Chromatography
Separation Techniques

Rogatsky et al., J Chromat Separation Techniq 2012, 3:8
DOI: 10.4172/2157-7064.1000158

Research Article Open Access

Application of Fused-Core Particle Column in Two Dimensional Reversed Phase - Reversed Phase LC/MS Analysis of Biological Samples. Impact of Extra-Column Volume

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Abstract

We demonstrate successful applicability of fused–core column in analysis of biological samples (diluted urine and SPE–pre-purified plasma or serum) using 2 dimensional chromatography. Implementation of fused-core material in the second dimension of 2D chromatographic platform allowed improving S/N above 4 fold in comparison to traditional porous C18 column. Sample load onto the first dimension (5 µm) eliminates limitations of sample volume to be injected. The main limitation factor of the fused-core material–low loading capacity was overcome by using 2D chromatography. We emphasize importance of reduction of post-column (extra column) volume in LC/MS applications utilizing fused-core columns.

Keywords: Two dimensional chromatography; Extra-column volume; Fused-core; LC/MS

Introduction

Fused core particle in 1D chromatography

Fused-core particle columns are a growing field. Within the last decade, we have observed several different breakthroughs in the chromatographic columns market. First introduced were the sub-2 µm UPLC columns. In recent years, there has also been significant progress in the field of HILIC column chemistry. Now we see widespread growth in the field of fused-core particle columns, with different column manufacturers offering various choices of stationary phases, chemistries, pore sizes and column dimensions. 2.6-2.7 micron fused-core columns generate backpressures similar to those of a standard fully porous-3 µm column. The great advantage of fused core material is that these columns allow achievement of UHPLC-like resolution, without dramatic increase of the backpressure, therefore negating the necessity to purchase a UPLC/UHPLC system [1,2]. The relatively low pressure generated by fused core particles and the thin (about 0.5 µm) layer of stationary phase allows operation of fast flow rates without the loss of resolution by using standard HPLC equipment. That advantageous thin layer of stationary phase, however, could be a disadvantage in some applications due to its lower loading capacity (in comparison to fully porous materials). While fused-core particle columns demonstrate their efficiency in analysis of pure formulations, in an analysis of complex biological samples, its 0.5 µm stationary phase shell could be insufficient. The volume of the porous surface layer of 2.7 µm fused-core particle is 1/3rd of the volume of a fully porous 3 µm particle. Biological samples, even after initial off-line sample preparation, remain too dirty and too complex, which in certain cases will result in column overload [3], peak asymmetry [4] or instability of analyte retention time and higher signal suppression due to the insufficiency of the loading capacity.

Fused core particle in 2D chromatography

In this paper, we report an on-line two-dimensional (2D) column switching method aimed to minimize the drawbacks of fused-core particle columns applied to complex biological samples. This method was applied to the analysis of C-peptide, a large (3020 Da) endogenous peptide in human plasma. C-peptide and insulin are produced from cleavage of proinsulin in a 1:1 molar ratio; therefore, C-peptide can be used as a biomarker of insulin secretion. Direct, highly rugged and accurate analysis of insulin is considerably more challenging due to its higher clearance (and thus lower plasma concentration), and the high variance of measurement by established immunoassays. Sensitive LC/MS analysis has been proposed as a candidate reference method for C-peptide analysis, and has become an invaluable tool for improving our understanding of and diagnosis of diabetes [5,6]. While urinary C-peptide analysis is quite simple [7,8], plasma concentrations (especially fasting) do not exceed 2 ng/ml in non diabetic and Type 2 diabetic subjects. For Type 1 diabetes, C-peptide concentrations are in the sub 0.5 ng/ml range. LC/MS analysis of C-peptide therefore requires maximization of the power of both chromatography and mass spectrometry steps. In our application, we have successfully combined the separation power of 2D chromatography, the high resolution power of fused-core column architecture and the high selectivity of mass spectrometry to achieve better sensitivity.

Experimental

Reagents and materials

All organic solvents were HPLC grade and were obtained from Fisher (Suwanee, GA). MiliQ grade water was produced by Millipore system (Millipore corp, Bedford, MA).

