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Investigation of carryover of peptides in nano-liquid chromatography/mass spectrometry using packed and monolithic capillary columns

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ABSTRACT

This article relates on reversed-phase column technology as the main cause of carryover in the LC–MS/MS analysis of proteomics samples. The separation performance and column carryover was investigated using four capillary columns with different morphologies by monitoring the remaining traces of tryptic peptides of bovine serum albumin in subsequent blank LC–MS runs. The following trend in column carryover was observed: capillary column packed with 3 μm porous C18 particles ≫ 2.7 μm fused-core C18 packed column > silica C18 monolith ≫ poly(styrene-*co*-divinylbenzene) monolith. This is mainly related to the intrinsic properties of the different chromatographic materials, related to surface area and the presence and size of mesopores (stagnant zones where mass transfer is controlled by diffusion). Both isocratic and gradient wash steps with 2-propanol/acetonitrile mixtures were not effective to reduce column carryover. An isocratic wash step using a high acetonitrile percentage or blank gradient reduced carryover with approximately 50%. Nevertheless, it is important to note that effects of column carryover were still observed in a fifth subsequent gradient blank. Although the polymer monolith clearly outperformed the silica materials in terms of carryover, this material exhibited also the lowest loadability, which may be a disadvantage when profiling proteomics mixtures with a broad dynamic range.

1. Introduction

The development of highly sensitive LC–MS/MS methods that allow the detection of low abundant diagnostic and/or prognostic biomarkers in complex proteomics samples is one of the key steps to facilitate identification of clinically significant candidates [1]. Detection sensitivities in the low attomol range have been achieved in peptide-mapping experiments in a nanoLC–MS/MS workflow interfaced *via* electrospray (ESI) [2]. The high sensitivity is an effect of using high-efficiency columns yielding narrow and therefore high peaks while operating at low volumetric flow rates (in the range of 100–500 nL/min). Employing small I.D. columns (<25 μm) while adjusting the volumetric flow rates has resulted in greatly enhanced ionization efficiencies when employing an electrospray-ionization source [3].

Carryover is defined as the presence of an analyte detected in the adjacent chromatographic run originating from the previous injection(s) [4]. In label-free quantitation experiments carryover may lead to false identification of compounds or may result in false quantitation levels [5]. Also, carryover may lead to ion-suppression of low abundant peptides when co-elution occurs with analytes from the previous run [6]. In MS/MS experiments, when the precursor-ion selection is based on signal intensity, high abundant ions as a result from carryover will be selected for fragmentation where low abundant ions in the same MS scan-cycle will be discarded. The lack of fragmentation of low abundant ions can lead to lower protein sequence coverage or low abundant species are not identified. In the field of biomarker discovery where identified biomarkers must be validated over multiple injections, the presence of carryover can result in a poor repeatability in the quantitation of candidates.

Carryover can be divided into different categories, *i.e.*, "constant" carryover which is visualized as a small peak of similar size in each adjacent chromatographic blank and is most likely caused by a contamination in the injected solvent and "classic" carryover, where a peak is redundant in adjacent blanks, although the area reduces each run. Numerous potential sources of carryover have been identified in the LC system, *e.g.*, dead volume between the tubing connections, the injection needle and/or loop or mechanical damage (scratches) to the stator of the autosampler valve [7,8]. The mass spectrometer can also contribute to carryover by contamination of the orifice and by the slow removal of ions from the collision cell also known as "cross-talk" [9]. Depending on the column chemistry, column dimension, the mobile-phase composition, and the nature of the sample injected, the separation column can contribute to classic carryover due to complexation, *e.g.*, with residual silanol

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Table 1
Summary of stationary-phase properties. Capillary-column dimensions were 100 μm I.D. × 50 mm.

Column	Characteristic size	Chemistry	Surface area (m^2/g)	End capping
Porous particles	3 μm particles (100 Å pores)	C18	300	Yes
Fused-core particles	2.7 μm particles (90 Å pores)	C18	150	Yes
Silica monolith	1 μm macropore and 1 μm skeletons with mesoporesize 12 nm (120 Å)	C18	230	Yes
Polymer monolith	0.5 μm macropores and 0.3–0.6 μm microgloubules (no mesopores)	Phenyl	~7–50	No

groups, and slow diffusion of molecules from stagnant pores. The trap column used in a 'pre-concentration' setup can also contribute to carryover effects; however it is likely to be less compared with the carryover observed on the separation column. Trap columns are used for pre-concentration of diluted samples or desalting of samples containing non-MS compatible salts and are loaded with a high flow-rate. The high flow-rates allow effective washing of the trap column with a wash solvent of high elution power during the equilibration time of the separation column.

