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ARTICLE

Simultaneous Determination of Tryptophan and 5-hydroxytryptophan in Dietary Supplements using Capillary Zone Electrophoresis and Capacitively Coupled Contactless Conductivity Detection

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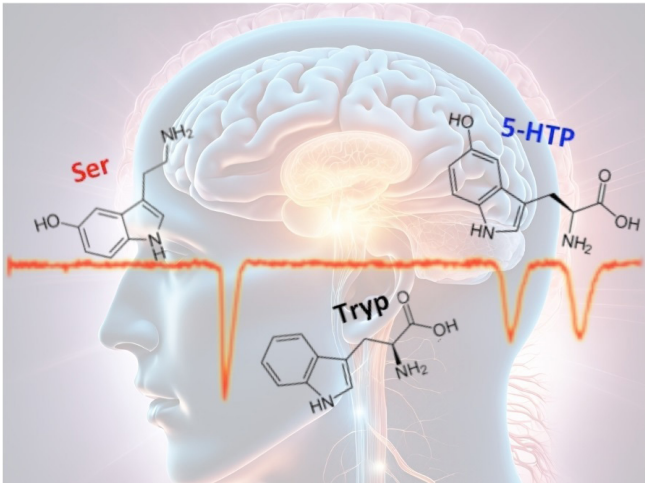

Serotonin is a neurotransmitter that plays several roles in human health, mainly related to well-being sensation and physiological processes. It is a metabolite of tryptophan and 5-hydroxytryptophan, which are used as dietary supplementation. A simple method was developed to separate these three compounds using capillary electrophoresis equipped with capacitively coupled contactless conductivity detection, where the optimized conditions were background electrolyte consisting of a 3.0 mol L^{-1} acetic acid solution pH 2.13, separation voltage at +27 kV, hydrodynamic injection at 11 kPa and 5.0 s, 40 cm (total) and 20 cm (effective) length fused-silica capillary (30 µm inner diameter). By using these conditions, complete resolution of these three compounds was achieved in less than 6 minutes with efficiencies higher than 8.6×10^4 plates m^{-1}. The limits of quantification were 66 µmol L^{-1} for serotonin and tryptophan and 132 µmol L^{-1} for 5-hydroxytryptophan. The method was applied on the determination of tryptophan and 5-hydroxytryptophan in supplements, and on the evaluation of the stability of the formulations under forced degradation studies.

Keywords: capillary electrophoresis, contactless conductivity detection, supplements, degradation, neurotransmitter

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INTRODUCTION

Serotonin (Ser) is a neurotransmitter that plays a key role in human health, being involved in many physiological processes. The synthesis of serotonin proceeds from tryptophan (Tryp), and the 5-hydroxytryptophan (5-HTP) acts as an intermediary.[1] In this way, Tryp and 5-HTP are sold as dietary supplementation because their consumption is related to the increase of Ser in the brain, leading to health improvements, mainly related to well-being sensation.[2] 5-HTP costs more than Tryp, which can lead to adulteration or contamination with Ser. Furthermore, the concentration of Tryp and 5-HTP can differ from that advertised. Thus, it is important to have a reliable method for screening these compounds in dietary supplements commercially available.

Some analytical methodologies have been proposed to evaluate the levels of Ser in biological matrices. Conversely, few methods describe the determination of Ser in the presence of Tryp and 5-HTP. Maffei stated that most of the methods for qualitative analysis of 5-HTP is based on HPLC.[3] In this direction, one can find examples using chromatography separation with mass spectrometry,[4-8] fluorescence,[9,10] and electrochemical[11] detectors, or electrochemical methods such as voltammetry.[12,13] However, dealing with their measurements in dietary supplementation is scarce.[14]

Separation in capillary zone electrophoresis (CZE) is based on the migration velocities differences of ionic species in the presence of high electric field. In the CZE separation process, the electroosmotic flow (EOF) plays a central role, acting on the resultant velocity vector and impacting the peak resolution. Coelho et al.[15] developed a CZE method for the determination of 5-HTP in supplements using a diode array detector and phthalate as an internal standard. By using borate buffer at pH 10.0 and anodic EOF, it was possible to separate 5-HTP and Tryp with an analysis time of 6.5 minutes.

