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Multivariate optimization of a gas–liquid chromatographic analysis of fatty acid methyl esters of blackcurrant seed oil

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SUMMARY

A method was investigated and optimized for the analysis of fatty acid methyl esters using blackcurrant (*Ribes nigrum*) seed oil as a model. This was done with a total of eight runs, utilizing experimental design to explore the experimental domain, a multivariate ranking function to rank the chromatograms and response-surface methods to locate an optimum.

INTRODUCTION

As a part of our general programme on the analysis of natural lipid mixtures, we wanted to investigate the multivariate optimization of various types of lipid analyses. The objective of this particular study was to use this approach in the routine analysis of fatty acid methyl esters (FAMEs) from vegetable oils by gas–liquid chromatography (GLC).

Numerous reports on the analysis of FAMEs by GLC have appeared[1–4] and there is a wide selection of suitable commercially available capillary columns with different types of stationary phases. Owing to the many options and applications, a systematic approach to find the best performance in a specific case was needed. In order to investigate optimization based on multivariate analysis, we wanted a model system containing naturally occurring fatty acids and some unusual isomers, and blackcurrant (*Ribes nigrum*) seed oil was selected for this purpose. This oil has a relatively high content (12–20%)[5] of γ-linolenic acid [18:3 ($n-6$)], which is an isomer of the common α-linolenic acid [18:3 ($n-3$)].

Sample introduction

The use of an on-column injection technique in FAME analysis places high demands on sample purity, as particles or by-products from the derivatization of the acylglycerols may be deposited on the column inlet and in time cause degradation of the bonded phase. By installing a precolumn, *i.e.*, a retention gap, between the injector and the column inlet, these problems can be overcome.

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Precolumns

The retention gap is a deactivated and uncoated section of fused silica, which causes little or no retention of the solute molecules. The uncoated precolumn should be deactivated with a reagent that promotes good wettability of the inner column surface by the injected solvent, otherwise droplets containing solute molecules may form, causing peak broadening and split peaks[6–10]. The length of the retention gap is determined by the maximum length of the flooded zone reached by the solvent when injected below its boiling point. The length of the flooded zone is determined by the volume and type of solvent injected. Involatile sample impurities will be adsorbed on the column wall within the flooded zone of the retention gap, which in time might cause an increase in retention of the precolumn[11]. Eventually, the involatile components will migrate onto the analytical column if the retention gap is not exchanged or shortened periodically.

The connection between the retention gap and the analytical column is a critical part of the installation. By use of a conical glass seat, a so-called press-fit connector[12], a seal is established between the connector and the polyimide sheath of the fused-silica tubing. The connection will grow stronger with time and for most applications it may be considered as an integrated part of the column.

Experimental design

Factorial design allows for a very effective coverage of the experimental domain in comparatively few experiments. By varying all of the experimental factors simultaneously, according to a predetermined plan, the main effects of the variables and their interactions can be determined. Many different types of experimental design have been described, differing mainly in the type or accuracy of information wanted. Fractional factorials at two levels are very economical in the initial step for screening large numbers of potentially important factors. When the important factors have been determined as described above, full factorials at three levels or a central composite design can be used to optimize the experimental conditions[13,14].

The multivariate ranking function

The above approach implies the need for an objective measure of experimental behaviour, *i.e.*, a means of ranking the resulting chromatograms quantitatively, and for this a multivariate chromatographic ranking function was used. In this study the "chromatographic resolution statistic" (*CRS*) was applied[15]. This function is devised to take resolution, peak distribution and total analysis time into account and thus provides an objective and quantitative criterion for the ranking of chromatograms.

Optimization

Response-surface methods can be used to obtain a graphical representation of the response (optimization parameter) over the experimental domain. A model can be developed that relates the design variables to a measure of experimental behaviour. This can be done by using regression methods, either linear or non-linear, depending on the nature of the inherent relationship. In the case where the effects of more than one experimental variable are being considered, multivariate regression methods must be used. We have used multiple linear regression to model the experimental data and to generate the response surface in the optimization step[16].

