Wang, Qiqin et al., 2016. Separation of N-derivatized di- and tri-peptide stereoisomers by micro-liquid chromatography using a quinidine-based monolithic column – Analysis of l-carnosine in dietary supplements. Journal of Chromatography A, 1428, pp.176–184.

Title: Separation of N-derivatized di- and tri-peptide stereoisomers by micro-liquid chromatography using a quinidine-based monolithic column - Analysis of L-carnosine in dietary supplements

Author: Qiqin Wang Elena Sánchez-López Hai Han Huihui Wu Peijie Zhu Jacques Crommen Maria Luisa Marina Zhengjin Jiang

PII: S0021-9673(15)01300-X
DOI: http://dx.doi.org/doi:10.1016/j.chroma.2015.09.016
Reference: CHROMA 356838

To appear in: *Journal of Chromatography A*

Received date: 11-6-2015
Revised date: 2-9-2015
Accepted date: 5-9-2015

Please cite this article as: Q. Wang, E. Sánchez-López, H. Han, H. Wu, P. Zhu, J. Crommen, M.L. Marina, Z. Jiang, Separation of N-derivatized di- and tri-peptide stereoisomers by micro-liquid chromatography using a quinidine-based monolithic column - Analysis of L-carnosine in dietary supplements, *Journal of Chromatography A* (2015), http://dx.doi.org/10.1016/j.chroma.2015.09.016

Separation of N-derivatized di- and tri-peptide stereoisomers by micro-liquid chromatography using a quinidine-based monolithic column - Analysis of L-carnosine in dietary supplements

Qiqin Wang[1,2#], Elena Sánchez-López[2#], Hai Han[1], Huihui Wu[1], Peijie Zhu[1], Jacques Crommen[1,3], Maria Luisa Marina[2,*], Zhengjin Jiang[1,*]

[1]*Department of Pharmacy and Guangdong Province Key Laboratory of Pharmacodynamic Constituents of Traditional Chinese Medicine & New Drug Research, Jinan University, Guangzhou 510632, China*

[2] *Department of Analytical Chemistry, Physical Chemistry and Chemical Engineering, Faculty of Biology, Environmental Sciences and Chemistry, University of Alcalá, Ctra. Madrid—Barcelona, Km. 33.600, Alcalá de Henares, 28871 Madrid, Spain*

[3]*Laboratory of Analytical Pharmaceutical Chemistry, Department of Pharmaceutical Sciences, University of Liege, CHU B36, B-4000 Liege, Belgium*

* Corresponding authors:

E-mail address: jzjjackson@hotmail.com (Zhengjin Jiang). Tel.:+86 2085223604

Email address: mluisa.marina@uah.es (M.Luisa Marina). Tel.: +34 91 8854935; fax: +34 91 8854971

[#] *These authors contributed equally to this work.*

Abstract

In the present study, a new analytical methodology was developed enabling the enantiomeric determination of *N*-derivatized di- and tri-peptides in dietary supplements using chiral micro-LC on a monolithic column consisting of poly(O-9-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-*co*-2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate) (poly(MQD-*co*-HEMA-*co*-EDMA)). After optimization of the mobile phase conditions, a baseline resolution of the stereoisomers of 24 out of 53 *N*-derivatized di- and tri-peptides was obtained. 3,5-Dinitrobenzoyl- and 3,5-dichlorobenzoyl-peptide stereoisomers were separated with exceptionally high selectivity and resolution. The monolithic column was then applied to the quantitative analysis of L-carnosine and its enantiomeric impurity in three different commercial dietary supplements. Method validation demonstrated satisfactory results in terms of linearity, precision, selectivity, accuracy and limits of detection and quantification. The determined amounts of L-carnosine in commercial formulations were in agreement with the labeled content for all analyzed samples, and the enantiomeric impurity was found to be below the limit of detection (LOD), showing the potential of the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column as a reliable tool for the quality control of L-carnosine in dietary supplements by micro-LC.

Keywords: carnosine / dietary supplements / enantioseparation / quinidine-based monolithic column / small peptides

43

1. Introduction

The stereochemistry of small peptides (di- and tripeptides) can profoundly influence their biological and pharmacological activities. For example, $_{L}$-carnosine (β-alanyl-$_{L}$-histidine) is widely distributed in mammalian tissues [1-2] and exhibits numerous biochemical properties including pH buffering [2-3], antioxidant capacity [3-4], inhibition of forming advanced glycoxidation and lipoxidation end-products [3], etc. On the contrary, its optical antipode $_{D}$-carnosine cannot be hydrolyzed by carnosinases (a specific serum dipeptidase that rapidly hydrolyzes $_{L}$-carnosine in vivo), making it possible to cross the blood-brain barrier and maintain the same radical-quenching activity as $_{L}$-carnosine in vitro [5-6]. Hence, investigations of the stereochemical composition of small peptides are an important aspect in the field of peptide analysis [7-12]. To the best of our knowledge, most reports describing studies of the discrimination of small peptide stereoisomers have usually focused on the separation of either enantiomers or diastereomers, while papers dealing with both kinds of stereoisomers are limited [7-13]. This could be related to an obvious lack of appropriate functionalities to interact with the chiral selectors (CSs) and the complexity of the stereochemical composition of small peptides [11,13]. In order to enhance detection sensitivity and improve the intermolecular interaction between small peptides and CSs, additional interaction sites could be introduced through the derivatization of amino groups, using reagents such as 2,4-dinitrophenyl (DNP) fluoride, 3,5-dinitrobenzoyl (3,5-DNB), 9-fluorenylmethoxycarbonyl (FMOC), 3,5-dinitrobenzyloxycarbonyl (3,5-DNZ) or carbazole-9-carbonyl (CC) chlorides, etc [14]. Furthermore, a wide array of analytical techniques and methods were developed for the determination of small peptide stereochemical composition [11].

High-performance liquid chromatography (HPLC) is the most commonly employed

separation technique for analyzing the stereochemical composition of small peptides [11,13,14], besides capillary electrophoresis (CE). In previous studies, different chiral stationary phases (CSPs) have been evaluated for the separation of all stereoisomers of small peptides by HPLC, such as those containing crown ethers [15-17], macrocylic antibiotics [18-19], cinchona alkaloids [13-14] or cyclodextrins [20-21] as chiral selectors. In particular, cinchona alkaloid based CSPs have received great attention due to their excellent stereoselectivity towards *N*-derivatized small peptides [13-14]. Therefore, it is of high interest to systemically evaluate their enantio- and diastereoselectivity toward a wide range of *N*-derivatized small peptides since only a few peptide stereoisomers have been tested with these CSPs in HPLC [13-14,22-26], capillary electrochromatography (CEC) [27], mass spectrometry (MS) [28-29], and CE [30].

In the last two decades, monolithic columns have proved to be an effective alternative to packed columns and have attracted considerable interest owing to their facile preparation methodology and good column characteristics, such as permeability and efficiency [31-34]. In our recent study [31], the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column was systemically re-optimized according to Lämmerhofer *et al.* [35-36] with some modifications. This column exhibited excellent enantioselectivity and good efficiency in the micro-LC mode for a wide range of *N*-derivatized amino acids [31]. However, the applicability of the poly(MQD-*co*-HEMA-*co*-EDMA) monolith for the stereoselective analysis of small peptides have not been reported so far.

In the present study, the enantio- and diastereoselective potential of the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column was systematically evaluated for the micro-LC separation of a series of *N*-derivatized di- and tri-peptide

stereoisomers. The influence of the apparent pH of the mobile phase, the organic solvent content, and the buffer concentration on the chiral separation of di- and tri-peptide derivatives has also been investigated. Finally, a method based on this monolithic column was developed and evaluated for the quantitative analysis of the dipeptide carnosine, labeled as a pure enantiomer ($_L$-carnosine) in several dietary supplements.

2. Materials and methods

2.1. Reagents and samples.

All reagents employed were of analytical grade. Acetonitrile (ACN), methanol (MeOH), acetic acid, and chloroform ($CHCl_3$) were obtained from Scharlau (Barcelona, Spain). Boric acid and pentane were purchased from Sigma (St. Louis, MO, USA), while ammonium acetate was from Merck (Darmstadt, Germany). 9-Fluorenylmethoxycarbonyl (FMOC) chloride and triethylamine were obtained from Fluka (Buchs, Switzerland). Propylene oxide, 3,5-dinitrobenzoyl (3,5-DNB) chloride, 3,5-dichlorobenzoyl (3,5-DClB) chloride, 3,5-dimethoxybenzoyl (3,5-DMB) chloride, DL-Alanyl-DL-Alanine (DL-Ala-DL-Ala), DL-Alanyl-DL-Leucine (DL-Ala-DL-Leu), DL-Alanyl-DL-Methionine (DL-Ala-DL-Met), DL-Alanyl-DL-Valine (DL-Ala-DL-Val), DL-Alanyl-DL-Phenylalanine (DL-Ala-DL-Phe), DL-Leucyl-DL-Valine (DL-Leu-DL-Val), DL-Leucyl-Glycine (DL-Leu-Gly), DL-Leucyl-DL-Phenylalanine (DL-Leu-DL-Phe), DL-Leucyl-Glycyl-Glycine (DL-Leu-Gly-Gly), DL-Leucyl-Glycyl-DL-Phenylalanine (DL-Leu-Gly-DL-Phe), Glycyl-DL-norLeucine (Gly-DL-norLeu), Glycyl-DL-norValine (Gly-DL-norVal), and $_L$-carnosine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). DL-Leucyl-DL-Alanine

(DL-Leu-DL-Ala) was obtained from Bachem (Switzerland). $_{D}$-Carnosine was a generous gift from Flamma S.p.A (Chignolo di'Isola, Bergamo, Italy). Dietary supplements containing $_{L}$-carnosine were commercially available and obtained in several health food shops in Alcalá de Henares, Spain. Distilled water was deionized using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Apparatus.

