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Determination of Nucleotides in Infant Formula by Ion-Exchange Liquid Chromatography

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Nucleotide-supplemented infant formula has been shown to positively modify the composition of intestinal microflora, emulating the attribute of human milk. Quantification of nucleotides in infant formula is of interest because of its applicability in quality and safety assessments. There is no standard method for the analysis of nucleotides in infant formula. In the present study, ion-exchange liquid chromatography (IELC)- and centrifugal ultrafiltration (CUF)-based protocols were developed for routine determination of additive nucleotides in infant formula. Five target nucleotides, guanosine 5′-monophosphate (GMP), inosine 5′-monophosphate (IMP), uridine 5′-monophosphate (UMP), cytidine 5′-monophosphate (CMP), and adenosine 5′-monophosphate (AMP) were measured by IELC with a mobile phase of 50 mM diammonium hydrogen phosphate buffer, pH 4.0, with UV detection at 254 nm. The calibration was linear over the range 0.5−50 μg/mL; $R^2 = 0.999$. The calculated LOD and LOQ were 0.01−0.05 μg/mL and 0.05−0.5 μg/mL, respectively. Recovery values (spiked concentration levels: 0.5, 5, and 10 μg/mL) ranged from 85.0 ± 1.4% to 92.3 ± 2.1% using only CUF preparation. This was applied to measure the concentration of five nucleotides in common infant formulas.

KEYWORDS: Nucleotides; infant formula; centrifugal ultrafiltration; ion-exchange liquid chromatography

INTRODUCTION

Nucleotides are the structural units in nucleic acids, coenzymes in biochemical pathways, as well as being sources of chemical energy. They may become essential and be used as additives for newborns if the endogenous supply is inadequate (e.g., during rapid growth or after injury) (*1–3*). These supplemented diets have been shown to elicit an enhanced immune response in infants (*4, 5*). Nucleotide-supplemented infant formula has been shown to positively modify the composition of intestinal microflora, emulating the attribute of human milk (*6*).

It has been shown that nucleotides are present at higher concentrations in human milk compared with bovine milk (*7*). There is a clear difference in the nucleotide monophosphate profile between bovine and human milk. The nucleotide monophosphates for infant formulas are guanosine 5′-monophosphate (GMP), inosine 5′-monophosphate (IMP), uridine 5′-monophosphate (UMP), cytidine 5′-monophosphate (CMP), and adenosine 5′-monophosphate (AMP). Surveys of these nucleotide levels have been reported using various analytical methods including detection, separation, and sample preparation (*8–11*).

For sample preparation, extraction of nucleotides from milk is usually achieved after initial protein precipitation and/or denaturalization with perchloric acid (PCA), trichloroacetic acid (TCA), or acetic acid (AA) (*8, 10–14*). Various cleanup procedures of extracted solution have been achieved by ion exchange and/or C_{18}-based solid-phase extraction, and phenylboronate affinity chromatography (*10–15*). It is not necessary to eliminate biological fluids but necessary to eliminate interferences in infant formula and milk samples before cleanup procedure although the preferred sample extraction/cleanup technique depends on the analytical method (*10*). There is no standard method available for the sample preparation of nucleotides in infant formula.

Liquid chromatography (LC) is generally used for determination of nucleotides in infant formula after sample extraction/cleanup. Three main modes of LC have been appraised for measurement of these nucleotides: reversed-phase liquid chromatography (RPLC), ion-pair RPLC (IP-RPLC), and ion-exchange liquid chromatography (IELC). In RPLC analysis, nucleotide separation with a C_{18}-based column is limited because of the inherently poor interaction of the highly polar nucleotides. Thus, RPLC with mass spectrometry has been used to analyze nucleotides and their metabolites in biological materials (*16, 17*).

