ND	ND	
PCB 169 whole milk (ng ml ⁻¹)	ND	ND	ND	ND	43.93%
PCB 169 fat basis (µg g ⁻¹)	ND	ND	ND	ND	
PCB 189 whole milk (ng ml ⁻¹)	ND	ND	ND	ND	53.64%
PCB 189 fat basis (µg g ⁻¹)	ND	ND	ND	ND	
Total PCB (as Aroclor 1260)					
Whole milk (ng ml ⁻¹)	7.8862	3.9658	18.5610	26.8423	
Fat basis (µg g ⁻¹)	0.6368	0.3238	0.6426	0.7075	
Lipid result (%m/v)	1.2384	1.2250	2.8888	3.7942	
(%m/m)	1.2049	1.9111	2.8177	3.6999	
Lipid recovery from spike	94.93%				

Despite some spattering, recoveries were much improved over previous runs and some recoveries were very high. This may have been due to the age of the standards with adsorption onto the glass occurring for the analytes giving the apparently very high (> 100%) recoveries.

Results for sample 9 seemed low but duplicates agreed well (except for the "total PCB" result) and so the subject may well carry only a low body burden of these materials. Sample 10 showed more usual levels of contamination but with less good agreement between replicates on account of spattering in sample 10A.

As before, the white precipitate was seen when the samples were evaporated down to low solvent volumes.

Samples were cleaned up on the HPLC system over two injections and the residue fractions were taken quite early.

The GC-MS results, which were obtained using the OV-17 column, showed some band broadening in all the milk samples for beta-HCH and less so for the internal standard. Quantitation was adequate, though not ideal, for beta-HCH but the slight distortion of the internal standard peak should not have compromised the quantitation of the other analytes.

The blank was again quite high for certain analytes. This was due to using a new batch of adsorbent which had only been washed once.

Lipid recovery was good (94.93%).

7.2.6. Run 6: Human milk samples 11 and 12.

The results for this final run are given in Table 20.

Insufficient milk was provided in sample 12 for a duplicate analysis to be performed.

Recoveries for this run were much better and perhaps too high and the possibility of adsorption of some analytes to glass in the standards was not ruled out. The recovery data for three analytes were lost due to a fault in the GC-MS system during data recording.

Spattering in the Kuderna-Danish evaporators was completely avoided by using only a modest temperature and once the sample was contained solely within the receiver tube, the latter was removed and the final stage of the evaporation was carried out over air.

Agreement between the duplicates for sample 11 was good for all analytes except HCB. Volatilisation losses in the evaporator may account for this discrepancy.

Table 20.

Results of the analysis of human milk samples 11 and 12 for OCP and PCB residues.

T=trace (= < 0.5 ng ml⁻¹ in whole milk.)ND=not detected (= < 0.1 ng ml⁻¹ in whole milk.)NOTE: Although data are reported with up to four decimal places, this is not intended to imply such a high degree of accuracy or precision.

Analyte	Sample 11		Sample 12		Spike recovery
	A	B	A	B	
					*
HCB whole milk (ng ml ⁻¹)	16.6237	12.7688	2.5197		91.80%
HCB fat basis (µg g ⁻¹)	0.4691	0.3743	0.0500		
α-HCH whole milk (ng ml ⁻¹)	T	T	T		103.91%
α-HCH fat basis (µg g ⁻¹)	T	T	T		
β-HCH whole milk (ng ml ⁻¹)	16.5869	18.7091	5.1037		111.00%
β-HCH fat basis (µg g ⁻¹)	0.4681	0.5484	0.1012		
γ-HCH whole milk (ng ml ⁻¹)	1.8833	1.1750	T		104.23%
γ-HCH fat basis (µg g ⁻¹)	0.0531	0.0344	T		
δ-HCH whole milk (ng ml ⁻¹)	ND	ND	ND		108.39%
δ-HCH fat basis (µg g ⁻¹)	ND	ND	ND		
o,p'-DDE whole milk (ng ml ⁻¹)	T	ND	ND		99.40%
o,p'-DDE fat basis (µg g ⁻¹)	T	ND	ND		
p,p'-DDE whole milk (ng ml ⁻¹)	47.0902	49.6803	34.3173		**
p,p'-DDE fat basis (µg g ⁻¹)	1.3289	1.4563	0.6807		
o,p'-DDT whole milk (ng ml ⁻¹)	T	T	T		138.40%
o,p'-DDT fat basis (µg g ⁻¹)	T	T	T		
p,p'-DDT whole milk (ng ml ⁻¹)	5.7747	5.6422	4.1942		140.15%
p,p'-DDT fat basis (µg g ⁻¹)	0.1630	0.1654	0.0832		
Dieldrin whole milk (ng ml ⁻¹)	4.8203	4.5508	1.5732		111.94%
Dieldrin fat basis (µg g ⁻¹)	0.1360	0.1334	0.0312		
Endrin whole milk (ng ml ⁻¹)	ND	ND	ND		**
Endrin fat basis (µg g ⁻¹)	ND	ND	ND		
PCB 118 whole milk (ng ml ⁻¹)	6.3361	6.3595	0.8052		**
PCB 118 fat basis (µg g ⁻¹)	0.1788	0.1864	0.0160		
PCB 156 whole milk (ng ml ⁻¹)	1.8452	1.6242	T		94.65%
PCB 156 fat basis (µg g ⁻¹)	0.0521	0.0476	T		
PCB 157 whole milk (ng ml ⁻¹)	T	T	T		79.59%
PCB 157 fat basis (µg g ⁻¹)	T	T	T		
PCB 169 whole milk (ng ml ⁻¹)	ND	ND	ND		61.93%
PCB 169 fat basis (µg g ⁻¹)	ND	ND	ND		
PCB 189 whole milk (ng ml ⁻¹)	ND	T	T		74.64%
PCB 189 fat basis (µg g ⁻¹)	ND	T	T		
Total PCB (as Aroclor 1260)					
Whole milk (ng ml ⁻¹)	169.68	171.82	21.27		
Fat basis (µg g ⁻¹)	4.7886	5.0368	0.4218		
Lipid result (%m/v)	3.5436	3.4114	5.0415		
(%m/m)	3.4716	3.3414	4.9527		
Lipid recovery from spike	103.42%				

* No duplicate of sample 12 was possible due to insufficient sample.

** Data lost due to GC-MS system breakdown.

Extracts were cleaned up over two injections on the HPLC system and residue fractions were taken slightly later than in Run 5 but in sufficient time to collect all residues.

The GC-MS determination was carried out using the 25 m OV-17 column. Peak shapes under GC-MS were all good except for slight broadening of beta-HCH in one milk sample.

Again, the blank determination for certain analytes was high indicating the need for more thorough washing of the adsorbent prior to use.

Absolute values for the residues in sample 11 were very high for most analytes and inspection of the subject's questionnaire revealed that she had lived abroad in countries where DCPs were used extensively as well as in industrialised countries where PCBs would have been common. The subject was also aged 34 and primiparous thus allowing considerable time for the accumulation of organochlorines in her body fat before lactation. Interestingly, the infant was born 7 weeks premature; however, any attempt to connect the high level of milk contamination to the premature birth of the infant would be conjecture.

The residue results for sample 12 were rather lower than

those for sample 11 and were more like the values expected.

Milk lipid values were reasonable and the duplicates agreed well. Lipid recovery was very good.

Overall, the method seemed to perform much better after modifying the procedure for using the Kuderna-Danish evaporators.

7.3. Conclusions.

As can be seen from the results above, the method performance improved with experience. Suggestions for modifications to the procedure are given in Section 8 but some points are worthy of note here. Firstly, it seems that the Kuderna-Danish evaporators were a major problem area and steps should be taken to control more effectively the problems associated with the use of this equipment. Secondly, the HPLC clean-up was not as robust as would be expected of a routine residue method and this aspect of the method needs further attention (see Section 8).

Generally, the results tended to fall within expected limits although the practical problems associated with these first few runs would indicate that the results obtained tended to be low except for Run 6.

Note: In several of the samples, the levels of some residues would exceed the acceptable daily intake levels for normal infants as defined by the FAO/WHO or other sources (37) (assuming an intake of 150 ml milk per kg infant body weight per day). The clinical significance of this is not known.

8. DISCUSSION AND CONCLUSIONS.

The methods, as described in the previous sections, appear to be workable but there remain a few question marks with regard to the performance and ruggedness of the complete procedure.

Recoveries appear to be quite variable from run to run although the true variability of the procedure is difficult to assess on account of the practical difficulties which arose during the first few attempts. There are several factors which are important in this context:

After adsorption of the sample onto the adsorbent and drying in the oven, samples were found to vary with regard to the amount of residual dampness. This was largely due to the differing sizes of glass mortar used for different samples resulting in different surface areas being exposed. Using uniformly sized (and preferably large) mortars would aid more consistent drying. Variability of water content could affect the extraction process.

The Kuderna-Danish evaporators used for the work incorporated Snyder columns containing only two ball-valves. This would reduce the degree of refluxing within the evaporators and could allow residues to escape by

volatilisation or droplet entrainment.

Spattering in the evaporators, particularly when the sample volume had been reduced until it was contained solely within the receiver tube, was also a major problem and it was ultimately found necessary to evaporate the last 10 ml or so of the sample over air. The benefits gained by completing the last stage of the volume reduction over air can be seen in the results for Run 6 (milk samples 11 and 12 Table 20 Section 7.2.6.). The recoveries obtained were high and the agreement between duplicate analyses of milk sample 11 (no duplicate of sample 12 was possible due to insufficient milk) was generally much improved over previous runs. Hence, this single modification to the procedure gave rise to a marked improvement in method performance and illustrates the difficulties that can be encountered in reducing the sample volume in residue analysis.

The timing of fraction collection from the HPLC clean-up system was also found to be critical, although the visual picture of the separation provided by the refractive index detector was helpful in this context. Separation of residues from the lipid was only just adequate and for milk samples of high fat content, the lipid band could easily encroach on the residue bands. This problem could be remedied by using a longer HPLC column or possibly by modifying the mobile phase composition, although a more

selective mobile phase may give rise to lipid solubility problems (see Section 4.2.3. for further discussion). Incorrect fraction timing could give rise to loss of residues or allow lipids to pass into the residue fraction.

Recoveries of delta-HCH and PCB 169 were routinely low. The reasons for this are not clear but HCH isomers can degrade to trichlorobenzenes and this may account for the loss of the delta isomer. Degradation of PCB 169 seems very unlikely and adsorption onto glassware may account for the loss of this material. The loss of endrin proved to be even more enigmatic since recoveries seemed to be either high or low. Degradation or adsorption may account for some of the loss although this would not explain the wide variability of the endrin recoveries. A more likely explanation would be a shift in retention on the HPLC system with varying lipid content. A high lipid concentration may cause the endrin to elute in the lipid band; this seems all the more likely in view of the fact that aldrin elutes mainly in the lipid band even at low lipid concentrations. Furthermore, endrin always seemed to be recovered when spike samples were cleaned up over two injections (i.e. over two 2 ml injections with a low concentration of lipid in each as opposed to a single, high lipid content injection).

The GC band broadening seen in some samples for beta-HCH

and the 4,4'-dibromobiphenyl internal standard was probably due to insufficient sample clean-up. Again, a longer HPLC column and also a longer retention gap would reduce this problem. The retention gap used was only 2m long which was barely adequate for the 10 μ l injections made and some flooding of the analytical column may have occurred despite the long injection time of 15 seconds. Extending the injection time to 20 seconds seemed to help but this was never rigorously established. It was considered to be strange that only beta-HCH and the 4,4'-dibromobiphenyl were affected. This would indicate that it was not simply a flooding problem since all compounds would then be affected. It seems more likely that the broadening was due to specific retention of the affected compounds by a contaminant in the sample solution.

High blank determinations for some of the analytes, particularly gamma-HCH, dieldrin and PCB 118, were encountered with some of the samples. The gamma-HCH and the dieldrin were present on account of the fibrous cellulose being old (1960s) and of plant origin and it is likely that the plants from which the cellulose was derived had been treated with these pesticides. The PCB 118 contamination arose as a consequence of the unfortunate need to carry out synthetic work in the same laboratory as the residue work which resulted in contamination of the adsorbent despite careful cleaning

of the work surfaces and equipment. Washing the adsorbent by thorough Soxhlet extraction reduced this problem but did not eliminate it and it would be desirable for a more effective washing procedure to be adopted. The procedure used employed heptane as the solvent and extraction was carried out overnight, but the thimble used was very large and permeation of the solvent into the core of the adsorbent may have been inadequate. In addition, the size of the Soxhlet apparatus made the cycling rate slow (about 4 to 5 cycles per hour).

It was also observed that the large GC injection volume and the design of the injector port were such that a small amount of "carry-over" from the previous injection was a regular occurrence and this added to the problem of high blank results. Thorough cleaning of the injector port cured this problem, but such cleaning could not be carried out during a run and so some carry-over from injection to injection was inevitable.

Another factor adding to the variability of results was the small number of standards analysed during a GC-MS run. Due to the limited time available for GC-MS work, only two standards (plus an Aroclor standard for "total PCB" determination) could be analysed during a run of two milk samples in duplicate, a blank and a spike. A run would routinely last about nine hours and drift in the sensitivity of the GC-MS system may well occur during

this time. Analysing so few standards during a nine hour run would not be sufficient to account for such drift.

As is usual, experience in using the method appeared to improve the quality of the results obtained, but problems still remain. The following improvements in the method should enhance method performance:

1. Wash the adsorbent more thoroughly before use and allocate a work area solely for residue work.
2. Use a 3-ball Snyder column in the Kuderna-Danish evaporators.
3. Evaporate the last portion of the crude extract over air in order to avoid spattering of the extract in the Kuderna-Danish receiver tube.
4. Use a longer HPLC column (say 400 mm) to improve lipid/residue separation and make fraction timing less critical.
5. Use a longer retention gap to prevent flooding of the analytical GC column and act as a more effective guard column.
6. Incorporate a back-flushing system for the in-line HPLC filter in order to avoid repeated dismantling of the HPLC clean-up system due to blockage.
7. Use a longer GC column and check the separation (either by GC or MS) of all 209 PCB congeners. A trifluoropropyl phase should achieve this but liquid crystalline phases would be worthy of investigation.

8. Analyse more standards during each GC-MS run.
9. A better design of GC injector port or, perhaps, a PTV or on-column injector would be a useful modification.
10. An automated data handling system with an improved peak measuring algorithm would also be a major improvement.

After making the above modifications to the method, the procedure should be ready for further ruggedness testing along the lines proposed by Youden and Steiner (31). As the method stands, it is probably adequate for routine use, but after modification and more thorough validation, the procedure should represent a significant advance over current methods in terms of precision, labour intensiveness and use of consumables and ought to be entirely acceptable as a routine procedure.

