Optimization of Separation of Fat-Soluble Vitamins by Supercritical Fluid Chromatography Using Serial Micropacked Columns

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The advantages of using two on-line coupled micropacked columns in supercritical fluid chromatography for analyzing fat-soluble vitamins are evaluated. A rotatable central composite experimental design is used to optimize the combination of some experimental variables (temperature gradient and pressure gradient) involved in the chromatographic separation. Relative standard deviations obtained under the conditions of the experimental design giving the highest response are also included. The analysis of fat-soluble vitamins occurring in real-life samples (a pharmaceutical preparation for newborns and an infant formula) is presented.

Keywords: *Fat-soluble vitamins; supercritical fluid chromatography; separation optimization; micropacked columns*

INTRODUCTION

Vitamins are a group of nutrients with widely varying chemical and physiological functions, which are broadly distributed in natural food sources. They are essential for the normal functioning of the human body, and some of them are involved in metabolic reactions.

In food science and technology, the analysis of vitamins is of great interest for the evaluation of several biochemical and nutritional aspects, but the thermal instability of vitamins and their low concentration in foods make the analysis difficult.

Nowadays it is generally accepted that chromatographic methods are an essential tool for the rapid and specific determination of vitamins in foods. In this respect, it is clear that the continuing development of chromatographic instrumentation (including coupled techniques) has allowed a substantial advance in the simultaneous and reliable determination of vitamins.

With respect to the improvement of a chromatographic separation, the use of an adequate optimization procedure is of great interest. On many occasions a simultaneous multivariate method is required since univariate techniques (sequential optimization of one parameter at a time) or intuitive approaches may be inefficient in the search for the optimum set of experimental conditions. So far, different multivariate optimization procedures have been successfully used in various forms of chromatography (Holderith et al., 1976; Otto and Wegscheider, 1983; Berridge, 1984; Crow and Foley, 1990; Tabera et al., 1991; Lanças et al., 1991; Villén et al., 1992; Señoráns et al., 1993) including the response surface methodology (Box and Hunter, 1957; Bayne and Rubin, 1986; Box and Draper, 1987).

Recently, the use of supercritical fluids (Smith, 1988; White, 1988; Lee and Markides, 1990) has been considered as an interesting alternative technique for the analysis of vitamins since it is a suitable procedure for the determination of extremely labile compounds (Rizzolo and Polesello, 1992). In this respect, supercritical fluid chromatography (SFC) has already shown its ability for analyzing vitamins in highly complex matrices (Matsumoto et al., 1986; White et al., 1988).

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Up to now, capillary and packed columns have been extensively used in SFC. Open capillary columns are relatively inert and possess high efficiencies, high permeabilities, and lasting stabilities, but their low sample capacities may be a serious inconvenience for the analysis of trace compounds. Packed columns improve the sensitivity of an analysis, but they often exhibit a high degree of activity. Moreover, the use of packed columns may result in an extremely high pressure drop across the column, thus involving significant differences in fluid densities along the column which may produce appreciable losses of resolution (Schoenmakers and Verhoeven, 1986; White and Houck, 1986; Pacholec et al., 1988).

In previous work we have evaluated the advantages and limitations of using in SFC micropacked columns packed with large particle size material by liquid chromatographic standards (Ibáñez et al., 1993a, 1994). It is evident that the use of such particle size results in lower column efficiency but, as a counterpart, the pressure drop across the micropacked column is similar to that in capillary columns and definitely lower than in packed columns. Moreover, typical values of expanded gas volume flow for micropacked columns are lower than those for standard packed SFC columns and, consequently, ionization detectors can be easily interfaced to these columns, whereas the use of packed columns can cause complications. The chromatographic characteristics of the micropacked columns are compatible with high sample volume loadability, its sample capacity being higher than that of capillary columns (Ibáñez et al., 1993b). The use of micropacked columns is also advantageous because a wide range of chromatographic selectivities and polarities is achievable.

