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ABSTRACT

A chiral methodology was developed in this work enabling for the first time the separation of the four stereoisomers of the amino acid 4-hydroxyproline (4-Hyp) in the format of capillary electrophoresis (CE). After a screening of different neutral cyclodextrins (CDs) in the electrokinetic chromatography (EKC) mode, methyl-γ-CD was selected as chiral selector to stereoselectively separate 4-Hyp (previously derivatized with 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl)) in a 75 mM phosphate buffer at pH 7.0. The effect of the concentration of the CD, the separation voltage, and the temperature on the chiral separation was investigated. A concentration of 10 mM for methyl-γ-CD, a voltage of 30 kV, and a temperature of 15 °C allowed the separation of the four stereoisomers of 4-Hyp in less than 21 min with resolutions between consecutive peaks of 1.5, 2.7, and 3.6. The injection of individual standard solutions of each stereoisomer enabled peak identification and the methodology was able to detect up to 0.1 % (1.3×10^{-11} mmol) of each stereoisomer. Analytical characteristics of the developed methodology were adequate to be applied to the analysis of nutricosmetic supplements. A good agreement was observed between the content determined for *trans*-4-L-Hyp and that indicated in the label for the product. No enantiomeric impurities were detected what shows the great potential of this method in the quality control of these products.

1. Introduction

Hydroxyproline (Hyp) is formed by post-translational modification of proline [1]. It is considered a conditionally essential amino acid since although it is produced by the body in situations of stress, growth, or trauma, its levels may decrease making necessary its supplementation [2,3]. Hyp has two conformations: 4-hydroxyproline (4-Hyp) and 3-hydroxyproline (3-Hyp) [4], being the first one the predominant form in collagen (the most abundant protein in animals, which has a relevant importance in the connective tissues maintenance) [5–8].

4-Hyp is a chiral compound with two chiral carbon atoms in its structure so it has four stereoisomers [9]: *trans*-4-hydroxy-L-proline (*trans*-4-L-Hyp), *trans*-4-hydroxy-D-proline (*trans*-4-D-Hyp), *cis*-4-hydroxy-L-proline (*cis*-4-L-Hyp), *cis*-4-hydroxy-D-proline (*cis*-4-D-Hyp) (see **Figure S1**). *cis*-4-L-Hyp regulates angiogenesis [10] and it is a biomarker of different types of cancer [11,12]. *cis*-4-D-Hyp is present in some fungi and bacterial species [9]. Although it is not synthesized in animal tissues [13], it can be found in liver, kidney, and some intestinal microorganisms [6,14] due to the transformation of *trans*-4-L-Hyp into *cis*-4-D-Hyp by the action of the 4-hydroxyproline-2-epimerase enzyme. *trans*-4-D-Hyp is obtained from free proline by some bacteria [9]. Lastly, *trans*-4-L-Hyp is the most relevant stereoisomer since it is present in collagen and elastin [6] as well as in several regulatory enzymes [15,16]. Since this stereoisomer provides benefits to the organism (for instance, skin, bone, and joint health) [17], it is employed in dietary supplements and cosmetic products.

The analysis of 4-Hyp is very relevant from different perspectives. In the clinical field, its determination is important because its levels depend on the physiological state and age [18]; for example, 4-Hyp is an osteoporosis biomarker in urine [19]. In Food Science, it can be used as

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an indirect collagen value in meat products [20]. Usually, the 4-Hyp stereoisomer analyzed is not specified but it is assumed that it corresponds to *trans*-4-L-Hyp since it is the most abundant in humans [9]. Nevertheless, bearing in mind the different biological functions of each 4-Hyp stereoisomers and even opposite physiological roles (*cis*-4-L-Hyp inhibits collagen generation but *trans*-4-L-Hyp promotes it [21]), the development of chiral methodologies allowing the stereoselective determination of 4-Hyp is of utmost relevance.

