Determination of the neurotoxin 3-*N*-oxalyl-2,3-diaminopropionic acid and other free amino acids in *Lathyrus cicera* and *L. sativus* seeds by reversed-phase high-performance liquid chromatography

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Short title: Determination of ODAP and other amino acids in *Lathyrus* by HPLC

Abstract

A method for determination of the neurotoxic non-protein amino acid 3-*N*-oxalyl-2,3-diaminopropionic acid (ODAP) and other free amino acids in *Lathyrus cicera* and *Lahtyrus sativus* is presented. Seed extracts were derivatized by reaction with diethyl ethoxymethylenemalonate (DEEMM) and analysed by reversed-phase high-performance liquid chromatography (RP-HPLC). Calibration curves showed very good linearity of the response. The limits of detection (LOD) and quantification (LOQ) were 0.15 and 0.50 μM, respectively. The method has a high intra- (RSD<0.42%) and inter-repeatability (RSD= 2.01-2.33%), and a remarkable accuracy with a 99% recovery in spiked samples. The method yielded similar results in comparison with a previously established colorimetric method. The method is very easy to carry out and allows for ready analysis of large number of samples using very basic HPLC equipment because the derivatized samples are very stable and have very good chromatographic properties.

Keywords: *Lathyrus cicera*; *Lathyrus sativus*; 3-*N*-Oxalyl-2,3-diaminopropionic acid; Free amino acids; Diethyl ethoxymethylenemalonate

Introduction

The genus *Lathyrus* (Leguminosae) includes 187 species and subspecies that are found in Eurasia, North America, temperate South America, and East Africa, which are used for animal feedstock, human consumption, and for ornamental purposes (McCutchan 2003). Many of these species can grow in poor soils and in drought conditions, and are especially resistant to diseases and frost, representing a good alternative to other more demanding crops (López Bellido 1994). *L. sativus* and *L. cicera* are the most common species in the genus. Their seeds represent a good source of protein for animals and their content in antinutritional factors is not higher than the content in other commonly used feed grain legumes (Hanbury et al. 2000). The seeds of *L. sativus* are also consumed by humans in some Asian an African countries including India, Nepal, Pakistan, Bangladesh and Ethiopia, and are also used for preparation of some traditional dishes in Spain and Portugal ('gachas', 'chicharada', etc.) (Campbell 1997; Caminero Saldaña and Grajal Martín, 2009).

Nevertheless, the presence of β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) in the seeds limits the expansion of these crops. β-ODAP is a non-protein amino acid responsible for the neurolathyrism syndrome in animals and humans. Neurolathyrism is characterized by weakness of the hind limbs and paralysis or rigidity of the muscles, but only appears after consumption of large quantities of the seeds for long periods of time. The mechanism of this neurotoxicity is still not completely understood, and there is a high interindividual and interspecies variation in susceptibility to β-ODAP. Recent studies have shown that the onset and development of neurolathyrism also depends on the existence of other conditions such as a poor overall nutritional status, low availability of sulphur amino acids and reduced thiols, oxidative

stress, and the presence of other antinutritional components (Vaz Patto 2009; Enneking 2011). To our knowledge, there are no varieties of *Lathyrus* free of β-ODAP although there are some varieties low in the toxin (Van Moorhem et al. 2011; Sanchez-Vioque et al. 2009). Some breeding programs have resulted in varieties with a combination of good characteristics including low β-ODAP, high yield, appropriate phenology, and stress tolerance (Kumar et al. 2011).

The most popular method for the analysis of ODAP is the spectrophotometric determination of the product resulting from the reaction between 2,3-diaminopropanoic acid and *o*-phthaldialdehyde (OPA) in the presence of mercaptoethanol, This reaction follows the conversion of ODAP to 2,3-diaminopropanoic acid by alkaline hidrolysis (Rao 1978). Nevertheless, this colorimetric method does not discriminate between β-ODAP and α-ODAP, which is a non-toxic isomer also present in the seed. Moreover, it cannot be used with the green parts of *Lathyrus* because pigments interfere with the determination. Several methods for a selective determination of β-ODAP have been proposed, including methods based on HPLC (Chen et al. 2000; Yang et al. 2005; Thippeswamy et al. 2007; Fikre et al. 2008), capillary zone electrophoresis (Arentoft and Greirson 1995; Zhao et al. 1999a; Onar et al. 2014), enzymatic reactions (Moges and Johansson 1994), and thin layer chromatography (Paradkar et al. 2003). Some of these methods also allowing for determination of other free amino acids.

