

Research Article

Separation of Cyclic Dipeptides (Diketopiperazines) from Their Corresponding Linear Dipeptides by RP-HPLC and Method Validation

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Simple, rapid, sensitive, precise, and accurate methods for detection and separation of seven diketopiperazines (DKPs), cyclo(Gly-Gly), cyclo(DL-Ala-DL-Ala), cyclo(L-Asp-L-Phe), cyclo(L-Asp-L-Asp), cyclo(Gly-L-Phe), cyclo(L-Pro-L-Tyr), and cyclo(L-Arg-L-Arg), from their corresponding linear dipeptides and related amino acids L-Phe and L-Tyr by reversed-phase high-performance liquid chromatography (RP-HPLC) were established. Moreover, for the racemic DKP cyclo(DL-Ala-DL-Ala) and dipeptide DL-Ala-DL-Ala, separation of the diastereomers was achieved. All methods can be performed within 15 min. For all DKPs, dipeptides, and amino acids, linear ranges with correlation coefficients R^2 greater than 0.998 were determined. Lowest limits of detection were found to be between 0.05 and 10 nmol per 10 μ L injection, depending on the substance. For all tested substances intrarun and interrun precision ranged from 0.5 to 4.7% and 0.7 to 9.9% relative standard deviation, and accuracy was between -4.2 and 8.1% relative error. Short-term and freeze-thaw stabilities were 93% or greater for all substances. Recovery rate after heat treatment was determined to be at least 97%. These methods will be useful for quantitative determination of DKPs and their potential biodegradation products: dipeptides and amino acids.

1. Introduction

Diketopiperazines (DKPs) are the smallest possible cyclic peptides composed of two α -amino acids. They are abundant natural compounds produced by various bacteria like *Streptomyces* sp. [1], *Pseudomonas aeruginosa* [2], or *Lactobacillus plantarum* [3], fungi, e.g., *Aspergillus flavus* [4] or *Alternaria alternata* [5], and marine sponges like *Dysidea herbacea* [6]. Recently, the interest in this substance class has increased due to their immense bioactivities including antibacterial activity [7], antifungal function [3], cytotoxicity [4], phytotoxicity [5], and inhibition of plasminogen activator inhibitor-1 [8]. DKPs were shown to act as quorum sensing molecules; e.g., cyclo(L-Pro-L-Tyr), used in this study, was identified in culture supernatant of *Pseudomonas aeruginosa* and was identified as an activator of an *N*-acylhomoserine lactone biosensor [2]. Besides their widespread biosynthesis in nature, DKPs occur as chemical degradation products of, for

example, amoxicillin, an aminopenicillin antibiotic [9], neuropeptide substance P [10], angiotensin converting enzyme inhibitor enalapril [11], or the sweetener aspartame with cyclo(L-Asp-L-Phe) as degradation product [12–17]. Amoxicillin and especially its degradation products can be detected in aquatic environment and food of animal origin, thus they are under discussion as a health problem due to their allergenic potential [9]. Analysis of these compounds can be performed with HPLC-MS/MS [9] or UHPLC-MS/MS [18]. Enalapril and two degradation products are determined with RP-HPLC and detection at 215 nm [11]. Aspartame and its degradation products can be analyzed via RP-HPLC and detection of peptide bond at 195 nm [12], 200 nm and 220 nm [13] or 210 nm [14], capillary electrophoresis and detection at 214 nm [15], HPLC coupled with MS/MS [16] or analysis of trimethylsilyl derivatives with GC [17]. Cyclo(Gly-Gly) and Gly are separated by RP-HPLC and detected at 200 nm [19]. Thus, there are some analysis methods of selected

DKPs available, but to the best of our knowledge there is no publication of a comprehensive study for separation of DKPs and their corresponding linear dipeptides and amino acids and method validation. However, this would be of great interest for studies on microbial, enzymatical, and chemical degradation of this biologically active substance class. Until now, there are only few bacterial strains reported which can hydrolyze DKPs to the corresponding linear dipeptides. One strain is *Paenibacillus chibensis* (DSM 329) hydrolyzing the aspartame degradation product cyclo(L-Asp-L-Phe) [20]. This strain was chosen as biological matrix for method validation in this work.

The aim of this study was to establish rapid methods for detection and separation of seven selected DKPs from their corresponding dipeptides and in some cases the amino acids. Moreover, these methods were validated for sensitivity, linearity, precision, accuracy, stability, and recovery.

