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Rapid isolation of polychlorinated biphenyls from milk by a combination of supercritical-fluid extraction and supercritical-fluid chromatography

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ABSTRACT

The extraction and isolation of polychlorinated biphenyls (PCBs) from sample matrices such as freeze-dried milk is a lengthy and expensive process that conventionally requires the isolation of microgram amounts of PCBs from large volumes of flammable organic solvents used in the extraction and chromatographic stages. Supercritical-fluid extraction (SFE) and chromatography (SFC) using carbon dioxide has been investigated as a replacement procedure using a Simplex optimisation approach to optimise the working conditions. The SFE conditions required to extract PCBs and milk fat from freeze-dried milk were investigated, and it was found that although extraction was rapid (10 min), the conditions required were identical so that there was no separation of PCBs and fat. However, SFC conditions have been developed that permit the resolution of PCBs from milk fat in 15 min. Thus, a combination of SFE and SFC have the potential to replace the existing methods of extraction and isolation with procedures that are quicker, cheaper, and avoid the use of flammable organic solvents.

INTRODUCTION

The high solvating power of supercritical fluids was reported in the last century [1], but practical applications, such as supercritical-fluid extraction (SFE) [2] and supercritical-fluid chromatography (SFC) [3], were first reported in 1962 and 1969, respectively. These papers have led to the application of supercritical fluids being investigated for the improvement of analytical methods by a large number of researchers [4–11]. A variety of organic compounds have been extracted from different matrices using carbon dioxide. Extraction using supercritical fluids can achieve better efficiencies than conventional Soxhlet extraction in a much shorter time period for some analytes and matrices [9]. This has been demonstrated for the extraction of polycyclic aromatic hydrocarbons from environmental solids by Hawthorne and Miller [10]. SFE also minimises the use of toxic organic solvents, and the need for

concentration steps before analysis of the extracts. SFE can be optimised for a particular extraction because the solvent strength of a supercritical fluid is directly related to its density which can be easily modified by changing the extraction pressure, and to a lesser extent, the temperature.

SFC has also been widely investigated, because of the high efficiency that is possible, and the ability of SFC to separate types of compounds which cannot be analysed by traditional gas chromatography [5]. Relatively non-volatile, thermally unstable and high-molecular-mass solutes can all be analysed by SFC. One such example is the analysis of thermally labile sulphonylureas reported by McNally and Wheeler [11]. Separations by SFC can be achieved and enhanced by varying the pressure, temperature, and mobile phase composition. The mobile phase composition can be varied by the addition of organic compounds as modifiers which change the eluting power of a supercritical mobile phase such as carbon dioxide. A range of organic solvents, including methanol and acetonitrile, have been investigated

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as supercritical fluid modifiers by Levy and Ritchey [7].

Current literature methods that are employed for the extraction of polychlorinated biphenyls (PCBs) from solid matrices and from liquids that have been either absorbed on porous inert solids, or freeze-dried, are usually lengthy. A typical procedure would require Soxhlet extraction (3–8 h or overnight) with an organic solvent followed by evaporation to a small volume (Kuderna–Danish apparatus), followed by a chromatographic clean-up step to isolate PCBs (either with organochlorine pesticides, or separated from them), from fats. All these steps require uncontaminated, clean glassware, and ensure that sample preparation for GC analysis is the most lengthy and expensive part of the analytical process. This report describes the application of SFE and SFC to replace the traditional extraction and isolation steps respectively, with considerable savings in time and cost.

EXPERIMENTAL

Supercritical-fluid extraction

SFE conditions were investigated and optimised for the extraction of PCBs from a typical matrix (*e.g.* freeze-dried milk + Florisil) using supercritical carbon dioxide (CO_2). A Simplex optimisation procedure was undertaken [12], with variations in the extraction temperature and back pressure used as the significant factors for the extraction.

A Jasco SFE system was kindly provided by Ciba-Corning Analytical (Halstead, UK). An ethylene glycol-filled cooler was used to maintain the head of the carbon dioxide pump, Model 880-PU at −10°C, with the flow-rate controlled from the electronic keypad. The extraction vessel, consisting of an empty 13 cm × 10 mm I.D. HPLC column with two screw-capped ends, was housed in a Model 860-CO column oven at a set temperature. The material to be extracted was placed in the HPLC column and the two ends screwed on tightly by hand. A Rheodyne switching valve was used to switch the flow of supercritical CO_2 through the extraction vessel once the sample had been loaded. A Model 875-UV ultraviolet detector with a high-pressure flow-cell was used to continuously monitor the extract at a specific wavelength. A Model 880-81 back pressure regulator kept the entire extraction system at a con-

stant back pressure, via an electronic feedback regulator that was flow independent. This arrangement provides greater stability throughout the system than the more frequently used capillary restrictors. The temperature of the back pressure regulator was also controlled to avoid the problem of the extract becoming plugged in the exit tubing. The extract was vented to the atmosphere through the back pressure regulator and collected in 1 ml of heptane in an ice-cooled 120 × 14 mm I.D. test tube. Throughout the optimisation of the extraction pressure and temperature, the liquid CO_2 flow-rate was 3.0 ml min^{-1}, and the UV detector was used at a wavelength of 254 nm and a range of 0.08 AUFS.