In this work, we present a method for determination of free amino acids, including α- and β-ODAP, in the seeds of *Lathyrus* sp., using precolumn derivatization by reaction with DEEMM and RP-HPLC with UV detection at 280 nm. DEEMM is a universal reagent for amino groups and has been used in amino sugar (Gómez-Sánchez et al. 1984) and amino acid (Alaiz et al. 1989) chemistry, as well as for amino acid analysis (Alaiz et al. 1992; Gomez-Alonso et al. 2007; del Campo et al. 2008; Rebane

and Horedes 2010; Rebane et al. 2011; Begoña, et al. 2013). This method has now been adapted so that it allows for determination of α- and β-ODAP and other free amino acids including homoarginine, a free amino acid which is also of interest because of its bioactive properties. The main advantages of this method are the stability of the derivatized amino acids, and its simplicity, accuracy, sensitivity, and repeatability.

Materials and methods

Plant material

L. sativus flour was purchased in a local market and *L. cicera* seeds were provided by the plant breeding project at the Centro Agrario de Albaladejito (Cuenca, Spain) or donated by Bank of Plant Germplasm of Cuenca, BPG-Cuenca (Cuenca, Spain). Seeds were collected at full maturity and were allowed to dry at room temperature before storage at -20 C in falcon tubes. From each sample, around 30 g of seeds were randomly selected and ground using a MM 301 mill (Retsch, Haan, Germany).

Reagents

Potasium tetraborate tetrahydrate, DL-2,3-diaminopropionic acid monohydrochloride, OPA, DEEMM, L-homoarginine hydrochloride, DL-2-aminobutyric acid (internal standard, I.S.), amino acid standards, water (HPLC grade), and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and 2-mercaptoethanol was purchased from Merck (Whitehouse Station, NJ, USA). Standard α- and β-ODAP were purified from *L. sativus* (Rao et al. 1964; Harrison et al. 1992).

Colorimetric determination of ODAP

ODAP was determined as described (Hussain et al. 1994). Flour samples (500 mg) were extracted twice by stirring in 5 mL ethanol:water (6:4, v/v) for 30 min. The supernatants recovered after centrifugation at 4000 rpm for 30 min were taken to 10 mL. Two mL of these supernatants were hydrolyzed in a screw cap tube by addition of 4 mL 3M KOH and incubation at 100°C for 30 min. The reaction with OPA was carried out by mixing an aliquot (0.25 mL) of the hydrolyzed extract, 0.75 mL distilled water, and 2 mL reagent (100 mg OPA and 0.2 mL 2-mercaptoethanol in 100 mL 0.5 M potasium tetraborate buffer pH 10.0). Absorbance at 425 nm was measured after incubation for 30 min at room temperature. The procedure included 3 blanks: 0.25 mL non-hydrolysed extract + 0.75 mL distilled water + 2 mL of the OPA reagent (OPA blank); 0.25 mL non-hydrolysed extract + 0.75 mL distilled water + 2 mL tetraborate buffer (sample blank); 0.25 mL hydrolysed extract + 0.75 mL distilled water + 2 mL tetraborate buffer (buffer blank). The final absorbance was given by: A = (A sample – A buffer blank) – 1/3 (A OPA blank – A sample blank). A calibration curve was made using DL-2,3-diaminopropionic acid monohydrochloride as standard and a conversion factor to ODAP of 1.69 (Aletor et al. 1994).

Chromatographic system

The HPLC system (Beckman-Coulter) consisted of a 126 solvent module, 166 detector, and IBM personal computer. Data acquisition and processing were carried out using 32

Karat 7.0 version software (Beckman-Coulter). Samples (20 μL) were injected in a reversed-phase column (Novapack C18, 300 x 3.9mm i.d., 4 μm, Waters).

Mobile phase A consisted of 25 mM glacial acetic acid and 0.02% sodium azide in water (w/v) adjusted to pH 6.0. Mobile phase B was acetonitrile. Mobile phases were filtered through a 0.45 μm membrane filter. For binary gradient elution, the program is shown in Table 1. The column was maintained at 18ºC and operated at a flow-rate of 0.9 mL/min.

Preparation of sample

Samples (4.0 mg in 1.5 ml eppendorf tubes) were stirred in ethanol:water (6:4 v/v, 1 ml) for 30 min at room temperature using a vortex mixer at full speed equipped with a tube holder (Labnet Biotecnica S.L., Madrid, Spain), and centrifuged at 8850 g for 10 min using an Eppendorf 5415R microcentrifuge (Hauppauge, NY, USA). Pellets were reextracted twice more, and the resulting supernatants were pooled and taken to dryness under nitrogen.

Precolumn derivatization

Samples were dissolved in 990 μL 1M borate buffer pH 9.0, followed by addition of 8 μL I.S. (0.424 g/L) and 2 μL DEEMM. The solution was thoroughly mixed and incubated at 50ºC for 50 min. Samples were filtered through 0.22 μm membranes before injection into the HPLC system (20 μL).

