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Optimization of the porous structure and polarity of polymethacrylate-based monolithic capillary columns for the LC-MS separation of enzymatic digests

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Abstract

The porous structure as well as the polarity of methacrylate ester based monolithic stationary phases have been optimized to achieve the separation of various peptide mixtures originating from enzymatic digests. The porous structure, determined by the size of both pores and microglobules, was varied through changes in the composition of porogenic solvents in the polymerization mixture, while the polarity was controlled through the incorporation of butyl, lauryl, or octadecyl methacrylate in the polymer backbone. Both the morphology and the chemistry of the monoliths had a significant effect on the retention and efficiency of the capillary columns. The best resolution of peptidic fragments obtained by digestion of cytochrome *c* with trypsin in solution was obtained in gradient LC-MS mode using a monolithic capillary column of poly(lauryl methacrylate-*co*-ethylene dimethacrylate) featuring small pores and small microglobules. Raising the temperature to 60°C enabled separations to be carried out at higher flow rates. Separations carried out at 60°C with a steeper gradient proceeded without loss of performance in half the time required for a comparable separation at room temperature. Our preparation technique affords monolithic columns with excellent column-to-column and run-to-run repeatability of retention times and pressure drops.

Keywords

Proteomics; Peptides; High-performance liquid chromatography; Column technology; Monolithic stationary phase; Temperature

1 Introduction

It has been speculated that an exact knowledge of the proteome together with the availability of well identified biomarkers would enable the monitoring of dynamic changes in living organisms and predict the onset of an illness even before its manifestation [1,2]. The most common top-down method used today for the characterization of proteins in complex samples typically comprises several steps. First, the intact proteins are separated by means of two-dimensional gel electrophoresis, a process introduced by O'Farrell more than 30 years ago [3]. The first dimension-isoelectric focusing-is based on differences in isoelectric point, followed by the second dimension - separation via polyacrylamide gel electrophoresis (PAGE) – involving differences in molecular size [4]. The separated proteins are then stained to

visualize them, the spots excised, destained, digested, desalted, and finally identified by mass spectrometry. Because this multistep method is labor intensive and sometimes difficult to reproduce, alternative techniques are being sought to both accelerate the process and enable the complete automation of the process.

One of the options available involves bottom-up proteomics with complete digestion of all proteins followed by chromatographic separation and mass-spectrometric characterization of the protein fragments. However, digestion of the complete proteome will lead to a very large number of peptides and their separation is one of the grand challenges of current analytical chemistry. The main advantage of chromatographic separation is its speed as well its ease of automation and its ability to be readily coupled to mass-spectrometric detection [5]. The quality of the chromatographic separation is directly proportional to the performance of the column. Therefore, the search for efficient columns has a high priority in the proteomic arena.

Porous polymer-based monolithic stationary phases were first introduced in liquid chromatography about 15 years ago and proved excellent for the rapid separation of large molecules [6–11]. Silica-based monoliths emerged a few years later and their performance was demonstrated with fast separations of small molecules [12–14]. Polymer-based monoliths are typically prepared *in-situ* from a liquid precursor [15,16], a feature that makes the monolithic stationary phases very attractive for their use in narrow bore capillaries and microfluidic devices. Both the chemistry and the porous properties of the monoliths, which have a direct effect on their chromatographic performance, are readily controlled by the composition of the polymerization mixture and conditions used for the *in situ* polymerization [17–20].

Although columns packed with microparticulate stationary phase remains the gold standard in peptide separations [21], excellent results have also been obtained using columns containing both C₁₈ silica and poly(styrene-co-divinylbenzene) monoliths [20,22–29]. In contrast, methacrylate-based monolithic columns only gave satisfactory results in the separation of proteins [30], but their use in the separation of peptides has met with limited success due to the weak retention and limited resolution of the columns [28,31].

This report describes the preparation of monolithic capillary columns from ethylene dimethacrylate and butyl, lauryl, or octadecyl methacrylate with various pore structures, and their testing in the separation of peptidic fragments obtained by digestion of cytochrome *c* with trypsin. The chromatographic performance of these new monolithic column stationary phases is then compared to that obtained with a commercial capillary column packed with ODS beads.