Supercritical-fluid chromatography

SFC was investigated for the separation of PCBs from fat. The PCBs and fat were both extracted from cow's milk by SFE. The same SFE system was used for the SFC work, with a few modifications. A second Model 880-PU HPLC pump was used to allow the addition of an organic modifier to the supercritical CO_2 mobile phase. The two phases were mixed in a Model 880-30 mixer module. Three different HPLC columns were investigated by replacement of the extraction vessel used in the SFE work, a 30-cm PLRP-S column (Polymer Labs., Church Stretton, UK), a 15-cm Hamilton PRP-1 column (Jones Chromatography, Hengoed, UK), and a 10-cm Brownlee Labs. RP-8 Spheri-5 column (Anachem, Luton, UK).

A second Rheodyne valve was fitted in series with the first Rheodyne valve to allow a 20-μl injection loop to be used to introduce the sample onto the column. A syringe was used to inject the samples onto the column, and the analytes were collected in the same way as described for SFE.

Gas chromatography–mass spectrometry

All the extracts were analysed by a Hewlett-Packard (Bracknell, UK) HP 5890 gas chromatograph with a 5970 MSD mass spectrometer on a 50-m OV-1 column (Hewlett-Packard). A selected ion monitoring (SIM) programme designed specifically for the analysis of PCBs according to their chlorination level was used [13]. A 5-μl aliquot of each extract was injected onto the column in the splitless mode. The injector temperature was set at 250°C, and the flow-rate of hydrogen carrier gas set at 1 ml

min^{-1}. A temperature programme with an initial temperature of 75°C for 2 min, followed by ramps of 30°C min^{-1} to 120°C, and then 10°C min^{-1} to 270°C, and a final temperature of 270°C for 35 min' was used. The SIM programme meant that at any given time during a run, the mass spectrometer was monitoring for four mass ions specific to two levels of PCB chlorination.

Every peak found by GC–MS was mathematically tested to prove whether or not it was due to a PCB by the method of Erickson et al. [13]. The peaks that failed this test were ignored, and only those peaks that passed were used to assess the levels of PCBs extracted [13].

Gas chromatography–electron-capture detection (ECD)

A Perkin-Elmer (Beaconsfield, UK) 8320B capillary GC–ECD system with a 25-m HT-5 column (SGE Pty., Milton Keynes, UK) was also used to analyse some of the extracts. The injector and detector temperatures were set at 250°C, and hydrogen was used as the carrier gas with nitrogen as the make-up gas. A temperature programme was used for the analyses, commencing at 75°C for 2 min, followed by ramps of 30°C min^{-1} to 120°C and then 1.5°C min^{-1} to 210°C, and a final temperature of 210°C for 10 min.

RESULTS AND DISCUSSION

Supercritical-fluid extraction

PCBs from cow's milk. The solvent strength of a supercritical fluid is determined by a number of factors, one of which is its density, so that the solvating ability of a particular supercritical fluid towards a particular species (*i.e.* PCBs) can be modified by changing the extraction pressure [10]. Also for carbon dioxide SFE, increasing temperature (at constant pressure) can enhance extraction efficiencies even though density is lower at the higher temperature.

A mixture of freeze-dried skimmed milk, equivalent to 10 ml of the original milk (fat content 0.1%, w/w) and Florisil was used as the initial extraction medium. The low level of fat from the milk ensured that the UV signal for extracted PCBs would be easily identified, and the Florisil aided the rapid penetration of the freeze-dried milk by the supercritical CO_2.

Initially, the level of the PCB spike required for the Simplex optimisation experiment was investigated. The spike was introduced into the powder contained in the extraction chamber with a syringe. Moderate extraction conditions were used and the concentration of PCBs added was increased gradually until a suitable response was found on the chart recorder. A 50-μl spike of 100 μg ml^{-1} Aroclor 1242 was found to give the required response, and this level of spike was used throughout the optimisation procedure.

An optimisation procedure is usually commenced at a set of conditions removed from the anticipated optimum. The variables investigated here were the extraction pressure and temperature, and the response measured was peak height. Peak areas gave inaccurate estimates of extraction efficiency due to the fluctuating nature of the baseline. The optimisation procedure was started at a high extraction temperature and a low back pressure as this was expected to give a poor extraction of PCBs from cow's milk, because the extracting strength of supercritical CO_2 usually increases with increasing pressure and usually decreases with increasing temperature, although solvation is not the only limiting process of SFE with CO_2. Matrix interactions are also an important consideration.