Validation of the method

Validation was carried out by determination of linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability, and accuracy (recovery) (Taverniers et al. 2004). Calibration curves were drawn by plotting the peak area ratios of analytes/internal standard against reference analyte concentrations (determined in triplicate). LOD and LOQ were calculated by injecting diluted standard solutions to determine the concentrations corresponding to a signal/noise ratio (S/N) of 3 and 10, respectively. The repeatability of the method was determined by the same analyst from the relative standard deviation (RSD) of the peak area based on 8 runs of a standard mixture over 1 day (intra-day repeatability), and from the RSD of the peak area based on 8 runs of a standard mixture on independent days (inter-day repeatability). Accuracy was tested by the standard procedure of adding three consecutive concentrations (5, 10 and 20 μM) of each type of stock solution (α- and β-ODAP and L-homoarginine hydrochloride) to a sample of seed. Non-spiked sample replicates (blank) were used to determine the initial α- and β-ODAP and L-homoarginine contents of the seed. The percentage recovery at each concentration was calculated as [(amount found in the sample spiked sample) – (amount found in the blank)/(amount added] x 100. Finally, evaluation of the method was completed by comparison with data obtained by a colorimetric method (OPA method).

Statistical analysis

The relative standard deviation (RSD) was calculated according to the formula RSD = s/μ x 100, where s is the standard deviation and μ is the average value. It was expressed

as a percentage. The Microsoft Office Excel 2003 data analysis package was used for statistical analysis.

Results

Twenty-six amino acids were separated and identified (Fig. 1A). The DEEMM derivatives of β-ODAP, α-ODAP and homoarginine eluted at 4.3, 6.0 and 16.5 min, respectively, and their peaks did not overlap with any other amino acid. Homoarginine was the major free amino acid in the seeds, although significant amounts of aspartic acid, glutamic acid, asparagine, arginine and β-ODAP (especially in *L. sativus*) were also found (Fig. 1B).

Analysis of 0.50 to 100 μM α- and β-ODAP and 0.50 to 450 μM homoarginine showed a linear response (r>0.999), low LOD (0.15 μM), and low LOQ (0.50 μM) (Table 2). Peak areas for derivatized α-ODAP, β-ODAP, and homoarginine were essentially unchanged for at least one week at room temperature as indicated by the low inter-day repeatability (RSD= 2.01-2.33%). The intra-day repeatability with a RSD below 0.42% was excellent. The accuracy of the method is also supported by the recovery of α-ODAP, β-ODAP, and homoarginine from seed extracts to which 5, 10 or 20 μM α-ODAP, β-ODAP, and homoarginine were added. About 99% recovery (RSD= 2-4%) was possible after extraction and derivatization (Table 3).

The contents of α- and β-ODAP in twenty different samples of *L.sativus* and *L. cicera* determined by the HPLC method and by the OPA colorimetric method (Hussain et al. 1994) were in general consistent (Table 4). Contents of α- and β-ODAP of *L. cicera* samples ranged from 0.017 to 0.058 g/100 g and from 0.092 to 0.302 g/100 g, respectively, that is, the content of β-ODAP was about 4 to 7 times higher than that of

α-ODAP. Sample of *L. sativus* showed a content of β-ODAP (0.53 g/100 g) clearly superior to those of *L. cicera*. However, homoarginine was the main free amino acid in *Lathyrus* sp whose contents ranged from 0.905 to 1.567 g/100 g. Other amino acids like aspartic and glutamic acids, asparagine and arginine also showed a large variability among samples.

Discussion

Precolumn derivatization of α-ODAP, β-ODAP, and standard non-protein amino acids by reaction with DEEMM resulted in derivatives that showed a very good chromatographic behavior in reversed-phase HPLC and were detected at 280 nm with no interference from the reagent (Fig. 1). Free amino acid profiles of samples showed a predominance of homoarginine, α- and β-ODAP, aspartic and glutamic acids, asparagine and arginine, and they were similar to others previously reported for these species (Fikre et al. 2008).

The α-ODAP, β-ODAP, and homoarginine DEEMM derivatives were very stable as can be deduced from the low inter-day repeatability of the method, which allows for the storage of samples for several days at room temperature before analysis thus facilitating their processing (Table 2). The low LOQ, the wide range of linearity and a high accuracy are also positive aspects of this method, especially in the screening of a large number of samples in breeding programs aimed to select seeds with a reduced content of β-ODAP.

The seeds of *Lathyrus cicera* showed a high variability in the contents of α-ODAP, β-ODAP probably due to a low degree of domestication of this species (Table 4). However, the ratio between β-ODAP and α-ODAP was within the range previously

established for these compounds (Khan et al. 1993; Moges and Johansson 1994) although the equilibrium between both depends on the temperature and the isomerization of β- to α-ODAP can be increased by heating, which constitutes a way to decrease the content of the neurotoxin (Zhao et al. 1999b).