2. Experimental

2.1. Chemicals and Reagents. The standards cyclo(L-Ala-L-Ala), L-Asp-L-Phe, L-Phe-L-Asp, cyclo(L-Pro-L-Tyr), L-Pro-L-Tyr, L-Tyr-L-Pro, cyclo(L-Asp-L-Asp), L-Asp-L-Asp, cyclo(Gly-L-Phe), Gly-L-Phe, L-Phe-Gly, and L-Arg-L-Arg acetate salt were purchased from Bachem Holding (Bubendorf, Switzerland), cyclo(Gly-Gly), Gly-Gly, and DL-Ala-DL-Ala were obtained from TCI Europe N.V. (Zwyndrecht, Belgium), cyclo(DL-Ala-DL-Ala), L-Ala-L-Ala, cyclo(L-Asp-L-Phe) and L-Phe were received from Sigma-Aldrich (St. Louis, USA), L-Tyr was purchased from Carl Roth Corporation (Karlsruhe, Germany), and cyclo(L-Arg-L-Arg) acetate salt was provided by Taros Chemicals Corporation (Dortmund, Germany).

Methanol ROTISOLV HPLC Gradient Grade (MeOH), peptone from casein, K_2HPO_4 , KH_2PO_4 , $Na_2HPO_4 \times 2H_2O$, $NaH_2PO_4 \times 2H_2O$ were supplied by Carl Roth Corporation (Karlsruhe, Germany). Yeast extract was obtained from Becton, Dickinson and Company (Franklin Lakes, USA). $MgSO_4 \times 7H_2O$ was purchased from Sigma-Aldrich (St. Louis, USA).

2.2. Instrumentation and Chromatographic Conditions. The HPLC analysis was performed using an Agilent 1200 system (Agilent Technologies, Santa Clara, USA) equipped with a quaternary pump, a degasser, an autosampler, a thermostated column compartment, and a variable wavelength detector. Separations were carried out with isocratic elution on a reversed-phase column NUCLEODUR Sphinx RP (4.6 mm ID \times 250 mm, 5 μ m particle size, Macherey-Nagel Corporation, Düren, Germany) connected with a C_{18} security guard column (3.0 mm ID \times 4 mm, Phenomenex, Torrance, USA). The mobile phase consisted of varying ratios of MeOH and 20 mM sodium phosphate buffer (pH 5.5). The buffer was filtered through a nitrocellulose membrane with 0.22 μ m pore size (Merck Millipore, Darmstadt, Germany). Best separation conditions were investigated by determination of retention time (t_R), separation factor (α), and peak resolution (R_s) for each standard with 10%, 20%, and 30% MeOH. After

determination of best separation conditions, the following compositions of mobile phase were used. Separation of cyclo(Gly-Gly) from Gly-Gly, cyclo(DL-Ala-DL-Ala) from DL-Ala-DL-Ala, cyclo(L-Asp-L-Asp) from L-Asp-L-Asp, and cyclo(L-Arg-L-Arg) from L-Arg-L-Arg was done with 10% MeOH and 90% buffer. 20% MeOH and 80% buffer were used to separate cyclo(L-Asp-L-Phe), L-Asp-L-Phe, L-Phe-L-Asp, and L-Phe. With 30% MeOH and 70% buffer cyclo(L-Pro-L-Tyr) was separated from L-Pro-L-Tyr, L-Tyr-L-Pro, and L-Tyr, and cyclo(Gly-L-Phe) from Gly-L-Phe, L-Phe-Gly, and L-Phe. The injection volume was 10 μ L and detection wavelength was 210 nm. The flow rate was 0.7 mL min⁻¹, and the column temperature was set to 20°C (for analysis with 10 and 20% MeOH) or to 30°C (for 30% MeOH). Differing from these standard conditions cyclo(L-Asp-L-Asp) and L-Asp-L-Asp were separated with 15°C column temperature and 0.3 mL min⁻¹ flow rate; cyclo(L-Arg-L-Arg) was detected at a wavelength of 268 nm.

Validation of each substance was performed under best separation conditions and run time was 15 min for each sample.

2.3. Preparation of Standard Solutions. Stock solutions of DKPs, dipeptides, and amino acids were prepared in 50 mM sodium phosphate buffer (pH 7.5) and stored at -20°C. The concentration of each stock solution was adjusted to 50 mM, except for 25 mM cyclo(L-Asp-L-Phe), 10 mM cyclo(Gly-L-Phe), and 5 mM L-Tyr. These stock solutions were serially diluted and used directly for determination of the linear range. Calibration curves were established based on seven concentrations within the linear range. For method validation the stock solutions were diluted to low, medium, and high concentration and were mixed (1:1) with crude extract of DSM 329.

2.4. Crude Extract Preparation. Crude extract was prepared from the bacterial strain *Paenibacillus chibensis* (DSM 329) purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strain was cultivated in modified complex medium (10 g L⁻¹ peptone, 10 g L⁻¹ yeast extract, 3 g L⁻¹ K_2HPO_4 , 1 g L⁻¹ KH_2PO_4 , 0.5 g L⁻¹ $MgSO_4 \times 7H_2O$, adjusted to pH 7.2) [20] at 30°C and 100 rpm for 24 h until reaching an OD₆₀₀ of 2.1. Cells were washed three times by centrifugation (4,816 \times g, 30 min, 4°C), discarding the supernatant and resuspension in 50 mM sodium phosphate buffer (pH 7.5). Washed cell pellet was resuspended in the same buffer to an OD₆₀₀ of approximately 17. Cells were disrupted by sonication for 25 min using alternate intervals of 30 sec pulsation on and 30 sec pulsation off and 35% amplitude (Sonopuls HD 3100 with ultrasonic probe MS 72, Bandelin electronic Corporation, Berlin, Germany). After centrifugation (4,816 \times g, 30 min, 4°C), the crude extract was inactivated by heating at 90°C (20 min, 1,500 rpm) with Thermomixer comfort (Eppendorf Corporation, Hamburg, Germany) and centrifuged (24,725 \times g, 20 min, 4°C). The protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, USA) according to