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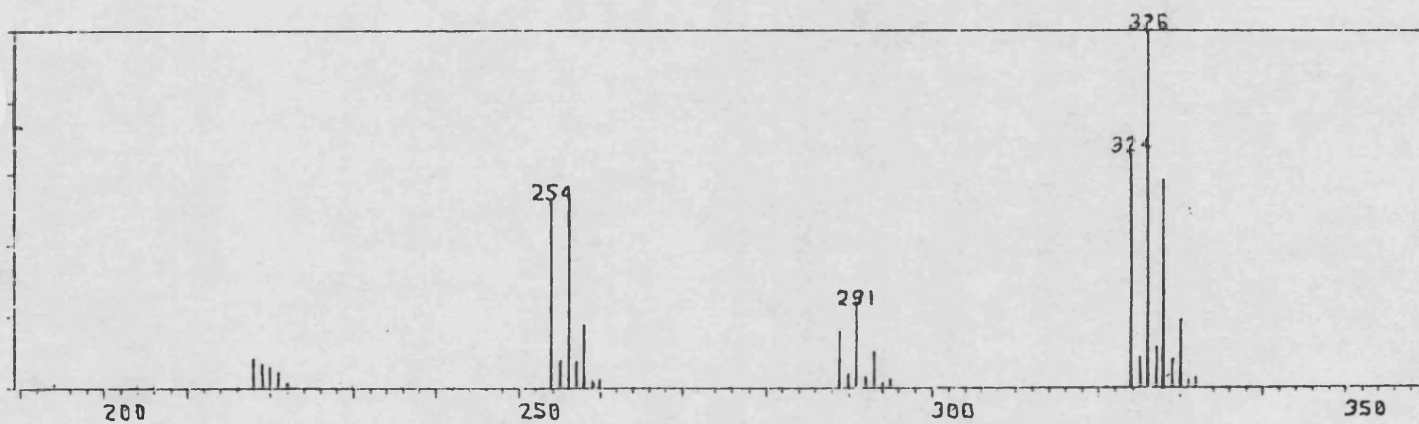
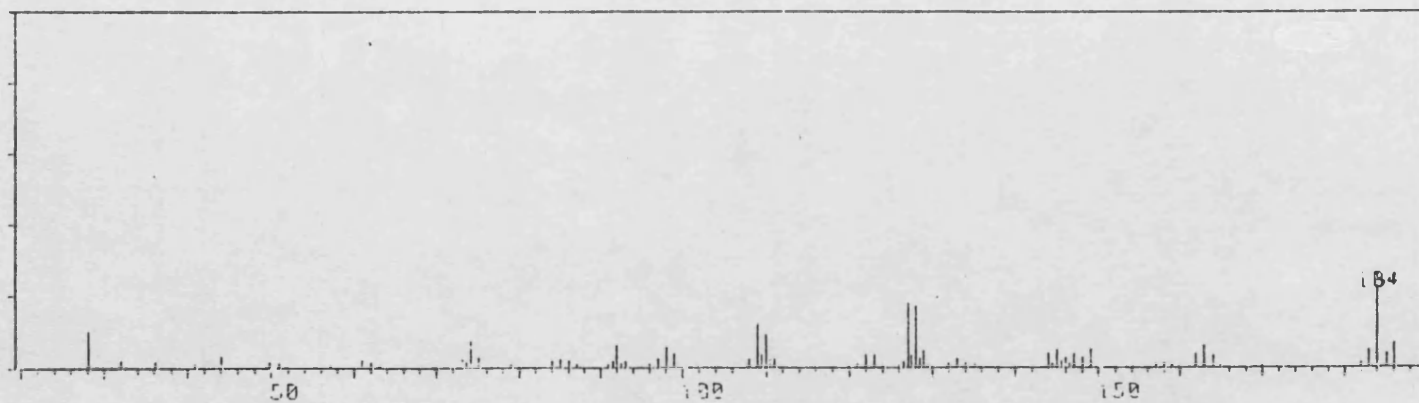
APPENDIX 1.

Proton NMR data and 70 eV EI mass spectra of PCBs 97,
118, 156, 157, 169 and 189.

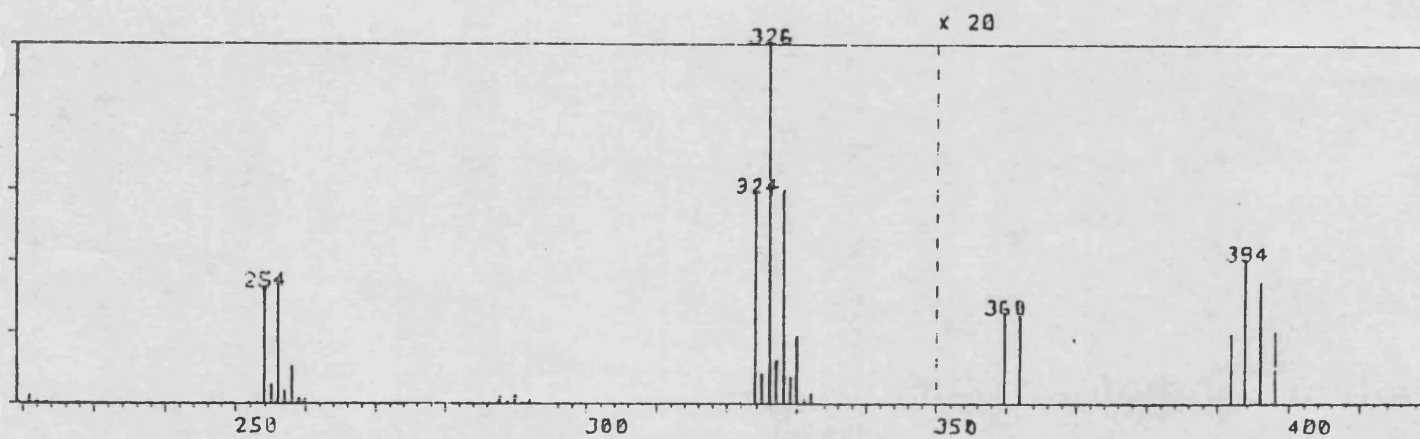
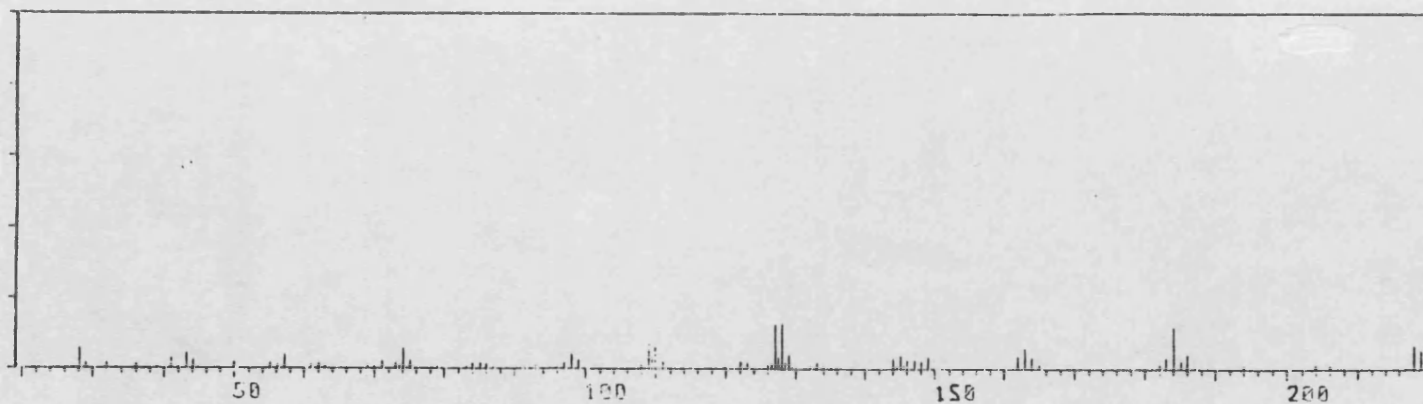
	H ₂	H ₃	H ₄	H ₅	H ₆	H ₂ '	H ₃ '	H ₄ '	H ₅ '	H ₆ '
PCB 97 2,2',3',4,5- pentachlorobiphenyl		7.60s *			7.36s *			7.54dd* ³ J=8.0Hz H _A ',H _B ' ⁴ J=1.6Hz H _A ',H _A '	7.28t ³ J=8.0Hz H _B ',H _A ' ³ J=8.0Hz H _B ',H _A '	7.14dd* ³ J=8.0Hz H _A ',H _B ' ⁴ J=1.6Hz H _A ',H _A '
PCB 118 2,3',4,4',5- pentachlorobiphenyl		7.59s*			7.41s*	7.50d ⁴ J=2.1Hz H _A ',H _A '			7.52d ³ J=8.4Hz H _B ',H _A '	7.24dd ³ J=8.4Hz H _A ',H _B ' ⁴ J=2.1Hz H _A ',H _A '
PCB 156 2,3,3',4,4',5- hexachlorobiphenyl					7.36s	7.47d ⁴ J=2.1Hz H _A ',H _A '			7.53d ³ J=8.4Hz H _B ',H _A '	7.23dd ³ J=8.4Hz H _A ',H _B ' ⁴ J=2.1Hz H _A ',H _A '
PCB 157 2,3,3',4,4',5'- hexachlorobiphenyl				7.46d* ³ J=8.4Hz H _B ,H _A	7.15d* ³ J=8.4Hz H _B ,H _B	7.41s				7.41s
PCB 169 3,3',4,4',5,5'- hexachlorobiphenyl	7.54s				7.54s	7.54s				7.54s
PCB 189 2,3,3',4,4',5,5'- heptachlorobiphenyl					7.36s	7.41s				7.41s

¹H NMR data for selected PCB congeners. The instrument used for the acquisition of the data was a JEOL GX 270 FT-NMR. The samples were dissolved in CDCl₃ with TMS as internal reference standard. Spectra were recorded at 20 °C using 32K data points and 64 scans. Resolution was 0.2Hz.

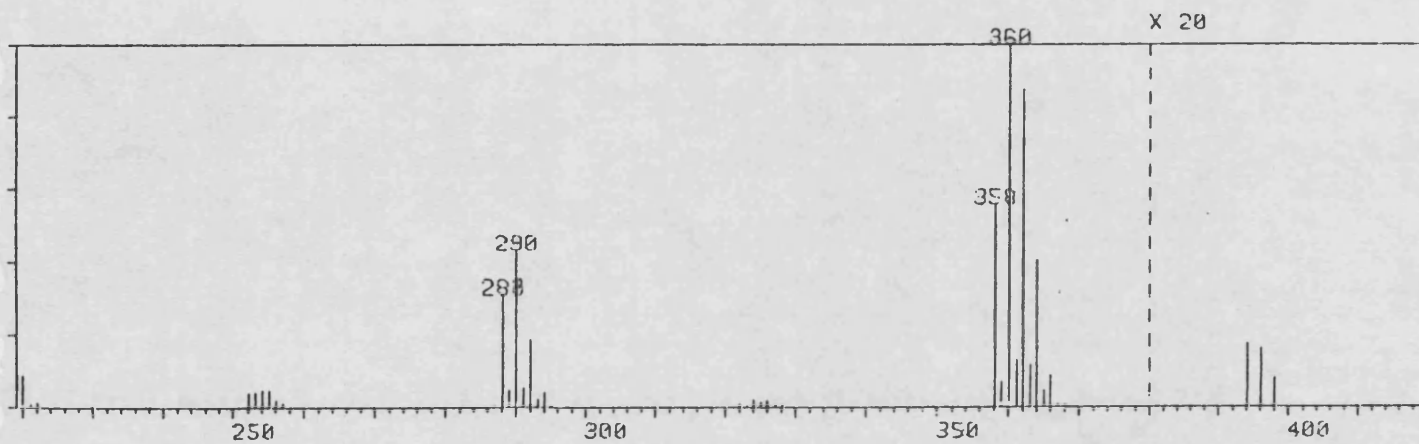
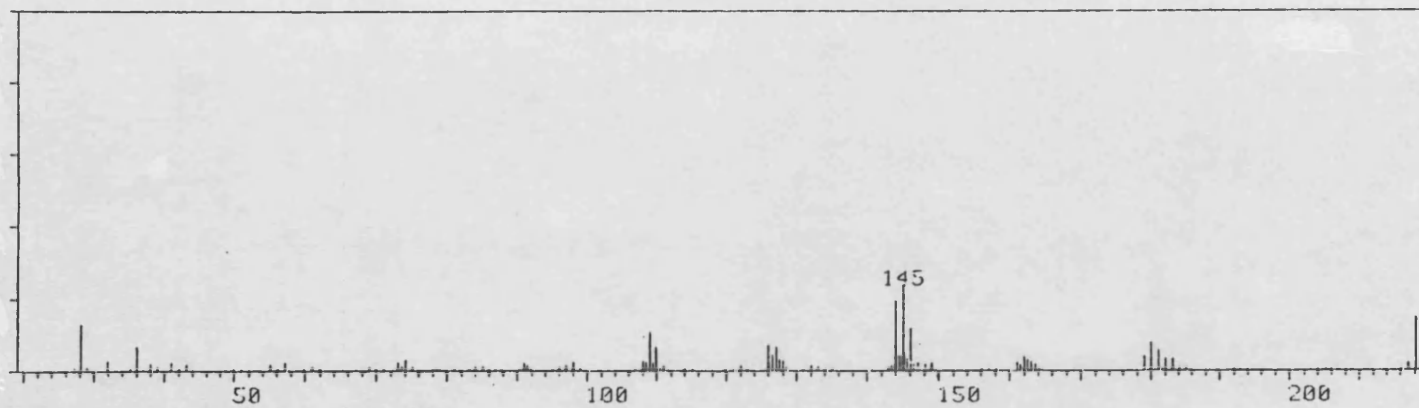
Assignments marked * were made using the empirical formula and data given by Yanagisawa et al. (52)



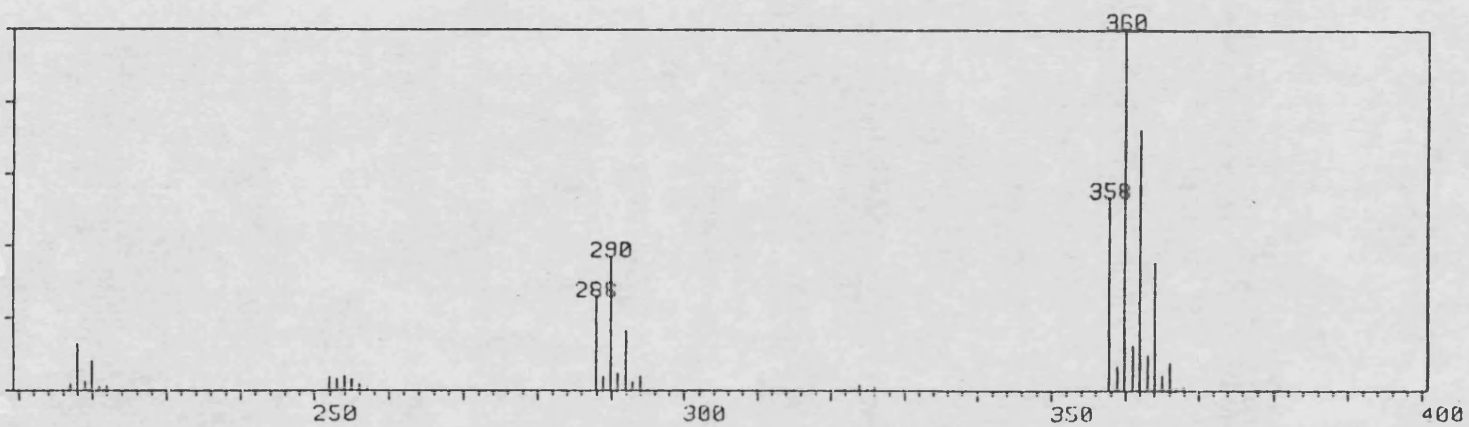
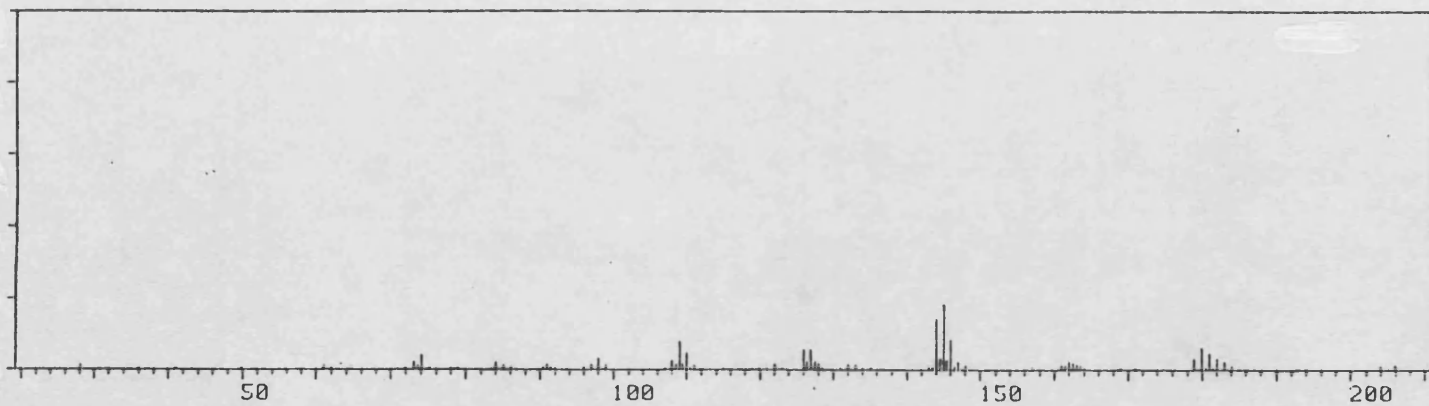
70 eV EI mass spectrum of PCB 97