Initial studies at 65°C and 120 kgf cm^{-2} and at 55°C and 120 kgf cm^{-2} (1 kgf/cm^2 = 0.098 MPa) resulted in no PCB peaks being observed during the extraction although subsequent GC–MS analysis of the two collected extracts showed the typical Aroclor 1242 pattern. This indicated that the extraction of the PCBs from the cow's milk had occurred, but
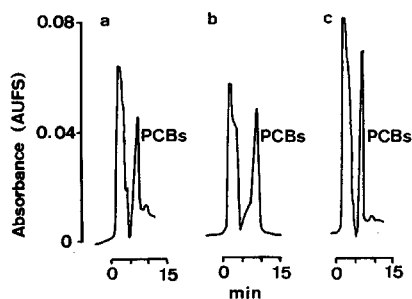

Fig. 1. SFE of PCBs from freeze-dried, skimmed milk with a spike of 50 μl of 100 μg ml^{-1} Aroclor 1242. Conditions: (a) 55°C and 160 kgf/cm^2; (b) 45°C and 240 kgf/cm^2; (c) 47°C and 220 kgf/cm^2.
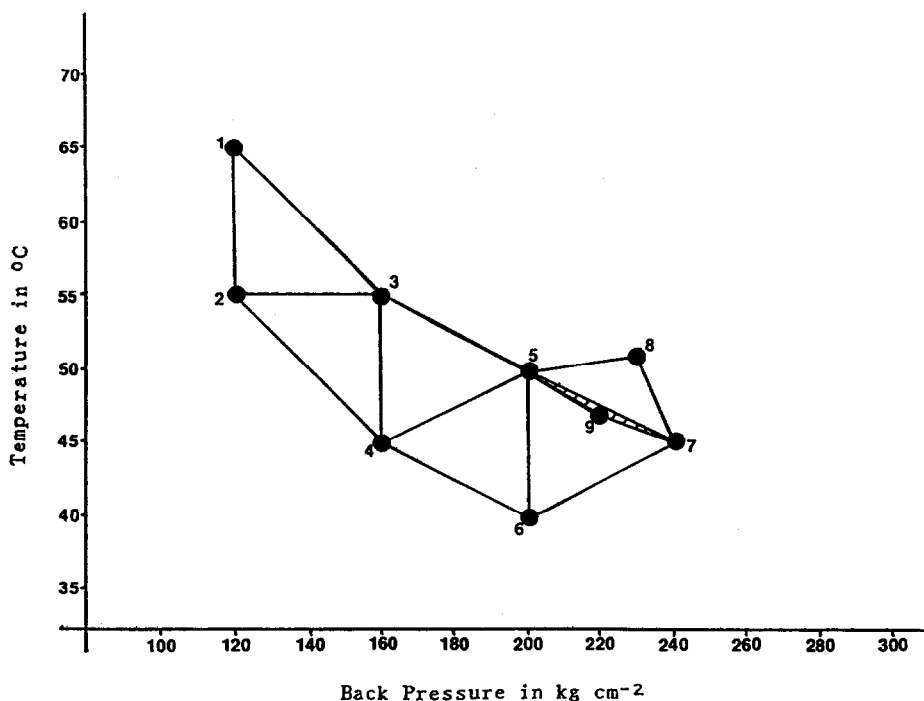
Fig. 2. Diagram of the Simplex optimisation procedure for the extraction of PCBs from freeze-dried, skimmed milk.

that the rate of extraction was too slow for a peak to be detected by the UV detector.

When conditions of 55°C and 160 kgf cm^{-2} were selected a peak was obtained on the chart recorder (Fig. 1a), and the first triangle of the Simplex optimisation was set up by measuring the peak height under these conditions, and at 45°C and 160 kgf cm^{-2} and at 50°C and 200 kgf cm^{-2}. The conditions giving the smallest peak height response were rejected, and the next set of experimental conditions was calculated according to Miller and Miller [14]. Fig. 1 shows three of the UV traces obtained during the optimisation. The Simplex optimisation was continued until a triangle was constructed where each of the three points gave a similar peak height response. This final triangle was made up of points 5, 7 and 9 and is shaded in Fig. 2. The optimum extraction conditions for the extraction of PCBs from cow's milk using supercritical CO_2 lie within this final triangle. The last point to be investigated, 47°C and 220 kgf m^{-2}, achieved an extraction of Aroclor 1242 from cow's milk in approximately 10 min, (Fig. 1c).

All of the extracts were collected, analysed by GC–MS, and all the extracts showed the characteristic Aroclor 1242 pattern, proving that the peak monitored by the UV detector was due to the Aroclor 1242 spike. No discrimination was seen in the extraction of PCBs according to the level of chlorination, *i.e.* all the different PCB congeners in Aroclor 1242 (monochlorinated to pentachlorinated) were extracted under each set of conditions. Fig. 3 shows a comparison of the chromatogram obtained from the extraction at 45°C and 240 kgf cm^{-2} and the chromatogram of Aroclor 1242 standard. Some very minor differences in the pattern of peaks are evident in Fig. 3a compared with Fig. 3b but these were not considered to be important at this stage of the study. A sample of Aroclor 1260 (pentachlorinated to nonachlorinated) was also shown to have been extracted under the conditions of 47°C and 220 kgf cm^{-2}.

