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# A novel hydrophilic interaction liquid chromatography method for the determination of underivatized amino acids in alimentary supplements

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ABSTRACT

Amino acids playing important roles in metabolic processes are often included in dietary supplements whose use has largely expanded over the last 20 years not only in patients with particular deficiencies, but also in athletes and even common people that want to enrich their regular daily diet. In the present study, a bare silica Kinetex core-shell 2.6 μm HILIC column was used for separation of some important hydrophilic amino acids and amino acids-like molecules *i.e.*, aspartic acid, creatine, carnitine, arginine and the tripeptide glutathione (GSH), by optimizing the chromatographic conditions for their determination in complex alimentary supplements. The contribution of partition, adsorption and ion exchange on the retention mechanism was studied by varying parameters such as water content and the counter-ion concentration in the mobile phase. Optimum conditions employed a Phenomenex Kinetex core-shell 2.6 μm HILIC (100 × 4.6 mm i.d.) column and a mobile phase of acetonitrile/potassium phosphate buffer (12.5 mM; pH = 2.8) 85:15, v/v, at the flow rate of 1.4 mL/min, using UV detection at 200 nm. A reference HPLC method for the selective determination of GSH by using 1,4-naphthoquinone as derivatization reagent was also introduced for comparative purposes.

The developed HILIC method was validated and applied to the analysis of the considered compounds in dietary supplements. Interestingly, in some of the real samples, oxidized glutathione which is an inactive impurity of GSH, was found at the level of about 20%. The proposed study confirms the importance of simple analytical methods for a rigorous quality control of dietary supplements containing unstable active ingredients.



## 1. Introduction

Amino acids (AAs) play diverse and crucial roles in metabolism, and nutritional studies have shown that their dietary supplementation modulates gene expression and enhances growth of the small intestine and skeletal muscle, or reduces excessive body fat [1]. Formulations based on L-carnitine, a conditionally essential amino acid-like molecule, and creatine, a bioenergetic compound important in muscle metabolism, are administered in promoting muscle protein synthesis [2]. Arginine and aspartic acid are often included as components of nutritional supplements offering great promise for improved health and wellbeing of humans and animals [1,3]. Glutathione (GSH), a major component of the cellular antioxidant system, can be partly absorbed from the small intestine by diet intake but it is also synthesized *de novo*, playing a role of exogenous and endogeneous antioxidant. The biological active site of GSH is represented by the thiol group of the cysteine residue and due to its high nucleophilicity, it serves as a free radical scavenger both under physiological conditions and in xenobiotic toxicity [4,5]. Food and Drug Administration does not establish standards for the contents of active substances in dietary supplements, however given their widespread use among the people of developed countries, manufacturers are now expected to guarantee the identity, purity, strength, and composition of the nutritional supplements. For example, the Good Manufacturing Practices (GMPs) aim to prevent the inclusion of the wrong ingredients as well as the addition of too much or too little of the dietary supplements ingredients [6].

The standard methods for analysis of AAs and amino acids-like molecules in these preparations, involve use of devoted systems based on ion-exchange chromatography or on pre-column derivatization followed by reversed phase HPLC [7,8]. In recent years determination using chromatography hyphenated to mass spec-

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trometry of free AAs in a variety of matrices has gained great progress mainly because of the opportunity for selective separation of the polar underivatized analytes by hydrophilic interaction liquid chromatography (HILIC) [8–11]. On the other hand, if sensitivity is not an issue, sophisticated and expensive detection techniques are not necessary and in such a case, HILIC approach shows the advantage of using low UV cutoff eluent (acetonitrile rich-mobile phase) allowing for the spectrophotometric detection of free AAs at low wavelength.

The purpose of the present study was to develop and validate a simple HPLC-UV method for analysis of underivatized aspartic acid, L-carnitine, creatine, arginine and GSH in dietary supplements formulations containing vitamins and other active ingredients. Since the analysis of GSH showed to be critical for the low content in the formulation and for its high susceptibility to oxidation that can jeopardize the stability, a reference reversed phase HPLC method for the selective determination of the active thiol by using 1,4-naphthoquinone (NPQ) as a pre-column derivatization reagent, was introduced.

## 2. Experimental

### 2.1. Materials

L-aspartic acid (≥99%), L-glutathione reduced (GSH,>97%), oxidized glutathione (GSSG,>97%), creatine monohydrate (≥99%), L-arginine (≥99.5%), boric acid and potassium phosphate monobasic (ACS reagent, purity≥99%), phosphoric acid (Ph.Eur, purity 85.0–88.0%), acetonitrile (ACN) and methanol (MeOH) Chromasolv® (HPLC≥99.9%) were obtained from Sigma-Aldrich (Milan, Italy). Carnitine tartrate was a gift from E-Pharma Trento S.p.A (Trento, Italy). 1,4-Naphthoquinone NPQ, quercetin (used as internal standard, IS) and triethylamine (TEA) were purchased from Carlo Erba (Milan, Italy). Deionized water was obtained with a Milli-Q system (Millipore, Milford, MA, USA). All the solutions were filtered using 0.22 μm filters (Millipore).