To date, just two articles have described the separation of *cis/trans* 4-Hyp diastereomers in collagen type I employing micellar electrokinetic chromatography (MEKC) [22] or HPLC with UV and mass spectrometry (MS) detection [23]. Other works reported the separation of only a pair of enantiomers of 4-Hyp [24,25]. Thus, gas chromatography (GC) with flame ionization detection (FID) was employed for the determination of the optical purity of 4-Hyp standards [24] while ultra-high performance liquid chromatography (UHPLC) with fluorescence detection [25] was applied to the quantification of DL-4-Hyp in collagen from pig and cod skins. In these works, two peaks were observed although it was not indicated if the separated enantiomers corresponded to the *cis* or *trans* diastereomers. The separation of three of the four stereoisomers of 4-Hyp was reported by Opekar *et al.* who developed a GC–MS method for the enantiomeric separation of eight secondary amino acids (including *cis/trans*-4-Hyp) in human biofluids, biologically active peptides, and collagen [26]. Under the best chromatographic conditions, *trans*-4-D-Hyp and *cis*-4-L-Hyp were resolved whereas *trans*-4-L-Hyp and *cis*-4-D-Hyp coeluted (at 22.9 min). Just two articles reported the separation of the four stereoisomers of 4-Hyp. Tojo *et al.* carried out the simultaneous enantiomeric determination of proline, *trans*-4-Hyp, and *cis*-4-Hyp in mouse serum and collagen-rich skin tissue in 60 min using two-dimensional micro-HPLC with fluorescence detection [27]. More recently, Wu *et al.* developed a quick method for differentiating DL-proline, DL-*cis*-4-Hyp, DL-*trans*-4-Hyp, DL-*cis*-*N*-*tert*-butoxycarbony-4-Hyp, and DL-*trans*-*N*-*tert*-butoxycarbony-4-Hyp enantiomers through trapped ion mobility spectrometry-mass spectrometry (TIMS-MS) [28] although the method was not applied to the analysis of real samples.

Although capillary electrophoresis (CE) has demonstrated to be a powerful technique in the field of chiral separations due to its high versatility, high-resolution power, and high separation efficiency [29], its potential in the stereoselective separation of the four stereoisomers of 4-Hyp has not been explored. However, a CE method could avoid the use of high-expensive analytical techniques or the use of tedious sample preparation procedures.

In this work, the first CE methodology enabling the separation of the four 4-Hyp stereoisomers derivatized with FMOC-Cl is presented. Electrokinetic chromatography using a CD as chiral selector was employed and different experimental variables, such as the nature of the chiral selector and its concentration, the separation voltage, the working temperature, and the injection time, were optimized to find the best results in terms of resolution. The developed methodology was further applied to the analysis of a 4-Hyp-based nutricosmetic supplement to demonstrate its potential to achieve the quality control of these products.

2. Materials and methods

2.1. Reagents and samples

Analytical grade reagents were employed in this work. Di-sodium hydrogen phosphate was from Supelco (Merck KGaA, Darmstadt, Germany), boric acid was from Sigma Aldrich (St. Louis, MO, USA) and sodium hydroxide was provided by Labkem (Barcelona, Spain). *cis*-4-hydroxy-L-proline (*cis*-4-L-Hyp), *cis*-4-hydroxy-D-proline (*cis*-4-D-Hyp), *trans*-4-hydroxy-L-proline (*trans*-4-L-Hyp), *trans*-4-hydroxy-D-proline (*trans*-4-D-Hyp) were from Sigma Aldrich.

Methyl-β-CD (degree of substitution (DS) 1.8) was provided by two commercial suppliers (Sigma Aldrich and Fluka (Buchs, Switzerland)). α-CD was from Sigma Aldrich, β-CD, γ-CD, and 2-hydroxypropyl-β-CD (DS 0.6) were from Fluka. Heptakis(2,3,6-tri-O-methyl)-β-CD, 2-Hydroxypropyl-γ-CD (DS 3.2 and 4.5), and methyl-γ-CD (batch CYL-1998, DS 12.6 and batch CYL-5028, DS 13.0) were purchased from Cyclolab (Budapest, Hungary). Acetonitrile (ACN) and *n*-pentane from Scharlau (Sentmenat, Barcelona, Spain) and 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) from Sigma Aldrich were employed for sample preparation. Nutricosmetic Tenseur Forte was acquired in an online cosmetic store from Portugal and contained hydroxy-L-proline and maltodextrin. No information related to the presence of *cis* or *trans* L-Hyp was given in this product. Milli-Q water System from Millipore (Bedford, MA, USA) provided the water used for solutions preparation.