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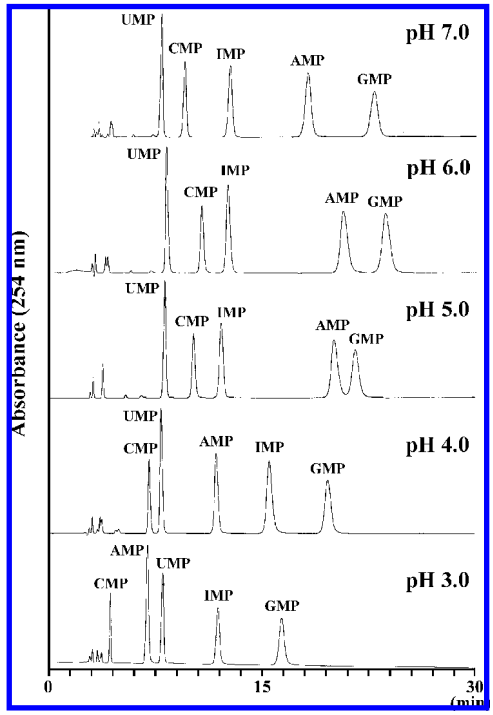

Figure 1. IELC chromatograms for separation of five nucleotides with pH factors. Column: CAPCELL PAK NH_2 UG 80 (4.6 mm × 250 mm, 5.0 μm, Shiseido Fine Chemicals Co., Tokyo, Japan). Mobile phase: 50 mM diammonium hydrogen phosphate in water adjusted to pH 3.0−7.0 with phosphoric acid. Flow rate: 1.0 mL/min.

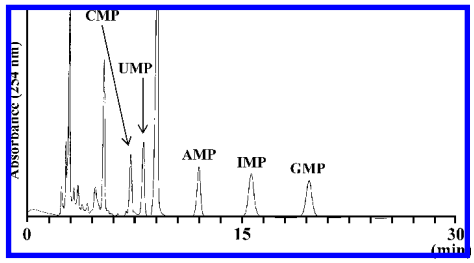

Figure 2. IELC chromatogram for recovery test of five nucleotides from infant formula. Concentration level is 5 μg/mL in the infant formula sample.

Table 1. Precisions of Intraday (Retention Time and Peak Area), LOD, and LOQ of Five Nucleotides[a]

analytes	RSD_{rt} (%)	RSD_{area} (%)	LOD (μg/mL)	LOQ (μg/mL)	calibration curve (0.5−50 μg/mL)
AMP	0.07	2.23	0.03	0.1	$y = 17223x - 6572$; $R^2 = 0.999$
CMP	1.09	2.83	0.03	0.1	$y = 9574x - 3297$; $R^2 = 0.999$
GMP	0.28	3.00	0.05	0.2	$y = 22262x - 12698$; $R^2 = 0.999$
UMP	0.08	2.39	0.01	0.05	$y = 16673x - 5019$; $R^2 = 0.999$
IMP	1.05	2.10	0.05	0.2	$y = 23553x - 13817$; $R^2 = 0.999$

[a] Concentration level is 5 μg/mL standard. Relative standard deviation of retention time ($n = 6$) was abbreviated as RSD_r (%). Relative standard deviation of peak area ($n = 6$) was abbreviated as RSD_{area} (%). Limit of detection (S/N = 3) was abbreviated as LOD. Limit of quantitation (S/N = 10) was abbreviated as LOQ.

A mass spectrometric detector could be accomplished with the low chromatographic retention and/or resolution of the extremely polar nucleotides and would be very expensive for developing a routine and useful method. The determination of nucleotides in milk and infant formula samples was reported using IP-RPLC with mobile phase as tetrabutyl ammonium hydrogensulphate ion-pair reagent (*7, 9, 14*). These IP-RPLC methods for simultaneous determination and separation of five nucleotides in infant formula samples were needed to making complicated mobile phase and gradient and spending long run time (over 60 min) (*9, 14*). Separation of polar metabolites such as nucleotides, coenzyme-A esters, sugar nucleotides, and sugar bisphosphates was attempted by IP-RPLC coupled with electrospray ionization mass spectrometry (*18*); however, it is necessary to use complicated ion-pair gradient modes with adjusting pH to obtain the efficient separation of these compounds. This IP-RPLC technique was confusing; various optimal ion-pair reagents were investigated, and an ion-pair reagent was found to be the contamination of the instrumental source. On the other hand, IELC is useful for analysis of nucleotides in infant formula because only pH is optimized in the mobile phase. Recently, the growing interest in ionic liquids and ion-exchange chromatography has resulted in an exponentially increasing production of analytical applications (*19*). Therefore, the IELC methods could be considered to optimize only the pH of the mobile phase without any specific reagents, gradient mode, and cost performance for routine analysis of five nucleotides in infant formula samples.

