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Rapid reversed-phase liquid chromatography separation of cyclolinopeptides with monolithic and microparticulate columns

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ABSTRACT

Three monolithic C_{18}-bonded silica gel columns i.e. Chromolith® SpeedROD (CSR), Chromolith® Performance (CP), and Chromolith® High Resolution (CHR), MerckKGaA Darmstadt, Germany and two particle-based columns i.e. ZORBAX Eclipse XDB-C_{18} (ZEX), Agilent and POROS R1/20 (POR), Applied Biosystems were compared for their performance in separating a mixture of flaxseed cyclolinopeptides (CLs). Gradient mobile phases of acetonitrile and water were optimized for each column. The performance of CHR column in profiling CL standards, measured as the resolution of individual CL, selectivity, and peak asymmetry exceeded the performance of traditional particle-packed columns and the other monolithic columns. The profiling of CLs in aqueous methanolic flaxseed extract was optimized for high-throughput analysis. A total analysis time of 1.5 min at a flow rate of 3.0 mL min^{-1} was achieved on a CSR column. Injection of over 2000 methanol extracts of flaxseed on a CSR column had no impact on backpressure or resolution of a standard CL mixture.

1. Introduction

Flax (*Linum usitatissimum*) seeds contain natural hydrophobic cyclic peptides (cyclolinopeptides/CLs), comprising eight or nine amino acid residues with molecular weights of approximately 1 kDa [1]. The discovery of CLs was described by Kaufmann and Tobschirbel [2] who isolated CLA (**1**, Table 1) from precipitated "slime" obtained as a byproduct in the processing of crude flaxseed oil. Since then, other CLs (**2**–**9**) have been reported in flaxseed and roots extracts [3–6].

Although their role in plants is unknown, CLs are biologically active. In in vitro studies, **1** inhibits activation and proliferation of T-lymphocyte cells [7], protects liver from specific poisonous agents [8], and suppresses immunity [7,9]. Additionally, CLs **2**, **5**, and **7** exhibit immunosuppressive activities [1,5]. These peptides have potential applications as therapeutic agents for suppressing haemolytic anemia and post adjuvant arthritis, postponing skin allograph rejection, and delaying hypersensitivity response [10,11].

Flax oil is readily oxidized due to its high content of polyunsaturated fatty acids [12]. Similarly, methionine-containing CLs such as **7** are known to oxidize as flaxseed oil ages [12]. Methionine (Met) can be oxidized to methionine sulfoxide (Mso) and further to methionine sulfone (Msn) via chemical and/or biological means [14–16]. Exposure to oxygen and heat are considered to be the major contributing causes of methionine oxidation. This makes isolation and purification of methionine-containing peptides vital because the reversible methionine oxidation and reduction is a well-established molecular mechanism for cellular regulation [13].

Separation and identification of CL from flaxseed oil has been achieved through conventional reverse phase-HPLC using particle packed columns [250 mm × 4 mm, 5 μm, LiChrospher 100 RP-18 column (MerckKGaA) and ZEX (Agilent)] that employ lengthy methods which are not suitable or convenient for high-throughput analysis [12,17]. Shorter elution times are possible by reducing column length and/or increasing the flow rate [18]. Shorter columns however have lower resolution while higher flow rate increases backpressure and potentially lowers column resolution. Use of a shorter column with small particle size (<3 μM) operating at high backpressure is one approach that may yield rapid separation [19].

Attempts to improve chromatographic stationary phases led to the introduction of monolithic macroporous columns in the early 1990s to allow for high-speed separation of analytes without compromising column efficiency [20]. These chromatographic polymer-based media possess different structures from conventional silica packed columns. Monolithic columns contain solid porous rod comprising interconnected skeletons and flow paths that make up the pores [21]. They are characterized by mesopores and macropores that offer significantly greater porosity, greater permeability and higher surface area compared to conventional columns packed with spherical beads [18]. The small

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Table 1
Amino acid sequences of flaxseed cyclolinopeptides.

CL	Amino acid sequence	Molecular weight	Number
CLA	*cyclo*-(Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile)	1040.34	1
1-Met-CLB	*cyclo*-(Met-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile)	1058.38	2
1-Abu-CLB	*cyclo*-(Abu-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile)	1012.29	3
1-Mso-CLB	*cyclo*-(Mso-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile)	1074.38	4
1-Msn-CLB	*cyclo*-(Msn-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile)	1090.38	5
1-Mso-CLD	*cyclo*-(Mso-Leu-Leu-Pro-Phe-Phe-Trp-Ile)	1064.34	6
1-Mso-CLE	*cyclo*-(Mso-Leu-Val-Phe-Pro-Leu-Phe-Ile)	977.26	7
1-Mso, 3-Mso-CLF	*cyclo*-(Mso-Leu-Mso-Pro-Phe-Phe-Trp-Val)	1084.35	8
1-Mso, 3-Mso-CLG	*cyclo*-(Mso-Leu-Mso-Pro-Phe-Phe-Trp-Ile)	1098.38	9

skeleton and large through-pores of monolithic columns results in improved flow-independent mass transfer properties and separation efficiency when compared to particle-based columns. Hence, monolithic columns possess low hydraulic resistance [22] and can withstand high flow rates, a condition usually associated with high back pressure problems. Monolithic columns are composed of synthetic organic material (acrylate, polystyrene, acrylamide), inorganic material (silica), or natural polymers (cellulose) [18,21] that can be cast in the forms of disks, rods, or tubes. Monolithic columns are commercially available through a number of manufacturers [22] and are used for fast separation of biological macromolecules, smaller biomolecules, preparative isolation, and separation of diastereomers [18].