Columns packed with microparticulate porous silica particles are typically considered as the gold standard LC–MS analysis of peptides. This is due to properties, such as high purity, large surface area, and outstanding mechanical strength. With the introduction of ultra-high-pressure liquid chromatography (UHPLC) there is a clear trend toward the use of sub-3 and sub-2 micron silica particles [10]. Large I.D. columns packed with fused-core particle technology have showed excellent performance with minimum reduced plate heights ($h = H/d_p$) often lower than 2 [11,12]. This has been attributed to a reduced eddy-dispersion (A-term contribution), resulting from a very narrow particle-size distribution, a reduced B-term contribution due differences in the porous zone diffusion coefficient, and a reduced C-term contribution due to reduced diffusion path length. As an alternative for packed-bed columns monolithic columns were introduced in the 1990s [13–16]. A monolith is a continuous macro-porous support structure prepared from silica or polymer precursors which is in capillary format covalently bonded to the wall to enhance the robustness. Much research effort has been directed to the optimization of the porous structure to achieve the best possible separation efficiency while maintaining high column porosity and hence a relatively low column pressure [17–19]. The feasibility of polymer monoliths for biomolecule separation (antibodies [20,21], intact proteins [22,23], oligonucleotides [24], and peptides [25]) have been demonstrated on many occasions. Silica monoliths have shown proven performance for the separation of small molecules [26]. Recently, we compared the kinetic performance of silica monoliths with that of columns packed with fused-core and porous-silica particles [27]. The capillary silica-monolithic column outperformed the packed columns both in terms of the efficiency (peak capacity) and permeability.

Depending of the mobile-phase conditions and column properties, such as morphology, presence of stagnant pores, and differences in surface chemistry, column carryover may affect the qualitative and quantitative analysis results. In the present study, the influence of using different stationary phases (columns packed with porous particles, fused-core silica particles, and silica and polymer monolithic columns) on classic carryover was evaluated by the LC–MS quantitation of tryptic peptides detected in the analytical blank after the injection of a concentrated tryptic digest from bovine serum albumin.

2. Materials and methods

2.1. Chemicals and materials

A tryptic digest of bovine serum albumin (BSA) was obtained from Bruker Daltonik (Bremen, Germany). LC/MS grade acetonitrile (ACN) was purchased from RCI Labscan Limited (Bangkok, Thailand) and formic acid (FA) was obtained from Fluka (Buchs, Switzerland). Water was purified using a MilliQ Gradient A10 system (Millipore, Molsheim, France).

The carryover study was conducted using an ACE C18, 50 mm × 0.1 mm I.D. column packed with porous 3 μm silica particles from Advanced Chromatography Technologies (Aberdeen, UK), a Halo C18, 50 mm × 0.1 mm I.D. column packed with 2.7 μm fused-core particles (0.5 μm porous shell fused to a solid core) from Advanced-materials-technology (Wilmington, USA), a MonoCap C18, 50 mm × 0.1 mm I.D. silica-monolithic column from GL Sciences Inc. (Tokyo, Japan) and a 50 mm × 0.1 mm I.D. poly(styrene-*co*-divinylbenzene) monolithic PepSwift column from Dionex Benelux (Amsterdam, The Netherlands). More detailed stationary-phase properties have been summarized in Table 1.

2.2. Instrumentation and LC–MS conditions

NanoLC–MS experiments were conducted using an UltiMate 3000 system (Dionex, Germering, Germany) coupled on-line to an ion-trap mass spectrometer (AmaZon, Bruker Daltonik, Bremen, Germany), using a CaptiveSpray ion source (Bruker-Michrom, Auburn, CA). The LC system comprised a quaternary low-pressure mixing gradient pump with a built-in membrane degasser unit, a temperature-controlled pulled-loop autosampler equipped with 1 μL sample-loop, and a thermostatted column compartment. The LC system was operated in the direct-injection configuration; as such, 20 μm I.D. fused-silica connection tubing was used to connect the column inlet to the autosampler and the column outlet to the mass spectrometer interface. The LC–MS instrument was controlled with Hystar V3.2 software. Data processing was performed using Data Analysis V4.0 SP2 software (Bruker Daltonik, Bremen, Germany). 1 μL injections of tryptic peptides from BSA were separated applying a linear aqueous ACN gradient containing 0.1% FA at a flow rate of 1 μL/min and thermostating the columns at a temperature of 60 °C. A wash step was performed applying 72% aqueous ACN containing 0.1% FA for 1.5 min and column equilibration at the gradient start composition was performed for 6 min. MS detection was performed in full-scan mode in the positive-ionization standard-enhanced scan mode (resolution of 0.3 μm at scan speed of 8100 μm/s) applying a capillary voltage of 1500 V with an end plate off-set of −500 V. The dry gas flow rate was set at 3 L/min with a dry temperature of 180 °C. The mass-range was set from 300 to 1200 m/z with an ion-current charge (ICC) of 100,000 and a maximum accumulation time of 50 ms. MS/MS spectra were acquired in the ultra-scan mode (resolution of 0.5 μm at scan speed of 32,000 μm/s). The two most abundant precursor ions were selected for fragmentation. The detected peptide fragments were searched against the Mascot database SwissProt version 2.3 (Matrix Science Ltd., London, United Kingdom) under taxonomy other mammalian, Carbamidomethyl (C) as fixed modification and ammonia-loss (N-term C), deamidated (NQ), Gln → pyro-Glu (N-term Q), oxidation (M) as variable modifications. The alkylation with iodoacetamide resulted in the addition of a 57.07 Da carbamidomethyl group on the cysteine (C) residues.

