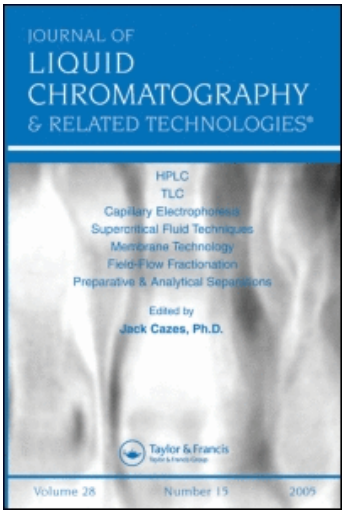

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John M. Roman[a]; Eric Abbott[a]; Xia Xu[a]; Stephen D. Fox[a]; Timothy D. Veenstra[a]; Haleem J. Issaq[a]
[a] Laboratory of Proteomics and Analytical Technologies, Advanced Technology Program, SAIC-Frederick, Inc., Frederick, Maryland, USA

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Optimization of Experimental Parameters for Packed Column Supercritical Fluid Chromatography

John M. Roman, Eric Abbott, Xia Xu, Stephen D. Fox, Timothy D. Veenstra, and Haleem J. Issaq

Laboratory of Proteomics and Analytical Technologies, Advanced Technology Program, SAIC-Frederick, Inc., Frederick, Maryland, USA

Abstract: The present research is a study of experimental parameters that affect the resolution of a test mixture of estrogens by packed column supercritical fluid chromatography. The parameters that were evaluated included fluid flow rate, effect on resolution and retention times of the type and concentration of four organic modifiers, namely methanol, ethanol, isopropanol, and acetonitrile. Also, the effect of column type on the resolution of a mixture of estrogen metabolites was studied. Two packing materials cyanopropyl silica and Betasil diol silica were selected for this study. The following percentages of each of the packing materials were used in columns connected in series for the separation of the test mixture:100.0% CPS, 37.5% CPS/62.5% Diol, 50% CPS/50% Diol, 62.5 CPS/32.5% Diol, and 100.0% Diol. The results indicated that connecting two columns having the same dimensions (a ratio of one-to-one CPS/Diol) in series gave the best resolution. There was no effect on retention times or resolution when the two columns were reversed, i.e., column order has no effect. Also, retention times were a function of the organic modifier; methanol gave the shortest retention times, with approximately the same separation factor as the other three modifiers.

Keywords: Optimization, Experimental parameters, Packed column, Supercritical fluid chromatography

Address correspondence to Dr. Haleem J. Issaq, Laboratory of Proteomics and Analytical Technologies, Advanced Technology Program, SAIC-Frederick, Inc., NCI-Frederick, P. O. Box B, Frederick, MD 21702, USA. E-mail: issaqh@mail.ncifcrf.gov

INTRODUCTION

Separations, using electrophoresis or chromatography, require the optimization of the experimental parameters before useful data can be acquired. These parameters include column dimensions, packing material type and size, mobile phase composition, flow rate, column temperature, volume and concentration of sample injection and detection wavelength in UV, and excitation and emission wavelengths when fluorescence is the mode of detection. Also, selection of either isocratic or gradient elution; if the gradient is linear the analyst has to select the slope (time) of the gradient. There have been many approaches, statistical and graphical, for the optimization of the experimental parameters in high performance liquid and gas chromatography.[1]

Supercritical fluid chromatography (SFC) is an analytical separation technique that was introduced over forty years ago by Klesper et al. as high pressure gas chromatography above critical temperatures.[2] Today, the columns used in SFC are packed columns similar to those used in HPLC separations. Furthermore, pSFC possesses many attributes common to HPLC such as the use of mobile phase modification, and gradient elution. However, the major difference between both analytical systems is the composition of the mobile phase. The mobile phase in HPLC is made up of a combination of organic solvents (normal-phase) or water with a polar organic modifier (reversed-phase). In pSFC, the mobile phase, is neither a liquid nor a gas, it is a fluid that is defined as that substance that exists above its critical temperature and pressure and possesses the properties of both the liquid and the gas. In most published SFC applications carbon dioxide, above its critical temperature and pressure, is used as the mobile phase. It is a nonpolar solvent with a polarity similar to that of short chain aliphatic hydrocarbons. Unlike water modified with organic solvents (reversed-phase chromatography), the generated carbon dioxide fluid viscosity is more like that of a gas than a liquid, resulting in low back pressures even at high flow rates.[3] To increase the solvent strength of fluids such as CO_2 and improve the resolution, modifiers such as polar organic solvents (methanol, ethanol) are commonly added to the carbon dioxide mobile phase. Also, analyses by pSFC are faster than HPLC due to the lower viscosity and higher diffusivity of carbon dioxide, resulting in faster separations and higher efficiencies.[4]

