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Enantiomeric separation of non-protein amino acids by electrokinetic chromatography

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ABSTRACT

New analytical methodologies enabling the enantiomeric separation of a group of non-protein amino acids of interest in the pharmaceutical and food analysis fields were developed in this work using Electrokinetic Chromatography. The use of FMOC as derivatization reagent and the subsequent separation using acidic conditions (formate buffer at pH 2.0) and anionic cyclodextrins as chiral selectors allowed the chiral separation of eight from the ten non-protein amino acids studied. Pyroglutamic acid, norvaline, norleucine, 3,4-dihydroxyphenilalanine, 2-aminoadipic acid, and selenomethionine were enantiomericaly separated using sulfated-α-CD while sulfated-γ-CD enabled the enantiomeric separation of norvaline, 3,4-dihydroxyphenilalanine, 2-aminoadipic acid, selenomethionie, citrulline, and pipecolic acid. Moreover, the potential of the developed methodologies was demonstrated in the analysis of citrulline and its enantiomeric impurity in food supplements. For that purpose, experimental and instrumental variables were optimized and the analytical characteristics of the proposed method were evaluated. LODs of 2.1×10^{-7} and 1.8×10^{-7} M for D- and L-citrulline, respectively, were obtained. D-Cit was not detectable in any of the six food supplement samples analyzed showing that the effect of storage time on the racemization of citrulline was negligible.

1. Introduction

Non protein amino acids are a group of compounds which are not found as protein constituents. A huge amount of non-protein amino acids (more than 800) from different origin and with diverse functions have been described. Many of them are the end products of secondary metabolism, whereas many others are intermediate of metabolic pathways [1]. Although these compounds have been studied to a lesser extent than protein amino acids, they have demonstrated to be relevant in the pharmaceutical, clinical, and food fields. In fact, some of them present relevant biological activities, for instance, DOPA is applied in the treatment of Parkinsonís disease [2] and norleucine is related to the inhibition of the oxidative stress associated with Alzheimer's disease (AD) [3]. Non-protein amino acids can also be present in foods as metabolic intermediates, as products formed during food processing or as additives in food to increase some nutritional and functional properties [4,5], and they have demonstrated to be relevant markers of the food quality and safety. For example, the study of these compounds allowed to detect adulterations of olive oil with seed oils [6–8], to evaluate food processing (fermentation, storage, thermal treatment) [9–12] as well as to determine the nutritional quality and toxicity of some foods [7,8,13].

A relevant factor that should be considered in the analysis of non-protein amino acids is their chiral behavior. In nature, they are generally composed by their L-forms, however the racemization into the D-form may be produced through different metabolic pathways, by different processing conditions employed by the food industry to improve food characteristics [14–16], synthesized in enzymatic pathways by the action of microorganisms or they can even be present in supplemented foodstuffs due to the fraudulent addition of racemic mixtures [17,18]. In fact, although the use of racemic mixtures of non-protein amino acids is allowed in the pharmaceutical industry [19] the use of D-enantiomers in the food industry is forbidden and the regulatory agencies prevent their use in the elaboration of foods and dietary supplements [20]. All these facts originate that nowadays the chiral analysis of non-protein amino acids is of high interest in different fields.

Abbreviatons: Aminoadipic, 2-aminoadipic acid; CD, cyclodextrin; Cit, citrulline; DOPA, 3,4-dihydroxiphenylalanine; EKC, electrokinetic chromatography; Hcy, homocysteine; Norleu, norleucine; Norval, norvaline; Orn, ornithine; Pipe, pipecolic acid; Pyro, pyroglutamic acid; SAM, S-adenosyl-L-methionine; SeMet, Selenomethionine.

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CE has shown to be a powerful tool to carry out chiral separations since it allows short analysis times, high separation efficiency, versatility and feasibility to incorporate a great variety of chiral selectors to obtain high resolutions. EKC is the CE mode usually employed to achieve chiral separations. From the first research works dealing with the use of EKC [21–23], this technique has experienced an enormous growth being nowadays one of the best options for analytical enantioseparations [24]. Regarding non-protein amino acids, just a few research works reported the enantiomeric determination of non-protein amino acids in foods by CE. Thus, EKC-UV methodologies were developed for the chiral separation of ornithine in fermented foods (beer, vinegar and wine) and food supplements [11,25–27], S-adenosyl-L-methionine (SAM) in fruit juices [28] whereas the coupling EKC-MS^2 enabled the evaluation of fermentation processes through the enantiomeric determination of ornithine in beer samples or to identify and quantitate L-carnitine in infant formulas and dietary supplements as well as its enantiomeric impurity (D-carnitine) [29,30]. Also, a CEC method with UV detection has been described to the determination of citrulline in food supplements [31].