A detailed description of the preparation of the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column for micro-LC by in situ copolymerization of MQD, HEMA, and EDMA, can be found in an earlier paper [31]. All micro-LC experiments were performed on a laboratory self-assembled system that consisted of a Shimadzu LC-10AS pump (Shimadzu, Kyoto, Japan), a UV-Vis detector model 200 (Linear instruments, Fremont, California, USA), and a Valco four-port injection valve with a 20 nL internal loop (Houston, TX, USA). To reduce the flow and pressure inside the capillary column, a T-shaped stainless steel piece (Cheminert, Valco Instruments Houston, Texas, USA) was employed to connect the pump to the injection valve via a split-flow capillary (dimensions: 150 mm × 25 μm I.D). Data acquisition and data handling were performed using a N2000 chromatography workstation (Science Technology, Hangzhou, China). The pH values were measured with a 744 pH-meter from Metrohm (Herisau, Switzerland).

2.3. Chromatographic conditions.

Unless otherwise stated, the mobile phase was a mixture of 0.1 M ammonium acetate/ACN (35/65, v/v). The apparent pH of the mixture was adjusted to the desired pH with acetic acid. The total flow rate was set to 20 μL/min. The mobile phase for the separation of FMOC-derivatized peptides was a mixture of ACN/MeOH (35/65, v/v) containing 0.1 M acetic acid and 4 mM of triethylamine. The separation of

144 FMOC-derivatized peptides was performed at a total flow rate of 10 μL/min. The
145 mobile phase was filtered through a 0.22-μm membrane and degassed before use. The
146 injection volume of all samples was 20 nL and the analytes were all monitored at a
147 wavelength of 254 nm, using on-column detection.

148 **2.4. Derivatization of small peptides in standard solutions and dietary**
149 **supplements.**

150 Three different L-carnosine dietary supplement products were obtained from health
151 food shops in Alcalá de Henares, Spain. Sample 1 was a supplement tablet containing
152 500 mg L-carnosine plus some excipients like cellulose, stearic acid and silica.
153 Samples 2 and 3 were capsules containing only 500 mg L-carnosine as the active
154 ingredient. A stock solution of each product was prepared by grinding tablets or the
155 content of emptied capsules to a fine powder and transferring a certain amount of
156 powder into a 50 mL conical flask to which 0.2 M borate buffer (pH 9.0) was added.
157 After weighing the flask, the solution was stirred magnetically for 30 min and
158 sonicated for 30 min to dissolve L-carnosine completely. After removal of the stir bar,
159 a slight amount of borate buffer was added into the flask in order to maintain its total
160 weight constant. The solution was shaken and undissolved ingredients were
161 removed by centrifugation and filtration. The theoretical L-carnosine concentration of
162 the solutions was 40 mM, on the basis of the label declarations.

163 The *N*-derivatized di- or tri-peptides were synthesized according to the literature [37],
164 except for the FMOC derivatives. For example, to 0.152 mmol of peptide standard or
165 dietary supplement suspended in 10 mL of dry $CHCl_3$, 0.167 mmol of
166 3,5-dinitrobenzoyl chloride and 0.172 mmol of propylene oxide were added. After
167 sonication for 1 h at room temperature, the solvent was evaporated under nitrogen, the
168 crude *N*-(3,5-dinitrobenzoy1) peptide was dissolved in methanol and the solution was

169 filtered to remove any insoluble residue [37]. As described previously [38-39],
170 peptides were amidated by 9-fluorenylmethoxycarbonyl chloride in aqueous solution
171 to afford FMOC derivatives. Briefly, 300 μL of 60 mM FMOC chloride were mixed
172 with 300 μL of 20 mM peptide standard or dietary supplement solution in 0.2 M
173 borate buffer (pH 9.0). The reacted mixture was kept at room temperature for 2 min,
174 and was then extracted with 600 μL of pentane to remove the reagent excess. Finally,
175 the FMOC-peptide solution was diluted 10 times with mobile phase prior to injection
176 [38-39].

177 **2.5. Data treatment and calculations**

178 The resolution (R_s) between adjacent peaks was determined according to the
179 following equation:

180

$$R_s = 1.18 \frac{t_{R2} - t_{R1}}{w_{1/2,1} + w_{1/2,2}}$$

181

182 where t_{R1}, t_{R2} are the retention times of the adjacent peaks, respectively, and $w_{1/2,1}$ and
183 $w_{1/2,2}$ are their corresponding widths at half-height [40].

184 The enantioselectivity (α) and the theoretical plate number (N) were obtained
185 according to standard equations from the literature [31,41-42]. Experimental data
186 analysis was performed using Excel Microsoft 2007 and Origin 7.5. A comparison of
187 the slopes of regression lines was based on F- and t-tests using Statgraphics Plus 5.0.

188

189 **3. Results and Discussion**

190 **3.1. Investigation of the enantio- and diastereoselectivity of the**
191 **poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column toward *N*-derivatized di-**
192 **and tri-peptides**

193 In order to systemically evaluate the discrimination power of the

poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column in the micro-LC mode for *N*-derivatized di- and tri-peptide stereoisomers, nine *N*-protected small peptides were employed as test analytes (DL-Ala-DL-Ala, DL-Ala-DL-Leu, and DL-Leu-DL-Ala, with three different derivatization agents, 3,5-DNB, 3,5-DClB, and 3,5-DMB chlorides). The structures of the different *N*-protecting groups and small peptides studied in this paper are presented in **Fig. S1**. The influence of the apparent pH of the mobile phase, the organic solvent and buffer concentrations were especially investigated.

The apparent pH is one of the most critical factors in the optimization of the mobile phase composition. Different apparent pH values were tested (4.0, 5.0, 5.3, and 6.0) in a mobile phase made of 0.05 M ammonium acetate/ACN (35/65, v/v). As can be seen in **Table S1**, the retention factors of the nine *N*-derivatized dipeptides dramatically increased as the mobile phase pH increased from 4.0 to 6.0. However, the increase in retention was somewhat more pronounced for the stereoisomers of the dipeptide derivatives in positions 1 and 4 in the chromatograms, so that the $\alpha_{1/2}$ values had a slight tendency to decrease with increasing pH while the opposite effect was observed for the $\alpha_{3/4}$ values (**Table S1**). Moreover, the stereoisomers of all dipeptide derivatives could not be completely separated at pH 6.0. The best compromise with respect to overall selectivity and resolution was obtained at pH 5.3. At this apparent pH, a baseline separation was observed for the four stereoisomers of 3,5-DNB-DL-Ala-DL-Leu, 3,5-DNB-DL-Leu-DL-Ala, 3,5-DClB-DL-Ala-DL-Leu, 3,5-DClB-DL-Leu-DL-Ala and 3,5-DMB-DL-Leu-DL-Ala. This positive effect might be attributed to optimum electrostatic interaction between the positively charged tertiary nitrogen of quinidine on the monolithic CSP and the negatively charged carboxylate function of the *N*-derivatized dipeptides at pH 5.3. Furthermore,

according to Lindner et al., the electrostatic interactions would keep the chiral selector and the analyte close enough, so that other intermolecular interactions, such as additional stereoselective hydrogen bonding, π-π and steric interactions, could take place and contribute to the separation of the dipeptide stereoisomers [13,26,43-46]. Therefore, a mobile phase apparent pH of 5.3 was selected for further experiments.

The organic modifier proportion in the mobile phase is another important parameter in the optimization of the separation of the stereoisomers of *N*-derivatized small peptides. As shown in **Table S2**, the selectivities remained almost constant while the retention factors and the resolution values for the stereoisomers of the nine tested analytes decreased when the ACN content was increased from 55 to 75%. These results indicated the existence of a hydrophobic retention on the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic stationary phase under these separation conditions. In order to make a trade-off between resolution and analysis time, a mobile phase containing 65% ACN was selected for further studies.