Fat from cow's milk. The Simplex optimisation of the extraction of fat from cow's milk followed the same procedure (Fig. 2) as that for the extraction of PCBs. A UV detector wavelength of 230 nm was
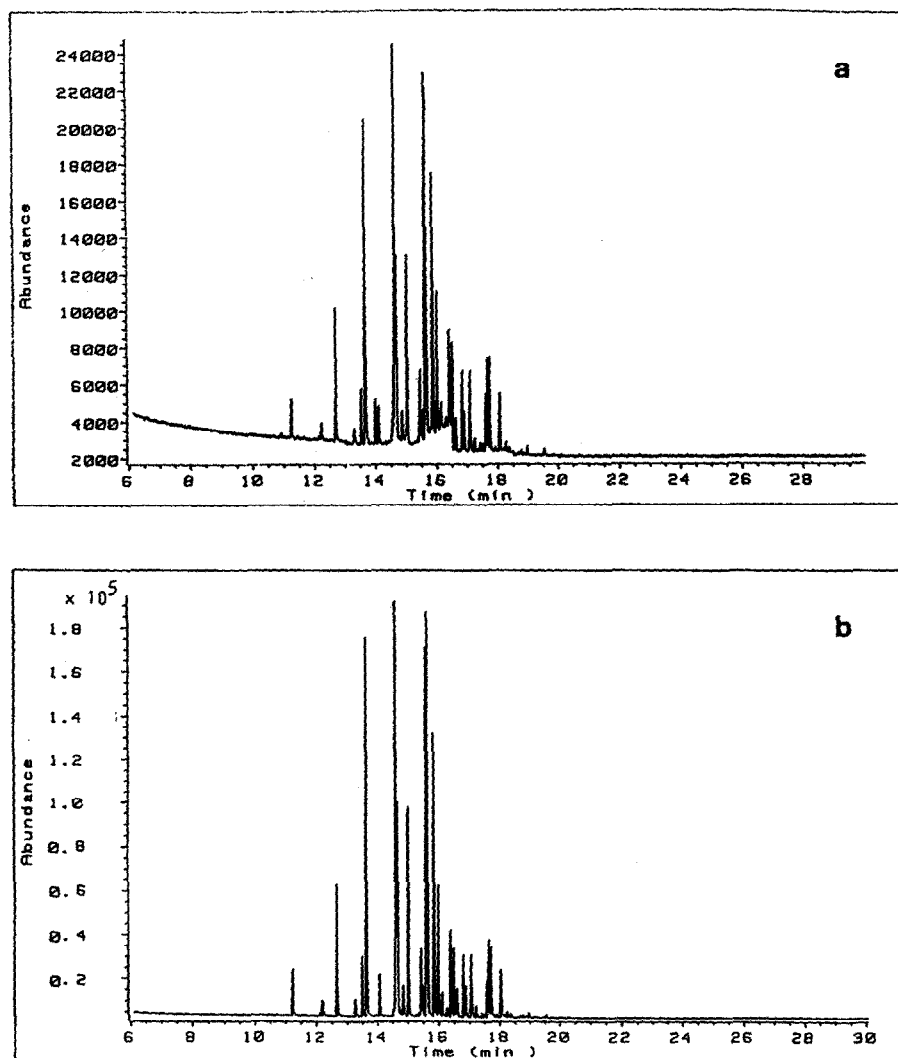
Fig. 3. Comparison of the GC–MS chromatogram of one of the SFE extracts from milk and an Aroclor 1242 standard. (a) GC–MS of extract at 45°C and 240 kgf cm^{-2}; (b) GC–MS of Aroclor 1242 standard.

used to monitor the fat. A mixture of freeze-dried unskimmed milk (fat content 4%, w/w) and florisil was used as the extraction medium.

Two features were immediately recognised as the optimisation procedure was carried out. Firstly, the peaks obtained for fat were not as sharp as previously found for the PCB peaks. Secondly, the differences in peak height recorded during the optimisation of fat extraction were smaller than for the PCB optimisation. Fig. 4 shows two of the UV traces obtained for the extraction of fat from cow's milk.