On the other hand, the possibility of using two or more serially connected packed columns to obtain an additional selectivity adjustment has been recently suggested by some authors (Berger and Wilson, 1993), but there is little experimental information on their use. In addition to this, it is evident that the longer the packed column, the higher the pressure drop will be, so that both instrumental and chromatographic restrictions can make a particular analysis difficult.

Conversely, the on-line coupling of micropacked columns is a very interesting option due to the low pressure drop characteristic of this type of columns. Up

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to now, only single micropacked columns have been used for SFC separation of fat-soluble vitamins in test mixtures (Ibáñez et al., 1995a), although, in this respect, further research remains to be done, mainly concerning the improvement of the selectivity adjustment required for a specific separation.

The aim of this work was to evaluate the possibility of on-line coupling of two micropacked columns loaded with different stationary phases. The optimization of the chromatographic separation of fat-soluble vitamins contained in foods via the use of a rotatable central composite experimental design (Bayne and Rubin, 1986) was also examined. To illustrate the potential of the technique, the SFC separation of fat-soluble vitamins occurring in real-life samples is presented.

EXPERIMENTAL PROCEDURES

SFC. Analyses were performed by using a Carlo Erba supercritical fluid chromatograph (SFC 3000) equipped with an injection valve and flame ionization detector (FID). The injection port and detector were maintained at 40 and 375 °C, respectively, in all experiments. Restrictor (mounted between the column and the FID) was drawn from 8 μm i.d. fused silica capillary tubing and coupled to the micropacked columns by means of a zero dead-volume connector. Supercritical grade carbon dioxide was used as mobile phase and was delivered by a Carlo Erba SFC 300 syringe pump, refrigerated at 0 °C.

Column. According to our previous experience on SFC of fat-soluble vitamins, the use of Carbowax 20M as stationary phase seemed to provide an acceptable selectivity, although vitamins E, K_1, A, and D_2 were only resolved in two peaks (E + K_1 and A + D_2) (Ibáñez et al., 1995a). The use of other single columns of different characteristics, at several experimental conditions, did not result in the desired separation. For this reason, in this work the combination of two different stationary phases was evaluated to try to improve the selectivity adjustment. Specifically, the micropacked column used in this study was prepared by placing on-line (by means of a zero-dead volume connector) two micropacked columns coated with SE-54 (1% vinyl, 5% phenyl, 94% methyl polysiloxane; Supelco, Bellefonte, PA) and Carbowax 20M [poly(ethylene glycol); Supelco], respectively. In each case, the above-mentioned support was loaded with 3% (w/w) of the corresponding phase. The use of Carbowax 20M in SFC does not demand its previous immobilization (Lee and Markides, 1990), but SE-54 was cross-linked by adding dicumyl peroxide (0.5 mg/100 mg) and subsequent heating of the column at 5 °C/min, under a nitrogen flow, from 100 to 160 °C (3 h). Micropacked columns were made from a pretreated stainless steel tube (Chrompack) (20 cm × 0.5 mm) and a spherical porous siliceous support from Merck (Volaspher A-2, 100−125 μm) according to a previously reported procedure (Reglero et al., 1985).

Experimental Design. The variables selected to perform the experimental design were pressure and temperature gradient rates. The initial and final pressures were, respectively, 90 and 300 atm, and the evaluated range of pressure program rate was established to be between 1 and 7 atm/min. For the temperature program, positive gradient, negative gradient, and isothermal conditions were studied. The temperature program ranged from −5 and 5 °C/min, starting from an initial temperature of 100 °C, 60 and 160 °C being the final temperatures. In each analysis, the initial conditions (100 °C and 90 atm) were held for 5 min.

The experimental data were obtained by carrying out 13 experimental runs (in randomized order) according to the rotatable central composite design. Subsequently, the second-order polynomial model given by eq 1 was fitted to experimental data:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

Here X_1 is the temperature program, X_2 the pressure program, y the dependent variable, and β the coefficients to be estimated.