The aim of this work was to develop novel EKC-UV methodologies based on the use of cyclodextrins as chiral selectors to carry out the chiral separation of ten non-protein amino acids (pyroglutamic acid, norvaline, norleucine, 3,4-dihydroxyphenilalanine (DOPA), selenomethionine, homocysteine, ornithine, 2-aminoadipic acid, citrulline and pipecolic acid) that have demonstrated to play an important role in some metabolic routes or to have some interesting properties and healthy benefits. L-Citrulline and L-ornithine, which can be synthetized during the metabolism of L-arginine [32], have been used in dietary supplements to favour the metabolism of corporal fatty excess and reduce ammonia levels [31,27]. D-Cit has demonstrated to have different *in vivo/in vitro* behavior compared with L-Cit [31] while D-orn has shown toxic effects at high levels and to be an inhibitor of urea synthesis by competition with the L-enantiomer [25]. L-Norvaline is also related with L-arginine metabolism since it is an inhibitor of arginase activity, reducing urea production and increasing NO production [32]. L-Pyroglutamic acid and selenomethionine are also of high relevance in human health since the first has a number of remarkable cognitive enhancing effects [33] (its L-enantiomer is bioactive to improve blood circulation in the brain, whereas D-pyroglutamic remains inactive [34]) and the second is considered the major source of selenium to humans which is indispensable for human health since its deficit could cause an adverse impact on immune system. While L-selenomethionine occurs naturally, L-selenomethionine is toxic at high levels [35]. Regarding pipecolic acid, it is considered to be a neuromodulator which play a role in the central inhibitory γ-aminobutyric acid system. The presence of its D enantiomer in fluids and tissues can be due to food intake or production by intestinal bacteria. [12]. Due to the scarce number of studies devoted to the enantiomeric separation of these non-protein amino acids by CE, the development of analytical methodologies with this aim presents a high relevance.

2. Materials and methods

2.1. Reagents and samples

All reagents employed were of analytical grade. Boric acid, sodium hydroxide, ammonium hydroxide, and pentane were obtained from Sigma-Aldrich (Madrid, Spain). Formic acid, hydrochloric acid, and acetonitrile were from Scharlau (Barcelona, Spain). The chiral selectors β-CD, Heptakis (2,3,6-tri-*O*-methyl)-β-CD, (2-Hydroxi)propyl-β-CD (DS ~ 3), γ-CD, carboxymethyl-β-CD were purchased from Fluka (Buchs, Switzerland), methyl-β-CD, Heptakis(2,6-di-*O*-methyl)-β-CD, sulfated-β-CD, succinyl-β-CD from Sigma-Aldrich (Madrid, Spain), and (2-hydroxi)propyl-γ-CD, methyl-γ-CD, acetylated-β-CD, acetylated-γ-CD, carboxyethylated-β-CD (DS ~ 3), phosphated-β-CD, sulfated-γ-CD (DS ~ 14), and sulfated-α-CD (DS ~ 12) from Cyclolab (Budapest, Hungary). Water used to prepare solutions was purified through a milli-Q System from Millipore (Bedford, MA, USA).

DL-Norvaline (Norval), DL-norleucine (Norleu), 3,4-dihydroxy-DL-phenylalanine (DOPA), DL-homocysteine (Hcy), DL-2-aminoadipic acid (Aminoadipic), DL-selenomethionine (SeMet), D-pipecolic acid (D-pipe) and D-Ornithine (D-Orn), were supplied from Sigma-Aldrich (Madrid, Spain), whereas D-Citruline/L-Citruline (D-Cit/L-Cit), D-Pyroglutamic acid/L-Pyroglutamic acid (D-Pyro/L-Pyro), L-Pipecolic (l-Pipe) and L-Ornithine (L-Orn) were obtained from Fluka (Buchs, Switzerland).

The derivatization reagent 9-fluorenylmethoxycarbonyl chloride (FMOC—Cl) was purchased from Sigma-Aldrich (Madrid, Spain).

The six different food supplements analyzed were acquired in different Madridís markets (Spain).

2.2. CE conditions

CE experiments were carried out with an Agilent 7100 CE system (Agilent Technologies, Waldbronn, Germany) equipped with a DAD working at 210 nm with a bandwidth of 4 nm. The instrument was controlled by HP^{3D}CE ChemStation from Agilent Technologies. Separations were performed using uncoated fused-silica capillaries of 50 μm ID (362.8 μm OD) with a total length of 58.5 cm (50 cm effective length) or with a total length of 48.5 cm (40 cm effective length) purchased from Polymicro Technologies (Phoenix, AZ, USA). The samples were injected by applying a pressure of 50 mbar from 4 to 20 s, and the electrophoretic separation was achieved using reverse-polarity mode (voltage of −20 kV) and working temperature from 15 to 25 °C.

Before its first use, new capillaries were rinsed (applying 1 bar) with 1 M sodium hydroxide for 30 min, followed by 5 min with Milli-Q water and conditioned with buffer solution for 60 min. At the beginning of each day the capillary was pre-washed (applying 1 bar) with 0.1 M sodium hydroxide during 10 min, Milli-Q water for 5 min, and buffer for 40 min, and BGE during 10 min. Between injections, the capillary was conditioned with 0.1 M hydrochloric acid (2 min), Milli-Q water (1 min) and BGE (5 min).

2.3. Preparation of solutions and samples

The borate buffer solution (200 mM, pH 9.0) required to dissolve the non-protein amino acids before the derivatization step was prepared by dissolving the appropriate amount of boric acid in Milli-Q water. The separation buffer solution was prepared by diluting the appropriate volume of formic acid with Milli-Q water and adjusting the pH to the desired value with hydrochloric acid before completing the volume with water to get the desired buffer concentration (100 mM). The BGE was obtained by dissolving the suitable amount of the appropriate chiral selector (CDs) in the separation buffer.

Stock standard solutions of each non-protein amino acid were prepared dissolving the appropriate amount in borate buffer (200 mM, pH 9.0). These solutions were stored at 4 °C until the derivatization step with FMOC.

To prepare sample solutions of the six dietary supplements analyzed, the content of four capsules was weighed, powdered and mixed homogeneously. Taking into account the labeled amount of Cit in each supplement, an appropriate amount of the powdered obtained was dissolved in Milli-Q water to obtain a standard solution of 50 mM. Dissolution was performed by ultrasonication for