Instruments

Our method used a 1100 series 2D liquid chromatography system (Agilent Technologies, Wilmington, DE, USA), equipped with two G1312A binary pumps, G1316A column compartment equipped with a 2-position, 6-port swirching valve, G1379A degasser and G1367A

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Received December 03, 2012; **Accepted** December 20, 2012; **Published** December 24, 2012

Citation: Rogatsky E, Browne S, Zheng BN, Stein DT (2012) Application of Fused-Core Particle Column in Two Dimensional Reversed Phase - Reversed Phase LC/MS Analysis of Biological Samples. Impact of Extra-Column Volume. J Chromat Separation Techniq 3:158. doi:10.4172/2157-7064.1000158

WP autosampler. LC/MS analysis was performed on an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Framingham, MA, USA) equipped with a Turboionspray source.

Liquid chromatography operating conditions

35 µl of sample was injected on the first dimension column, a 5 µ Jupiter C5, 300 Å, 2×100 mm (Phenomenex, Torrance, CA, USA). Water-Acetonitrile gradient containing 0.1% TFA was used. In the second dimension chromatography was used 2.1×30 mm Ascentis Express Peptide C18 160 Å 2.7 µm column from Supelco (Bellefonte, PA, USA). For second dimension was used mass spectrometry-compatible mobile phases consisting of 0.4% formic acid, 2% methanol and 2.5% isopropyl alcohol (IPA) and in water (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.45 ml/min. Both columns and switching valve operated at 40°C. C-peptide was eluted 19-28% B shallow gradient (1.9% B/min). Mass spectrometry conditions were identical to described previously [8].

Results and Discussion

UHPLC columns in 2D: pressure restrictions

The greatest advantage of 2D/multidimensional chromatography is greater peak capacity compared to single column applications. Among stand-alone columns, peak capacity is higher for sub-2 micron particle size and fused-core material columns, compared to their fully porous analogs. Integration of these types of columns into multidimensional platforms could potentially increase total peak capacity more, and in addition, improve operational throughput. However, there are intrinsic barriers to such implementation. Sub-2 micron particle size columns generate high pressure, typically above 800 Bar. This mandates special requirements for the HPLC pump. In addition, transfer of the analyte from the first dimension to the second UHPLC dimension can only be accomplished via online collection loop(s) [9], because trap column(s) between the first and second dimension, as well as the first dimension column, are not designed to tolerate such high pressures, generated by the second dimension. The column-switching valve between the first and second dimension, as well as trap column hardware, should have a 800-1000 Bar pressure limit, which increases system cost and limits available options. In contrast, the incorporation of a fused-core particle column in an on-line multidimensional platform is considerably easier and less problematic, compared to UHPLC columns.

Incorporation of fused-core column in the 2D RP-RP LC/MS platform

We have previously reported a 2D RP-RP LC/MS method designed for measurement of human C-peptide and endogenous peptide hormones in biological samples [10,11]. In the first dimension, we used a 2×100 mm, 5 µm Jupiter C5 column. The peptide analyte of interest eluted by a water-acetonitrile gradient, containing 0.1% TFA and transferred by switching valve into the second dimension column, 5 µm Polaris C18 2×50 mm, 180 Å. A fine separation by shallow water–acetonitrile gradient containing 0.4% formic acid, followed by mass spectrometer detection and quantitation performed. Using this platform, we have successfully analyzed thousands of samples; however, we reached the limits of instrumental sensitivity. Plasma samples collected from C-peptide deficient subjects (Type 1 Diabetes and fasting Type 2 Diabetes) frequently had peptide concentrations below our limit of quantification. Since mass spectrometry sensitivity already maximized, we attempted to increase the peak height (and S/N) on the chromatography side. Our efforts to implement a 1 mm ID column were unsuccessful, since analyte transfer from the first dimension and refocusing in the second dimension resulted in a high flow rate ~ 0.7

