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Quantitative aspects of the determination of compounds with widely varying polarity using capillary supercritical fluid chromatography

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ABSTRACT

The quantitative aspects of capillary supercritical fluid chromatography are discussed, focusing on the impact of the nature of the analytes and their chromatographic behaviour on quantification. In the experimental work model substances, mainly nitrogen containing, of varying polarity and basicity were chromatographed on a polar cyanopropyl–methyl- and a slightly polar phenyl–methyl-polysiloxane stationary phase using supercritical nitrous oxide as the mobile phase. Changes in the nature of the stationary phase were shown to decrease adsorption tendencies, resulting in improved peak shapes and thus better quantification. The precision of peak-area measurements using direct injection was 3–12% (relative standard deviation) in the concentration range 100–1000 ppm, the higher values being obtained for the non-optimum solute–stationary phase combinations, as indicated by the relative response plots. The detection limits obtained with a nitrogen-sensitive thermionic detector were in the range 2–29 ppm, corresponding to 0.1–1.4 ng. It was found that quantitative determinations of early eluting compounds is facilitated by using a precolumn-based injection system.

INTRODUCTION

Although the number of papers dealing with capillary supercritical fluid chromatography (SFC) is rapidly increasing, quantitative aspects are still rarely discussed. Table I lists papers on capillary SFC using the criterion that regression data were presented. In these papers, quantitative aspects are only briefly mentioned, and to our knowledge no paper has appeared in which quantification based on regression data is the main subject. Our previous paper [18] included a limited discussion on quantitative analysis, with a few model substances chromatographed on a slightly polar phenyl–methyl column. In this work we extended the number of model substances to include compounds of widely differing polarity and basicity. Their chromatographic behaviour was investigated on two capillary columns differing in polarity.

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TABLE I

QUANTITATIVE DETERMINATIONS REPORTED IN CAPILLARY SFC

Analyte[a]	Detection	Ref.
PAHs	Fluorimetric	1
PAHs (nitrated)	Thermionic	2
Benzothiophene	Flame photometric	3
Herbicides	UV absorbance; Flame ionization	4
PAHs	UV absorbance	5
PAHs	UV absorbance	6
Insecticides	Mass spectrometric	7
α-Keto acids	Thermionic	8
PAHs, hydrocarbons	UV absorbance; Flame ionization	9
Pesticides	UV absorbance	10
Caffeine	Fourier transform infrared spectrometric	11
Benzothiophene	Flame photometric	12
Trimyristin	Light scattering	13[b]
Thiols	Chemiluminescence	14
Pesticides	Radiofrequency plasma	15
Organosulphur compounds	Flame photometric	16
Steroids	Thermionic	17
Aromatic amines	Thermionic	18
Halogenated compounds	Electron capture	19
Mefloquine	Electron capture	20

[a] PAHs = polynuclear aromatic hydrocarbons.
[b] Packed capillary column.

EXPERIMENTAL

Equipment

The equipment has been described previously [18]. It consists of a syringe pump (Model 8500; Varian, Walnut Creek, CA, USA) and a gas chromatograph (Model 3710, Varian), equipped with either a thermionic nitrogen–phosphorus detector (Model TSD, Varian) or a flame ionization detector (Varian). The pump was modified to work under constant pressure, as described [21]. A digital voltmeter showed the pressure directly in bar, a digital pulse counter measured the flow-rate (3600 pulses correspond to 1 ml) and a table-top computer (ABC-80; Luxor, Motala, Sweden) equipped with a digital–analog converter was used for creating linear and parabolic pressure gradients. Samples were injected using a manual, 60-nl loop injector (Model C14W; Valco, Houston, TX, USA) or a precolumn injection system developed for trace analysis [22]. Collection and handling of chromatographic data were executed with a PC (Model V386A; Victor Svenska, Stockholm, Sweden) using the JCL6000 Chromatography Data System (Jones Chromatography, Hengoed, Mid-Glamorgan, UK). Chromatograms were also registered on a recorder (Model 1107; W + W Electronics, Basle, Switzerland).

The capillary columns were connected with a zero dead-volume union (Valco) to a 50- or 100-μm frit restrictor (Lee Scientific, Salt Lake City, UT, USA). The restrictor end was positioned 1–2 mm below the outlet of the detector tip and swept with make-up gas (25 ml/min of nitrogen) entering at the detector base.