The influence of the ammonium acetate concentration in the mobile phase on retention, selectivity and resolution was also studied by varying the buffer concentration from 0.05 to 0.15 M, while the other separation conditions were kept constant (**Table S3**). No significant influence on selectivity was observed for the stereoisomers of the nine *N*-derivatized small peptides over this concentration range, but their retention factors and resolution values increased with decreasing buffer concentration. These results seem to indicate that electrostatic interactions contribute to the retention of the *N*-derivatized small peptides. Finally, a 0.1 M concentration of ammonium acetate was chosen as a good compromise between resolution and analysis time.

Unless otherwise stated, the stereoisomers of the other *N*-derivatized di- and

tripeptides examined could be separated under the same chromatographic conditions as those selected for the nine tested dipeptides. In a few cases, better results were obtained by decreasing the ACN content in the mobile phase from 65 to 50 % or by replacing ACN with MeOH. For FMOC derivatives, chiral separations could only be achieved with a polar organic mobile phase consisting of a mixture of ACN and MeOH (see section 3.2).

As can be seen in **Table 1**, all stereoisomers of 24 out of the 53 *N*-derivatized di- and tripeptides could be baseline separated. In most cases, the stereoisomers of the *N*-derivatized di- and tri-peptides could be at least partially separated, except those of 3,5-DMB-DL-Leu-DL-Val, 3,5-DMB-DL-Leu-Gly-DL-Phe, 3,5-DMB-Gly-DL-norVal, 3,5-DMB-Gly-DL-norLeu, FMOC-DL-Ala-DL-Met, FMOC-DL-Leu-Gly-Gly, FMOC-DL-Leu-Gly-DL-Phe, FMOC-Gly-DL-norLeu and FMOC-Gly-DL-norVal. **Fig. 1** depicts representative chromatograms obtained under the selected conditions. It is worth noting that the four stereoisomers of the dipeptide derivatives have not equivalent peak areas: one pair of enantiomers is generally present in a larger amount than the other one, in accordance with previous observations [14,21]. It is then easy to see that the resolution of one enantiomeric pair (peaks in positions 1 and 4) is much higher than that of the other one (peaks in positions 2 and 3). This also explains why the separation of diastereomers (peaks 1-2 or 3-4) is often more difficult to obtain than enantioseparations.

Table 1 also shows that the highest enantioselectivity and resolution values were obtained for the stereoisomers of 3,5-DNB derivatives, followed by those of 3,5-DClB derivatives. These results demonstrate that the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column is well suited to the separation of the enantiomers and diastereomers of *N*-derivatized di- and tripeptides.

From these results several other conclusions can be drawn. First, the influence of *N*-protecting groups with different levels of π-acidity such as those possessing aromatic rings with different electron densities can be investigated. As a general observation, the π-acidity groups have a favorable impact on the chiral separation of the peptide derivatives. As could be expected, the selectivities for the stereoisomers of the peptide derivatives were found to decrease with decreasing electrophilic character of the *N*-protecting groups (3,5-DNB > 3,5-DClB > 3,5-DMB, cf. **Tables S3** and **1**). The higher selectivities obtained for 3,5-DNB derivatives (e.g. α (3,5-DNB-DL-Ala-DL-Leu) = 1.17, 1.69, 1.40) could be explained by the fact [45] that the electron-deficient 3,5-DNB group will undergo stronger π-π interaction with the electron-rich quinoline ring of quinidine than the 3,5-DClB (e.g. α (3,5-DClB-DL-Ala-DL-Leu) = 1.18, 1.37, 1.20) and 3,5-DMB (e.g. α (3,5-DMB-DL-Ala-DL-Leu) = 1.20, 1.24, 1.00) groups (**Table S3**). This additional chiral discrimination increment could facilitate the separation and lead to an increase in the resolution of the stereoisomers of *N*-derivatized peptides [45].

On the other hand, it is clear that the 3,5-DNB derivatives exhibit higher selectivities than the FMOC derivatives (**Table 1**). This trend was evidenced by comparison of the selectivities for a wide range of dipeptide derivatives, including 3,5-DNB-DL-Ala-DL-Leu (α = 1.17, 1.69, 1.40) and FMOC-DL-Ala-DL-Leu (α = 1.18, 1.16, 1.00) (see **Table 1**). However, 3,5-DNB-, 3,5-DClB- and 3,5-DMB-derivatives of carnosine could not be prepared according to the derivatization method used for other small peptides due to the low solubility of carnosine in most organic solvents [47]. Moreover, when carnosine was dissolved in aqueous solution, the corresponding derivatization agents reacted with water so that the products could not be obtained, FMOC-carnosine being the only derivative that

we could study.

From the results given in **Table 1**, another interesting observation can be made: the effect on chiral recognition when introducing one or two achiral glycine moieties into an amino acid or a dipeptide. For that purpose, 3,5-DClB protected DL-Leu, DL-Leu-Gly, DL-Leu-Gly-Gly, DL-Leu-DL-Phe, and DL-Leu-Gly-DL-Phe were chosen as test analytes under the usual chromatographic conditions (0.1 M ammonium acetate/ACN (35/65, v/v), apparent pH = 5.3). With the exception of 3,5-DClB-DL-Leu-Gly-DL-Phe, a successful separation was achieved for all pairs of enantiomers or diastereomers (**Table 1**). It is interesting to note that the retention factors, enantioselectivities, and resolution values decrease by addition of glycine moieties. For instance, the α values for some amino acid or peptide enantiomers or diastereomers as 3,5-DClB derivatives decrease as follows: DL-Leu (α = 2.95) > DL-Leu-Gly (α = 1.96) > DL-Leu-Gly-Gly (α = 1.21) in the one hand and DL-Leu-DL-Phe (α = 1.31, 1.23, 2.04) > DL-Leu-Gly-DL-Phe (α = 1.07, 1.16, 1.00) on the other hand. This finding is consistent with the conclusions of earlier studies that the reduction of selectivity may be attributed to a loss of enantiomer or diastereomer discriminating interactions as a consequence of the increment in the chain length [26,43-44].

Finally, in order to confirm the enantiomer elution order, $_{LL}$-form and $_{DL}$-form enantio-enriched 3,5-DNB-Ala-Leu and 3,5-DClB-Leu-Phe were selected as analytes. As shown in **Fig. S2**, the $_{LL}$-form of 3,5-DNB-Ala-Leu was eluted first and followed with $_{LD}$-, $_{DL}$- and $_{DD}$-form in turns. The same enantiomer elution order was also observed for 3,5-DClB-DL-Leu-DL-Phe.

3.2. Development of a micro-LC method for the individual quantification of carnosine enantiomers employing the poly(MQD-*co*-HEMA-*co*-EDMA)

monolithic column

The successful separation of the stereoisomers of most *N*-derivatized di- and tri-peptides on the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column encouraged us to apply this chiral discrimination system to the analysis of real samples. In a previous study, Su et al. developed a sensitive LC method for the simultaneous determination of carnosine enantiomers in rat plasma [6]. After derivatization with o-phthaladehyde and N-acetyl-L-cysteine, the carnosine enantiomers were separated on an ODS column and quantified using fluorescence detection. The lowest limit of quantification (LLOQ) was 40 ng/mL for each enantiomer [6]. In order to evaluate the applicability of the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column, a micro-LC method for the stereoselective determination of $_{L}$-carnosine in different dietary supplements was developed.

From initial experiments with a hydro-organic mobile phase (0.1 M ammonium acetate/ACN (35/65, v/v), apparent pH = 5.3), it became evident that the FMOC-DL-carnosine enantiomers could not be successfully separated. Therefore, a polar organic mobile phase consisting of ACN/MeOH (50/50, v/v) containing 0.1 M acetic acid and 4 mM triethylamine was chosen for method development. First of all, the effect of the ACN content in the mobile phase was tested in the range from 50 to 20 %. A decrease in the ACN content resulted in an increase in the retention of the FMOC-DL-carnosine enantiomers (from 12 to 18 min); however, a baseline separation of these enantiomers could not be achieved in the studied ACN concentration range and a mobile phase with an intermediate ACN content (35 %) was chosen. Other parameters such as acetic acid concentration and flow rate were also investigated. Different concentrations of acetic acid in the mobile phase were evaluated (0.05, 0.1 and 0.15 M), keeping the ratio of ACN/MeOH constant at 35/65

(v/v). However, the variation of acetic acid concentration did not lead to an important improvement in the enantioresolution of FMOC-DL-carnosine, so that the 0.1 M concentration was selected. Finally, the total flow rate was studied in the 10-30 µL/min range. By decreasing the total flow rate, a significant increase in enantioresolution was obtained. Therefore, using a mobile phase consisting of ACN/MeOH (35/65, v/v) containing 0.1 M acetic acid and 4 mM triethylamine at a total flow rate of 10 µL/min, a baseline separation of FMOC-DL-carnosine enantiomers could be achieved (**Fig. 2**).