To evaluate the influence of each set of experimental conditions on the separation achieved, a numerical value (response) should be assigned to each chromatogram (Schoenmakers, 1986). In this work we propose the use of two different chromatographic response functions, namely CRF_1 and CRF_2:

$$CRF_1 = \sum \alpha_{i+1,i} \quad (2)$$

$$CRF_2 = CRF_1 \times n \quad (3)$$

α is the relative retention, and n is the number of compounds resolved ($R_s \geq 1$) in the experimental run for which the response is being calculated. This criterion was, however, different regarding vitamins A, D_2, and D_3, the separation of which proved to be rather difficult. For these compounds, the achievement of a low degree of resolution was accepted and the value of n was established after peak separation was examined in fine detail by using an adequate chromatographic software (Maxima 820 chromatography software, version 3.30). In this way, the assigned response was considered to contribute more efficiently to the convenient progress of the optimization procedure (i.e., to direct the search for the optimum to improve separation of poorly resolved peaks).

Evidently, CRF_2 may give similar information to that achieved by means of CRF_1, but its use is strongly recommended in those cases in which a very large retention value that may occur in the chromatogram for a specific pair of peaks makes the search for the optimum difficult.

A minimum of four replicate injections were carried out for each set of conditions throughout the experimentation.

Samples. A synthetic mixture of fat-soluble vitamins containing K_3, K_1, A, A acetate, E, E acetate, D_2, and D_3 vitamins (4 mg/mL of each) (Sigma) was prepared with chloroform as solvent. The mixture was stored at 4 °C in dark flasks.

A pharmaceutical preparation for newborns (Protovit, Roche) and an infant formula (Almiron, Nestle) enriched with vitamins were also used. Preparation of real-life samples for subsequent SFC analysis was performed from samples of 1 g by using a Hewlett-Packard supercritical fluid extractor Model HP-7680A. To retain the liquid sample (pharmaceutical preparation) in the extraction chamber during the SFE process, a solid support (glass wool) was placed into the chamber prior to extraction.

Experimental conditions for SFE experimentation were as follows: temperature, 40 °C; density, 0.9 g/mL; time, 20 min; flow rate, 3 mL/min; and trap temperature, 30 °C. It should be noted, however, that further optimization (i.e., by applying an adequate experimental design) concerning SFE extraction of real samples should be performed to improve the efficiency of the sample preparation step.

RESULTS AND DISCUSSION

Table 1 shows the coded factor levels, physical values and design matrix used for the optimization of the SFC separation of fat-soluble vitamins in a micropacked column resulting from connecting two columns loaded with SE-54 and Carbowax 20M. As can be seen, the experimental design that has been used defines the temperature and pressure gradient rates corresponding to 13 different experimental runs. The values obtained for the response functions CRF_1 and CRF_2 in each one of the mentioned 13 runs are given in Table 2. It is clear that significant variations in the response values are produced in the experimental region investigated as values obtained for CRF_1 vary from 5.1 to 8.4 and CRF_2 ranges between 25.6 and 67.4. The highest response values are obtained if the SFC separation is carried out under the experimental conditions (see Table 1) defining run 11 (i.e., fixing both variables x_1 and x_2 at level 0, which means the use of a temperature

Table 1. Factor Levels, Physical Values, and Design Matrix for the Optimization of the SFC Separation of Fat-Soluble Vitamins in a Column Resulting from Coupling On-Line Two Micropacked Columns (SE-54 + Carbowax 20M)

factor	experimental variable	levels				
		−1.414	−1	0	1	1.414
x_1	temp prog (°C/min)	−5.000	−3.536	0.000	3.536	5.000
x_2	pres prog (atm/min)	1.000	1.879	4.000	6.121	7.000

run	x_1	x_2	run	x_1	x_2
1	−3.5	2.0	7	0.0	1.0
2	3.5	2.0	8	0.0	7.0
3	−3.5	6.0	9	0.0	4.0
4	3.5	6.0	10	0.0	4.0
5	−5.0	4.0	11	0.0	4.0
6	5.0	4.0	12	0.0	4.0
			13	0.0	4.0