Typical settings for the flame ionization detector were: hydrogen flow-rate 30 ml/min and air flow-rate 180 ml/min and for the thermionic detector 1.2 and 210 ml/min, respectively. For the thermionic detector the bead current was set at 3.27 A (580 scale divisions) and the bias voltage at 7 V. The optimization procedure of the thermionic detector has been described previously [18].

Nitrous oxide (medical grade, >99%; AGA, Stockholm, Sweden) was used as the mobile phase. Typical flow-rates from the pump (measured by the pulse counter) at room temperature and 100 bar were 6 μl/min.

Columns

The columns investigated were DB-17 (50% methyl–50% phenyl-polysiloxane), 10 m × 50 μm I.D., film thickness 0.10 μm (J&W Scientific, Folsom, CA, USA), SB-cyanopropyl-50 (50% methyl–50% cyanopropyl-polysiloxane), 10 m × 50 μm I.D., film thickness 0.25 μm (Lee Scientific) and SB-octyl-50 (50% *n*-octyl–50% methyl-polysiloxane), 10 m × 100 μm I.D., film thickness 0.5 μm (Lee Scientific).

Chemicals

All substances used as solutes in the direct injection mode are listed in Table II together with abbreviations.

2,6-TDA and MDA were obtained from Merck (Darmstadt, Germany). The carbamate derivatives 2,4-TDC and 2,6-TDC were prepared by reacting the corresponding diamine with ethyl chloromate (Sigma, St. Louis, MO, USA) according to the procedure described [23]. The amide derivative FMDA was prepared at the Department of Occupational Medicine (University Hospital of Lund). Methaqualone was a gift from R. Isaksson (Department of Organic Chemistry 3, University of Lund, Sweden). Cothinine was obtained from Pharmacia (Helsingborg, Sweden) and Raclopride from Astra (Södertälje, Sweden). Trioctylamine [>95% by gas chromatography (GC)] was obtained from Merck and dodecanoic acid dinitrile (purum) from Fluka (Buchs, Switzerland). Octadecane (Fluka) was used as a model substance in the precolumn injection experiments. Solvents were of the highest available purity.

RESULTS AND DISCUSSION

The analyte

In all quantitative work, the nature and concentration of the sample components must first be considered. In GC, a decision first is made between direct analysis or the development of a derivatization procedure. In SFC, the higher solubility of the solutes in the mobile phase compared with GC permits the direct analysis of more polar compounds. However, it must be stressed that the improved peak shapes that can be accomplished by derivatization give improved quantification possibilities also in SFC. The improved peak shape of, *e.g.*, MDA after derivatization to its amide derivative (Fig. 5 in ref. 18) resulted in an improvement in the precision [relative standard deviation (R.S.D.)] from *ca.* 13% to *ca.* 3% at concentrations of 25 ppm. Advantages of derivatization in SFC analysis have been recognized by others, *e.g.*, David and Novotny [8] in the determination of α-keto acids.

TABLE II

SURVEY OF COMPOUNDS TESTED

Compound	Abbreviation	Formula	Compound	Abbreviation	Formula
2,6-Toluenediamine	2,6-TDA	H_2N, CH_3, NH_2	Methaqualone	MQ	N, CH_3, N, CH_3, O
2,6-Toluenediethyldicarbamate	2,6-TDC	$C_2H_5O-\overset{O}{C}-HN$, CH_3, $NH-\overset{O}{C}-OC_2H_5$	Cothinine	COTH	N, N, O, CH_3
2,4-Toluenediethyldicarbamate	2,4-TDC	$H_5C_2O-\overset{O}{C}-HN$, $NH-\overset{O}{C}-OC_2H_5$, CH_3	Raclopride	RP	Cl, OH, $\overset{O}{C}-NH-CH_2$, N, C_2H_5, OCH_3, Cl
4,4′-Diaminodiphenylmethane	MDA	H_2N, CH_2, NH_2	Trioctylamine	TOA	$CH_3(CH_2)_7-N$, $(CH_2)_7CH_3$, $(CH_2)_7CH_3$
4,4′-Diaminodiphenylmethane (perfluorobutyro derivative)	FMDA	$F_7C_3-\overset{O}{C}-NH$, CH_2, $NH-\overset{O}{C}-C_3F_7$	Dodecanoic acid dinitrile	DODN	$N\equiv C-(CH_2)_{10}-C\equiv N$

Chromatographic behaviour

A prerequisite for good quantification is a symmetric peak shape for the components of interest and a good separation between them. The peak shape, determined primarily by the mutual interactions between the analyte and the stationary phase, may be improved by changing the stationary phase polarity. Fig. 1 shows the improvement for the polar compound raclopride on changing from a slightly polar to a strongly polar stationary phase. Even though the peak heights differ considerably, the peak areas are the same. The accuracy of electronic integration will be considerably better in the case of peak shapes as in Fig. 1b.