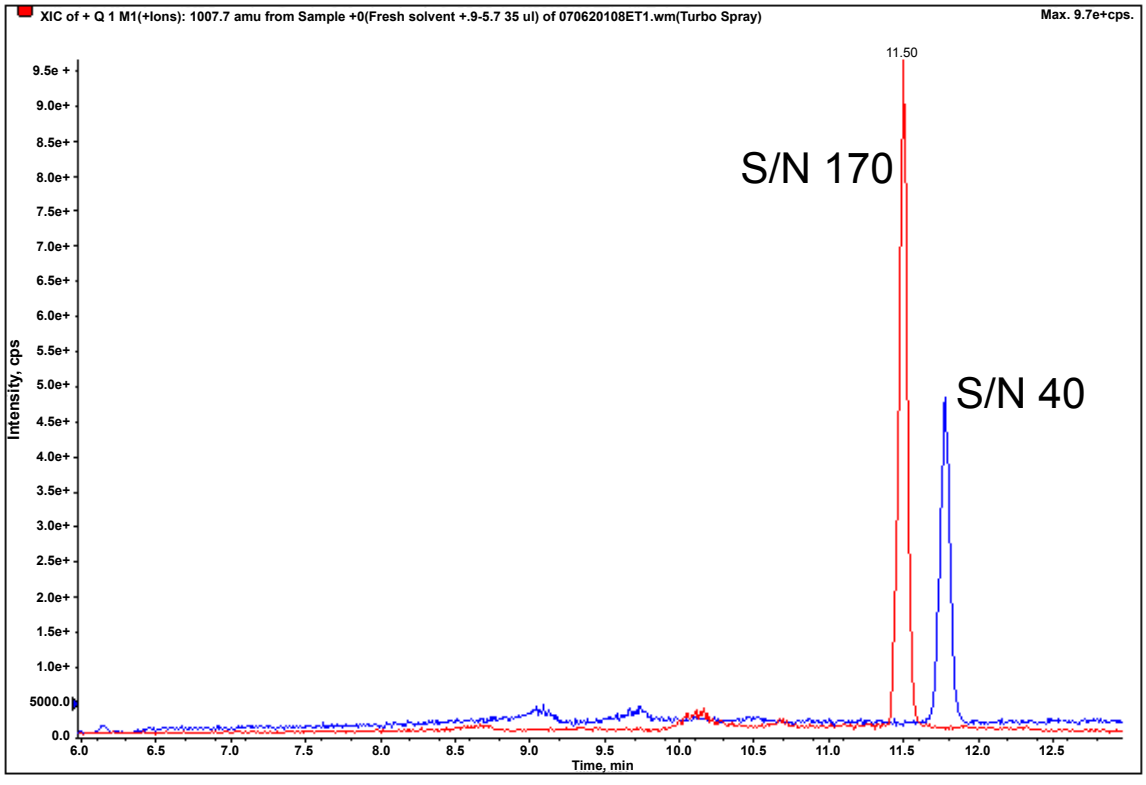
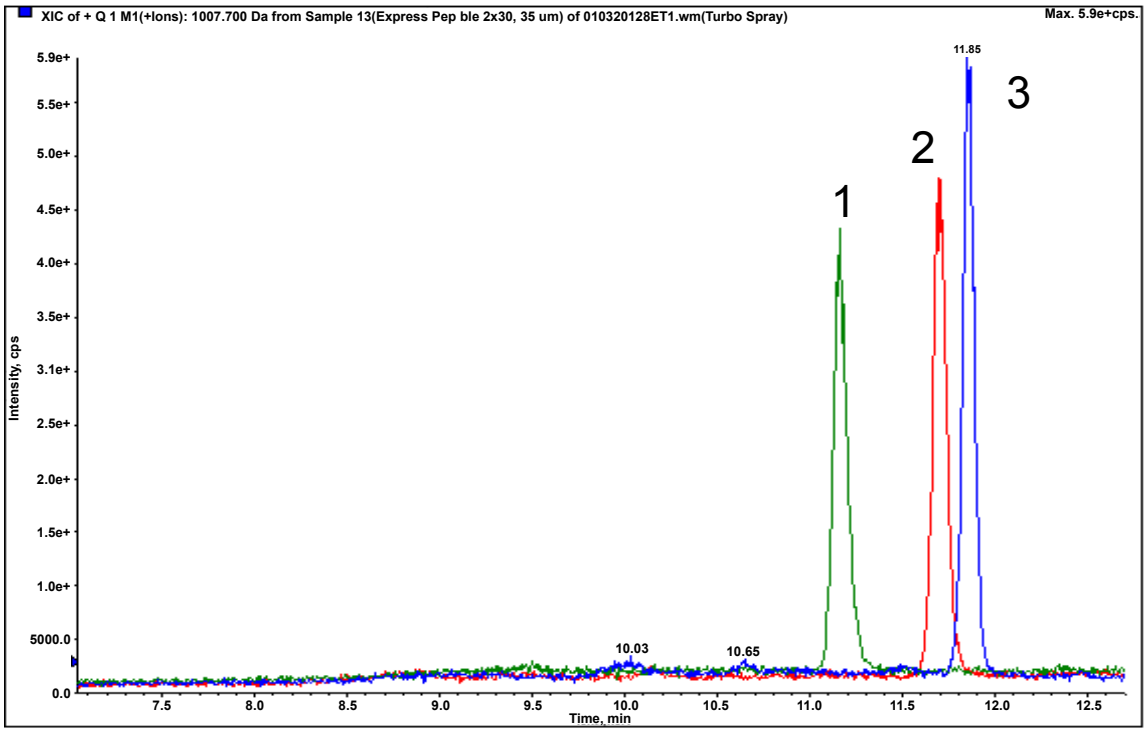