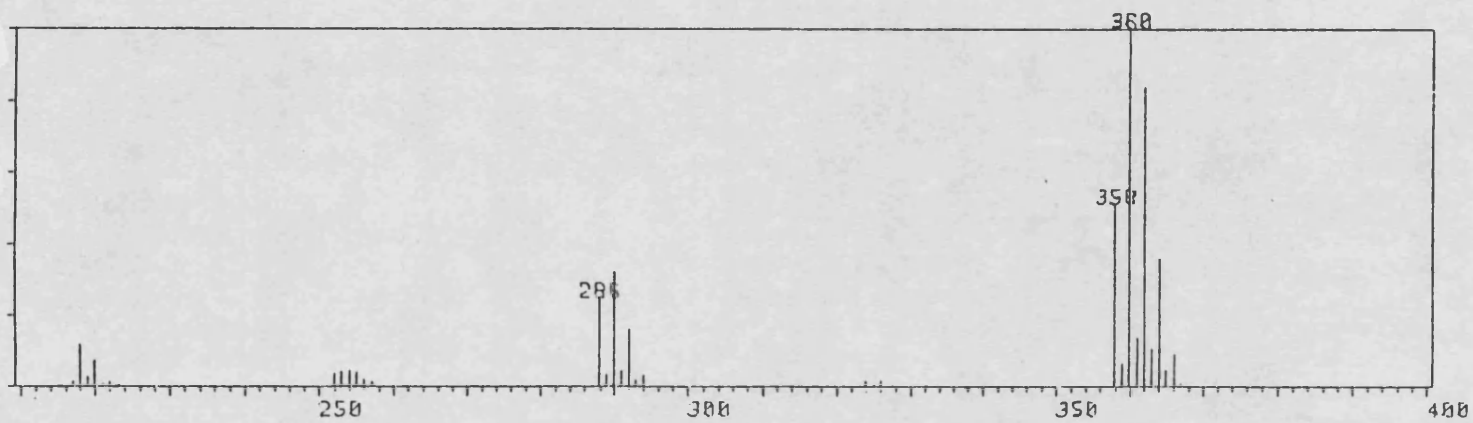
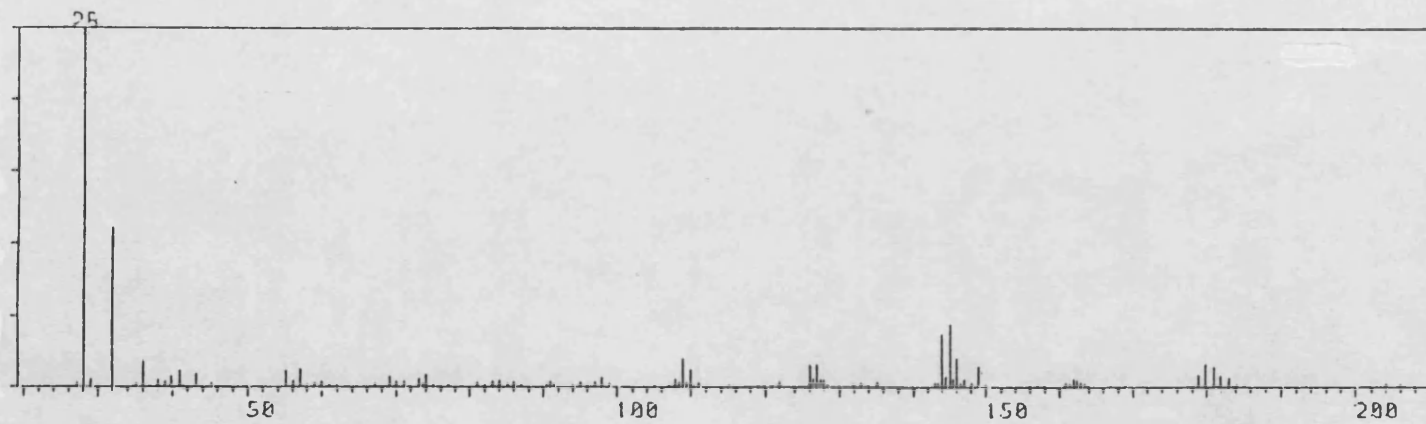
70 eV EI mass spectrum of PCB 118



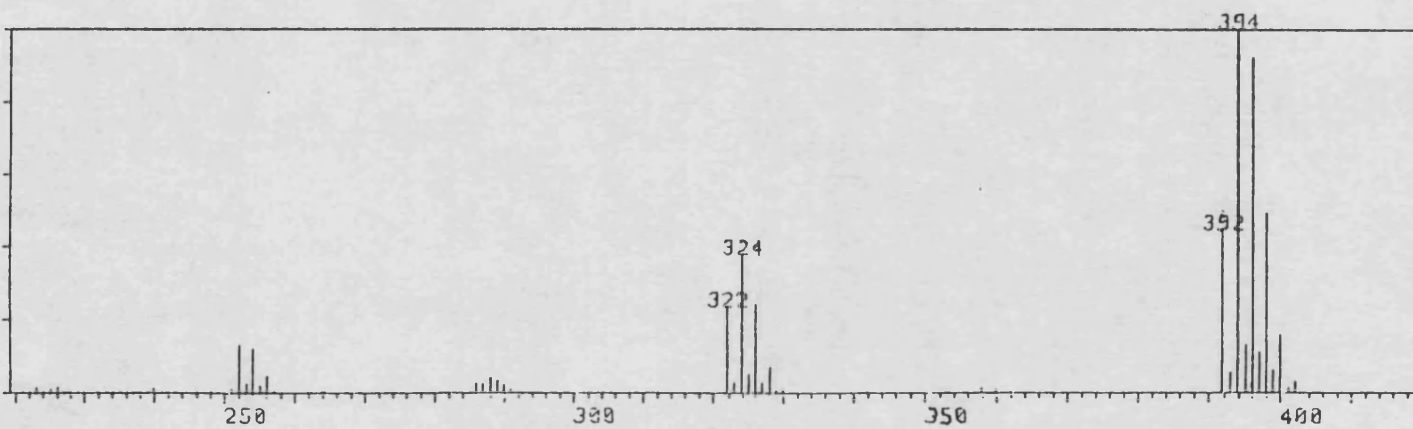
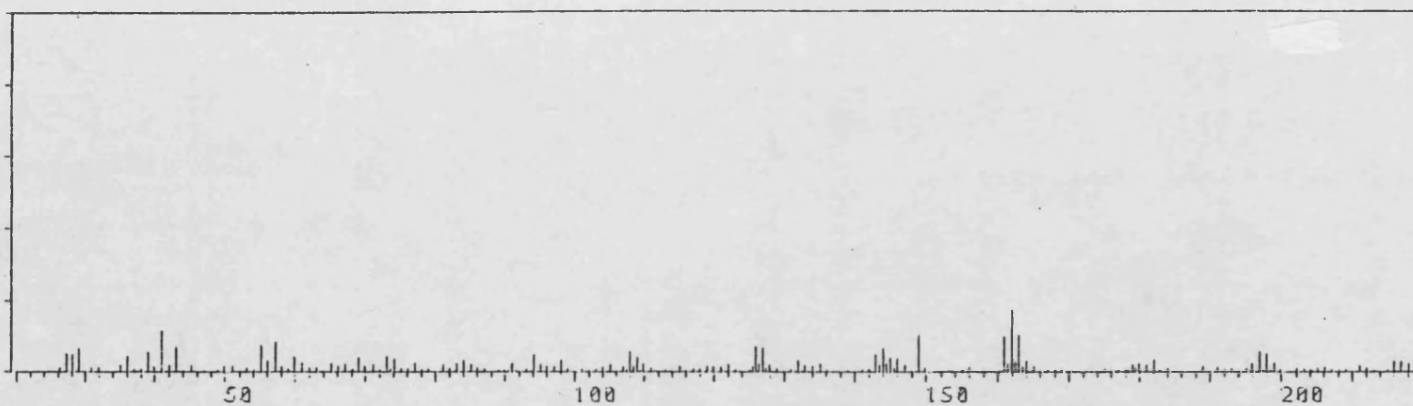
70 eV EI mass spectrum of PCB 156



70 eV EI mass spectrum of PCB 157



70 eV EI mass spectrum of PCB 169

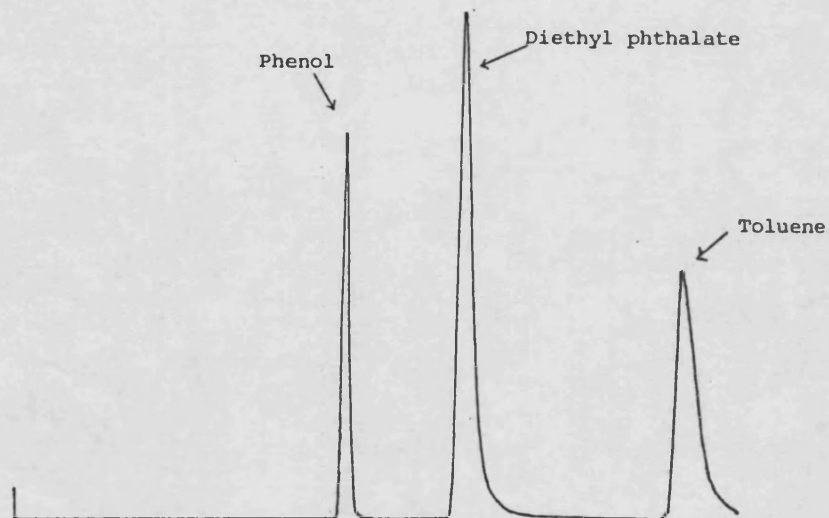


70 eV EI mass spectrum of PCB 189

APPENDIX 2.

Original test chromatogram and re-run of test mixture for
Polymer PLRP-S column after 18 months of use.

Serial No:	10M-RPS1-11-3	Eluent:	7:1 ACN/H ₂ O	Plates/metre (1/2ht):	46838
Particle Size (μm):	10	Flow Rate:(ml/min):	1	Plates/metre (5.σ):	38489
Pore Type:	100.	Col. Pressure (bar):	14	Symmetry (@ 10%):	1.39
Length/I.D.(mm):	300 x 7.5	Detector:	UV	Elution Time (Sec):	662.0
		Test Material:	TEST MIX	Width (1/2ht; sec):	13.14
		Inj. Vol. (μl):	0.5	Width (5σ; sec):	39.90



ORIGINAL TEST CHROMATOGRAM

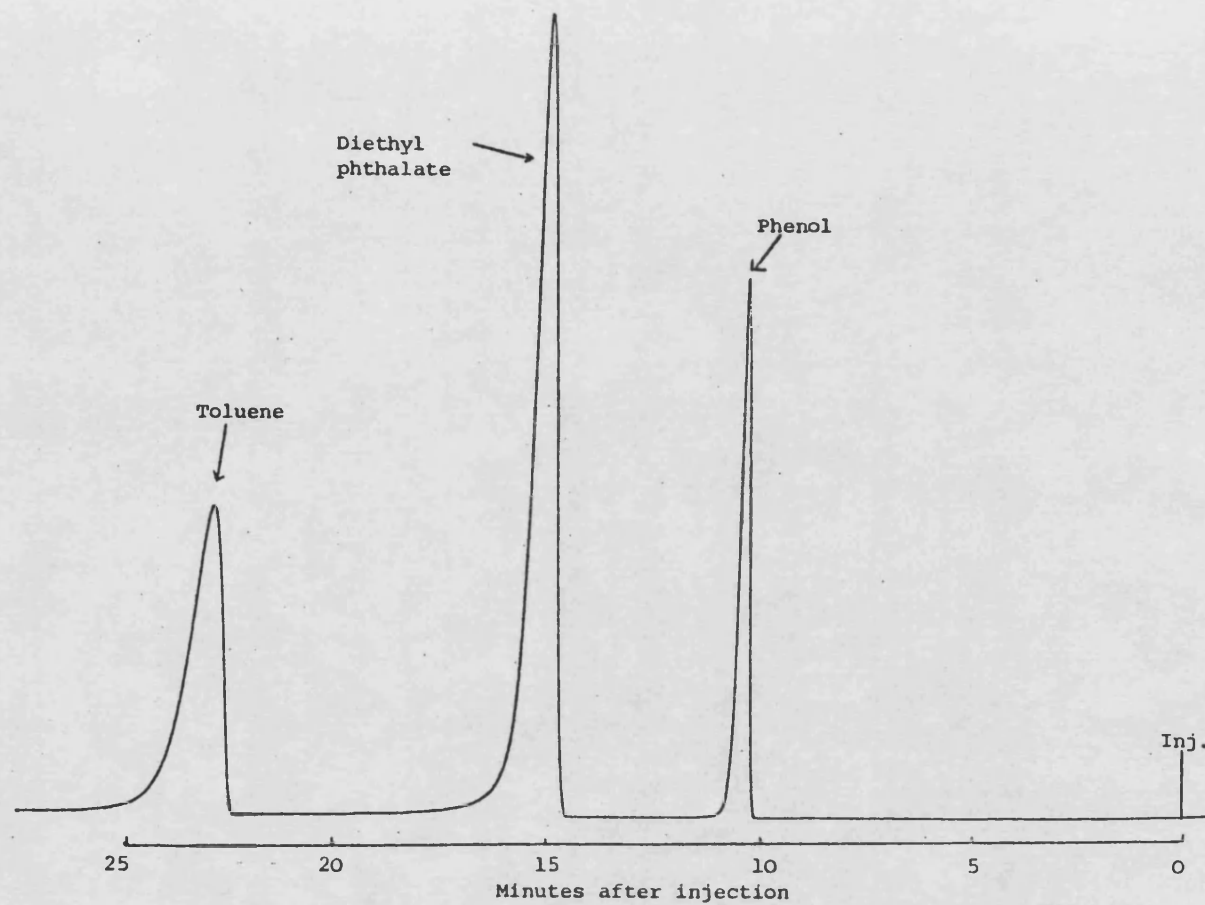
Elution times	(Secs.)
Phenol	662.0
Diethyl phthalate	897.0
Toluene	1334.0



POLYMER
LABORATORIES LTD.

Column: PLRP-S 10μ 100 A
Part No: 1112-6100

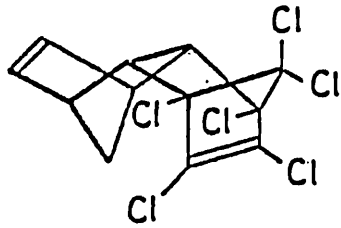
Column Test
Chromatogram



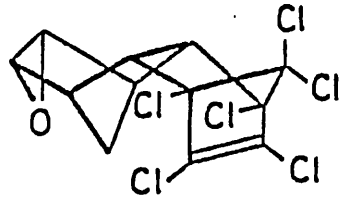
Test mixture run on Polymer PLRP-S column after about 18 months use.
Chromatographic conditions as for original test chromatogram.

APPENDIX 3.

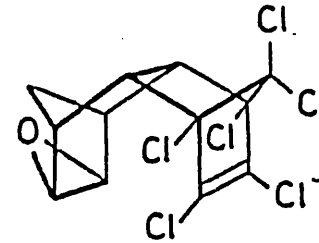
Structures of selected organochlorine compounds.



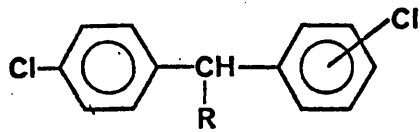
ALDRIN



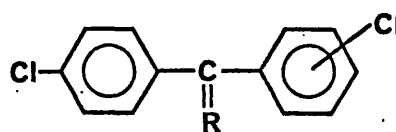
DIELDRIN



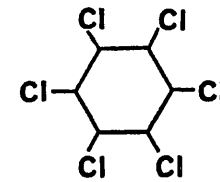
ENDRIN



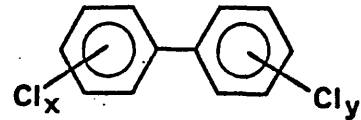
$R=CCl_3$ p,p' DDT
 o,p' DDT
 $R=CHCl_2$ p,p' TDE
 o,p' TDE



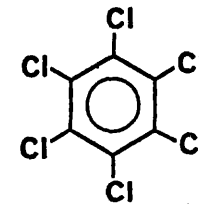
$R=CCl_2$ p,p' DDE
 o,p' DDE
 $R=CHCl$ p,p' TDE olefin
 o,p' TDE olefin



HCH isomers



POLYCHLORINATED BIPHENYL (PCB)



HEXACHLOROBENZENE (HCB)

APPENDIX 4.

Copy of questionnaire supplied to milk sample donors.