Table 2. Recovery Test of Nucleotides in Infant Formula by Various Extraction Procedures[a]

extraction	AMP	CMP	GMP	UMP	IMP
1 M PCA	95.3 ± 3.9%	80.6 ± 8.1%	88.9 ± 2.2%	96.1 ± 5.8%	88.5 ± 1.4%
1 M TCA	94.5 ± 1.7%	86.0 ± 5.6%	87.3 ± 2.0%	94.9 ± 2.9%	88.7 ± 1.3%
1 M AA	96.3 ± 3.8%	98.7 ± 6.7%	91.4 ± 1.9%	98.3 ± 5.1%	94.0 ± 4.3%
nonacid	91.7 ± 2.2%	89.5 ± 2.5%	89.4 ± 1.5%	92.3 ± 2.1%	91.2 ± 0.4%

[a] Spiked concentration level was 5.0 μg/mL ($n = 3$). Perchloric acid was abbreviated as PCA. Trichloroacetic acid was abbreviated as TCA. Acetic acid was abbreviated as AA.

The aim of this study is that a simple, routine, rapid, and useful technique is developed. Thus, we use centrifugal ultrafiltration (CUF) for sample preparation of nucleotides in infant formulas. CUF is a variety of membrane filtration in which centrifugation forces a liquid against a semipermeable membrane. Suspended solids and solutes of high molecular weight are retained, while the liquid and low molecular weight solutes pass through the membrane depending on the molecular weight cut off of the membrane used. This significance of the simple, rapid, accurate, and ecological CUF method was applied for rapid analysis of inositol phosphates in foods (*20*). This technique has been extensively used for the removal of high molecular weight species for various samples; however it is not applicable for nucleotides. In this study, we established a simple, routine, rapid, and useful analytical procedure for determining nucleotides in infant formula using CUF and IELC.

MATERIALS AND METHODS

Reagents. Adenosine 5′-monophosphate (AMP) was obtained from Oriental Yeast Co., Ltd., Tokyo, Japan. Guanosine 5′-monophosphate (GMP) was obtained from Wako Pure Chemical Co., Ltd., Osaka, Japan. Inosine 5′-monophosphate (IMP), uridine 5′-monophosphate (UMP), and cytidine 5′-monophosphate (CMP) were obtained from Sigma-Aldrich Co., Ltd., St. Louis, MO. Diammonium hydrogen phosphate (chemical grade) and phosphoric acid (chemical grade) were obtained from Wako Pure Chemical Co., Ltd., Osaka, Japan. Purified water was obtained from a Milli-Q purifying system (Millipore, Bedford, MA). For sample extraction, chemical-grade trichloroacetic acid (TCA), perchloric acid (PCA), and HPLC-grade acetic acid (AA) were obtained from Wako Chemical Co., Ltd.

Table 3. Precisions of Interday for Recovery Test of Five Nucleotides in Infant Formula[a]

conc levels (μg/mL)	AMP				CMP				GMP				UMP				IMP			
	day 1	day 2	day 3	AV ± SD[b]	day 1	day 2	day 3	AV ± SD	day 1	day 2	day 3	AV ± SD	day 1	day 2	day 3	AV ± SD	day 1	day 2	day 3	AV ± SD
0.5	102.1	100.2	98.4	100.2 ± 1.9%	100.4	101.4	103.3	101.7 ± 1.5%	97.7	94.5	93.3	95.2 ± 2.3%	102.7	92.8	91.7	95.7 ± 6.1%	104.0	95.2	94.4	97.9 ± 5.3%
5	95.4	92.4	91.3	93.0 ± 2.1%	94.6	94.2	92.4	93.7 ± 1.2%	91.7	92.5	88.8	91.0 ± 1.9%	95.4	94.9	94.1	94.8 ± 0.6%	91.4	90.2	91.5	91.0 ± 0.7%
10	92.6	97.8	92.8	94.4 ± 3.0%	88.7	95.2	94.7	92.9 ± 3.6%	91.6	95.7	90.2	92.5 ± 2.9%	95.2	99.7	94.4	96.4 ± 2.8%	96.2	94.0	92.0	94.0 ± 2.1%

[a] Average was abbreviated as AV. Standard deviation of recovery (days 1, 2, and 3) was abbreviated as SD. [b] $n = 3$ (3 days).