EXPERIMENTAL

Study design

A two-level full factorial design in three variables was used in this study. This design allows one to see main and first-order effects in eight (2^3) experiments.

The variables chosen in this study were injector temperature, oven programming rate and injection volume. The design matrix and experimental domain are shown in Table I.

Each row in the design matrix describes an individual run, these runs being executed in random order to avoid any time-dependent trends in systematic variation. This experimental design was applied to the analysis of the standard mixture and the blackcurrant seed oil.

TABLE I

CHROMATOGRAPHIC VARIABLES AND *CRS* VALUES

Injection temperature (°C)	*Rate (°C/min)*	*Injection volume (μl)*	*Design*	*CRS values*	
				Standard[a]	*Sample*[b]
130	3	1.5	+ + +	8.66	7.25
130	3	0.5	+ + −	9.35	6.84
80	3	1.5	− + +	15.24	12.38
130	1	1.5	+ − +	16.14	12.52
80	3	0.5	− + −	16.67	13.08
130	1	0.5	+ − −	17.54	13.28
80	1	1.5	− − +	30.12	—[c]
80	1	0.5	− − −	40.20	39.04

[a] Reference mixture ME 32.
[b] Blackcurrant seed oil, methyl esters.
[c] Missing value: see text.

Chromatographic resolution statistic

We used the chromatographic resolution statistic (*CRS*) to rank the chromatograms objectively and quantitatively in this study:

$$CRS = \{\sum[(R_i - R_{opt})^2/R_i(R_i - R_{min})^2] + \sum(R_i)^2/a\bar{R}^2\}\ (T_f/n)$$

where R_i = resolution element for the *i*th peak pair, R_{opt} = chosen value of optimum resolution, R_{min} = chosen value of minimum resolution, $\bar{R}^2$ = square of mean resolution, a = total number of resolution elements, T_f = retention time of final peak and n = total number of peaks.

The *CRS* function was initially applied to a standard mixture of methyl esters in equal amounts, in order to obtain a uniform peak height for subsequent resolution measurements.

Materials

Cold-pressed blackcurrant seed oil was obtained from Bio Lipid (Falköping,

Sweden). The blackcurrants had been grown in Sweden. A standard mixture of methyl esters (ME 32) containing methyl palmitate (16:0), methyl stearate (18:0), methyl oleate (18:1), methyl linoleate (18:2) and methyl linolenate (18:3) in equal amounts was purchased from Larodan (Malmö, Sweden). The concentration of the mixture injected was 1.0 mg/ml. Dimethyl carbonate, isooctane, hexane and methanol were all of analytical-reagent grade from Merck (Darmstadt, F.R.G.).

Derivatization procedure

The conversion of acylglycerols to methyl esters by alcoholysis is widely employed[17]. In this reaction, which is suitable for materials low in free fatty acids (<3%), an alkali methanolysis procedure is used. It requires the use of two