CONFIDENTIAL

Specimen questionnaire for mothers participating in the study

Name of Mother Reference No.

Date of birth (mother)

Weight of mother

Height of mother

Do you smoke?

Are you taking any drugs at present? (Please list them)

Address

Date of birth (baby)

Weight of infant at birth

Please list any problems associated with baby (e.g. health problems, rashes, irritability etc.)

Number of other children: please fill in the table

Child No.	date of birth	was it breast-fed (yes/no)	how long for?
1			
2			
3			
4			
5			

1
2
3
4
5

Please give brief details of places you have lived since birth,
 e.g. birth-1965 Weston, Bath
 1965 -1976 On a farm, Wiltshire
 1976 -present Oldfield Park, Bath

DATES

LOCATION

Please list your places of work and occupation since leaving school

Employer

Occupation

Have you ever been exposed to agricultural and/or garden chemicals, for example, crop-spraying. Have you or do you work, live, or otherwise spend time near land where pesticides, insecticides, or herbicides are in regular use. If so please give details e.g. at what age, type and duration of exposure.-

Thank-you for your time and co-operation. This data will be treated in the strictest confidence.

LJN:ethics

APPENDIX 5.

Materials sources.

MATERIALS SOURCES.

All OCPs were obtained as Certified Reference Materials from the National Physical Laboratory, Teddington, Middlesex, UK.

Aroclors 1242, 1248, 1254 and 1260 were provided by Dr. M. Cooke, Department of Inorganic Chemistry, University of Bristol, Bristol, UK.

All solvents used were of HPLC grade and were supplied by Fisons, Loughborough, UK.

Silica gel 60 (70-230 mesh ASTM) "Merck" (7734) for column chromatography was obtained from BDH, Poole, Dorset UK.

Florisil (60-100 mesh ASTM) was obtained from Aldrich Chemical Co., Billingham, Dorset, UK.

Sodium sulphate (anhydrous) was obtained from BDH, Poole, Dorset, UK.

Keiselguhr was obtained from BDH, Poole, Dorset, UK.

Fibrous cellulose was supplied by Whatman, Maidstone, UK.

Cholesterol was obtained from Sigma Chemical, Poole.

Dorset, UK.

Witepsol H15 was supplied by J.M. Loveridge, Southampton, UK.

Hypersil Phenyl HPLC packing material was obtained from Shandon Southern, Runcorn, UK.

Zorbax ODS preparative HPLC column was supplied by Du Pont (UK) Ltd., Stevenage, Hertfordshire, UK.

Polymer PLRP-S semi-preparative HPLC column was supplied by Polymer Laboratories Ltd., Shropshire, UK.

Hamilton PRP-1 HPLC column was supplied by Hamilton Company, PO Box 10030, Reno, Nevada, USA.

OV-1 and OV-17 capillary GC columns were obtained from Alltech England, Carnforth, Lancashire, UK.

DB-210 (50% trifluoropropyl) capillary GC column was obtained from J & W Scientific, Rancho Cordova, California, USA.

CP Sil 5 column was obtained from Chrompack UK, London SW 16, UK.

Note

Clean-up and separation of chlorobiphenyl isomers after synthesis by Cadogan coupling using preparative high-performance liquid chromatography

M. P. SEYMOUR, I. W. DUNCAN, T. M. JEFFERIES* and L. J. NOTARIANNI

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(Received July 8th, 1986)

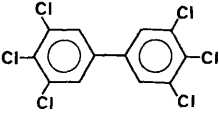
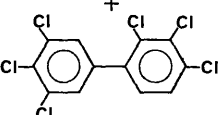
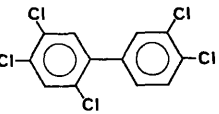
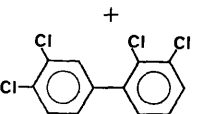
The analysis of environmental samples for polychlorinated biphenyl (PCB) content is a common procedure. There are 209 possible PCB congeners with many exhibiting widely differing toxicities¹. Levels of PCB contamination are generally expressed as a "total PCB" figure, usually obtained by a gas chromatographic (GC) pattern-matching technique or by a perchlorination method. Doubts have been expressed as to the accuracy and precision of these approaches to the quantification of PCBs^{2,3}. Furthermore, these methods provide little or no information with regard to the identities of the individual congeners present in the sample, thus rendering the analysis of little value for toxicological evaluation. Consequently, the trend is now towards congener-specific analyses for PCBs using capillary GC methods^{2,4}. Such methods require the use of individual congeners either as surrogate standards^{5,6} or, preferably, as individual standards for each chlorobiphenyl analyte². Unfortunately, less than half of the 209 congeners are available commercially and so workers frequently have to resort to in-house synthesis of their own standards^{7,8}. Syntheses are relatively straightforward and for most chlorobiphenyls are achieved by the coupling of a chloroaniline-generated radical to a chlorobenzene in an excess of the chlorobenzene^{7,9}. Unfortunately, the yields of such syntheses are low and produce a number of by-products. Additionally the use of a chlorobenzene with non-equivalent hydrogens gives rise to a mixture of chlorobiphenyl isomers. The clean-up of the chlorobiphenyls produced by this type of coupling (Cadogan coupling¹⁰) is laborious and the separation of chlorobiphenyl isomers can be extremely difficult, often being achieved by using repeated preparative thin-layer chromatography (TLC)⁷. Described below is a clean-up procedure followed by a preparative high-performance liquid chromatographic (HPLC) separation method for selected pairs of chlorobiphenyl isomers produced by Cadogan coupling.

EXPERIMENTAL AND RESULTS

The coupling reactions were chosen such that no more than two chlorobiphenyl isomers would be produced. The Cadogan coupling was performed in an excess of the selected chlorobenzene and after the reaction had taken place the first step in the clean-up procedure was the removal of this excess. This was done by

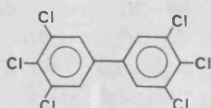
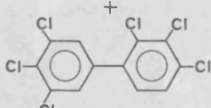
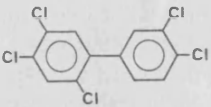
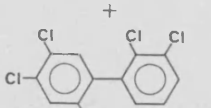
distillation under vacuum. The residue, which was a deep red-brown colour, was dissolved in a minimum of hexane and was transferred to the top of a 300 mm × 50 mm I.D. column of silica (Kieselgel 60, 70–230 mesh ASTM, Merck; wet-packed in hexane) and eluted with hexane. The chlorobiphenyls and any remaining chlorobenzene were virtually unretained by the silica and were the first components of the residue to elute from the column. Most of the by-products of the reaction appeared to be coloured compounds ranging from a deep red-brown to a light yellow colour. These were separated by the silica-hexane system with the darker pigments being retained at the top of the column and the lighter ones eluting close to the chlorobiphenyls. It was found necessary to collect a little of the light-yellow band with the chlorobiphenyl fraction in order to obtain maximum recovery of the chlorobiphenyls. The chlorobiphenyl fraction was then evaporated to dryness using a rotary evaporator.

TABLE I
PCB ISOMER PAIRS STUDIED AND MOBILE PHASE USED FOR THEIR SEPARATION

<i>Identity</i>	<i>Congener number (after Ball- schmitter and Zell¹¹)</i>	<i>Synthesis starting materials</i>	<i>Mobile phase for separation</i>
 3,3',4,4',5,5'- Hexachlorobiphenyl	169	3,4,5-Trichloro- aniline + 1,2,3-trichloro- benzene	THF-water 75:25 (v/v)
 2,3,3',4,4',5'- Hexachlorobiphenyl	157		
 2,3',4,4',5- Pentachlorobiphenyl	118	2,4,5-Trichloro- aniline + 1,2-dichlorobenzene	THF-water 65:35 (v/v)
 2,2',3',4,5- Pentachlorobiphenyl	97		

distillation under vacuum. The residue, which was a deep red-brown colour, was dissolved in a minimum of hexane and was transferred to the top of a 300 mm × 50 mm I.D. column of silica (Kieselgel 60, 70–230 mesh ASTM, Merck; wet-packed in hexane) and eluted with hexane. The chlorobiphenyls and any remaining chlorobenzene were virtually unretained by the silica and were the first components of the residue to elute from the column. Most of the by-products of the reaction appeared to be coloured compounds ranging from a deep red-brown to a light yellow colour. These were separated by the silica-hexane system with the darker pigments being retained at the top of the column and the lighter ones eluting close to the chlorobiphenyls. It was found necessary to collect a little of the light-yellow band with the chlorobiphenyl fraction in order to obtain maximum recovery of the chlorobiphenyls. The chlorobiphenyl fraction was then evaporated to dryness using a rotary evaporator.

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 2,2',3',4,5- Pentachlorobiphenyl	97		

The isolated isomer fractions were evaporated to dryness in a rotary evaporator (starting at a low temperature to remove the THF, then increasing the temperature to remove the water). The individual isomers were then recrystallised from methanol and dried. Identities and purities were confirmed using capillary GC, ^1H and ^{13}C NMR and mass spectrometry. Purities as determined by capillary GC are shown in Table II.

TABLE II

PURITY OF PCB ISOMERS BY CAPILLARY GC AFTER CLEAN-UP SEPARATION AND RECRYSTALLISATION

PCB	Purity* (%)
97	99.0
118	>99.5
157	99.4
169	98.0

* Assuming equal response factors for all peaks in the chromatogram.

DISCUSSION

Chlorinated biphenyls are very non-polar substances and are not retained by silica. Reversed-phase systems provide more retention and therefore better conditions for attempting a separation of chlorobiphenyl isomers. The isomers produced by the Cadogan synthesis are inevitably very similar, containing the same number of chlorines and the same substitution pattern in one of the rings (the ring provided by the chloroaniline, see Fig. 2). This leaves relatively few differences to exploit in order to achieve a separation. However, although the chlorines in the ring provided by the chlorobenzene will have the same pattern with respect to each other in all the chlorobiphenyl products, the pattern of substitution of the chlorines with respect to the ring bridge will be different (see Fig. 2). This has two main consequences; firstly, increasing the degree of *ortho* substitution will affect the dihedral angle between the rings and restrict the rotation about the ring bridge^{1,2}. Secondly, the difference in the

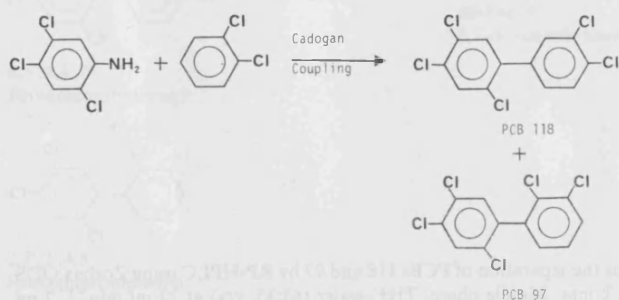


Fig. 2. Synthesis of PCBs 118 and 97 by Cadogan coupling.

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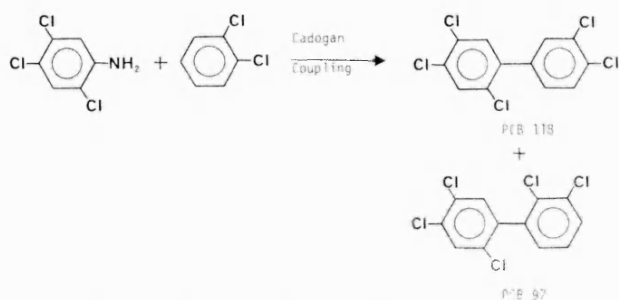


Fig. 2. Synthesis of PCBs 118 and 97 by Cadogan coupling.

since eliminating such contaminants by recrystallisation could prove to be very difficult.

CONCLUSION

The use of a preparative ODS column with a THF-water mobile phase has been shown to exhibit useful selectivity for the separation of chlorobiphenyl isomers after synthesis and preliminary clean-up. This HPLC approach is deemed to be more convenient and less time-consuming than repeated preparative TLC.

It should be borne in mind that certain chlorobiphenyl congeners are very toxic both by ingestion and skin contact. Appropriate precautions should be taken to prevent contamination of workers, and equipment should be decontaminated after use.

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Limitations in the Use of Nickel Boride Dechlorination for the Analysis of Polychlorinated Biphenyls

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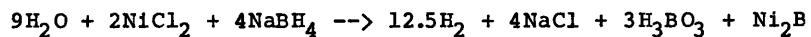
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The determination of polychlorinated biphenyls (PCBs) in environmental samples presents the analyst with many problems. PCBs occur as complex mixtures in the environment at variable concentrations ranging from the low ppb to the ppm level. Most sample extracts need to be subjected to an extensive cleanup procedure prior to GC analysis. An electron-capture detector is the most commonly used detector but cleanup procedures may still let electron-capturing species remain in the extract, so the identities of the eluting peaks must be confirmed.

In order to overcome some of these problems, perchlorination of the PCBs has been used; this gives rise to one GC peak (decachlorobiphenyl) which is well removed from most interfering peaks, but this technique has been found to be qualitatively and quantitatively unreliable (De Kok et al. 1982; Duinker et al. 1980). Dechlorination has also been proposed (Dennis et al. 1979; De Kok et al. 1980; Kennedy et al. 1982) but we find that this also is unsatisfactory and do not recommend its use.

MATERIALS AND METHODS

A nickel boride dechlorination method (Dennis & Cooper 1977; Dennis et al. 1979) was employed whereby a precipitate of nickel boride is produced *in situ* according to the reaction:



(Dennis & Cooper 1975)

A total of fifteen experiments were performed; they fell into three groups:

i) Solutions of each of three Aroclors (1242, 1248 and 1260, 5 mL of approximately 150 µg/mL each) were placed in 250-mL round bottomed (r.b.) flasks. 2-Propanol (25 mL) and 2M NiCl₂ (aq. 0.5 mL) were added.

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To each of these was added, dropwise, 30 mmol of NaBH_4 dissolved in 4 mL of distilled water. Thus, each reaction mixture comprised approximately:

PCB : Ni^{2+} : NaBH_4
2.8 μmol :1000 μmol :30000 μmol

in 25 mL of 2-propanol + 4.5 mL of water + 5 mL of n-heptane.

The mixture was allowed to react, with stirring, for 2 h at room temperature after which time 100 mL of distilled water was added. The reaction products were then extracted with n-heptane and made up to a known final volume.

ii) Solutions of each of the three Aroclors in n-heptane (5 mL of 15 $\mu\text{g}/\text{mL}$) were placed in 250-mL r.b. flasks and evaporated just to dryness over air. NaBH_4 powder (500 mg) was added to each. 2-Propanol (0.5 mL) and distilled water (0.5 mL) were added; then 2M NiCl_2 (aq. 0.3 mL) was added dropwise. So, each reaction mixture comprised approximately:

PCB : Ni^{2+} : NaBH_4
0.3 μmol : 600 μmol :13000 μmol

in 5 mL of 2-propanol + 0.8 mL of water.

The mixture was allowed to react, with stirring, at room temperature for 75 min. Distilled water (150 mL) was then added to the flasks followed by concentrated HCl dropwise until no further hydrogen was produced. The mixture was then steam extracted for 3 h by using a Nielson-Kryger unit (Veith & Kiwus 1977) into n-heptane and diluted to a known final volume.

iii) A solution of PCB (4 mL of 131 $\mu\text{g}/\text{mL}$ Aroclor 1260 + 125 $\mu\text{g}/\text{mL}$ Aroclor 1242) in n-heptane was pipetted into a 25-mL r.b. flask. This was evaporated just to dryness in a rotary evaporator at 45°C. NaBH_4 powder (1.0 g) was added followed by 8 mL of 2-propanol. 2M NiCl_2 (aq. 1 mL) was then added, dropwise, so the reaction mixture comprised approximately:

PCB : Ni^{2+} : NaBH_4
3.3 μmol :2000 μmol :27000 μmol

in 8 mL 2-propanol + 1 mL of water.

The reaction was allowed to proceed for 45 min at room temperature. After this time the reaction mixture was transferred quantitatively to a 100-mL separating funnel with about 70 mL of distilled water and extracted with n-heptane. The extract was made up to a known final volume.

All organic solvents were of high purity and analyses were performed by using capillary GC with a 25 m 0.35 mm i.d. SE-30 equivalent column. Flame ionisation detection was employed. Quantitative work was performed using either 4-chloro-m-cresol or 4,4'-dibromobiphenyl as internal standard.