Furthermore, in order to obtain a reliable derivatization method, the conditions affecting the reaction between DL-carnosine and FMOC chloride, such as the FMOC chloride/DL-carnosine concentration ratio, the buffer pH and the reaction time were also investigated. After evaluating the effect of the FMOC chloride/DL-carnosine concentration ratio on the average peak area, a ratio of 3 was found to be optimum and selected for further experiments (**Fig. S3**). The influence of the reaction time was also studied in the range from 0.5 to 5 min. At times higher than 1 min, the reaction was found to reach the same yield as that at 5 min; therefore, a reaction time of 2 min was selected. The next step was to study the effect of the buffer pH employed in the reaction medium. As this reaction needs a basic medium to take place, the tested pH values ranged from 8 to 10. The maximum reaction rate was achieved at pH 9.0, this result being consistent with the findings of a previous study [48]. These results demonstrate that the developed derivatization method can be used for a quantitative application.

3.3. Validation of the developed method

Before proceeding with the analysis of $_{L}$-carnosine in real samples, the analytical characteristics of the developed method such as selectivity, linearity, precision,

accuracy, LOD, the limit of quantification (LOQ), and the stability of the standard solution of the FMOC derivative, were studied.

The developed method has proved to be selective for carnosine enantiomers as no interfering peaks originating from the sample matrices were found; the small peak observed in all samples at the front of the $_{L}$-carnosine peak is most probably an impurity of $_{L}$-carnosine, also present in trace amounts in the reference standard (**Fig. 2**). However, the analysis time was fairly long and the peaks were rather broad. This might be due to the extra-column volume contributions. Concentration ranges for calibration curves were 0.19-15 mM for $_{L}$-carnosine and 0.019-1.5 mM for $_{D}$-carnosine, respectively. As can be seen in **Table 2**, linearity was adequate in both cases since R^2 values were > 99.0 %, confidence intervals for the slopes did not include the zero value and confidence intervals for the intercepts included the zero value (at a 95 % confidence level). Moreover, the p-value of the t-test between the standard calibration and standard addition calibration methods allowed to conclude that no matrix interferences existed since for both enantiomers, this value was above 0.05 (at a 95 % confidence level) (**Table 2)**. Thus, external standard calibration method has proved to be adequate for the quantification of carnosine enantiomers in the dietary supplement products.

Method accuracy was evaluated by measuring the recoveries obtained from solutions of $_{DL}$-carnosine spiked samples, injected in triplicate, at three different percentages of the labeled content (80, 100 and 120%). Mean recoveries were acceptable as they were near 100% (see **Table 2**). Method precision was evaluated in terms of instrumental and method repeatabilities, and intermediate precision. RSD values (%) obtained for areas and retention times are shown in **Table 2**. It can be concluded that RSD (%) obtained for peak areas are acceptable as they were < 5 % for instrumental

repeatability and < 10% for method repeatability and intermediate precision. The limit of detection for $_{D}$-carnosine was calculated as equivalent to 3 times the *S*/*N* ratio, which corresponds to 6.25 μM, while the limit of quantification was determined as equivalent to 10 times the *S*/*N* ratio, resulting in a value of 20.8 μM (**Table 2**). It is worth noting that both LOD and LOQ values for $_{D}$-carnosine were determined in the presence of a large excess of $_{L}$-carnosine (see **Figs. 2A** and **B**).

Finally, the stability of FMOC-$_{L}$-carnosine stored at 4 ºC was evaluated by injecting a solution containing 2 mM FMOC-$_{L}$-carnosine in triplicate in three different days. RSD values were 0.8 and 4.8 % for retention times and peak areas, respectively.

3.4. Quantification of carnosine in dietary supplements

The developed and validated quantitative method was applied to the analysis of carnosine enantiomers in dietary supplement products. Three different products, containing in principle 500 mg of $_{L}$-carnosine per tablet or capsule, were analyzed. **Table 3** shows the results obtained for the analyzed samples. In all cases, the concentration of $_{D}$-carnosine was below the detection limit (see **Figs. 2A** and **C**) of the present method and the amount of $_{L}$-carnosine was in agreement with the labeled content for all the analyzed products (**Table 3**).

4. Conclusions

In this research, the poly(MQD-*co*-HEMA-*co*-EDMA) chiral monolithic column has been evaluated in terms of performance and stereoselectivity towards a wide range of *N*-derivatized di- and tri-peptides, using a laboratory self-assembled micro-LC system. A baseline separation of all stereoisomers of 24 out of the 53 small peptide derivatives studied was obtained with this CSP, demonstrating its excellent enantioselectivity and diastereoselectivity. The effects of the apparent pH of the mobile phase, the organic

solvent content and the buffer concentration on the retention and separation of the stereoisomers of *N*-derivatized di- and tri-peptides seem to confirm that electrostatic and hydrophobic interactions are both responsible for the retention of these acidic analytes while chiral recognition is more related to other kinds of intermolecular interactions, such as hydrogen binding, π-π and steric interactions. Finally, a method was developed to quantify carnosine enantiomers in real samples and its analytical characteristics (linearity, specificity, accuracy, precision, LOD, and LOQ) were evaluated. The applicability of the validated method has been demonstrated by analyzing carnosine in several dietary supplements.

Acknowledgments

We gratefully appreciate the financial support from the National Natural Science Foundation of China (Grants: 81273477 and 81202499), the Ministry of Economy and Competitiveness (Spain) (research project CTQ2013-48740-P) and the Comunidad of Madrid (Spain) and European funding from FEDER program (research project S2013/ABI-3028, AVANSECAL-CM*)*. Qiqin Wang gratefully acknowledges funding from "Short-term abroad research program" and "Excellent doctoral climb program" within Jinan University. Elena Sánchez-López thanks the University of Alcalá for her pre-doctoral grant.

References:

[1] J. Liao, I. Lin, K. Huang, P. Kuo, S. Wu, T. Wu, Carnosine ameliorates lens protein turbidity formations by inhibiting calpain proteolysis and ultraviolet c-induced degradation, J. Agric. Food Chem. 62 (2014) 5932-5938.

[2] V. Lanza, F. Bellia, R. D'Agata, G. Grasso, E. Rizzarelli, G. Vecchio, New glycoside derivatives of carnosine and analogs resistant to carnosinase hydrolysis: Synthesis and

445 characterization of their copper (II) complexes, J. Inorg. Biochem. 105 (2011) 181-188.
446 [3] A.A. Boldyrev, G. Aldini, W. Derave, Physiology and pathophysiology of carnosine, Physiol.
447 Rev. 93 (2013) 1803-1845.
448 [4] R. Kohen, Y. Yamamoto, K.C. Cundy, B.N. Ames, Antioxidant activity of carnosine,
449 homocarnosine, and anserine present in muscle and brain, Proc. Natl. Acad. Sci. USA 85 (1988)
450 3175-3179.
451 [5] G.I. Grasso, F. Bellia, G. Arena, G. Vecchio, E. Rizzarelli, Noncovalent interaction-driven
452 stereoselectivity of copper (II) complexes with cyclodextrin derivatives of $_{L}$- and $_{D}$-carnosine,
453 Inorg. Chem. 50 (2011) 4917-4924.
454 [6] D. Su, K. Bi, C. Zhou, Y. Song, B. Wei, L. Geng, W. Liu, X. Chen, Enantioselective separation
455 and determination of carnosine in rat plasma by fluorescence LC for stereoselective
456 pharmacokinetic studies, Chromatographia 71 (2010) 603-608.
457 [7] B. Sungthong, R. Iványi, S.C. Bunz, C. Neusüß, G.K.E. Scriba, CE-MS characterization of
458 negatively charged α-, β- and γ-CD derivatives and their application to the separation of dipeptide
459 and tripeptide enantiomers by CE, Electrophoresis 31 (2010) 1498-1505.
460 [8] I. Ali, Z.A. Al-Othman, A. Al-Warthan, L. Asnin, A. Chudinov, Advances in chiral separations
461 of small peptides by capillary electrophoresis and chromatography, J. Sep. Sci. 37 (2014)
462 2447-2466.
463 [9] R. Ludewig, S. Nietzsche, G.K.E. Scriba, A weak cation-exchange monolith as stationary
464 phase for the separation of peptide diastereomers by CEC, J. Sep. Sci. 34 (2011) 64-69.
465 [10] G.K.E. Scriba, Recent developments in peptide stereoisomer separations by capillary
466 electromigration techniques, Electrophoresis 30 (2009) S222-S228.
467 [11] C. Czerwenka, W. Lindner, Stereoselective peptide analysis, Anal. Bioanal. Chem. 382 (2005)
468 599-638.
469 [12] V. Kašička, Recent developments in capillary and microchip electroseparations of peptides
470 (2011-2013), Electrophoresis 35 (2014) 69-95.
471 [13] C. Czerwenka, N.M. Maier, W. Lindner, Liquid chromatographic-mass spectrometric
472 separation of oligoalanine peptide stereoisomers: influence of absolute configuration on
473 enantioselectivity and two-dimensional separation of diastereomers and enantiomers, J.

474 Chromatogr. A 1038 (2004) 85-95.

475 [14] C. Czerwenka, M. Lämmerhofer, W. Lindner, Micro-HPLC and standard-size HPLC for the
476 separation of peptide stereoisomers employing an ion-exchange principle, J. Pharm. Biomed. Anal.
477 30 (2003) 1789-1800.

