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Digital subtraction chromatography: an LC-technique to reduce disturbing peaks in bioanalysis

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Abstract: A method of reducing disturbing peaks in LC-chromatograms is described. The technique, here called digital subtraction chromatography (DSCh), makes use of the new generation of integrating chromatographic data processors. These processors are able to store the LC-detector signal and then subtract one chromatogram from another with the resulting chromatogram reintegrated and reprinted. The possibility of getting a useful chromatogram by subtracting a zero sample chromatogram from a sample with disturbing peaks is demonstrated. The theoretical background of the method is given together with a discussion of the optimisation of the chromatographic conditions. A bioanalytical application is given.

Keywords: *Liquid chromatography; removal of disturbing peaks; baseline correction; chromatographic data processor; bioanalysis.*

Introduction

Modern LC-techniques have become powerful tools in the bioanalytical chemistry. Their main advantage is the often limited requirements on sample preparation in combination with a high separation capacity. Limitations are mainly concerned with the detector, although there are several different kinds available commercially, like UV, RI, fluorescence, electrochemical and MS detectors.

The main efforts in the development of LC-bioanalysis have concerned the separation step. Nevertheless, especially in the bioanalysis of compounds present in trace amounts, there are difficulties in separating the analyte peak from endogenous background peaks. In the present work a new approach to these problems is presented.

The modern computerised chromatographic integrators can store the output signal from the detector, generated under several analytical conditions, in a digital form. Subtracting one chromatogram from another can be done using the stored data with the resulting chromatogram integrated and printed. This computerised processing of chromatographic data has been described already in 1974 [1], where baseline drifts caused by solvent peak tailing or temperature gradients in GC were compensated.

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For instance a chromatogram with interfering peaks present, a blank sample, which, in principle, is equal to the real sample except for the searched substance, could be analysed. A perfect blank sample is a chromatogram where all peaks, except for the analyte peaks, are identical with the sample chromatogram. The best way to obtain this is to make an artificial blank out of the sample. This can be done by splitting the sample into two parts as late as possible in the clean-up procedure. In one of the aliquots the analytes can then be quantitatively transformed by a derivatisation process. This sample will then serve as a blank. It is sufficient if this sample is a representative blank in the most interesting part of the chromatogram. By subtracting the blank sample chromatogram from the real sample, a resulting chromatogram showing the peak of the searched compound presented on a straight baseline may be obtained.

A further essential requirement of the technique is the use of as stable chromatographic conditions as possible. In this work, the possibilities of optimising the chromatographic conditions for this digital subtraction chromatography technique are studied. The effects of a variation of different essential parameters, as column temperature, modifier content, flow rate and injection technique, are investigated. This work is a further part of the development of methods for the bioanalysis of trace amounts of benzylpenicillin [2].

Experimental

Apparatus

Most of the instruments used in the investigation were manufactured by the Shimadzu Co., Tokyo, Japan. The pump was an LC 5A connected to a variable UV-detector, SPD-2A, operated at 280 nm. The detector signal was processed by an integrating chromatographic data processor, CR3-A. The injector was a Rheodyne Model 7125 (Berkely, CA, USA), equipped with a 2.0 ml loop. The columns were heated by a Thermomix Model 1419 oven from B. Braun, FRG. A maximator pump, MSF 72, from Schmidt, Kranz and Co., (Sorg/Südharz, FRG) was used for column packing.

Chemicals

Chloramphenicol (CAP) and chloramphenicol succinate (CAP-S) were obtained from Carlo Erba (Milan, Italy). Benzylpenicillin was kindly supplied from Novo Industri AS (Copenhagen, Denmark). Cloxacillin was obtained from commercial Ekvacillin^{T}, Astra Läkemedel AB (Södertälje, Sweden). All other chemicals were of analytical grade from E. Merck.

Chromatographic technique

The mobile phase was prepared from a stock solution of sodium dihydrogen phosphate and disodium hydrogen phosphate with a pH of 4.9. Appropriate volumes of the stock solution were added to volumetric flasks which were made up to volume with distilled water. The ionic strength (μ) was arranged to be 0.1. The eluent was then degassed under vacuum in an ultrasonic bath prior to use.

One precolumn (40 × 4.0 mm) and one analytical column (150 × 4.0 mm) were used in series. The stationary phase was LiChrosorb RP-18, 5 μm (E. Merck, Darmstadt, FRG). The columns were constructed from stainless steel and were equipped with modified Swagelok connectors and filters of 2 μm porosity (100 μm thick). Columns were packed by a balanced density technique [3] modified as described previously [4].

The columns were heated by a water jacket arranged according to Wiese and Martin [4] and connected to a circulating thermostat bath.

An electric switch was mounted on the injector enabling the data processor to start at the same time as sample injections. Also it was possible to start the integrator manually.

Chromatographic studies

The samples were aqueous solutions containing a mixture of CAP-S and CAP. The pH was kept at 4.9 where CAP-S is stable [5]. The CAP-S was a mixture of CAP-1-S and CAP-3-S, the peak of CAP-3-S was studied, it being the largest [5].