Homoarginine was largely the most abundant free amino acid in all analyzed samples. Both positive and negative physiologic effects have been attributed to this amino acid and hence the importance of its accurate determination in the seeds of *Lathyrus* sp. The role of homoarginine as a substrate for the synthesis of nitric oxide, an important mediator of vascular tone and the inflammatory response, has been highlighted to the point that it is being proposed as a component in the diet that can promote cardiovascular health (Rao 2011). The conversion of homoarginine to lysine by the mammalian liver has been described and could help to increase the supply of this essential amino acid to the diet (Bell 2003). Nevertheless, Breitner (1988) postulated that the presence of homoarginine in gene activator-repressor histones may be a direct cause of most cancers. Homoarginine is also a modulator of nitric oxide which mediates glutamate neurotoxicity (Dawson et al. 1991).

As compared to other methods reported for determination of α-ODAP, β-ODAP, and homoarginine, the major advantages of this method are its simplicity and the stability of reagents and derivatized amino acids, which allows for accurate, easy determination of α-ODAP, β-ODAP, and homoarginine in a large number of samples using unsophisticated equipment such as a basic HPLC system with an UV detector. Other HPLC methods, which are in general superior to earlier spectrophotometric method (Rao 1978), require more sophisticated equipment and are more time consuming. Pre-column derivatization using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and UV detection has been applied for the HPLC analysis of α-ODAP, β-

ODAP, and homoarginine in plant tissues (Chen et al. 2000). The α-ODAP, β-ODAP, and homoarginine were eluted at 17.16, 13.83 and 25.55 min, using ternary gradient. These derivatives decomposed easily when they were irradiated by UV light (254 nm) at room tempearature. Analysis of dilutions of α- and β-ODAP from 0.32 to 32 nmol showed a linear response in this range with r >0.999, and LOD for both isomers were 0.01 nmol. Phenyl isothiocyanate has also been used as a pre-column derivatization reagent for HPLC determination of α-ODAP, β-ODAP, and homoarginine in plant tissue (Fikre et al. 2008), but the authors do not show data validation. Pre-column derivatization using *o*- phthalaldehyde and UV (340 nm) and fluorescence detection has been applied for the HPLC analysis of β-ODAP in plant tissues (Thippeswamy et al. 2007). The β-ODAP was eluted at 13.60 min. A linear relationship (r >0.999) between peak area and β-ODAP concentration was observed over a range of 2.4-1250 μM, when detected at 340 nm, and 0.15-625 μM, when detected by fluorescence. The LOD by fluorescence was 0.125 μM. Finally, *para*-nitrobenzyloxycarbonyl chloride has also been used as a pre-column derivatization reagent for HPLC determination of α-ODAP, β-ODAP, and homoarginine in plant tissue (Yan et al. 2005). The α-ODAP, β-ODAP, and homoarginine were eluted at 17.5, 12.1 and 4.8 min, respectively. Analysis of dilutions of α-, β-ODAP and homoargine from 10 to 250 μM showed a linear response in this range with r >0.999, and LOD of 0.44, 0.48 and 0.48 μM, respectively.

Conclusions

RP-HPLC of DEEMM derivatives allows for determination of ODAP and other non-protein amino acids that are present in the seeds of *Lathyrus*. As compared to other methods, this procedure has a number of advantages that taken together prove the value

of this new approach. Thus, this method is an extension of a well established method for determination of amino acids that is easy to carry out in any standard HPLC device, and can be used to easily process a high number of samples because the derivatized amino acids are stable even at room temperature. The analysis is based on the very good chromatographic and absorption characteristics of the DEEMM derivatives, which allow for very good resolution of the peaks, as well as very good sensitivity and repeatability.

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Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

Conflict of Interest

Cristina Megías declares that she has no conflict of interest. Isabel Cortés Giraldo declares that she has no conflict of interest. Manuel Alaiz declares that he has no conflict of interest. Julio Girón Calle declares that he has no conflict of interest. Javier Vioque declares that he has no conflict of interest. Omar Santana Méridas declares that he has no conflict of interest. David Herraiz Peñalver declares that he has no conflict of interest. Raul Sánchez Vioque declares that she has no conflict of interest.

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Figure captions

Fig. 1. HPLC analysis of the DEEMM derivatives of an amino acid standard mixture (A) and a seed extract (B). 1=Asp; 2=β-ODAP; 3=Glu; 4=α-ODAP; 5=Asn; 6=Ser; 7=Gln; 8=His; 9=Gly; 10=Thr; 11=Arg; 12=Ala; 13=homoarginine; 14=Pro; 15=I.S.; 16=Tyr; 17=ammonium ion; 18=Val; 19=Met; 20=Cys-Cys; 21=Ile; 22=Trp; 23=Leu; 24=Phe; 25=Cys; 26=Lys

Table 1

Elution gradient for the analysis of α-ODAP, β-ODAP, and other non protein amino acids. Solvent A: 25 mM glacial acetic acid, 0.02% sodium azide in water (w/v) pH 6.0. Solvent B: acetonitrile

Time (min)	Solvent A (%)	Solvent B (%)
0	96	4
3	88	12
13	88	12
30	69	31
35	69	31
40	96	4

Table 2

Precision, calibration parameters, and sensitivity of the HPLC determination of α-ODAP, β-ODAP, and homoarginine as determined by analysis of standards

Compound	Repeatability (%)		Calibration		LOQ[d] (μM)	LOD[e] (μM)
	Intra-day[a]	Inter-day[b]	R	Linear range[c] (μM)		
α-ODAP	0.35	2.01	0.9991	0.50-100	0.50	0.15
β-ODAP	0.34	1.97	0.9992	0.50-100	0.50	0.15
Homoarginine	0.41	2.33	0.9994	0.50-450	0.50	0.15

[a] RSD of peak area based on 8 runs of a standard mixture over 1 day.