Table 4. Result of Measuring Five Nucleotides in Common Infant Formula Samples by Developed Method

sample no.	supplementation of nucleotides	conc nucleotides (μg/g) AMP	CMP	GMP	UMP	IMP
1	added	31.8	69.5	16.2	24.5	15.2
2	added	18.8	93.1	19.4	18.4	19.1
3	added	17.0	142.1	13.7	40.7	31.7
4	added	9.9	66.8	5.2	22.2	10.0
5	added	24.1	134.9	52.7	32.5	62.9
6	added	26.2	35.4	56.6	9.3	33.3
7	added	31.0	41.2	54.5	11.6	32.8
8	nonadded	ND[a]	33.6	ND	ND	ND
9	nonadded	ND	39.8	ND	ND	ND
10	nonadded	ND	78.0	ND	ND	ND

[a] ND < 0.5 μg/g.

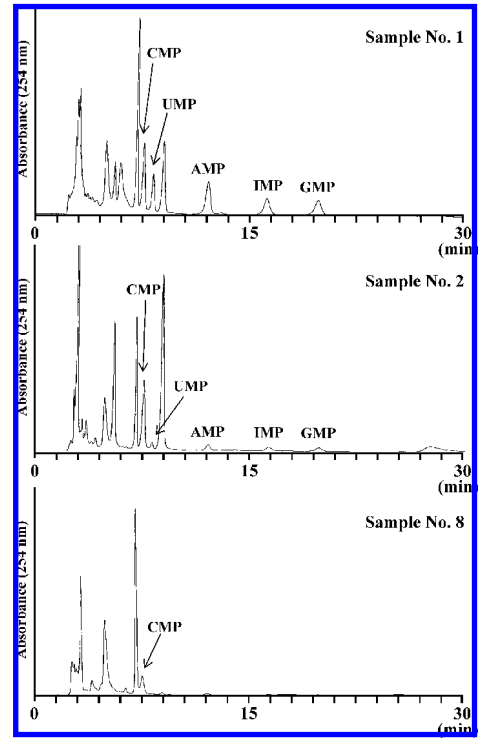

Figure 3. IELC chromatograms for monitoring five nucleotides from common infant formulas. Sample number is shown in **Table 4**.

Standard Solutions. Concentrated solution (1.0 mg/mL) of each nucleotide was prepared in pure water. These solutions of nucleotides were mixed and diluted as required by the addition of pure water.

Sample Preparation of Nucleotide Extraction from Infant Formula. For investigation of the optimal sample preparation and recovery tests, a nucleotide-free infant formula sample was obtained from Wakodo Co. Ltd., Tokyo, Japan. In addition, nucleotide-supplemented and unsupplemented infant formula samples were obtained from a local store in Nagoya, Japan. These 5.0 g samples were weighed in glass 100 mL beakers. Then, 50 mL of water was added and mixed. Necessary standards were added at this stage. The mixture was vortex-mixed for 1 min, stored at 4 °C for 10 min, and then centrifuged at 3500 rpm (rcf 2328*g*) for 15 min (Kubota 5420, Kubota Co., Tokyo, Japan). The supernatants obtained were applied in the CUF step. Nucleotides in the infant formula samples were pretreated using Amicon Ultra-4 (Ultracel-3K, regenerated cellulose 3000 M.W. for volumes <4 mL; Milliore Co., Ltd., Billerica, MA). Sample solution (0.5 mL) was eluted through CUF cartridges by centrifugation at 3500 rpm for 15 min and measured by IELC.

IELC Separation and Analysis of Nucleotides. Liquid chromatography (LC) was performed using a LC-20AT pump, SPD-20A detector, CTO-20AC column oven, and C-R8A recorder system

(Shimadzu Co., Kyoto, Japan). The column selected was a CAPCELL-PAK NH_2 UG 80 (4.6 mm × 250 mm, 5.0 μm, Shiseido Fine Chemicals Co., Tokyo, Japan). The mobile phase for IELC analysis consisted of 50 mM diammonium hydrogen phosphate in water and a pH adjusted with phosphoric acid. The flow rate was 1.0 mL/min. The column temperature was 40 °C. A sample volume of 10 μL was injected. The elution of nucleotide was monitored by UV absorbance at 254 nm.