The injected samples contained 100 ng each of CAP-S and CAP in volumes from 10 to 350 μl and 5 μg of cloxacillin in a volume of 10 μl. The void volumes (V_m) of the columns were measured by the injection of water. Symbols and equations for chromatographic parameters used were according to Kirkland [6].

The mean pump flow rate during each analysis was measured 15 times by a volumetric pipette connected to the outlet from the detector cell. The time to fill the volume between two marks of the pipette was measured by means of a stop watch.

Digital subtraction technique

Normally, four samples were injected and analysed with no time lag between the analyses. These mother chromatograms were stored by the CR3-A. Then the three resulting chromatograms were printed by subtracting the mother chromatograms Nos 2–1, Nos 3–2 and Nos 4–3 at the highest sensitivity (lowest attenuation) possible. A mean value of the quotient between the amplitude of the resulting peak after subtraction and the mother peak was then calculated, as discussed more in detail in the Results and Discussion, see Fig. 1.

The retention times required in some calculations were obtained from the values printed by the data-processor.

The effect of displaced retention times in the chromatograms was studied by a manual retardation of the start of the CR3-A compared to the injection of the sample.

Bioanalytical application of the method

Pooled human milk from healthy women was used in this study. Milk samples of 9 ml were extracted and concentrated 40 times according to a clean-up scheme for the analysis of benzylpenicillin in milk [2]. The final aqueous phase of 225 μl from each sample was split equally and transferred to two 1.5 ml conical polypropylene tubes. This pair of samples was regarded to be identical.

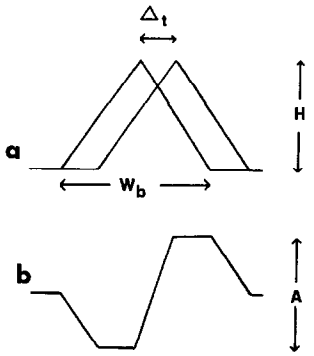

Figure 1
Idealised chromatographic peaks. Symbols, see text. (a) Two identical peaks with different retention times drawn over each other. (b) The resulting chromatogram obtained by subtracting the left peak from the right peak in (a).

In a preliminary study, chromatograms obtained from the analyses of a pair of identical samples were processed according to the DSCh-technique. The operation for chromatogram memorisation of two analyses was set on the integrator. Then the total amounts of the identical samples were analysed after each other. A resulting chromatogram was then obtained by subtracting the first analysis from the second in each pair. This was done by the background compensation programme of the integrator.

In a second study, one of the samples of the identical pairs was spiked with the mercuric mercaptide of benzylpenicillinic acid [4] (0.6 ng benzylpenicillin/ml milk). The volume of the aliquot half was adjusted with a blank solution used to prepare the derivative of Pc-G. These pairs of control and spiked samples were then analysed and processed by the DSCh-technique as described above.

Results and Discussion

Digital storage technique

The analogue signal from the detector was stored by the CR3-A chromatographic data processor in a digitalised form. The digital values were stored as μV with the time between the values dependent upon the chosen data processor parameter–peak width factor [7]. This technique forms a chromatogram consisting of points in a coordinate system, where the x-axis is time and the y-axis voltage. The start of the analysis is fixed at time zero and the y-values are allowed to vary from 1 V down to −5 mV.

By storing two chromatograms in this digital form it was possible to subtract the y-value at every fixed x-value in one chromatogram from the y-value at the corresponding x-values in a second chromatogram. The resulting chromatogram was reintegrated and reprinted as in normal LC-analysis.

If two identical chromatograms are processes by the digital subtraction chromatography technique described above, the result will be a straight line with the start in origon corresponding to the x-axis.

This ideal straight line will never exist in practice, as two chromatograms are never identical and the detector drift will alienate the straight line from the x-axis. It is possible, however, to optimise the technique by carefully selecting the chromatographic conditions.

Basic studies

In Fig. 1, two idealised chromatographic peaks with different retention times (a) and the resulting subtracted chromatogram (b) are demonstrated. A quotient (Q) between the total amplitude (A) between the two resulting positive and negative peaks after the subtraction and the height (H) of the original peaks can be calculated from the expression:

$$Q = \frac{A}{H} \tag{1}$$

and since $A_{max} = 2H$

$$0 \leq Q \leq 2 \tag{2}$$

The peaks in Fig. 1 were simplified to triangles. The difference in retention times for the two peaks is (Δ_t) and the base width for each of the peaks is (W_b). It was shown

empirically that when Q is plotted versus Δ_t/W_b for peaks like those in Fig. 1, a straight line is obtained according to the equation:

$$Q = \frac{4 \times \Delta_t}{W_b} \tag{3}$$

when $0 \leq Q \leq 2$.

This expression, equation 3, is valid for ideal triangles, but it is well known that a chromatographic peak will have an approximate Gaussian form. One protolyte (CAP-S) and one non-protolyte (CAP) were used as test substances. Their chemical and chromatographic properties have been studied earlier [5]. The pH of the mobile phase was kept close to the pK_a of CAP-S in order to see if this fact influenced the pH dependent retention of this compound compared to the non-protolyte CAP.