Carryover was determined for eleven high abundant peptides as shown in Table 2 listed in elution order as observed on the 3 μm porous-particulate column. In case the peptide showed multiple

Table 2
Overview of peptides used to determine carryover. Numbering corresponds to the elution order as observed on the 3 μm particulate column.

#	Amino acid sequence	*m*/*z*	MW (Da)
1	K.YICDNQDTISSK.L	722.4 (2+)	1385.6
2	K.AEFVEVTK.L	922.5 (1+); 461.8 (2+)	921.5
3	K.YLYEIAR.R	927.5 (1+); 464.3 (2+)	926.5
4	R.KVPQVSTPTLVEVSR.S	820.5 (2+); 547.4 (3+)	1638.9
5	R.RPCFSALTPDETYVPK.A	941.0 (2+); 627.7 (3+)	1822.9
6	R.RHPEYAVSVLLR.L	720.5 (2+); 480.7 (3+)	1438.8
7	K.QTALVELLK.H	1014.6 (1+); 507.9 (2+)	1013.6
8	K.LGEYGFQNALIVR.Y	740.5 (2+)	1478.8
9	R.HPYFYAPELLYYANK.Y	945.0 (2+); 630.4 (3+)	1887.9
10	K.TVMENFVAFVDK.C	700.4 (2+)	1398.7
11	K.QTALVELLK.H* +Gln-pyro-Glu (N-term Q)*	997.6 (1+); 499.4 (2+)	996.6

* The N-terminal Gln and Glu was modified to pyro-Glu by Gln cyclase which resulted in a loss of 17.0 Da.

charge states, the summation of all charge states were used to create the extracted ion chromatogram (EIC). The EIC of each peptide was utilized to obtain the peak area which was processed by GraphPad Prism version 5.03, December 10, 2009 (GraphPad Software Inc., CA, USA).

2.3. Sample preparation

A tryptic digest from BSA dissolved in 10% ACN containing 0.1% FA was diluted to 500 fmol/μL and 200 fmol/μL in aqueous 0.1% FA. Calibration standards of BSA digest solution were prepared ranging in concentration from 10 fmol/μL to 5 attomol/μL. The limit of detection (LOD) and limit of quantification (LOQ) were determined using the calibration standards. All dilutions contained 5% ACN and were prepared in glass autosampler vials to minimize the loss of hydrophobic peptides.

3. Results and discussion

The carryover detected in an analytical blank is the summation of system carryover and column carryover. System carryover was investigated by the injection and LC–MS analysis of 500 fmol tryptic digest from BSA, while bypassing the separation column, followed by a gradient blank. No detectable amounts of system carryover were observed (data not shown).

3.1. Assessment of column performance

Tryptic digests of complex protein mixtures result in a large number of peptides ranging from hydrophilic (early eluting) to hydrophobic (late eluting). The assessment of column performance and carry-over effects on these highly complex mixtures is challenging in terms of reproducibility due to possible co-elution of peptides with different pK_a values, which leads to ion suppression during the ESI process. Therefore, a less complex tryptic digest from a single, very well characterized protein (Bovine Serum

