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COLUMN SWITCHING TECHNIQUES IN THE LIQUID CHROMATOGRAPHIC ANALYSIS OF ORGANOCHLORINE PESTICIDES IN MILK

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SUMMARY

A system is described for the analysis of organochlorine pesticides in milk by high-performance liquid chromatography. Sample clean-up, separation of pesticides from milk fat and quantification of both fat and pesticides are carried out on-column using column switching and backflushing techniques. The combination of liquid chromatography with electron-capture detection results in a detection limit of ~ 0.1 ppm pesticide in milk fat.

INTRODUCTION

The use of persistent organochlorine compounds, such as DDT, lindane and polychlorobiphenyls, has led to widespread contamination of the environment. Increasing awareness of the possible health hazards caused by these compounds has led public health authorities to propose tolerance levels for organochlorine compounds in food and beverages.

The importance of milk as a raw material for dairy products prompted a study of the feasibility of monitoring milk production for pesticide contamination. The routine examination of large numbers of samples would require automatic instrumentation capable of separating and determining the individual organochlorine compounds.

This separation is complicated by the presence of numerous complex compounds in the milk which can interfere with the analysis and by the extremely high sensitivity needed to ensure that the pesticides fall within the agreed limits.

Traditional laboratory methods for the analysis of organochlorine compounds in milk consist of three distinct stages. Initially the fat (with the fat-soluble pesticides) is extracted from the milk. The pesticide residues are then separated from the fat and from other compounds which may also interfere with the subsequent chromatographic analysis. This separation is normally achieved using a combination of liquid–liquid

partitioning and adsorption chromatography[1]. Finally the resulting solution is analysed by gas chromatography (GC) with electron-capture detection.

We have developed a laboratory prototype of an automatic instrument, the milk pesticide monitor, that combines these steps so that with an input of raw milk, an analysis is performed for both fat and pesticides, under the control of a microcomputer based on the Philips MCCS system. The full instrument has been described elsewhere[2], and the present paper gives a detailed account of the chromatographic analysis. Both "clean-up" and measurement of fat and pesticides are carried out by high-performance liquid chromatography (HPLC).

Larose[3] described the use of HPLC for the separation of γ-BHC from fats in a fish extract, prior to determination by GC. In this case the lack of a sufficiently sensitive detector prevented the quantification of γ-BHC during the HPLC stage. HPLC has also been used for the analysis of substituted ureas[4], and carbamates[5] in milk extracts after prior separation of fats by liquid–liquid partition. An excellent review of the application of HPLC to pesticide analysis has been published by Moye[6]. Organochlorine pesticides have been determined both by liquid–solid adsorption chromatography[7] and reversed-phase chromatography[8] using a UV detector. The low concentration of pesticides in environmental samples precludes the use of this detector in the present application. Sufficient sensitivity can, however, be obtained using an electron capture detector (ECD) modified for liquid chromatography (LC)[9]. Guiochon and co-workers[10,11] have shown that detection limits in HPLC can be further improved by using large sample injection volumes. In practice this is limited by the accompanying loss in column efficiency.

SYSTEM DESIGN

Sequence of operation

It is necessary to separate the pesticides from the fat and to measure the concentration of individual pesticides and fat in the extract. The organochlorine pesticides, of interest in this application, range in polarity from hexachlorobenzene to dieldrin and can be conveniently separated on a microparticulate silica column using *n*-hexane as mobile phase[9].

In the milk pesticide monitor, the chemical extraction and chromatographic stages are operated in parallel, and as the extraction cycle time is 30 min, chromatographic analysis must also be completed in this time. Using a column chosen to separate the early eluting pesticides (150 mm × 3.1 mm I.D. packed with Partisil 10) the dieldrin peak is eluted after 25 min (Fig. 1).

In such a system fat is strongly adsorbed at the head of the column and cannot be directly determined. However, it can be readily removed by backflushing with a more polar solvent and subsequently measured.

Elution of the fat could be achieved by using gradient elution, but after each analysis the column would require regeneration, preferably by reversal of the gradient profile, so that the analysis time would be effectively doubled. Furthermore, gradient elution is difficult to automate.