Figure 1: Increase of S/N from 40 to 170 with direct replacement to 2×30 mm Ascentis Express Peptide 2.7 µm, 160 Å.

ml/ml, which was too high for a 1 mm ID column (data not shown). We replaced the second dimension column (5 µm Polaris C18 2×50 mm 180 Å) with a 2×30 mm fused-core C18 Ascentis Express Peptide, 160 Å. Peak height was increased almost two fold, and S/R increased 4 fold (Figure 1). Lesser loading capacity (compared to fully porous particles)–the main limitation factor of the Fused-Core material–was overcome by using 2D chromatography. Only a small, (<0.2 ml) C-Peptide containing fraction was loaded onto the second column. The majority of fused-core particle columns are available on the market with 100 Ascentis Express Peptide C18 160 Å 2.7 µm pore size and are optimal for the separation of "small molecule" analytes below 500 Da. Peptide-separation columns are more uncommon in a family of fused-core particle materials. Ascentis Express Peptide, 160 Ascentis Express Peptide C18 160 Å 2.7 µm introduced in 2010, and to our knowledge, is the first wide-pore fused-core material. Recently, a second company has introduced a fused-core peptide column. This new product, when used in our platform, demonstrated higher LC/MS noise and a lack of S/N enhancement (Figure 2).

Extra-column volume effects: impact of tubing volume

Fused-core particle columns are a cost effective means of extending the capabilities of a standard analytical HPLC, instead of purchasing a UPLC/UHPLC. The maximum potential improvement in peak shape by fused core columns, however, may not be achieved by a simple "plug and play" replacement of traditional 5 or 3 µm columns. This is due to the considerably larger gradient dead volumes and especially of post-column dead volumes of older model HPLC systems. Since fused-core columns are operated at higher flow rates without loss of resolution, compared to traditional fully porous columns, this somewhat neutralizes the negative impact of pre-column delay/dead volume. In contrast, minimization of post-column volume is critical for achieving high performance with fused-core columns [12] as compared to their fully porous analogs. Neglecting the necessity of minimizing post-column volume will cause chromatographic peak broadening. Therefore, the understanding, control and minimization of post-column volume is a critical step in fused-core method optimization, especially in LC/MS applications. While post column volume in HPLC/UV stackable modules is easy to minimize (or it has already been optimized in UHPLC/UPLC systems), the use of the mass spectrometer as a detector is more complicated in terms of post column volume. We would like to emphasize that a mass spectrometer is like a stand-alone detector, having 2-4 times longer tubing between column and source inlet, compared to the distance between column and detector in optimized HPLC/UV (Table 1). We have tested 66 cm length tubing connecting a second dimension fused core column (Acsentis Express Peptide 2×30, Supelco) and mass spectrometer. Tubing ID range of 125-175 µm, which is typical for HPLC, tested. We noticed a 25% loss of S/N with sub-optimal but standard tubing use.



Peak 1: Aeris Peptide C18, 3.6 µm 2.1×50 mm (fused-core); Peak 2: XBridge C18, 3.5 µm 2.1×30 mm; Peak 3: Ascentis Express Peptide C18, 2.7 µm 2.1×30 mm (fused-core).