Capacitively coupled contactless conductivity detection (C^4D) has demonstrated to be a versatile detection strategy when applied to CZE, since presents low cost of implementation, sensitivity comparable to UV-vis absorption, and easiness of operation.[16,17] Therefore, we report, the simultaneous determination of Ser, Tryp, and 5-HTP in food supplement samples by using CZE-C^4D. The method was evaluated regarding injection repeatability, linearity, limits of detection and quantification, and recovery. Additionally, we evaluated the stability of the supplements under stress conditions such as temperature, light, and hydrolysis.

MATERIALS AND METHODS

Chemicals and materials

Analytical grade standard L-tryptophan (Tryp), 5-hydroxy-L-tryptophan (5-HTP), serotonin (Ser), and sodium tetraborate decahydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid glacial, sodium phosphate dibasic, and sodium phosphate were acquired from Synth (Diadema, SP, Brazil). All chemicals were used as received. All the stock solutions were prepared fresh daily in Milli-Q ultrapure water (18.2 MΩ cm).

Three different dietary supplement samples were purchased at local drugstores. According to the label information, sample SP1 contains 100 mg/capsule of Tryp, sample SP2 contains 100 mg/capsule of 5-HTP, and sample SP3 contains both analytes in a concentration of 100 mg/tablet, each. The amount of the powder was dissolved in deionized water (50.00 mL), followed by sonication for 2 min. The prepared samples were diluted 20-fold, and then filtered using a membrane filter (pore size of 0.45 µm) from Merck (Darmstadt, Germany) before injection into the CE-C^4D system.

Instrumentation and method

A lab-made capillary electrophoresis (CE) system was used for the analysis, consisting on a hydrodynamic injector, a high-voltage power supply (Spellman, NY, USA), and a C^4D operating at 500 kHz and 4.0 $V_{peak\text{-}to\text{-}peak}$.[18] Details of the instrument used can be found elsewhere.[17,18] All experiments were run at room temperature, measured at 25 ± 1 °C.

A fused-silica capillary with a total length of 40 cm and an effective length of 20 cm, 30 µm i.d., and 300 µm o.d. was used. Before analysis, the capillary was flushed with deionized water (DI) for 10 min, 0.1 mol L^{-1} NaOH for 10 minutes, DI again for 10 minutes, and finally with background electrolyte (BGE) for another 10 min. Borate, phosphate, and acetic acid BGEs were tested. The standard solutions and samples were introduced hydrodynamically at 11 kPa for 5 s at the anodic end of the capillary. Separations were carried out at +27 kV using cathodic EOF.

RESULTS AND DISCUSSION

Development of the method and optimization

With a good knowledge of the pK_a and electrophoretic mobilities (μ_{ep}) values of the target species, we can predict the behavior of each compound against the pH of the BGE (Figure 1). The effective electrophoretic mobility (μ_{ef}) versus pH of each compound demonstrated that in the pH range commonly used in CZE, all species are amphoteric and can be separated using an electrical driven-technique without any derivatization or modification of the capillary internal wall.

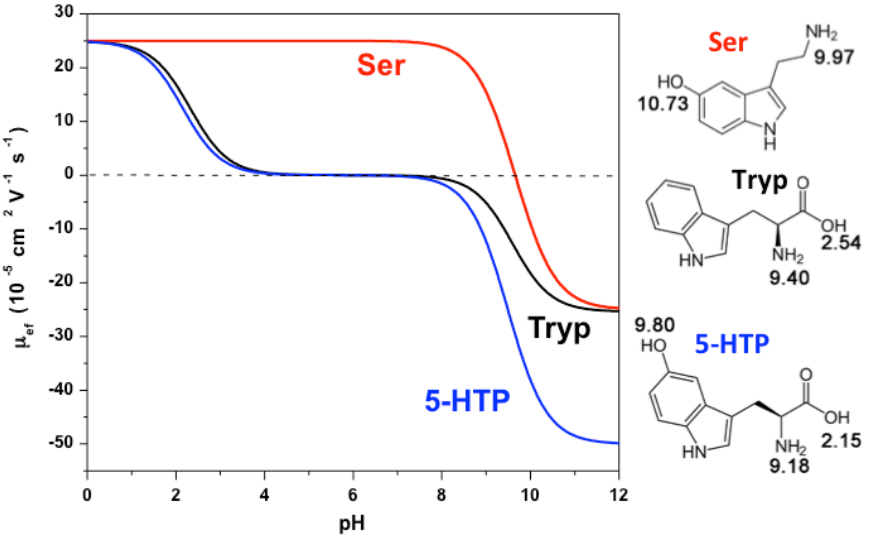

Figure 1. Effective electrophoretic mobilities (μ_{ef}) versus pH curves, molecular structures, and pK_a values of Ser, Tryp, and 5-HTP. pKa data were obtained from Chemicalize, ChemAxon Ltd. Mobility data were obtained from PeakMaster 5.3 (free download from https://web.natur.cuni.cz/gas/peakmaster.html). The electrophoretic mobilities of Ser and 5-HTP were estimated having the Tryp as base ($Tryp^+$ and $Tryp^-$) and can differ from the actual ones. No correction of ionic strength was applied.