RESULTS AND DISCUSSION

Experiments in group (i) gave seven main peaks plus a very small amount of reduced biphenyl (probably phenylcyclohexane) for all three Aroclors studied (see Fig. 1). The proportions of the peaks varied slightly depending on the Aroclor used but biphenyl was the main product in every case. The six peaks eluting after biphenyl are mono- and dichlorobiphenyls - probably ortho isomers since, due to steric effects, ortho chlorines are more difficult to remove from the biphenyl nucleus than meta and para chlorines. The presence of the chlorinated biphenyls also confirms the observation of Kozloski (1985) that n-alkanes reduce the efficiency of the nickel boride catalyst. There was no significant residue of higher chlorinated biphenyls left in the sample. Recoveries were not determined.

Group (ii) experiments gave biphenyl in good yield but left a small amount of monochlorobiphenyl remaining and also produced significant amounts of reduced biphenyl (see Fig. 2). The recovery results are shown in Table 1.

Table 1. Recoveries from group(ii) dechlorination experiments.

PCB Source	Mean recovery of biphenyl expressed as percentage of theoretical yield.	Mean total recovery expressed as percentage of theoretical yield of biphenyl.*
Aroclor 1242	74 %	83 %
Aroclor 1248	75 %	80 %
Aroclor 1260	58 %	73 %

* Based on total peak areas.

We have previously validated the efficiency of the steam extraction method for biphenyls and PCBs (**unpublished results**) and found it to be almost 100% efficient at the levels being considered when extracting from aqueous media.

The production of reduced biphenyl in significant amounts demonstrates that the activity of the catalyst is not immediately destroyed by the addition of the concentrated HCl. The nickel boride dissolves under the action of the acid after boiling for about three min in the steam extraction apparatus, to give a pale green solution (presumably of NiCl_2). In the time between heating the reaction mixture and the dissolution of the catalyst, the conditions in the flask seem to be suitable for reducing the biphenyl nucleus. Under these conditions, it may be expected that any chlorines remaining on the biphenyl ring system would be removed. That this is not so may be due to the chloride ions from the acid in some way attacking the aromatic system, assisted by the catalytic surface.

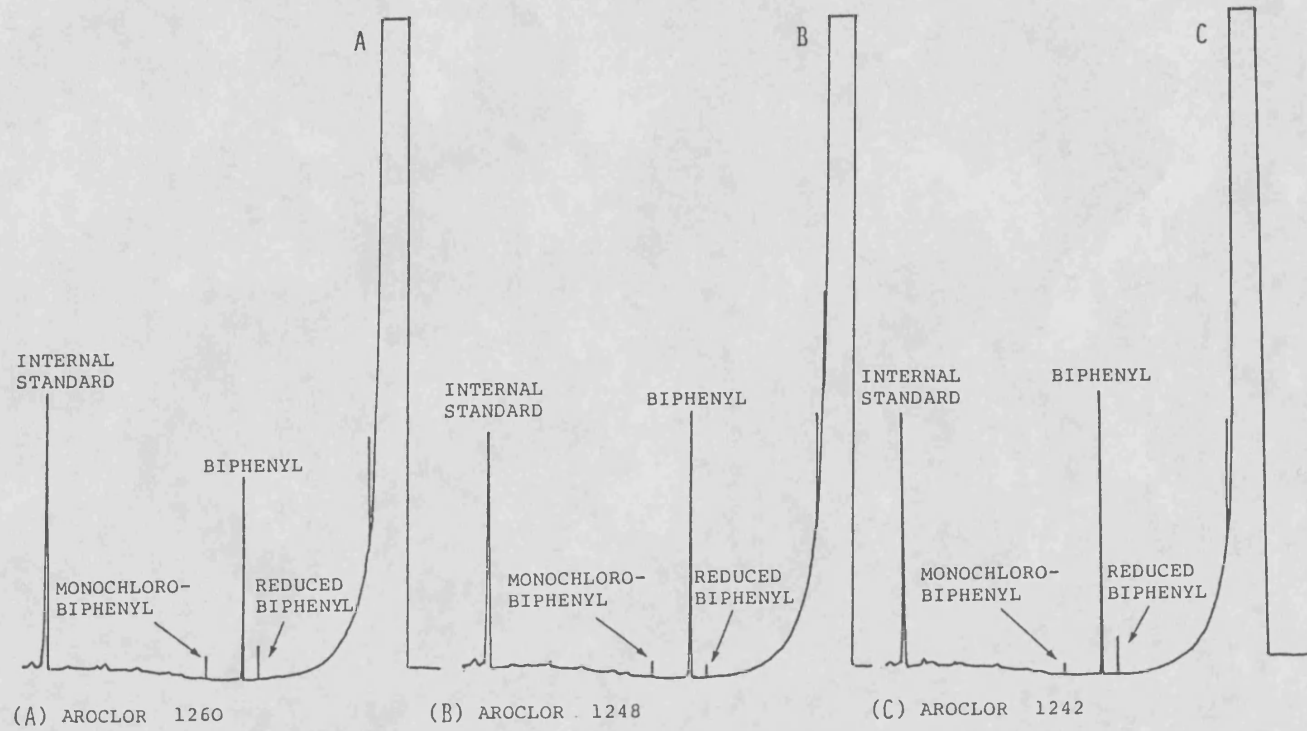


Figure 1 Group (i) Dechlorination Experiments

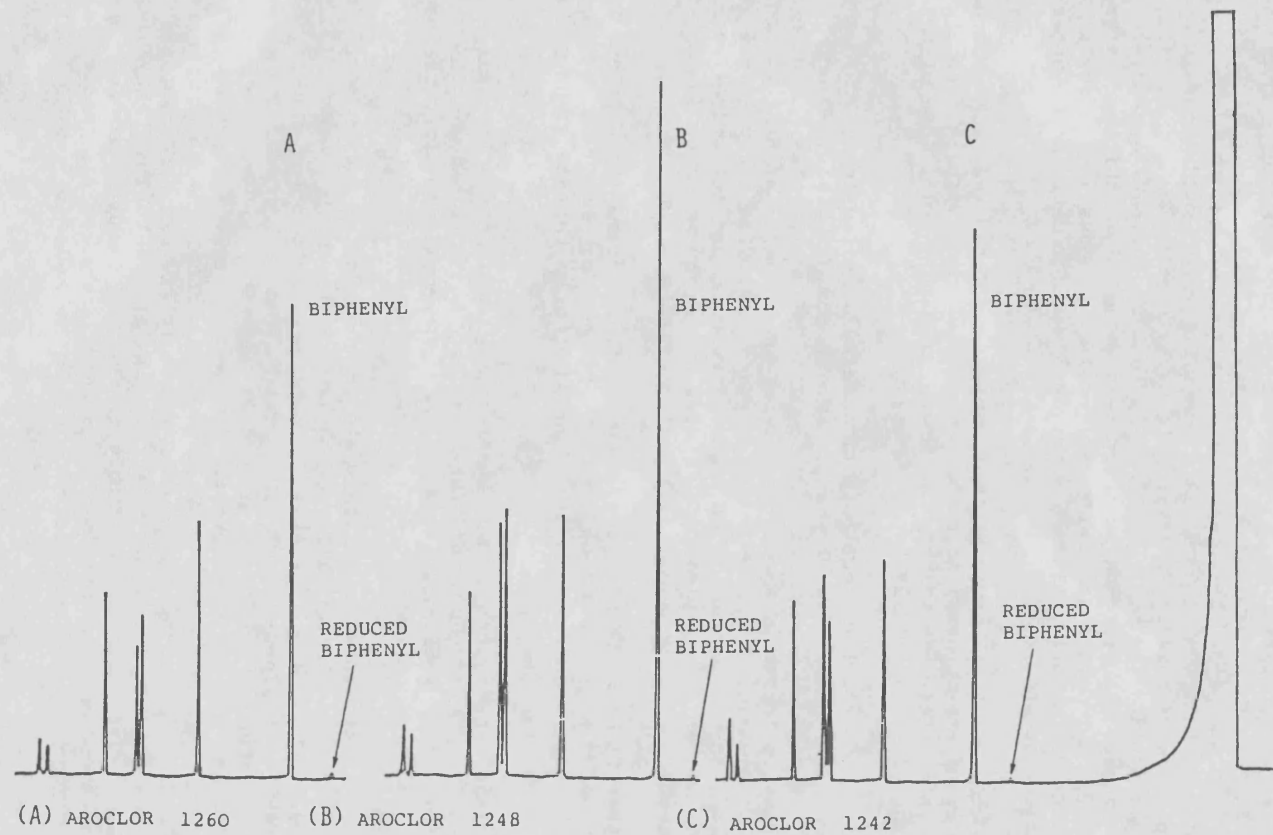


Figure 2 Group (ii) Dechlorination Experiments

Experiments in group (iii) illustrate the fact that it is possible to obtain biphenyl as the sole product although a small amount of a monochlorobiphenyl (about 3% of total peak area) did remain in one sample (see Fig. 3). Recoveries for replicates varied from 75% to 82% of theoretical yield of biphenyl.

It can be seen that this dechlorination method has many shortcomings. The procedure is sensitive to reaction conditions and extraction methods. It is noteworthy that De Kok et al. (1982) found dechlorination with LiAlH_4 also gave variable and possibly inaccurate results. The best recovery obtained in this laboratory was 85% of theoretical biphenyl yield. The loss may be due to a number of factors including incomplete dechlorination, incomplete extraction and loss of biphenyl by volatilisation.

Biphenyl is a volatile compound and we have found that any attempt to evaporate biphenyl solutions to dryness, particularly over air or in a rotary evaporator, can result in almost total loss of the biphenyl, particularly at low levels.

Another drawback of dechlorination methods is the increase in the limit of detection. Biphenyl is not significantly electron-capturing so GC-ECD cannot be used. Consequently, a flame ionisation detector must be employed or, if using an HPLC method, a UV detector. These detectors may not be sufficiently sensitive to detect the low levels of biphenyl produced by dechlorination of environmental samples. GC-MS does give a much lower limit of detection but may not be routinely available to the analyst.

Biphenyl itself is also an environmental contaminant and unless it is removed (e.g. by oxidation) from samples prior to the dechlorination step, it could artificially inflate the result obtained for PCBs.

Overall, the method is not satisfactory for the routine analysis of PCBs in environmental samples. The procedure is not sufficiently robust or reliable and the reaction requires the use of expensive reagents in considerable quantities.

The current trend in PCB analysis is towards the use of accurate congener-specific capillary GC methods (Ballschmitter & Zell 1980; Bush et al. 1983; Safe et al. 1985). This allows the analyst to produce congener-by-congener assay results for toxicological evaluation. It is known that different PCB congeners display different toxicological profiles (Poland & Glover 1977; Safe 1984) and so analyses which provide the toxicologist with congener-specific data on the chlorinated biphenyls are far more meaningful than a method, such as perchlorination or dechlorination, which merely gives a single (possibly inaccurate) result for total PCB concentration. Congener-specific methods are also less involved in that the chemical modification of the PCBs is avoided thus eliminating one step in the assay procedure.

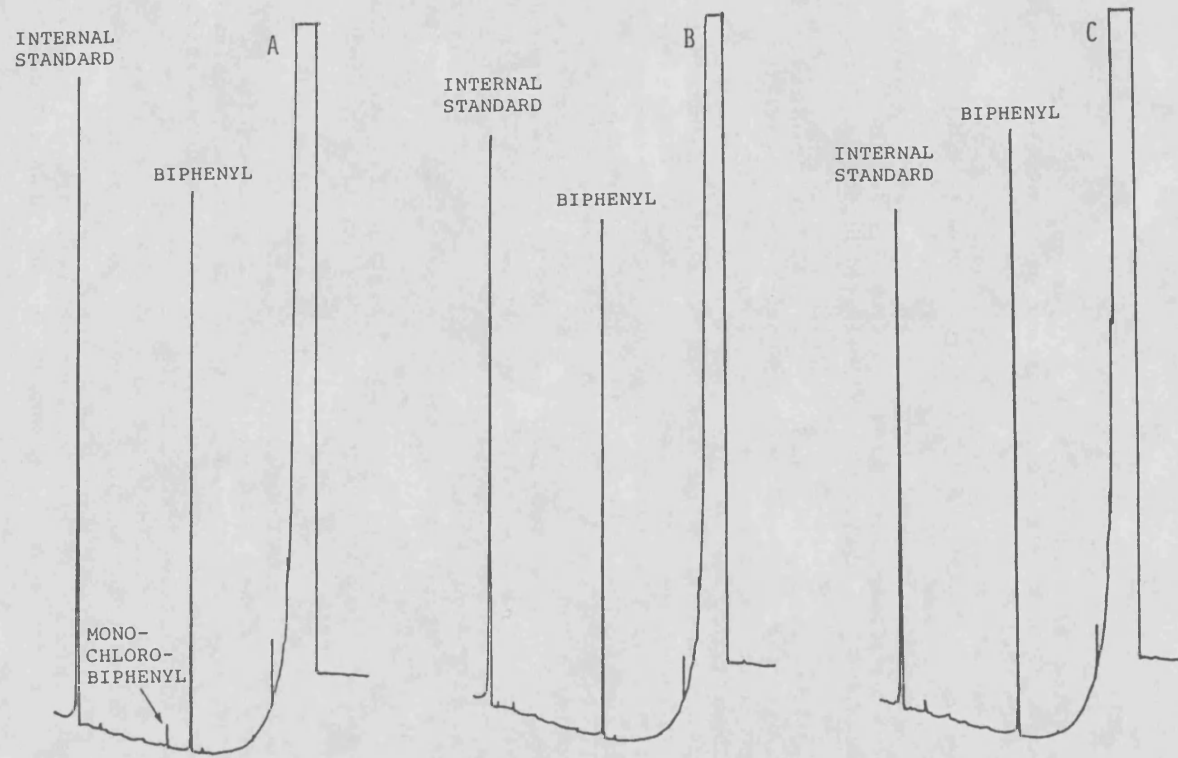


Figure 3 Replicate Group (iii) Dechlorination Experiments

We believe that dechlorination methods of PCB analysis should, on the whole, be rejected for the reasons outlined above, in favour of congener-specific analyses which, in the absence of major interferences, seem to provide more accurate and meaningful data.

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Large-scale Separation of Lipids From Organochlorine Pesticides and Polychlorinated Biphenyls Using a Polymeric High-performance Liquid Chromatographic Column

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The separation of organochlorine pesticides and polychlorinated biphenyls from a high concentration of lipid has been achieved using a polymeric column designed for high-performance liquid chromatography. The method, which can be used as a clean-up step in residue analysis procedures, has a high lipid capacity but low solvent consumption. Recoveries of both lipid and selected organochlorine compounds were routinely over 90% at p.p.m. levels.

Keywords: Polymeric high-performance liquid chromatographic columns; organochlorine pesticides; polychlorinated biphenyls; lipids; clean-up procedure

The analysis of lipid-containing matrices for residues of organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) is a common procedure.¹⁻³ All the methods require the separation of the residues from the lipids prior to the determination of the OCPs and PCBs by gas chromatography. The removal of the lipids is usually carried out by low-resolution column chromatography using an adsorbent such as silica, alumina or Florisil as the stationary phase.^{3,4} Low-resolution gel permeation chromatography has also been used by several workers in order to achieve this type of separation.⁵⁻⁹ However, the advent of high-performance polymeric columns has provided the opportunity to achieve separation whilst taking advantage of the higher resolution and considerably lower solvent consumption offered by HPLC technology.

Experimental

Apparatus

The column used was a 300 × 7.5 mm i.d. Polymer PLRP-S (10 μm, 100 nm pore diameter) column (Polymer Laboratories, Shropshire, UK). This was mounted in a Du Pont Instruments 830 Preparative HPLC unit with a Rheodyne injection valve fitted with a 2.0-ml injection loop. Detection was achieved using an LDC Spectromonitor III refractive index detector set at 2 × 10⁻⁴ ΔRI f.s.d.

Materials

The heptane and propan-2-ol used were both of HPLC grade (Fisons, Loughborough, UK). The OCPs were obtained from the National Physical Laboratory (Teddington, Middlesex, UK). The triglycerides were obtained in the form of the suppository base Witepsol H15 (J. M. Loveridge, Southampton, UK), which is a mixture of triglycerides of saturated fatty acids designed to melt at body temperature. The cholesterol was supplied by Sigma Chemical (Poole, Dorset, UK).

Method

A mobile phase composed of 20% V/V propan-2-ol in heptane was prepared and filtered through a 0.5-μm PTFE membrane filter.