Figure 2: Column comparison in the second dimension under the same conditions.

Tubing color	Internal diameter	Volume/cm, µl	Volume of 66 cm, µl	S/N
red	0.005'=125 µm	0.123	8.1	180
purple	0.006'=150 µm	0.182	12	145
yellow	0.007'=175 µm	0.248	16	135

Table 1: Impact of postcolumn volume on S/N.

Extra-column volume effects, impact of mass spectrometer hardware

An additional contribution to post-column [delay] volume is made by the mass spectrometer itself. This volume varies by brand and is difficult to minimize, both due to potential warranty issues as well as to most unsophisticated users treating the mass spectrometer as a black box. For example, the majority of mass spectrometers include a column-switching valve and a low volume in-line filter prior to the source. This set-up designed to prevent source contamination, resulting from the build-up of non-volatile salts eluted in the column void volume. A typical example of LC/MS analysis would be of diluted urine in a single column application. In many cases, however, the sample introduced into the LC/MS column is previously desalted/pre-cleaned and therefore the source divert valve can be excluded from the flow path. The use of multidimensional chromatography, or orthogonal sample preparation techniques, greatly improves sample cleanliness, and samples can be successfully analyzed without the use of the traditional divert valve (Figure 3).

In another example, the Applied Biosystems/MDS SCIEX standard Turbo Electrospray source is equipped with a 50 cm length×125 µm PEEK tubing contributing 6.35 µl of post-column volume. For electrospray ionization, a high voltage, up to 5000 V is applied in the source, and this tubing serves as a safety feature, preventing backwards migration of electrical charge from the source towards the column and LC instrument. We have conducted experiments replacing this standard tubing (part of a PM kit) with the same length of PEEKsil tubing with an ID of 100 µm (black color code for a standard PEEK) and 125 µm (red code for standard PEEK). The advantage of PEEKsil tubing over PEEK in SCIEX source, in fused-core column applications, was reported by Carl Sanchez at the HPLC symposium 2010, Boston MA. He suggested that PEEKSIL tubing (SGE, Houston, TX) which is factory pre-cut, has higher edge quality compared to that achieved by manual tube cutting. This contributes to better peak shape by enabling true zero-dead volume tubing connections, which will result in better S/N. We obtained ~ 20% S/N increase and tested further the phenomenon of S/N. Standard 50 cm length×125 µm PEEK tubing was replaced by 100 and 125 µm PEEKsil tubing and source settings were re-optimized. Replacement of 125 µm ID tubing (6.136 µl volume for 50 cm) to 100 µm tubing (3.927 µl volume for 50 cm) decreased volume by 64%. However, the real difference in S/N was observed between the same ID tubing: 125 µm ID PEEK and PEEKsil tubings, and not between 100 µm and 125 µm PEEKsil tubing as was expected (Figure 4). PEEK tubing generated higher mass spectrometry background noise. We assume that the explanation of such a great positive impact of PEEKsil tubing in noise is due to at least 5 times higher manufacturing tubing tolerances, compared to PEEK. As a result, mobile phase flow in the source is less turbulent which leads to better solvent evaporation and aids in droplets formation in the source.

Conclusion

Due to the thinner porous layer of the stationary phase in fused-core particle columns, the total column loading capacity is also smaller when compared to standard fully porous particle columns. As a result, fused-core particle columns are more susceptible to sample overload and binding competition, which often may occur when complex biological samples are used for analysis. These undesirable effects may impact peak shape, assay reproducibility, column performance and/or lifetime. We overcome these intrinsic limitations of fused core material through the development of a 2D RP-RP platform which utilizes a fully porous C18 column in the first dimension and a fused-core C18 column in the second. This 2D column switching design eliminates the typical drawbacks of fused-core columns and maximizes their advantages in LC/MS analysis of complex biological samples. Efficient use of fused-core columns required careful optimization of post-column volume.