Considering the μ_{ef} values, the best separation condition will be achieved in a BGE with pH around 9-10. In other words, the species will have a higher mobility difference, promoting a higher resolution between the peaks. We next sought to evaluate the BGE composition to find the best separation conditions for Ser, Tryp, and 5-HTP concerning pH and the voltage applied. Thus, phosphate and borate buffer (both at pH 9.3) were investigated (Figure 2).

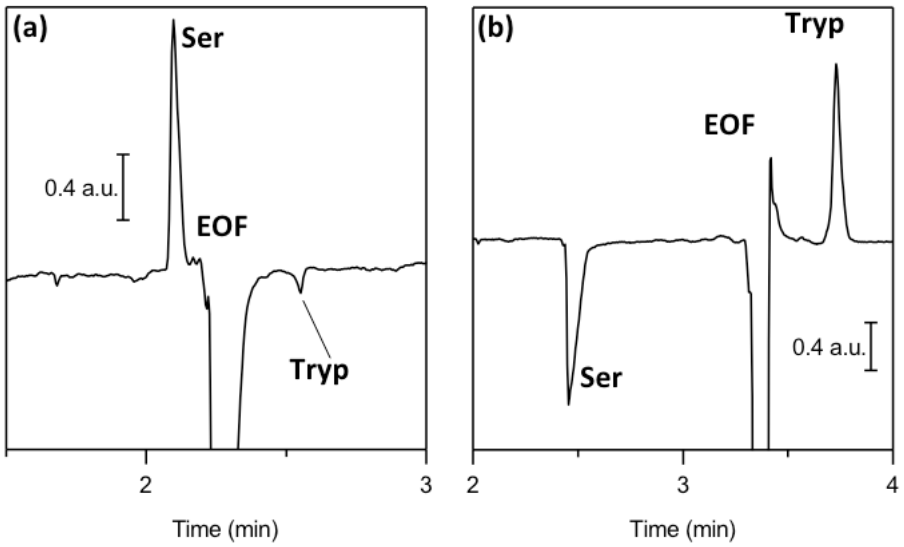

Figure 2. Electropherograms provided by the injection of a standard solution containing 200 µmol L^{-1} of Ser, Tryp, and 5-HTP in (1) 20 mmol L^{-1} borate buffer (pH 9.3) and (2) 20 mmol L^{-1} phosphate buffer (pH 9.3) using CE-C^4D. a.u. stands for arbitrary units. Capillary: 40 cm length, 20 cm effective length, and 30 µm i.d.; separation voltage, +25 kV (inlet site); hydrodynamic injection, 11 kPa for 5 s.

Ser is positively charged at pH 9.3, while Tryp and 5-HTP are negative species. This way, it was expected that both Tryp and 5-HTP would migrate after the EOF. However, it was not possible to observe 5-HTP for both BGE, probably because its μ_{ef} overcomes the mobility of the EOF (μ_{eo}) in magnitude, migrating in opposite direction and leaving the capillary without reaching the detector (Figure 2). It is interesting to note the peak shape of the species in these two BGE. Since we used C^4D, positive or negative peaks appear due to the differences on the conductivity between sample and BGE zones. For Ser, positive and negative peaks are obtained in borate and phosphate, respectively. Since Ser is a cation in this pH, these mobility differences are related to the sodium present on the BGE. A small amount of sodium hydroxide is used to adjust the pH of the borate buffer, standing for a small conductivity. Conversely, in phosphate buffer the concentration of sodium is much higher, giving a higher conductivity. In turn, Tryp is an anion at pH 9.3 and the differences on mobility stand directly to the borate and phosphate mobilities. For example, in Figure 2a, one can see that the peak of Tryp has low intensity because the conductivity of the sample zone is close to that of the BGE, which impacts also the sensitivity.