### 2.2. Solutions

Potassium phosphate monobasic buffer solution (pH = 2.8; 12.5 mM) used as a component of the mobile phase in HILIC separations, was prepared by adding phosphoric acid up to the desired pH value. All standard solutions were freshly prepared and stored at 2–8 °C during the day; routine stock solutions of aspartic acid, GSH, creatine, arginine and carnitine tartrate (0.93 mg/mL, 0.1 mg/mL, 4 mg/mL, 2 mg/mL, 0.4 mg/mL, respectively) were prepared in potassium phosphate monobasic buffer (pH = 2.8; 12.5 mM).

TEA phosphate buffer solution (pH 3.0; 50 mM) used as a component of the mobile phase in RP-HPLC of derivatized GSH, was prepared by adding phosphoric acid to an aqueous TEA solution up to the desired pH value. Borate buffer solutions (pH 7.5; 100 mM) were prepared dissolving boric acid in water and adjusting the pH with sodium hydroxide. Stock solution of GSH (12 mg/mL) used in the derivatization method was prepared in borate buffer (pH 7.5; 100 mM) and the corresponding IS stock solution (2.4 mg/mL of quercetin) was prepared in MeOH. Before derivatization, GSH stock solution was diluted in MeOH in order to obtain a mixture whose final composition contained 80% (v/v) MeOH. The derivatization reagent solution was prepared by dissolving NPQ (12 mg/mL) in MeOH/borate buffer 92:8 (v/v).

### 2.3. Instrumentation

The liquid chromatograph consisted of a PU-1580 pump equipped with the LG-1580-02 ternary gradient unit and a UV-diode-array detector (UV-DAD) model MD-910 (Jasco Corporation, Tokyo, Japan). The data were collected on a PC equipped with the integration program Borwin-PDA. The solvents were degassed on line with a degasser model DG 2080-53 (Jasco Corporation). Manual injections were carried out using a Rheodyne model 7725i injector with 20 μL sample loop. A column inlet filter (0.5 μm x 3 mm i.d.) model 7335 Rheodyne was used. Reacti-Therm/Heating/Stirring module (Pierce, Rockford, IL, USA) was used for the derivatization reaction. Sonarex Super RK 102 (35 KMZ) Bandelin (Berlin, Germany) with thermostatically controlled heating (30–80 °C) was used for ultrasonication.

### 2.4. Derivatization procedure

A 250 μL aliquot of a GSH solution (standard or sample) in the presence of IS, was treated with 50 μL of NPQ solution. The derivatization reaction was carried out under magnetic stirring in a micro-reaction vessel (3.0 mL) at room temperature (25 ± 2 °C) and pH 7.5 for 2 min. Then, a 250 μL aliquot of the mobile phase was added. Finally, 20 μL of the obtained solution was injected into the chromatograph.

### 2.5. Chromatographic conditions

HILIC separations were performed on a Phenomenex Kinetex core-shell 2.6 μm HILIC (100 × 4.6 mm i.d.) column with a mobile phase consisting of ACN/potassium phosphate monobasic (pH 2.8; 12.5 mM) 85:15, v/v, at a flow rate of 1.4 mL/min. The analysis was also performed by applying a gradient elution under the following conditions: ACN (eluent A)/potassium phosphate monobasic (pH 2.8; 12.5 mM) (eluent B) at 85:15 (v/v) for 11 min; from 11 to 20 min a linear change to 75% of eluent A and 25% of eluent B was applied then this final composition was maintained up to 30 min (flow rate = 1.4 mL/min). Detection was carried out by the UV-diode array system setting the wavelength at 200 nm for all analytes except for creatine that was determined at 260 nm.

In analysis of GSH by derivatization method, separations were performed in RP-HPLC conditions on a Phenomenex Synergi 4 μm MAX-RP (250 mm × 4.6 mm i.d.) column with a mobile phase consisting of MeOH/TEA phosphate buffer (pH 3.0; 50 mM) 65:35, v/v, at a flow-rate of 0.4 mL/ min. Detection was carried out at the wavelength of 420 nm.

### 2.6. Analysis of alimentary supplements

The composition of the commercially available formulation includes in addition to the considered analytes (*i.e.,* aspartic acid, GSH, creatine tartrate, arginine and carnitine), maltodextrines, citric acid, potassium phosphate, orange essence, magnesium oxide, sodium saccharin, aspartame, vitamin C, ferric pyrophosphate, selenium, silicon dioxide, colorants E106a, vitamins B1, B6, B12, E, folic acid.

#### 2.6.1. HILIC method

The content of 5 sachets (about 30 g) of the formulation was transferred in a mortar and was finely ground; an amount of powder equivalent to 0.9 g was transferred to a 50 mL volumetric flask and mixed with 40 mL of potassium phosphate monobasic buffer (pH = 2.8; 12.5 mM). The mixture was stirred and kept at room temperature in ultrasonic bath for 15 min; afterwards the suspension was completed to the volume with phosphate buffer and filtered through a 0.22 μm cellulose acetate membrane. Aliquots of the obtained solution were diluted 1 to 10 with a mixture of the aqueous phosphate buffer/ACN (25/75, v/v); before injection into the chromatograph, the samples were ultrasonicated (15 min at room temperature) and filtered through 0.22 μm membrane.