The extraction temperature and pressure conditions found to be optimal for the extraction of PCBs from cow's milk and fat from cow's milk were shown to be essentially the same. This illustrated that although SFE using carbon dioxide can readily extract both fat and PCBs from cow's milk, this approach is not selective enough to allow the extraction of PCBs, the analyte of interest, from cow's milk free of fat. A second step was, therefore, required to separate the PCBs from the fat.
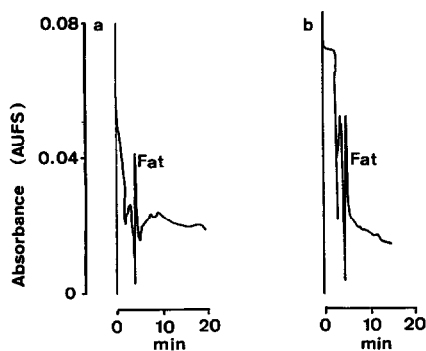
Fig. 4. SFE of fat from freeze-dried milk with a spike of 50 μl of 100 mg ml^{-1} Witepsol S55. Conditions: (a) 55°C and 160 kgf cm^{-2}; (b) 47°C and 220 kgf cm^{-2}.

Supercritical-fluid chromatography

SFC of PCBs. For a number of years a polymeric PLRP-S column (300 × 7.5 mm I.D.) has been routinely used in this laboratory as a semi-preparative step to isolate PCBs (+ pesticides) from milk fat using heptane–2-propanol (35:65) as the mobile phase [15]. This column was selected for this work to provide a direct comparison between HPLC and SFC, by using the same flow-rate (2.3 ml min^{-1}). It was noted that this column could not be used at pressures greater than 3000 p.s.i. (= 211 kgf cm^{-2}).

A 20-μl sample of 100 μg ml^{-1} Aroclor 1242 in heptane was injected onto the column at 45°C and 160 kgf cm^{-2} with a mobile phase of CO_2–2-propanol (90:10). Several peaks were observed on the chart recorder, but no PCBs were detected when the extract was analysed by GC–MS and GC–ECD. An injection of blank heptane gave the same set of peaks on the chart recorder, indicating that the PCBs were probably not being extracted. Therefore, stronger extraction conditions were employed to try to elute the PCBs from the PLRP-S column. The back pressure was increased to 200 kgf cm^{-2} and the mobile phase changed to 100% CO_2, but no PCBs were detected by GC–MS or GC–ECD. Under HPLC conditions, 100% heptane would elute PCBs with the solvent front. This implies that heptane is more non-polar than supercritical CO_2 under the conditions stated. This contrasts with the statement of Mourier *et al.* [16] that the polarity of supercritical CO_2 is close to that of hexane. The conclusion drawn from these results was that the Aroclor was not eluting from the column under

SFC conditions and therefore, a column expected to show less retention of PCBs (10-cm RP-8 Spheri-5 column) was selected.

When a 20-μl injection of 100 μg ml^{-1} Aroclor 1242 in heptane was made at 50°C and 180 kgf cm^{-2}, with a mobile phase of 100% CO_2 at 1 ml min^{-1}, a peak was detected which GC–MS analysis proved was due to the Aroclor 1242. However, although a number of different chromatographic conditions were tested, including a mobile phase of CO_2–2-propanol (40:60) at 50°C and 140 kgf cm^{-2} and a flow-rate of 0.5 ml min^{-1}, it was not possible to increase the retention of the Aroclor 1242 to any degree. Therefore, separation of Aroclor 1242 from fat using this column would not be possible.

These results showed that an HPLC column with more retention for PCBs than the RP-8 column, but less retention than the 30-cm PLRP-S column was needed. In view of previous experience with the polymeric PRP-1 material, a 15-cm column was tested under a range of experimental conditions. At 50°C and 160 kgf cm^{-2} with a mobile phase of CO_2–2-propanol (80:20), flow-rate 1 ml min^{-1}, a set of peaks were detected when 20 μl of 100 μg ml^{-1} Aroclor 1242 in heptane were injected (Fig. 5). The extract was collected as two fractions, (i and ii, Fig. 5). When analysed by GC–ECD, fraction i was shown not to contain PCBs, while fraction ii showed the Aroclor 1242 pattern.

The back pressure was reduced to 140 kgf cm^{-2}, and several different mobile phase compositions were investigated to improve the results chromatographically. A mobile phase of CO_2–2-propanol

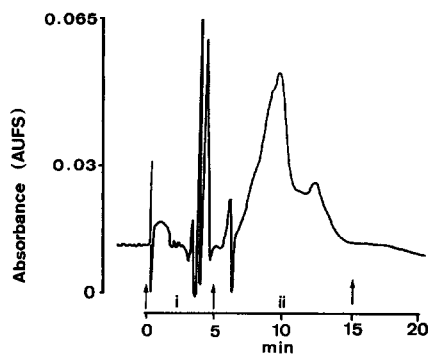Fig. 5. SFC of a 20-μl spike of 100 μg ml^{-1} Aroclor 1242 on a 15-cm PRP-1 column with a mobile phase of CO_2–2-propanol (80:20) at 50°C and 160 kgf cm^{-2}.