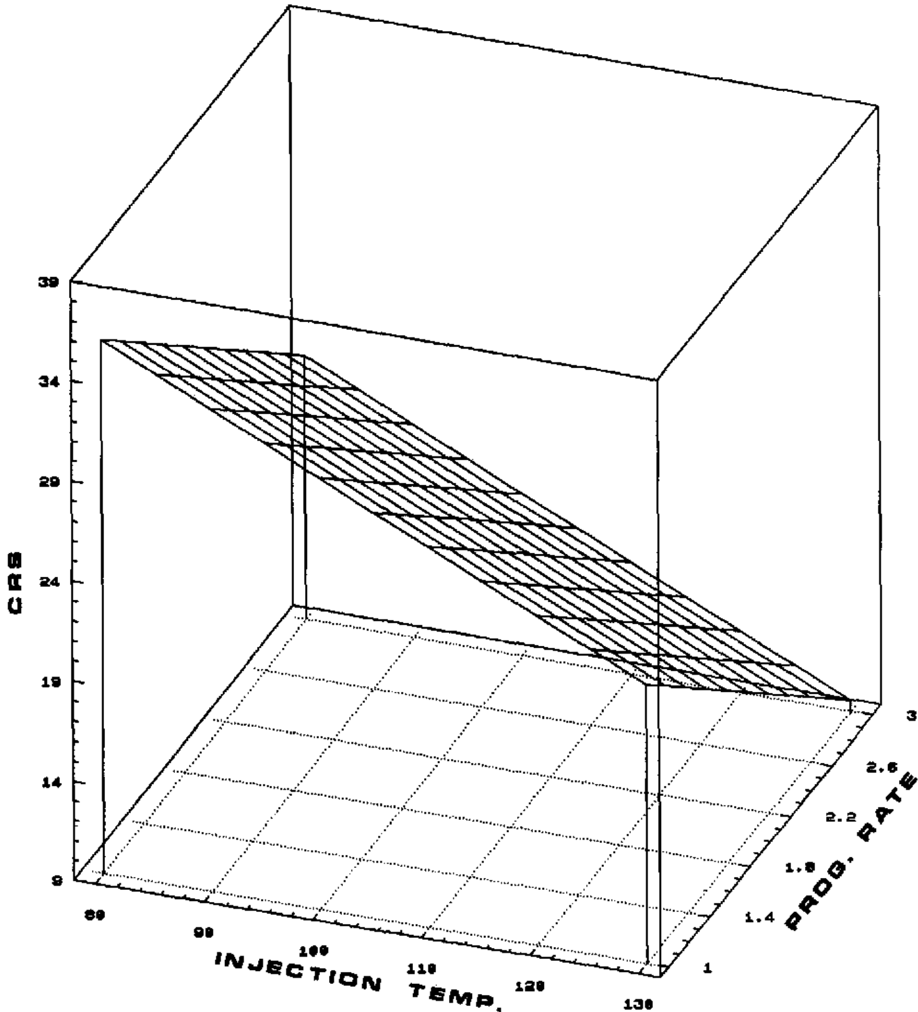

Fig. 1. Response surface of the design variables injection temperature and temperature programming rate plotted against the *CRS* values.

pre-prepared stock solutions: solution A, dimethyl carbonate–isooctane (1:1, v/v); and solution B, 2.3 g of sodium, cut clean under hexane, added in small pieces to 200 ml of methanol. Solution B was stable for several weeks at room temperature.

A 10-mg amount of oil was placed in a test-tube with a PTFE-lined screw-cap. The oil was dissolved in 2 ml of solution A, then 1 ml of solution B was added. The test-tube was sealed and shaken vigorously for 30 s, then left for 15 min to ensure complete methylation of the acylglycerols. After addition of 3 ml of water, the tube was shaken vigorously for 5 s and thereafter centrifuged at 2 *g* for 1 min. The organic (upper) layer containing the methyl esters was recovered and diluted 1:4 with isooctane for analysis by GLC.

Gas–liquid chromatography

The analysis of the methyl esters was conducted on a Varian (Walnut Creek, CA, U.S.A.) Model 3500 capillary gas chromatograph, equipped with a Varian temperature-programmable on-column injector and a capillary flame ionization detector.