The parameters that need to be optimized in pSFC are similar to those in HPLC and TLC methodology with one significant difference, the mobile phase for HPLC and TLC may have more than one component. It is not unusual, that combination of three or four solvents is used to achieve the required separation. For example, in gradient RP-HPLC two basic solvents are employed; water or buffer as solvent A, and water/methanol, or water/tetrahydrofuran as solvent B. In TLC it is not unusual to use three or four different organic solvents. Current published literature indicates that in pSFC the mobile phase is made up of carbon dioxide with a modifier, for example methanol. Gradient elution in pSFC, as in HPLC gradient elution,

is achieved by increasing the amount of modifier within a specified time period.

In a previous study from this laboratory, fifteen estrogen metabolites were resolved by pSFC using two columns in series, a diol column and a CPS column having the same dimensions.[5] This study will evaluate the effect of selected experimental parameters, mainly different polar modifiers (methanol, ethanol, isopropanol and acetonitrile), mobile phase flow rate and gradient composition on the separation of a test mixture of estrogen metabolites. Also, since supercritical CO_2 possesses the properties of a gas, it was of interest to see if supplementary sheath gas (nitrogen) is needed and what effect the sheath gas flow will have on the signal when mass spectrometry is the mode of detection. In addition, we will examine the effect of combining two different columns each packed with different material, separately and in combination, on the overall resolution of the test mixture. A graphical model will be developed for the selection of the two column packing-materials' ratios that will result in optimum resolution of the estrogen mixture.

EXPERIMENTAL

Materials and Reagents

The estrogens used in this study were purchased from Steraloids, Inc. (Newport, RI). All estrogens and their metabolite standards have reported chemical and isotopic purity of $\geq 98\%$ and were used without further purification. All other chemicals and solvents were reagent and HPLC grade, respectively.

Instrumental Setup

The supercritical fluid chromatograph (SFC) system was purchased from Berger SFC Analytix (Newark, DE). The instrument is equipped with an autosampler injector (LEAP Technologies, Cary, NC), and a mass spectrometer detector model LCQ Deca XP (Thermo Finnigan, San Jose, CA). Mass spectrometric analysis was in positive ionization mode with the following parameters: ion source voltage, 5 kV; heated capillary temperature, 350°C; capillary voltage, 7 V; sheath gas flow rate, 5 units; auxiliary gas flow rate, 0 units; tube lens offset, −15 V. MS/MS full scan data for the protonated molecules [MH^+] of EM-Dansyl were obtained at a relative collision energy of 45% as follows: 2-OHE_1 and 4-OHE_1 m/z 753 → 125–800; 2-OHE2 m/z 755 → 125–800; 16-ketoE_2 and 16R-OHE_1 m/z 520 → 125–600; E_3, 16-epiE_3, and 17-epiE_3 m/z 522 → 125–600; E_1 m/z 504 → 125–550; E_2 m/z 506 → 125–550; 3-$MeOE_1$, 2-$MeOE_1$, and 4-$MeOE_1$ m/z 534 → 125–600; 2-$MeOE_2$ and 4-$MeOE_2$ m/z 536 → 125–600.