In Fig. 2 the approximate Gaussian peaks of CAP are shown. The chromatograms were obtained from two analyses of a mixture of CAP-S and CAP, where the chart speed was increased during the elution of CAP. The retention times for the peaks were

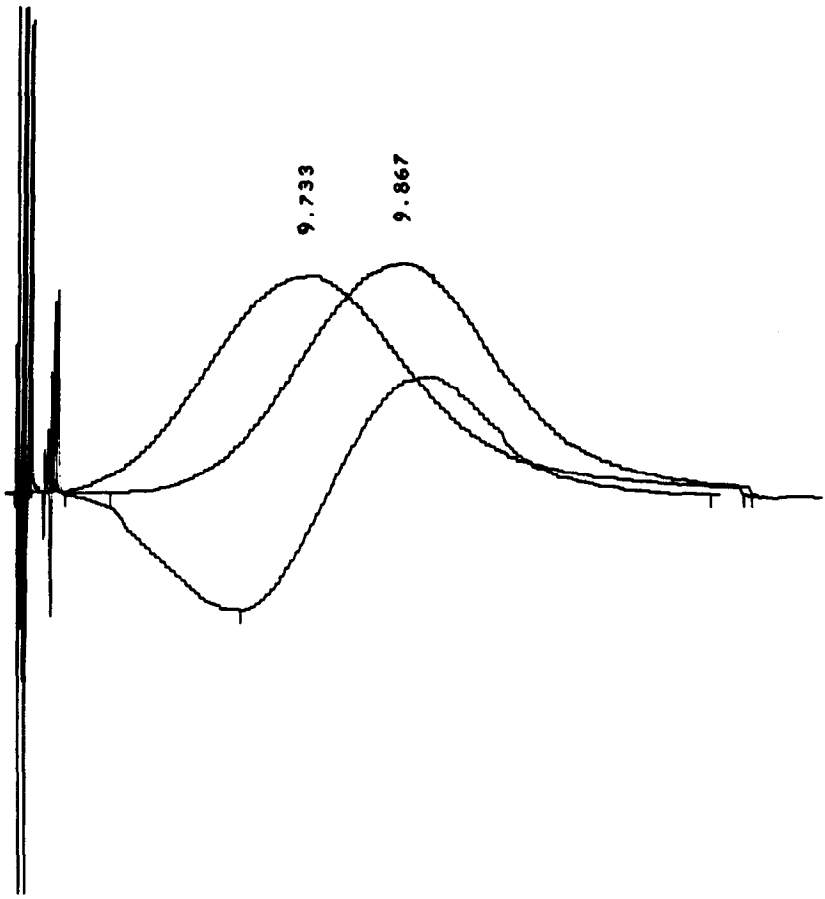

Figure 2
True chromatographic peaks obtained from two analyses of CAP. The chromatograms are printed from the same start point and the resulting chromatogram is printed by the digital subtraction chromatography technique. See also text.

displaced as described in the experimental section and the resulting subtracted chromatogram also was printed.

By varying the Δ_t of the peaks in Fig. 2, a straight line ($r = 0.995$) was obtained (Fig. 3) according to the expression:

$$Q = \frac{a \times \Delta_t}{W_b} + b \tag{4}$$

The equation that satisfies the experimental data gives $b = 0.056 \pm 0.105$, $a = 4.26 \pm 0.72$ ($P < 0.005$). As can be seen, the relationship for true chromatographic peaks was similar to that of triangles when Δ_t was the dominating variable (equation 3). As will be discussed later, where the studies were focused on the lowest Q-values close to origin, there may be more variables than Δ_t influencing the Q-value.

Column temperature dependency

The column temperature is known to be a powerful regulator of the capacity factor (k'), especially for substances with longer retention times [8]. In the LC-system used in Fig. 4, the retention time for CAP was decreased linearly ($r = 0.994$) by 14.6 s/°C in the range 22–70°C.

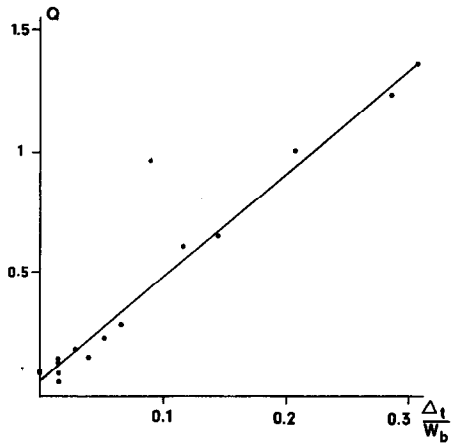

Figure 3
Plots according to equation 4. Mobile phase: 30% (v/v) methanol in phosphate buffer, pH 4.9, $\mu = 0.1$. Support: LiChrosorb RP-18 (5 μm); columns: 40 × 4.0 mm + 150 × 4.0 mm; flow rate: 0.8 ml/min; column temperature: 50°C; sample: CAP, 10 μl.