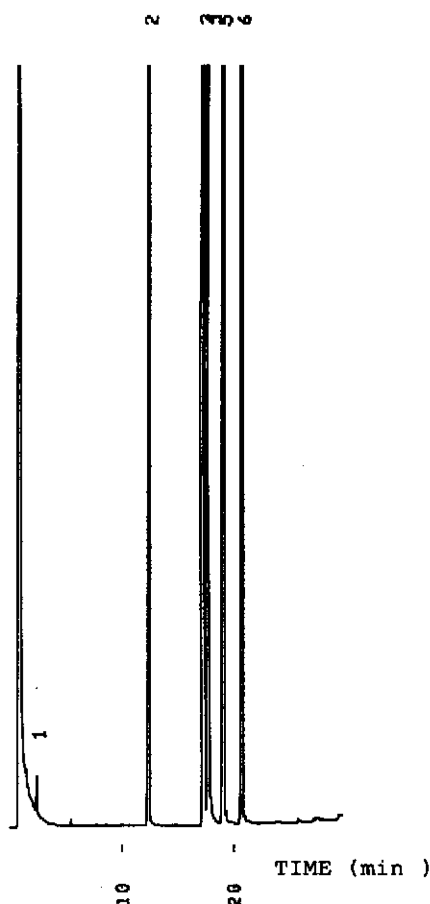

Fig. 2. Gas chromatogram of a five-component mixture of methyl esters. Design: (+ + +). Conditions as in Table I. Peaks, from left ro right: 16:0, 18:0, 18:1, 18:2, 18:3.

A DB-WAX fused-silica capillary column (30 m × 0.32 mm I.D.) coated with polyethylene glycol with film thickness 0.25 μm (J&W Scientific, Folsom, CA, U.S.A.) was used.

Between the injector body and the analytical column a precolumn, *i.e.*, retention gap (1.5 m × 0.32 mm I.D.), deactivated with diphenyltetramethyldisilyl (DPTMS), purchased from MEGA Capillary Columns Laboratory (Legnano, Italy), was installed. The two sections of fused silica were joined together with a press-fit connector (Mikro Kemi, Uppsala, Sweden).

Temperature programming was started at either 80 or 130°C, the latter representing hot on-column injection[18–20]. The temperature programming rate was set at 1 or 3°C/min and the volume injected was either 0.5 or 1.5 μl (Table I). The carrier gas was helium and the flow-rate was 3.0 ml/min throughout the investigation.

The temperature of the detector was 250°C. The detector signals were recorded with a C-R3A electronic integrator (Shimadzu, Kyoto, Japan).

RESULTS AND DISCUSSION

The overall objective of this study was to develop an optimum GLC method for the analysis of the fatty acid methyl esters of lipid materials of various origin, *e.g.*, blackcurrant seed oil. To this end the utility of experimental design in method