478 [15] U. Conrad, B. Chankvetadze, G.K.E. Scriba, High performance liquid chromatographic
479 separation of dipeptide and tripeptide enantiomers using a chiral crown ether stationary phase, J.
480 Sep. Sci. 28 (2005) 2275-2281.

481 [16] B. Esquivel, L. Nicholson, L. Peerey, Enantiomeric resolution of underivatized small peptides
482 by HPLC with a chiral crown ether stationary phase, JJ. High Resolut. Chromatogr. 14 (1991)
483 816-823.

484 [17] M. Hilton, D.W. Armstrong, Evaluation of the enantiomeric separation of dipeptides using a
485 chiral crown ether LC column, J. Liq. Chromatogr. 14 (1991) 3673-3683.

486 [18] M.G. Schmid, M. Hölbling, N. Schnedlitz, G. Gübitz, Enantioseparation of dipeptides and
487 tripeptides by micro-HPLC comparing teicoplanin and teicoplanin aglycone as chiral selectors, J.
488 Biochem. Biophys. Methods 61 (2004) 1-10.

489 [19] A. Berthod, Y. Liu, C. Bagwill, D.W. Armstrong, Facile liquid chromatographic
490 enantioresolution of native amino acids and peptides using a teicoplanin chiral stationary phase, J.
491 Chromatogr. A 731 (1996) 123-137.

492 [20] S. Chen, The HPLC resolution of *N*-2,4-dinitrophenylated amino acids and peptides
493 stereoisomers on naphthylethylcarbamate-*β*-cyclodextrin bonded phase using the
494 acetonitrile-based mobile phase: evidence for the chiral recognition pattern, Amino acids 27 (2004)
495 277-284.

496 [21] S. Chen, The facile HPLC enantioresolution of amino acids, peptides on
497 naphthylethylcarbamate-*β*-cyclodextrin bonded phases using the acetonitrile-based mobile phase
498 after their pre-column derivatization with phenyl isothiocyanate: factors that affect the resolution,
499 Amino acids 26 (2004) 291-298.

500 [22] S. Schiesel, M. Lämmerhofer, A. Leitner, W. Lindner, Quantitative high-performance liquid
501 chromatography-tandem mass spectrometry impurity profiling methods for the analysis of
502 parenteral infusion solutions for amino acid supplementation containing L-alanyl-L-glutamine, J.

503 Chromatogr. A 1259 (2012) 111-120.

504 [23] S. Wernisch, O. Trapp, W. Lindner, Application of cinchona-sulfonate-based chiral
505 zwitterionic ion exchangers for the separation of proline-containing dipeptide rotamers and
506 determination of on-column isomerization parameters from dynamic elution profiles, Anal. Chim.
507 Acta. 795 (2013) 88-98.

508 [24] S. Wernisch, W. Lindner, Versatility of cinchona-based zwitterionic chiral stationary phases:
509 Enantiomer and diastereomer separations of non-protected oligopeptides utilizing a multi-modal
510 chiral recognition mechanism, J. Chromatogr. A 1269 (2012) 297-307.

511 [25] A. Mucha, M. Lämmerhofer, W. Lindner, M. Pawelczak, P. Kafarski, Individual
512 stereoisomers of phosphinic dipeptide inhibitor of leucine aminopeptidase, Bioorg. Med. Chem.
513 Lett. 18 (2008) 1550-1554.

514 [26] C. Czerwenka, P. Polásková, W. Lindner, Peptide enantiomer separations: Influence of
515 sequential isomerism and the introduction of achiral glycine moieties on chiral recognition, J.
516 Chromatogr. A 1093 (2005) 81-88.

517 [27] B. Preinerstorfer, D. Lubda, A. Mucha, P. Kafarski, W. Lindner, M. Lämmerhofer,
518 Stereoselective separations of chiral phosphinic acid pseudodipeptides by CEC using silica
519 monoliths modified with an anion-exchange-type chiral selector, Electrophoresis 27 (2006)
520 4312-4320.

521 [28] J.M. Bobbitt, L. Li, D.D. Carlton Jr., M. Yasin, S. Bhawal, F.W. Foss Jr., S. Wernisch, R.
522 Pell, W. Lindner, K.A. Schug, Diastereoselective discrimination of lysine-alanine-alanine peptides
523 by zwitterionic cinchona alkaloid-based chiral selectors using electrospray ionization mass
524 spectrometry, J. Chromatogr. A 1269 (2012) 308-315.

525 [29] C. Czerwenka, W. Lindner, Enantiomer discrimination of peptides by tandem mass
526 spectrometry: influence of the peptide sequence on chiral recognition, Rapid Commun. Mass
527 Spectrom. 18 (2004) 2713-2718.

528 [30] C. Czerwenka, M. Lämmerhofer, W. Lindner, Electrolyte and additive effects on enantiomer
529 separation of peptides by nonaqueous ion-pair capillary electrophoresis using
530 *tert.*-butylcarbamoylquinine as chiral counterion, Electrophoresis 23 (2002) 1887-1899.

531 [31] Q. Wang, J. Feng, H. Han, P. Zhu, H. Wu, M.L. Marina, J. Crommen, Z. Jiang,

532 Enantioseparation of N-derivatized amino acids by micro-liquid chromatography using
533 carbamoylated quinidine functionalized monolithic stationary phase, J. Chromatogr. A 1363 (2014)
534 207-215.
535 [32] Z. Jiang, N.W. Smith, P.D. Ferguson, M.R. Taylor, Hydrophilic interaction chromatography
536 using methacrylate-based monolithic capillary column for the separation of polar analytes, Anal.
537 Chem. 79 (2007) 1243-1250.
538 [33] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, Octadecylsilylated porous
539 silica rods as separation media for reversed-phase liquid chromatography, Anal. Chem. 68 (1996)
540 3498-3501.
541 [34] F. Svec, Porous polymer monoliths: Amazingly wide variety of techniques enabling their
542 preparation, J. Chromatogr. A 1217 (2010) 902-924.
543 [35] M. Lämmerhofer, E.C. Peters, C. Yu, F. Svec, J.M.J. Fréchet, Chiral monolithic columns
544 for enantioselective capillary electrochromatography prepared by copolymerization of a monomer
545 with quinidine functionality. 1. Optimization of polymerization conditions, porous properties, and
546 chemistry of the stationary phase, Anal. Chem. 72 (2000) 4614-4622.
547 [36] M. Lämmerhofer, F. Svec, J.M.J. Fréchet, Chiral monolithic columns for enantioselective
548 capillary electrochromatography prepared by copolymerization of a monomer with quinidine
549 functionality. 2. Effect of chromatographic conditions on the chiral separations, Anal. Chem. 72
550 (2000) 4623-4628.
551 [37] W.H. Pirkle, T.C. Pochapsky, Chiral molecular recognition in small bimolecular systems: A
552 spectroscopic investigation into the nature of diastereomeric complexes, J. Am. Chem. Soc. 109
553 (1987) 5975-5982.
554 [38] H. Wan, P.E. Andersson, A. Engström, L.G. Blomberg, Direct and indirect chiral separation of
555 amino acids by capillary electrophoresis, J. Chromatogr. A 704 (1995) 179-193.
556 [39] E. Domínguez-Vega, A.L. Crego, K. Lomsadze, B. Chankvetadze, M.L. Marina,
557 Enantiomeric separation of FMOC-amino acids by nano-LC and CEC using a new chiral
558 stationary phase, cellulose tris(3-chloro-4-methylphenylcarbamate), Electrophoresis 32 (2011)
559 2700-2707.
560 [40] E. Domínguez-Vega, A.B. Martínez-Girón, C. García-Ruiz, A.L. Crego, M.L. Marina, Fast