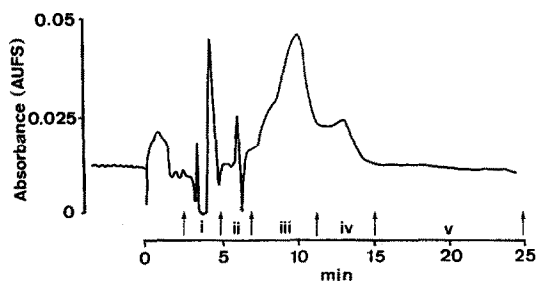Fig. 6. SFC of Aroclor 1242; fraction collection with mobile phase of CO_2–2-propanol (80:20) at 50°C and 140 kgf cm^{-2}.

(80:20) gave the best peaks shapes. This mobile phase showed a chromatogram with a series of peaks between 6 and 20 min. The exact location of the PCBs in the elution pattern was identified by the collection of five fractions during a run (Fig. 6). These fractions were analysed by both GC–ECD and GC–MS, and PCBs were found to be present in fractions iii and iv; fractions i, ii and v did not contain any PCBs.

SFC of fat. Witepsol S55 is a typical lipid material similar in composition to milk fat [15], and was used to investigate the SFC behaviour of fat. The 15-cm PRP-1 column was again used for the SFC of fat because the results for PCBs indicated that this column showed the most promise for achieving the required separation of PCBs from fat. The level of Witepsol S55 spiked needed was investigated, and a spike of 20 μl of 100 mg ml^{-1} Witepsol S55 in heptane was adopted. The chromatographic conditions optimised for PCBs [CO_2–2-propanol (80:20), 1 ml min^{-1}, 50°C, 140 kgf cm^{-2}], were then used for the fat in order to provide a direct comparison with the PCB results. When the fat spike was injected, a peak was detected at about 4 min, the same elution time as a blank injection of heptane. The identity of the fat peak was confirmed by injecting fat samples of different concentrations, and peaks of different heights were observed. The fat peak eluted at about 4 min, which was before the PCBs eluted under the same chromatographic conditions, and this indicated that the separation of fat from PCBs was possible on this column.

SFC of fat + PCBs. To confirm the above findings a combined sample containing fat and PCBs was prepared by mixing 50 μl of 100 μg ml^{-1} Aroclor 1242 with 50 μl of 100 mg ml^{-1} Witepsol S55.
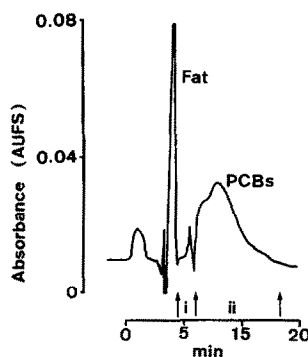

Fig. 7. SFC of fat and PCBs on a 15-cm PRP-1 column with a mobile phase of CO_2–2-propanol (80:20) at 50°C and 140 kgf cm^{-2}.

This was injected onto the 15-cm PRP-1 column at 50°C and 140 kgf cm^{-2}. Two mobile phase compositions were investigated to achieve the best possible separation of fat from PCBs, namely CO_2–2-propanol (80:20) and (60:40).

With each of the mobile phases, the mixture of fat and PCBs showed one large peak at about 4 min, followed by a series of peaks between 6 and 15 min. The previous work indicated that the first peak was due to the solvent and fat, and that the later peaks were due to the Aroclor 1242. The "fat" and "PCB" fractions were collected for each mobile phase, (Figs. 7 and 8). The identity of the peak at 4 min was confirmed by varying the fat concentrations injected onto the column. Two "PCB" fractions, (i, ii, Fig. 7) were collected with the CO_2–2-propanol
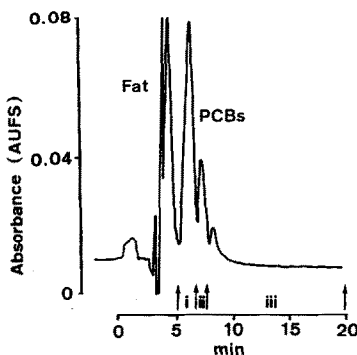

Fig. 8. SFC of fat and PCBs on a 15-cm PRP-1 column with a mobile phase of CO_2–2-propanol (60:40) at 50°C and 140 kgf cm^{-2}.