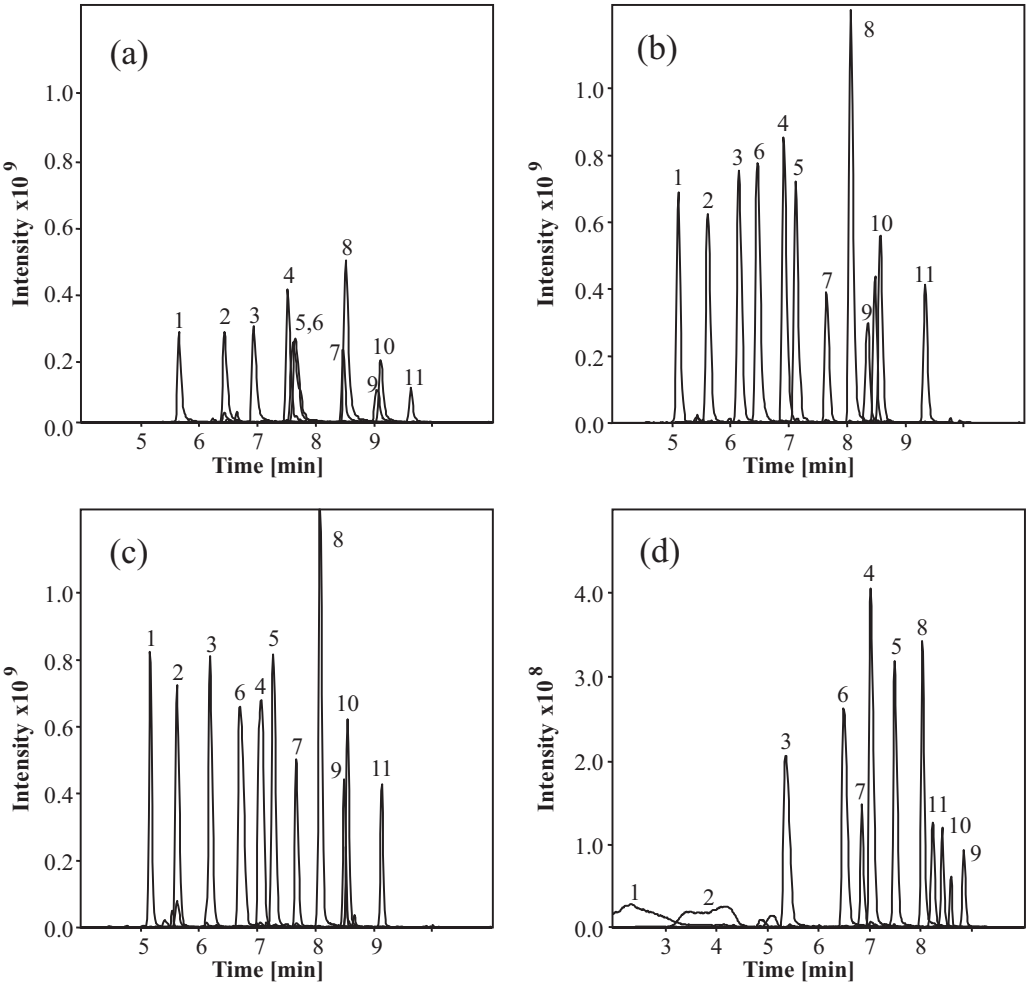

Fig. 1. Extracted ion chromatograms of 11 tryptic BSA peptides measured on (a) a capillary column packed with 3 μm porous particles, (b) a capillary column packed with 2.7 μm fused-core particles, (c) a silica monolithic capillary column, and (d) a polymer monolithic capillary column. Gradient conditions: flow rate = 1 μL/min; gradient time = 7.5 min. Injected amount on silica columns is 500 fmol tryptic peptides, 200 fmol for the polymer monolith.

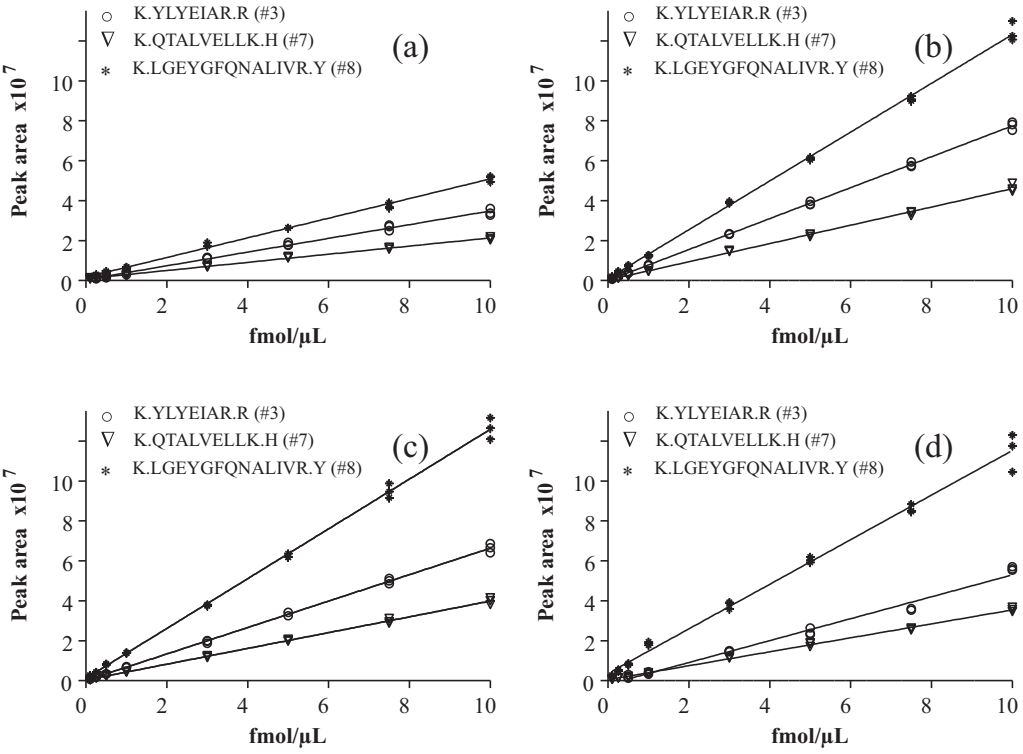

Fig. 2. Calibration curves for the triplicate injections of 11 tryptic BSA peptides measured on (a) a capillary column packed with 3 μm porous particles, (b) a capillary column packed with 2.7 μm fused-core particles, (c) a silica monolithic capillary column, and (d) a polymer monolithic capillary column in the concentration range from 100 attomol/μL to 10 fmol/μL. Peptide identification: (○) K.YLYEIAR.R (#3); (▽) K.QTALVELLK.H (#7); and (*) K.LGEYGFQNALIVR.Y (#8).