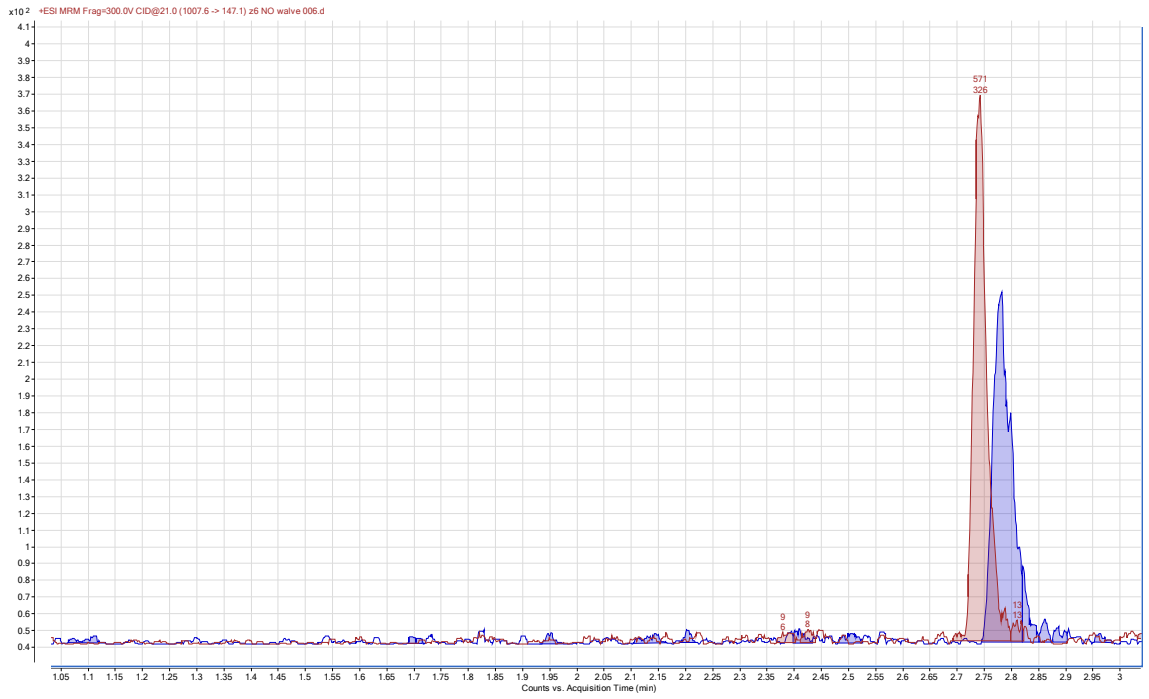


Figure 3: Impact of divert valve bypass: 56% gain of peak height (from 209 to 326).