2.2. CE conditions

The experiments were carried out in an Agilent 7100 CE system (Agilent Technologies, Waldbronn, Germany) provided with HP^{3D} CE ChemStation software from Agilent Technologies. Diode-array detection (DAD) at 200 nm and a bandwidth of 4 nm was employed. Uncoated fused-silica capillaries (80.5 cm total length, 72 cm effective length, 50 μm ID (362.8 μm OD) were from Polymicro Technologies (Phoenix, AZ, USA). A 75 mM sodium phosphate buffer (pH 7.0) was employed and the background electrolyte (BGE) contained 10 mM methyl-γ-CD. Final experimental conditions for the electrophoretic separation were based on the use of a temperature of 15 °C, an applied voltage of 30 kV, and sample injection at 50 mbar pressure for 10 s.

Capillary pretreatment and capillary daily washing consisted of rinsing with sodium hydroxide 1 M for 30 min, Milli-Q water for 5 min, and 60 min of buffer solution. Capillary washing between analyses was performed using sodium hydroxide 0.1 M for 2 min, Milli-Q water for 2 min, and BGE for 4 min. The pressure applied in these procedures was 1 bar.

2.3. Preparation of solutions and samples

Sodium phosphate and borate buffers were prepared by diluting the required amount of reagents in Milli-Q water. pH was adjusted adding 1 M sodium hydroxide. Electrophoretic buffer was prepared with sodium phosphate (75 mM, pH 7.0). BGE for the electrophoretic separation was obtained by disolving methyl-γ-CD at a 10 mM concentration in the electrophoretic buffer.

Boric acid buffer (200 mM, pH 9.0) was used to separately dissolve each stereoisomer (*cis*-4-L-Hyp, *cis*-4-D-Hyp, *trans*-4-L-Hyp, *trans*-4-D-Hyp) to obtain 40 mM stock standard solutions. Samples were prepared weighing and mixing the content of twenty capsules of Tenseur Forte product. The 4-L-Hyp amount declared in the product specifications was considered to elaborate stock sample solutions (0.4 mg/mL). Boric acid buffer (200 mM, pH 9.0) was employed to dissolve the required amount of the sample by sonicating it for 15 min to extract 4-L-Hyp. Finally, the solutions were centrifuged (10 min, 4000*g* at 25 °C) and the supernatant was taken.

Derivatization was carried out following a procedure previously described in the literature [30]. 200 μL of the standard solutions or sample were mixed with 200 μL of FMOC-Cl dissolved in ACN. FMOC-Cl concentration was three times the sample concentration. After 2 min, the reaction was stopped by adding 500 μL of *n*-pentane. The last step was to dilute 60 μL of the sample in 540 μL of Milli-Q water.

All vials were sonicated for 5 min before CE analysis.

2.4. Data treatment

Chemstation software from Agilent Technologies was employed to obtain migration times, peak areas, and resolutions between peaks. Corrected peak areas (peak area/peak migration time) were used. Graphs and statistical analyses were performed using Statgraphiscs Centurion XVI, Excel Microsoft, and Origin 8.0 software.