Albumin – BSA) was selected as a model to represent a 'typical' proteomics sample containing both hydrophilic and hydrophobic peptides. The linear gradient window was optimized for the different column types such that all peptides eluted in a comparable retention window. The gradient composition was 0–40% aqueous ACN containing 0.1% FA for the column packed with porous particles, 0–34% for the silica monolithic column, and 0–32% for the fused-core particulate column and the polymer monolithic column. The resulting extracted ion chromatograms (overlays of 11 tryptic peptides measured on the four different column types) are depicted in Fig. 1. The injected amount for the packed columns and silica monolith was 500 fmol tryptic peptides on column. This amount was reduced to 200 fmol for the polymer monolithic capillary column, following the loadability recommendations of the manufacturer.

The elution order on the silica material columns was comparable, whereas the elution order on the polymer monolith was slightly different due to the different base material (polystyrene). For example, the retention time of peptide R.HPYFYAPELLYYANK.Y shifted to higher value on the polymer monolithic column, which may be explained by enhanced π–π interactions. Also, the first two peptides elute as broad peaks on the polymer monolithic column. Due to the low surface area, these hydrophilic peptides are not retained after injection at the column head and elute (partially) in isocratic mode during the gradient delay. More hydrophobic peptides (or when using column structures with higher retention capacity) are focused at the start of the column (on-off retention mechanisms) and only elute after applying a higher acetonitrile content.

The chromatographic performance of the four columns was evaluated by the peak capacity (n_c) which is the maximum number of chromatographic peaks that can be separated with a resolution of 1 in the gradient window. The peak capacity was calculated from the peak width (w) measured at 4σ (13.4% of peak height for a Gaussian peak) and the gradient time t_G according to the following formula:

$$n_c = 1 + \frac{t_G}{w} \quad (1)$$

When applying a 7.5 min gradient the average 4σ peak widths were determined for the porous particulate, fused-core particulate, silica monolith and PS-DVB monolithic column at 9.3, 8.4, 7.0 and 8.8 s, respectively. The resulting peak capacity ranged between 51 and 67. The difference in column performance affects the limit of detection (LOD) and limit of quantitation (LOQ). This is due to higher signal intensities when peak widths become narrower (with fixed area). The noise measured in the MS is based on signal intensity; therefore the LOD and LOQ were calculated based on peak height (with a signal-to-noise ratio of 3 and 6, respectively). The LOD and LOQ of all 11 peptides calculated on the four columns are shown in Supplementary information (Table S1).

3.2. Assessment of column carryover

To assess column carryover, calibration curves were constructed based on the concentration of the chromatographic peak which is reflected by the peak area. Calibration curves were recorded by triplicate injections of 1 μL tryptic peptides from BSA in a concentration range from 5 attomol/μL to 10 fmol/μL on the different column types. Fig. 2 shows the resulting calibration curves for peptide K.YLYEIAR.R (#3), K.QTALVELLK.H (#7) and K.LGEYGFQNALIVR.Y (#8) on the porous particulate column (a), fused-core particulate column (b), silica-monolithic column (c), and the polymer-monolithic column (d) with the triplicate injections shown as data points in the graph. Stable ionization efficiencies were observed during the carryover experiments on each individual column as reflected by the high linearity with r-squared values between 0.952 and 0.999 over the tested dynamic