2 Experimental

2.1 Chemicals and materials

Butyl methacrylate (BuMA, 99%), lauryl methacrylate (LaMA, 99%), stearyl methacrylate (StMA, 65%), ethylene dimethacrylate (EDMA, 98%), 2,2'-azobisisobutyronitrile (98%, AIBN), 3-(trimethoxysilyl)propyl methacrylate (99%), methanol (99%), basic alumina, 1,4-butanediol, 1-propanol, tris(hydroxymethyl)aminomethane (tris), urea (98%), iodoacetamide, 1,4-dithio-DL-threitol (>95%), trypsin (sequencing-grade modified), cytochrome *c* from bovine heart, water (for LC-MS) and formic acid (for LC-MS) were purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (New Jersey, USA). All monomers (BuMA, LaMA, StMA, and EDMA) were purified by passing them over activated basic alumina. Afterwards, BuMA and EDMA were distilled under reduced pressure. All other reagents were used as received.

Polyimide-coated 375- μ m O.D. and 100- μ m I.D. fused-silica capillary tubing was obtained from Polymicro Technologies (Phoenix, AZ, USA). A 15-cm $\times$ 75- μ m I.D. fused-silica

capillary column packed with 3 μm , 100 Å C_{18} particles was purchased from Dionex (Sunnyvale, CA, USA).

2.2 Preparation of the monolithic columns

Monolithic columns were prepared in fused-silica capillaries by *in-situ* polymerization. Prior to the polymerization reaction, the inner wall of the fused-silica capillaries was vinylized following a previously published protocol [32] that enables covalent attachment of the monolith to the capillary wall. Typical polymerization mixtures comprised 24% monovinyl monomer (BuMA, LaMA, or StMA), 16% crosslinker (EDMA), 60% porogens (1,4-butanediol and 1-propanol in different ratios), and AIBN (free radical initiator, 1% with respect to monomers). After purging the mixture with nitrogen for 10 min, surface-modified capillaries were filled with the polymerization mixture and then sealed with a septum. The polymerization was carried out for 24 h in a water bath thermostated to 70°C. The monolith was then washed with methanol at a flow rate of 0.5 $\mu\text{L}/\text{min}$ for 30 min using a syringe pump.

The monolithic columns were attached to the HPLC system and conditioned with 80% aqueous acetonitrile at a flow rate of 0.5 $\mu\text{L}/\text{min}$ for 10 min and then in 0.05 vol% formic acid solution in water for another 10 min.

2.3 Protein digestion

To 100- μL cytochrome *c* solution containing 1 mg of protein in 6 mol/L urea was added 100 mmol/L tris buffer pH 7.8, 5 μL 200 mmol/L 1,4-dithio-DL-threitol in 100 mmol/L tris buffer (reducing reagent) and allowed to react for 1 h at room temperature. Alkylation of the liberated thiol functionalities was achieved by adding 20 μL 200 mmol/L iodoacetamide in 100 mmol/L tris buffer to the above mixture and maintaining it for 1 h. Then, 20 μL of dithiothreitol solution was added and allowed to react at room temperature for 1 h to quench unreacted iodoacetamide, followed by diluting the reaction mixture with 775 μL of water to decrease the urea concentration. Trypsin solution (100 μL) prepared by dissolving 20 μg of trypsin in 25 μL of ice-cold tris buffer and 75 μL of ice-cold water was added and the digestion carried out overnight at 37°C. Annotation of fragments found in the digest is shown in Table 1.

2.4 Instrumentation

Porosity measurements were carried out in both low- and high-pressure modes using Pascal 140 and Pascal 440 mercury-intrusion porosimeters (CE Instruments, Milan, Italy), respectively. Scanning electron micrographs were obtained using a S-4300 SE/N scanning electron microscope (Hitachi, Pleasanton, CA, USA).