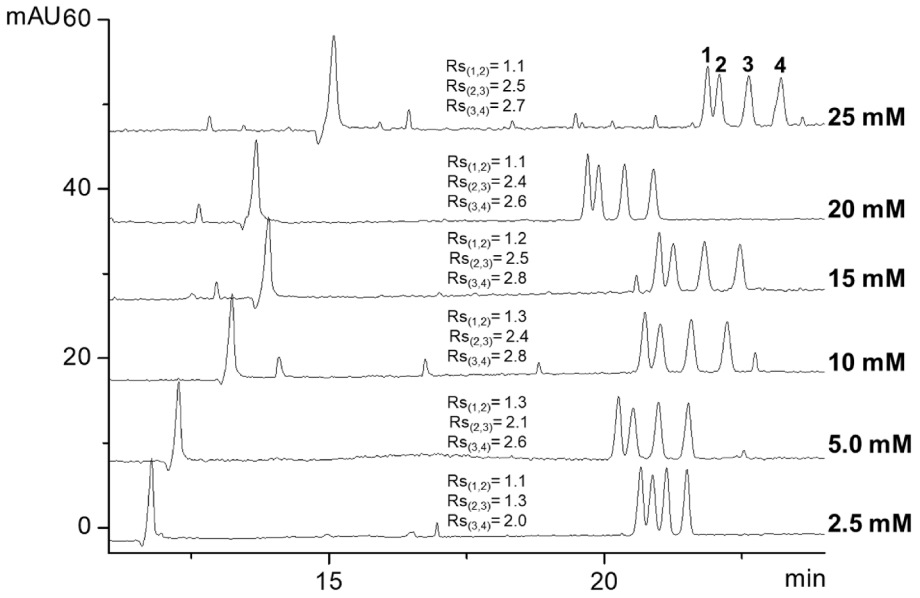

Fig. 1. Effect of the concentration of methyl-γ-CD (DS 12.6) on the chiral separation of 4-Hyp (0.125 mM each stereoisomer). Experimental conditions: 75 mM phosphate buffer (pH 7.0); uncoated fused-silica capillary, 80.5 cm (72 cm effective length) × 50 μm I.D.; applied voltage, 30 kV; temperature, 15 °C; injection, 50 mbar × 5 s; UV detection, 200 ± 4 nm.

3. Results and discussion

3.1. Development of an EKC methodology enabling the stereoselective separation of 4-Hyp

Taking into account the pKa values for 4-Hyp (1.64 for the carboxylic group, 10.62 for the amine group, and 14.86 for the hydroxyl group) and that its derivatization with FMOC-Cl takes place through the amine group, a negative charge is expected for FMOC-4-Hyp in a wide pH range. For this reason, a 75 mM phosphate buffer (pH 7.0) was chosen in order to have enough cathodic electroosmotic flow (EOF) enabling to detect the anionic analyte in the cathodic capillary end. Other initial conditions employed to start the method development for the stereoselective separation of 4-Hyp were a separation voltage of 30 kV and a temperature of 15 °C. Under these initial conditions, a screening of neutral CDs was carried out to select an adequate CD able to originate as much chiral discrimination as possible. Eigth different CDs at a 5 mM concentration, one of them with two different DS values and other acquired in two different commercial suppliers, were tested. **Table S1** groups the resolution obtained between consecutive peaks and the migration time obtained for each stereoisomer with the different CDs assayed. As this Table shows, the best results were obtained for methyl-γ-CD in terms of resolution between consecutive peaks (1.3, 2.1, and 2.6 Rs values in an analysis time of less than 22 min). Therefore, the effect of the concentration of this CD on the separation was investigated. Fig. 1 shows the electropherograms obtained when the concentration of methyl-γ-CD was varied from 2.5 to 25 mM. Taking into account the resolution between consecutive peaks, especially between the two first peaks and the second and third peaks corresponding to 4-Hyp, a 10 mM CD concentration was selected. Then, under these conditions, the effect of the voltage on the separation was studied. **Figure S2** shows that a decrease in the separation voltage caused a non-desirable increase in migration times so the value of 30 kV was kept as the best one. In a similar way, when the effect of the temperature was investigated (see **Figure S3**), it was shown that 15 °C was the value enabling the best resolution between the two first peaks although, as expected, a decrease in migration times was observed when increasing the temperature due to a decrease in the solution viscosity. Regarding injection conditions, although in all previous experiments a hydrodynamic injection at 50 mbar for 5 s was employed, an injection time of 10 s was selected given

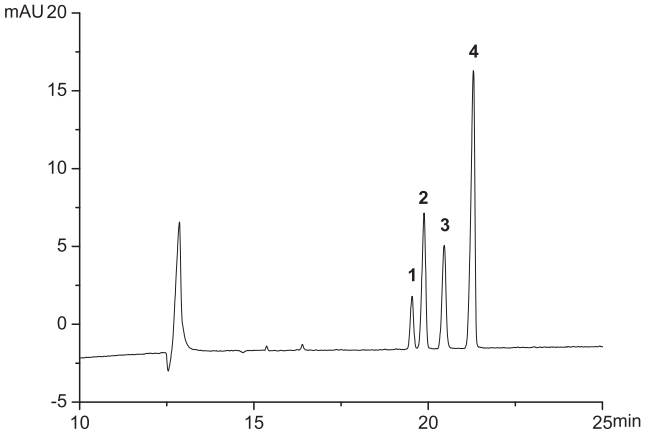

Fig. 2. Electropherograms corresponding to the chiral separation of 4-Hyp stereoisomers. Experimental conditions: 75 mM phosphate buffer (pH 7.0) containing 10 mM methyl-γ-CD (DS 13.0), injection, 50 mbar × 10 s. Other conditions as in Fig. 1. Peaks: 1, *cis*-4-L-Hyp (0.03 mM); 2, *cis*-4-D-Hyp (0.09 mM); 3, *trans*-4-L-Hyp (0.06 mM); 4, *trans*-4-D-Hyp (0.18 mM).