The other condition that presents the possibility of an effective separation is under a pH range of around 2-3. In this case, acetic acid solutions in three concentrations (6.0, 3.0, and 2.0 mol L^{-1}) were verified, as well as the separation voltage (+15, +25, and +27 kV) and injection time (1.0 to 8.0 s).

Considering resolution between Tryp and 5-HTP, background noise, ionic strength, buffer capacity, and detectability, the best performance was obtained using BGE at 3.0 mol L^{-1} (pH 2.13) at 25±1 °C. The separation voltage of +27 kV (faster analysis) and injection at 11 kPa for 5 s (better peak shape and resolution) were selected for the next experiments. Table S1 (Supplementary Information) summarizes the optimized condition for the proposed CZE-C^4D method.

Analytical performance

The analytical performance of the proposed method was evaluated. The run-to-run repeatability was investigated by performing a sequence of ten consecutive injections of a solution containing Ser (420 µmol L^{-1}) and equimolar amounts of Tryp and 5–HTP (630 µmol L^{-1}) (Figure 3). The RSD values for the three compounds using CZE–C^4D were lower than 2.1% and 6.0% for migration times and peak areas,

respectively. The obtained values are acceptable according to the ANVISA guidelines.[19] In addition, all the compounds were separated in less than 6 min with baseline resolution (Rs > 2.5). The separation efficiencies were higher than 8.6×10^4 plates m^{-1}. The performance parameters of the method can be accessed in more detail in Table I.

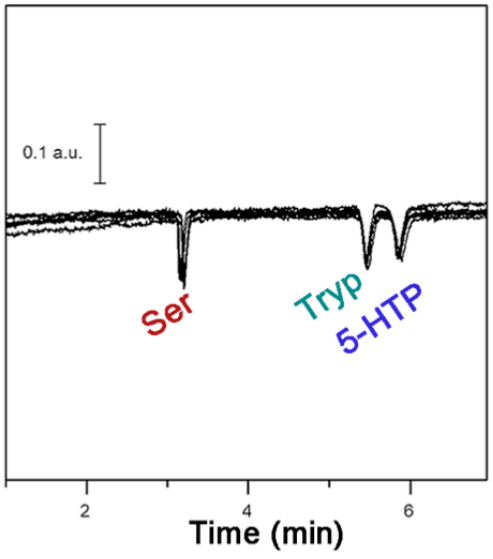

Figure 3. Electropherograms obtained from ten sequential injections of a standard solution containing 420, 630, and 630 µmol L^{-1} of Ser, Tryp, and 5-HTP, respectively. Conditions: BGE: 3.0 mol L^{-1} acetic acid (pH 2.13), capillary: 40 cm length, 20 cm effective length, and 30 µm i.d.; separation voltage, +27 kV (inlet site); hydrodynamic injection, 11 kPa for 5 s.

The method demonstrated suitable analytical features for the determination of Ser, Tryp, and 5-HTP. Determination coefficients ($R^2 > 0.990$), calibration parameters, and linear ranges (Table I) were reached for the three target compounds in the linearity study using external calibration curves (Figure 4). It is important to highlight that these results were obtained without using an internal standard method (commonly used in CE methods). The limit of detection can be obtained using an empirical approach, which consists of successive measures of more dilute standard solutions until a signal-to-noise of 3:1 is achieved.

Table I. Analytical features of proposed method CZE-C^4D (n=10)

Parameter	Ser	Tryp	5-HTP
RSD peak Area (%)	1.0	6.0	3.2
RSD migration time (%)	2.1	0.1	1.6
Migration time (min)	3.2 ± 0.1	5.50 ± 0.01	5.8 ± 0.1
N (plates m^{-1})	90065	86316	91757
Resolution (R_s)	17.5 ± 1.3[a]	2.5 ± 0.2[b]	-
Intercept	$(3.2 \pm 0.4)10^{-4}$	$(-1.9 \pm 0.7)10^{-4}$	$(-6 \pm 2)10^{-4}$
Slope	$(1.80 \pm 0.01)10^{-5}$	$(1.52 \pm 0.03)10^{-5}$	$(1.69 \pm 0.08)10^{-5}$
Linearity (R^2)	0.9990	0.997	0.990
Work range (µmol L^{-1})	70 – 700	70 – 700	130 – 780
LOD (µmol L^{-1})	20	20	40
LOQ (µmol L^{-1})	66	66	132

Resolution between [a] Ser and Tryp; [b] Tryp and 5-HTP.