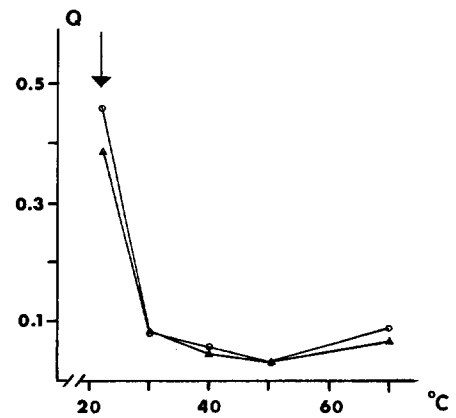

Figure 4
Effect of the column temperature on the Q-value. Mobile phase: 25% (v/v) methanol in phosphate buffer pH 4.9, $\mu = 0.1$. Columns, support and flow rate: See Fig. 3; different column temperatures; samples: ○ = CAP-S, △ = CAP, 10 μl; arrow indicates a non-thermostated column at room temperature.

Fluctuations of room temperature will influence the micro climate around an unthermostated column and this may cause the retention times to vary between the analyses. A difference of 0.1°C in the column temperature between two analyses would then, according to the calculations above, give a Δ_t of 1.46 sec. CAP has a W_b of 39 s at $k' = 6.5$. According to equation 5, the Q for CAP would then be 0.21 at a variation of 0.1°C. In Fig. 4, the advantage of a thermostated column is demonstrated. Compared to the non-thermostated condition at room temperature, the thermostated range (30–70°C) exhibits a low Q-value, probably due to a reduced Δ_t.

In Fig. 5, the Q-value was plotted versus the variations of k' caused by the variation in column temperature. It can be seen that it was not the long retention times with the non-thermostated column that caused the increase in the Q-values. The hold-up time in the column was longer for CAP at 30°C ($k' \approx 9.7$) than for CAP-S at room temperature ($k' \approx 7.5$), though the Q-value was much lower for CAP.

Organic modifier dependency

Methanol was used as organic modifier throughout the study. In order to check if W_b was proportional to k' within the tested range, the peak heights were plotted against $1/k'$. A straight line ($r = 0.996$) was obtained for CAP when $1 < k' < 13$. This showed with a good approximation that W_b increased proportionally to increasing k'. Also this is in agreement with other studies [8]. The increasing Q-values at higher methanol concentrations of the eluent as demonstrated in Figs 6 and 7 therefore could not be caused by variation in W_b. In order to investigate if the short retention times, caused by the high methanol content of the mobile phase, were responsible for the high Q-values, cloxacillin with a higher k' than CAP also was tested. Periodic variations of the pump flow may be more pronounced at shorter analysis times than at longer times. By studying Fig. 7 and comparing it with Fig. 6 it can be seen that it was impossible to find any correlation between the Q-value and retention time (compare also Figs 4 and 5). It was rather an effect of the very special column conditions applicable to each mobile phase. This will be discussed later in this work.

In Fig. 6 it can be seen that a moderate methanol content up to about 35% (v/v) gave the best result. In trace analysis there must be a compromise between a good peak response of the analysed substance and the magnitude of the disturbing peaks. If the

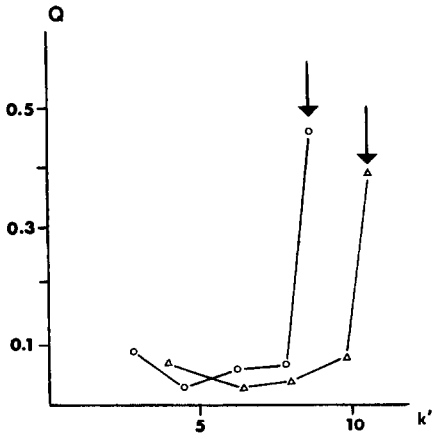

Figure 5
Q as a function of the column temperature-related capacity factors (k'), compare Fig. 4. Chromatographic conditions and samples, see Fig. 4. Arrows indicate a non-thermostated column at room temperature.

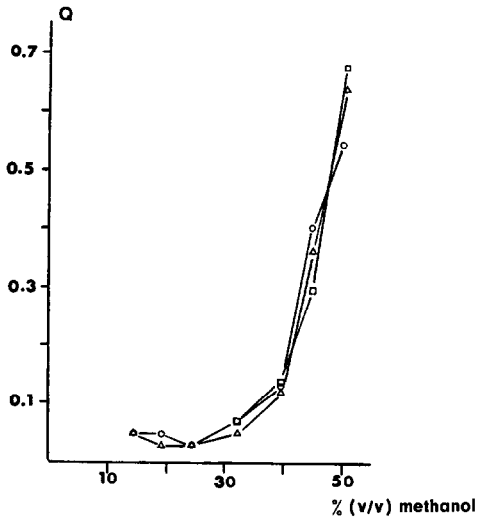

Figure 6
Effect of the methanol concentration of the mobile phase on the Q-value. Mobile phase: different methanol concentrations in the phosphate buffer pH 4.9, $\mu = 0.1$; columns, support and flow rate: see Fig. 3; column temperature: 50°C; samples: ○ = CAP-S, Δ = CAP, □ = Cloxacillin, 10 µl.