Perfusion columns that contain a special matrix geometry that has very large (400–800 nm) pores connected to a network of smaller (30–100 nm) pores [23] have been developed to meet the need for rapid separations. A combination of diffusion and flow of the mobile phase through the pores carries solutes into and out of the perfusion matrix. This unique flow pattern is advantageous in aiding the stationary-phase mass transfer and reducing band broadening especially for large molecules such as proteins even at higher flow rates.

The primary objective of this work was to develop an HPLC method for high-throughput screening of CLs in flaxseed varieties. To achieve this, the resolution, selectivity, capacity factor and symmetry of five commercial chromatographic columns were evaluated. A mixture of four CL standards (**2**–**5**) was used for all chromatographic investigations (Table 1). Column longevity of CSR was determined while screening flaxseed varieties to determine CL content.

2. Experimental

2.1. Reagents and samples

HPLC-grade acetonitrile (CH_3CN) and methanol (MeOH) were purchased from Fischer Scientific (Fair Lawn, NJ, USA). Water (H_2O) was purified to 18.2 MΩ cm on a Milli-Q Integral system (Millipore, Molsheim, France). A standard of **2** was obtained by solvent partitioning of flaxseed oil using 95% aq. ethanol (1:1, v/v). The organic layer was concentrated and was subjected to flash column chromatography (FCC) on silica gel 60 (40–63 μm particle size, EMD Chemicals). Sequential elution was performed using (a) ethyl acetate (EtOAc, 25%, v/v) in *n*-hexane; (b) EtOAc (50%, v/v) in *n*-hexane; (c) EtOAc (75%, v/v) in *n*-hexane; (d) EtOAc (100%); (e) MeOH (5%, v/v) in dichloromethane (CH_2Cl_2); (f) MeOH (7.5%, v/v) in CH_2Cl_2; and (g) MeOH (10%, v/v) in CH_2Cl_2. A crude mixture comprising cyclolinopeptides **1** and **2** (fraction d) was extracted with diethyl ether, the combined extract was filtered, and was subsequently concentrated using a rotary evaporator. The resulting crude extract was dissolved in MeOH (1:8, w/v) and was filtered. Compound **2** was purified from the mixture on a HPLC system (BioCAD® Sprint) using a reverse phase preparative column (GL Sciences Inc. Inertsil Prep – ODS, 10 μm particle size silica gel, 250 mm × 30 mm I.D). The mobile phase consisted of H_2O–CH_3CN (45:55 for 9 min, to 10:90 in 30 min, and to 45:55 in 1 min). Standards of **4** and **5** were extracted from cyclolinopeptide-laden silica gel [17]. Firstly, the cyclolinopeptide-containing silica gel was extracted with *n*-hexanes (1:2, w/v) with periodic stirring (30 min) to remove residual oil. The resulting defatted silica was eluted with (a) EtOAc (50%, v/v) in *n*-hexanes and (b) MeOH (10%, v/v) in CH_2Cl_2. Fraction b was concentrated and the resulting residue was taken in EtOAc (500 mL) and was washed with a saturated solution of sodium bicarbonate (500 mL) and brine. The organic phase was dried, filtered, and subjected to FCC on silica gel 60 eluting with (a) EtOAc (80%, v/v) in *n*-hexane; (b) EtOAc (100%, v/v); (c) MeOH (2.5%, v/v) in CH_2Cl_2; (d) MeOH (5%, v/v) in CH_2Cl_2. Fraction d contained a mixture of **4** and **5**, and was subsequently purified using preparative HPLC as described above.

Compound **3** was prepared by reductive elimination of sulfur from the methionyl residue using Raney Nickel (16 h at 240 °C) and was purified using an Agilent 1200 series HPLC system. The mobile phase consisted of H_2O–CH_3CN (39:61 for 3 min, to 10:90 in 0.5 min, to 39:61 in 0.5 min, and to 39:61 in 1 min) at a flow rate of 5.0 mL min^{-1}. A Chromolith® Semiprep (100 mm × 10 mm I.D, Merck KGaA Darmstadt) column was used in this purification. The standards were subjected to HPLC, High Resolution HPLC–MS and HPLC MS/MS analyses (Fig. 1). Flaxseed extract was obtained by 80% aq. MeOH (1:8, w/v) extraction of ground (degummed) flaxseed at 60 °C for 2 h, into which an internal standard, **3** was added. The extract was oxidized with hydrogen peroxide to convert all Met residues to similar chemical forms, that is, Mso. The reaction was quenched with sodium thiosulfate to prevent over oxidation of Met to Msn.

2.2. HPLC apparatus

All chromatographic separations were performed on Agilent 1200 series HPLC system (Agilent Technologies Canada, Mississauga, ON) equipped with a quaternary pump, autosampler, photodiode-array detector (wavelength range 190–300 nm), and a degasser. The column compartment temperature was 23 °C, unless otherwise stated. Eluting peaks were detected at wavelengths of 214 nm with a 10 nm bandwidth against a reference signal at 300 nm with a 10 nm bandwidth using Chemstation LC 3D^{TM} system software (Agilent Technologies Canada Inc., Mississauga, ON). Injection volume was maintained at 10 μL for all investigations. The mobile phase consisted of a gradient of water-acetonitrile as detailed in Section 3. A flow rate of 2.0 mL min^{-1} was used unless otherwise stated. The HR-HPLC–MS and HPLC–MS/MS were performed on an Agilent HPLC 1200 series directly connected to a Bruker microTOF-Q II Mass Spectrometer (Hybrid Quadrupole-TOF MS/MS, Bruker Daltonik GmbH, Bremen, Germany) with Apollo II ESI ion source. A Chromolith FastGradient RP-18e column (3 μm particle size silica, 50 mm × 2.0 mm I.D, Merck KGaA, Darmstadt, Germany) was used to separate compounds prior to MS analysis.