Sample Preparation

The estrogens in this study were derivatized with dansyl chloride, to increase their ionization efficiency, according to published procedures.[6,7] The estrogens were dissolved in methanol containing 0.1% ascorbic acid and dried under a stream of nitrogen then resuspended in 150 μL of 0.1 M sodium bicarbonate buffer (pH 8.4) and 150 μL of dansyl chloride solution (1 mg/mL in acetone). After vortexing, the solutions were heated at 60°C for 10 min to form the dansyl derivatives. After derivatization, samples were analyzed by pSFC/electrospray ionization tandem mass spectrometry (ESI)-MS/MS.

pSFC General Procedure

To determine the column separation and system parameter strategy for system optimization, the resolution of a mixture of estrogens was selected. A set of default conditions was altered one parameter at a time. As a default configuration, 5 μL injections of derivatized samples containing 400 pg/μL of each estrogen were separated using a cyanopropyl silica (CPS) column, or a diol column (columns were purchased from ThermoElectron Corp., Bellafonte, PA). The columns' dimensions were 2.1 mm i.d. × 250 mm packed with 5 μm particles. The columns were maintained at a temperature of 35°C during the analysis. To elute the metabolites, a methanol/carbon dioxide linear gradient of pure carbon dioxide to 30% methanol/carbon dioxide in 15 min was used at a flow rate of 2 mL/min. The backpressure regulator was set at 100 bar.

Changes in modifier type and concentration, CO_2 flow rate; gradient composition and columns packed with different materials were tested one at a time. The selected modifiers included MeOH, EtOH, IPrOH, and acetonitrile. Flow rate effects were examined at 1.0, 2.0, and 3.0 mL/min. Linear gradients of modifier in CO_2 from 0–30% were tested at rates of 1.0, 2.0, and 3.0% MeOH/min. CPS and Betasil Diol-100 columns of dimensions 2.1 mm i.d. × 250 mm or 2.1 mm i.d. × 150 mm were placed in series to combine for a total length of 400 mm with different relative percentages. The evaluated percentages were 100.0% CPS, 37.5% CPS/Diol, 50% CPS/Diol, 62.5% CPS/Diol, and 100.0% Diol. Column orders were also reversed to determine what effect, if any, they would have on separation.

Standard Curve

Standard curves were created after determining the optimal experimental conditions, using injections of 2 ng of deuterated estrogen metabolites working internal standards and varying amounts of estrogen metabolites, ranging from 1 ng to 4 ng.

RESULTS AND DISCUSSION

Column Selection

Column selection is an important experimental parameter along with the mobile phase. Before optimizing other parameters it was decided to see which column (packing material) gives the best separation. After preliminary evaluation of columns packed with different materials, it was decided to use columns packed with CPS and Betasil Diol-100 that are commercially available. Each column failed in resolving all the estrogens in the test mixture, Figure 1, however, estrogens that were not resolved using the CPS column were resolved using the diol column. Therefore, it was decided to use a graphical procedure that was used for binary mobile phase optimization in reversed phase HPLC,[8] to see which combination of both columns in series (percentages of packing materials) will lead to the separation of all the estrogens in the test mixture. For this experiment, two sets of two different length columns, 2.1 mm i.d. × 250 mm and 2.1 mm i.d. × 150 mm were packed with 5 μm particles, one set was packed with CPS and the other set with diol silica. Different columns (length and packing) were connected in series to give a total length of 400 mm. By doing so, we were able to get the following percentages of each material; 100, 66.5, 33.5, and 0.0% CPS in diol. The results, Figure 1, show that the best separation between all the components of the mixture was around 50–55% CPS/diol. In our previous study the ratio of 1 to 1 CPS to diol was successfully used to separate fifteen estrogen metabolites by pSFC/MS.[5]

