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Packed column supercritical fluid chromatography of isomeric polypeptide pairs

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ABSTRACT

The characterization and determination of peptides is of great importance in the pharmaceutical industry as is the ability to rapidly perform targeted determinations of bioactive peptides in complex matrices. The purpose of the presented work is to assess the feasibility of packed column supercritical fluid chromatography (SFC) for the separation of two-pairs of water soluble peptides of identical mass, composition and charge that differ only in amino acid sequence. Upon evaluating a variety of conditions, trifluoroacetic acid (HTFA) in conjunction with methanol as the modifier proved to be, in general, the most successful mobile phase additive for elution of the two isomeric peptide pairs from all nitrogenous stationary phases. In contrast, water and ammonium acetate gave distorted peak shapes and therefore proved to be less satisfactory as neutral additives. The basic additive, iso-propylamine (IPAm), coupled with HA-Pyridine yielded the highest resolution factor for the complete study. Aminopropyl and HA-Pyridine columns with 5 μm particle size and 60 Å pore size were found to be best for resolution of each peptide pair. Bare silica and phenyl-hexyl stationary phases did not afford any separation. The primary roles of the carbon dioxide and methanol modifier are believed to provide (a) stationary phase solvation and (b) peptide solubility and transport; while, HTFA is postulated to fully protonate each peptide and form ion pairs between its conjugate base and cationic peptide analyte. The separation process, therefore, is best viewed as ion pair supercritical fluid chromatography (IP-SFC). For the case where IPAm gave good resolution on the HA-Pyridine column, the peptides are probably in the neutral state.

1. Introduction

The characterization and determination of peptides is of great importance in the pharmaceutical industry as is the ability to rapidly perform targeted determinations of bioactive peptides in complex matrices. Traditionally, peptides are purified by using reversed phase HPLC [1]. Candidate peptides for purification can be highly diverse in molecular mass, polarity, hydrophilicity/hydrophobicity, and amino acid sequence. For example, hydrophobic peptides often contain impurities that are difficult to resolve using standard HPLC techniques, and the analysis is further hampered by insolubility in the solvents that are typically used in HPLC methods. Even when chromatographic challenges and solubility problems are overcome, it can still be a difficult task to determine peptide purity. Issues in these cases include poor resolution, slow speed of analysis, long method development times, and use of large volumes of conventional solvents [2].

Supercritical fluid chromatography (SFC) can provide advantages in many of these areas where traditional HPLC of peptides has encountered challenges. Solubility problems, for example, encountered in reversed phase HPLC solvents are avoided since SFC is compatible with methanol, trifluoroethanol, chloroform, and many other solvents used to dissolve hydrophobic peptides and proteins. In addition, there may be scale up and preparative advantages for SFC versus HPLC. Hydrophilic peptides, on the other hand, have been studied less and may prove problematic for SFC since the mobile phase is predominantly non-polar CO_2. Thus, much of the work to date concerning SFC of peptides has dealt with hydrophobic peptides.

One of the earliest examples of separation of a peptide by SFC involved the hydrophobic cyclic undecapeptide, cyclosporine A, with a molecular mass of 1202 Da [3]. A microbore column (10 cm × 1 mm) packed with 5 μm octadecylsilica particles was employed at 75 °C using methanol-modified CO_2 as the mobile phase and positive ion chemical ionization mass spectrometry (CIMS) using methanol or 2-propanol modifier as the CI reagent gas [4]. Cyclosporine A was observed to yield almost exclusively the protonated molecule (m/z 1203) with an elution time of approximately 4.5 min by SFC/ESI-MS.

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Table 1
Peptide sequence and concentration of injected solution. The amino acid residues that are interchanged are shown in red.

Product number	Lot number	Peptide sequence	Molecular mass (Dalton)	Concentration (mg/mL)
819714	CI-05-00551	Ac-GGLGLALGVLKK-NH_2	1166.4	0.57
819784	CI-05-00547	Ac-GVLGLALGGLKK-NH_2	1166.4	0.79
819791	CI-05-00541	Ac-GFLGLALGGLKK-NH_2	1214.5	0.91
819785	CI-05-00544	Ac-GGLGLALGFLKK-NH_2	1214.5	0.91

More recently, SFC/ESI-MS has been used for separation/detection of the pentadecapeptide gramicidin D [5]. In each case the N-terminus and C-terminus of gramicidin which are water insoluble were blocked by formyl and ethanolamine groups. Three forms of gramicidin (B, C, and A) were separated using a cyanopropylsilica column (4.6 mm × 50 mm, 5 μm) in under five minutes; whereas by HPLC-MS it was difficult to achieve a notable separation of the three in less than 30 min [6].