TABLE 1: Retention factor (k) for DKPs, dipeptides, and amino acids and separation factor (α) and resolution (R_s) of two adjacent peaks at chosen separation conditions.

Separation conditions	Substance	Retention factor k	Separation factor α	Resolution R_s		
10% MeOH, 20°C, 0.7 mL min ⁻¹	Cyclo(Gly-Gly)	1.03	1.63	5.47		
	Gly-Gly	0.63				
	Cyclo(L-Ala-D-Ala)	2.34				
	10% MeOH, 15°C, 0.3 mL min ⁻¹	Cyclo(L-Ala-L-Ala) and cyclo(D-Ala-D-Ala)	2.75	1.18	3.26	
		L-Ala-L-Ala and D-Ala-D-Ala	0.67			
		L-Ala-D-Ala	0.84	1.25	1.83	
		Cyclo(L-Ala-D-Ala)	2.34			
		L-Ala-D-Ala	0.84			
		Cyclo(L-Arg-L-Arg)	1.08			
20% MeOH, 20°C, 0.7 mL min ⁻¹		L-Arg-L-Arg	0.79	1.36	1.55	
		Cyclo(L-Asp-L-Asp)	0.61	1.15	1.04	
	L-Asp-L-Asp	0.53				
	20% MeOH, 20°C, 0.7 mL min ⁻¹	Cyclo(L-Asp-L-Phe)	3.33	1.14	2.31	
		L-Phe	2.93			
		L-Asp-L-Phe	2.10	1.40	5.45	
		L-Phe	2.93			
		L-Asp-L-Phe	2.10			
	30% MeOH, 30°C, 0.7 mL min ⁻¹	L-Phe-L-Asp	1.32	1.59	5.88	
		Cyclo(L-Pro-L-Tyr)	3.75	2.26	9.44	
L-Tyr-L-Pro		1.66				
30% MeOH, 30°C, 0.7 mL min ⁻¹		L-Tyr-L-Pro	1.66	1.76	4.23	
		L-Tyr	0.95			
		L-Pro-L-Tyr	0.93			
		30% MeOH, 30°C, 0.7 mL min ⁻¹	L-Tyr	0.95	1.02	0.22
			Cyclo(Gly-L-Phe)	4.36	2.31	16.12
			L-Phe-Gly	1.89		
			L-Phe-Gly	1.89	1.01	0.17
L-Phe			1.87			
Gly-L-Phe			1.84			
30% MeOH, 30°C, 0.7 mL min ⁻¹	L-Phe	1.87	1.02	0.27		

Hold-up time = 2.167 min (manufacturer information).

the manufacturers' instructions. The obtained crude extract was diluted with the described buffer to a final protein concentration of 0.3 mg mL⁻¹.

2.5. Method Validation. The detection of DKPs, dipeptides, and amino acids was validated for sensitivity, linearity, precision, accuracy, stability, and recovery according to the bioanalytical method validation guidelines of the FDA [21]. Validation of each substance was done under best separation conditions, which were determined in this study.

Intrarun precision and accuracy were assessed using five replicates at low, medium, and high concentration, within consecutive runs. Interrun precision was determined using these experimental conditions on three different days. Precision was expressed as relative standard deviation, RSD (%) = 100 * (standard deviation/mean). Accuracy was calculated as relative error, RE (%) = 100 * (mean measured concentration - true concentration/true concentration). The acceptance limits were set at a maximum of $\pm 15\%$ at medium and high concentrations and $\pm 20\%$ at low concentration. For each substance the mean value of low, medium, and high concentrations was calculated for precision and accuracy.

Short-term temperature stability and freeze-thaw stability were determined with freshly spiked samples at low and high concentration in triplicate. Stability was calculated by comparing the peak areas obtained by direct injection, after storage at 21°C for 24 h, and after three freeze and thaw cycles. For determination of freeze and thaw stability samples were frozen at -20°C for 24 h, completely thawed and refrozen for 21 h. The freeze-thaw cycle was repeated two times and samples were analyzed on the third cycle.

Recovery was determined by using four replicates at low, medium, and high concentrations. Evaluation was done by comparing the peak areas of samples spiked after heat treatment with samples spiked before heat treatment. For heat treatment samples were incubated at 90°C and 1,400 rpm for 10 min using Thermomixer comfort (Eppendorf Corporation, Hamburg, Germany).