Individual solutions of each of the following sixteen OCPs were prepared in heptane: α-hexachlorocyclohexane (α-HCH), β-HCH, γ-HCH (lindane), δ-HCH, *p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDE, *o,p'*-DDE, *p,p'*-TDE, *o,p'*-TDE, *p,p'*-TDE olefin, chlordane, aldrin, dieldrin, endrin and hexachlorobenzene (HCB). The concentration of pesticide in each solution was approximately 0.03% m/V, except for β-HCH, which, because of its poor solubility, was used as a saturated solution.

The following PCB and lipid solutions were also prepared in heptane: 0.5% m/V Aroclor 1260 + 15% m/V triglycerides + 0.5% m/V cholesterol; 0.5% m/V Aroclor 1242 + 15% m/V triglycerides + 0.5% m/V cholesterol; 45 p.p.m. Aroclor 1242 + 48 p.p.m. Aroclor 1248 + 102 p.p.m. Aroclor 1260 + 15% m/V triglycerides; 15% m/V triglycerides + 0.5% m/V cholesterol.

The mobile phase was pumped through the column at a flow-rate of 2.3 ml min⁻¹ with the injection system and column held at 35 °C in order to assist the solubility and reduce the viscosity of the lipid solutions. The column eluate was passed through the refractive index detector. A 2.0-ml volume of each solution was injected and the output of the RI detector was recorded on a chart recorder. The volume range after the point of injection over which each material eluted is given in Table 1. (For example, α-HCH begins to elute 16.8 ml after

Table 1. Elution fractions of various organochlorine pesticides and polychlorinated biphenyls. Column total exclusion volume approximately 8.7 ml

Material	Elution fractions/ml
Triglycerides, 15% + cholesterol, 0.5%	9.2-14.7
PCBs (Aroclor 1242 with Aroclor 1260), 0.5%	16.1-23.9
α-HCH	16.8-21.2
β-HCH	16.8-20.7
γ-HCH (lindane)	18.4-23.5
δ-HCH	15.4-17.9
<i>p,p'</i> -DDT	15.8-19.6
<i>o,p'</i> -DDT	14.7-18.9
<i>p,p'</i> -DDE	15.2-19.8
<i>o,p'</i> -DDE	15.2-18.6
<i>p,p'</i> -TDE	16.6-21.2
<i>o,p'</i> -TDE	15.6-20.9
<i>p,p'</i> -TDE olefin	16.1-21.2
Chlordane	In lipid-17.9
Aldrin	In lipid-17.3
Endrin	15.2-18.6
Dieldrin	15.0-18.9
HCB	14.7-19.1
Heptane	8.7-13.3

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the point of injection and has completely eluted 21.2 ml after injection at a flow-rate of 2.3 ml min^{-1} .) The lipid and PCB fractions for the p.p.m. level of Aroclor 1242/1248/1260 + 15% *m/V* triglyceride solution were collected for recovery studies. The recoveries of the lipid were determined gravimetrically. The recoveries of the PCBs were determined by a GC pattern-matching technique using 4-chloro-*m*-cresol as an internal standard.

Results and Discussion

All the compounds were eluted within 24 ml and all except aldrin and the technical mixture chlordane were completely separated from the lipid. This means that all the OCPs studied, except aldrin and chlordane, and all the PCB mixtures studied can be separated completely from up to 300 mg of lipid injected in a volume of only 2.0 ml with a total elution volume of less than 24 ml. This represents a considerable saving in consumables over conventional adsorbent or gel permeation clean-up methods. In addition, this procedure using a polymeric column allows the lipid fraction to be determined gravimetrically. This is not usually possible with adsorption chromatography clean-up procedures.

The use of the RI detector and the chart recorder provided a visual picture of the chromatographic process. The limit of detection of the RI detector was far above that which would be required to detect the trace levels of OCPs and PCBs encountered in most lipid-containing environmental samples, but the lipids were easily detected with the peak going well off-scale on the chart recorder. The use of the detector and recorder permitted the lipid and OCP - PCB fractions to be collected at exactly the right times without having to measure elution volumes. Fig. 1(a) shows a typical chromatogram for an injection of 2.0 ml of Aroclor 1242 (0.5% *m/V*) + cholesterol (0.5% *m/V*) + triglycerides (15% *m/V*) and Fig. 1(b) shows the result for a similar injection using Aroclor 1260 instead of 1242. The sharp rise and fall of the unretained lipid band should be noted. The PCB peak in both Fig. 1(a) and (b) is broad. This is due not only to the large injection volume

employed but also to the fact that the Aroclors are mixtures of a number of chlorobiphenyl compounds, each showing slightly different retention characteristics. The more highly chlorinated PCBs found in Aroclor 1260 tend to elute slightly earlier (*i.e.*, nearer the lipid) than the less chlorinated PCBs found in Aroclor 1242. Slight tailing is evident in both Aroclor peaks.

The recoveries of lipids in four replicate samples were determined to be 96.3, 96.6, 98.0 and 98.9%. The recoveries of PCBs were typically found to lie in the range 91–101% with no discernible discrimination between the different PCB congeners. Our reason for developing this procedure was to provide a more rapid and economic method for the isolation of OCP and PCB residues in human milk extracts than those methods currently available. The results so far indicate that at the 1–10 p.p.b. level in 10 ml of milk (10–100 ng), we are obtaining recoveries greater than 70% for the entire extraction, HPLC isolation and capillary GC - ECD analytical procedure. Unfortunately, endrin has been noted to be "lost" occasionally. If this is due to the HPLC step, it may be that the high fat concentration causes the endrin to elute faster and so to remain in the lipid fraction. This effect has not been noted with any other compound and the fact that it does not always occur suggests that collecting the OCP - PCB fraction closer to the lipid fraction may cure the problem.

Fig. 2 shows the results obtained by injecting 2.0 ml of the 0.03% *m/V* *p,p'*-DDT solution. The large off-scale peak on the chromatogram is a solvent front due to the heptane, not to any lipid in the sample. Again, there is slight tailing in the *p,p'*-DDT peak and the peak is broad, but it is fairly well retained and would be well removed from any lipid in the sample.

The elution volumes given in Table 1 show that, using this system, all the OCPs and PCBs studied (except for aldrin and chlordane) should be completely resolved from up to 300 mg of lipid. The PLRP-S polymeric column employed in this study is described by Polymer Laboratories as a spherical, rigid macroporous polystyrene - divinylbenzene adsorbent. Its homogeneous aromatic structure and high surface area create an extremely hydrophobic packing with a pH range of 1–13

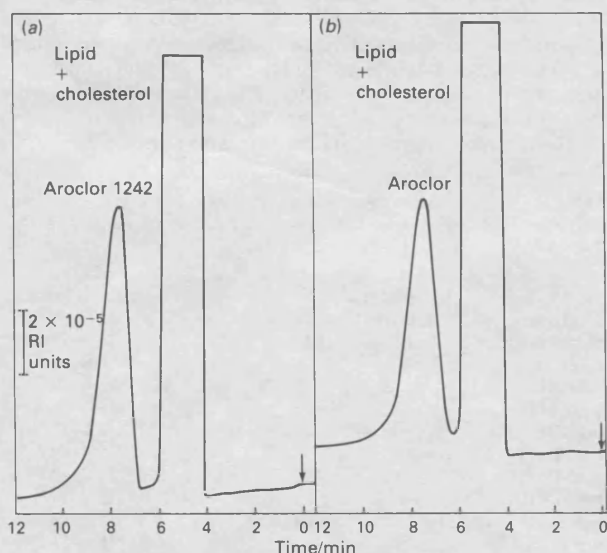


Fig. 1. (a), Injection of 2.0 ml of Aroclor 1242 (0.5% *m/V*) + cholesterol (0.5% *m/V*) + triglycerides (15% *m/V*) in mobile phase. Column, $300 \times 7.5 \text{ mm i.d.}$; polymer, PLRP-S; mobile phase, 20% propan-2-ol in heptane; flow-rate, 2.3 ml min^{-1} (b) Injection of 2.0 ml of Aroclor 1260 (0.5% *m/V*) + cholesterol (0.5% *m/V*) + triglycerides (15% *m/V*) in mobile phase. Chromatographic conditions as in Fig. 1(a)

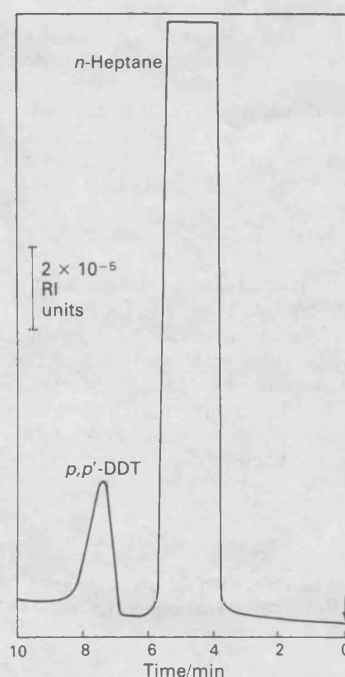


Fig. 2. Injection of 2.0 ml of *p,p'*-DDT (0.03% *m/V*) in heptane. Chromatographic conditions as in Fig. 1(a)

and a wide eluent compatibility. It is recommended for separations requiring reversed-phase conditions. However, we have employed it under normal-phase conditions because our sample was predominantly lipid. From previous experience with polymeric resins such as Amberlite XAD-4 (BDH Chemicals, Poole, UK) we knew that aromatic compounds were retained by these materials from aqueous solutions. Our objective was to achieve no retention of the lipid and slight retention of the OCPs and PCBs. The high lipid content of our sample essentially limited the choice of mobile phase to hydrocarbon, chlorohydrocarbon or THF-based systems. Chlorohydrocarbons were rejected because our original analytical procedure required GC with electron-capture detection. Hydrocarbons were selected because they permitted more polar solvents to be added in order to influence selectivity. Heptane was preferred to hexane because of its reduced volatility, and propan-2-ol was used as the more polar component because of its miscibility. It was found that increasing the propan-2-ol content increased the retention of the OCPs and PCBs but slightly reduced the ability of the mixture to dissolve large quantities of lipid. The composition of 20% V/V propan-2-ol in heptane provided a good separation of the OCP and PCB residues from a large quantity of lipid in a small elution volume. A discussion of the mechanism of retention of the residues is beyond the scope of this paper but almost certainly involves elements of adsorption, partitioning and size exclusion. It may well be possible to attain greater selectivity by using other polar solvents such as THF or dichloromethane and so to give more scope for the fractionation of the OCPs and PCBs should this be required.

Conclusions

It has been demonstrated that the use of a 300 × 7.5 mm i.d. Polymer PLRP-S high-performance column with 20% V/V propan-2-ol in heptane as the mobile phase is a useful clean-up procedure for the separation of lipids from OCP residues. The

system offers the advantages of low solvent consumption, high lipid capacity with the option of recovering the lipid fraction and the convenience of being able to monitor the separation with a refractive index detector. The disadvantages of the system include the inconvenience of having to process one sample at a time and the possibility of leaving a small residue of sample in the injection syringe. This latter problem, in our experience, appears to be negligible when using such large injection volumes.

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Routine Determination of Organochlorine Pesticides and Polychlorinated Biphenyls in Human Milk Using Capillary Gas Chromatography - Mass Spectrometry*

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The analysis of human milk for residues of polychlorinated biphenyls and organochlorine pesticides is a laborious and expensive procedure. This paper describes an approach to this analysis which is significantly less labour-intensive and more cost effective than traditional methods. These advantages were achieved by the adsorption of the milk on to a polar substrate prior to Soxhlet extraction, using a polymeric HPLC column for the clean-up of the extract followed by a highly selective capillary GC - MS analysis for the determination of the residues.

Keywords: *Polychlorinated biphenyl determination; organochlorine pesticide determination; residue analysis; human milk; capillary gas chromatography - mass spectrometry.*

Over the past few years there has been an increasing awareness of the need to monitor environmental levels of organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs). These compounds are known to display various types and degrees of acute toxicity,¹ but with a few exceptions acute toxicity is not usually a problem at environmental levels of contamination. The OCPs and PCBs generally occur in human milk at the low p.p.b. level, and few organochlorine compounds, except perhaps the chlorinated dibenzodioxins and dibenzofurans, exhibit significant acute toxicity at these concentrations. However, OCPs and PCBs are very persistent and we are all constantly exposed to low levels of these pollutants, mainly through our diet.² Because of this persistence and the ability of these compounds to accumulate in the body, together with the ubiquity of the contamination, the possibility of chronic toxicity, whatever form it may take, cannot be ruled out. It is largely for these reasons that government authorities around the world, in addition to individual laboratories, have directed considerable effort towards the monitoring of organochlorine compounds in the environment. It is important to monitor the contamination of human milk for two principal reasons. Firstly, the stability and high lipophilicity of these compounds mean that relatively high levels can accumulate in human milk lipids.³ Secondly, the infant receiving the milk has only poorly developed defences against an assault by chemicals and so may be more susceptible to any toxic effects. This does not mean that acute poisoning of infants through breast feeding is to be expected, but it may be speculated that this early exposure to toxic materials may pre-dispose the individual to certain disease states in later life.

Experimental

Extraction Procedure

The extraction of organohalogen residues may be accomplished by a number of methods. These procedures are often lengthy and require large amounts of solvent.⁴ As an alternative to methods based on the use of separating funnels or centrifugation, freeze-drying of the milk followed by Soxhlet extraction of the dried powder may be used. However, this may give a relatively dirty extract and the

freeze-drying process may result in the partial loss of some of the residues.⁵ In an attempt to avoid these problems, we adsorbed the milk on to a polar substrate and removed the water by gentle evaporation. The nature of the adsorbent used is very important. It must be very permeable to the solvent in order to allow efficient extraction through the thimble. It must also retain as much of the milk matrix as possible, *i.e.*, the protein, carbohydrates, electrolytes, residual water, etc., but not retain the organochlorine residues or lipids. We found that a 1 + 1 by volume mixture of fibrous cellulose (Whatman) and 60-100 mesh Florisil adequately fulfilled our requirements. The Florisil retained much of the unwanted material and the fibrous cellulose, with its diversity of particle sizes and geometries, served to make the Soxhlet bed permeable in addition to having some retentive properties of its own. In our method, a known amount of milk (about 10 ml) was added to two-thirds of a 30 × 80 mm Soxhlet thimble-full of the cellulose - Florisil adsorbent in a glass mortar and was mixed in for about 1 min. This was then placed in a fan oven at 60 °C for about 30 min in order to evaporate the water. The evaporation was aided by occasional re-mixing of the adsorbent substrate.

During the drying process, the milk constituents and organochlorine residues bound to the adsorbent allowing the water to evaporate but retaining the residues. The dried material was then transferred quantitatively with hexane washings directly into the Soxhlet thimble and was extracted overnight with hexane at a cycling rate of about 30 to 40 cycles h⁻¹ providing a solid - liquid extraction instead of the more usual liquid - liquid extraction. During the extraction, the milk components began to turn brown but much of this straw brown colour was retained by the adsorbent, although some of it passed into the extract. The extract was then passed through a hexane-washed filter-paper into a Kuderna - Danish evaporator and was reduced in volume to about 1.3 ml. This small volume of extract contained the OCP and PCB residues, a high proportion of milk lipids and residues of other milk components.

Clean-up Procedure

The clean-up procedure has to separate the organochlorine residues from all other material in the extract, and in addition it should permit the recovery of the lipid material in order to allow a gravimetric determination of the fat content to be carried out. There are three main approaches to sample clean-up in current use: acetonitrile partitioning, column chromatography using various stationary phases and solvent systems and gel permeation chromatography (GPC).⁶ Gel

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permeation chromatography has the advantages that the lipid is recovered and the column is re-usable. Solvent consumption, however, is high and fractionation of the residue components, if required, may be quite limited.