Thurbide and Zhang also reported the separation of monomeric gramicidin A, gramicidin B, and gramicidin C on a poly(styrene-divinylbenzene) column with 30% methanol modified CO_2 [7]. Subsequent to this investigation, it was observed that packed column SFC could be used to monitor the monomeric and four dimeric species of gramicidin as a function of solvent, incubation time, concentration, and temperature [8]. Later, the technique was used to monitor the binding of calcium ions to gramicidin double-helical dimers in a series of three typical alcohols ranging in polarity [9].

Another report has dealt with the separation of polypeptides containing a variety of acidic and basic residues with up to 40-mers by packed column SFC-MS [10]. Trifluoroacetic acid was employed as the additive in a methanol/CO_2 mobile phase to both suppress deprotonation of peptide carboxylic acid groups and to protonate peptide amino groups. A 2-ethylpyridine-silica column was used for the study. Five polypeptides (angiotensin I acetate, angiotensin II acetate, angiotensin III acetate, urotensin II hydrochloride, and sauvagine) with various mobile phase modifier/additive combinations were examined. In general all the polypeptides gave the sharpest peak shapes when 13 mM HTFA in methanol was used as modifier. Even sauvagine which contains over 40 residues and has a molecular mass of 4500 Da including four basic amino acid groups and seven acidic amino acid groups, eluted with a sharp symmetrical peak shape when 13 mM HTFA in methanol was employed.

We believe that packed column SFC not only holds great promise for the characterization of hydrophobic but also hydrophilic peptide mixtures. In addition, coupled with reversed-phase HPLC, the normal-phase elution characteristics of SFC may provide a rapid, orthogonal second dimension in 2-D separations. In order for SFC to demonstrate its potential in this area, chromatographic resolution of isomers that differ in ionic charge and amino acid sequence must be demonstrated. We therefore wish to report a study concerned with a packed column SFC separation of two pairs of specially synthesized, end-capped polypeptides wherein each pair has the same molecular weight and amino acid content, but the sequence within each pair has two amino acid fragments interchanged.

Table 2
pcSFC gradient schedule.

Time (min)	CO_2 (%)	Modifier (%)
0	95	5
1.0	95	5
10.0	50	50
15.0	50	50
17.0	95	5

2. Experimental

2.1. Peptides and sample preparation

End-capped 12mer peptides were synthesized by CPC Scientific Inc. (San Jose, CA) for this research wherein the N-terminus was capped with an acetyl group and the C-terminus was capped with an amide group. One pair of the linear capped peptides (i.e. CI-05-00551 and CI-05-00547) exhibited a molecular mass of 1166.4 Da and the other pair of capped peptides (i.e. CI-05-00544 and CI-05-00541) had a molecular mass of 1214.5 Da. Each peptide pair had the same composition and minimal variation in the amino acid sequence. The former pair (1166.4 Da) differed only in the placement of one of the glycines (G) and the lone valine (V) in the chain. The latter peptide pair (1214.5 Da) differed only in the placement of one of the glycines (G) and phenylalanine (F). Each peptide chain contains two lysine units and the second associated amine group on these residues can no doubt be positively charged in an acidic environment. Thus, the peptides supplied by CPC Scientific Inc. are stated to (1) be salts with trifluoroacetate as the counterion and (2) exhibit water solubility of 1 mg/mL. The amino acid sequence along with the quoted molecular mass and sample concentration for each end-capped cationic peptide chain are given in Table 1. Samples for chromatographic injections were prepared by pipetting a 50:50 mixture of each peptide pair, for example 0.5 mL of CI-05-0051 + 0.5 mL of CI-05-0547.