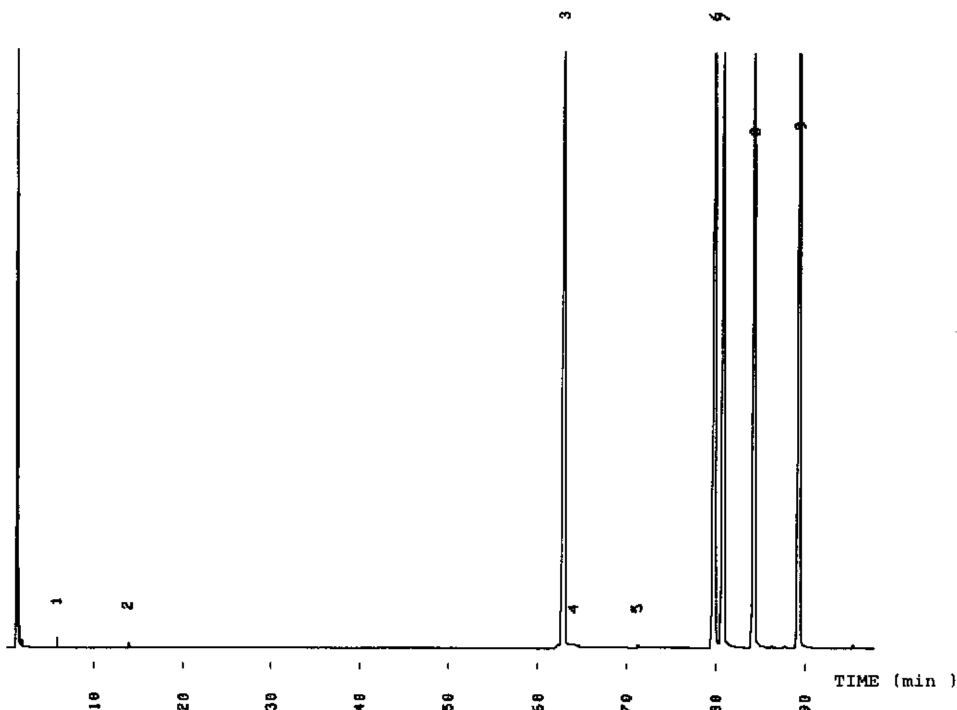

Fig. 3. Gas chromatogram of a five-component mixture of methyl esters. Design: (– – –). Conditions as in Table I. Peaks, from left to right: 16:0, 18:0, 18:1, 18:2, 18:3.

development was explored by employing a two-level full factorial experimental design in three variables to elucidate the effect of the selected experimental variables on the chromatography.

The results of this study can be summed up in the response surface plot (Fig. 1), where the *CRS* values for each individual chromatographic run are plotted against two