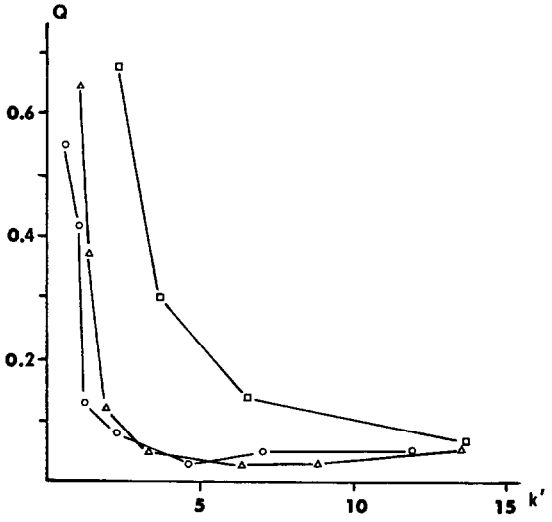

Figure 7
Q as a function of the organic modifier-related capacity factors (k'), compare Fig. 6. Chromatographic conditions and samples: see Fig. 6.

disturbing peaks are small, the increased Q-values, obtained at 45–50% (v/v) methanol content in the mobile phase, can be accepted. This is demonstrated later in the chromatograms of biological samples.

The curve in Fig. 6 is displaced to the left in parallel during the life-time of the column. The quality of the support in the column as well as the packing technique also affect the curve.

Flow rate dependency

The linear velocity of the mobile phase for the columns used in this work was 1.8 mm/sec at a flow rate of 0.8 ml min^{-1}. This was close to the optimal linear velocity

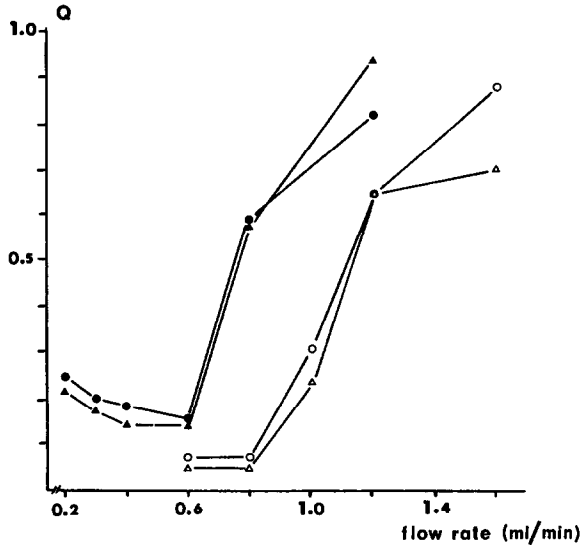

Figure 8
Effect of the flow rate on the Q-value for two different mobile phases. Mobile phases: ● and ▲ = 50% (v/v) methanol in phosphate buffer pH 4.9, ○ and △ = 25% (v/v) methanol in phosphate buffer pH 4.9, μ = 0.1. Different flow rates. Column, support and column temperature: see Fig. 3. Samples: ○ and ● = CAP-S, △ and ▲ = CAP, 10 µl.

through the packing material in the column, where changes of the flow rate just have a weak influence on the column efficiency [9]. That made it possible to increase the W_b, counted as time units, just by decreasing the flow rate and without influencing the peak height noticeably. If Δ_t is constant, an increasing W_b would, according to equation 5, decrease the Q-value.

In trace analysis, where a high methanol concentration has to be chosen in the eluent in order to have a short k' to obtain a visible peak, a decreased flow rate may reduce the negative influence on the Q-value caused by high methanol contents.

In Fig. 8, the relationship between the flow rate and Q is demonstrated for two different mobile phases. As W_b is inversely proportional to the flow rate, the fast decrease of the Q-values in Fig. 8 must — according to equation 5 — be caused by a more complex mechanism than merely the increase of W_b.

Once again it can be seen that it was the chromatographic conditions in each experiment that influenced the result. In Fig. 9, the Q-values were plotted versus the elution times for CAP-S and CAP at two different mobile phases. In order to save analysis time, it may be better to decrease the flow rate at a high methanol concentration of the eluent with the connected high Q-values (Fig. 6) than to increase the flow rate at a low methanol concentration, if the Q-values are then acceptable (Fig. 9).

Composition of the injection sample

In bioanalysis it is not always, for practical reasons, possible to choose the condition of the injected sample. Some experiments were performed to investigate how different parameters of the injection technique affected the Q-value.