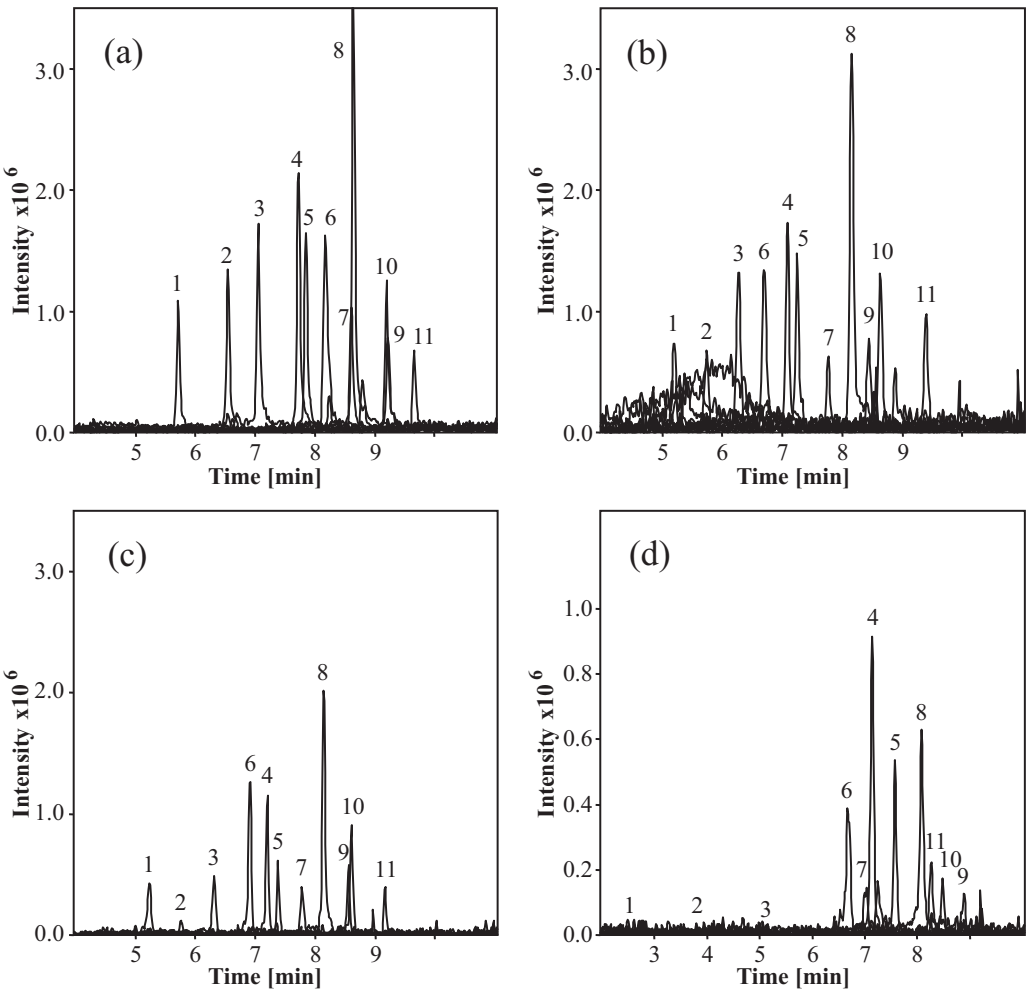

Fig. 3. Extracted ion chromatogram of 11 tryptic BSA peptides detected in the first gradient blank after the injection of 500 fmol tryptic BSA peptides on (a) a capillary column packed with 3 μm porous particles, (b) a capillary column packed with 2.7 μm fused-core particles, (c) a silica monolithic capillary column, and (d) 200 fmol tryptic BSA peptides on a polymer monolithic capillary column.

range for all eleven peptides on the four columns. Although differences in peak area were observed due to different ionization efficiencies for different peptides, the general trend remained similar on the different column types. Statistical results for the eleven peptides determined on the porous particulate, fused-core column and the silica and polymer-monolithic column are shown in Supplementary information (Table S2).

After the injection and separation of 500 fmol tryptic BSA peptides on the silica columns and 200 fmol on the polymer-monolith column (Fig. 1), column carryover was calculated in adjacent gradient blanks by the observed peak area detected by MS.

Fig. 3 shows the EIC of 11 BSA peptides detected in the first gradient blank for the different column types. For the polymer monolith, the *y*-axis was scaled (2.5 times lower) due to the lower amount of standard injected, to allow direct comparison with the other columns. Carryover was observed predominantly on the column packed with porous particles followed by the fused-core particulate column and silica monolith as shown in Fig. 4 (determined in triplicate). A possible explanation for the higher carryover observed for peptide R.HPYFYAPELLYYANK.Y (# 9) on the polymer monolithic column is the presence of six aromatic amino acids and consequently enhanced π–π interactions with the stationary phase. Column carryover was also determined on the silica columns after the injection of 200 fmol tryptic BSA peptides which resulted in comparable amounts of carryover as observed after the injection of 500 fmol tryptic BSA peptides and is shown in Supplementary information (Fig. S3).

3.3. *Effect of washing procedures on column carryover*

To reduce column carryover, different wash steps have been described in literature [28,29]. Mitulovic et al. described a wash procedure for the autosampler by a repetitive routine including aspiration and dispense of a 2,2,2,-trifluoroethanol (TFE) solution, followed by a column cleaning step by addition of TFE to the mobile phase during a gradient blank [30]. However, without proper removal of residual TFE from the column, a loss of peptides was observed in the MS. Excessive system and column washing was required to remove residues of TFE, which is therefore not practical for routine analysis of multiple samples. The following alternative wash steps were examined for the removal of carryover:

I. Gradient blank similar as performed for the elution of tryptic peptides from BSA.
II. Isocratic wash with 80 (v/v)% aqueous acetonitrile containing 0.1% FA for 10 min.
III. Gradient blank from 0.1% aqueous FA to 33:33:34 (v/v)% water: 2-propanol:acetonitrile in 7.5 min.
IV. Isocratic wash with 33:33:34 (v/v)% water:2-propanol: acetonitrile for 10 min.