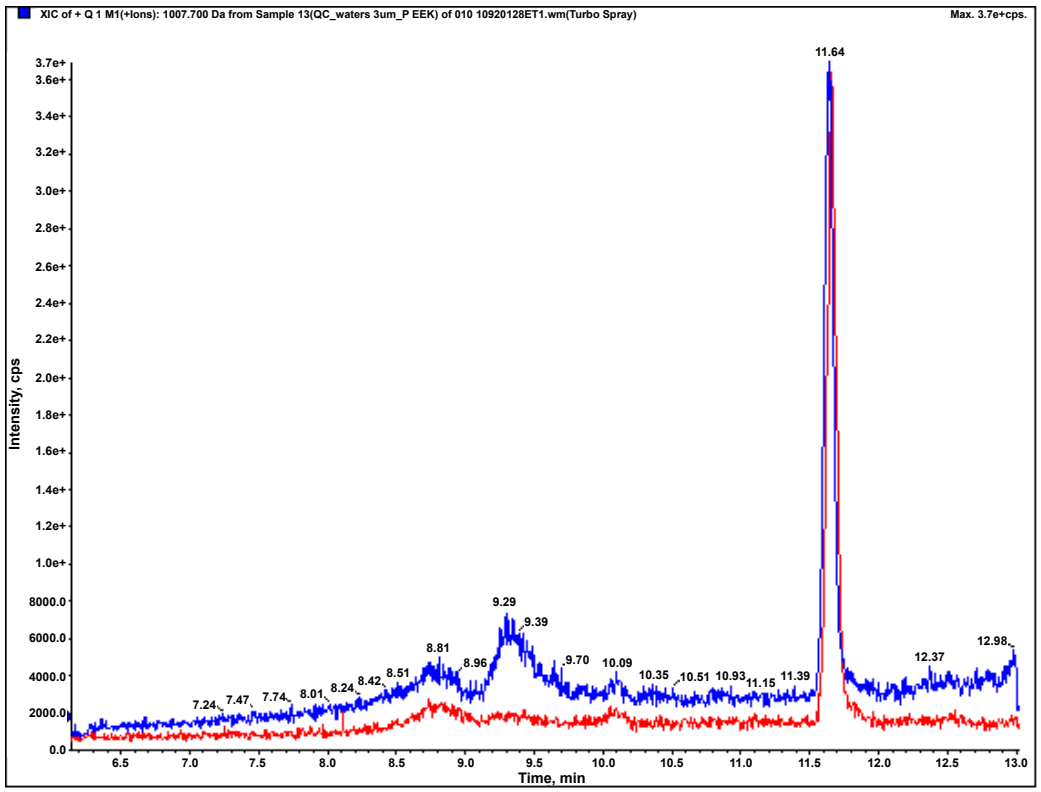


Figure 4: Greater background noise when standard PEEK tubing 125 µm was used, compare to same ID PEEKsil.

Acknowledgement

We would like to thank Robert Freeman and SGE Analytical Science for samples of custom-made PEKSIL tubing and helpful technical discussions.

References

1. Destefano J, Langlois T, Kirkland J (2008) Characteristics of superficially-porous silica particles for fast HPLC: some performance comparisons with sub-2-microm particles. J Chromatogr Sci 46: 254-260.
2. Abrahim A, Al-Sayah M, Skrdla P, Bereznitski Y, Chen Y, et al. (2010) Practical comparison of 2.7 microm fused-core silica particles and porous sub-2 microm particles for fast separations in pharmaceutical process development. J Pharm Biomed Anal 51: 131–137.
3. Zheng J, Patel D, Tang Q, Markovich R, Rustuma AM (2009) Comparison study of porous, fused-core, and monolithic silica-based C18 HPLC columns for Celestoderm-V Ointment analysis. J Pharm Biomed Anal 50: 815–822.
4. Ruta J, Zurlino D, Grivel C, Heinisch S, Veuthey J, et al. (2012) Evaluation of columns packed with shell particles with compounds of pharmaceutical interest. J Chromatogr A 1228: 221–231.
5. Stoyanov A, Rohlfing C, Connolly S, Roberts M, Nauser C, et al. (2011) Use of cation exchange chromatography for human C-peptide isotope dilution–mass spectrometric assay. J Chrom A 1218: 9244-9249.
6. Rogatsky E, Balent B, Goswami G, Tomuta V, Jayatillake H, et al. (2006) Sensitive quantitative analysis of C-peptide in human plasma by 2-dimensional liquid chromatography-mass spectrometry isotope dilution assay. Clin Chem 52: 872-879.
7. Fierens C, Thienpont L, Stöckl D, Willekens E, Leenheer A (2003) Application of a C-peptide electrospray ionization-isotope dilution–liquid chromatography–tandem mass spectrometry measurement procedure for the evaluation of five C-peptide immunoassays for urine. J Chromatogr B Analyt Technol Biomed Life Sci 792: 249-259.
8. Rogatsky E, Tomuta V, Cruikshank G, Vele L, Jayatillake H, et al. (2006) Direct sensitive quantitative LC/MS analysis of C-peptide from human urine by two dimensional reverse phase/reverse phase high-performance liquid chromatography. J Sep Sci 29: 529-537.
9. Cacciola F, Jandera P, Blahova E, Mondello L (2006) Development of different comprehensive two dimensional systems for the separation of phenolic antioxidants. J Sep Sci 29: 2500-2513.
10. Rogatsky E, Stein DT (2006) Two dimensional reverse phase - reverse phase chromatography: a simple and robust platform for sensitive peptide quantitative analysis by LC/MS. Hardware design. J Sep Sci 29: 538-546.
11. Rogatsky E, Tomuta V, Jayatillake H, Cruikshank G, Vele L, et al. (2007) Trace LC/MS analysis of polypeptide biomarkers: impact of 1D and 2D chromatography on matrix effects and sensitivity. J Sep Sci 30: 226-233.
12. Fekete S, Fekete J (2011) The impact of extra-column band broadening on the chromatographic efficiency of 5 cm long narrow-bore very efficient columns. J Chromatogr A 1218: 5286-5291.