A simple way to increase the injected sample amount in bionanalyses is to inject a larger sample volume. By choosing a favourable pH and/or a low organic modifier concentration in the injected sample solution, it is in many cases possible to get

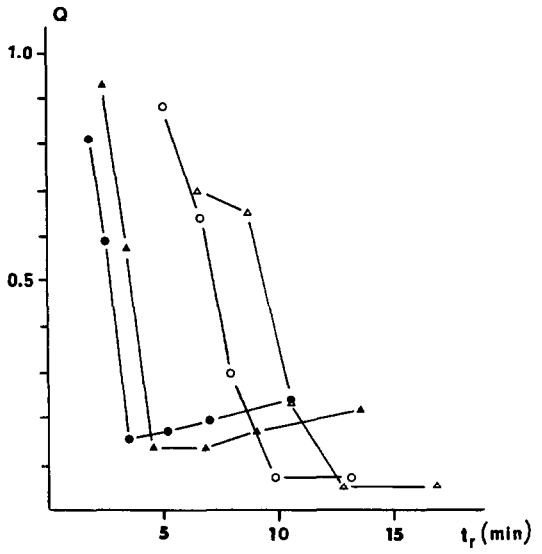

Figure 9
Q as a function of the retention times obtained at different flow rates for two different mobile phases, compare Fig. 8. Chromatographic conditions and samples: see Fig. 8.

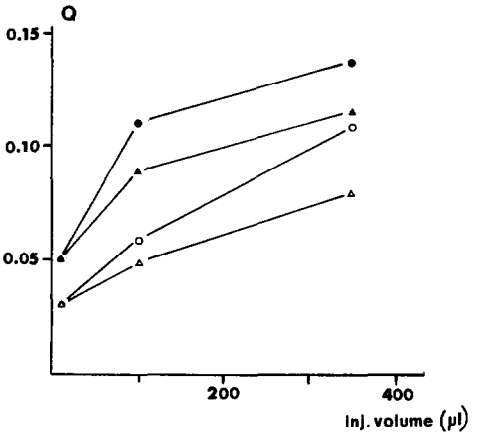

Figure 10
Effect of the injection volume on the Q-value for two different mobile phases. Mobile phases: ● and ▲ = 32.5% (v/v) methanol in phosphate buffer pH 4.9, ○ and △ = 25% (v/v) methanol in phosphate buffer pH 4.9, μ = 0.1. Columns, support, column temperature and flow rate: see Fig. 6. Samples: ○ and ● = CAP-S, △ and ▲ = CAP, in different volumes, buffer, pH 4.9.

conditions where an increased sample volume has a negligible effect on the peak heights [10].

As can be seen in Fig. 10, a larger injection volume increased the Q-value. If the disturbing peaks are high, it is advisable to concentrate the sample to a smaller volume to achieve a lower Q-value.

If the whole prepared sample volume is to be injected onto the column, the volumes may differ between two analyses. The displacement of the organic modifier from the column packing, caused by the injection of an aqueous solution, will increase the retention times somewhat, depending upon magnitude of the sample volume. If two injection volumes of an aqueous solution are not equal, the Δ_t will be affected. In Fig. 11 it is shown that the Q-value increased with increasing differences between the injected sample volumes. A small percentage difference was acceptable, however.

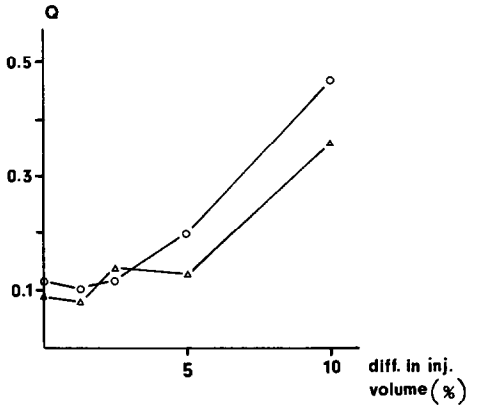

Figure 11
Effect of differences in injection volumes between the subtracted analyses on the Q-value. Mobile phase: 32.5% (v/v) methanol in phosphate buffer pH 4.9, μ = 0.1. Columns, support, column temperature and flow rate: see Fig. 6. Samples: ○ = CAP-S, △ = CAP in 200–220 µl buffer pH 4.9.

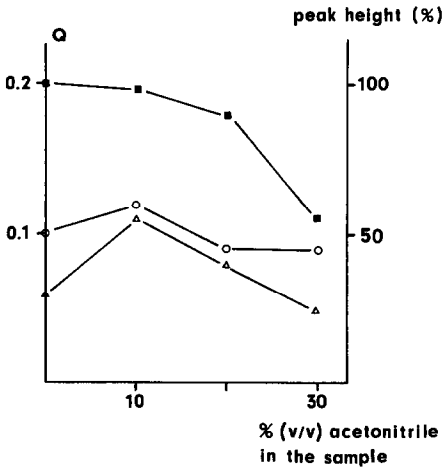

Figure 12
Effect of different acetonitrile concentrations in the sample on the Q-value. Mobile phase: 25% (v/v) methanol in phosphate buffer pH 4.9, μ = 0.1. Columns, support, column temperature and flow rate: see Fig. 6. Samples: ○ = CAP-S, △ = CAP in 200 µl of different mixtures of buffer pH 4.9 and acetonitrile. ■ = Peak heights of CAP.