An alternative approach to the rapid separation of a mixture of wide-ranging polarities is that of column switching, as described by Deans[12] for GC and more recently by Huber *et al.*[13] for LC. Reference to Figs. 1 and 2 indicates that a short

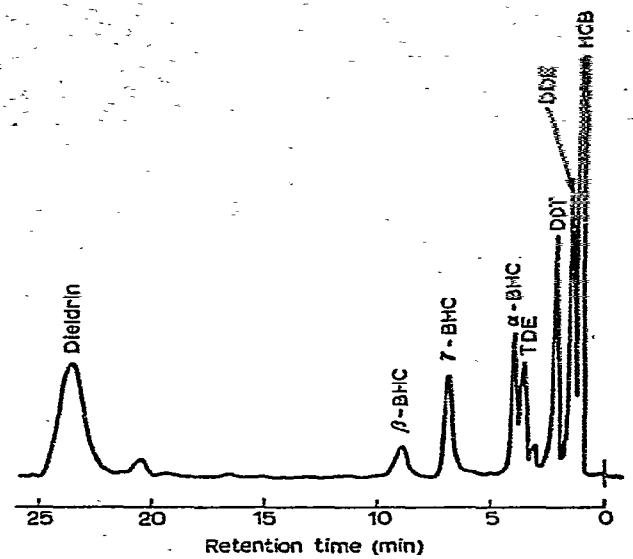

Fig. 1. Chromatogram of a standard pesticide mixture on the analytical column (150 mm × 3.1 mm I.D. packed with 10-μm Partisil). Flow-rate, 1 ml/min; ECD attenuation, × 256; standing current, $1 \cdot 10^{-10}$ A; purge, 30 ml nitrogen/min.

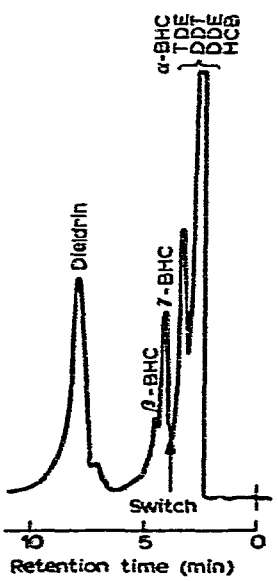

Fig. 2. Chromatogram of a standard pesticide mixture on the precolumn (50 mm × 2.1 mm I.D. packed with 5-μm Partisil). Flow-rate, 1 ml/min; ECD attenuation, × 256; standing current, $1 \cdot 10^{-10}$ A; purge, 30 ml nitrogen/min.

column (50 mm × 2.1 mm I.D., packed with Partisil 5) would be sufficient to separate the strongly retained pesticides (γ-BHC, β-BHC, heptachlor epoxide and dieldrin), whereas the longer column is necessary to resolve the early eluting compounds