the increase in sensitivity obtained under these conditions without harming the resolution as shown in **Figure S4**. Finally, as the batch of methyl-γ-CD with DS 12.6 that was initially used was outdated, the results obtained when using both CD batches (DS 12.6 vs DS 13.0) were compared in terms of reproducibility, and even better resolution was found with the one with DS 13.0 displaying very similar migration times (see **Figure S5**). Under the optimized conditions, Rs values of 1.5, 2.7, and 3.6 between consecutive peaks were obtained in less than 21 min. Solutions of each stereoisomer were individually injected into the system to identify the peak corresponding to each of the 4-Hyp stereoisomers. Fig. 2 shows the chiral separation, under the optimized conditions, displaying the migration order of the four stereoisomers, being the *trans*-4-L-Hyp the third-migrating enantiomer.

Table 1
Analytical characteristics of the developed EKC method for the stereoselective determination of 4-Hyp in dietary supplements.

		cis-4-L-Hyp	*cis*-4-D-Hyp	*trans*-4-L-Hyp	*trans*-4-D-Hyp
External standard calibration method[a]					
Linear range		0.005–0.2 mM	0.005–0.2 mM	0.005–0.2 mM	0.005–0.2 mM
Slope ± t·sb		43 ± 2	43 ± 2	43 ± 2	43 ± 2
Intercept ± t·sa		0.1 ± 0.2	0.1 ± 0.2	0.1 ± 0.2	0.1 ± 0.2
R		0.999	0.999	0.999	0.999
p-value of ANOVA[b]		0.0812	0.0528	0.0653	0.0716
Matrix interferences[c]		*Confidence Interval (Slope ± t·sb)*	*Confidence Interval (Slope ± t·sb)*	*Confidence Interval (Slope ± t·sb)*	*Confidence Interval (Slope ± t·sb)*
	Sample	47 ± 8	47 ± 8	50 ± 10	45 ± 13
Precision	*Concentration level*	*RSD (%)*	*RSD (%)*	*RSD (%)*	*RSD (%)*
Instrumental repeatability[d]	0.03 mM	t, 0.3; Ac 1.2	t, 0.4; Ac 1.4	t, 0.4; Ac 1.3	t, 0.4; Ac 1.6
(n = 6)	0.06 mM	t, 1.9; Ac 2.9	t, 1.9; Ac 3.4	t, 2.0; Ac 1.9	t, 2.1; Ac 5.2
	0.10 mM	t, 2.3; Ac 3.9	t, 2.6; Ac 3.7	t, 2.7; Ac 1.6	t, 2.8; Ac 2.6
Method repeatability[e]	0.03 mM	t, 0.5; Ac 4.2	t, 0.5; Ac 5.7	t, 0.5; Ac 4.9	t, 0.5; Ac 1.0
(n = 9)	0.06 mM	t, 2.0; Ac 2.1	t, 2.0; Ac 2.2	t, 2.1; Ac 2.5	t, 2.1; Ac 1.1
	0.10 mM	t, 2.4; Ac 2.2	t, 2.4; Ac 1.9	t, 2.5; Ac 1.7	t, 2.6; Ac 2.8
Intermediate precision[f]	0.03 mM	t, 0.5; Ac 3.5	t, 1.5; Ac 2.9	t, 0.6; Ac 2.9	t, 0.6; Ac 1.3
(n = 9)	0.06 mM	t, 1.3; Ac 2.9	t, 1.4; Ac 2.8	t, 1.3; Ac 3.0	t, 1.4; Ac 0.9
	0.10 mM	t, 1.4; Ac 1.3	t, 1.3; Ac 1.7	t, 1.4; Ac 1.2	t, 1.4; Ac 1.0
Accuracy[g]		*%Mean Recovery*	*%Mean Recovery*	*%Mean Recovery*	*%Mean Recovery*
sample		104 ± 3	103 ± 3	105 ± 11	101 ± 4
LOD[h] **(mM)**		9.1×10^{-4}	1.1×10^{-3}	1.1×10^{-3}	1.1×10^{-3}
LOQ[i] **(mM)**		2.6×10^{-3}	3.5×10^{-3}	3.5×10^{-3}	3.5×10^{-3}