Table 2. Response Values (CRF_1 and CRF_2) Obtained for the 13 Experimental Runs Carried out According to a Rotatable Central Composite Experimental Design for the Fat-Soluble Vitamins Separation Using a Column Resulting from Coupling On-Line Two Micropacked Columns (SE-54 + Carbowax 20M)

run	CRF_1[a]	CRF_2[a]
1	7.019	49.133
2	8.089	56.623
3	6.970	48.790
4	5.125	25.625
5	6.032	36.192
6	5.298	26.490
7	6.464	38.784
8	5.955	35.730
9	8.229	65.832
10	8.205	65.640
11	8.420	67.360
12	8.152	65.216
13	8.139	65.112

[a] Chromatographic response function values calculated from the peaks corresponding to K_3, A acetate, E acetate, E, K_1, A, D_2, and D_3 vitamins.

program and a pressure program equal to 0 °C/min and 4 atm/min, respectively).

The empirical models fitted to experimental data obtained from the two mentioned response functions are given, respectively, by eqs 4 and 5. Only the statistically significant coefficients ($P \leq 0.05$) are retained in each model.

$$CRF_1 = 8.229 - 0.467X_2 - 1.066X_1^2 - 0.794X_2^2 - 0.729X_1X_2 \quad (4)$$

$$CRF_2 = 65.832 - 14.560X_1^2 - 11.602X_2^2 \quad (5)$$

The coefficients of determination (R^2) obtained for eqs 4 and 5 (respectively, 0.850 and 0.721) seem to indicate that the proposed model is adequate to explain the variability of the experimental data.

Figures 1 and 2 show the response contour plots (respectively, for CRF_1 and CRF_2) resulting from joining those points having identical response values. These plots allow prediction of the chromatographic resolution to be achieved when the experimental conditions in the studied region are changed. It is interesting to emphasize that the two response functions which have been evaluated provide similar information concerning the location of the optimum. As can be seen, those combinations of experimental variables involving the selection

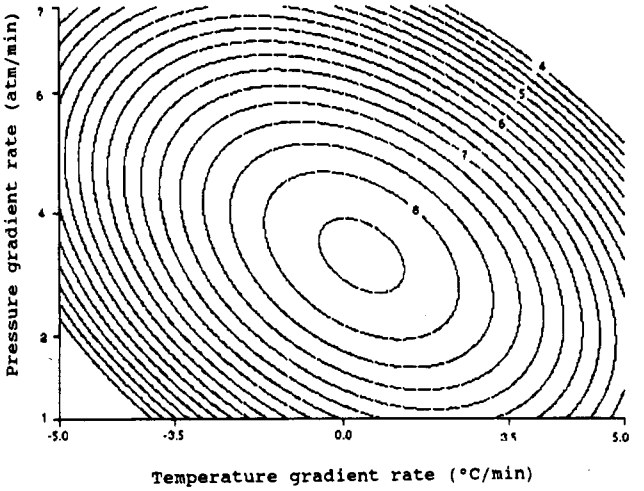

Figure 1. Response contour plots obtained from eq 4 for optimization of the SFC separation of fat-soluble vitamins. Column, 0.2 m × 500 μm i.d.; stationary phase, SE-54 + Carbowax 20M (on-line coupling).

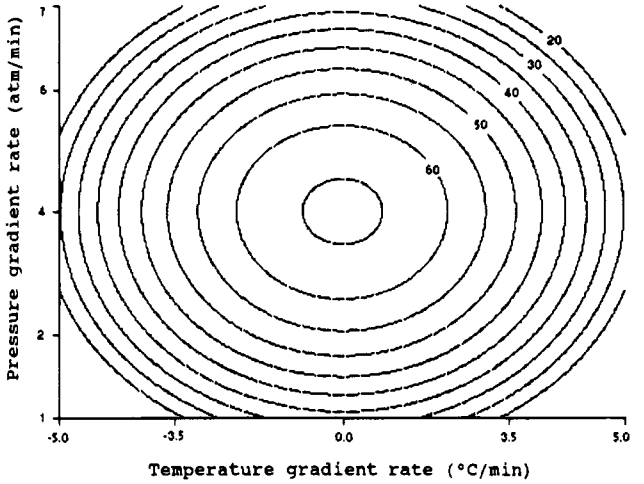

Figure 2. Response contour plots obtained from eq 5 for optimization of the SFC separation of fat-soluble vitamins. Column was as in Figure 1.