[b] RSD of peak area based on 8 runs of a standard mixture on independent days.

[c] Concentration range between the limit of quantification and the upper linear limit.

[d] Limit of quantification: signal/noise ratio = 10.

[e] Limit of detection: signal/noise ratio = 3.

Table 3

Recovery (mean and RSD) of the HPLC method for determination of free amino acids in *L. cicera* (sample 20 of the Table 4)

Compound	Initial content (μM)	First concentration added (5 μM)		Second concentration added (10 μM)		Third concentration added (20 μM)	
		Content I (μM)	Recovery	Content II (μM)	Recovery	Content III (μM)	Recovery
α-ODAP	4.22	9.17	99.00 (3.98)	14.13	99.10 (3.05)	24.09	99.35 (2.64)
β-ODAP	24.04	29.01	99.40 (3.15)	33.97	99.30 (2.44)	43.93	99.45 (2.36)
Homoarginine	116.54	121.50	99.20 (2.79)	126.46	99.20 (2.32)	136.37	99.15 (2.35)

Table 4

Contents (g / 100 g seed dry weight) of α-ODAP, β-ODAP, homoarginine and other free amino acids in *L. sativus* and *L. cicera* seeds. Data are the mean of three determination ± standard deviation

Sample	Species	BPG-CU [a] code	α-ODAP (HPLC)	β-ODAP (HPLC)	ODAP [b] (OPA [c])	Homoarginine
1	*L. cicera*	-	0.026 ± 0.001	0.173 ± 0.003	0.210 ± 0.007	1.261 ± 0.032
2	*L. cicera*	-	0.027 ± 0.001	0.174 ± 0.003	0.211 ± 0.008	1.479 ± 0.063
3	*L. cicera*	-	0.018 ± 0.001	0.092 ± 0.003	0.120 ± 0.001	1.292 ± 0.014
4	*L. cicera*	-	0.018 ± 0.001	0.095 ± 0.002	0.121 ± 0.003	1.253 ± 0.093
5	*L. cicera*	-	0.017 ± 0.000	0.115 ± 0.002	0.140 ± 0.001	0.905 ± 0.019
6	*L. cicera*	-	0.024 ± 0.002	0.143 ± 0.004	0.172 ± 0.005	1.117 ± 0.028
7	*L. cicera*	-	0.026 ± 0.001	0.151 ± 0.002	0.182 ± 0.001	1.108 ± 0.055
8	*L. cicera*	-	0.026 ± 0.001	0.161 ± 0.001	0.190 ± 0.003	1.286 ± 0.017
9	*L. cicera*	-	0.027 ± 0.001	0.164 ± 0.004	0.201 ± 0.001	1.130 ± 0.064
10	*L. sativus*	-	0.067 ± 0.001	0.528 ± 0.006	0.591 ± 0.007	1.206 ± 0.016
11	*L. cicera*	BCU000260	0.026 ± 0.001	0.137 ± 0.006	0.154 ± 0.001	1.280 ± 0.041
12	*L. cicera*	BCU000149	0.032 ± 0.002	0.123 ± 0.001	0.161 ± 0.013	1.089 ± 0.076
13	*L. cicera*	BCU000114	0.023 ± 0.000	0.141 ± 0.001	0.170 ± 0.001	1.077 ± 0.050
14	*L. cicera*	BCU000160	0.041 ± 0.003	0.206 ± 0.010	0.250 ± 0.001	1.375 ± 0.061
15	*L. cicera*	BCU000136	0.042 ± 0.003	0.232 ± 0.011	0.271 ± 0.001	1.269 ± 0.081
16	*L. cicera*	BCU000222	0.051 ± 0.001	0.246 ± 0.009	0.290 ± 0.005	1.274 ± 0.010
17	*L. cicera*	BCU000155	0.042 ± 0.000	0.256 ± 0.003	0.310 ± 0.002	1.317 ± 0.049
18	*L. cicera*	BCU000147	0.058 ± 0.003	0.268 ± 0.013	0.333 ± 0.013	1.172 ± 0.043
19	*L. cicera*	BCU000172	0.047 ± 0.002	0.273 ± 0.010	0.330 ± 0.001	1.432 ± 0.055
20	*L. cicera*	BCU000181	0.053 ± 0.001	0.302 ± 0.007	0.352 ± 0.001	1.567 ± 0.033