[a] Eight standard solutions at different concentration levels for each stereoisomer injected in triplicate for three consecutive days.
[b] p-value for ANOVA to confirm that experimental data fit properly to linear model.
[c] Comparison of the confidence intervals for the slopes corresponding to the standard additions and the external standard calibration methods.
[d] Six consecutive injections of standard solutions at three concentration levels (0.03, 0.06 and 0.1 mM of each stereoisomer).
[e] Three replicates of standard solutions at three concentration levels (0.03, 0.06 and 0.1 mM of each stereoisomer) injected in triplicate on the same day.
[f] Three replicates of standard solutions at three concentration levels (0.03, 0.06 and 0.1 mM of each stereoisomer) injected in triplicate during three consecutive days.
[g] Calculated as the mean recovery obtained when the dietary supplement containing 0.06 mM of *trans*-4-L-Hyp (nominal concentration) was spiked with known concentrations of the standard solution (50, 75, 100 and 120 % of the nominal concentration).
[h] LOD and LOQ experimentally obtained for a S/N ratio = 3 or a S/N ratio = 10, respectively.
[i] LOD and LOQ experimentally obtained for a S/N ratio = 3 or a S/N ratio = 10, respectively.

3.2. Application of the developed method to the analysis of 4-Hyp-based nutricosmetic supplements

The developed method was applied to the chiral analysis of dietary supplements. With this aim, its analytical characteristics were evaluated according to the International Council on Harmonization (ICH) guidelines Q2(R1) [31].

Method linearity was investigated by injecting in triplicate for three consecutive days eight standard solutions at different concentration levels ranging from 0.005 to 0.02 mM for each 4-Hyp stereoisomer. Corrected peak areas were plotted as a function of the concentration of each isomer and correlation coefficients of 0.999 were obtained in all cases. As shown in Table 1, for a 95 % confidence level, confidence intervals for the slopes did not include the zero value while in the case of the intercept they included the zero for the four stereoisomers. An ANOVA test confirmed that data obtained fitted to a linear model since p-values were higher than 0.05 in every case.

Matrix interferences were evaluated by comparing the confidence intervals of the slopes corresponding to the standard additions and the external standard calibration methods. Additions of 50, 75, 100, and 120 % of the nominal concentration of the dietary supplement analyzed (0.06 mM of *trans*-L-4-Hyp) were achieved. There were not statistically significant differences between the slopes obtained by both calibration methods (at a 95 % confidence level) so it could be stated that no matrix interferences existed and that the external calibration method can be used for quantitative analysis of dietary supplements. Since the response relative factor (RRF), calculated as the slope of each minor component/slope *trans*-L-4-Hyp (major component), was 1.0 in all cases, the responses of the four stereoisomers can be considered equivalent as established by the European Pharmacopeia [32]. Thus, the percentage of the minor stereoisomers can be established from the ratio between their areas and the area of *trans*-L-4-Hyp.

Instrumental and method repeatability and intermediate precision were determined to evaluate method precision. Instrumental repeatability was obtained from six repeated injections of three standard solutions of 4-Hyp at three concentration levels for each stereoisomer (0.03, 0.06, 0.10 mM). RSD values for corrected peak areas ranged from 1.2 to 5.2 % while they were lower than 2.8 % for migration times. Three replicates of standard solutions for each stereoisomer at three concentration levels (0.03, 0.06, 0.10 mM) were injected in triplicate on the same day to evaluate method repeatability. Values obtained for RSD corresponding to corrected peak areas ranged from 1.0 to 5.7 and they were lower than 2.6 % for migration times. Finally, three replicates of standard solutions of each stereoisomer at three concentration levels (0.03, 0.06, 0.10 mM) were injected in triplicate during three consecutive days to evaluate intermediate precision. RSD values ranged from 0.9 to 3.5 % for corrected peak areas and they were lower than 1.5 % for migration times.

Method accuracy was determined as the mean recovery obtained when known amounts of each stereoisomer were added to the dietary supplement analyzed containing 0.06 mM of the *trans*-L-4-Hyp (nominal concentration). Added amounts of stereoisomers represented percentages of 50, 75, 100, and 120 mM relative to the nominal concentration of *trans*-L-4-Hyp. As shown in Table 1, recoveries close to 100 % were obtained in all cases.