The resolution, R_s, and the relative peak sizes, *i.e.*, the concentration ratio between two neighbouring peaks are also of considerable importance for quantitative accuracy and integrator performance. Both of these aspects have been discussed by Guiochon and Guillemin [24]. In SFC the resolution can be controlled by simultaneous variation of three parameters, namely temperature, pressure and mobile phase composition. This offers unique possibilities to tailor the separation. In practice, an optimization with respect to temperature and pressure is normally sufficient. With pressure programming normally little additional work is needed to improve the resolution greatly. For example, from an R_s value of 1.0 (at 120 bar and 150°C) the resolution was improved to 2.3 on applying a slightly concave parabolic pressure gradient for the pair MQ and COTH in a mixture of three model compounds (MQ, COTH and RP). This would decrease the error in the relative peak-area measurements from *ca.* 2% to virtually zero [24]. It should be noted that the elution order was changed when the pressure gradient was applied. The chromatograms in Fig. 2 illustrate the separation.

Instrumental aspects

Injection. In capillary SFC, a sample introduction system must be capable of introducing very small, repeatable sample volumes on the analytical column. We have used two different systems, manual direct valve injection of 60-nl samples and a device using precolumn enrichment and subsequent transfer of the analytes to the analytical column using the mobile phase. The sample solvent in this study was usually methanol, which was found [18] to give smaller solvent peaks than other solvents in the chromatographic system used.

With direct injections, no overloading effects were observed with analyte concentrations up to 2000 ppm (w/w) in methanol solution (*i.e*, injection of 95 ng), even at pressures as low as *ca.* 100 bar. The solubility of the solvent in the mobile phase and possible adsorption of the analyte in the injection valve determine the injection time needed to transfer the whole sample onto the analytical column. For example, we found previously that with 2,6-TDC in methanol at 90 bar, *ca.* ten loop volumes of mobile phase were needed, corresponding to an injection time of *ca.* 5 s [18]. The solubility can be improved and the adsorption tendency supressed by using higher mobile phase pressures, giving shorter injection times. On the other hand, too high pressures may destroy the separation between the compounds of interest, which is why a compromise must be chosen. However, the extra band broadening resulting from the longest injection time (5 s) needed in our experiments had no noticeable effects on the quantification.