561 derivatization of the non-protein amino acid ornithine with FITC using an ultrasound probe prior
562 to enantiomeric determination in food supplements by EKC, Electrophoresis 30 (2009)
563 1037-1045.
564 [41] Q. Zhang, J. Guo, F. Wang, J. Crommen, Z. Jiang, Preparation of a *β*-cyclodextrin
565 functionalized monolith via a novel and simple one-pot approach and application to
566 enantioseparations, J. Chromatogr. A 1325 (2014) 147-154.
567 [42] V. Piette, M. Lämmerhofer, W. Lindner, J. Crommen, Enantiomer separation of *N*-protected
568 amino acids by non-aqueous capillary electrophoresis and high-performance liquid
569 chromatography with *tert*.-butyl carbamoylated quinine in either the background electrolyte or the
570 stationary phase, J. Chromatogr. A 987 (2003) 421-427.
571 [43] C. Czerwenka, M. Lämmerhofer, N.M. Maier, K. Rissanen, W. Lindner, Direct
572 high-performance liquid chromatographic separation of peptide enantiomers: Study on chiral
573 recognition by systematic evaluation of the influence of structural features of the chiral selectors
574 on enantioselectivity, Anal. Chem. 74 (2002) 5658-5666.
575 [44] C. Czerwenka, M. Zhang, H. Kählig, N.M. Maier, K.B. Lipkowitz, W. Lindner, Chiral
576 recognition of peptide enantiomers by cinchona alkaloid derived chiral selectors: Mechanistic
577 investigations by liquid chromatography, NMR spectroscopy, and molecular modeling, J. Org.
578 Chem. 68 (2003) 8315-8327.
579 [45] C. Czerwenka, M. Lämmerhofer, W. Lindner, Structure-enantioselectivity relationships for
580 the study of chiral recognition in peptide enantiomer separation on cinchona alkaloid-based chiral
581 stationary phases by HPLC: Influence of the N-terminal protecting group, J. Sep. Sci. 26 (2003)
582 1499-1508.
583 [46] C. Czerwenka, N.M. Maier, W. Lindner, Enantiomer discrimination by mass spectrometry:
584 noncovalent interactions of an N-derivatized dipeptide with various cinchona alkaloid derivatives
585 and comparison with enantioselective liquid-phase separations, Anal. Bioanal. Chem. 379 (2004)
586 1039-1044.
587 [47] E. Husson, C. Humeau, C. Harscoat, X. Framboisier, C. Paris, E. Dubreucq, I. Marc, I.
588 Chevalot, Enzymatic acylation of the polar dipeptide, carnosine: Reaction performances in organic
589 and aqueous media, Process Biochem. 46 (2011) 945-952.

590 [48] A. Jámbor, I. Molnár-Perl, Quantitation of amino acids in plasma by high performance
591 liquid chromatography: Simultaneous deproteinization and derivatization with
592 9-fluorenylmethyloxycarbonyl chloride, J. Chromatogr. A 1216 (2009) 6218-6223.

Figure captions

Fig. 1. Separation of *N*-derivatized small peptide stereoisomers on the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column. Separation conditions: column dimensions: 210 mm × 100 μm I.D.; mobile phase: (a). 0.1 M ammonium acetate/ACN (35/65, v/v) (apparent pH = 5.3) for all chromatograms except f, total flow rate: 20 μL/min, backpressure: 24 bar; (b). ACN/MeOH (35/65, v/v), 0.1 M acetic acid, 4 mM triethylamine (apparent pH = 6.0) for chromatogram f, total flow rate: 5 μL/min, backpressure: 6 bar; UV detection wavelength: 254 nm; injection volume: 20 nL.

Fig. 2. A. Chromatogram of D-carnosine at a concentration (6.25 μM) corresponding to LOD in the presence of a large excess of L-carnosine (0.25 mM).

B. Chromatogram of D-carnosine at a concentration (20.8 μM) corresponding to LOQ in the presence of a large excess of L-carnosine (0.25 mM).

C.Chromatograms of 1) FMOC-D,L-carnosine standard solution (concentration 1 mM); 2) dietary product #3; 3) dietary product #2; 4) dietary product #1.

Separation conditions: column dimensions: 210 mm × 100 μm I.D.; mobile phase: ACN/MeOH (35/65, v/v), 0.1 M acetic acid, 4 mM triethylamine (apparent pH = 6.0), total flow rate: 10 μL/min, backpressure: 10 bar; UV detection wavelength: 254 nm; injection volume: 20 nL.

Table 1. Retention and separation of *N*-derivatized peptide stereoisomers on the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column. Separation conditions: mobile phase, [a] 0.1 M ammonium acetate/ACN (35/65, v/v) (apparent pH = 5.3), total flow rate: 20 μL/min; backpressure: 24 bar; [b] 0.1 M ammonium acetate/ACN (50/50, v/v) (apparent pH = 5.3), total flow rate: 10 μL/min; backpressure: 20 bar; [c] 0.1 M ammonium acetate/MeOH (35/65, v/v) (apparent pH = 5.3), total flow rate: 10 μL/min; backpressure: 16 bar; [d] ACN/MeOH (35/65, v/v), 0.1 M acetic acid, 4 mM triethylamine (apparent pH = 6.0), total flow rate: 10 μL/min; backpressure: 10 bar; [e] ACN/MeOH (35/65, v/v), 0.1 M acetic acid, 4 mM triethylamine (apparent pH = 6.0), total flow rate: 5 μL/min; backpressure: 6 bar; UV detection wavelength: 254 nm; injection volume: 20 nL.

Table 2. Analytical characteristics of the developed micro-LC method for the individual determination of carnosine enantiomers in dietary supplements.

a) Six standard solutions at different concentration levels injected in triplicate.

b) Addition of four known amounts of D- and L-carnosine to a dietary supplement sample containing a

constant concentration of L-carnosine.

c) Accuracy was evaluated as the recovery obtained from three dietary supplement sample solutions spiked with standard D,L-carnosine solution at three different percentages (80, 100, and 120 %) of the labelled content (n = 9).

d) Six consecutive injections of a dietary supplement sample solution containing 2 mM L-carnosine (as labeled amount) spiked with 0.5 mM L- and D-carnosine (n = 6).

e) Six dietary supplement sample solutions containing 2 mM L-carnosine (as labeled amount) spiked with 0.5 mM L- and D-carnosine (n = 6).

f) Three dietary supplement sample solutions containing 2 mM L-carnosine (as labeled amount) spiked with 0.5 mM L- and D-carnosine in 3 different days (n = 9).

g) LOD: equivalent to 3 × S/N.

h) LOQ: equivalent to 10 × S/N.

Table 3. Analysis of three different dietary supplement products with a labeled content of 500 mg of $_{L}$-carnosine per tablet or capsule. ND: Not detected (<LOD). Experimental conditions as in **Fig. 2**.

Table 1. Retention and separation of *N*-derivatized peptide stereoisomers on the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column.

Sample	k_1	k_2	k_3	k_4	$\alpha_{1/2}$	$\alpha_{2/3}$	$\alpha_{3/4}$	$R_{s\,1/2}$	$R_{s\,2/3}$	$R_{s\,3/4}$	*N1*
3,5-DNB-DL-Ala-DL-Ala[a]	2.03	2.26	4.86	5.15	1.12	2.15	1.06	1.16	7.10	0.52	18336
3,5-DNB-DL-Ala-DL-Leu[a]	1.76	2.05	3.46	4.86	1.17	1.69	1.40	1.55	5.40	3.59	18168
3,5-DNB-DL-Ala-DL-Val[a]	1.97	2.41	3.96	4.97	1.23	1.64	1.26	2.04	5.13	2.39	16208
3,5-DNB-DL-Ala-DL-Met[a]	1.50	1.50	2.55	2.55	1.00	1.69	1.00	0.00	4.49	0.00	14467
3,5-DNB-DL-Ala-DL-Phe[b]	4.72	5.19	7.72	15.44	1.10	1.49	2.00	1.38	6.35	12.33	21294
3,5-DNB-DL-Leu-DL-Ala[a]	1.85	2.14	5.00	6.58	1.16	2.34	1.32	1.50	8.95	3.12	19315
3,5-DNB-DL-Leu-DL-Val[a]	2.03	2.03	3.38	3.38	1.00	1.67	1.00	0.00	4.32	0.00	12000
3,5-DNB-DL-Leu-DL-Phe[a]	2.61	3.12	4.91	12.35	1.20	1.57	2.52	1.63	4.06	7.91	13696
3,5-DNB-DL-Leu-Gly[a]	1.93	4.96			2.57			7.98			13248
3,5-DNB-DL-Leu-Gly-Gly[a]	1.49	1.96			1.32			2.14			13425
3,5-DNB-DL-Leu-Gly-DL-Phe[a]	2.01	2.01	2.50	2.74	1.00	1.24	1.09	0.00	1.24	0.59	4439
3,5-DNB-Gly-DL-norLeu[a]	3.20	4.20			1.31			2.44			11783
3,5-DNB-Gly-DL-norVal[a]	2.91	3.79			1.30			2.93			16301
3,5-DClB-DL-Ala-DL-Ala[a]	2.16	2.43	3.96	3.96	1.12	1.63	1.00	1.27	3.94	0.00	20127
3,5-DClB-DL-Ala-DL-Leu[a]	1.75	2.06	2.83	3.39	1.18	1.37	1.20	1.68	3.38	1.92	19290
3,5-DClB-DL-Ala-DL-Val[a]	2.48	2.48	3.25	3.25	1.00	1.31	1.00	0.00	2.68	0.00	15440
3,5-DClB-DL-Ala-DL-Met[a]	1.81	1.81	2.50	2.50	1.00	1.38	1.00	0.00	2.93	0.00	14901
3,5-DClB-DL-Ala-DL-Phe[a]	2.92	3.41	4.17	5.99	1.17	1.22	1.44	1.68	2.10	3.79	15153
3,5-DClB-DL-Leu-DL-Ala[a]	1.91	2.24	3.81	4.79	1.18	1.70	1.25	1.52	5.35	2.23	15182
3,5-DClB-DL-Leu-DL-Val[a]	2.22	2.22	2.87	2.87	1.00	1.29	1.00	0.00	1.34	0.00	2429
3,5-DClB-DL-Leu-DL-Phe[a]	2.60	3.42	4.20	8.56	1.31	1.23	2.04	2.53	2.04	6.68	11381