A common way to prepare biological samples for injections onto a column is to precipitate the proteins with an organic solvent, preferably acetonitrile [11]. The solvent content of the injected sample often has to be reduced in order to have a good column efficiency [5]. In Fig. 12 it is demonstrated that the Q-value was not affected by an increasing amount of acetonitrile in the injected sample. It should be noted that the poor peak heights at the high solvent concentrations in the sample did not affect the Q-value much by an increased W_b.

Further observations

No differences between the Q-values were obtained for the non-protolyte (CAP) and the protolyte (CAP-S). Lower ionic strengths of the mobile phases were tested but CAP-S was not affected with reference to the Q-value.

The measurements of the flow rate from the pump showed no typical pattern of variation. The flow rate varied less than the peak retention times printed on the chromatograms, and no correlation between the mean flow rate during the time of analysis and the corresponding retention times could be found (r = 0.08). A dual piston pump (ConstaMetric III, LDC, Milton Roy Co., FL, USA) with better specified flow stability than the LC5-A was also tested, but no effect on the Q-values could be

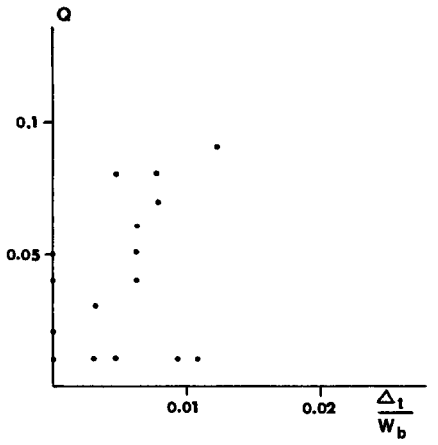

Figure 13
Plots according to equation 4 under optimised conditions. Compare Fig. 3. Conditions: see Fig. 3.

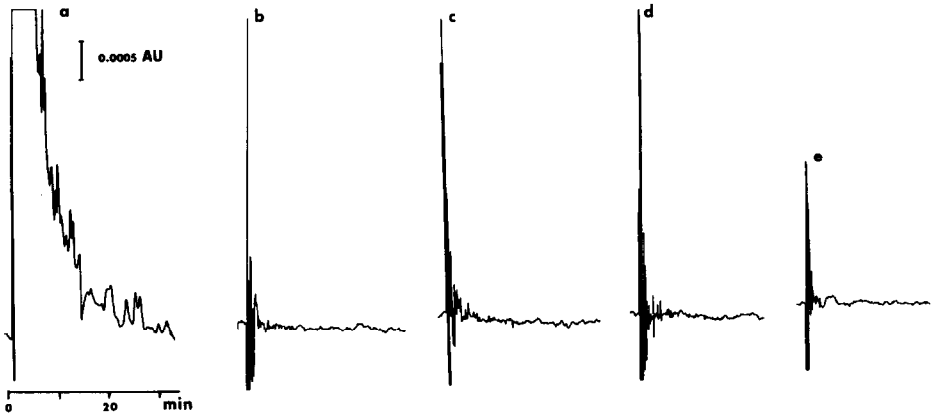

Figure 14
Bioanalyses of extracts of pooled human blank milk processed by the digital subtraction chromatography technique. Mobile phase: 45% (v/v) methanol, 2% (w/v) imidazole, 50 μM mercury(II)chloride in phosphate imidazole buffer pH 4.9. Columns, support, column temperature, flow rate: see Fig. 6. Samples: 150 μl aliquots of an aqueous human milk extract. Chromatograms: a: example of a mother chromatogram. b–e: examples of resulting chromatograms obtained by subtracting pairs of mother chromatograms.

observed. The time intervals between the analyses to be subtracted were insignificant, the time of analysis for the human milk extracts demonstrated later in the biological application for instance was about 30–40 min.

A Q-value of zero was never reached in this study, since the quotient between the resulting baseline noise and the studied peaks gave $Q \approx 0.01$. Three detector time constants, 0.25, 0.55 and 1.55 sec, were tested. No effect on the Q-value was observed. The use of a time constant 1.55 sec, however, gave a smoother chromatogram.

During the work of this study it has been understood that each injection of a sample gave rise to a unique behaviour of the molecules in the sample zone. This may affect such chromatographic parameters as asymmetry factors (Asf), plate height (H) etc. The variations caused by this unique behaviour of each injection may be more or less pronounced owing to the chromatographic conditions. This may explain why the

variations of the Q-values were independent of the hold-up times on the column, as mentioned earlier.

In Fig. 13, the Q-values were obtained under optimised chromatographic conditions as a moderate methanol content of the mobile phase, a thermostated column, a low sample volume, a flow rate of 0.8 ml/min and an automatic start of the integrator at the time of injection. The plot in Fig. 13 of Q versus $\Delta_t W_b$ according to equation 4 shows a very poor correlation ($r = 0.33$). It should be pointed out that Δ_t is calculated from the retention times printed by the chromatographic data processor. In Fig. 2 it can be seen that the top of the peaks are more or less plateau shaped. Depending on small variances of this shape the printed retention times may differ for peaks which otherwise are identical. This could be an explaination why different values of (Δ_t/W_b) gave the same value for Q and vice versa in Fig. 13.