In the precolumn injection mode, a solvent evaporation step reduces the size of

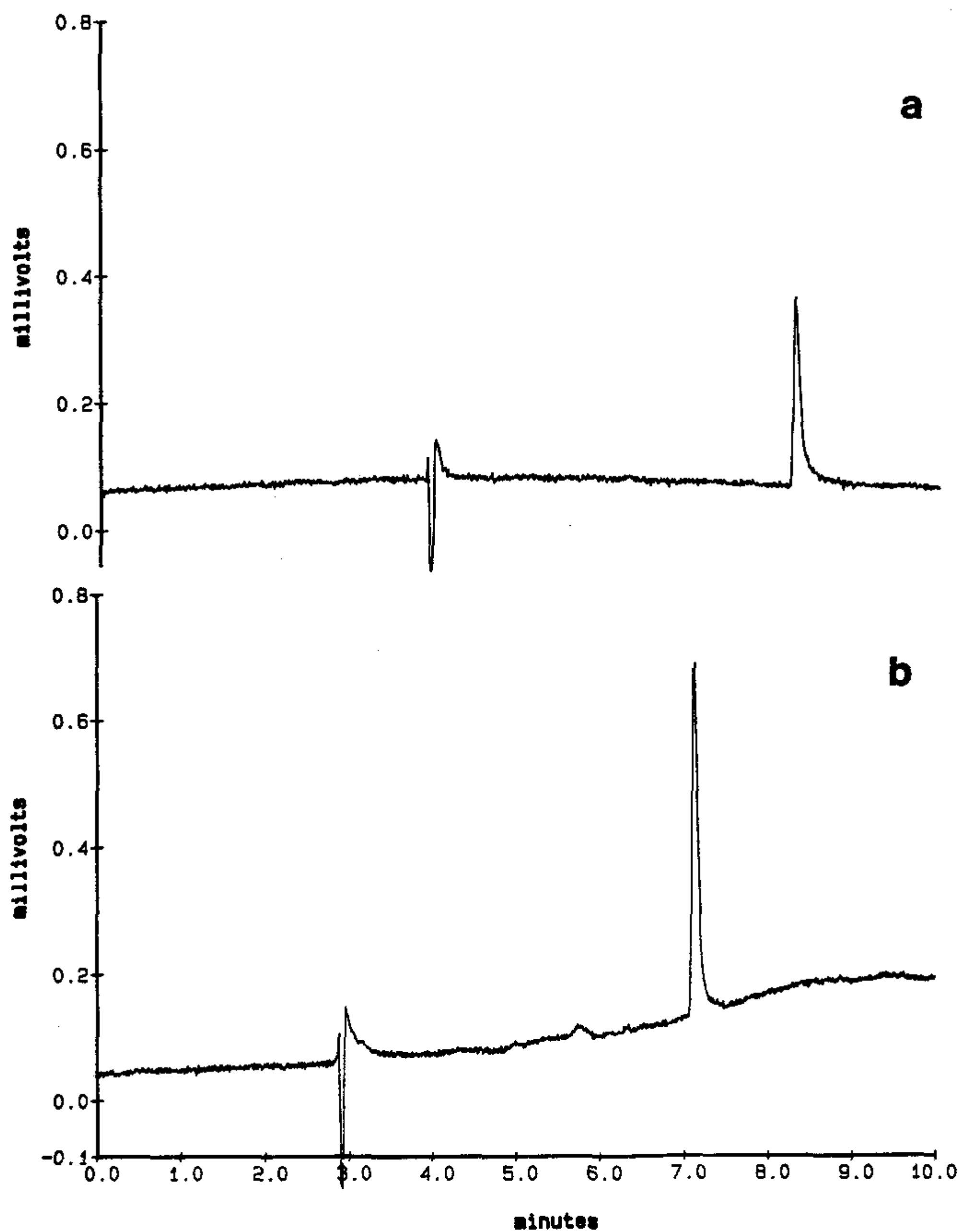

Fig. 1. Chromatograms of Raclopride. Columns, (a) DB-17, (b) SB-cyanopropyl-50; concentration, 250 ppm; sample solvent, methanol; injection volume, 60 nl; pressure programme: 100–180 bar at 10 bar/min; temperature, 150°C.

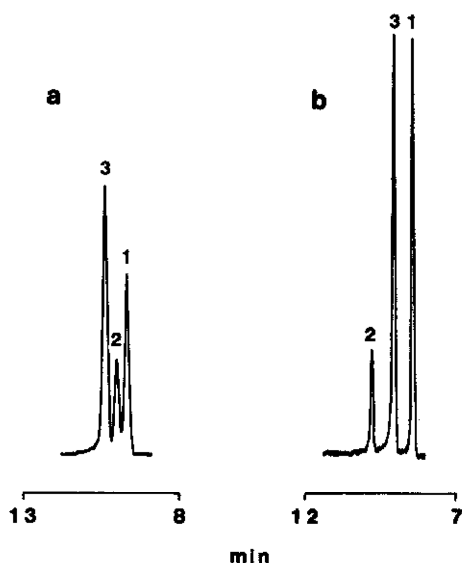

Fig. 2. Chromatograms of some model substances. Column: SB-cyanopropyl-50; concentration, 250 ppm; attenuation, (a) $4 \cdot 10^{-12}$ A f.s., (b) $2 \cdot 10^{-12}$ A f.s.; sample solvent, methanol; injection volume, 60 nl; system pressure, (a) 120 bar, (b) 120–180 bar, concave parabolic pressure gradient during for 10 min, 0.01 bar/min starting rate; temperature, (a) 150°C, (b) 115°C. Peaks: 1 = DODN; 2 = MQ; 3 = COTH (for abbreviations, see Table I).

the solvent peak in addition to giving a lower noise level during the entire chromatographic run. This permits determinations of early eluting analytes even at low concentrations. By increasing the sample size on the precolumn, enrichments are possible to the extent that analyses below 1 ppm are feasible. Fig. 3 shows a comparison between the two modes of injection, direct or via a precolumn, using flame ionization detection. The precolumn injection device is described in detail in a separate paper [22].