2.5 Chromatography

HPLC experiments were carried out using a Dionex UltiMate 3000 instrument. Mass spectra were recorded using a quadrupole/time-of-flight mass spectrometer equipped with a nanoelectrospray interface (UltroTOFQ, Bruker Daltonics, Billerica, MA, USA). The capillary column was connected to the injector via 10- μm I.D. connection capillary and attached to a New Objective PicoTip emitter (FS360-20-10-D-20, Woburn, MA, USA). An emitter voltage of +3 kV was applied for detection in positive ion mode. High-temperature LC experiments were carried out using a home-made column oven constructed from copper tubing and a flexible electric heating tape (Thermolyne, Barnstead, Dubuque, IA, USA) connected to a Type 45500 stepless input controller (Barnstead, Dubuque, IA, USA). Typically, a volumetric flow rate of 500 nL/min was used for all separations while the steepness of the linear mobile phase gradient of acetonitrile in 0.05 vol% aqueous formic acid was adjusted to obtain a retention time of about 32 min for the late eluting peptide T12. The injected volume was 1 μL .

3 Results and discussion

3.1 Control of porous structure

Good control over the morphology of a chromatographic stationary phase is mandatory to prepare columns with good resolution, derived from column efficiency and retention. For packed columns, an increase in efficiency can be achieved by using particles of smaller size. Similarly, the efficiency of monolithic stationary phases depends on the domain size, represented by the combined average size of the macropores and microglobules. Previous studies have shown that the composition of the polymerization mixture and reaction conditions strongly affect the morphology of monolithic columns [18,33,34].

In this study we prepared methacrylate-based monolithic stationary phases for which the polarity, and therefore the retention properties, were tuned by incorporating BuMA (C₄), LaMA (C₁₂), and StMA (C₁₈) monomers in the polymer matrix. Furthermore, the percentages of 1,4-butanediol and 1-propanol in the porogenic solvent were systematically varied to affect the size of both pores and microglobules (domain size) in the monoliths. For example, decreasing the percentage of 1,4-butanediol in the polymerization mixture from 28 to 22% in the preparation of poly(butyl methacrylate-*co*-ethylene dimethacrylate) monoliths led to a sharp reduction in pore size at the maximum of the distribution curve from 4.5 μ m to 300 nm (see Table 2 for details). Scanning electron microscopy also confirmed that a significant decrease in the size of the pores and microglobules had been achieved.

Similar changes in the porous structure were also observed for poly(lauryl methacrylate-*co*-ethylene dimethacrylate) when the fraction of 1,4-butanediol was lowered from 22 to 16%. Figure 1 compares the morphologies of monoliths prepared with 17% and 16% of 1,4-butanediol in the polymerization mixture. Clearly, changing the percentage of 1,4-butanediol by a mere 1% can lead to a significant decrease in the size of both pores and microglobules. This decrease in pore size is of course accompanied by an increase in back pressure from 5.2 to 8.5 MPa as measured by the LC system. The SEM micrographs of the monoliths also confirm their perfect attachment to the walls of the capillaries.

In contrast, changing the 1,4-butanediol porogen content from 22% to 14% did not lead to any increase in back pressure in monolithic capillary columns prepared from polymerization mixtures containing stearyl methacrylate and ethylene dimethacrylate. The SEM micrographs of Figure 2 reveal only an insignificant decrease in the size of the microglobules. However, the microglobules create larger agglomerates, the morphology is less homogeneous and the pore size is slightly larger. The agglomeration results from the hydrophobic interactions of nuclei formed at the onset of phase separation. Polymer molecules that include monomer units with highly hydrophobic C₁₈ side chains tend to agglomerate in the relatively polar environment of the polymerization mixture, which contains a large proportion of porogenic alcohols.

3.2 Effect of polarity and morphology on separation

The tryptic digest of cytochrome *c* was used as a model peptide mixture to characterize the separation properties of the monolithic capillary columns differing in both morphology and polarity. In order to reduce external band broadening in the connection capillary between the injector and the column and to desalt the digest prior to MS detection, the peptides were first trapped at the top of the column from their solution in 0.05% aqueous solution of formic acid which was also used as the mobile phase at the start of the gradient. To allow a direct comparison of different columns, a volumetric flow rate of 500 nL/min was used for all separation while the steepness of the linear mobile phase gradient was adjusted to always obtain a retention time of about 32 min for the late eluting peptide T12.

First, we investigated the effect of the alkyl chain length of the monovinyl monomer on the chromatographic performance of the monolithic columns. We found that monolithic poly(butyl methacrylate-*co*-ethylene dimethacrylate) columns did not separate the hydrophilic peptides T4 and T14 from unidentified non-retained species. The performance of poly(stearyl methacrylate-*co*-ethylene dimethacrylate) monolithic column was also poor. Once again, peptides T4 and T14 were not resolved and broad peaks were observed most likely as a result of the large sizes of both pores and microglobules. Since the morphology and consequently the chromatographic performance of these columns could not be improved using a variety of mixtures of 1,4-butanediol and 1-propanol in the porogenic solvent, these columns were abandoned.