In order to avoid the use of large volumes of solvent, we used high-performance polymeric columns with a view to reducing the solvent consumption but maintaining separation.⁷ With a sample size approaching 2 ml, the column has to be at least semi-preparative in size and we used a Polymer Laboratories PLRP-S 300 × 7.5 mm column with 10- μ m particle size and 100-nm diameter pores. Using 20% propan-2-ol in heptane as the mobile phase at a flow-rate of 2.3 ml min⁻¹ and keeping the injection system and column at 35 °C to reduce the viscosity of the injected sample, the total elution volume for an injection was less than 24 ml. The lipid was eluted first and collected for a gravimetric determination of fat content. The elution of the lipid may be monitored using a refractive index detector. The OCPs and PCBs were eluted in the 11 ml immediately after the lipid and this volume was collected as one fraction. There is little scope for fractionation with such a small elution volume and no attempt was made to fractionate the residues. To this 11 ml a known volume of an internal standard (1 ml of 50 ng ml⁻¹ 4,4'-dibromobiphenyl) was added for the GC - MS determination only, and this was evaporated to about 1 ml over clean, dry air. The extract was then ready for GC determination.

Gas Chromatography - Mass Spectrometric Determination

The GC - MS equipment used for this work was a Dani 3800 gas chromatograph coupled to a VG 7070E (VG Analytical) mass spectrometer. The column was a 15 m × 0.25 mm i.d. DB-210 (0.25 μ m film) (J & W Scientific) with a 2 m retention gap of 0.25 mm i.d. deactivated fused silica. The carrier gas was helium at a linear gas velocity of 70 cm s⁻¹. The injector port was maintained at 270 °C and the oven programme was 80 °C for 2.5 min then 1.5 °C min⁻¹ to 200 °C. The injection was of 10 μ l of sample introduced over 15 s with a splitless time of 2 min. The mass spectrometer was used in the positive ion - multiple ion monitoring mode (see Table 1 for ion masses monitored) with 70 eV EI ionisation and a dwell time of 100 ms on each mass. Resolution (10% valley) was 500 with "flat-top peak" tuning. The specific analytes determined were α -HCH, β -HCH, γ -HCH, δ -HCH, *o,p'*-DDT, *p,p'*-DDT, *o,p'*-DDE, *p,p'*-DDE, dieldrin, endrin, PCB 118, PCB 156, PCB 157, PCB 169 and PCB 189. The numbering system for PCBs is after Ballschmitter and Zell.⁸

With a large number of analytes, as in this example, the question of chromatographic selectivity arises. We found that a 50% trifluoropropyl column (OV-210 or similar) gives very good selectivity amongst organochlorine pesticides, however, the selectivity between PCB isomers was more important for our work. Fig. 1 shows a mixture of OCPs together with a large number of PCBs in the form of Aroclors chromatographed on DB-210 column (J & W Scientific). The chromatogram shows that all the OCPs are resolved from each other and that the PCBs are well scattered. On very non-polar stationary phases such as Apiezon greases, OV-1 or SE-54 the PCBs tend to elute in clusters depending on the level of chlorination, with the lower chlorinated compounds eluting earliest. This means that there is a reduced likelihood that PCBs with the same number of chlorines, *i.e.*, isomers, will be resolved as they tend to elute in tight groups. A mass spectrometer cannot usually distinguish between PCB isomers as their mass spectra are essentially identical, but it can distinguish between PCBs with different numbers of chlorines. Fig. 2 shows the mass spectra for three different PCB congeners. Figs. 2(a) and 2(b) are for different hexachlorobiphenyls; it can be seen that they are essentially indistinguishable but give virtually no interference in the molecular ion cluster of the pentachlorobiphenyl shown in Fig. 2(c). Hence, in this example, hexachloro

Table 1. Selected ion mass monitored for determination by GC - MS

Analyte	<i>m/e</i> monitored
HCHs	183
DDTs	235
DDEs	246
"Drins"	263
HCB	284
PentaCBs	326
HexaCBs	360
HeptaCBs	394

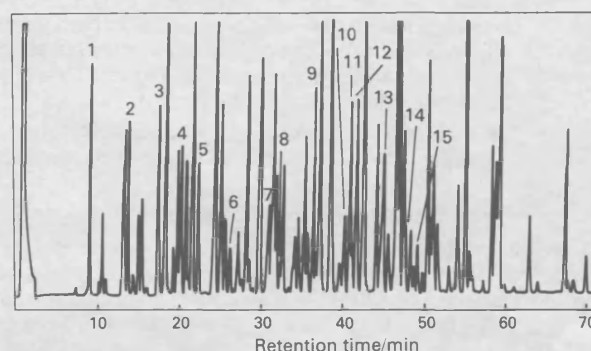


Fig. 1. Chromatogram of mixture of PCBs (as mixed Aroclors), selected OCPs and 4,4'-dibromobiphenyl separated on a 15 m × 0.25 mm i.d. DB-210 column (0.25 μ m film) (J & W). Injection volume, 2.5 μ l. Splitless mode; 100 °C for 100 s, 1.2 °C min⁻¹ to 190 °C. H₂ carrier at 70 cm s⁻¹; electron capture detection; injector temperature, 270 °C; ECD temperature, 350 °C. 1, HCB; 2, α -HCH; 3, γ -HCH; 4, aldrin; 5, δ -HCH; 6, 4,4'-dibromobiphenyl; 7, *o,p'*-DDE; 8, *p,p'*-TDE olefin; 9, *p,p'*-DDE; 10, *o,p'*-TDE; 11, *o,p'*-DDT; 12, dieldrin; 13, endrin; 14, *p,p'*-TDE; and 15, *p,p'*-DDT

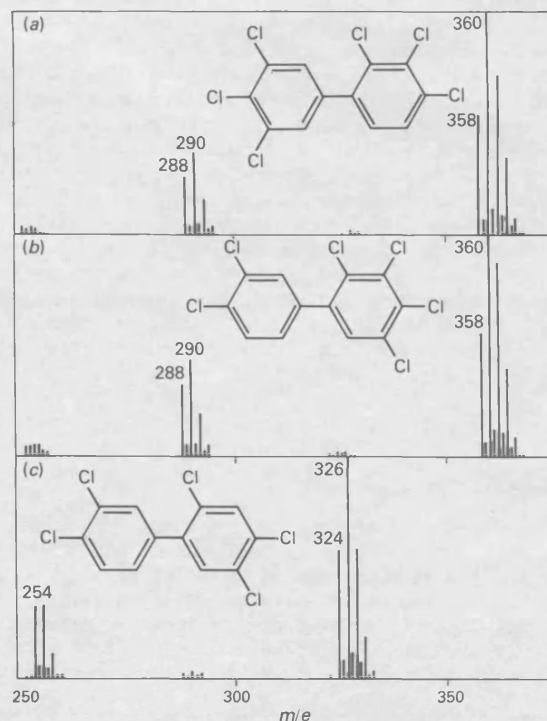


Fig. 2. (a) Mass spectrum of 2,3,3',4,4',5'-hexachlorobiphenyl; (b) mass spectrum of 2,3,3',4,4',5'-hexachlorobiphenyl; and (c) mass spectrum of 2,3',4,4',5-pentachlorobiphenyl

isomers can readily be distinguished from pentachloro isomers and similarly for other isomer groups differing by one chlorine. Polychlorinated biphenyls differing by two chlorines can, however, cause problems. The loss of two chlorines is a favoured fragmentation pathway for PCBs⁹ and therefore a PCB containing $n + 2$ chlorines can lose two of the chlorines and hence give rise to partial interference in the molecular ion cluster of a PCB containing n chlorines. This is illustrated in Fig. 3(a) by the heptachlorobiphenyl fragment ions giving rise to peaks in the mass fragmentogram produced by monitoring $m/e = 326$. Consequently, the selectivity between PCB isomers and between isomer groups differing by two chlorines has to come from the GC component of a GC-MS determination. Hence, a more polar, more selective stationary phase has to be used in order to scatter the PCB isomers more effectively, giving a greater chance of separating the members of each isomer group. A 50% trifluoropropyl column provides good selectivity by virtue of the unique properties of the fluorinated side-chain, whilst maintaining good column stability and efficiency.

Table 1 shows the ion masses monitored for each analyte group. Five specific PCB congeners are included in the list of analytes and these particular congeners can be quantified using this method. Unfortunately, most PCB congeners are not available commercially and congener-specific determinations may well involve in-house synthesis of standards for some congeners.^{10,11}

Results

Fig. 3 shows a 1 + 1 + 1 + 1 mixture by mass of Aroclors 1242, 1248, 1254 and 1260 chromatographed on a 15 m DB-210 column. Figs. 3(a) and (b) are two mass fragmentograms produced from the same injection and the time scales are superimposable. Fig. 3(a) was produced by monitoring $m/e = 326$ (the base peak in the pentachlorobiphenyl molecular ion

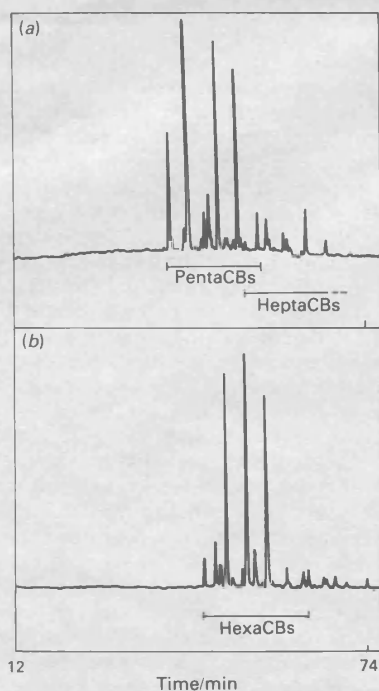


Fig. 3. Mass fragmentograms from a single injection of a 1 + 1 + 1 + 1 mixture of Aroclors 1242 + 1248 + 1254 + 1260 (approx. 100 p.p.b. of each). GC-MS conditions as in text. (a) Monitoring $m/e = 326$ (molecular ion for pentachlorobiphenyls). Pentachlorobiphenyl window approx. 16 min. (b) Monitoring $m/e = 360$ (molecular ion for hexachlorobiphenyls). Hexachlorobiphenyl window approx. 18 min

cluster) and Fig. 3(b) was produced by monitoring $m/e = 360$ (the base peak in the hexachlorobiphenyl molecular ion cluster). It can be seen that the selectivity of the 50% trifluoropropyl column scattered the pentachloro isomers over a window about 16 min wide and the hexachloro isomers over a window about 18 min wide. Despite the fact that many pentachloro- and hexachlorobiphenyls co-elute, the selectivity of the mass spectrometer prevented any major interference between the isomer groups. Heptachlorobiphenyls produce fragment ions at $m/e = 326$ and could therefore interfere with the pentachlorobiphenyls, but there is little overlap between the pentachloro- and heptachlorobiphenyl elution windows and hence the chance of interference is small. Using a 15-m column, the total analysis time for all OCPs and PCBs studied was about 70 min. The use of a 2-m retention gap not only served as a guard column, but also permitted the use of large injection volumes, which reduced the limits of detection of the method.

We can also quantify "total PCBs" by our method. There is some controversy over total PCB determination as many of the methods used, such as pattern-matching, are only semi-quantitative¹⁰ and suffer from poor accuracy, poor precision¹² or both. Moreover, a total PCB value is not very meaningful in a toxicological context and tells the analyst nothing about the distribution of congeners within that value. For completeness's sake, we can produce a total PCB value by measuring the total areas of the peaks for penta-, hexa- and heptachlorobiphenyls and comparing this area with that produced by an Aroclor 1260 standard. Aroclor 1260 is commonly used as a standard for pattern-matching analyses of human milk and penta-, hexa- and heptachloro compounds account for about 92% by mass¹³ of Aroclor 1260. Consequently, our total PCB value is expressed as ng ml^{-1} of Aroclor 1260. It is advisable to monitor ions for all ten levels of PCB chlorination in order to detect any unusual PCB distributions but we use only the penta-, hexa- and heptachlorobiphenyls for quantification.

Fig. 4 shows the chromatographic trace of a milk sample processed by our method and analysed using a Perkin-Elmer 8320B gas chromatograph with a 25 m OV-17 column and electron capture detection (ECD). The chromatogram is fairly complex but certain compounds can be identified and by virtue of the high resolution some can be quantified. Generally, however, there is too much overlapping of peaks to permit the accurate determination of the majority of analytes and fractionation would have to be carried out in this instance prior to determination by GC-ECD. Fig. 5 shows three of the mass fragmentograms produced by the same sample determined on our GC-MS multiple ion monitoring system using the 50% trifluoropropyl column with a retention gap. It can be seen that the signal to noise ratio is much improved and that the analyte identification is much more confident. The resolution of all our target analytes is at least adequate and accurate determination is possible in all instances. Fractionation is unnecessary, thus saving time in both sample preparation and GC analysis as all the compounds are determined in one run. In Fig. 5(a) there is slight interference of one of the pentachlorobiphenyl peaks by two hexachlorobiphenyls. This is because the two hexachlorobiphenyls concerned are present at much higher concentrations than the pentachloro compounds and hence the very minor fragments at $m/e = 326$ produced by hexachlorobiphenyls show up, but this interference is of little practical significance.

Table 2 shows the recoveries and relative standard deviations for the procedure using GC-ECD and GC-MS analyses. The recovery experiments were carried out by spiking the adsorbent with an accurately known amount of each of the analytes (approximately 50 ng) plus an accurately known amount of lipid (approximately 300 mg of a mixed triglyceride suppository base). A 10-ml volume of glass-distilled water was added and the extraction and clean-up procedures were carried out as for the milk samples. The

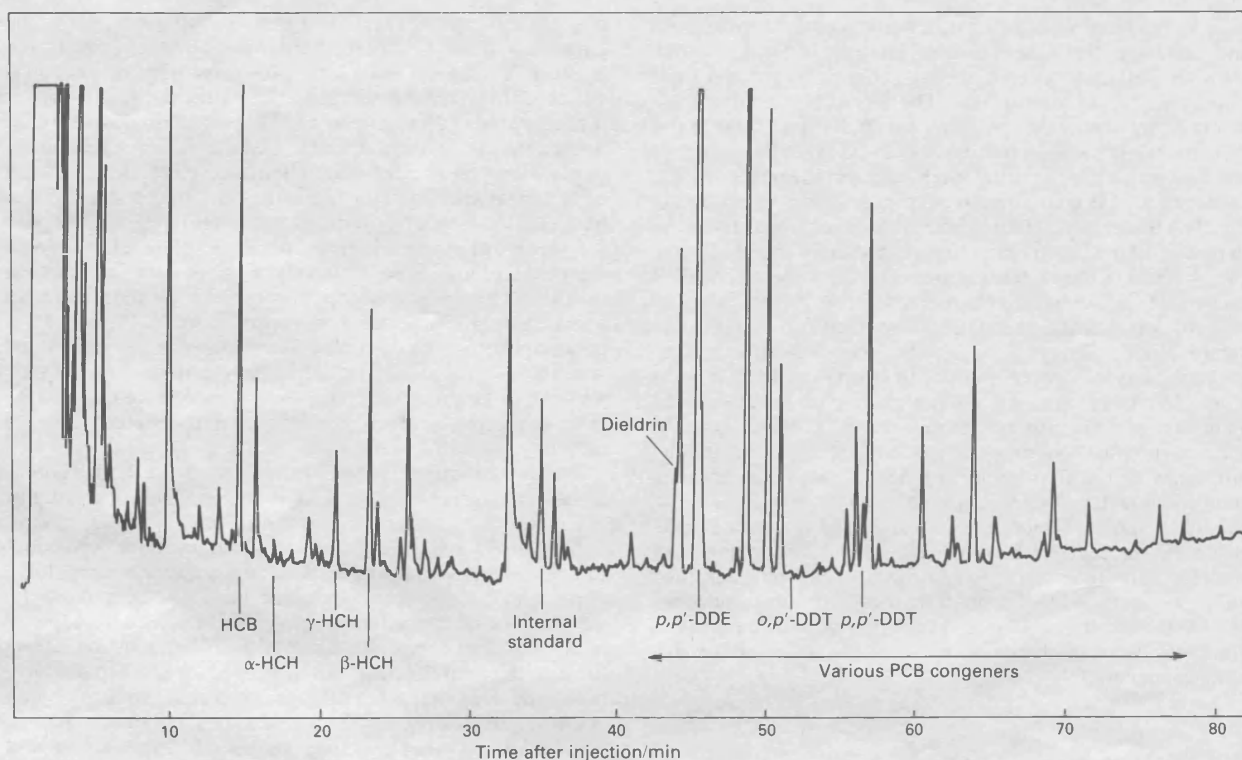


Fig. 4. Chromatogram of milk extract separated on a Perkin-Elmer Model 8320B GC - ECD fitted with an OV-17, 25 m \times , 0.25 mm i.d. (0.2 μ m film) column. Injection volume 2.5 μ l. Splitless mode; 100 $^{\circ}$ C for 2 min, 30 $^{\circ}$ C min^{-1} to 120 $^{\circ}$ C, 1.3 $^{\circ}$ C min^{-1} to 240 $^{\circ}$ C. H_2 carrier at 70 cm s^{-1} ; injector temperature, 270 $^{\circ}$ C; ECD temperature, 350 $^{\circ}$ C

recoveries are all at least adequate except for δ -HCH, which is rather low and probably not usable. The recovery for PCB 169 is also low but is probably just acceptable. The relative standard deviations are, on the whole, fairly low for this type of analytical procedure and therefore the method appears to be fairly precise.