Detection. In this work, both flame ionization detection (FID) and nitrogen-sensitive thermionic detection (TID) were used, in both instances with nitrous oxide as the mobile phase.

In the precolumn experiments in which we have used nitrous oxide in combination with FID we found that the baseline increased linearly with increasing pressure, *i.e.*, increased flow-rate of nitrous oxide to the detector. The same behaviour was reported by Ashraf-Khorassani *et al.* [25], who attributed the baseline increase to the increase in oxidizing agent (nitrous oxide) in the flame. However, there is also a possibility that the increase may be due to hydrocarbon impurities in the nitrous oxide. A fact which may point in this direction is that the change in flame composition will be small as make-up gas (nitrogen) at 25 ml/min is used compared with a change in flow-rate from *ca.* 0.5 to *ca.* 1 ml/min during a pressure-programmed run. This problem will be investigated further. Fortunately, this does not seem to influence the linearity of the detector response. As the baseline is the same in different runs with the same set of experimental conditions, the baseline rise can be corrected for by using a chromatographic data system.

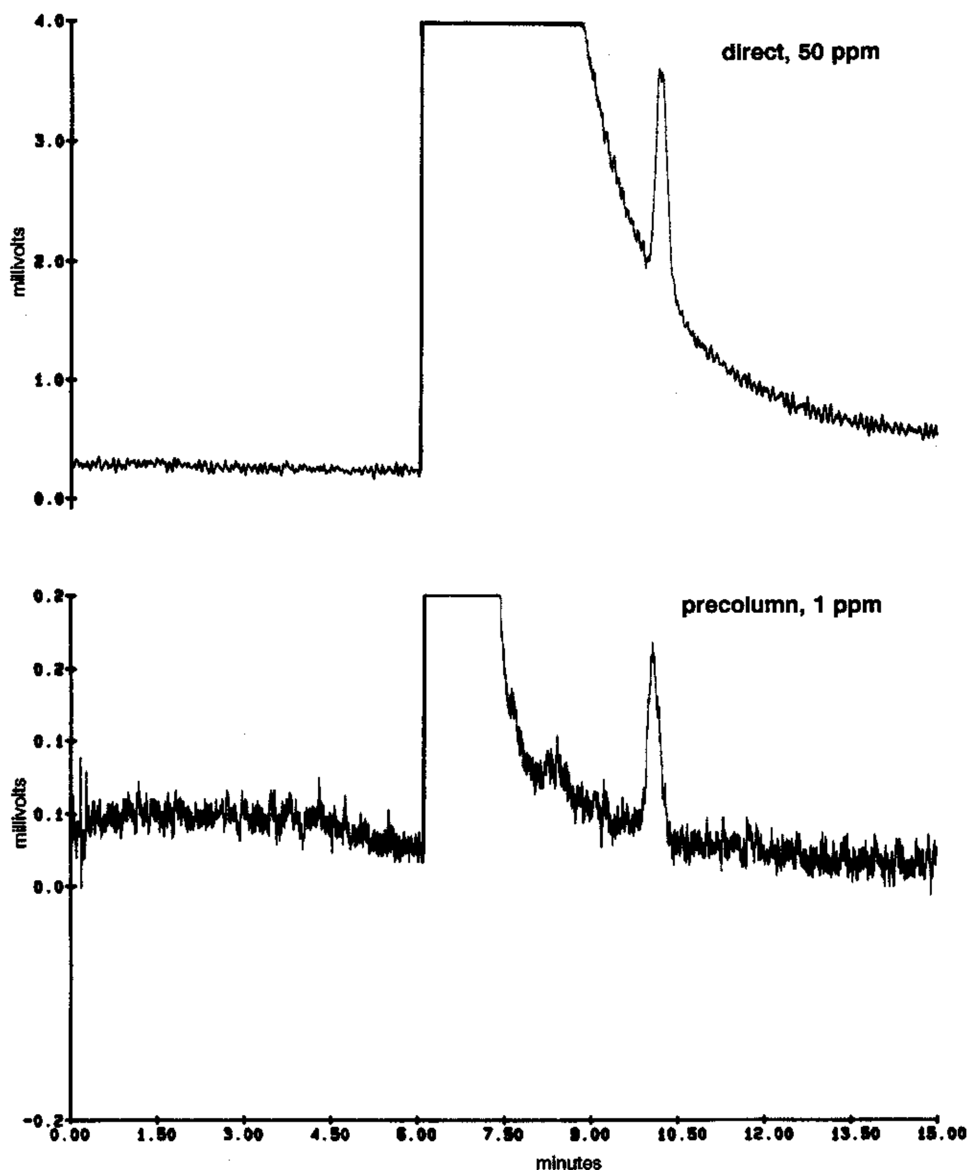

Fig. 3. Chromatograms of octadecane using direct and precolumn system injection. Column, SB-octyl-50; sample solvent, acetone; injection volume, 60 nl, direct injection; precolumn injection, 10 μl; system pressure, 100 bar, temperature, 170°C.