3,5-DClB-DL-Leu-Gly[a]	2.05	4.02			1.96			6.50			14840
3,5-DClB-DL-Leu-Gly-Gly[a]	1.45	1.75			1.21			1.70			16183
3,5-DClB-DL-Leu-Gly-DL-Phe[a]	1.89	2.02	2.35	2.35	1.07	1.16	1.00	0.29	0.80	0.00	2519
3,5-DClB-Gly-DL-norLeu[a]	3.18	3.68			1.16			1.71			17742
3,5-DClB-Gly-DL-norVal[a]	3.03	3.47			1.14			1.59			18315
3,5-DClB-Leucine[a]	2.57	7.57			2.95			8.71			

Sample	k_1	k_2	k_3	k_4	$\alpha_{1/2}$	$\alpha_{2/3}$	$\alpha_{3/4}$	$R_{s\,1/2}$	$R_{s\,2/3}$	$R_{s\,3/4}$	*N1*
3,5-DMB-DL-Ala-DL-Ala[a]	1.28	1.42	1.87	2.00	1.11	1.32	1.07	0.97	2.41	0.51	22949
3,5-DMB-DL-Ala-DL-Leu[c]	2.89	3.51	4.57	5.67	1.22	1.30	1.24	1.94	2.68	2.22	13784
3,5-DMB-DL-Ala-DL-Val[a]	1.41	1.41	1.68	1.68	1.00	1.19	1.00	0.00	1.37	0.00	12493
3,5-DMB-DL-Ala-DL-Met[a]	1.49	1.49	1.94	1.94	1.00	1.30	1.00	0.00	2.22	0.00	13401
3,5-DMB-DL-Ala-DL-Phe[b]	2.29	2.66	3.07	3.86	1.16	1.16	1.26	1.89	1.92	3.12	26371
3,5-DMB-DL-Leu-DL-Ala[b]	1.41	1.65	2.50	3.03	1.18	1.51	1.21	1.77	4.99	2.49	23756
3,5-DMB-DL-Leu-DL-Val[a]	1.33	1.33	1.33	1.33	1.00	1.00	1.00	0.00	0.00	0.00	3398
3,5-DMB-DL-Leu-DL-Phe[b]	2.96	3.66	4.08	7.48	1.24	1.11	1.83	2.74	1.50	8.35	20081
3,5-DMB-DL-Leu-Gly[b]	1.56	2.63			1.69			6.18			23578
3,5-DMB-DL-Leu-Gly-Gly[b]	0.84	0.99			1.17			1.34			23237
3,5-DMB-DL-Leu-Gly-DL-Phe[a]	1.37	1.37	1.37	1.37	1.00	1.00	1.00	0.00	0.00	0.00	16662
3,5-DMB-Gly-DL-norLeu[a]	1.31	1.31			1.00			0.00			21807
3,5-DMB-Gly-DL-norVal[a]	1.30	1.30			1.00			0.00			24991
FMOC-DL-Ala-DL-Ala[d]	3.06	3.59	3.79	4.18	1.17	1.06	1.10	1.63	0.57	1.09	15935
FMOC-DL-Ala-DL-Leu[d]	2.02	2.38	2.77	2.77	1.18	1.16	1.00	1.49	1.40	0.00	13579
FMOC-DL-Ala-DL-Val[d]	3.14	3.14	3.58	3.58	1.00	1.14	1.00	0.00	1.02	0.00	8901
FMOC-DL-Ala-DL-Met[d]	4.74	4.74	4.74	4.74	1.00	1.00	1.00	0.00	0.00	0.00	9119
FMOC-DL-Ala-DL-Phe[d]	4.75	5.29	6.00	6.51	1.11	1.13	1.08	1.25	1.44	0.95	15297
FMOC-DL-Leu-DL-Ala[d]	1.88	2.23	2.44	2.44	1.19	1.09	1.00	1.55	0.80	0.00	14367
FMOC-DL-Leu-DL-Val[d]	2.38	2.38	3.64	3.64	1.00	1.53	1.00	0.00	3.04	0.00	6749
FMOC-DL-Leu-DL-Phe[e]	2.59	2.94	3.81	4.69	1.14	1.29	1.23	1.57	3.25	2.74	19382
FMOC-DL-Leu-Gly[e]	2.91	3.28			1.13			1.57			24751
FMOC-DL-Leu-Gly-Gly[d]	3.24	3.24			1.00			0.00			14842
FMOC-DL-Leu-Gly-DL-Phe[d]	3.01	3.01	3.01	3.01	1.00	1.00	1.00	0.00	0.00	0.00	2241
FMOC-Gly-DL-norLeu[d]	3.13	3.13			1.00			0.00			4713
FMOC-Gly-DL-norVal[d]	3.33	3.33			1.00			0.00			4813
FMOC-DL-Carnosine[d]	6.87	8.31			1.21			1.55			6339

645

646

647 Separation conditions: mobile phase, [a] 0.1 M ammonium acetate/ACN (35/65, v/v) (apparent pH = 5.3), total flow
648 rate: 20 µL/min; backpressure: 24 bar; [b] 0.1 M ammonium acetate/ACN (50/50, v/v) (apparent pH = 5.3), total
649 flow rate: 10 µL/min; backpressure: 20 bar; [c] 0.1 M ammonium acetate/MeOH (35/65, v/v) (apparent pH = 5.3),
650 total flow rate: 10 µL/min; backpressure: 16 bar; [d] ACN/MeOH (35/65, v/v), 0.1 M acetic acid, 4 mM
651 triethylamine (apparent pH = 6.0), total flow rate: 10 µL/min; backpressure: 10 bar; [e] ACN/MeOH (35/65, v/v), 0.1
652 M acetic acid, 4 mM triethylamine (apparent pH = 6.0), total flow rate: 5 µL/min; backpressure: 6 bar; UV
653 detection wavelength: 254 nm; injection volume: 20 nL.

654

655 **Table 2.** Analytical characteristics of the developed micro-LC method for the

656 individual determination of carnosine enantiomers in dietary supplements

	L-carnosine	D-carnosine
External standard calibration method [a)]		
Range	0.1875 – 15 mM	0.01875 – 1.5 mM
Slope ± $t \cdot s_{slope}$	152000 ± 13000	210000 ± 20000
Intercept ± $t \cdot s_{intercept}$	-13000 ± 101000	2000 ± 17000
R^2	99.8 %	99.5 %
Standard addition calibration method [b)]		
Study of matrix interferences (p-value of t-test)	0.162	0.663
Accuracy [c)]		
Mean recovery (%)	105 ± 13	108 ± 7
Precision		
Instrumental repeatability [d)]		
t, RSD (%)	0.8	1.8
A, RSD (%)	3.3	4.8
Method repeatability [e)]		
t,RSD (%)	0.7	1.5
A, RSD (%)	7.2	8.0
Intermediate precision [f)]		
t, RSD (%)	2.4	5.0
A, RSD (%)	7.2	7.8
LOD [g)]	6.25 µM	6.25 µM
LOQ [h)]	20.8 µM	20.8 µM

657
658 i) Six standard solutions at different concentration levels injected in triplicate.
659 j) Addition of four known amounts of D- and L-carnosine to a dietary supplement sample
660 containing a constant concentration of L-carnosine.
661 k) Accuracy was evaluated as the recovery obtained from three dietary supplement sample
662 solutions spiked with standard D,L-carnosine solution at three different percentages (80, 100,
663 and 120 %) of the labelled content (n = 9).
664 l) Six consecutive injections of a dietary supplement sample solution containing 2 mM
665 L-carnosine (as labeled amount) spiked with 0.5 mM L- and D-carnosine (n = 6).
666 m) Six dietary supplement sample solutions containing 2 mM L-carnosine (as labeled amount)
667 spiked with 0.5 mM L- and D-carnosine (n = 6).
668 n) Three dietary supplement sample solutions containing 2 mM L-carnosine (as labeled amount)
669 spiked with 0.5 mM L- and D-carnosine in 3 different days (n = 9).
670 o) LOD: equivalent to 3 × S/N.
671 p) LOQ: equivalent to 10 × S/N.
672

673

674 **Table 3.** Analysis of three different dietary supplement products with a labeled content
675 of 500 mg of L-carnosine per tablet or capsule.