3. Results and Discussion

3.1. Separation. Best separation conditions of DKPs, their corresponding linear dipeptides, and the amino acids L-Phe and L-Tyr are summarized in Table 1. These two aromatic

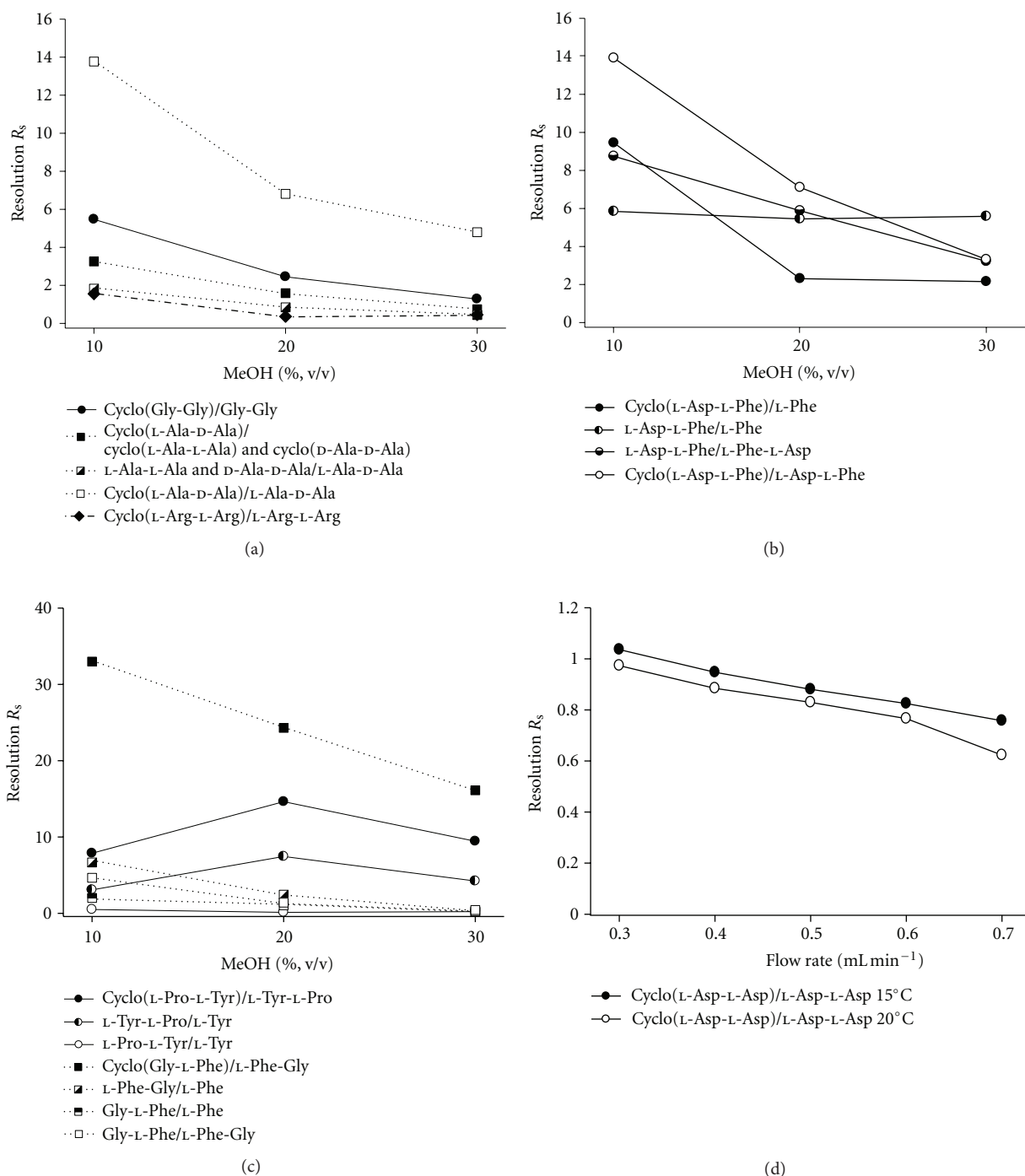


FIGURE 1: Resolution R_s of two adjacent peaks of DKPs, dipeptides, and amino acids depending on MeOH concentration (a–c) or flow rate (d); substances with (a) 10% MeOH, (b) 20% MeOH, and (c) 30% MeOH chosen as best separation conditions; (d) cyclo(L-Asp-L-Asp) and L-Asp-L-Asp at flow rate from 0.3 to 0.7 mL min⁻¹ at 15 and 20°C column temperature and 10% MeOH.

amino acids were analyzed due to their detectability at 210 nm.

Separation factors α and peak resolutions R_s were calculated for two adjacent peaks at different MeOH concentrations (Figures 1(a)–1(c)). For some separations the order of peaks changed by altering MeOH concentration, resulting in variation of peaks which are adjacent.

The aim was to identify a method for each DKP, dipeptide and the two amino acids with resolution greater than 1.5 and longest retention time of maximum 15 min.