TID has been shown to give a linear response in the normal concentration range with nitrous oxide [8,17,18] as the mobile phase in both isobaric and pressure-programmed runs. As the TID response in general is linear, the occurrence of non-linear effects in some chromatographic systems is therefore attributed to the injection system or to the column.

It is advantageous to use a TID instead of FID, as the response with substances containing nitrogen is often about ten times higher and the selectivity is high towards hydrocarbons. This means that the separation of a nitrogen compound from a non-nitrogen-containing substance will not be as critical as when using FID. Further, bleeding of the stationary phase will generally be suppressed to a large extent, as will the signal from small amounts of hydrocarbon contaminants in the mobile phase. The stable baseline with TID also in pressure-programmed runs (see Fig. 2) is favourable compared with UV detection, where the changes in refractive index may result in baseline drift. Sometimes, however, we have found a small baseline drift with TID in pressure-programmed runs, especially on the cyanopropyl column (see Fig. 1b). This can probably be attributed to stationary phase bleeding or elution of compounds with very long retention times, as the situation is drastically improved after a reconditioning period at high operating pressure.

Quantification

Precision. Generally, the precision of peak-area measurements, using direct injection, was 3–12% (R.S.D.) in the concentration range 100–1000 ppm, corresponding to an injected amount of 5–50 ng (Fig. 4). As several non-optimum solute–stationary phase combinations were included in our investigations, extra band broadening, *e.g.*, due to adsorption, often occurs on the column. This means that the