Validation of Analytical Method. Nucleotides were quantified by a six-point calibration curve (0.5−50 μg/mL). The area of each nucleotide was plotted against the analyte concentration. The linearity of this response was checked by calculating the correlation coefficient (R^2) and repeatability. Precision (intra- and interday) was calculated from the analysis of nucleotide standard solutions and recovery test samples by the same operator. Precision (intraday) within the laboratory was tested using a standard solution (5 μg/mL, $n = 3$) of retention time and peak area for IELC analytical method. Interday reproducibility was performed for three days (days 1, 2, and 3) using three concentrations of recovery test solutions for CUF preparation. The limits of detection (LOD) and quantity (LOQ) were calculated from the signal-to-noise ratio (S/N). The S/N values for LOD and LOQ were three and ten times, respectively.

RESULTS AND DISCUSSION

IELC Separation and Analysis of Nucleotides. Recently, HPLC with UV detection for determination of nucleotides was reported (*21*). This chromatographic condition was achieved on a two bonded column (spherical silica packing and C_{18} reversed phase) using a mobile phase of a *tetra*-butyl ammonium hydrogen sulfate solution/acetonitrile/methanol (9.6/0.3/0.1, v/v/v) for separation of AMP and GMP without IMP, UMP, and CMP (*21*). Therefore, this HPLC with UV cannot be used for simultaneously determination of five nucleotides. Consequently, we tried to use a simpler IELC with an NH_2-based column separation and UV absorbance detector for determining nucleotides in infant formula than reversed phase mode. IELC separation of nucleotides was investigated using a mobile phase comprising 50 mM diammonium hydrogen phosphate buffers at a pH range 3.0−7.0 with phosphoric acid. Nucleotides could be separated on an NH_2-based column due to their polarities, enabling separation of five major nucleotides by IELC analysis of nucleotide standard solutions, and the chromatogram showed five main peaks at an absorbance of 254 nm (**Figure 1**). The condition of the mobile phase in pHs 3.0, 4.0, 5.0, 6.0, and 7.0 showed good separation of the five nucleotides and a total analytical time of 30 min (**Figure 1**). For appropriating this retention mechanism, it is clear that analyte groups with different charge have distinct elution ranges of pH. Considering that these nucleotides with different charges occupy different pH in the chromatogram, such analytes have the different peak capacity for an ion-exchange separation from pH ranges. The theory of the peak capacity in ion-exchange separations has been sketched out in a previous publication (*22*). On the other hand, when we tried to measure the five nucleotides from infant formula samples, only the mobile phase condition at pH 4.0 showed good separation between analytes and sample matrixes (**Figure 2**). Because it is impossible and difficult, the separation of CMP and the matrix (retention time: 9.0 min) peaks was completed in the mobile phase condition at pH 5.0−7.0. Moreover, the peaks of CMP and the front eluted materials (retention time: 4.5−6.0 min) could not be separated in the mobile phase condition at pH 3.0. Therefore, we decided to measure five nucleotides at 254 nm in IELC with the mobile phase adjusted to pH 4.0 and to measure infant formula samples.

To achieve successful IELC separation and analysis of the nucleotides, we investigated the precision of intraday (retention time and peak area), LOD, and LOQ of five nucleotides (**Table 1**). The repeatability test of retention time was that the RSDs ($n = 6$, intraday) of the five nucleotide standards were 0.07−1.09%. The RSD of peak area ranged 2.10−1.09% ($n = 6$). The calibration graphs obtained for the five nucleotide standards were linear over the calibration range (0.5−50 μg/mL) and showed good correlation values ($R^2 = 0.999$). The calculated LOD and LOQ were 0.01−0.05 μg/mL (0.1−0.5 ng) and 0.05−0.2 μg/mL (0.5−2 ng), and the S/N was three and ten times, respectively. **Table 1** shows that IELC was a sensitive and accurate method for determining the five nucleotides. This method could be used to evaluate nucleotides in infant formula.