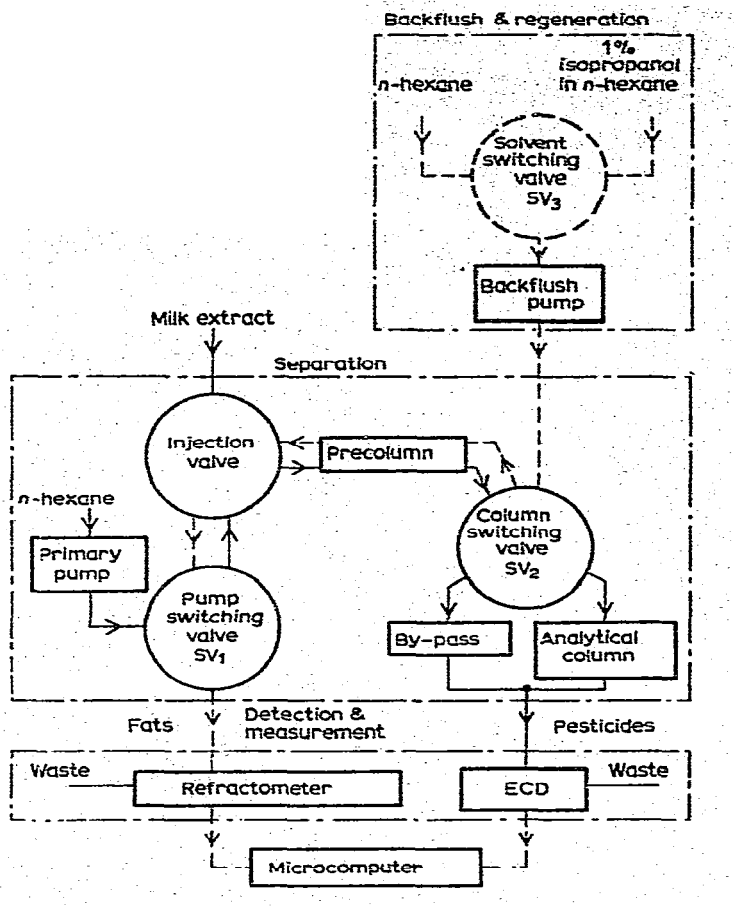

Fig. 3. Schematic diagram of the HPLC system.

(HCB, *p,p'*-DDE, *p',p'*-DDT, *p,p'*-TDE and *α*-BHC). A system of flow-switching was therefore devised, as shown in Fig. 3, in order to optimise the analysis.

The switching sequence is illustrated in Fig. 4. In the first period T_1 (~ 80 sec) the fat is retained on the precolumn and the early eluting pesticides (HCB to *α*-BHC) are led from the precolumn into the analytical column and stored. At the end of T_1 the flow is switched. In period T_2 (~ 680 sec) the late eluting compounds *γ*-BHC, *β*-BHC, heptachlor epoxide and dieldrin, having been separated in the precolumn, are led via the bypass coil to the ECD. The flow is then switched again and in period T_3 (~ 225 sec) the early eluting compounds are separated on the analytical column and detected. At the end of period T_3 the primary pump is stopped, the backflush pump is started, and the valves are switched to the appropriate positions. During period T_4 (~ 180 sec) 1% isopropanol in *n*-hexane is pumped back through the precolumn to remove the adsorbed fat. Finally, during period T_5 (600 sec) the precolumn is regenerated with *n*-hexane, which is also pumped back through the column by the backflush pump.

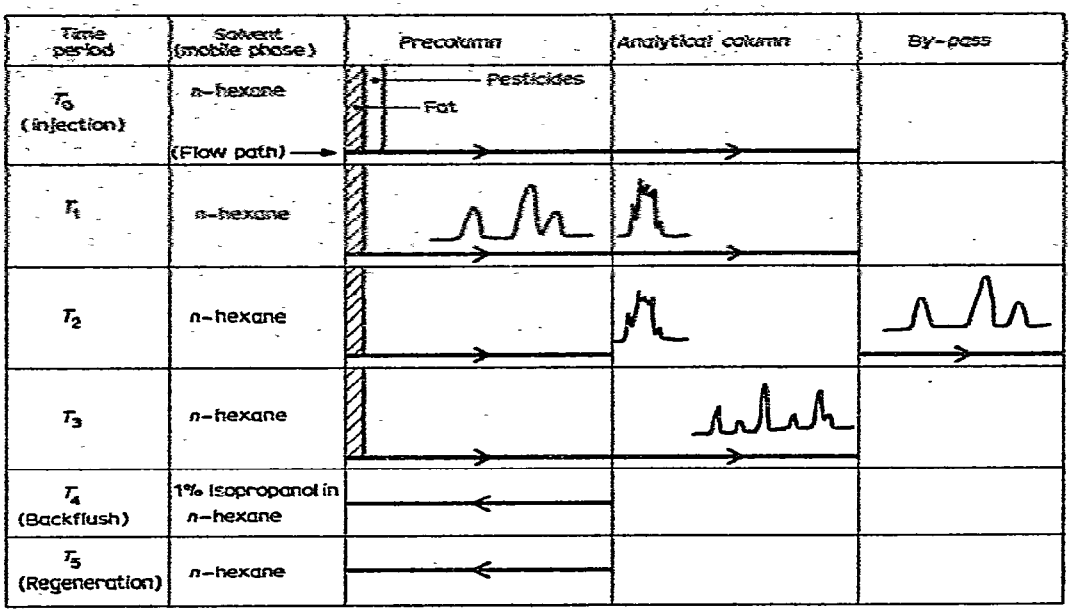

Fig. 4. Schematic representation of the switching sequence.