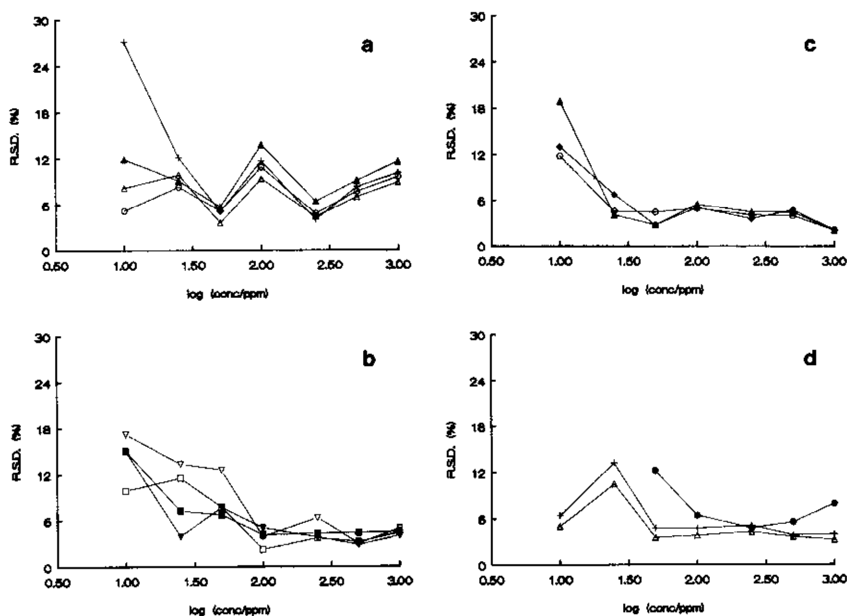

Fig. 4. Relative standard deviation *versus* logarithm of concentration. Columns, (a,b) DB-17, (c,d) SB-cyanopropyl-50; sample solvent, methanol; injection volume, 60 nl; pressure programme, (a) 100–180 bar at 8 bar/min, (b) 110–170 bar at 3 bar/min, (c) conditions as in Fig. 2b, (d) 90 bar isobaric for 3 min, 90–210 bar at 15 bar/min; temperature, (a,d) 150°C, (b) 135°C, (c) 115°C. (a) + = MQ, ▲ = COTH, △ = TOA, ○ = DODN; (b) ▽ = DMA, ▼ = FMDA, □ = 2,6-TDA, ■ = 2,6-TDC; (c) ▲ = COTH, ◆ = 2,4-TDC, ○ = DODN; (d) ● = RP, + = MQ, △ = TOA (for abbreviations, see Table I).

precision values obtained often contain significant contributions from this extra band broadening in the column and also from the injection procedure. The best precision (*ca.* 3%) was obtained, as expected, for near-optimum systems at high analyte concentrations. Automation of the direct injection procedure would probably further improve the precision.

When comparing the precision using manual direct injection with that using other sample introduction systems for capillary SFC, *viz.*, split injection [26,27], delayed split injection [28], time-split injection [28,31] and extraction injection [32], only time-split injection seems to be superior in this respect, capable of giving values lower than 2%. For example, Richter *et al.* [26] achieved an R.S.D. of 1.8–1.9% at concentrations of 200 ppm of C_{24}–C_{30} hydrocarbons, substances which ought to behave ideally on the column. For the precolumn injection system (still under development) [22], R.S.D. values of *ca.* 7% (five injections) at concentrations of 50 ppm have been obtained.

Detection limits. Detection limits, using direct injection, determined as twice the noise level for the model substances, are listed in table III. The results show, as expected, that the best precision in the low concentration range is obtained for the substances with the lowest detection limits; consider, *e.g.*, DODN in Fig. 4. However, the peak shape also has a large influence on precision. This can be seen for MDA and FMDA (also in Fig. 4). Although the detection limits for the two analytes are the same, the precision in the low concentration range is considerably worse for MDA, which gives a peak with marked tailing. With the precolumn injection system concentrations below 0.3 ppm can be determined. With a nitrogen-sensitive detector this value is expected to be *ca.* 10 times lower. Further work on these aspects is in progress.

Linearity. The merits of plotting normalized relative response *vs.* concentration instead of normal regression lines with response *vs.* concentration have been discussed previously [18]. The former approach, which gives more detailed information about possible adsorption effects in the low concentration range, was also used here.

Fig. 5 demonstrates the behaviour of different model substances at various concentrations on two columns with different polarity. All plots of response *vs.* con-

TABLE III

DETECTION LIMITS (ppm)

Compound	Columns	
	DB-17	SB-cyanopropyl-50
MQ	12	4.6
COTH	6.7	2.6
RP	–	29
TOA	7.1	6.4
DODN	3.0	1.8
2,6-TDA	2.1	–
2,6-TDC	5.6	–
2,4-TDC	–	9.1
MDA	5.3	–
FMDA	5.3	–