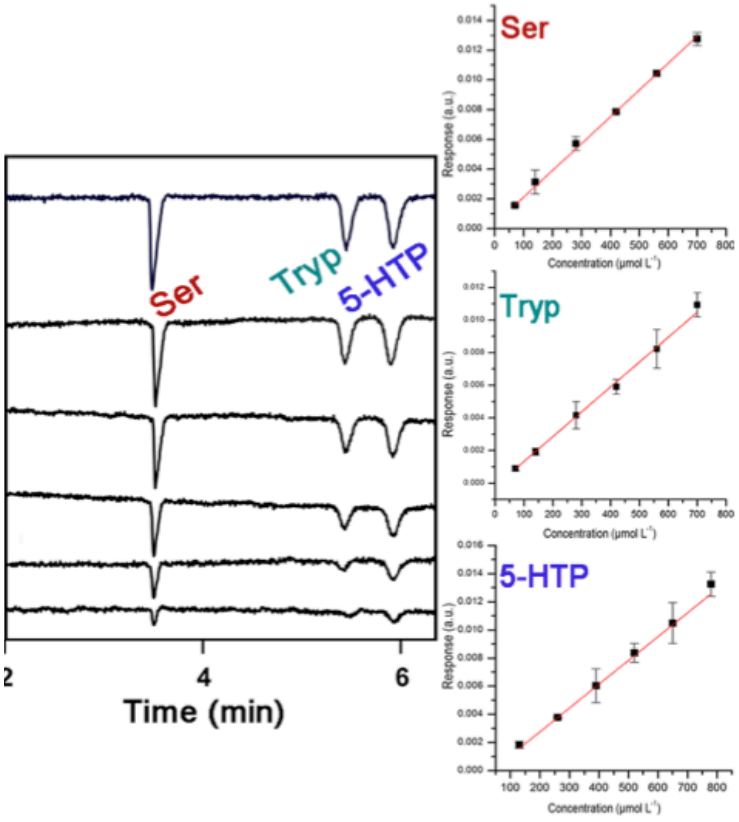

Figure 4. Electropherograms obtained from the injection of standard solutions containing Ser (70 – 700 μmol L^{-1}), Tryp (70 – 700 μmol L^{-1}), and 5-HTP (130 – 780 μmol L^{-1}). Conditions as in Figure 3. On the right, respective calibration curves are also shown.

Application of the optimized method to commercially available samples

The efficiency of the method was verified by the separation and quantification of the target compounds in three different food supplement samples. The results attained from the proposed method were statistically compared with results provided by the supplement labels at 95% confidence level (Table II). The *t*-test was used, and calculated *t*-values were smaller than the critical value (4.30; n = 3), which indicated no statistical difference between the proposed method and the results provided by the label. Furthermore, recovery studies were also performed by spiking the sample with known amounts of the target compounds (Figure 5). Recovery values of 89% to 102% were obtained, demonstrating no matrix interference and reliable accuracy for food supplement samples (Table S2).

Table II. Results (average ± SD) obtained in the analysis of food supplement samples using the proposed CE-C^4D method

Sample		Label values (mg/capsule)	CZE-C^4D (mg/capsule)	Error[c] (%)
SP1	Tryp	100	101 ± 1	-1
	5-HTP	-	-	
SP3	Tryp	-	-	
	5-HTP	100	95 ± 2	5
SP2	Tryp	100	101 ± 2	-1
	5-HTP	100	99 ± 2	1

[c] Relative difference between the label values and results achieved by the proposed method.