For separation of cyclo(Gly-Gly) from Gly-Gly and cyclo(L-Arg-L-Arg) from L-Arg-L-Arg best separation conditions were achieved with 10% MeOH mixed with 90% 20 mM sodium phosphate buffer (pH 5.5), 20°C

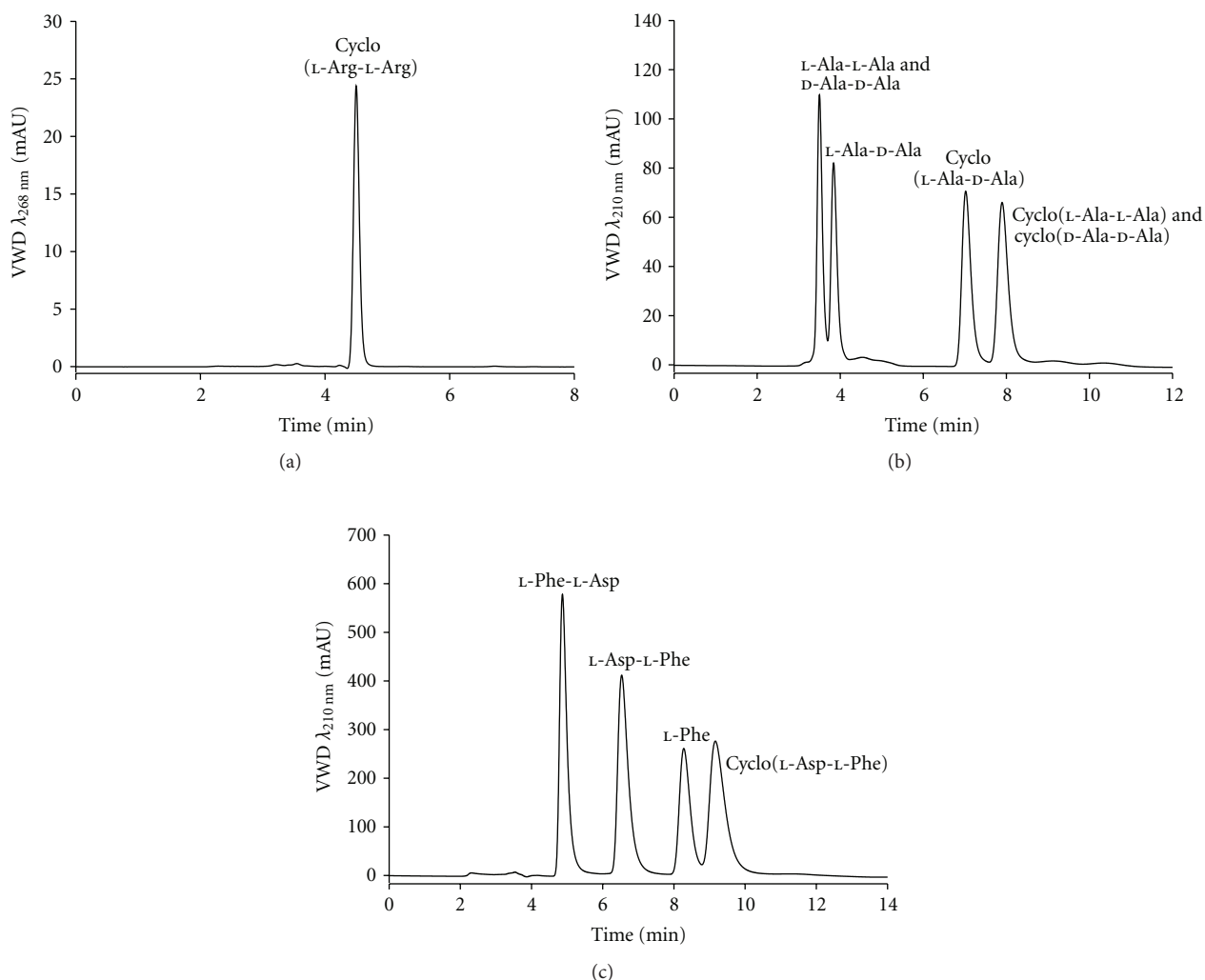


FIGURE 2: HPLC chromatograms of 1 mM standards of (a) cyclo(L-Arg-L-Arg) at 10% MeOH, 268 nm; (b) L-Ala-L-Ala and D-Ala-D-Ala, L-Ala-D-Ala, cyclo(L-Ala-D-Ala) and cyclo(L-Ala-L-Ala) and cyclo(D-Ala-D-Ala) at 10% MeOH, 210 nm; (c) L-Phe-L-Asp, L-Asp-L-Phe, L-Phe and cyclo(L-Asp-L-Phe) at 20% MeOH, 210 nm; all separations were performed at: 20°C column temperature and 0.7 mL min⁻¹ flow rate.

column temperature, and a flow rate of 0.7 mL min⁻¹ (Table 1, Figure 1(a)). In contrast to all used substances which were measured at a wavelength of 210 nm, cyclo(L-Arg-L-Arg) was detected by absorption of the guanidino groups ($\lambda = 268$ nm). Detection of cyclo(L-Arg-L-Arg) at 210 nm resulted in four peaks and it remained unclear which peak belonged to cyclo(L-Arg-L-Arg). On the contrary, detection of cyclo(L-Arg-L-Arg) at 268 nm showed one symmetric peak (Figure 2(a)).