Design and construction

The primary pump (Model 20 LC, Pye Unicam) delivered *n*-hexane at a fixed flow-rate of 1 ml/min (~ 1200 lb./in.^{2}) and the backflush pump (Model HKP-25, Labotron) delivered either *n*-hexane or 1% isopropanol in *n*-hexane through the precolumn only, at a fixed flow-rate of 3 ml/min (1500 lb./in.^{2}). The solvents, *n*-hexane (Distol Reagent, Fisons, Loughborough, Great Britain) and isopropanol (Analar Reagent, BDH, Poole, Great Britain) were used without further purification.

Injections of milk extract were performed using a loop valve (Model CV-6-HPA, Valco Instruments), fitted with a 100-μl loop.

The column switching valve, SV_2, could be switched to any of three positions, enabling flow to be directed from the precolumn to the analytical column, from the precolumn to the bypass coil or from the backflush pump to the precolumn. The valve chosen for this function was specified as leak tight to 4500 lb./in.^{2} with a total internal volume of 15 μl (Model C74451-A1380-A3, Siemens).

The pump-switching valve, SV_1, served to switch flow from the primary pump to the injection valve or from the injection valve to the refractometer. In this case we used a three-port two-way switching valve, that was leak tight to 2500 lb./in.^{2} (Model 32.000, Spectroscopic Accessory Co.).

The solvent switching valve, SV_3, was used to direct liquid from either of the two solvent reservoirs to the backflush pump. This operated at atmospheric pressure and a miniature PTFE/CTFE valve (part No. 3L2, Hamilton) was employed.

All valves were operated pneumatically under control of the micro-computer.

The precolumn (50 mm × 2.1 mm I.D.) was packed with 5-μm Partisil silica (Whatman Biochemicals, Springfield Mill, Great Britain) and the analytical column (150 mm × 3.1 mm I.D.) with 10-μm Partisil. Columns were packed using a modi-

fication of the balanced-density slurry method described by Majors[14]. The analytical column bypass was a coil of stainless-steel tubing (30 m × 0.25 mm I.D.), the dimensions being chosen to provide a flow restriction similar to that of the analytical column.

The ^{63}Ni ECD (Model GCV, Pye Unicam) was connected to the chromatographic system by a heated stainless-steel transfer tube and maintained at 300°. It was operated in the constant current mode, as described elsewhere[3]. Peak areas were measured using a computing integrator (Model DP 88, Pye Unicam).

The refractive-index detector, a Refracto Monitor Model 1107 (Laboratory Data Control) was thermostatted by circulating water through the cell housing.

RESULTS AND DISCUSSION

Precolumn performance

The precolumn serves three functions. It separates the pesticides into two groups, gives adequate resolution of individual components of the later eluting group, and adsorbs the milk fat until it is removed by backflush. The most stringent requirement is the second, and the resolution, R_s, between any two peaks can be expressed[15] as

$$R_s = \tfrac{1}{4}(\alpha - 1)\sqrt{N}\left[\frac{k'}{(1 + k')}\right] \quad (1)$$

where k' is the mean capacity factor for the two solute peaks and N is the mean theoretical plate number for the two solute peaks. α is the separation factor for the two peaks, *i.e.*

$$\alpha = \frac{k_2'}{k_1'}$$

Rearrangement of eqn. 1 gives a definition of the number of theoretical plates required to give the desired resolution between two peaks.

Of the pesticides to be separated on the precolumn, the two giving the smallest α value (1.18), and hence most difficult to separate, are β-BHC and heptachlor epoxide.

The error in peak area assignment by the perpendicular drop method for two overlapping peaks has been theoretically determined by Proksch *et al.*[16] as a function of resolution for peaks of various relative sizes. In the context of the present work, the maximum anticipated area ratio for detected peaks is 4.