Although peptides T4 and T14 remained unresolved, the poly(lauryl methacrylate-*co*-ethylene dimethacrylate) columns exhibited very good retention and consequently afford better resolution. Thus, these monoliths appeared best suited for the analysis of the complex mixture containing smaller peptide fragments.

Figure 3 shows the effect of morphology of the monolithic poly(lauryl methacrylate-*co*-ethylene dimethacrylate) capillary columns on the separation of tryptic digest of cytochrome *c*. A monolithic column prepared from the polymerization mixture containing 16% 1,4-butanediol features large pores and therefore small resistance to flow. The separation shown in Figure 3A was obtained with this column; it is characterized by broad peaks and poor resolution of the different peptide fragments. In contrast, a significant improvement in performance is achieved with a monolithic column prepared from a polymerization mixture containing 14.5% 1,4-butanediol as shown in Figure 3B. This monolith consisting of a significantly larger number of microglobules has a smaller domain size. As a result, this monolith has a larger surface area that amplifies the retention. In addition, the smaller domain size minimizes the eddy dispersion (A term in Van Deemter equation) and improves mass transfer (C_m term). The cumulative effect is an increase in both column efficiency and resolution of peptides. However, this improvement is obtained at the expense of permeability. While the pressure drop observed at the start of the gradient for the former column was 8.5 MPa, it grew to 26.5 MPa for the latter although the column length was reduced from 30 to 15 cm. However, it should be noted that despite the shorter column length, the peak width in chromatogram 3B is narrower over a wide range of retention factors from 1.7 to 7.7.

For comparison, the total ion chromatogram of the cytochrome *c* digest was also recorded with a 15-cm long column packed with 3- μ m porous C_{18} silica particles (Figure 4). The elution patterns are similar for both the lauryl methacrylate monolithic column (Fig. 3B) and the packed column (Fig. 4). Only the elution order of fragments T7-8 and T8 is reversed. The performance of both columns is very similar with a slightly narrower peak width for the latter that, however, requires a pressure 3 MPa higher to achieve the same volumetric flow rate. This is likely due to the smaller size of interparticular voids in the packed column. Furthermore, the inherent porosity of the silica particles affords a larger surface area enabling more frequent interactions.

3.3 Effect of temperature

The chromatographic runs shown so far required times longer than 30 min. This length of run time is undesirable for high-throughput proteomic studies. The separations can be accelerated by using steeper gradient. For example, decreasing the gradient time from 46 to 20 min at the same flow rate of 500 nL/min shortens the analysis time from 32 to 19 min. However, fragments T8 and T7-8 were not completely resolved since the gradient volume was smaller in the latter separation. Using a flow rate more than 50% higher to achieve the same gradient volume is not an option since the back pressure would exceed the limit of the HPLC instrument used.

Other approach to accelerating the separation involves the use of higher temperatures. Figure 5 shows the separation of the cytochrome *c* digest achieved with the monolithic poly(lauryl methacrylate-*co*-ethylene dimethacrylate) capillary column operating at a temperature of 60° C. The higher column temperature leads to narrower peaks due to an improvement in the diffusion rate of the peptides that diminishes the mass-transfer contribution to band broadening. Furthermore, lower retention at higher temperature also decreases the overall analysis time. Since the mobile phase has a lower viscosity at higher temperature, a flow rate of 700 nL/min could be used without exceeding the 35 MPa limit tolerated by the HPLC instrument. As a result, the analysis time is reduced to 15 min, only half that required for the separation at the room temperature.

3.4 Stability and repeatability

A good run-to-run and column-to-column repeatability is the key for wide acceptance of chromatographic columns. We tested repeatability of chromatographic performance of the poly(lauryl methacrylate-*co*-ethylene dimethacrylate) monolithic columns in more detail. First, we determined the stability of the retention times and pressure drop during fifteen consecutive separations of tryptic cytochrome *c* digest using a single monolithic column. Table 3 shows very low variations characterized by RSD values of 0.5 and 0.2% for retention times of fragments T9-10 and T12-13, respectively. No significant changes in back pressure were observed.