(80:20) mobile phase, and these were analysed by both GC–MS and GC–ECD. Three "PCB" fractions (i–iii, Fig. 8) were collected and analysed with the CO_2–2-propanol (60:40) mobile phase. The GC–MS traces obtained are shown in Figs. 9 and 10. The extract from using CO_2–2-propanol (80:20) mobile phase showed PCBs only in fraction ii (Figs. 7 and 9b), whereas the extracts from using CO_2–2-propanol (60:40) mobile phase showed PCBs in fractions (i–iii), (Figs. 8 and 10). The separation of the fat from the PCBs was achieved with both mobile phases, with the CO_2–2-propanol (80:20) mo-

bile phase giving the better separation of the fat from the PCBs. This was because the fat peak with the 80:20 mobile phase was sharper than with the 60:40 mobile phase. This is extremely important because in our experience, the presence of trace levels of fat in PCB extracts has a deleterious effect on the column performance of the GC–MS. This leads to an increase in baseline noise and a reduction in MS sensitivity. The 60:40 mobile phase also shows some chromatographic resolution occurring between PCBs, probably based upon differences in levels of biphenyl chlorination. In Fig. 10, an increase in the
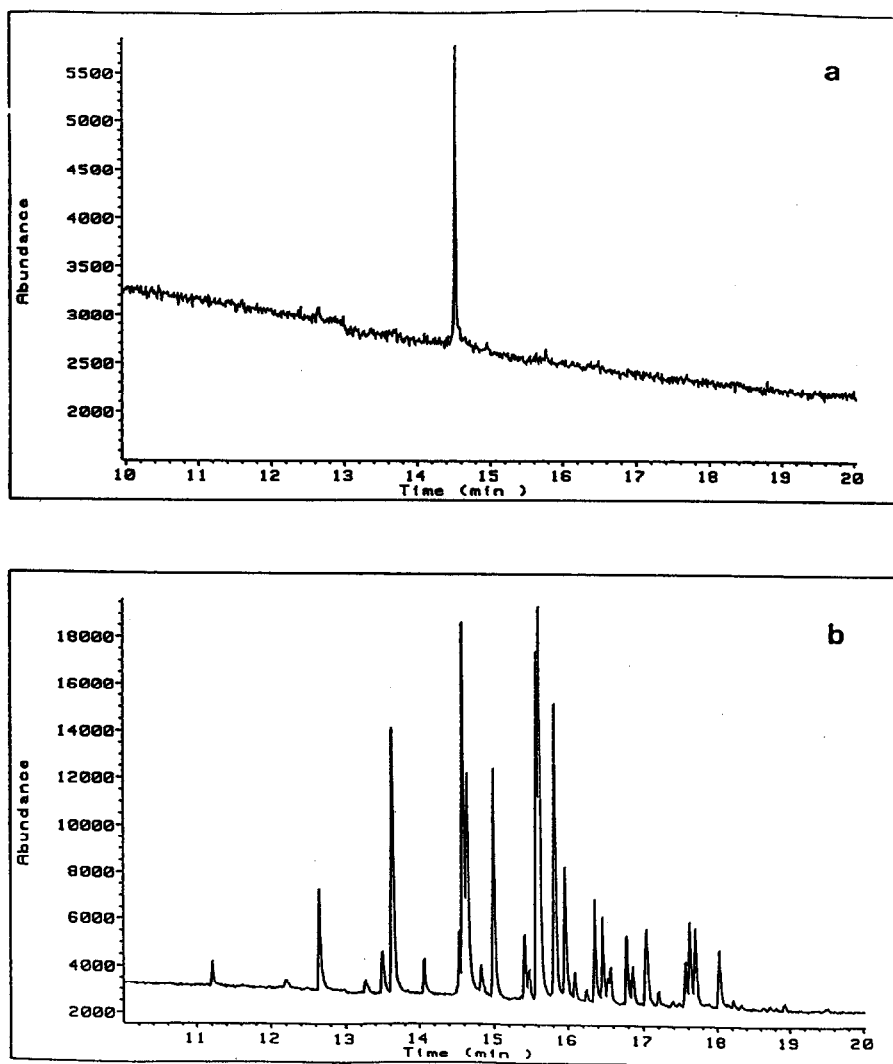