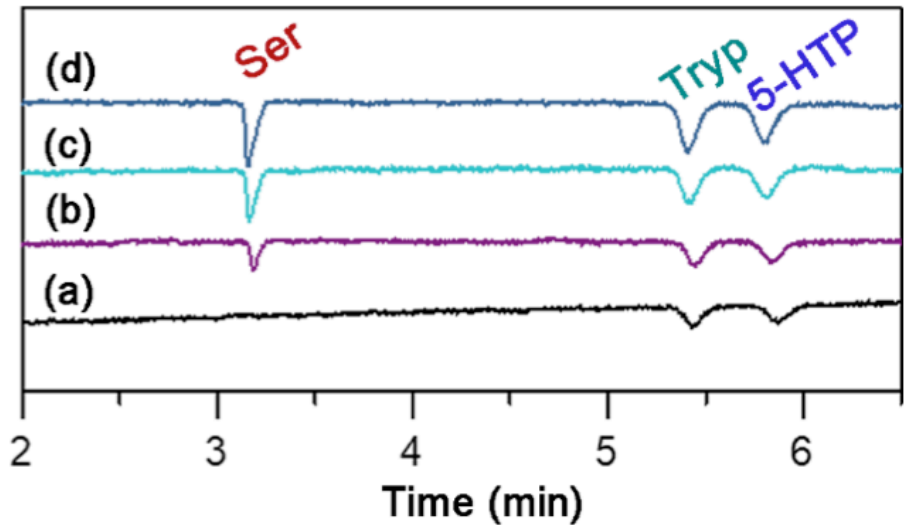

Figure 5. Electropherograms obtained for (a) diluted sample solution containing 236 and 242 μmol L^{-1} of Tryp and 5-HTP, respectively, and sample solution spiked with standard solution to give added concentrations of Ser, Tryp, and 5-HTP of (b) 140, 130 and 130 μmol L^{-1}, (c) 280, 260 and 260 μmol L^{-1}, (d) 420, 390, 390 μmol L^{-1}, respectively. Conditions as in Figure 3.

Forced degradation study

The proposed CE-C^4D method may also be employed to evaluate the degradation rate of the components of supplement formulations. The following stress testing conditions were applied to the active compound based on the available guidance provided by The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH).[20]

These studies were carried out with standard solutions containing 1 mmol L^{-1} of Tryp and 5-HTP. The stability of the analytes was monitored after being exposed to several conditions: photolytic (Figure S1a), acidic hydrolysis (Figure S1b), alkaline hydrolysis (Figure S1c), and thermal stress (50 °C) (Figure S1d). Standard solutions were evaluated before and after the stress assays with the purpose of supervising the degradation process. According to the electropherograms (Figure S1), the degradation was promoted in all conditions for both analytes. The degradation percentage (DP) of Tryp and 5-HTP (Table III) was calculated using the following equation:

$$DP = \frac{(A_{degraded} - A_{control})}{A_{control}} \times 100$$

where:

$A_{degraded}$ = peak area of the compound in the degraded solution

$A_{control}$ = peak area of the compound before degradation

Table III. DP of Tryp and 5-HTP after 24 h under stress assays

Experimental approach	Tryp (%)	5-HTP (%)
Photolytic	29	45
Thermal (50 °C)	0.5	16
H_2SO_4 (0.1 mol L^{-1})	76	80
NaOH (0.1 mol L^{-1})	75	90

Sulfuric acid was used as a protic acid to catalyse the cleavage of a chemical bond via nucleophilic substitution reaction. Sodium hydroxide was used to promote a nucleophilic substitution reaction in which the attacking nucleophile is a hydroxide ion. Hydrolytic study under basic conditions involves catalysis of ionizable functional groups present within the Tryp and 5-HTP molecular structures. As can be observed in hydrolysis assays, the degradation was pronounced (DP > 75%). Therefore, Tryp and 5-HTP were found to be unstable under these conditions.

Raising the temperature and photo exposition may increase the rate of reaction. Therefore, dietary supplement products are susceptible to degradation at photothermal expositions. Based on the results in Table III, the degradation percentage of Tryp and 5-HTP exposed to UV light and or heat for 24 h were pronounced, except for Tryp under heat, where a DP of 0.5% was found.

The mechanisms and routes for the degradation are out of the scope of this work, because the C^4D does not provide structural information. Bellmaine, Schnellbaecher, and Zimmer[21] reported a comprehensive review about the reactivity, where it is possible to find several possible degradation products of Tryp and impurities that can be present on commercial formulations. But it is worthwhile to note that no interfering peaks were observed during forced degradation studies. This can be due to the limit of sensitivity of the detector or even to the formation of negatively charged or neutral products that will not appear in the electropherogram under the conditions established for the separation.