The column-to-column repeatability determined from stability of retention times and pressure drop was demonstrated with three monolithic columns prepared from three different batches of the polymerization mixture. The average values of retention time and pressure drop summarized in Table 4 for three consecutive separations were calculated for each column. The RSD values were again very low amounting 0.6 and 0.8% for retention times of T9-10 and T12-13, respectively. The variation in the back pressure was also very low (RSD = 2%). These results confirm the good repeatability of the preparation of our monolithic columns.

4 Concluding remarks

This study demonstrates that a careful optimization of porous structure of monolithic poly(butyl methacrylate-*co*-ethylene dimethacrylate), poly(lauryl methacrylate-*co*-ethylene dimethacrylate) and poly(octadecyl methacrylate-*co*-ethylene dimethacrylate) stationary phases enables the preparation of capillary columns useful for the chromatographic separation of complex peptide mixtures. The use of three monovinyl methacrylate monomers differing in the length of their lateral alkyl chain helps to demonstrate the effect of polarity of the monolith on retention. Since the porous structure is not only a function of the composition of the porogenic solvent but also of the chemistry of the monomers used, a series of optimization experiments must be carried out to achieve the desired quality of separations of complex mixtures. Of the three types of monoliths studied here, poly(lauryl methacrylate-*co*-ethylene dimethacrylate) columns provided the best results with sufficient retention and resolution in the separation of a complex mixture of peptidic fragments. Since the polymer monoliths are stable in both acidic and basic pH solutions as well as at higher temperatures, they are attractive for separations involving the type of extreme conditions that are unsuitable for typical columns packed with bonded microparticulate silica. Although this study concerned only the separation of a tryptic digest of cytochrome *c*, it is likely that the optimized monolithic poly(lauryl methacrylate-*co*-ethylene dimethacrylate) capillary columns can be used for the separations of a broad range of digests or peptide mixtures and may find numerous application in the broad field of proteomic research.

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Abbreviations

AIBN	2,2'-azobisisobutyronitrile
BuMA	butyl methacrylate
EDMA	ethylene dimethacrylate
LaMA	lauryl methacrylate
StMA	stearyl methacrylate

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Figure 1.

SEM micrographs of the cross section of 100-μm I.D. poly(lauryl methacrylate-*co*-ethylene dimethacrylate) monolithic capillary columns prepared using different proportions of 1,4-butanediol and 1-propanol in porogenic solvent. Conditions: Polymerization mixture 24% LaMA, 16% EDMA, 60% porogenic solvents, AIBN (1% with respect to monomers); percentage of 1,4-butanediol in polymerization mixture 17 (A) and 16% (B), polymerization at 70°C for 24 h.

Figure 2.

SEM micrographs of the cross section of 100- μ m I.D. poly(stearyl methacrylate-*co*-ethylene dimethacrylate) monolithic capillary columns prepared using different proportions of 1,4-butanediol and 1-propanol in porogenic solvent. Conditions: Polymerization mixture 24% StMA, 16% EDMA, 60% porogenic solvents, AIBN (1% with respect to monomers), percentage of 1,4-butanediol in polymerization mixture: 22 (A), 20 (B), 14% (C), polymerization at 70°C for 24 h.

Figure 3.

Effect composition of porogenic solvent on the separation of tryptic digest of cytochrome *c* using monolithic poly(lauryl methacrylate-*co*-ethylene dimethacrylate) capillary columns. Conditions: Polymerization mixture: LaMA 24%, EDMA 16%, 1,4-butanediol + 1-propanol 60% (butanediol 16 (A) and 14.5% (B)), AIBN (1% with respect to monomers), polymerization at 70°C for 24 h; column length 30 (A) and 15 cm (B); flow rate 500 nL/min, gradient from 0 to 50% acetonitrile in aqueous 0.05% formic acid in 40 (A) and 46 min (B), column temperature 25°C, sample volume 1 μ L. For peak identification see Table 1.

Figure 4.