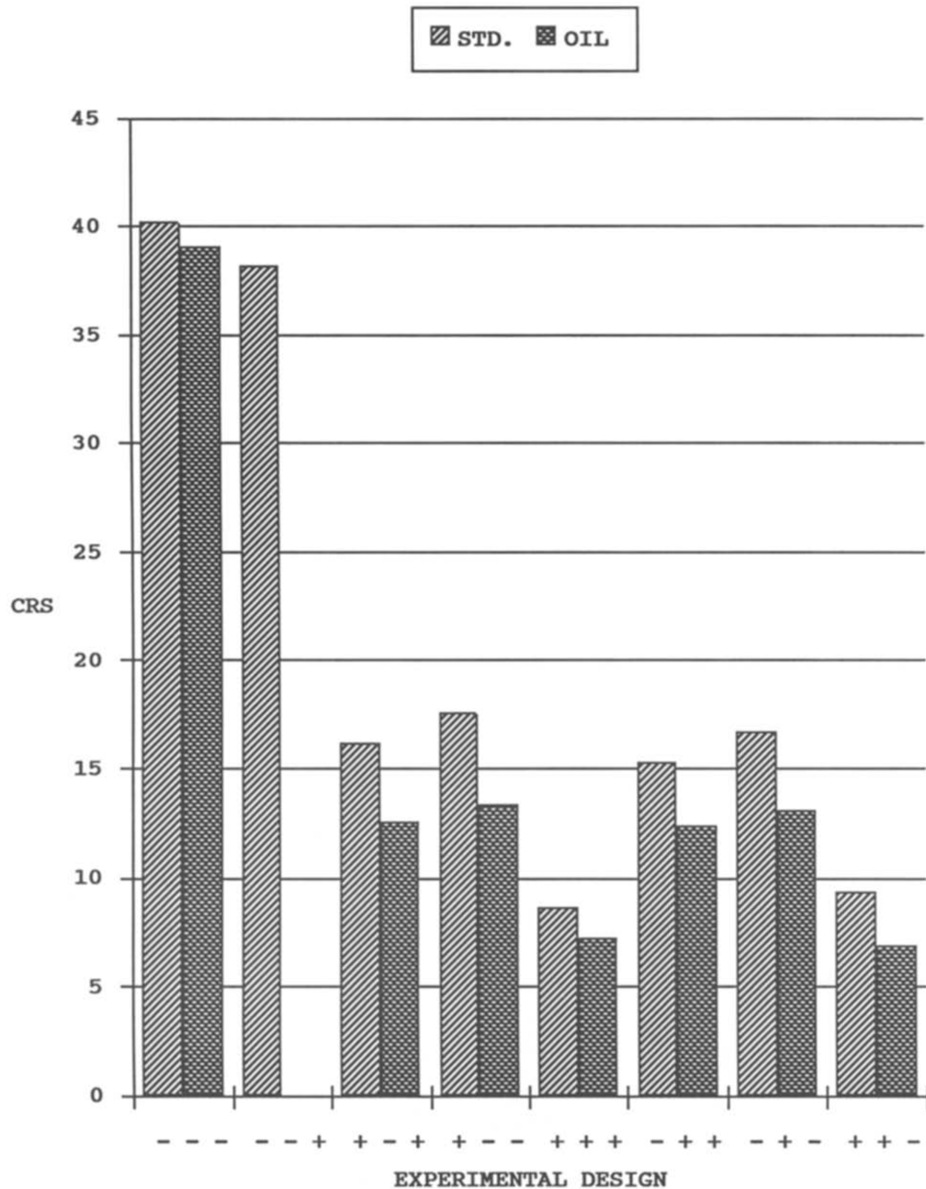

Fig. 4. Histogram over the *CRS* values of all runs from the standard mixture and blackcurrant seed oil. Missing value: see text.

of the design variables, injection temperature and temperature programming rate. The independent variables (Table I) are related to the dependent variable (*CRS* values) by a multiple linear regression model. This model explained 80% of the total variance present in the data.

The chromatograms at the extreme points of the experimental design in the standard runs are shown in Fig. 2 (design: + + +) and Fig. 3 (design: − − −). The *CRS* function as it is described, penalizes lengthy analysis times which account for the high *CRS* value (40.20) of run (− − −) in Fig. 3. However, this can be compensated for by adjusting the $1/T_f$ term, *i.e.*, total analysis time. The results of the *CRS* function for all the chromatographic runs are shown graphically in Fig. 4. The missing value for the oil sample in this figure (design: − − +) where the oleic acid isomers[18:1 ($n-9$) and 18:1 ($n-7$)] were only partially resolved, is due to the fact that the *CRS* function is not defined as R_i approaches R_{min}.