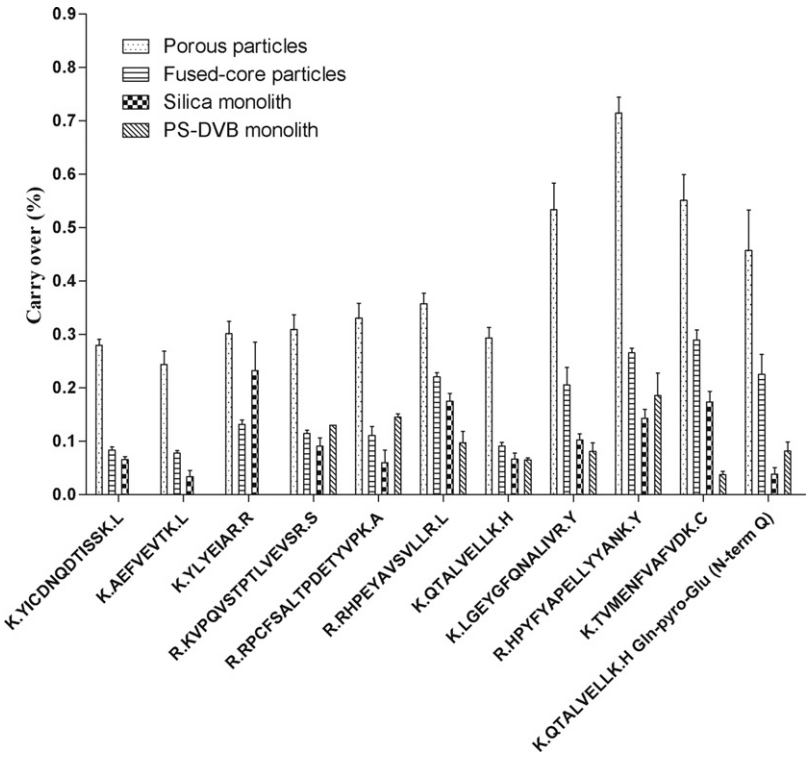

Fig. 4. Carryover determined for the first gradient blank after the injection of 500 fmol tryptic BSA peptides on the silica material columns and 200 fmol on the polymer monolithic column with the standard deviation from the triplicate injections shown as vertical bars.

Fig. 5 shows the resulting extracted ion chromatograms of selected peptides of the gradient blank after the gradient elution of 500 fmol tryptic peptides from BSA and the subsequent wash step obtained on the column packed with 3 μm porous particles. Although the use of 2-propanol often is advised for cleaning, the isocratic and gradient wash with the 2-propanol mixture were both not effective in the removal of carryover. When using acetonitrile in an isocratic or gradient wash step, the MS signal of the gradient blank was reduced by a factor of two, compared to the wash steps using 2-propanol.

Fig. 6 shows five consecutive gradient blanks recorded after the LC–MS analysis of 500 fmol tryptic peptides from BSA on the porous particulate column. Each consecutive gradient blank reduced the amount of carryover although even after four gradient blanks, all eleven peptides could still be identified in the fifth gradient blank. A similar experiment was conducted for all column types including the polymer-monolithic column where no quantifiable amount of carryover was observed in the third gradient blank after the injection of 200 fmol tryptic peptides from BSA. The fourth gradient blank recorded on the different columns is depicted in Supplementary information (Fig. S4). Fig. 7 illustrates the carryover determined in triplicate for all 11 peptides on all four columns measured in five consecutive gradient blanks after the injection of 500 fmol tryptic peptides from BSA. The difference in the observed carryover between the 11 peptides on the fused-core particulate column varied between 0.08% and 0.29%. As such the standard

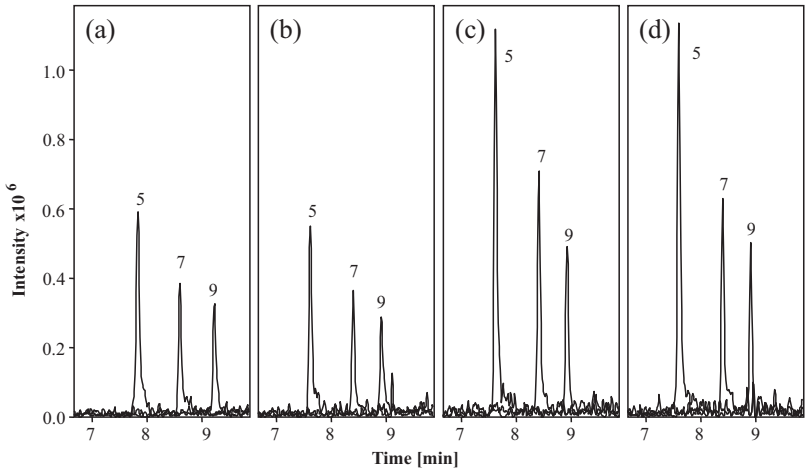

Fig. 5. Effect of different wash conditions on column carryover using a capillary column packed with 3 μm porous particles. Extracted ion chromatograms of peptides R.RPCFSALTPDETYVPK.A (#5), K.QTALVELLK.H (#7), and R.HPYFYAPELLYYANK.Y (#9) of gradient blank after 500 fmol injection of tryptic BSA peptides and a wash step: (a) 7.5 min gradient of 0–40% aqueous ACN containing 0.1% FA, (b) isocratic wash with 80 (v/v)% aqueous acetonitrile containing 0.1% FA for 10 min, (c) 7.5 min gradient from 0.1% aqueous FA to 33:33:34 (v/v)% water:2-propanol:acetonitrile, and (d) isocratic wash with 33:33:34 (v/v)% water:2-propanol:acetonitrile for 10 min.