Fig. 9. GC–MS chromatograms of the "PCB" fractions collected in Fig. 7. (a) "PCB" fraction i; (b) "PCB" fraction II.
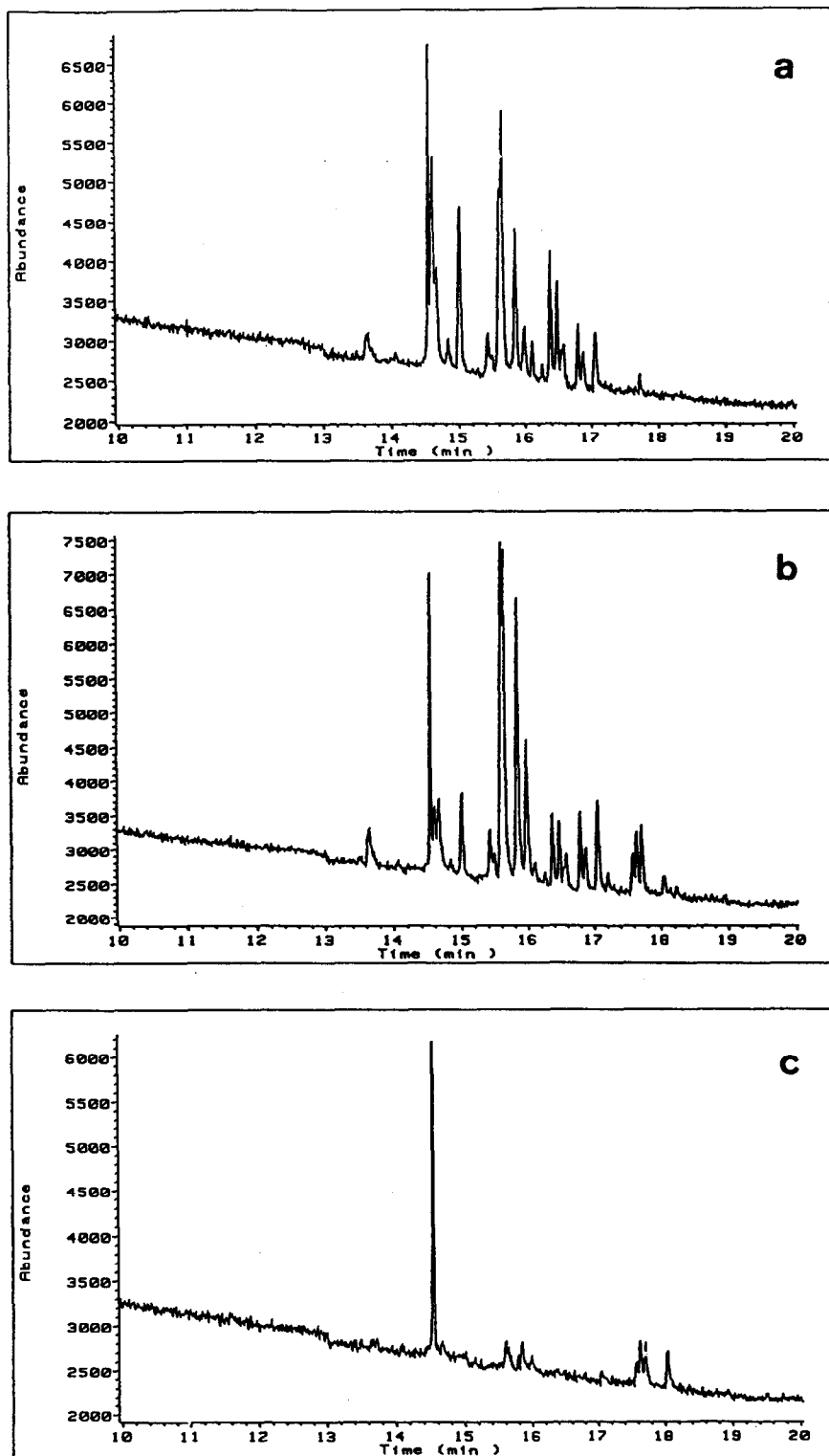
Fig. 10. GC–MS chromatograms of the "PCB" fractions collected in Fig. 8. (a) "PCB" fraction i; (b) "PCB" fraction ii; (c) "PCB" fraction iii.

occurrence of higher-molecular-mass PCBs can be seen in (c) and (b) compared to (a). The peak at 14.5 min in Figs. 9 and 10 is not a PCB, but probably a silyl fragment from the stationary phase of the capillary column.

These experimental results proved that it was possible to separate fat from PCBs by SFC on a 15-cm PRP-1 column. SFC should be a preferable separation method to HPLC for PCBs in fatty foods because SFC does not require the use of large amounts of hazardous, toxic organic solvents or the further concentration of samples before analysis that are inherent drawbacks of HPLC methodology. Studies are continuing to develop the method into a routine combined SFE–SFC procedure, optimised for the maximum percentage recovery of PCBs. In this report, recoveries were not accurately determined because a combined SFE–SFC procedure will almost certainly require different conditions to be developed. On a semi-quantitative basis, recoveries were typically about 60%.

CONCLUSIONS

The study shows the effectiveness of supercritical fluids for the extraction of PCBs and fat from cow's milk, and for the chromatographic separation of the extracted PCBs and fat. The current methods for the extraction of PCBs from cow's milk in the literature usually employ a Soxhlet extraction step, followed by a chromatography step to separate the PCBs from fat and other co-extractants.

This traditional approach has three main disadvantages when compared to methods employing supercritical fluids: (i) a large volume of toxic organic solvents is needed; (ii) the time taken for the extraction is lengthy; (iii) additional pre-concentration steps are needed prior to the final analysis, increasing the possibility of sample loss or contamination.

The proven speed, efficiency, and ease of use of supercritical fluids for extraction and chromatographic separation, coupled with the increased availability of supercritical-fluid equipment points to the increased utilisation of supercritical fluids on a routine basis for the analysis of trace organics in milk and, probably, a wide range of other matrices.

ACKNOWLEDGEMENTS

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