By combining the information derived from eqn. 1 with that published by Proksch we can obtain a correlation between the plate number of the column and the maximum anticipated error in peak area assignment. Fig. 5 shows the corresponding graph for two peaks ($\alpha = 1.18$) with an area ratio of 4. If we assume that an error of 10% is permissible for any peak, the precolumn is required to provide at least 380 theoretical plates. This requirement enables us to determine the maximum sample volume for a given length of precolumn. In practice, after equilibration, a 50 mm × 2.1 mm I.D. precolumn packed with Partisil 5 generated 400 plates for a 100-μl injection volume. A short precolumn was preferable for the purpose of rapid regeneration.

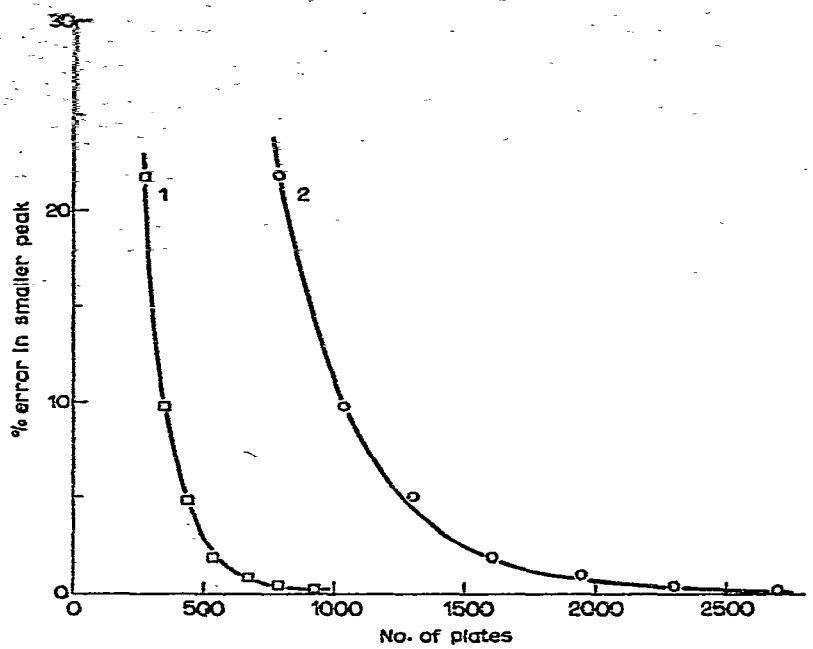

Fig. 5. Errors in the quantification of overlapping peaks as a function of column plate number. 1 = β-BHC and heptachlor epoxide (precolumn only); 2 = p,p'-TDE and α-BHC (precolumn and analytical column).

Analytical column performance

The analytical column is required to separate the early eluting pesticides, and of these the pair giving the smallest α value (1.15) is p,p'-TDE and α-BHC. The graph of maximum anticipated error in area assignment *versus* the number of theoretical plates is shown in Fig. 5. In this case for a maximum error of 10% the analytical column is required to develop 1300 theoretical plates. In practice, the precolumn and analytical column (150 mm × 3.1 mm I.D. column packed with 10-μm Partisil) gave 1700 plates for a 100-μl injection.

The plate efficiencies quoted for both precolumn and analytical column were determined using the full switching system, including the dead and swept volumes inherent in the valves and connection tubing.

Analytical sensitivity

The LC–ECD system was calibrated for the organochlorine compounds of interest, and typical calibration graphs are shown in Fig. 6. After an initial cycle to condition the precolumn, the retention times were acceptably constant (Table I).

Milk-fat solutions of known concentrations were used to calibrate the refractive-index detector, and the graph (Fig. 7) indicates that the detector is linear to 0.5% (w/w) fat.