[a] BPG-CU, Bank of Plant Germplasm of Cuenca

[b] α-ODAP + β-ODAP

[c] OPA is the *o*-phthalaldehyde method (colorimetric method)

Table 4 (continued)

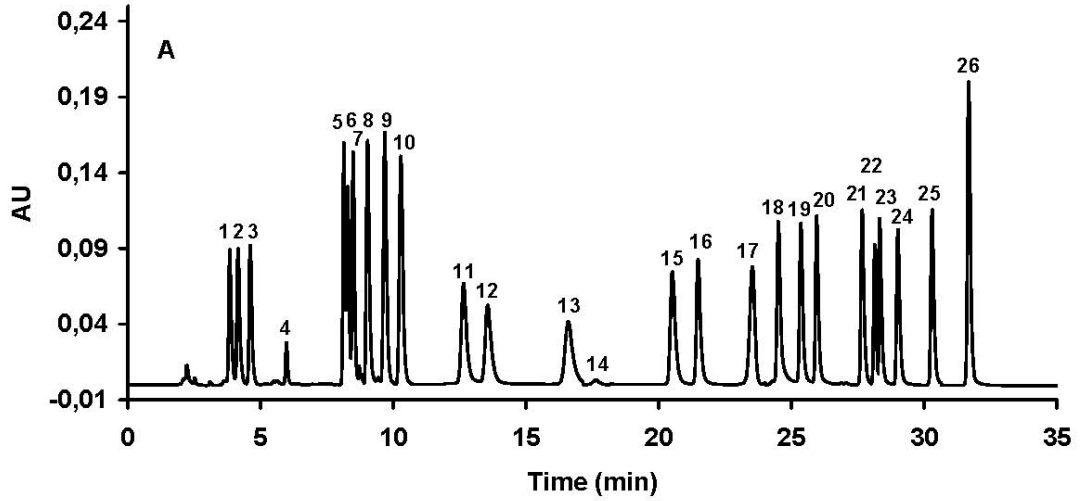
Asp	Glu	Asn	Ser	Gln	His	Gly
0.087 ± 0.000	0.114 ± 0.002	0.136 ± 0.003	0.000 ± 0.000	0.000 ± 0.000	0.004 ± 0.000	0.006 ± 0.000
0.094 ± 0.009	0.145 ± 0.012	0.148 ± 0.014	0.000 ± 0.000	0.000 ± 0.000	0.006 ± 0.000	0.006 ± 0.000
0.080 ± 0.000	0.088 ± 0.013	0.175 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	0.002 ± 0.000	0.006 ± 0.000
0.080 ± 0.003	0.076 ± 0.002	0.177 ± 0.008	0.000 ± 0.000	0.000 ± 0.000	0.002 ± 0.000	0.005 ± 0.000
0.076 ± 0.001	0.129 ± 0.000	0.134 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	0.007 ± 0.000	0.004 ± 0.000
0.085 ± 0.001	0.130 ± 0.004	0.110 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	0.010 ± 0.000	0.006 ± 0.000
0.076 ± 0.004	0.107 ± 0.010	0.109 ± 0.005	0.000 ± 0.000	0.000 ± 0.000	0.003 ± 0.000	0.005 ± 0.000
0.071 ± 0.001	0.113 ± 0.002	0.065 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	0.002 ± 0.000	0.005 ± 0.000
0.065 ± 0.005	0.106 ± 0.007	0.064 ± 0.004	0.000 ± 0.000	0.000 ± 0.000	0.002 ± 0.000	0.003 ± 0.000
0.059 ± 0.002	0.084 ± 0.003	0.108 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	0.004 ± 0.000	0.009 ± 0.000
0.101 ± 0.004	0.105 ± 0.004	0.058 ± 0.002	0.000 ± 0.000	0.000 ± 0.000	0.016 ± 0.001	0.003 ± 0.000
0.084 ± 0.004	0.145 ± 0.007	0.090 ± 0.005	0.000 ± 0.000	0.000 ± 0.000	0.012 ± 0.001	0.008 ± 0.001
0.086 ± 0.003	0.102 ± 0.004	0.079 ± 0.003	0.000 ± 0.000	0.000 ± 0.000	0.005 ± 0.001	0.002 ± 0.000
0.066 ± 0.004	0.103 ± 0.008	0.112 ± 0.007	0.000 ± 0.000	0.000 ± 0.000	0.009 ± 0.001	0.005 ± 0.000
0.056 ± 0.002	0.090 ± 0.006	0.074 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	0.008 ± 0.001	0.005 ± 0.000
0.063 ± 0.001	0.111 ± 0.002	0.119 ± 0.003	0.000 ± 0.000	0.000 ± 0.000	0.014 ± 0.000	0.005 ± 0.000
0.057 ± 0.004	0.092 ± 0.001	0.153 ± 0.006	0.000 ± 0.000	0.000 ± 0.000	0.014 ± 0.000	0.006 ± 0.000
0.055 ± 0.002	0.108 ± 0.003	0.070 ± 0.002	0.000 ± 0.000	0.000 ± 0.000	0.011 ± 0.000	0.007 ± 0.000
0.061 ± 0.003	0.087 ± 0.004	0.107 ± 0.006	0.000 ± 0.000	0.000 ± 0.000	0.006 ± 0.000	0.003 ± 0.000
0.072 ± 0.003	0.113 ± 0.002	0.109 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	0.008 ± 0.000	0.003 ± 0.000