LODs and LOQs were experimentally determined as the minimum concentration of each stereoisomer originating an S/N ratio of 3 and 10 times, respectively. Values of LODs were 9.1×10^{-4} mM for *cis*-4-L-Hyp and 1.1×10^{-3} mM for *cis*-4-D-Hyp, *trans*-4-L-Hyp, and *trans*-4-D-Hyp, which corresponded to amounts injected of 7.5×10^{-12} mmol and $9.2 \times$

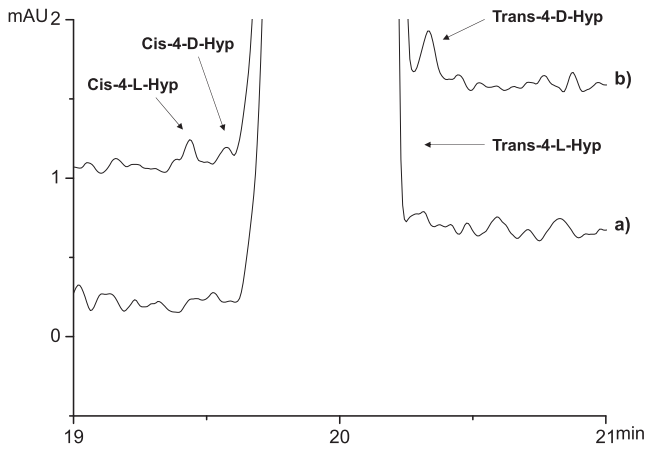

Fig. 3. Electropherograms obtained in the analysis of a dietary supplement at a concentration 1.5 mM of *trans*-4-L-Hyp: (a) non-spiked dietary supplement, and (b) dietary supplement spiked with 0.1 % (1.3×10^{-11} mmol) of stereoisomers *cis*-4-L-Hyp, *cis*-4-D-Hy, and *trans*-4-D-Hyp. Experimental conditions as in Fig. 2.

10^{-12} mmol, respectively, while LOQs were 2.6×10^{-3} mM for *cis*-4-L-Hyp and 3.5×10^{-3} mM for the other three stereoisomers, corresponding to amounts injected of 2.2×10^{-11} mmol and 2.9×10^{-11} mmol. Moreover, the relative LOD (RLOD) was 0.1 % for each enantiomeric impurity relative to the majority enantiomer in the nutricosmetic supplement.

Since the analytical characteristics of the developed methodology were found appropriate to be applied to the analysis of nutricosmetic supplements, a 4-Hyp-based supplement was injected in the EKC system. Fig. 3 shows the electropherograms obtained when the sample was analyzed and when the same sample spiked with a 0.1 % (1.3×10^{-11} mmol) of each enantiomeric impurity was injected. A content of 519 ± 7 mg/capsule was obtained for *trans*-4-L-Hyp which corresponded to a percentage of 104 ± 1 % with respect to the labeled content (500 mg/capsule). No enantiomeric impurities were detected showing that legal regulations were accomplished.

4. Conclusions

The first separation of the four stereoisomers of 4-Hyp in the format of CE is presented in this work. An EKC-UV methodology was developed using methyl-γ-CD as chiral selector. 4-Hyp was derivatized with FMOC-Cl and stereoselectively separated with a phosphate buffer at pH 7.0 containing 10 mM methyl-γ-CD in less than 21 min and with resolutions between consecutive peaks of 1.5, 2.7, and 3.6. The analytical characteristics of the developed methodology were evaluated in terms of linearity, precision, accuracy, LODs, and LOQs and found appropriate to apply it to the determination of 4-Hyp in nutricosmetic supplements. In addition, relative LODs of 0.1 % were obtained enabling to assess the absence of enantiomeric impurities. A good agreement was observed between the content of *trans*-4-L-Hyp determined in the nutricosmetic supplement analyzed and the labeled one. No enantiomeric impurities were detected in the sample. Results obtained show the potential of the developed methodology to carry out the quality control of 4-Hyp-based nutricosmetic supplements.

CRediT authorship contribution statement

Samuel Bernardo-Bermejo: Investigation, Methodology, Formal analysis, Validation, Data curation, Visualization, Writing – original draft. **Sandra Adámez-Rodríguez:** Investigation, Formal analysis, Validation, Data curation, Visualization, Writing – original draft. **Elena Sánchez-López:** Conceptualization, Investigation, Methodology, Formal analysis, Validation, Resources, Supervision, Writing – review & editing, Project administration. **María Castro-Puyana:** Conceptualization, Methodology, Formal analysis, Validation, Resources, Supervision, Writing – original draft, Writing – review & editing, Project administration. **María Luisa Marina:** Conceptualization, Methodology, Validation, Resources, Supervision, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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