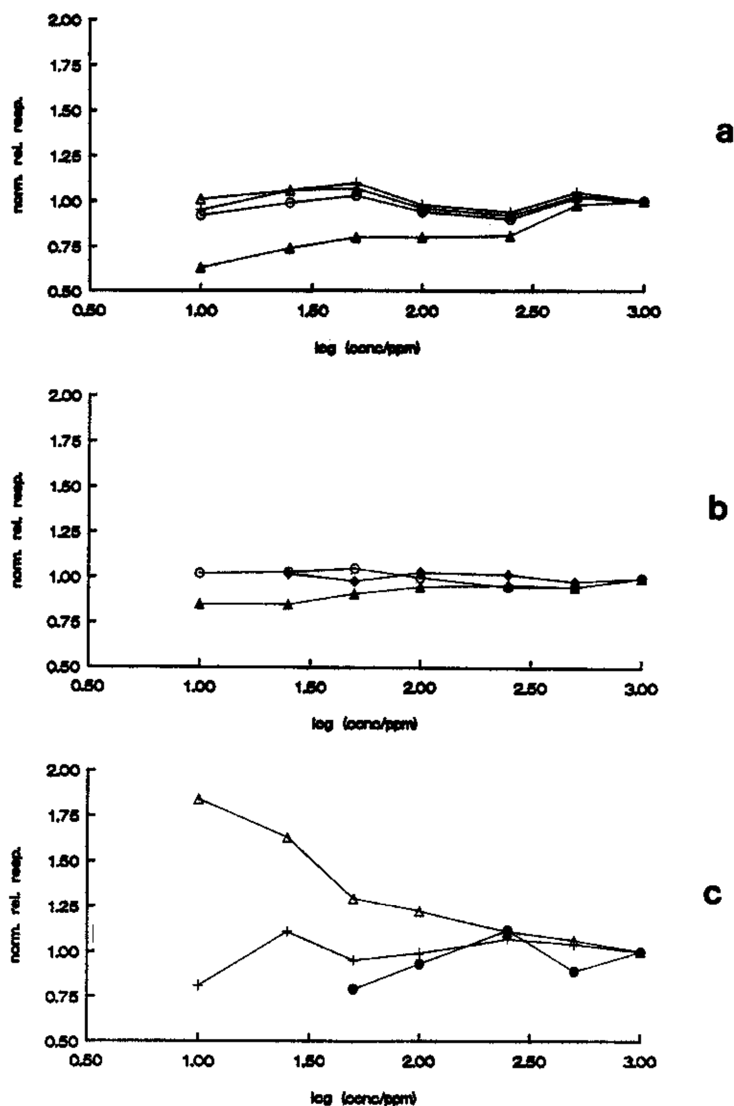

Fig. 5. Normalized relative response *versus* logarithm of concentration. Columns, (a) DB-17, (b,c) SB-cyanopropyl-50. Chromatographic conditions, (a) as in Fig. 4a, (b) as in Fig. 2b, (c) as in Fig. 4d. (a) + = MQ, ▲ = COTH, △ = TOA, ○ = DODN; (b) ▲ = COTH, ◆ = 2,4-TDC, ○ = DODN; (c) ● = RP, + = MQ, △ = TOA (for abbreviations, see Table I).

centration seem by visual inspection to be straigth lines and their linear correlation coefficients are all above 0.9995, except for COTH (0.9991) on the DB-17 column and RP (0.997) on the cyanopropyl column. In two of the systems considered, COTH on the DB-17 column and TOA on the cyanopropyl column, the regression lines deviate significantly from the origin at the 95% confidence level, indicating non-optimum behaviour. The sensitivity for RP is markedly lower compared with the other investigated substances. The noise therefore has a greater influence on the peak-area

determinations, which, not unexpectedly, resulted in this lower correlation coefficient.

Deviations from optimum behaviour are much more obvious in relative response plots. COTH has a low relative response in the low concentration range, indicating adsorption of this compound on the slightly polar DB-17 column (Fig. 5a), an effect which decreases considerably on the more polar cyanopropyl column (Fig. 5b).

TOA on the cyanopropyl column (Fig. 5c) illustrates the problem of quantification when the analyte elutes close to the solvent front. The positive error in the response of trioctylamine is more pronounced in relative terms at lower concentrations and results in a significant positive intercept of the normal regression line. A possible explanation for this behaviour may be a change in the detector sensitivity for substances coeluting with the solvent.

Apart from the deviations discussed above, fairly straight lines, parallel to the abscissa, were obtained for all other systems in the relative response plots. These straigth lines indicate near-optimum chromatographic behaviour, suggesting that quantitative determinations based on regression plots are possible in the concentration range considered.

Linear regression lines and calibration graphs for 2,6-TDA, 2,6-TDC, MDA and FMDA have been presented previously [18]. Two of the substances, the free amines 2,6-TDA and MDA, gave negative intercepts, indicating adsorption, but only with MDA was this deviation significant at the 95% confidence level.

CONCLUSIONS

We have shown that quantitative measurements using SFC with the conventional 60-nl injection loop in general are possible at concentrations above *ca.* 50 ppm, correponding to an injection of 2 ng in methanol. When analysing substances that elute early and are suitable for nitrogen-sensitive detection, accurate quantitative measurements can be performed at concentrations of a few ppm (*ca.* 100 pg). However, for trace analysis below this level, techniques for introduction of larger sample volumes still need to be improved.

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