Best separation of cyclo(DL-Ala-DL-Ala) from corresponding dipeptide DL-Ala-DL-Ala was determined with 10% MeOH as well (Table 1, Figure 1(a)). Moreover, for these racemic substances separation of the diastereomers could be performed. Cyclo(L-Ala-D-Ala) was separated from the enantiomeric pair cyclo(L-Ala-L-Ala) and cyclo(D-Ala-D-Ala), and L-Ala-D-Ala was separated from the enantiomeric pair L-Ala-L-Ala and D-Ala-D-Ala (Figure 2(b)). By measuring the enantiomers cyclo(L-Ala-L-Ala) and L-Ala-L-Ala attribution of the peaks was achieved.

It was not possible to separate cyclo(L-Asp-L-Asp) and L-Asp-L-Asp at 10% MeOH, 20°C column temperature, and a flow rate of 0.7 mL min⁻¹. Under these conditions only an R_s value of 0.62 was determined (Figure 1(d)). 10% is the lowest MeOH concentration which can be used for this column, but by decreasing column temperature to 15°C and flow rate to 0.3 mL min⁻¹ the resolution was improved to 1.04 (Table 1, Figure 1(d)).

Good separation of cyclo(L-Asp-L-Phe), L-Asp-L-Phe, L-Phe-L-Asp, and L-Phe and retention times lower than 15 min were achieved with 20% MeOH, 20°C column temperature, and a flow rate of 0.7 mL min⁻¹ (Table 1, Figure 2(c)). Even better resolution was obtained with 10% MeOH (Figure 1(b)), but under these conditions retention times were longer than 15 min.

For cyclo(L-Pro-L-Tyr), L-Pro-L-Tyr, L-Tyr-L-Pro, and L-Tyr best separation and short retention times were observed with 30% MeOH, 30°C column temperature, and a flow

TABLE 2: Linear range, linear equation, correlation coefficient R^2 , and limit of quantification (LOQ) for DKPs, dipeptides, and amino acids.

Substance	Linear range (mM)	Linear equation	R^2	LOQ (nmol per 10 μ L injection)
Cyclo(Gly-Gly)	0.010–5.00	$y = 1732x + 107$	0.9980	5.00
Gly-Gly	0.010–5.00	$y = 1355x + 124$	0.9989	10.00
Cyclo(L-Ala-D-Ala)	0.025–5.00	$y = 2188x + 93$	0.9991	5.00
Cyclo(L-Ala-L-Ala) and cyclo(D-Ala-D-Ala)	0.025–5.00	$y = 2271x + 96$	0.9991	0.25
L-Ala-L-Ala and D-Ala-D-Ala	0.025–5.00	$y = 1821x + 111$	0.9996	5.00
L-Ala-D-Ala	0.025–5.00	$y = 1642x + 60$	0.9996	5.00
Cyclo(L-Arg-L-Arg)	0.100–50.00	$y = 162x + 5$	1.0000	1.00
L-Arg-L-Arg	0.010–10.00	$y = 1611x + 86$	0.9996	5.00
Cyclo(L-Asp-L-Asp)	0.005–2.50	$y = 6855x + 307$	0.9992	5.00
L-Asp-L-Asp	0.010–5.00	$y = 5192x + 225$	0.9990	5.00
cyclo(L-Asp-L-Phe)	0.005–5.00	$y = 8227x + 162$	0.9998	0.05
L-Asp-L-Phe	0.005–5.00	$y = 8258x + 132$	0.9999	0.05
L-Phe-L-Asp	0.005–2.50	$y = 8389x + 122$	0.9994	0.50
L-Phe (20% MeOH)	0.005–5.00	$y = 5845x + 131$	0.9997	0.10
Cyclo(L-Pro-L-Tyr)	0.005–5.00	$y = 8256x + 116$	0.9999	0.05
L-Pro-L-Tyr	0.005–2.50	$y = 7164x + 87$	0.9997	1.00
L-Tyr-L-Pro	0.010–10.00	$y = 6127x + 172$	0.9997	5.00
L-Tyr	0.010–2.50	$y = 4929x + 43$	1.0000	10.00
Cyclo(Gly-L-Phe)	0.005–5.00	$y = 7662x + 165$	0.9997	0.05
Gly-L-Phe	0.005–2.50	$y = 8360x + 106$	0.9995	0.10
L-Phe-Gly	0.005–2.50	$y = 7392x + 109$	0.9993	0.10
L-Phe (30% MeOH)	0.005–2.50	$y = 6121x + 60$	0.9997	0.10