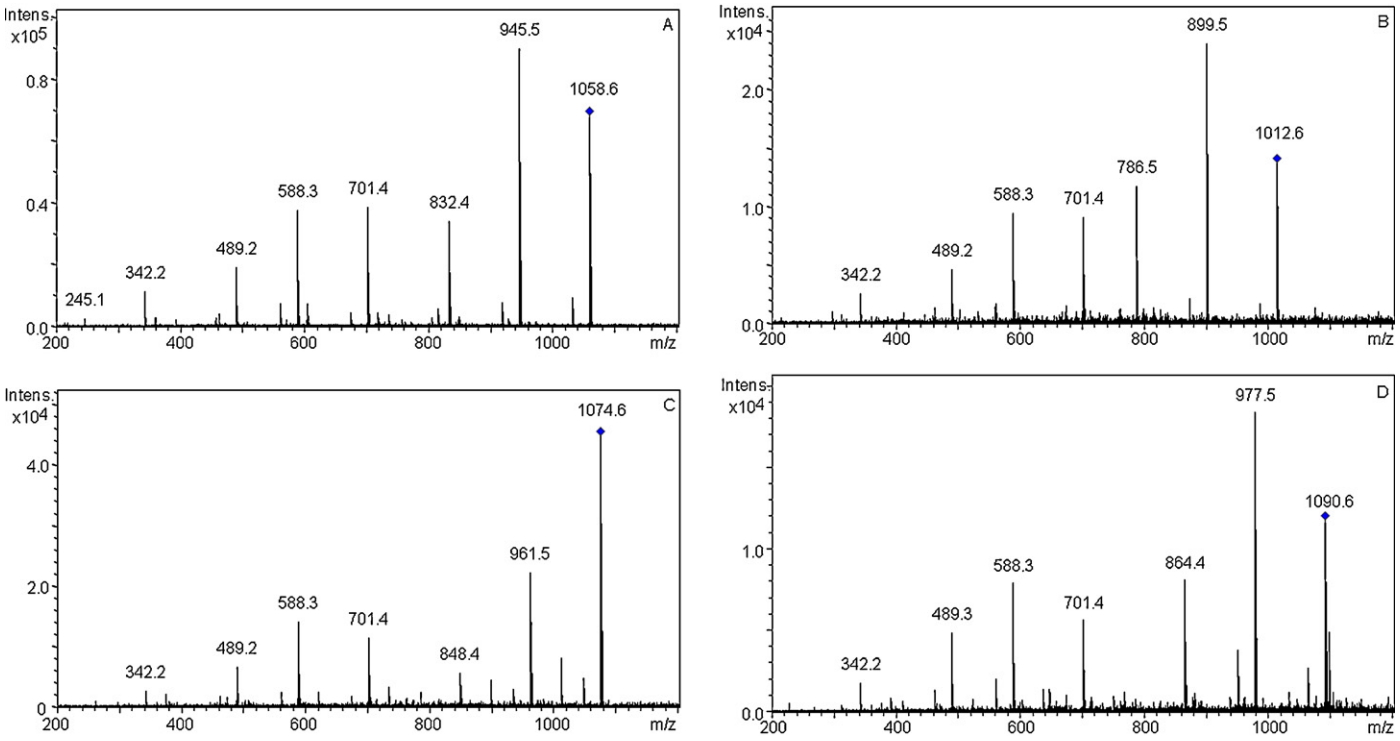

Fig. 1. MS/MS spectra of standards: (A) 1-Met-CLB (**2**), (B) 1-Abu-CLB (**3**), (C) 1-Mso-CLB (**4**), and (D) 1-Msn-CLB (**5**). Samples were introduced to the ESI source through an HPLC column.

The mobile phase consisted of a linear gradient of 0.1% formic acid in H_2O and 0.1% formic acid in CH_3CN (60:40 for 2 min, to 10:90 in 8 min, to 60:40 in 0.5 min, to equilibration for 5.5 min) at a flow rate of 0.40 mL min^{-1}.

2.3. Stationary phase

Three monolithic C_{18}-bonded silica rod columns (CSR, 50 mm × 4.6 mm I.D; CP, 100 mm × 4.6 mm I.D; CHR, 100 mm × 4.6 mm I.D) and a particle-packed column (POR, 100 mm × 4.6 mm I.D) were tested for their utility in separating a CL mixture. For reference purposes, a particle-packed column (ZEX, 150 mm × 4.6 mm I.D) was also employed. The main characteristics of the columns studied are provided in Table 2.

2.4. Sample preparation

Standards of **2**–**5** were each weighed (MSA225S000DU, Sartorius, Germany) and dissolved in methanol (5.0 mL) in separate volumetric flasks to make stock solutions of 0.4 mg mL^{-1}. The flasks were subsequently stoppered and sealed with Parafilm™ (Pechiney Plastic Packaging, Chicago, IL) to limit solvent evaporation. A mixture of CL was prepared by combining 300 μL of each CL stock solution giving a total CL concentration of 0.4 mg mL^{-1} with each CL being 0.1 mg mL^{-1}.