The limits of quantification are all at least 0.1 ng ml^{-1} in whole milk, or better for individual compounds; this is a conservative figure for most analytes, particularly hexachlorobenzene where the limit of quantification is well into the parts per 10^{12} range.

Discussion

The analysis of human milk for OCP and PCB residues poses considerable problems. There are 209 possible PCB congeners, although only a selection of these have been found in human milk samples. In addition, milk is known to contain many OCPs. Most of the individual residues are present in human milk at the low ng ml^{-1} level and have to be separated from about a 10^7 -fold excess of lipid, in addition to the other constituents of milk, prior to quantification. All the methods used consist of three main steps: extraction, clean-up and GC quantification.

The simplicity of our extraction and clean-up procedures provide considerable savings in terms of labour and consumables. The use of an adsorbent substrate in the Soxhlet extraction step not only obviates the need for freeze-drying but also retains much of the milk matrix thus producing a relatively clean extract. The HPLC clean-up using a semi-preparative polymeric column provides a lipid fraction of low volume for a gravimetric determination of lipid content, and an organochlorine residue fraction which, after the addition of an internal standard and further reduction in volume, is suitable for GC - MS determination.

The quantification of organochlorine residues by gas chromatography is most frequently carried out using electron capture detection. The ECD is very sensitive to organochlorine compounds but exhibits only partial selectivity for these substances and an extensive clean-up procedure therefore has to be incorporated in the sample work-up. An alternative to the ECD is the Hall electrolytic conductivity detector. This detector can be made to be very selective for organochlorine compounds and can therefore tolerate less clean samples. The detector of choice, however, is the mass spectrometer. It is very sensitive, exhibits a very wide linear dynamic range and offers unrivalled selectivity. This means that sample clean-up can be minimised and that fractionation of complex multi-residue samples can generally be avoided.

The GC - MS determination may be implemented in many different ways according to the requirements of the analyst and the facilities available. A 50% trifluoropropyl column provides good selectivity amongst OCPs and effectively scatters PCB isomers for MS detection. A 15 m \times 0.25 mm i.d. (0.25 μ m film) column is adequate for the purpose but there is scope for improvement, perhaps using a slightly more efficient or more selective column; liquid crystalline phases may be useful in this context. The use of a retention gap not only acts as a guard column but also allows large injection volumes to be used.

Various mass spectrometric conditions can be employed for this type of determination. This work was carried out using the positive ion 70 eV electron impact mode but techniques such as negative ion chemical ionisation show considerable promise and offer greater sensitivity and selectivity. An added bonus in using GC - MS is that the selectivity is so great that HPLC grade solvents can be used for the sample work-up instead of the considerably more expensive pesticide grades.

The procedure appears to be simpler and more cost effective than other methods for the determination of OCP

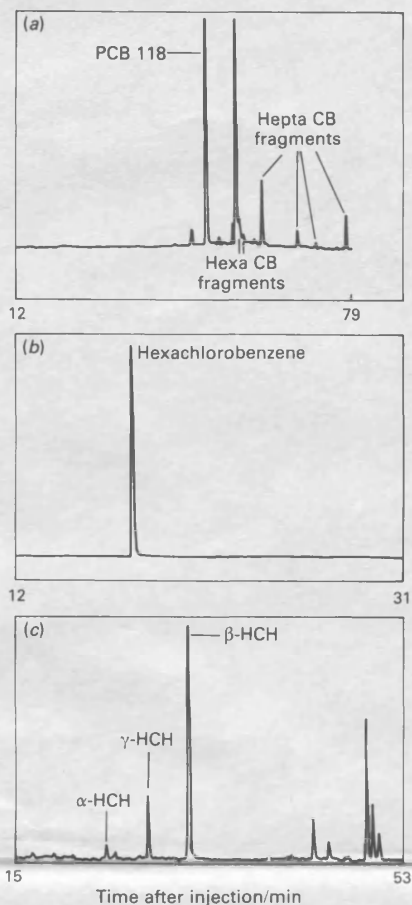


Fig. 5. Mass fragmentograms produced from the same milk sample as shown in Fig. 4. GC - MS conditions as in text. (a) Monitoring $m/e = 326$; (b) monitoring $m/e = 284$; and (c) monitoring $m/e = 183$

and PCB residues in human milk. The method is robust and easy to perform but requires routine access to a capillary GC - MS system. Not all laboratories may be able to employ such equipment on a regular basis, but as the cost of such instrumentation falls, it may be hoped that an increasing number of laboratories will have access to this particular technique.

We thank the Pharmaceutical Society of Great Britain for providing one of us (M. P. S.) with a scholarship in support of this work.

Table 2. Recoveries and relative standard deviations for selected analytes determined from spikes (see text) using GC - ECD and GC - MS. Mean recovery of lipid ($n = 5$) = 94.2%, relative standard deviation (RSD), 2.22%

Analyte	Mean recovery (GC - ECD), %	RSD (GC - ECD), %	Mean (GC - ECD), %	RSD (GC - ECD), %
HCB	83.2*	2.54*	99.81	13.86
α -HCH	87.2	4.30	106.92	6.83
β -HCH	88.4	5.56	90.89	9.39
γ -HCH	90.9	3.53	106.51*	4.99*
δ -HCH	63.2	9.38	67.30	8.41
<i>o,p'</i> -DDE	91.5	2.89	86.69	6.28
<i>p,p'</i> -DDE	100.1†	2.84†	92.68*	9.16*
<i>o,p'</i> -DDT	94.6	5.11	91.66	10.55
<i>p,p'</i> -DDT	97.8	6.01	98.88	10.12
Dieldrin	87.8	4.64	94.09	10.36
Endrin	98.9	5.49	96.03	8.51
PCB 156	92.0	3.20	99.62	8.31
PCB 157	91.2	2.03	95.36	8.74
PCB 169	79.7	7.56	77.52	11.79
PCB 189	89.8	2.94	80.71	13.38

* $n = 4$.

† $n = 3$.

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An Analysis of PCBs and Organochlorine Pesticides by Capillary Gas Chromatography—A Modern Approach to PCB/OCP Residue Analysis of Human Milk

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The contamination of our environment with persistent, toxic compounds has become an issue of increasing importance over the past few years. Two groups of compounds in this category are the organochlorine pesticides (OCPs) and the polychlorinated biphenyls (PCBs).

Accurate and precise analyses for OCP and PCB residues are difficult and complex because of three principal factors: the complexity of the residue matrices, the low levels of contamination and the large number of individual compounds in the analyte group.

The aim of our work is to quantify selected OCPs and selected PCB congeners as well as total PCBs in samples of human milk.

Experimental

Traditional methods of analysis for OCPs and PCBs in milk samples are complex and labour intensive, requiring the use of efficient extraction processes plus extensive clean-up and fractionation procedures.¹ Such clean-up and fractionation steps often make use of one or more column chromatography stages per sample employing different solvents or column packing materials in order to achieve sample fractionation.² Each fraction (several per sample) has then to be analysed,

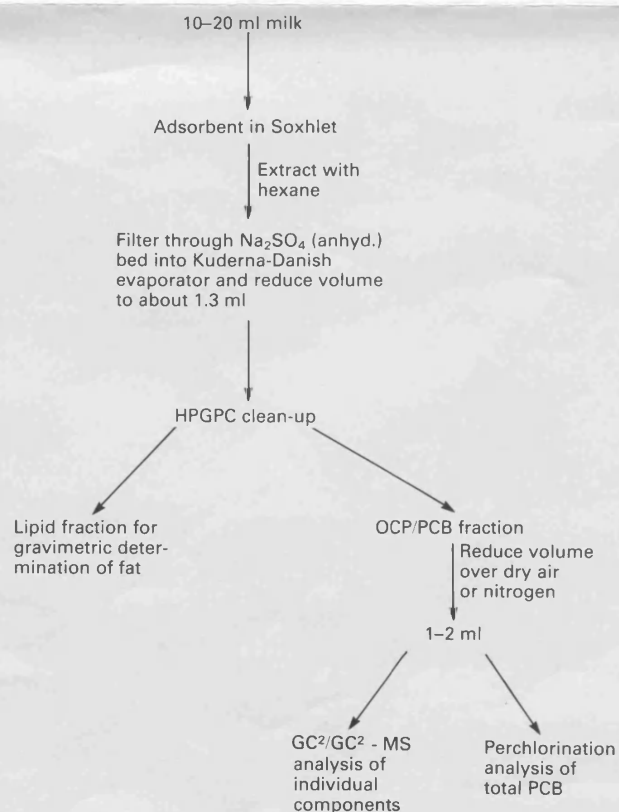


Fig. 1. Procedure for the analysis of human milk for organochlorine pesticides and polychlorinated biphenyl residues

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usually by a gas-chromatographic method. In our approach to the problems of this analysis, the usual column chromatography based procedures have been replaced by more modern and less laborious techniques (Fig. 1).

The sample of milk is adsorbed on to a hydrophilic substrate in a Soxhlet thimble. The milk is continuously extracted with hexane for several hours, after which time the extract is filtered through an anhydrous sodium sulphate bed into a Kuderna-Danish evaporator. The extract is then reduced in volume to about 1.3 ml.

At this point, the extract contains a very high proportion of human milk lipids which have to be removed prior to gas chromatographic (GC) analysis. This is achieved using a high-performance gel permeation column (Polymer PLRP-S 300 × 7.5 mm i.d., 10 μm particle size, 100 nm pore size). The sample is injected on to the column through a valve injection system. The lipids are all eluted within the first 10 ml or so followed by the PCBs and the majority of the OCPs in the next 12 ml. Thus, the total elution volume for the clean-up procedure is only about 22 ml. In contrast to column chromatography clean-ups, this HPGPC technique not only uses far less solvent but also allows the collection of the lipid fraction of the milk for a gravimetric determination of fat content. The OCP/PCB fraction is then reduced over air or nitrogen to the required final volume for GC analysis.

The sample, at this point, routinely contains very many individual OCP and PCB components and not even the high resolving power of capillary GC is capable of separating more than a few of the analytes sufficiently for accurate quantification to take place (Fig. 2). In Fig. 2, for example, even some of the sharpest peaks are due to two or more co-eluting components.

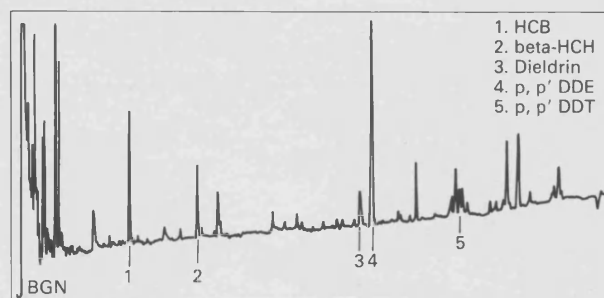


Fig. 2. OCP/PCB fraction of human milk injected on to a 25 m OV-17 0.25 mm i.d. 0.2 μm film capillary with hydrogen as carrier gas and electron-capture detection. Some selected OCPs are identified. Most of the later eluting peaks are PCBs. Other peaks are: 1, HCB; 2, beta-HCH; 3, dieldrin; 4, *p,p'*-DDE; 5, *p,p'*-DDT

In order to overcome this problem, the sample is usually fractionated by column chromatography with a number of different mobile phases. As an alternative to this, we decided to employ the considerable selectivity afforded by capillary GC - MS in order to achieve specificity for our chosen analytes. Our philosophy was to obtain as much selectivity for the analytes as possible using a selective capillary GC phase and then to enhance this by utilising the considerable specificity and sensitivity offered by mass spectrometry with selected ion

monitoring as the detection mode. Further sensitivity can be gained, if required, by employing large injection volumes on a retention gap.

Taking this approach completely avoids the fractionation of the cleaned-up sample and permits the determination of the selected analytes in one GC run. For the purposes of this analysis, a trifluoropropyl capillary column was found to provide a high degree of selectivity amongst OCPs and PCBs, thus helping to minimise the number of co-eluates.

Considering the PCBs alone, there are 209 possible congeners and a significant number of these have been shown to accumulate in human milk.³ Different PCB congeners exhibit different toxicological profiles⁴ and ideally, any toxicological evaluation of PCB contamination would be based on congener-specific analysis. Such analyses have been performed³ but present the analyst with several obstacles, not least of which is the acquisition of analytical standards for each congener. As a compromise, we intend to use capillary GC - MS with SIM to analyse for selected congeners that we have synthesised in-house. Obviously, total PCBs can also be determined by a congener-specific method, but other less demanding methods for total PCBs, such as pattern matching and perchlorination techniques, have also been used. These, however, can be rather inaccurate and do not generate any congener-specific data, making toxicological evaluation of the result all but impossible. Despite this, in the interests of completeness, it is desirable to produce data regarding total PCB contamination of the sample and to this end we decided to employ a perchlorination method, which involves conversion of all chlorinated biphenyls to decachlorobiphenyl by the action of antimony pentachloride at elevated temperature.⁵ This allows total PCBs to be quantified as one substance (decachlorobi-

phenyl) by gas chromatography. The procedure also enhances sensitivity when using electron capture detection as all the PCBs are essentially "concentrated" into one, highly electron-capturing peak.

Discussion

Overall, we have aimed at a method of milk analysis that is very much less labour-intensive than traditional methods and which uses highly selective and sensitive capillary GC techniques. By taking this approach, it is possible to analyse a large number of samples in a relatively short time with a considerable saving on consumables.

The major drawbacks are that the method requires specialised equipment, such as a high-performance gel permeation column and, more particularly, a capillary GC - MS system. At present the latter constraint may put this type of analytical methodology out of reach of many laboratories but GC - MS systems should become more accessibly priced in the near future, making the routine use of this powerful technique cost effective for complex analytical problems.

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ROM-overlay Programmes for the Spectra-Physics 4270 Integrator. Application to HPLC Column Efficiency and Suitability Testing

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The use of HPLC for the quantitative analysis of drug substances has increased dramatically in recent years. In order to comply with Good Laboratory Practice and pharmacopoeial requirements, it is necessary to test the chromatographic system to ensure its suitability for the particular assay. Use of the Spectra-Physics 4270 integrator as part of the chromatographic system has facilitated the introduction of suitability testing. ROM-overlay programmes have been written, which tailor the integrator output to the particular suitability test and eliminate operator measurement and calculation.

Suitability Testing and Programme Structures

In order to assess the suitability of a chromatographic system for a particular assay, column efficiency, resolution of components, tailing factor of the analyte peak and standard deviation for replicate injections must all be determined. The relative standard deviation of replicate injections can be obtained by using the integral statistics package of the SP 4270. Other parameters require programme modification.

A measure of column efficiency is provided by calculating the number of theoretical plates, N , in the column with the

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1998 REM      SP4270 ROM overlay programme
1999 REM      ARESO written by Peter Jones, Wyeth Research (UK) Ltd.
2000 REM      This programme calculates the efficiency of each peak in
2001 REM      the chromatogram. Resolution is also calculated.
2002 REM      Gaussian peaks are assumed.
2003 REM      This programme will only work when PH=2, TB=0 and
2004 REM      MN=2 or MN=5.
2005 REM
4160 C(G)=((PST(G)*PSH(G))/(0.0141468*PSA(G)))**2*16
4180 !TAB 22#8.3PST(G)TAB 32#10PSA(G)TAB 42#9PSH(G)TAB 54#2.04PSF(G)TAB
57#9C(G);
4181 D(G)=((PST(G+1)-PST(G))/(PST(G)+PST(G+1)))*((SQRC(G))/2): !TAB 67#
5.3D(G)
4182 RETURN
4385 !TAB 30"RT      "#5.03H(2);
4390 !TAB 46"HEIGHT"TAB 54"BC"TAB 57"EFFICIENCY"TAB 69"R#"
4395 GOTO 3170

```

Fig. 1. ROM-overlay programme incorporating resolution and column efficiency values into the report of the SP 4270