CONCLUSIONS

We successfully describe an efficient CE-C^4D method for the quantification of Ser, Tryp, and 5-HTP in supplements, with errors lower than 5% for all analyzed samples. The developed method is a simple alternative to content analysis in food supplementation because of the low volume of reagents and samples used compared to other reference procedures. Moreover, it is evident the versatility of the C^4D when applied to CE separations of ionic or ionizable species without the need for derivatization procedures.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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SUPPLEMENTARY MATERIAL

Table S1. Optimized conditions for the proposed CE-C^4D procedure

Parameter	Evaluated Range	Optimized Value
BGE concentration (mol L^{-1})	2.0–6.0	3.0
pH of BGE	2.13–9.30	2.13
Separation Voltage (kV)	15-27	27
Injection time (s) (11 kPa)	1.0-8.0	5.0

Table S2. Recovery values of Ser, Tryp, and 5-HTP for the proposed CZE-C^4D procedure

Ser			Tryp			5-HTP		
Added (µM)	Found (µM)	Recovery (%)	Added (µM)	Found (µM)	Recovery (%)	Added (µM)	Found (µM)	Recovery (%)
-	-	-	-	236	-	-	242	-
140	144	103	130	361	90	130	347	81
280	299	107	260	479	87	260	481	92
420	407	97	390	633	95	390	614	95
Average ± SD		102±5			91±4			89±7

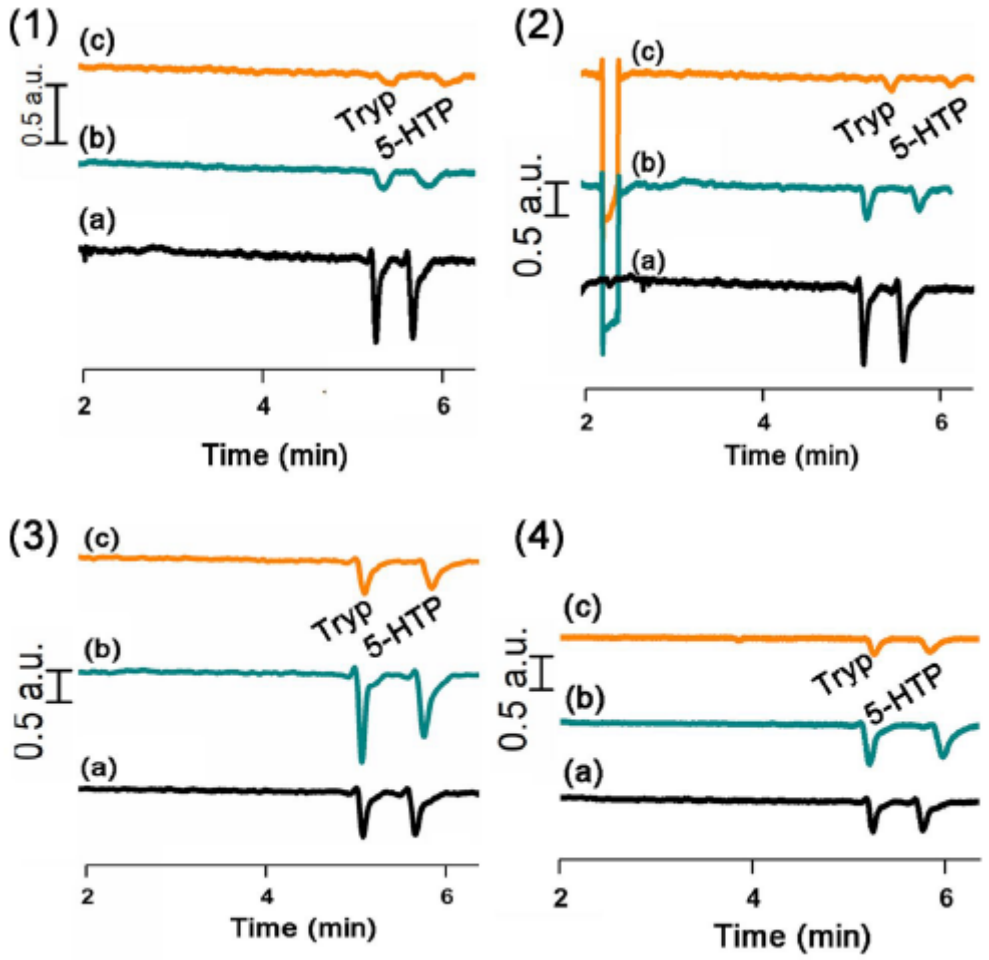

Figure S1. Electropherograms of (a) standard solutions containing 1 mmol L^{-1} of Tryp and 5-HTP; (c) standard solutions after each stress assay: (1) acidic hydrolysis, (2) alkaline hydrolysis, (3) thermal stress (50 °C), and (4) photolytic; (b) solutions after each stress assay spiked with standard solutions of 1 mmol L^{-1} Tryp and 5-HTP.