rate of 0.7 mL min⁻¹ (Table 1). Under these conditions one drawback was that L-Pro-L-Tyr was not separated from L-Tyr ($R_s = 0.22$) (Figure 1(c)); nevertheless, the main aim to separate DKP from dipeptides and amino acid was achieved. The same conditions were chosen for separation of cyclo(Gly-L-Phe) from Gly-L-Phe, L-Phe-Gly, and L-Phe (Table 1). Under these conditions separation of cyclo(Gly-L-Phe) from the corresponding dipeptides and L-Phe was possible, but not the separation of the two dipeptides and the amino acid from each other. For separation of all four substances best conditions were 10% MeOH, 20°C column temperature and a flow rate of 0.7 mL min⁻¹ with $R_s = 2.01$ for Gly-L-Phe and L-Phe, and $R_s = 4.66$ for Gly-L-Phe and L-Phe-Gly (Figure 1(c)). This resulted in a method time of about 30 min.

All DKPs could be separated from their corresponding dipeptides with a peak resolution R_s higher than 1.5, with the exception of cyclo(L-Asp-L-Asp) and L-Asp-L-Asp with R_s below 1.5.

Method validation was done under best separation conditions (see Table 1). The developed methods were validated

by using crude extract of *Paenibacillus chibensis* (DSM 329) spiked with the studied substances. This strain was chosen as biological matrix because of its reported ability to hydrolyze the DKP cyclo(L-Asp-L-Phe) [20].

3.2. Linearity and Sensitivity. The analytical results for linear range, linear equation, correlation coefficient R^2 , limit of detection (LOD) shown as lower limit of linear range and limit of quantification (LOQ) obtained from this investigation are summarized in Table 2.

LOD is in accordance with the lower limit of the linear range and is between 0.005 mM (e.g., cyclo(L-Pro-L-Tyr), cyclo(Gly-L-Phe)) and 0.100 mM for cyclo(L-Arg-L-Arg). The maximum of the linear range varied between 2.50 mM and 50.00 mM depending on the substance. For cyclo(Gly-Gly) a LOD of 5 μ g mL⁻¹ was described [19]; this equates 0.04 mM, which is higher than the LOD of 0.010 mM for cyclo(Gly-Gly) shown in this study. For cyclo(L-Asp-L-Phe) an LOD of 10 ng per 20 μ L injection [13] and 2.5 ng per 10 μ L injection [14] was described. These values equate 0.002 mM

TABLE 3: Precision, accuracy, stability, and recovery for DKPs, dipeptides, and amino acids in crude extract of DSM 329.

Substance	Intrarun precision (RSD %)	Interrun precision (RSD %)	Accuracy (RE %)	Short-term stability (%)	Freeze-thaw stability (%)	Recovery (%)
Cyclo(Gly-Gly)/Gly-Gly	1.1/0.9	0.7/2.6	4.7/7.7	100/99	97/98	97/99
Cyclo(L-Ala-L-Ala) and cyclo(D-Ala-D-Ala)/cyclo(L-Ala-D-Ala)	1.1/0.7	3.5/0.9	-0.3/8.1	99/101	99/98	99/99
L-Ala-L-Ala and D-Ala-D-Ala/L-Ala-D-Ala	1.3/1.1	3.9/1.7	6.6/2.9	100/97	101/100	100/100
Cyclo(L-Arg-L-Arg)/L-Arg-L-Arg	1.5/0.5	6.4/6.7	3.9/-0.3	102/99	101/99	100/98
Cyclo(L-Asp-L-Asp)/L-Asp-L-Asp	1.0/1.1	1.3/1.9	5.1/2.1	100/101	100/98	101/98
Cyclo(L-Asp-L-Phe)/L-Phe	1.1/1.5	3.0/3.1	2.2/2.3	102/100	98/98	100/98
L-Asp-L-Phe/L-Phe-L-Asp	2.7/2.1	9.9/1.9	2.3/0.3	94/103	99/98	99/101
Cyclo(L-Pro-L-Tyr)/L-Tyr	4.7/1.9	5.4/4.4	0.7/6.4	100/101	93/94	99/98
L-Pro-L-Tyr/L-Tyr-L-Pro	1.4/1.3	4.5/2.0	4.3/-0.1	98/98	99/98	99/98
Cyclo(Gly-L-Phe)/L-Phe	2.9/1.2	1.4/4.0	-4.2/1.8	97/100	100/100	99/100
Gly-L-Phe/L-Phe-Gly	1.3/0.9	3.4/2.7	0.3/-2.0	98/101	100/100	103/98

and 0.001 mM and thus are slightly lower than 0.005 mM detected in this study, but they are in the same range. We determined a broader linear range for cyclo(L-Asp-L-Phe) (0.005 to 5.00 mM) compared to 0.5 to 10.0 $\mu\text{g mL}^{-1}$ [14] (equates 0.002 to 0.038 mM) or 5 to 100 $\mu\text{g mL}^{-1}$ [15] (0.02 to 0.38 mM).