2.5. Calculations of chromatographic parameters

Chemstation LC 3D™ system software (Agilent Technologies Canada Inc., Mississauga, ON) was used to calculate peak parameters.

$$R = \frac{1.18(t_R1 - t_R2)}{W_{1/2}1 + W_{1/2}2} \quad (1)$$

$$k' = \frac{t_R - t_0}{t_0} \quad (2)$$

$$\alpha = \frac{t_R2 - t_0}{t_R1 - t_0} \quad (3)$$

where t_R is the retention time, $W_{1/2}$ is peak width at half height, and t_0 is column void volume. Eqs. (1)–(3) were used to calculate peak resolution, capacity factor, and selectivity, respectively.

Table 2
Characteristics of the chromatographic columns compared in this study.

	Chromolith® SpeedROD	Chromolith® Performance	Chromolith® High Resolution	ZORBAX Eclipse XDB-C_{18}™	POROS R1/20
Macropore size (μm)	2.0	2.0	1.15	-	-
Mesopore size (nm)	13	13	15	8	50–1000
Particle size (μm)	–	–	–	5.0	20
Specific surface area (m^2/g)	300	300	250	180	
Material	Silica based monolithic skeleton	Silica based monolithic skeleton	Silica based monolithic skeleton	Silica particles	Poly(styrene-divinylbenzene) particles
Manufacturer	Merck KGaA	Merck KGaA	Merck KGaA	Agilent	Applied Biosystems

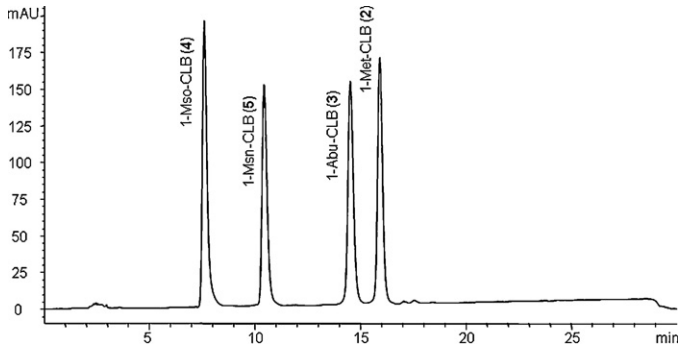

Fig. 2. Chromatogram of a mixture of CLs and internal standard 1-Abu-CLB (**3**) on the ZEX column at a flow rate of 0.5 mL min^{-1}. Solvent program is as listed in Table 3.

3. Results and discussion

3.1. Study of cyclolinopeptide standards mixture using monolithic and microparticulate columns

Compounds **2**–**5** were chosen because they differ by only the oxidation state and/or presence of the Met residue. Moreover, 1-Met-CLB (**2**) and 1-Msn-CLB (**5**) crystallize readily and, based on NMR observation, only occur in a *cis*-trans prolyl configuration [24,25]. Chromatographic separation of the CL mixture was performed on a commercial particle-packed ZEX column using water and acetonitrile gradients as summarized in Table 3. Within the 30 min analysis, the peptides were effectively separated with **4**, **5**, **3**, and **2** having retention times of 7.6, 10.4, 14.5, and 15.9 min, respectively (Fig. 2).

Rapid HPLC analysis is commonly accomplished by increasing the mobile phase flow rate. Since analysis time is inversely related to flow rate, rapid equilibration between analytes and the stationary phase allows the reduction of the analysis time, almost by half, when the flow rate is doubled. However, flow rate is also proportional to the increase in column backpressure. In an attempt to enable faster HPLC analysis time without compromising the pressure drop across the column, we employed a silica monolithic column, the CSR (Table 2), to separate a mixture of CL. The gradient elution was optimized at a flow rate of 2.0 mL min^{-1} (Table 3). Under these chromatographic conditions, the greater porosity of CSR enabled an increase in flow rate from 0.5 mL min^{-1} on ZEX column to 2.0 mL min^{-1} without any significant changes in backpressure. Moreover, the column length is proportional to analyte t_R, thus reduced column length results in the reduction of analysis time from 30 to 6 min. Compounds **4**, **5**, **3**, and **2** eluted at 2.79, 3.23, 3.74, and 3.95 min, respectively, from the CSR column (Fig. 3A). It was observed that changes in flow rate and gradient elution lead to a decline in the selectivity and more prominently, resolution of each CL (Table 4). On the other hand, capacity factor of individual CLs significantly improved when the CSR column was compared to the ZEX column. In spite of the drawbacks mentioned above, CSR column can still be considered desirable as it effectively resolved components of the CL mixture in shorter run times, which is required for high throughput analysis.

The performance of the CSR, CP and CHR and a particle-packed column, POR were subsequently compared. CP has double the column length (100 mm) of the CSR (50 mm). When applying an identical gradient elution previously used on CSR, the CP showed 1.4 times increase in resolution with no significant differences in selectivity (Table 5). As expected, t_R of **4**, **5**, **3**, and **2** increased to 3.46, 3.95, 4.53, and 4.74 min, respectively. The retention time increase was accompanied by a reduction in band broadening (Fig. 3B). On the other hand, CHR provided improvements in both speed and resolution with respect to the other two monolith columns. With the same gradient, resolution of individual CL was