2.2. Instrumentation and chromatographic conditions

The system that was used for this study was a Thar Analytical method station with a ZQ mass spectrometer manufactured by TharSFC/Waters (Pittsburgh, PA). The system was operated with Empower chromatography software. Each column was pre-equilibrated with 95:5 CO_2-modifier for 3 min. The flow rate was 2 mL/min and the back pressure regulator was set at 100 bar. The oven temperature was 40 °C. The primary modifier was 100% methanol and the additives were 0.2% (v/v) trifluoroacetic acid (HTFA) in methanol, 0.2% (v/v) iso-propylamine (IPAm) in

Table 3
Columns tested in the study.

Column	Dimensions (mm)	Particle Size (μm)	Pore Size (Å)	Column vendor
2 Ethyl pyridine	4.6 × 250	5 and 3	60	Princeton
Amino propyl	4.6 × 250	5	60	Princeton
HILIC diol	4.6 × 250	5	200	Phenomenex
4-Ethyl pyridine	4.6 × 250	5	60	Princeton
HA-Pyridine	4.6 × 250	5	60	Princeton
Bare silica	4.6 × 250	5	200	Phenomenex
Luna phenyl-hexyl	4.6 × 250	5	100	Phenomenex

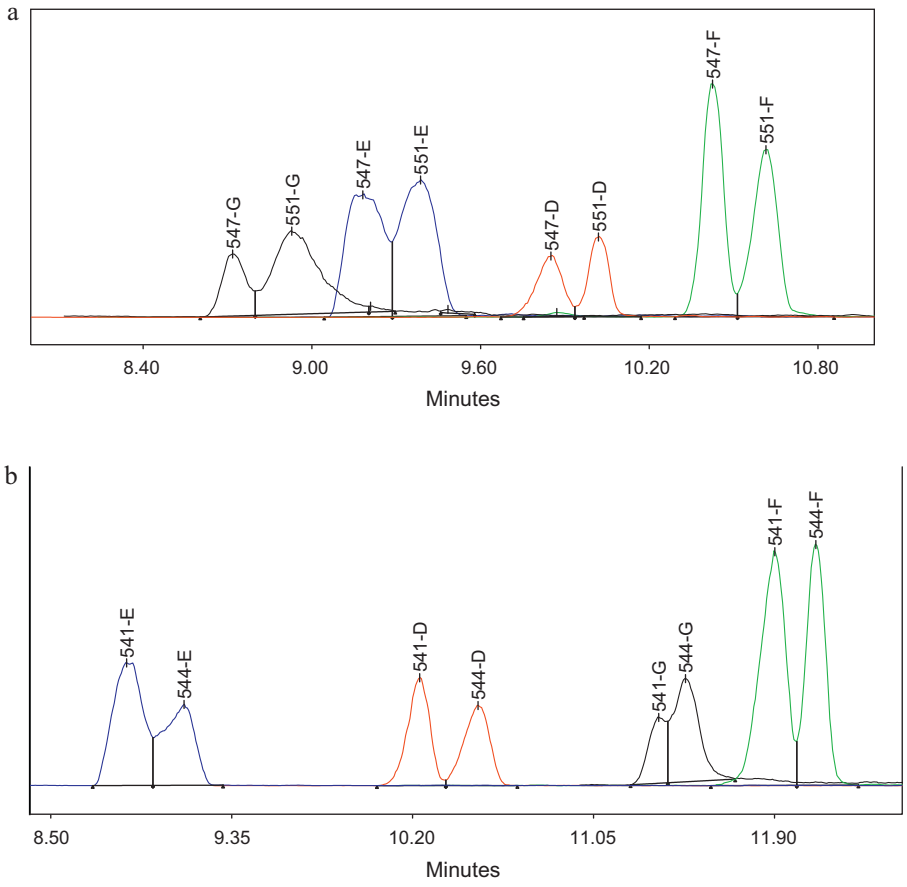

Fig. 1. Overlaid chromatograms of peptide pair #547/551(1a) and #544/541 (1b) on HA-PYR, 2EP, 4EP and AMINO (5 μm) with 0.2% HTFA as additive. D = HA-PYR, E = 4EP, F = AMINO, G = 2EP (For illustration purposes the scale and *x*-axis of the chromatograms have been altered).

methanol, and 10 mM NH_4OAc (AA) in methanol. The linear gradient schedule that was used throughout this study is shown in Table 2. A list of all the columns investigated in this study can be found in Table 3.