LOQ was defined as the lowest concentration which could still be measured with a maximum of 20% RSD for precision and $\pm 20\%$ RE for accuracy. The minimal LOQ was identified for cyclo(L-Asp-L-Phe), L-Asp-L-Phe, cyclo(L-Pro-L-Tyr), and cyclo(Gly-L-Phe) with 0.05 nmol per 10 μL injection and ranged to 10.00 nmol per 10 μL injection for Gly-Gly and L-Tyr (Table 2).

The linear equations calculated in this study strongly varied depending on the substance (Table 2). In general, the slopes for all aromatic substances were higher than for most other used substances, due to the higher UV absorption of the aromatic ring. The correlation coefficient R^2 for each substance was greater than or equal to 0.9990 within the defined linear range, except for cyclo(Gly-Gly) and Gly-Gly with 0.9980 and 0.9989, which are still acceptable values (Table 2).

3.3. Precision and Accuracy. The results for intrarun and interrun precision and accuracy are summarized in Table 3. The lowest relative standard deviation for intrarun precision was 0.5% for L-Arg-L-Arg. The highest RSD (%) for intrarun precision was measured for cyclo(L-Pro-L-Tyr) with 4.7%. For interrun precision the lowest RSD (%) value was 0.7%, detected for cyclo(Gly-Gly) and the highest RSD (%) value was 9.9% for L-Asp-L-Phe.

For substances which showed higher measured concentration than true concentration, the highest relative error for accuracy was 8.1%, detected for cyclo(L-Ala-D-Ala). The best accuracy was 0.3% RE, measured for L-Phe-L-Asp

and Gly-L-Phe. For samples which showed lower measured concentration than true concentration, best accuracy was -0.1% RE obtained for L-Tyr-L-Pro and highest RE (%) value was -4.2%, measured for cyclo(Gly-L-Phe). For all other substances accuracy was between -4.2 and 8.1% RE.

3.4. Stability and Recovery. Data measured for short-term and freeze-thaw stability and for recovery are shown in Table 3. After short-term storage at 21°C for 24 h at least 97% of each substance were still detectable except for L-Asp-L-Phe, with 94% stability. For freeze-thaw stability also 97% or more were determined for all substances apart from cyclo(L-Pro-L-Tyr) and L-Tyr, with a stability of 93% and 94%.

For all investigated DKPs, dipeptides and amino acids the recovery rate after heat treatment at 90°C for 10 min was at least 97% or greater. Thus, this procedure is efficient for enzyme inactivation in crude extract and all substances can be detected in sufficient amount.

4. Conclusions

In this work RP-HPLC methods were established for separation of seven DKPs from their corresponding linear dipeptides and in some cases the related amino acids within 15 min analysis time. This approach allows a rapid quantitative analysis of these molecules. Baseline separation with a peak resolution greater than 1.5 was achieved for separation of the six following DKPs from their corresponding linear dipeptides: cyclo(Gly-Gly), cyclo(DL-Ala-DL-Ala), cyclo(L-Arg-L-Arg), cyclo(L-Asp-L-Phe), cyclo(L-Pro-L-Tyr), and cyclo(Gly-L-Phe). Furthermore, for the racemic DKP cyclo(DL-Ala-DL-Ala) and dipeptide DL-Ala-DL-Ala a method to separate the diastereomers was established and could be applied for enantioselective analysis of these

molecules. Cyclo(L-Asp-L-Asp) could also be separated from its corresponding dipeptide, but not with baseline separation.

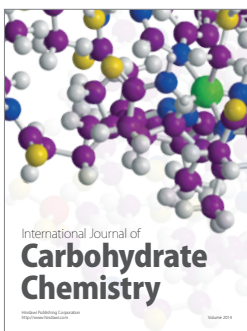
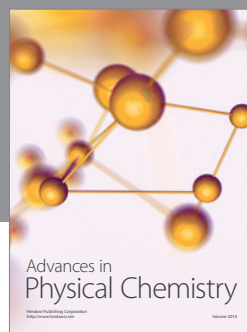
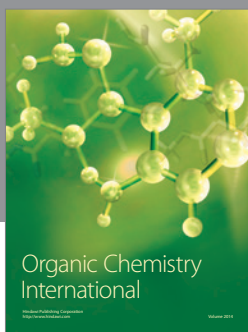
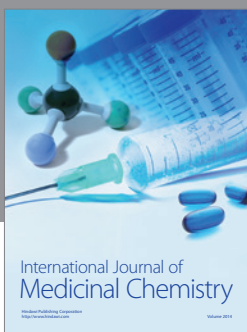
All methods were successfully validated in terms of sensitivity, linearity, intra- and interrun precision, accuracy, stability, and recovery, demonstrating their usefulness as analytical methods for the detection of DKPs, dipeptides, and amino acids in bacterial crude extract. The methodology could be extended to other analytes of interest and find application, for example, for a screening of strains exhibiting the ability to hydrolyze cyclic dipeptides to their corresponding linear dipeptides.

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