of two extreme values (i.e., the highest and/or lowest values of the experimental region) result in the lowest response. On the contrary, the highest responses are achieved when experimental values for the temperature and pressure gradient rates are close to the mean value of the respective ranges (i.e., $x_1 = 0$ °C/min and $x_2 = 4$ atm/min). Figure 3 gives the chromatogram obtained under best conditions in the experimental region studied. As can be seen, further optimization may be necessary in specific cases, but it is also clear that if vitamins A, D_2, and D_3 do not appear together in the sample to be analyzed, an acceptable separation may be achieved.

Table 3 shows the relative standard deviations (RSD), calculated from five replicates, for both the absolute and relative (normalized) peak areas. RSD values ranging from 0.3% (for vitamin K_1) to 6.1% (for vitamin A) were obtained.

It is worthwhile to note that, whereas the response contour plot of Figure 2 has a horizontal orientation, the contour plot of Figure 1 has a slanted one, from top left to bottom right. This is a consequence of the interaction term (X_1X_2) in eq 4, which causes the rotation of the natural axes of the plot with respect to the coordinate axes. If in this case (Figure 1) the experimentation would have been performed at a fixed

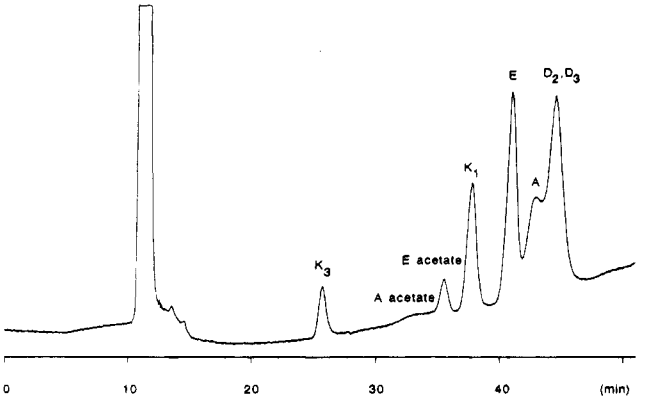

Figure 3. Chromatogram of fat-soluble vitamins SFC separation under the conditions of the experimental design giving the highest response (temperature gradient rate, 0 °C/min; pressure gradient rate, 4 atm/min). Column was as in Figure 1.

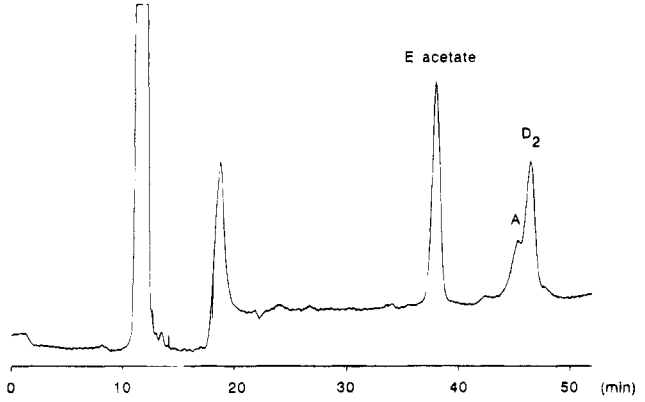

Figure 4. Fat-soluble vitamins separation obtained from a pharmaceutical preparation for newborns under best conditions in the experimental region studied. Column was as in Figure 1.