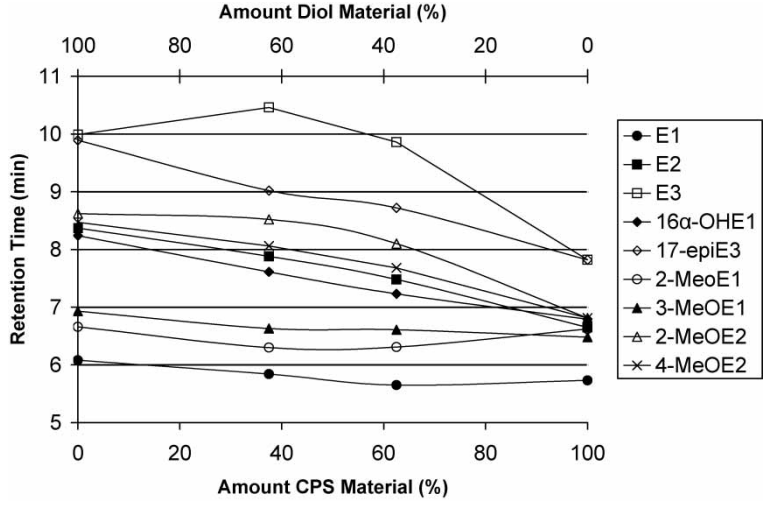

Figure 1. Graphical representation of retention times of nine estrogens using mixed CPS/diol columns.

Table 1. Effect of type of organic modifier on estrogen separation at a flow rate of 2 ml/min. linear gradient

	MeOH		EtOH		i-PrOH		ACN	
Compound	t_r	α	t_r	α	t_r	α	t_r	α
E_1	6.37	—	8.49	—	10.91	—	9.14	—
E_2	8.06	1.27	10.54	1.24	12.08	1.11	12.39	1.36
4-MeOE_2	8.85	1.10	11.41	1.08	12.73	1.05	13.30	1.07
E_3	10.17	1.15	13.12	1.15	13.60	1.07	—	—

Organic Modifier Selection

The next parameter that we studied was the organic modifier. We selected MeOH, EtOH, i-PrOH, and acetonitrile (ACN), using a linear gradient of 0% organic modifier to 30% organic modifier in CO_2 in 15 minutes. The retention times were shortest when MeOH was used, followed by EtOH, and i-PrOH. ACN as a modifier didn't elute E_3 off of the column before the gradient reached 30%, which begins to negate the benefit of shorter retention times and less use of organic solvent. All the metabolites eluted off the column faster, and the signal intensity was stronger with MeOH making it the preferred modifier for the analysis of estrogens and their metabolites. Although the separations were comparable, the retention times were not using the three organic modifiers (Table 1).

Gradient

The gradient that gave optimum separation was selected after three linear gradients were performed; 1%, 2%, and 3% methanol change/min. for 15 min. The 3% gradient appeared to elute metabolites off of the column and made it difficult to resolve metabolites of the same mass group. The use of a 1% gradient provided the highest α values, but produced retention times that were 3–5 min longer than the 2% gradient, Table 2. If maximum

Table 2. Effect of organic modifier (MeOH) increase in a linear gradient on the separation of estrogens

	1%/min. MeOH		2%/min. MeOH		3%/min. MeOH	
Compound	t_r	α	t_r	α	t_r	α
E_1	7.86	—	6.01	—	5.21	—
E_2	11.63	1.48	8.18	1.36	6.79	1.30
4-MeOE_2	12.05	1.04	8.53	1.04	6.93	1.20
E_3	16.83	1.40	11.05	1.30	8.68	1.25

Table 3. Effect of mobile phase flow rate on separation of estrogens using a linear gradient of 2% methanol/min

	1 mL/min		2 mL/min		3 mL/min	
Compound	t_r	α	t_r	α	t_r	α
E_1	9.84	—	6.15	—	4.27	—
E_2	12.47	1.27	8.24	1.34	6.49	1.52
4-MeOE_2	12.49	1.00	8.36	1.01	6.62	1.02
E_3	15.07	1.21	10.95	1.31	9.32	1.41

separation is the goal, then the use of a 1% gradient is optimal. However, a faster run with comparable separation can be obtained with a 2% gradient.

Flow Rate

The 3 mL/min flow rate produced the highest α values, but caused compounds of the same mass group to elute simultaneously. The 1 mL/min flow rate had lower α values than the 2 mL/min run and resulted in longer retention times, Table 3. For monitoring the major metabolites, such as estrone, estradiol, and estriol, a 3 mL/min flow rate is acceptable. For separation of compounds with the same mass and similar retention times, the 2 mL/min flow rate is optimal.

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