3. Results and discussion

The peptides selected for this investigation were specifically chosen because their separation is considered to be challenging. Each pair of peptides in this study have the same molecular mass, same amino acid residue content, are positively charged, and exhibit significant water solubility. Their elution from a packed column with a compressible CO_2-based mobile phase would not have been predicted 20 years ago. Even more unlikely would have been the report that the resolution of isomeric peptide pairs of the same molecular mass was achieved on packed column supercritical fluid chromatography (pcSFC). However, since that time, it has been demonstrated in numerous instances that mobile phase additives coupled with highly polar stationary phases can lead to successful chromatography of highly polar ionic analytes of large molecular weight. With this in mind, the goal of this research was to demonstrate the feasibility of pcSFC for chromatographic separation of individual capped peptides of identical mass, composition, and charge that differ only in amino acid sequence. The challenges presented by this study, thus, dictated that experiments be extensively repeated to ensure the highest accuracy. Columns were re-equilibrated under controlled conditions, and data were re-confirmed with virgin columns for comparison with previously used columns. Furthermore, repeat experiments separated by three months were conducted for consistent data comparison.

The four peptides used in this study each contained two lysine residues with a dangling aliphatic amino function which could be protonated. The peptides were received as salts of trifluoroacetate and therefore are positively charged. Elution and separation of the four peptides was best achieved when trifluoroacetic acid (0.2%, v/v) in methanol was used as the modifier. It is theorized that each of the peptides would be fully protonated in the mobile phase by HTFA to yield a dication and two trifluoroacetates would act as the dianion, thereby giving rise to ion pairing SFC (IPSFC). Ammonium acetate and isopropylamine were also studied as additives, but they each showed poor separaton of the peptide pairs on most of the columns tested except the HA-Pyridine column. The lack of separation in this case maybe due to poor ion pairing of the peptide with the ammonium acetate or the isopropylamine. In general, injection of each peptide universally yielded single sharp peaks (indicative of analyte purity) with a ZQ single quadrupole mass spectrometer in the electrospray mode on all columns tested, except the phenyl-hexyl column. The chromatograms presented are extracted ion chromatograms for the mass of each specific peptide. Next, a solution of each peptide pair was made and injected onto the same columns in an effort to chromatographically separate each pair into their two isomeric constituents. Partial to nearly baseline separation of each isomeric pair was obtained on the HA-Pyridine, aminopropyl, 4-ethyl pyridine, and 2-ethyl pyridine (5 μm) columns when 0.2% HTFA with methanol was used as modifier (Fig 1). The proprietary HA-Pyridine stationary phase which is a hybrid of Diol and pyridine chemistries also showed partial separation of the isomeric peptide pairs when 0.2% isopropylamine and 10 mM ammonium acetate was used as modifier (Fig. 2). The employment of water as a mobile phase additive did