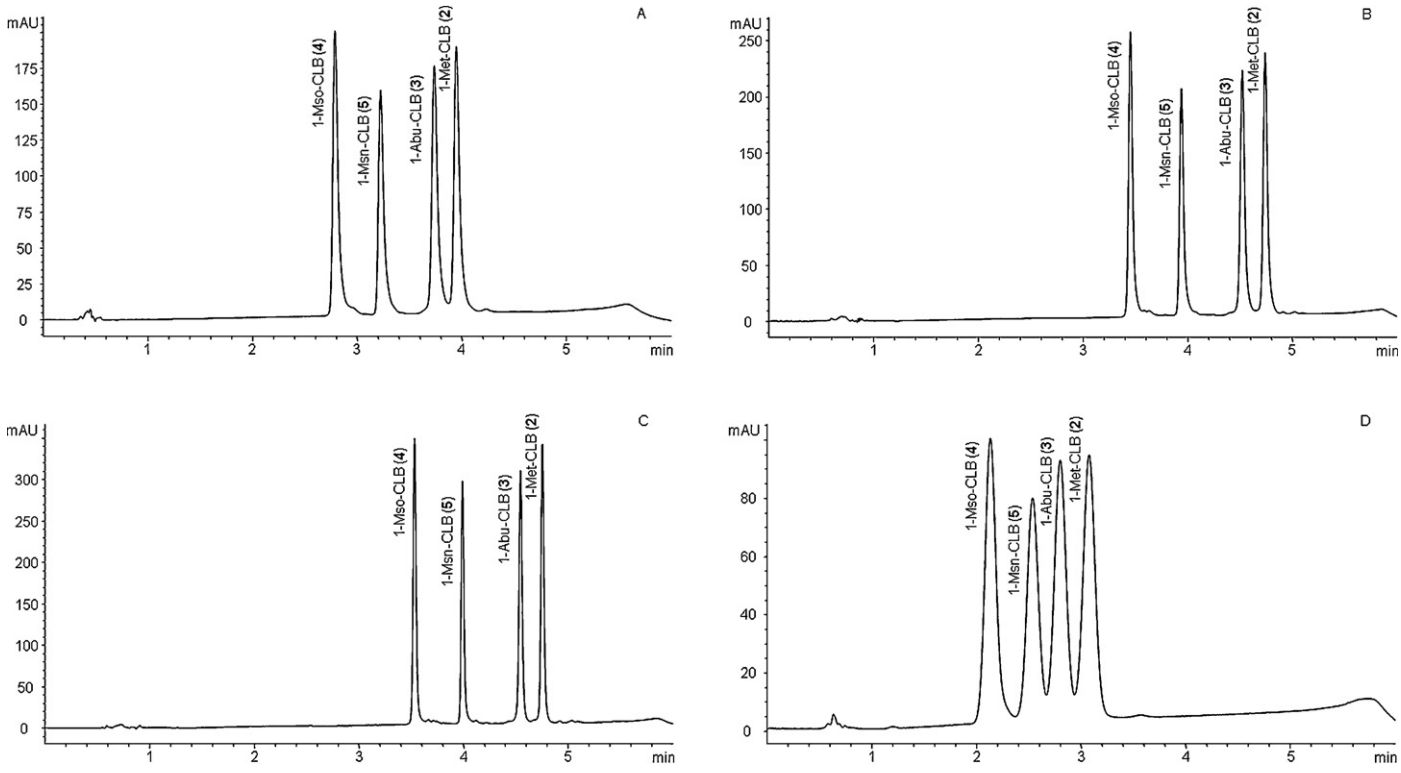

Fig. 3. Chromatograms of a mixture of CLs and 1-Abu-CLB (**3**) obtained using different columns: (A) Chromolith® SpeedROD, (B) Chromolith® Performance, (C) Chromolith® High Resolution, and (D) POROS. Gradient elution is as listed under SpeedROD in Table 3 at a flow rate of 2.0 mL min^{-1} for all chromatographic separations.

Table 3
Optimized solvent gradients for the chromatography columns using water (solvent A) and acetonitrile (solvent B).

ZORBAX Eclipse XDB-C_{18}™		Chromolith® SpeedROD		Chromolith® Performance		Chromolith® High Resolution		POROS R1/20	
Time (min)	Solvent B (%)	Time (min)	Solvent B (%)	Time (min)	Solvent B (%)	Time (min)	Solvent B (%)	Time (min)	Solvent B (%)
0	55	0	30	0	60	0	60	0	35
6.0	55	4.0	70	3.0	90	3.0	90	5.0	45
24.0	90	4.5	90	3.25	60	3.25	60	5.5	90
25.0	55	5.0	30	4.0	60	4.0	60	6.0	35
30.0	55	6.0	30					7.0	35

Table 4
Chromatographic data of CL on different stationary phases under optimized elution gradients.