Sample Preparation of Nucleotides in Infant Formula. Acid precipitation, solid-phase extraction, affinity chromatography, and ion-exchange chromatography have been reported for the determination of nucleotides from different types of infant formula and/or milk (*10*). In this study, the aim is that a more useful, simple, and accurate sample preparation for quantification of nucleotides added to infant formula is developed than reported methods. Thus, CUF was applied to the extraction/cleanup procedure of nucleotides in infant formula. CUF is easy to use and has a shorter operation time, resulting in high recovery and reproducibility, thereby saving analytical time and solvents (*23, 24*). CUF has a variety of sizes of membrane filtration. Suspended solids and solutes of high molecular weight are retained, while low molecular weight solutes pass through the membrane depending on the molecular weight cut off of the membrane used. The smaller size for cutoff is MW 3000 in Millipore Co. This size can be used for preparation of target nucleotides (MW 323−363). The preparation of nucleotides from milk is usually achieved after initial protein precipitation with various acid solutions. Then, samples are typically centrifuged and/or filtered for other cleanup procedures and analysis of nucleotides from matrixes. Our investigation for initial protein removal prior to CUF was carried out using various acid solutions (1 M PCA, TCA, and AA in water). The result of the recovery test (5 μg/mL) is shown in **Table 2**. The reproducibility of the CUF preparation of special CMP without initial protein precipitation was better than using with acid solutions (**Table 2**). Moreover, acid neutralization was advocated before extract storage because of the potent loss of nucleotides during long-term storage in acid (*25*). If extract samples were stored, it is needed to adjust neutralization. In this study, we suggest that initial protein precipitation with various acid solutions is not needed for CUF preparation and IELC analysis of nucleotides in infant formula. CUF is simply an alternative technique to acid precipitation to remove high molecular weight proteins. On the other hand, the chromatogram of five nucleotides by sample preparation with CUF showed the good separation between analytes and sample matrixes from infant formula (**Figure 2**). Moreover, for establishing this efficient CUF preparation of nucleotides in infant formula, we compared the CUF preparation to usual sample preparation with liquid−liquid extraction for a quality test (*26*). Recovery and total experimental time were investigated based on referenced extraction techniques (*26*). In the results, recovery and total time were 80% lower and over 3 h/two samples, respectively. On the other hand, recovery and total time were 89.4−92.3% and only 30 min/eight samples using the CUF preparation. On the basis of our experimental and compared results, a simple, routine, and useful extraction/cleanup procedure was developed using direct CUF without protein precipitation from infant formula.

Validation of Analytical Methods for Determination of Nucleotides in Infant Formula. For routine determination of nucleotides in infant formula, validation of the analytical method had the necessary precision of interday reproducibility. **Table 3** shows that the recovery tests using 0.5, 5, and 10 μg/mL in infant formula were evaluated for three days ($n = 3$). The results of interday reproducibility of the CUF and IELC methods suggest the utility of these methods for routine determination of the five nucleotides from infant formula. Overall results indicate that this approach of CUF preparation and IELC analytical method is an accurate technique for the routine and quality test of nucleotides in infant formula.

Measurement Five Nucleotides in Common Infant Formula Samples. Nucleotides in human milk and nucleotide supplementation of infant formula have been of interest recently, because nucleotides are thought to be important for many biological benefits (*27, 28*). Therefore, there is a clear need for a routine and reference method to measure intact and profile nucleotides in human milk and infant formula. The concentration of five nucleotides in common infant formula samples are shown in **Table 4**. IELC chromatograms are shown in **Figure 3**.

In conclusion, these results suggest that this approach of the CUF sample preparation and IELC analytical method could be applied to routine measurement of AMP, GMP, IMP, UMP, and CMP in infant formula. On the basis of these data, there is a clear difference in the nucleotide monophosphate profile between different commercially available infant formula samples.

ABBREVIATIONS USED

IELC, ion-exchange liquid chromatography; CUF, centrifugal ultrafiltration; GMP, guanosine 5′-monophosphate; IMP, inosine 5′-monophosphate; UMP, uridine 5′-monophosphate; CMP, cytidine 5′-monophosphate; AMP, adenosine 5′-monophosphate; UV, ultraviolet; LOD, limit of detection; LOQ, limit of quantity; PCA, perchloric acid; TCA, trichloroacetic acid; AA, acetic acid; RPLC, reversed-phase liquid chromatography; IP-RPLC, ion-pair RPLC; S/N, signal-to-noise ratio; RSD, relative standard deviation; MW, molecular weight.

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