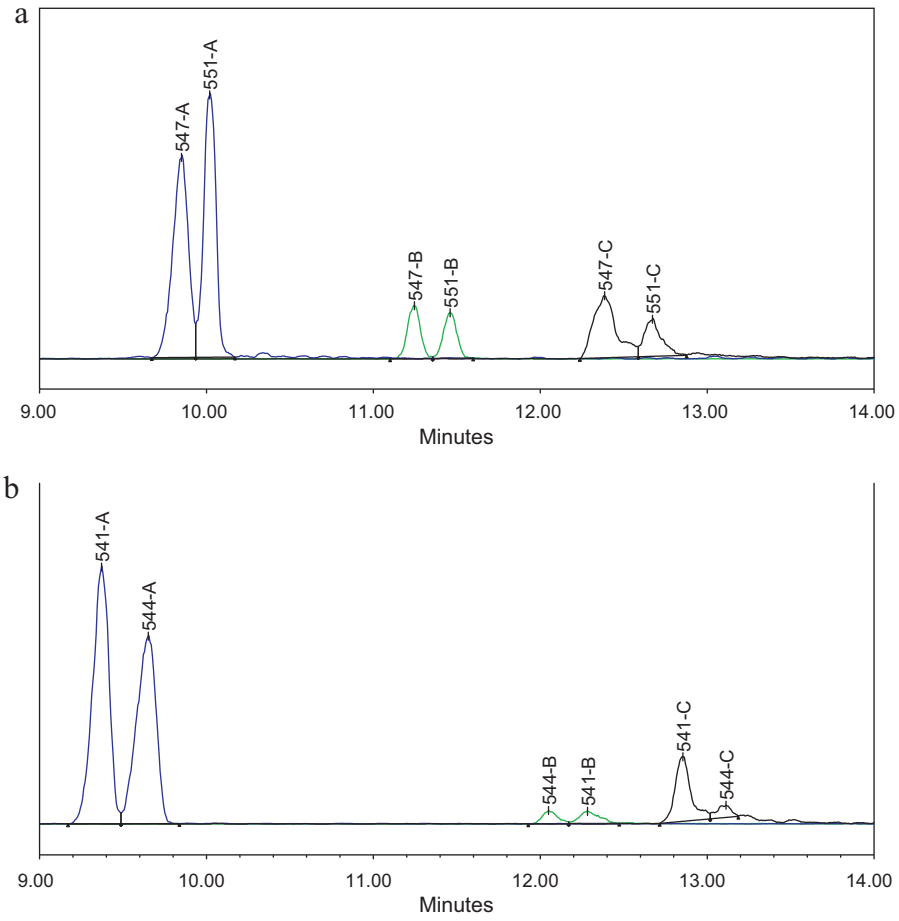

Fig. 2. Comparison of 3 additives on the HA-Pyridine column. (a) Peptide pair #547/551. (b) Peptide pair #541/544. A = 0.2% HTFA-MeOH, B = 0.2% IPAm-MeOH, C = 10 mM NH_4OAc-MeOH (For illustration purposes the scale and *x*-axis of the chromatograms have been altered).

not enhance the separation. With HTFA, the HA-Pyridine phase was slightly favored over the aminopropyl phase which in turn was more favored than the ethyl pyridine phase for separation of peptides #541/544 as evidenced by the resolution factors for each peptide pair seen in Table 4. On the other hand, peptides #547/551 were better resolved on the amino propyl column than the HA-Pyridine column. Phenyl-hexyl, Hilic Diol, and Bare Silica columns did not exhibit the same degree of resolution for the isomeric peptide pairs under the same chromatographic conditions as was employed with the basic phases.

Supplemental experiments were performed to determine if chromatographic resolution of the peptide pairs could be enhanced by making small changes to the original column set such as changing particle size and phase chemistry. The first set of experiments was performed to determine the effect of smaller particle size on resolution of the peptide binary mixture. The 2-ethyl pyridine column was chosen to be tested with 3 μm particles instead of 5 μm. To better understand the effect of small phase chemistry changes on the separation of the peptide pairs, the 2-ethylpyridine column was substituted with a 4-ethylpyridine column. Results of the above two experiments showed that small modifications to the stationary phase (i.e. 4-ethyl substituted for 2-ethyl) with identical particle/pore size and chromatographic conditions resulted in similar resolution of the peptide peaks.

With respect to the smaller particles, resolution of the peptide pairs was in fact better on the 5 μm/60 Å 2-ethylpyridine column than with the 3 μm/60 Å 2-ethylpyridine column of equal length. Since the flow rates of the two experiments were the same, the

Table 4
Resolution factors for resolved isomeric peptides.

Column	Modifier	R_S between CI-05-00551 and CI-05-00547	R_S between CI-05-00541 and CI-05-00544
HA-Pyridine	0.2% TFA in methanol	1.03	1.3
HA-Pyridine	0.2% Isopropyl amine in methanol	1.39	1.06
HA-Pyridine	10 mM Ammonium acetate in methanol	1.3[a]	1.35[a]
2 Ethyl pyridine – 3 μm	0.2% TFA in methanol	0.04	0.5
4-Ethyl pyridine	0.2% TFA in methanol	0.68	0.9
Amino propyl	0.2% TFA in methanol	1.18	0.93
2-Ethyl pyridine – 5 μm	0.2% TFA in methanol	0.82	0.36
HILIC diol	0.2% TFA in methanol	0.21	No separation
LUNA phenyl-hexyl	0.2% TFA in methanol	No separation	No separation
Bare silica	0.2% TFA in methanol	No separation	No separation

[a] Distorted peak shape.

