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Analytical Methods

Determination of free α-lipoic acid in foodstuffs by HPLC coupled with CEAD and ESI-MS

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ABSTRACT

A simple and rapid method for determination of free α-lipoic acid in different food matrices has been developed. It consists of extraction of α-lipoic acid with 0.5% glacial acetic acid in methanol by sonication, quantitative analysis of the extract by isocratic RP-HPLC (acetonitrile/methanol/50 mM potassium dihydrogen phosphate buffer adjusted to pH 3 with phosphoric acid: 350/65/585, v:v:v) at a flow rate of 0.45 ml/min coupled with coulometric electrode array detection at potentials between +300 and +700 mV and qualitative analysis by LC–ESI-MS in the negative ion mode for confirmation. Egg, dried egg powder, mayonnaise, fine peas and potatoes were analysed and free α-lipoic acid contents ranged from 0.1 to 4.2 μg/g with recoveries between 70% and 94%. Limits of quantitation were between 0.1 and 0.3 μg/g. This newly developed method can be used to establish a database for the content of free α-lipoic acid in different foodstuffs.

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1. Introduction

Alpha lipoic acid (α-LA), 1,2-dithiolane-3-pentanoic acid, is a versatile cofactor and universal antioxidant (Biewenga, Haenen, & Bast, 1997) present naturally in all pro-karyotic and eukaryotic cells. It is a redox-active compound capable of maintaining a healthy redox cellular state and hence possesses important therapeutic potential in conditions where oxidative stress is involved (Bustamante et al., 1998). In addition, α-LA is a model antioxidant which not only operates in both membrane and aqueous phases but also plays a significant role in the synergism of other antioxidants such as vitamin C (Packer, Witt, & Tritschler, 1995). It has been administered as a therapeutic agent in the treatment of various oxidative stress related diseases in which the pro and antioxidant balance is disrupted (Bilska & Wlodek, 2005; Packer, Tritschler, & Wessel, 1997).

In biological systems, α-LA can occur either in a free form, associated to proteins by hydrogen bonds or in a protein-bound form via amide linkage between the ε-amino group of a lysine residue and the carboxylic group of α-LA (Bradford, Howell, Aitken, James, & Yeaman, 1987; Reed, Koike, Levitch, & Leach, 1958), but it also forms mixed disulphides [Reed, DeBusk, Hornberger, & Gunsalus, 1953) and disulphide polymers (Thomas & Reed, 1956). The antioxidative activity of free α-LA has been studied in detail, but the antioxidant activity of the protein-bound lipoic acid has hardly been elucidated (Matsugo, Yasui, & Ozaki, 2003).

Different approaches have been made to determine α-LA in health food, dietary supplements, in food from animal and vegetable sources (Navari-Izzo, Quartacci, & Sgherri, 2002) and in biological samples. These include direct extraction or extraction after strong acid, base or mild enzymatic hydrolysis and quantitation of released α-LA (acid and base hydrolysis) or lipoyllysine (enzymatic hydrolysis). In health food or dietary supplements free α-LA could be extracted nearly quantitatively (96–99%) with different solvents and determined by HPLC/UV (Sun & Chen, 2006), HPLC with coulometric electrode array detection (CEAD) (Durrani, Schwartz, Schmid, & Sontag, 2007; Sen, Roy, Khanna, & Packer, 1999) and electrospray ionisation mass spectrometry (ESI-MS) (Durrani et al., 2007), capillary electrophoresis/UV (Sitton, Schmid, Gubitz, & Aboul-Enein, 2004), and differential pulse voltammetry at a glassy carbon electrode (Corduneanu, Garnett, & Brett, 2007). Because of the relatively high concentration of α-LA and the simple sample matrices determination of α-LA poses no problem for the analyst.

The determination in food or biological samples is more complex because of the different forms in which α-LA may occur (free or weakly bound to protein by hydrogen bonds, covalently linked to proteins). Swatiditat and Tsen (1973) showed that α-LA can be extracted from wheat flours and germs by a mixture of chloro-

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form/methanol/water and determined by thin layer chromatography. The ready extractability is explained by the presence of weakly bound α-LA. Acid hydrolysis, performed by the same authors, yielded lower concentrations of α-LA.

In order to cleave covalently protein-bound α-LA more drastic conditions have to be chosen. Shih and Steinsberger (1981) hydrolysed eggs and livers of chicken with 12 M sulphuric acid for 6 h at 125 °C, extracted the hydrolysate with benzene and determined α-LA after methylation by GC with a flame ionisation detector. The overall recovery using ^{14}C-lipoic acid was found to be 34%. Mattulat and Baltes (1992) reduced the concentration of sulphuric acid to 2 M and hydrolysed liver, kidney and meat samples at 120 °C for 7 h, extracted α-LA and determined it after derivatization with GC–MS. The recovery of this method was between 60% and 70%.

Base hydrolysis has also been used to release α-LA from complex samples. For that, samples were heated at 110 °C for 3 h in 2 M potassium hydroxide solution containing 4% bovine serum albumin. Then, α-LA was converted to its S,S-diethoxycarbonyl methyl ester and quantified by GC with a flame photometric detector. Lipoic acid could be determined selectively and the recoveries in biological and food samples like chicken, pork, beef, cuttlefish cow's milk, egg yolk and egg white were between 50% and 60%. Although α-LA is more resistant under base than under acid conditions and the addition of albumin during the extraction prevents the oxidation of α-LA to thiosulphinate or thiosulphonate it was estimated that 20–30% of α-LA present in the sample is lost during base hydrolysis (Kataoka, Hirabayashi, & Makita, 1993, 1997; Kataoka, 1998).