Analyte	R	α	t_R (min)	RSD (%) t_R	k'	$W_{1/2}$	Symmetry	Stationary phase
1-Mso-CLB (**4**)			2.03	0.17	2.04	0.24	0.60	
1-Msn-CLB (**5**)	7.2	1.56	10.42	0.10	3.17	0.23	0.64	ZORBAX Eclipse
1-Abu-CLB (**3**)	9.96	1.52	14.51	0.14	4.81	0.26	0.80	XDB-C_{18}™
1-Met-CLB (**2**)	3.33	1.12	15.89	0.17	5.36	0.25	0.78	
1-Mso-CLB (**4**)			2.79	0.02	5.20	0.06	0.57	
1-Msn-CLB (**5**)	4.09	1.19	3.23	0.03	6.17	0.06	0.52	Chromolith®
1-Abu-CLB (**3**)	4.68	1.18	3.74	0.07	7.31	0.07	0.75	SpeedRod
1-Met-CLB (**2**)	1.77	1.06	3.95	0.07	7.78	0.07	0.61	
1-Mso-CLB (**4**)			1.05	0.04	0.52	0.03	0.63	
1-Msn-CLB (**5**)	3.55	1.64	1.28	0.04	0.85	0.04	0.68	Chromolith®
1-Abu-CLB (**3**)	5.96	1.71	1.70	0.07	1.46	0.04	0.79	Performance
1-Met-CLB (**2**)	2.13	1.15	1.85	0.07	1.67	0.04	0.78	
1-Mso-CLB (**4**)			1.06	0.15	1.06	0.03	0.63	
1-Msn-CLB (**5**)	4.52	1.68	1.29	0.16	1.29	0.03	0.77	Chromolith®
1-Abu-CLB (**3**)	8.06	1.72	1.70	0.14	1.70	0.03	0.83	High Resolution
1-Met-CLB (**2**)	2.95	1.15	1.85	0.12	1.85	0.03	0.84	
1-Mso-CLB (**4**)			3.41	0.47	1.71	0.21	0.82	
1-Msn-CLB (**5**)	2.5	1.84	3.41	0.53	4.33	0.23	1.02	POROS R1/20
1-Abu-CLB (**3**)	1.95	1.37	2.91	0.45	3.54	0.23	0.96	
1-Met-CLB (**2**)	2.41	1.36	4.39	0.22	5.86	0.25	1.00	

$n = 3$. R, peak resolution; α, selectivity; t_R, retention time; RSD (repeatability), relative standard deviation; k', capacity factor; $W_{1/2}$, peak width at half height.

1.5–2 fold higher than that observed with the CSR. No significant changes in selectivity and retention times were noted (Fig. 3C).

When the same gradient elution was applied to the perfusion column (POR), both resolution and capacity factor decreased by half with respect to the CSR (Table 5). Band broadening (2-fold) was also observed across all CL peaks (Fig. 3D). Consequently, individual CL from the mixture produced peaks without baseline resolution between the peaks. Surprisingly, the retention times of individual CL on the perfusion column were significantly shorter than observed on monolith columns. This suggests that, although both columns have the same dimension, the perfusion column contains a greater porosity than the monolith columns.

In order to develop a rapid screening method for flaxseed extracts, an improved HPLC gradient was developed for each column. These changes were then optimized for the CP, CHR, and POR (Table 3). Optimization focused on reduction of runtimes while maintaining or improving chromatographic resolution. The analysis time for CP and CHR were reduced from 6 min to 4 min from the initial CSR gradient. As shown in Fig. 4, each CL eluted at similar times on both Chromolith® columns. Resolution of **3** on CHR

Table 5
Chromatographic data of CL on different stationary phases under elution gradient of that of Chromolith® SpeedROD.

Analyte	R	α	t_R (min)	RSD (%) t_R	k'	$W_{1/2}$	Symmetry	Stationary phase
1-Mso-CLB (**4**)			2.79	0.02	5.20	0.06	0.57	
1-Msn-CLB (**5**)	4.09	1.19	3.23	0.03	6.17	0.06	0.52	Chromolith®
1-Abu-CLB (**3**)	4.68	1.18	3.74	0.07	7.31	0.07	0.75	SpeedRod
1-Met-CLB (**2**)	1.77	1.06	3.95	0.07	7.78	0.07	0.61	
1-Mso-CLB (**4**)			3.46	0.17	4.01	0.05	0.82	
1-Msn-CLB (**5**)	5.87	1.17	3.95	0.15	4.70	0.05	0.65	Chromolith®
1-Abu-CLB (**3**)	6.63	1.18	4.53	0.13	5.54	0.05	0.92	Performance
1-Met-CLB (**2**)	2.48	1.06	4.74	0.11	5.86	0.05	0.75	
1-Mso-CLB (**4**)			3.53	0.06	3.58	0.03	0.90	
1-Msn-CLB (**5**)	8.68	1.16	4.00	0.09	4.42	0.03	0.76	Chromolith®
1-Abu-CLB (**3**)	9.44	1.17	4.55	0.15	4.99	0.04	1.13	High Resolution
1-Met-CLB (**2**)	3.56	1.05	4.76	0.18	5.18	0.03	0.88	
1-Mso-CLB (**4**)			2.14	0.18	2.33	0.12	0.87	
1-Msn-CLB (**5**)	2.00	1.28	2.80	0.02	2.97	0.13	0.98	POROS R1/20
1-Abu-CLB (**3**)	1.25	1.14	2.62	0.11	3.37	0.12	0.97	
1-Met-CLB (**2**)	1.26	1.13	3.08	0.10	3.80	0.13	0.97	

$n = 3$. R, peak resolution; α, selectivity; t_R, retention time; RSD (repeatability), relative standard deviation; k', capacity factor; $W_{1/2}$, peak width at half height.