smaller particle column may have been operated at a non-optimum flow rate [11]. The Van Deemter plot for a packed column has a rather wide optimum flow rate window and this small difference may not have been significant. It should be noted at this point that the coupling of two 2-ethylpyridine columns to increase the number of theoretical plates and extending the mobile phase gradient also did not give rise to better resolution of the peptide pairs. Thus, one suspects that plate number, gradient, modifier, etc. are not the sole factors for determining resolution of these peptide pairs, but rather the type of stationary phase is more critical [12]. Proceeding to 4-ethylpyridine was apparently not the right direction since 4-ethylpyridine (5 μm/60 Å) yielded no enhancement in resolution of the isomeric peptides. Equally unsuccessful was the employment of ammonium acetate (salt) as an additive. To further demonstrate the point that the type of stationary phase is more critical for isomeric peptide pair separation, our work on the proprietary HA pyridine phase purchased from Princeton Chromatography Inc., resulted in nearly baseline separation of the isomeric peptide pairs (Fig. 1) with all three additives although peak shapes with ammonium acetate as additive were highly distorted. On the other hand, with IPAm and HA-Pyridine the peaks were nearly baseline separated. This observation gives credence to the notion that stationary phase chemistry is a determining factor in isomeric peptide SFC resolution. Clearly, additional study of the proprietary HA pyridine phase is warranted.

To place our study in context, a similar series of synthetic peptides have recently been shown to be difficult to separate even in reversed phase HPLC mode [13]. In the reported study, peptides with 12 amino acid residues were considered wherein a single internal residue was sequentially substituted with amino acid units of varying hydrophobicity and polarity. These model peptides were used to evaluate a variety of RP-HPLC stationary phases (i.e. polar embedded, polar endcapped, etc.). The differences between our 12mers versus the literature 12mers are considerably more subtle; yet, resolution via ion pairing SFC is readily facilitated via several stationary phases in less than 20 min. The future of pcSFC is exceedingly bright for separation of ionic and water soluble analytes. Conversion of a neutral analyte into an ion actually may enhance efficient chromatographic separation by favoring an ion-pairing mechanism [14]. Work is underway to determine if comparable resolution can be achieved with unprotected peptides that again differ solely in amino acid sequence.

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References

[1] J.W. Eschelbach, J.W. Jorgenson, Anal. Chem. 78 (2006) 1697–1706.
[2] H.J. Issaq, K.C. Chan, G.M. Janini, T.P. Conrads, T.D. Veenstra, J. Chromatogr. B 817 (2005) 35–47.
[3] C.M. White, D.R. Gere, D. Boyer, F. Pacholec, L.K. Wong, in: P. Sandra (Ed.), Eighth Symposium on Capillary Chromatography, vol. II, 1987, p. 1027.
[4] H.T. Kalinoski, B.W. Wright, R.D. Smith, Biomed. Environ. Mass Spectrom. 15 (1988) 239–242.
[5] B. Bolaños, M. Greig, M. Ventura, W. Farrell, C.M. Aurigemma, H. Li, T.L. Quenzer, K. Tivel, J.M.R. Bulund, P. Tran, C. Pham, D. Phillipson, Int. J. Mass Spectrom. 238 (2004) 85–97.
[6] J.A. Orwa, C. Govvaerts, E. Roets, A.V. Schepdael, J. Hoogmartens, Chromatographia 53 (2001) 17.
[7] K.B. Thurbide, J. Zhang, Anal. Bioanal. Chem. 382 (2005) 1227–1233.
[8] J. Zhang, K.B. Thurbide, J. Chromatogr. 1101 (2006) 286–292.
[9] J. Zhang, K.B. Thurbide, J. Chromatogr. A 1171 (2007) 1–4.
[10] J. Zhang, J.D. Pinkston, P.H. Zoutendam, L.T. Taylor, Anal. Chem. 78 (2006) 1535–1545.
[11] T. Berger, B. Berger, LCGC 28 (2010) 344–357.
[12] T. Berger, J. Chromatogr. A 785 (1997) 3–33.
[13] C.T. Mant, D. Cepeniene, R.S. Hodges, J. Sep. Sci. 33 (2010) 3005–3021.
[14] R.W. Stringham, J. Chromatogr. A 1070 (2005) 1630–1670.