Table 4 (continued)

Thr	Arg	Ala	Pro	Tyr	Val	Met
0.006 ± 0.000	0.073 ± 0.000	0.009 ± 0.000	0.000 ± 0.000	0.001 ± 0.000	0.008 ± 0.001	0.000 ± 0.000
0.009 ± 0.000	0.037 ± 0.000	0.014 ± 0.001	0.000 ± 0.000	0.002 ± 0.000	0.008 ± 0.000	0.000 ± 0.000
0.013 ± 0.000	0.100 ± 0.000	0.014 ± 0.000	0.000 ± 0.000	0.003 ± 0.000	0.011 ± 0.000	0.000 ± 0.000
0.011 ± 0.000	0.088 ± 0.006	0.009 ± 0.000	0.000 ± 0.000	0.002 ± 0.000	0.010 ± 0.000	0.000 ± 0.000
0.003 ± 0.000	0.025 ± 0.001	0.011 ± 0.000	0.000 ± 0.000	0.002 ± 0.000	0.005 ± 0.000	0.000 ± 0.000
0.006 ± 0.000	0.040 ± 0.000	0.009 ± 0.000	0.000 ± 0.000	0.004 ± 0.000	0.008 ± 0.000	0.000 ± 0.000
0.005 ± 0.000	0.046 ± 0.002	0.010 ± 0.001	0.000 ± 0.000	0.002 ± 0.000	0.005 ± 0.000	0.000 ± 0.000
0.005 ± 0.000	0.021 ± 0.002	0.009 ± 0.001	0.000 ± 0.000	0.003 ± 0.000	0.005 ± 0.001	0.000 ± 0.000
0.004 ± 0.000	0.013 ± 0.001	0.007 ± 0.000	0.000 ± 0.000	0.002 ± 0.000	0.005 ± 0.000	0.000 ± 0.000
0.009 ± 0.000	0.107 ± 0.002	0.018 ± 0.001	0.000 ± 0.000	0.002 ± 0.000	0.006 ± 0.000	0.000 ± 0.000
0.007 ± 0.000	0.012 ± 0.000	0.004 ± 0.000	0.000 ± 0.000	0.001 ± 0.000	0.004 ± 0.000	0.000 ± 0.000
0.013 ± 0.000	0.168 ± 0.012	0.036 ± 0.002	0.000 ± 0.000	0.003 ± 0.000	0.013 ± 0.000	0.000 ± 0.000
0.005 ± 0.000	0.034 ± 0.002	0.005 ± 0.000	0.000 ± 0.000	0.001 ± 0.000	0.007 ± 0.000	0.000 ± 0.000
0.025 ± 0.001	0.154 ± 0.002	0.005 ± 0.000	0.000 ± 0.000	0.002 ± 0.000	0.008 ± 0.000	0.000 ± 0.000
0.021 ± 0.001	0.149 ± 0.003	0.010 ± 0.001	0.000 ± 0.000	0.002 ± 0.000	0.006 ± 0.000	0.000 ± 0.000
0.025 ± 0.000	0.134 ± 0.003	0.012 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.009 ± 0.000	0.000 ± 0.000
0.026 ± 0.001	0.176 ± 0.007	0.012 ± 0.000	0.000 ± 0.000	0.001 ± 0.000	0.007 ± 0.000	0.000 ± 0.000
0.021 ± 0.000	0.140 ± 0.009	0.019 ± 0.001	0.000 ± 0.000	0.002 ± 0.000	0.007 ± 0.000	0.000 ± 0.000
0.019 ± 0.000	0.142 ± 0.007	0.012 ± 0.001	0.000 ± 0.000	0.002 ± 0.000	0.012 ± 0.001	0.000 ± 0.000
0.024 ± 0.000	0.165 ± 0.005	0.013 ± 0.001	0.000 ± 0.000	0.002 ± 0.000	0.015 ± 0.001	0.000 ± 0.000

Table 4 (continued)