Mild enzymatic hydrolysis of bovine, rat and rabbit tissues using protease resulted in the liberation of lipoyllysine, which could be determined photometrically by enzymatic NADH oxidation (Akiba, Matsugo, Packer, & Konishi, 1998). The levels of enzymatically released lipoyllysine were also determined by HPLC and electrochemical detection (Packer, 1997). The highest contents of lipoyllysine in animal products were found in bovine kidney, heart and liver while spinach and broccoli were the best of the investigated plant sources. Tests with lipoyllysine bound to BSA gave about 100% recovery (Lodge et al., 1997) indicating quantitative cleavage of the linkage to the protein. Another method based on enzymatic hydrolysis (Satoh, Shindoh, & Min, 2008) included liberation of lipoyllysine with several proteases, reduction of disulphide bond, labelling with a fluorophore and analysis by HPLC with fluorescence detection. The recoveries in animal tissues were between 98.9% and 107.1%.

Applying strong acid or alkaline conditions to release α-LA from the sample results in low recoveries because α-LA is partly destroyed. Likewise, evidence exists that the content of lipoyllysine determined by mild enzymatic hydrolysis may be underestimated because some lipoyllysine may remain inaccessible to protease digestion (Lodge et al., 1997). Nevertheless, the papers mentioned above offer the possibility to approximately determine the sum of free plus protein-bound lipoic acid, and the content of protein-bound lipoic acid by means of lipoyllysine, respectively.

Yet, there is still a lack of information about the content of free α-LA, which is thought to be the most important therapeutic form (Biewenga et al., 1997). We have already been successful in developing a rapid method for quantification of α-LA acid in dietary supplements using HPLC with CEAD and ESI-MS detection (Durrani et al., 2007). The aim of the current study was to extend this method by a simple, modified sample preparation in order to extract free α-LA from complex food matrices without destroying and extracting the protein-bound α-LA. In addition, several food samples should be analysed in order to test the applicability of the method to different food matrices.

2. Experimental

2.1. Reagents and chemicals

All the reagents and chemicals were of analytical grade. Deionised water, prepared using a Barnstead Easypur LF (Dubuque, Iowa, USA) water purification system, was used in all experiments. DL-α-LA (purity ⩾ 99.0%), ±α-lipoamide (99–100%), bisphenol A (99%) and diatomaceous earth (suitable for most routine filtrations, ⩾95% SiO_2 basis, powder) were purchased from Sigma–Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate, p.A, *ortho*-phosphoric acid (85%), glacial acetic acid, HPLC-gradient grade acetonitrile and methanol were delivered from Merck (Merck, Darmstadt, Germany).

2.2. Preparation of solutions

2.2.1. Stock solutions

A primary stock solution of α-LA was prepared by dissolving 5.0 mg of α-LA in 100 ml methanol. The other standard solutions were prepared by serial dilution with acetonitrile/water (30:70, v:v). The internal standard (BPA) solution (1 mg/ml methanol) was diluted with acetonitrile/water (30:70, v:v) to 0.5 μg/ml. All solutions were stored in tightly sealed amber glass vials at 4 °C.

2.2.2. Mobile phase

Mobile phase I: acetonitrile/methanol/50 mM potassium dihydrogen phosphate (pH 3, adjusted with phosphoric acid) in a ratio of 350/65/585, (v:v:v) were mixed, filtering through a 0.45 μm Sartolon polyamide filter (Satorius AG, Göttingen, Germany) and degassing for 20 min by sonication prior to use.

Mobile phase II: consisted of 0.1% glacial acetic acid in water/acetonitrile (55:45, v:v).

2.3. Instrumentation and chromatographic conditions

2.3.1. HPLC–CEAD

The HPLC system consisted of a Merck Hitachi pump L-6200 (Merck, Darmstadt, Germany), a Basic Marathon autosampler (Spark, Holland B.V., Emmen, The Netherlands), an ACE 3 C 18 column (150 × 3.0 mm, particle size 3 μm, Advanced Chromatography Technologies, Aberdeen, Scotland) equipped with an ACE 3 C 18 pre-column and a coulometric electrode array detector controlled by Coul Array Win/Software (ESA, Chelmsford, USA). The potentials of the electrodes were set at +300, +400, +450, +500, +550, +600, +650, +700 mV against palladium reference electrodes. Twenty microlitres of the standard solutions or sample extracts were injected into the HPLC system.

Chromatographic separations were achieved isocratically using mobile phase I at a flow rate of 0.45 ml/min. Current/voltage curves of α-LA and bisphenol A (internal standard) were obtained by plotting the peak heights against the potential of the working electrodes.

2.3.2. HPLC–ESI-MS

The HPLC 1100 series system (Agilent Technology, Palo Alto, CA, USA) consisted of a G1312A binary pump, a G1322A mobile phase vacuum degassing unit, a G1313A autosampler, an ACE 3 C 18 column (150 × 2.1 mm, particle size 3 μm) with a pre-column of the same material (Advanced Chromatography Technologies, Aberdeen, Scotland). Mass spectrometric detection was performed on an HCT^+ ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionisation (ESI) source. For data acquisition Hystar 3.1 software for chromatography and

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