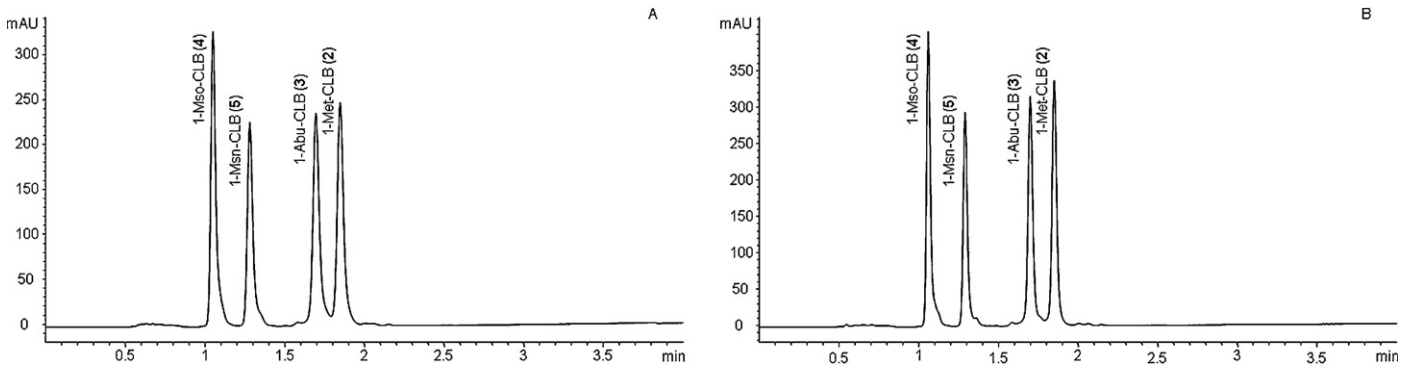

Fig. 4. Chromatograms of a mixture of CLs and 1-Abu-CLB (**3**) obtained from different columns: (A) Chromolith® Performance and (B) Chromolith® High Resolution. Optimized gradient elution is as listed under Performance/High Resolution in Table 3 at flow rate of 2.0 mL min^{-1} for all chromatographic separations.

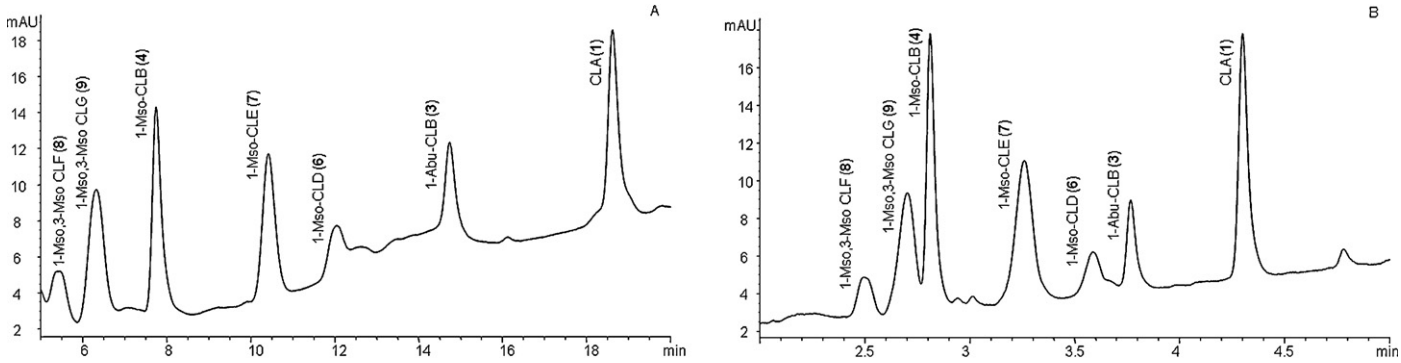

Fig. 5. Chromatograms of methanol extract of flax seed showing mixture of 1-Mso, 3-Mso-CLF (**8**), 1-Mso, 3-Mso-CLG (**9**), 1-Mso-CLB (**4**), 1-Mso-CLE (**7**), 1-Mso-CLD (**6**), and CLA (**1**) with internal standard, 1-Abu-CLB (**3**) obtained using different columns: (A) ZORBAX Eclipse XDB-C_{18} and (B) Chromolith® SpeedROD. Gradient elution is as listed in Table 3 at a flow rate of 2.0 mL min^{-1}.

increased 1.7-fold, while peak width of all CLs declined 2-fold in comparison to the CSR column (Table 4). Meanwhile, selectivity of individual peptides increased 1.5-fold for CP and CHR compared to CSR.

To meet the goal of reducing the run times on CSR and optimization on POR column, the gradient elution developed for CP and CHR (Table 3) was applied. These chromatographic conditions led to significant loss of resolution (data not shown) on the CSR column. On the other hand, a single broad fused peak visible on the HPLC-DAD chromatogram using the POR column signified that this elution gradient was not useful i.e. no separation of CL mixtures was achieved (data not shown). Further modification of this solvent gradient by decreasing the organic solvent concentration coupled with the maintenance of 2.0 mL min^{-1} flow rate led to separation of CLs in a 7 min run using the POR column, albeit with poor resolution (data not shown). These observations suggested that gradient elution on POR column is not the best option for rapid HPLC analysis of CL mixtures.

3.2. Study of aqueous methanolic flax seed extract using monolithic and microparticulate columns

The optimized method for each of the five columns (Table 3) was tested for applicability in profiling of CLs occurring in aqueous methanolic extracts of flaxseed varieties. Such extracts comprise CLs **1**, **4**, and **6**–**9**. From these analyses, we observed that ZEX and CSR columns could separate the crude peptide mixture to relatively well-resolved peaks under 30 and 6 min, respectively (Fig. 5A and B). On the other hand, **8** and **9** were not retained and eluted at void volume in CP, CHR and POR, indicating the poor suitability of the gradients for this application (data not shown).

We, however, attempted further modifications of gradient elution for CSR and CHR to suit their applicability in rapid screening of flaxseed CLs in crude extracts. As a result, the total HPLC analysis time on CHR from 4.0 to 2.5 min was achieved with improved resolution of all peptide peaks (data not shown). The CSR, with half of the column length of the CHR at a flow rate of 3.0 mL min^{-1} and 28 °C achieved similar resolution in 1.5 min (Fig. 6), making it the better choice in rapid screening of flaxseed CLs.