Samples	D-carnosine content (%)	L-carnosine content (mg/capsule)	L-carnosine content (%)
#1	ND	525 ± 9	105 ± 2
#2	ND	509 ± 38	102 ± 8
#3	ND	529 ± 27	106 ± 5

676 ND: Not detected (<LOD). Experimental conditions as in **Fig. 2**.

677
678
679
680
681

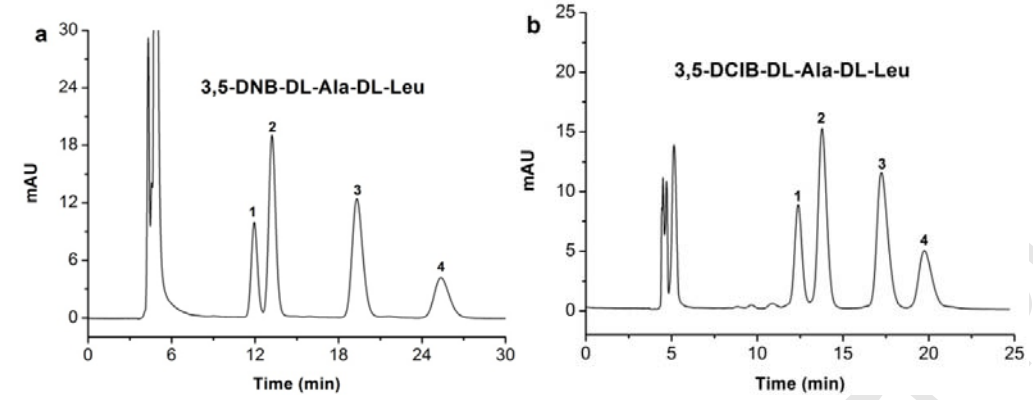
682 ## *Highlights*

683 ♦ *N*-derivatized peptides were successfully enantioresolved on the
684 poly(MQD-*co*-HEMA-*co*-EDMA) monolithic column.
685 ♦ The monolith exhibited good enantioselectivity and diastereoselectivity for
686 *N*-derivatized di- and tri- peptides.
687 ♦ The monolith shows potential as a reliable tool for the quality control of
688 L-carnosine in dietary supplements.

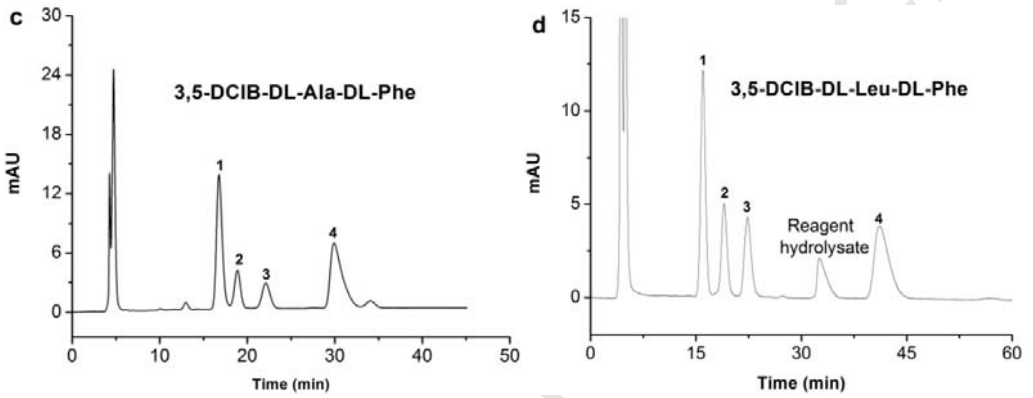
689
690
691

692 **Figures:**

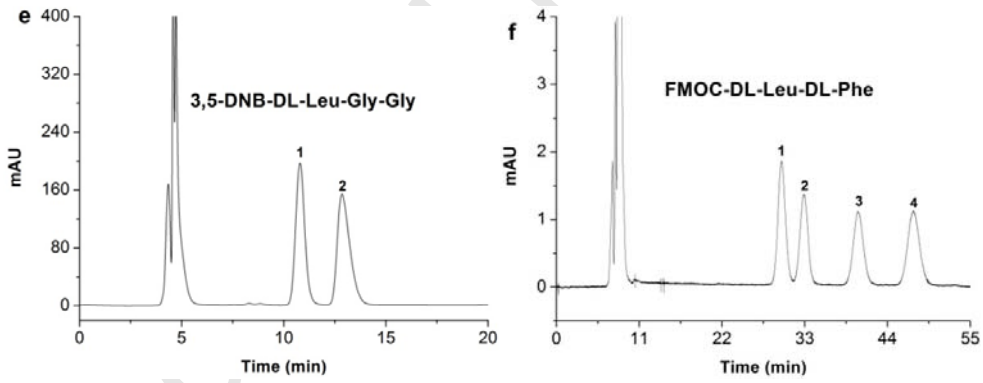
693



694



695



696

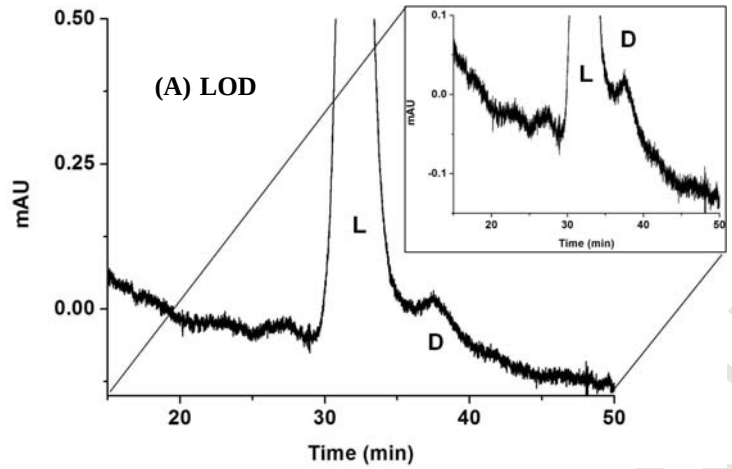
697

698

699

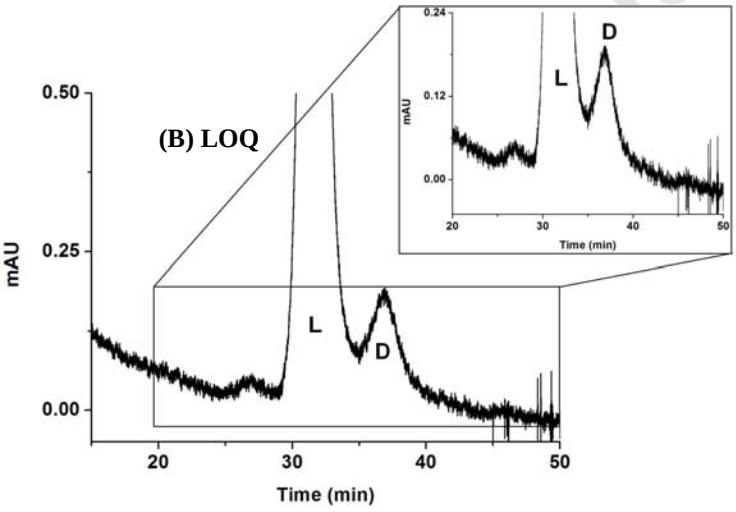
700

701

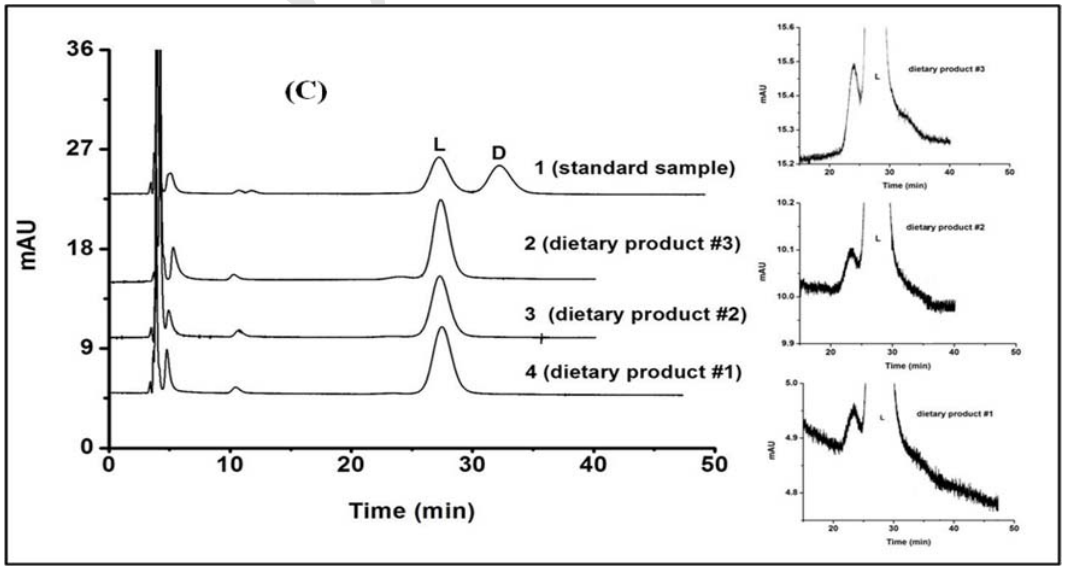


702

703



704



705

706

707