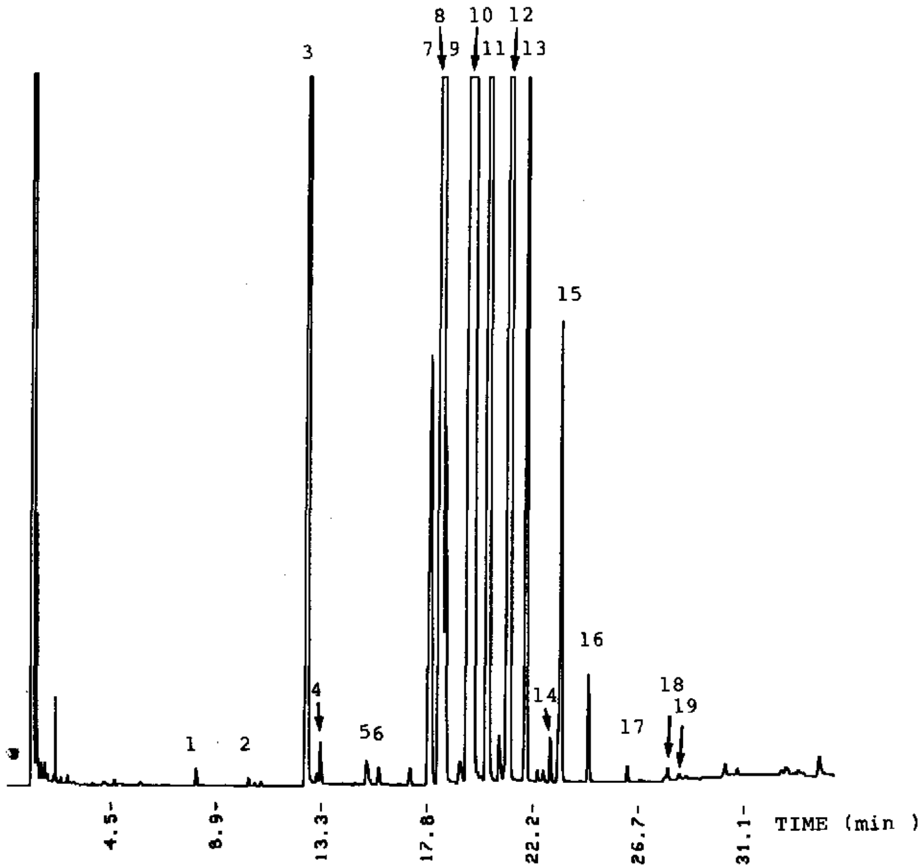

Fig. 5. Gas chromatogram of the methyl esters of blackcurrant seed oil. Design: (− + +). Conditions as in Table I. Peaks: 1 = 14:0; 2 = 15:0; 3 = 16:0; 4 = 16:1 ($n-7$); 5 = 17:0; 6 = 17:1; 7 = 18:0; 8 = 18:1 ($n-9$); 9 = 18:1 ($n-7$); 10 = 18:2 ($n-6$); 11 = 18:3 ($n-6$); 12 = 18:3 ($n-3$); 13 = 18:4 ($n-3$); 14 = 20:0; 15 = 20:1 ($n-9$); 16 = 20:2 ($n-6$); 17 = 20:4 ($n-6$); 18 = 22:0; 19 = 22:1.

According to the definition of the *CRS* function, the best chromatographic performance is attained when the function shows a minimum. The minimum in this experimental domain is at the maximum of the two design variables, *i.e.*, injection temperature and temperature programming rate 130°C and 3°C/min, respectively (Fig. 1). The chromatogram at this optimum point (design: + + −) for blackcurrant seed oil clearly reflects this behaviour (Fig. 5). All peaks are well resolved with adequate separation for the oleic acid isomers [18:1 ($n-9$) and 18:1 ($n-7$)], in a total analysis time of 32 min.

Optimum conditions are obtained for the oil sample with an injected volume of 0.5 μl, programming rate 3°C/min and initial column and injector temperature 130°C. The injection volume was found to be the least important of the design variables, its only noticable contribution being in the blackcurrant seed oil optimization.

The experimental domain can easily be extended by including in the design other variables that are believed to pertain to the chromatographic problem. This will make the procedure more time consuming, however, because more runs are demanded as the number of runs required for a full factorial at k levels and n factors is runs $= k^n$.

In Table II the fatty acid composition of the cold-pressed blackcurrant seed oil is given and in Fig. 5 a chromatogram of this oil, run with the optimized method, with a retention gap installed, is shown. The level of 18:3 ($n-6$) is lower than that reported

TABLE II

FATTY ACID COMPOSITION (%) OF BLACKCURRANT SEED OIL

Fatty acid	*Composition (%, w/w)*	*S.D. (n = 4)*	*Peak No.*
14:0	tr[a]		1
15:0	tr		2
16:0	5.3	0.02	3
17:0	tr		5
18:0	1.5	0.01	7
20:0	0.1	0.00	14
22:0	tr		18
16:1 ($n-7$)	tr		4
17:1[b]	0.2	0.04	6
18:1 ($n-9$)	14.7	0.02	8
18:1 ($n-7$)	0.7	0.01	9
20:1 ($n-9$)	1.0	0.01	15
22:1[b]	tr		19
18:2 ($n-6$)	47.0	0.09	10
20:2 ($n-6$)	0.2	0.01	16
18:3 ($n-6$)	12.2	0.02	11
18:3 ($n-3$)	13.2	0.02	12
18:4 ($n-3$)	2.7	0.01	13
20:4 ($n-6$)	tr		17
Unidentified	0.9	0.29	

[a] tr = Trace (<0.10%).

[b] Positions not determined.

by other workers[5]. This might be due to climatological factors affecting the growth of the blackcurrants used.

CONCLUSIONS

By utilizing experimental design, a multivariate evaluation procedure such as the *CRS* function and response surface methods, a limited number of experiments are needed to reach at least a local optimum. This is a very efficient and time-saving approach in method development or in the validation of already established analytical procedures.

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