3.3. Longevity of Chromolith® SpeedROD column

The longevity of CSR column was studied in a high-throughput screening of aqueous methanolic CL extracts of flax varieties. As

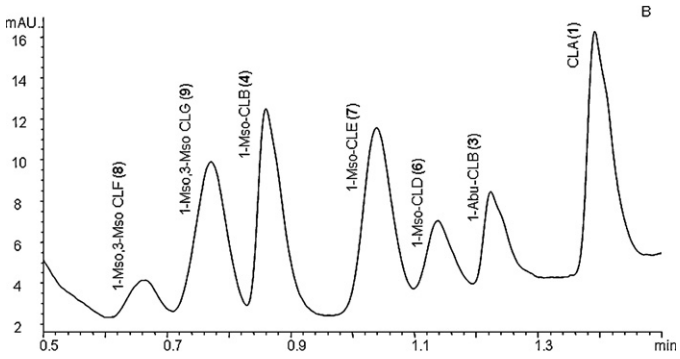

Fig. 6. Chromatograms of methanol extract of flax seed showing mixture of 1-Mso, 3-Mso-CLF (**8**), 1-Mso, 3-Mso-CLG (**9**), 1-Mso-CLB (**4**), 1-Mso-CLE (**7**), 1-Mso-CLD (**6**), and CLA (**1**) with internal standard, 1-Abu-CLB (**3**) obtained using Chromolith® SpeedROD at a flow rate of 3.0 mL min^{-1} with the following gradient elution: 0 min 45% B, 1.0 min 80% B, 1.1 min 90% B, 1.2 min 45% B, and 1.5 min 45% B.

Table 6
Chromatographic data of CL on Chromolith® SpeedROD column over multiple injections under optimized elution gradient.

Analyte	R	α	t_R (min)	k'	$W_{1/2}$	Symmetry	Back-pressure	Injection
1-Mso-CLB (**4**)			2.79	5.20	0.06	0.62	71.5	0
1-Msn-CLB (**5**)	4.83	1.19	3.24	6.20	0.05	0.65		
1-Abu-CLB (**3**)	5.47	1.18	3.75	7.33	0.06	0.77		
1-Met-CLB (**2**)	2.16	1.07	3.97	7.82	0.06	0.72		
1-Mso-CLB (**4**)			2.80	5.22	0.05	0.58	71.7	500
1-Msn-CLB (**5**)	5.31	1.19	3.25	6.22	0.05	0.58		
1-Abu-CLB (**3**)	6.14	1.19	3.77	7.38	0.05	0.72		
1-Met-CLB (**2**)	2.48	1.06	3.98	7.84	0.05	0.66		
1-Mso-CLB (**4**)			2.80	5.22	0.05	0.57	74.8	1000
1-Msn-CLB (**5**)	5.19	1.19	3.24	6.20	0.05	0.58		
1-Abu-CLB (**3**)	6.14	1.19	3.76	7.36	0.05	0.72		
1-Met-CLB (**2**)	2.48	1.07	3.97	7.82	0.05	0.67		
1-Mso-CLB (**4**)			2.81	5.24	0.05	0.57	78.3	1500
1-Msn-CLB (**5**)	5.19	1.19	3.25	6.22	0.05	0.56		
1-Abu-CLB (**3**)	6.14	1.19	3.77	7.38	0.05	0.69		
1-Met-CLB (**2**)	2.48	1.06	3.98	7.84	0.05	0.64		
1-Mso-CLB (**4**)			2.81	5.24	0.05	0.58	78.3	2000
1-Msn-CLB (**5**)	5.19	1.19	3.25	6.22	0.05	0.58		
1-Abu-CLB (**3**)	6.14	1.19	3.77	7.38	0.05	0.71		
1-Met-CLB (**2**)	2.48	1.06	3.98	7.84	0.05	0.66		

$n = 3$. R, peak resolution; α, selectivity; t_R, retention time; RSD (repeatability), relative standard deviation; k', capacity factor; $W_{1/2}$, peak width at half height.

mentioned previously, CSR column was chosen out of the five columns due to its excellent performance in separating CLs of flaxseed extracts within a shorter runtime as compared to ZEX column. Column longevity was evaluated by comparing HPLC parameters for 2000 injections of flaxseed extracts. Periodically the mixture of CL standards used in method development (Table 6) was injected to determine column performance. An increase in resolution, peak broadening, capacity factor, column efficiency, and back pressure were observed after the first 500 injections. However, no significant statistical variations in chromatographic parameters were observed, making monolithic column a suitable HPLC column for high throughput screening of CL.

4. Conclusion

The chromatographic performance of a conventional reversed phase HPLC column was compared to reversed phase monolithic and perfusion columns to evaluate their capability for separating a CL mixture and for high-throughput HPLC analysis. An optimized gradient was developed for each of the columns tested. The monolithic columns performed better than the perfusion column in these analyses. Shorter and rapid analyses were achieved with CP and CHR compared to other columns. We further established that increasing temperature from 23 °C to 28 °C at a flow rate of 3.0 mL min^{-1} significantly increased the performance of CSR by allowing for shortening of analysis time to just 1.5 min without compromising the resolution. Longevity of monolithic column CSR was suitable for high-throughput screening as chromatographic parameters were constant over thousands of injections. The monolithic columns are suitable for high throughput screening of CL.

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