A chromatogram of a "spiked" milk extract, containing $1 \cdot 10^{-9}$ g of each pesticide, is shown in Fig. 8. Minimum detectable concentrations (MDC) were calculated to lie in the range $\leqslant 0.5 \cdot 10^{-9}$ g/100 μl injected volume. With the extraction procedure employed in the milk pesticide monitor, it can then be shown that the

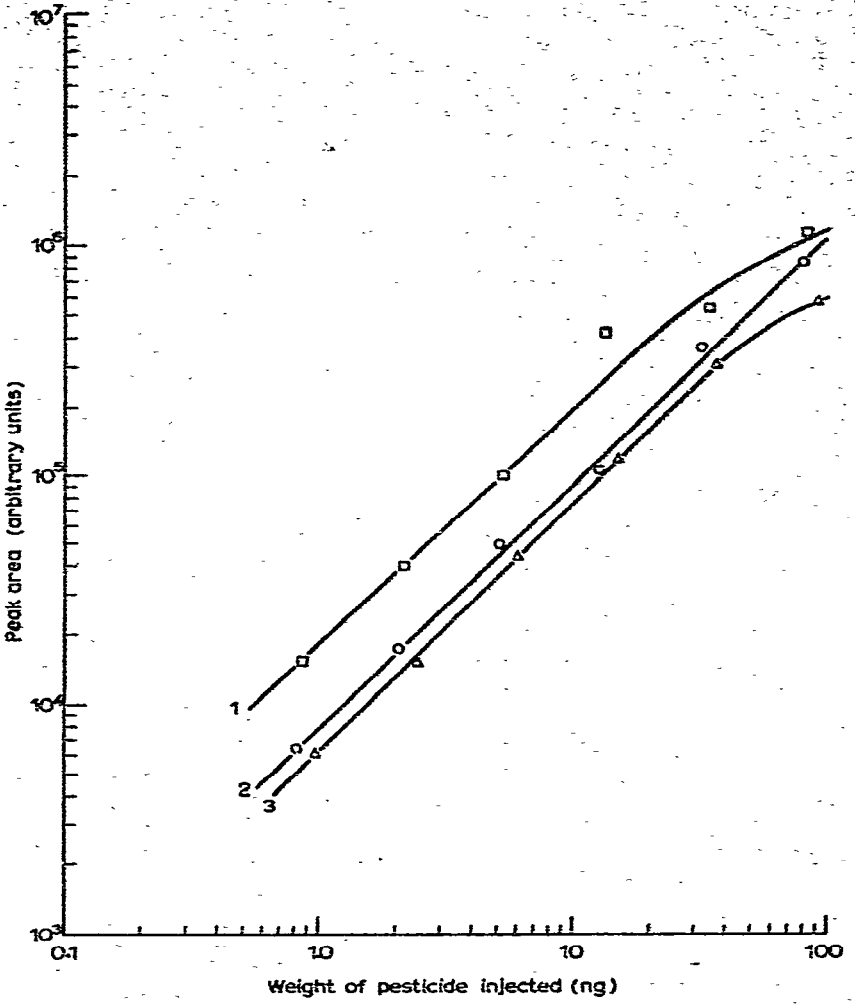

Fig. 6. Calibration of the ECD for organochlorine pesticides. Flow-rate, 1 ml/min; standing current, $1 \cdot 10^{-10}$A; purge, 30 ml nitrogen/min. 1 = *p,p'*-DDT; 2 = dieldrin; 3 = *β*-BHC.

TABLE I

RETENTION TIMES OF PESTICIDES ON SWITCHED COLUMNS

Pesticide	*Retention time (sec)* ± *S.D.*
γ-BHC	267 ± 5
β-BHC	289 ± 7
Heptachlor epoxide	325 ± 9
Dieldrin	610 ± 19
HCB	754 ± 4
p,p'-DDE	766 ± 4
p,p'-DDT	825 ± 5
p,p'-TDE	962 ± 5
α-BHC	1004 ± 4