Table 3. Relative Standard Deviations (RSD) Obtained (n = 5) for a Column Resulting from Coupling On-Line Two Micropacked Columns (SE-54 + Carbowax 20M) under the Conditions of the Experimental Design Giving the Highest Response (Run 11; x_1 = 0.0 °C/min; x_2 = 4.0 atm/min)

solute	RSD (%) absolute areas	RSD (%) normalized areas
K_3	1.3	0.4
A	3.7	2.1
E acetate	4.2	5.7
K_1	0.3	1.4
E	0.5	1.2
A	6.1	4.4
$D_2 + D_3$	1.5	3.2

4.0 °C/min, the maximal CRF would have been attained at ca. 2 atm/min. Then, by fixing this value and exploring the other variable (as in the "one factor at a time" approach), the maximal CRF obtained would not have been higher than 8.0. Hence, if interaction between experimental factors is assumed, a multivariate approach is needed to guarantee the optimum location.

To illustrate the applicability of the proposed column for the analysis of real-life samples, Figures 4 and 5 give the separation of fat-soluble vitamins corresponding to the SF extracts resulting from a pharmaceutical preparation for newborns and an infant formula, respectively. It should be emphasized that the overall procedure (including sample preparation and chromatographic separation) proposed in this work takes less than 1 h. The low signal-to-noise values obtained for some peaks

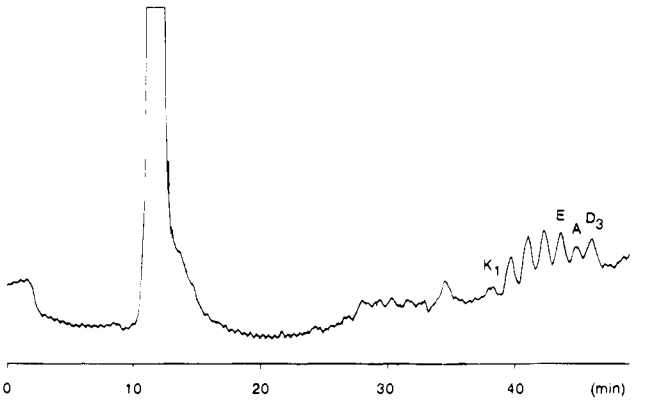

Figure 5. Fat-soluble vitamins separation obtained from an infant formula under best conditions in the experimental region studied. Column was as in Figure 1.

under the experimental conditions used suggest that the technique lacks sensitivity for accurate quantitation. However, it should be taken into account that the chromatographic characteristics of the micropacked column are compatible with high sample capacities. Consequently, relatively high concentrations of solutes can be injected without bringing about significant loss of separation efficiency (Ibáñez et al., 1993b). Therefore, it would be possible to improve the sensitivity of the analysis simply by performing the SFE step starting from sample weights higher than that used in analyses shown in Figures 4 and 5 (i.e, higher than 1 g).

It is worth remarking that the analysis time and the chromatographic resolutions achievable in these analyses are similar to those obtained by other authors with SFC capillary columns (White et al., 1988). Nevertheless, the possibility of performing such analyses with micropacked columns is of interest, as previously mentioned, if sample capacities higher than those characteristic of capillary columns are required. Moreover, the possibility of easily (on-line) coupling SFE–SFC is a further advantage of the proposed columns with respect to packed columns (Ibáñez et al., 1995b).

Concerning the determination of vitamins in foods, it is evident that HPLC is the most commonly used method as standard technique for the qualitative and quantitative analysis. In any case, it seems to be clear that the use of supercritical fluids for the analysis of compounds sensitive to light and oxidation deserves further consideration. Supercritical CO_2 allows the separation of solutes in an oxygen-free environment, which is essential for separating labile compounds, using a nonflammable, nonpolluting, and inexpensive mobile phase. Similarly, the universal nature and sensitivity of FID may be advantageous in some cases over the more specific detection methods available for LC. Also, interfacing SFC with mass spectrometry (SFC–MS) may be superior to LC–MS concerning the selectivity, applicability, and sensitivity demanded for specific applications.

Summarizing, the use of SFE and SFC may be a valuable tool in vitamin food analysis to overcome potential limitations of commonly applied techniques.

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