Total ion chromatogram of tryptic digest of cytochrome *c* obtained using commercial column packed with 3- μm C₁₈ silica particles. Column length 15 cm, gradient from 0 to 50% acetonitrile in aqueous 0.05% formic acid in 40 min, sample volume 1 μL , flow rate 500 nL/min, column temperature 25°C. For peak identification see Table 1. Peaks labeled with asterisk could not be identified.

Figure 5.

High-temperature separation of tryptic digest of cytochrome *c* using a poly(lauryl methacrylate-*co*-ethylene dimethacrylate) monolithic capillary column. Conditions: Polymerization mixture 24% LaMA, 16% EDMA, 14.5% 1,4-butanediol, 45.5% 1-propanol, and AIBN (1% with respect to monomers), polymerization at 70°C for 24 h; column length 15 cm, flow rate 700 nL/min, gradient from 0 to 50% acetonitrile in aqueous 0.05% formic acid in 20 min, column temperature 60°C.

Table 1

List of peptidic fragments separated from tryptic digest of cytochrome *c* using a poly(lauryl methacrylate-*co*-ethylene dimethacrylate) monolithic column and a commercial packed column and detected by mass spectrometer. For separation conditions see Fig. 3.

Retention time, min		m/z	Fragment no.	Sequence
monolithic	packed			
3.5–5.5	11.2	678.6 (1+)	T14	YIPGTK
3.5–5.5	12.2	634.6 (1+)	T4	IFVQK
12.4	16.5	529.1 (3+)	T9-10	KTGQAPGFSYTDANK
13.9	19.1	729.0 (2+)	T10	TGQAPGFSYTDANK
15.8	-	779.8 (1+)	T15	MIFAGIK
19.6	21.2	479.0 (3+)	T7-8	HKTGPNLHGLFGR
18.4	21.6	585.0 (2+)	T8	TGPNLHGLFGR
21.6	25.9	482.9 (2+)	T19	EDLIAYLK
26.4	27.3	545.4 (3+)	T4-5	IFVQKCAQCHTVEK
30.4	29.5	713.9 (3+)	T12-13	GITWGEETLMEYLENPKK
31.7	32.1	671.2 (3+)	T12	GITWGEETLMEYLENPK

Table 2

Porous properties of poly(butyl methacrylate-co-ethylene dimethacrylate) monoliths. Polymerization conditions: BuMA 24%, EDMA 16%, porogenic solvents 60%, AIBN (1% with respect to monomers), 70°C, 24 h.

Composition of porogen, %		D_p , mode ^a nm	D_p , mean ^b nm	S_g ^c m ² /g
1,4-butanediol	1-propanol			
22	38	312	343	67
24	36	718	850	54
26	34	1969	2726	47
28	32	3772	4808	42
30	30	4301	3969	52

^a Pore size at the peak of distribution profile;

^b Mean pore size;

^c Specific surface area.

Table 3

Repeatability of retention times of peptides T9-10 and T12-13 and pressure drop of a single poly(lauryl methacrylate-*co*-ethylene dimethacrylate) monolithic capillary column. Polymerization conditions: 24% LaMA, 16% EDMA, 15% 1,4-butanediol, 45% 1-propanol, AIBN (1% with respect to monomers), 70°C, 24 h. Chromatographic conditions: linear gradient from 0 to 50% acetonitrile in aqueous 0.05% formic acid solution in 30 min, flow rate 500 nL/min.

	$t_{R,1}^a$, min	$t_{R,2}^a$, min	P^b , MPa
Mean (n = 15)	11.95	23.76	20.07
RSD (%)	0.5	0.2	1.1
Confidence interval (95%)	11.92–11.98	23.73–23.78	19.95–20.18

^a Retention time of peptides T9-10 and T12-13, respectively;

^b Pressure drop.

Table 4

Column-to-column repeatability of retention times of peptides T9-10 and T12-13, respectively and pressure drop for poly(lauryl methacrylate-*co*-ethylene dimethacrylate) monolithic capillary columns prepared from three batches of the same polymerization mixture. For polymerization and LC conditions see Table 2.

	$t_{R,1}^a$, min	$t_{R,2}^a$, min	P^b , MPa
Mean (n = 3)	11.91	23.56	20.00
RSD (%)	0.6	0.8	2.0
Confidence interval (95%)	11.81–11.97	23.35–23.76	19.54–20.46

^a Retention time of peptides T9-10 and T12-13, respectively;

^b Pressure drop.