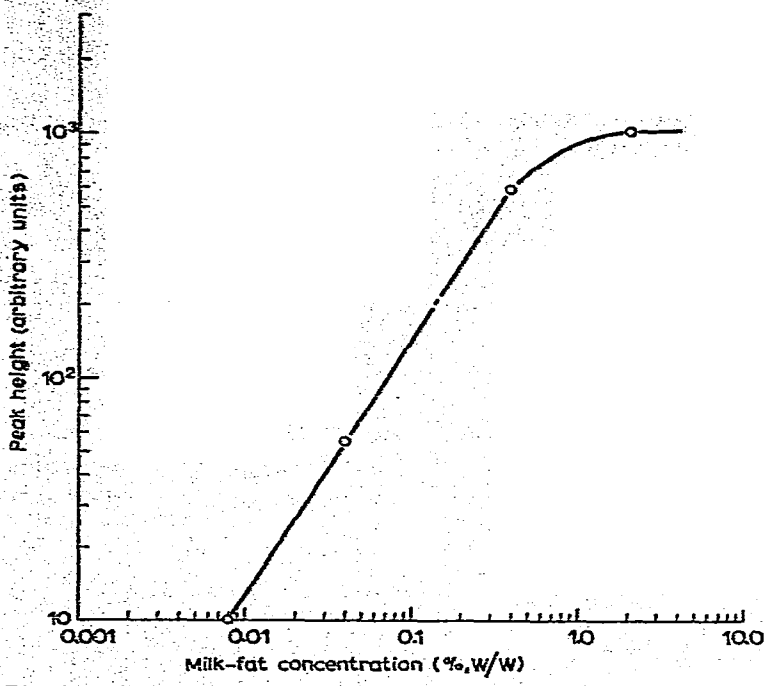

Fig. 7. Calibration of the refractometer for milk fat.

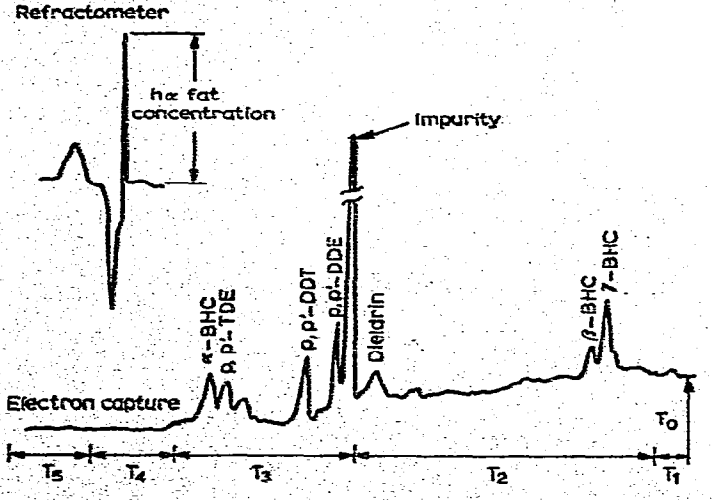

Fig. 8. Chromatogram of "spiked" milk fat using the switching system. Flow-rate, 1 ml/min; ECD attenuation, × 32; standing current, $1 \cdot 10^{-10}$A; purge, 30 ml nitrogen/min; refractometer attenuation, × 64; backflush flow, 3 ml/min. (T_0) Injection; (T_1) precolumn and analytical column; (T_2) precolumn and by-pass; (T_3) precolumn and analytical column; (T_4) backflush; (T_5) precolumn regeneration.

MDC of pesticides in the milk fat is in the range $\leqslant 0.1$ mg/kg of fat, which is below the tolerance levels suggested by the Food and Drug Administration for pesticide residues in dairy products.

CONCLUSIONS

The HPLC system described forms part of a fully automated prototype instrument for the analysis of pesticide residues in raw milk. Sensitivity is of the same order as other more conventional methods, and no stringent time- and labour-consuming clean-up step is necessary. The determination of fat in the injected sample makes prior isolation of fat unnecessary.

The use of column switching and backflushing allows the full advantages of HPLC to be realised in the analysis of a sample containing components of differing polarity and present in a wide range of concentrations. The short precolumn can be rapidly regenerated, and as the analytical column is only swept with a single solvent, it is extremely stable. The latter point is particularly important, as the pesticides are identified from their retention times.

The long-term stability and the effect of varied fat samples on the performance of the precolumn could only be proved by extended practical experience, but this approach to pesticide monitoring has not yet been put into full-scale operation. However, using a standard pesticide–milk fat solution, the variations in retention time were acceptable (as shown in Table I) and the technical feasibility has been confirmed as far as laboratory tests allow.

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