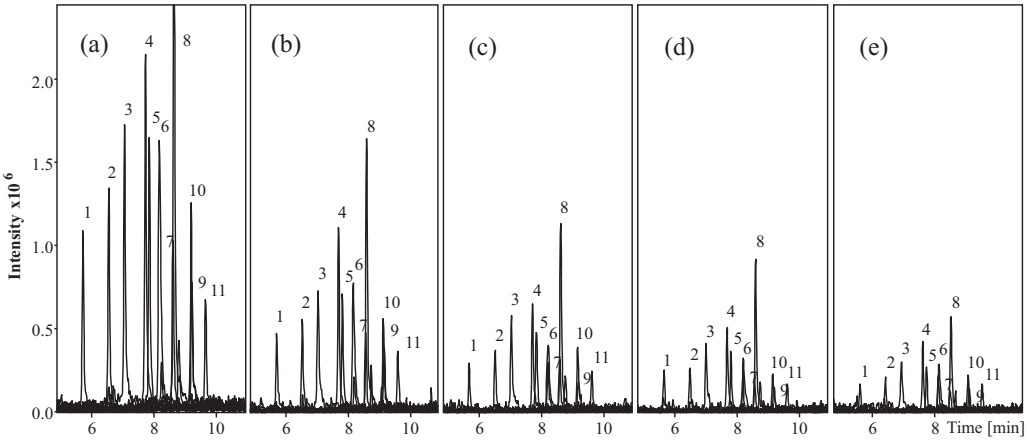

Fig. 6. Consecutive gradient blanks on a capillary column packed with 3 μm porous particles after 500 fmol injection of tryptic BSA peptides.

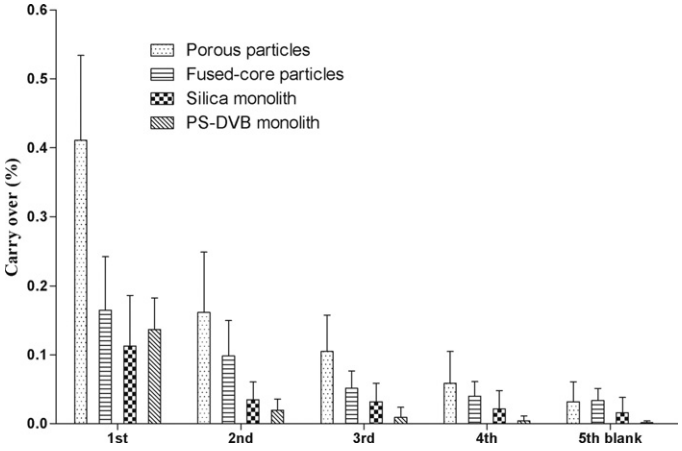

Fig. 7. Carryover calculated in 5 consecutive gradient blanks after the injection of 500 fmol tryptic BSA peptides for the different column types.

deviation does not represent the accuracy of the method, therefore the standard error of the mean (SEM) is shown instead. The SEM (σ_m) is calculated by the standard deviation (σ) between the carryover from the 11 peptides divided by the root of the sample size ($\sqrt{n}$). The main cause in carryover could be attributed to surface area. The surface area of porous particles is significantly larger than that of fused-core particles and silica monoliths. Other parameters which may affect carryover is the size of the mesopores (stagnant zones where mass transfer is dominated by diffusion) and the quality of end capping (residual silanol groups). The polymer monolith exhibited the lowest percentage of carryover, mainly due to low surface area and biocompatible polymer base material and absence of mesopores. In addition, the poly(styrene-*co*-divinyl benzene) backbone provides approximately the same retention behavior as a C4 column, instead of C18. It should be noted that after the injection of 500 fmol tryptic peptides from BSA on the polymer monolithic column, which is 2.5-fold above the manufacturer loadability specification, carryover was still below 0.05% for all peptides in the second gradient blank.

4. Concluding remarks

Carryover effects resulting in the elution of peptides from previous injection(s) is an underestimated phenomenon in proteomic research, and may lead to false identification scores and wrong quantitation results. The analytical column represents the main source of carryover in the HPLC system, and carryover effects can still be observed after executing five gradient blanks. These effects could be related to the surface area and the presence (and size) of mesopores. Significant carryover effects were observed on a capillary column packed with porous 3 μm C18 particles, and the effects are reduced for a 2.7 μm fused-core C18 particulate capillary column and silica C18 monolithic capillary column. After the injection of 500 fmol tryptic peptides from BSA on the PS-DVB monolithic column, which is 2.5-fold above the manufacturer loadability specification, a carryover below 0.05% for all 11 tryptic peptides from BSA was observed in the second gradient blank. Column selection may depend on the application (impurity profiling or peptide mapping) and is a trade-off between column carryover effects but also efficiency of the column and corresponding peak height, which will influence the method sensitivity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.11.016.

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