The use of more sophisticated chromatographic data processors with the possibility of adjusting the retention times manually in the reanalysing and subtraction steps, therefore may not reduce the Q-value very much under optimised conditions. In the first place it may be better to minimise the variations of Q, created in the column, by developing uniformly packed columns, a precise injection technique and, as demonstrated in this work, optimised chromatographic conditions.

Application of the method to a biological sample

The possibility of reducing chromatographic peaks in a bioanalysis by the DSCh-technique was investigated in a preliminary study. By analysing two identical samples, the resulting subtracted chromatogram should be as close to a straight line as possible. In Fig. 14, the chromatographic results of four such subtractions are shown. The samples were aliquots of an extract of human milk [2]. One of the original chromatograms (a) is also demonstrated. The methanol content of the mobile phase was 45% v/v and the Q-values for the peaks, with retention times >8 min in Fig. 14, were calculated to be about 0.15–0.25. These Q-values were as expected according to the chromatographic conditions, e.g. the composition of the mobile phase, injection volume etc. As can be seen, the baselines after subtraction are acceptable for retention times >8 min.

As discussed in the introduction of this work, the best way to obtain a blank sample for actual application of the DSCh-technique, is to make an artificial blank. In this basic study it was, for practical reasons, chosen to make an artificial sample simply by adding a derivative of benzylpenicillin [4] to one half of a split extract of blank milk. The identity of the two split sample halves to be analysed by the DSCh-technique could then be kept, except for the analysed compound.

In Fig. 15, the chromatographic results of subtractions of blank samples from spiked samples are demonstrated. An example of a blank sample chromatogram and a chromatogram of the spiked blank sample also is shown. The peak at the retention time of 10.7 min corresponded well with the added amount of the mercuric mercaptide of penicillenic acid. The relative standard deviation (RSD) of the peak area measurements was $\pm 9.6\%$, $N = 8$.

Conclusions

As shown in this work, it is possible to use digital subtraction chromatography in order to increase the quantitative as well as the qualitative strength of the determination of a compound presented in chromatograms with interfering peaks. The samples must,

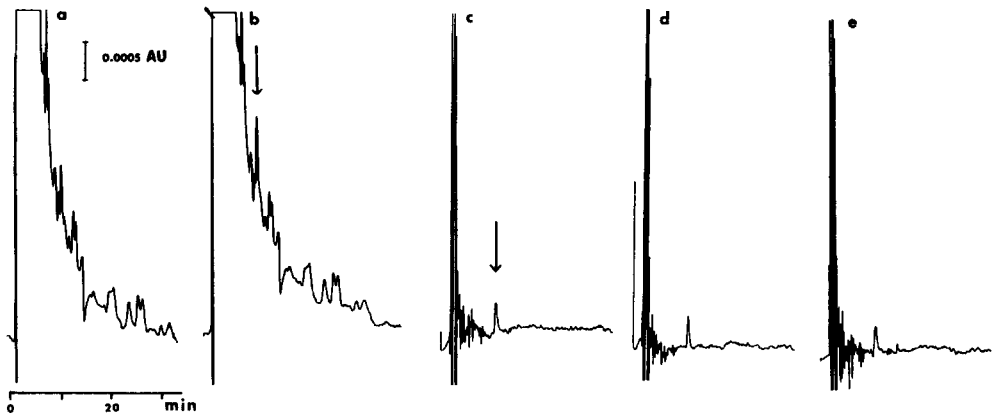

Figure 15
Bioanalyses of spiked extracts of pooled human blank milk from which blank samples are subtracted according to the DSCh-technique. Conditions: see Fig. 14. Samples: blank milk extracts as in Fig. 14(a) and blank milk extracts spiked with the mercuric mercaptide of benzylpenicillenic acid corresponding to 0.6 ng benzylpenicillin/ml milk (b). Chromatograms: a: example of a blank sample. b: example of a spiked blank sample. c–e: examples of resulting subtracted chromatograms. Arrow indicates the derivatives of benzylpenicillin.

Table 1
Effects of various chromatographic parameters on the DSCh-performance

Chromatographic parameter		DSCh*-performance
Thermostated column	yes	+
	no	−
Methanol content of the eluent	<35%	+
	>35%	−
Flow rate	low	+
	moderate	+
	high	−
Sample volume	small	+
	large	−
Differences in sample volumes	small	+
	large	−
Acetonitrile content of the sample	low	+ −
	high	+ −

*+ = Good performance quality; − = bad performance quality; + − = no difference.

however, be carefully prepared and the chromatographic conditions should, if possible, be optimised. This means not too high methanol content of the mobile phase, a thermostated column, a moderate flow rate and small and precise injection volumes. These conclusions are summarised in Table 1. The technique is used in a method where trace amounts of benzylpenicillin in milk are determined [2] and where the preparation of an artificial blank sample in one of the halves of a split sample is also demonstrated.

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