Cys-Cys	Ile	Trp	Leu	Phe	Cys	Lys
0.000 ± 0.000	0.001 ± 0.000	0.000 ± 0.000	0.036 ± 0.000	0.005 ± 0.000	0.001 ± 0.000	0.007 ± 0.000
0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.003	0.024 ± 0.000	0.004 ± 0.000	0.000 ± 0.000	0.008 ± 0.000
0.000 ± 0.000	0.002 ± 0.000	0.000 ± 0.003	0.026 ± 0.000	0.008 ± 0.000	0.001 ± 0.000	0.009 ± 0.000
0.000 ± 0.000	0.002 ± 0.000	0.000 ± 0.003	0.022 ± 0.000	0.008 ± 0.000	0.000 ± 0.000	0.008 ± 0.000
0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.003	0.009 ± 0.000	0.002 ± 0.000	0.000 ± 0.000	0.005 ± 0.000
0.000 ± 0.000	0.001 ± 0.000	0.000 ± 0.003	0.026 ± 0.000	0.007 ± 0.000	0.000 ± 0.000	0.006 ± 0.000
0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.003	0.024 ± 0.000	0.004 ± 0.000	0.000 ± 0.000	0.006 ± 0.000
0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.003	0.022 ± 0.000	0.005 ± 0.000	0.000 ± 0.000	0.006 ± 0.000
0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.003	0.019 ± 0.001	0.005 ± 0.000	0.002 ± 0.000	0.004 ± 0.000
0.000 ± 0.000	0.002 ± 0.000	0.000 ± 0.003	0.021 ± 0.001	0.016 ± 0.000	0.000 ± 0.000	0.007 ± 0.000
0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.003	0.032 ± 0.001	0.003 ± 0.000	0.000 ± 0.000	0.004 ± 0.000
0.000 ± 0.000	0.003 ± 0.000	0.000 ± 0.003	0.015 ± 0.001	0.009 ± 0.000	0.000 ± 0.000	0.009 ± 0.000
0.000 ± 0.000	0.003 ± 0.000	0.000 ± 0.003	0.042 ± 0.002	0.003 ± 0.000	0.001 ± 0.000	0.006 ± 0.000
0.000 ± 0.000	0.002 ± 0.000	0.000 ± 0.003	0.010 ± 0.002	0.006 ± 0.000	0.000 ± 0.000	0.008 ± 0.000
0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.003	0.009 ± 0.000	0.005 ± 0.000	0.000 ± 0.000	0.007 ± 0.000
0.000 ± 0.000	0.002 ± 0.000	0.000 ± 0.003	0.012 ± 0.000	0.004 ± 0.000	0.000 ± 0.000	0.008 ± 0.000
0.000 ± 0.000	0.002 ± 0.000	0.000 ± 0.003	0.014 ± 0.000	0.005 ± 0.000	0.002 ± 0.000	0.009 ± 0.000
0.000 ± 0.000	0.002 ± 0.000	0.000 ± 0.003	0.012 ± 0.000	0.006 ± 0.000	0.002 ± 0.000	0.009 ± 0.000
0.000 ± 0.000	0.001 ± 0.000	0.000 ± 0.003	0.011 ± 0.001	0.005 ± 0.000	0.000 ± 0.000	0.006 ± 0.000
0.000 ± 0.000	0.001 ± 0.000	0.000 ± 0.003	0.017 ± 0.001	0.005 ± 0.000	0.000 ± 0.000	0.008 ± 0.000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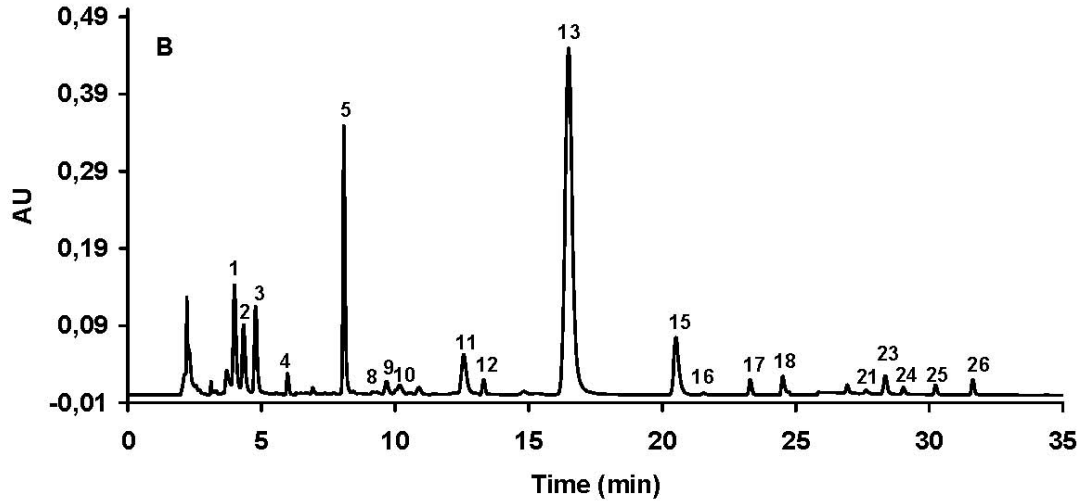
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