DISSERTATION

Determination of alpha lipoic acid content in dietary supplements and foodstuffs using high performance liquid chromatography with different detection modes

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PREFACE

This PhD research thesis is based upon the work done in the research group of Food Chemistry from December 2004 till March 2008 under the supervision of Univ. Prof. Dr. Gerhard Sontag at the Department of Analytical and Food Chemistry, University of Vienna, Währingerstraße 38, 1090-Vienna, Austria.

DEDICATED

TO

MY LATE FATHER

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
ASE	Accelerated solvent extraction
BCKADC	Branched chain α-ketoacid dehydrogenase complex
BPA	Bisphenol A
Br-AMN	2-Bromoacetyl-6-methoxynaphthalene
BZ-CL	Benzyl chloride
CE	Capillary electrophoresis
CEAD	Coulometric electrode array detector
c-Fos	A cellular proto-oncogene
CH_2Cl_2	Dichloromethane
CH_2N_2	Diazomethane
CI	Chemical ionization
DAD	Diode array detector
DBZ	Dibenzyl
DEOC	S,S-Diethoxycarbonyl
DHLA	Dihydrolipoic acid
D-Phe	D-Phenylalanine
EC	Electrochemical
ECF-HCl-Me	Elemental chlorine free hydrochloric acid-methanol
EI	Electron ionization
EI-MS	Electron-ionization-mass spectrometry
ESI	Electrospray ionization
ESI-MS	Electrospray ionization mass spectrometry
FAB	Fast atom bombardment
FID	Flame ionization detector
FL	Fluorescence
FPD	Flame photometric detector
GC	Gas chromatography
GCS	Glycine cleavage system
GSH/ GSSG	Glutathione/Glutathione disulphide
Gy	Gray (unit of radiation)
HDV	Hydrodynamic voltammograms
HPLC	High performance liquid chromatography

ICC	Ion charge control
ID	Internal diameter
IS	Internal standard
LA/DHLA	Redox couple of lipoic acid and dihydrolipoic acid
LAM	Lipoamide
LC-MS	Liquid chromatography- Mass spectrometry
LD	Laser desorption
LD_{50}	Lethal dose
LLys	ε-Lipoyllysine
LOD	Limit of detection
LOQ	Limit of quantification
m/z	mass-charge ratio
MRM	Multiple reaction monitoring mode
MS	Mass spectrometry
MTBSTFA	N-Methyl-N-(tert-butyldimethylsilyl)-trifluoroacetanide
$NaBH_4$	Sodium borohydride
NADH	Nicotinamide adenine dinucleotide (reduced form)
NF-kB	Nuclear factor-kappa B
ODS	Octadecyl silane
OPA	*Ortho*-Pthalaldehyde
PDC	Pyruvate dehydrogenase complex
POF	Pyruvate oxidation factor
QC	Quality control
RP	Reversed phase
Rs	Resolution
RSD	Relative standard deviation
SBD-F	4-Fluoro-2,1,3-benzoxadiazole-7-sulfonate
SIM	Selected ion monitoring
SPE	Solid phase extraction
tert. BDMS	Tert-butyl-dimethyl silyl
TSP	Thermospray
UAE	Ultrasound-assisted extraction (Ultrasonication)
UV	Ultraviolet
α-KADC	α-Keto-acid dehydrogenase complex

α-KGDC	α-Ketoglutarate dehydrogenase complex
α-LA	α-Lipoic acid

LIST OF PUBLICATIONS

SCIENTIFIC PUBLICATIONS

1. DURRANI, A. I., SCHWARTZ, H., SCHMID, W. and SONTAG, G. (2006): Quantitative determination of alpha lipoic acid in different dietary supplements using a rapid HPLC-CEAD method. Published in Proceedings of the „Österreichische Lebensmittelchemikertage“ pp 246-250. Editors: Cichna-Markl, M., Sontag G. and Stidl. R., Copyright Gesellschaft Österreichischer Chemiker, ISBN: 3-900-554-59-5.
2. MIHOVILOVIC, M. D., MÜLLER, B., MARKUS, S., DURRANI, A. I., STANETTY, P., DAZINGER, G. and KIRCHNER, K. (2006): Microbial Baeyer-Villiger Oxidation of Ketones by Cyclohexanone and Cyclopentanone Monooxygenase – A Computational Rational for Biocatalyst Performance**.** Monatshefte für Chemie 137: 785–794.
3. DURRANI, A. I., SCHWARTZ, H., SCHMID, W. and SONTAG, G. (2007): Alpha lipoic acid in dietary supplements: development and comparison of HPLC-CEAD and HPLC-ESI-MS methods. J Pharm Biomed Anal **45**(4): 694-699.
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5. DURRANI, A. I., SCHWARTZ, H., NAGL, M. and SONTAG, G. (2008): Rapid determination of free α-lipoic acid in foodstuffs using ultrasonication followed by quantitative determination using HPLC with coulometric electrode array and mass spectrometric detection. J. Food Chem., IN PROCESS.

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4. DURRANI, A. I., SCHWARTZ, H., SCHMID, W. and SONTAG, G (2007): Quantitative analysis of α-lipoic acid in egg yolk using HPLC with CEAD and ESI-MS detection modes. 3rd International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic.
5. DURRANI, A. I., SCHWARTZ, H., NAGL, M. and SONTAG, G. (2008): Determination of α-lipoic acid in food and supplements. First Symposium of the Vienna Research Platform of Nutrition and Food Sciences. Vienna, Austria.
6. DURRANI, A. I., SCHWARTZ, H., NAGL, M. and SONTAG, G. (2008): Quantitative determination of α-lipoic acid in some Austrian food samples

using HPLC with coulometric electrode array detection. Österreichische Lebensmittelchemikertage Eisenstadt, Austria.

ABSTRACT

Alpha lipoic acid (α-LA), 1,2-dithiolane-3-pentanoic acid, is a universal antioxidant present naturally in all prokaryotic and eukaryotic cells. Endogenously synthesized α-LA functions as a cofactor for several important mitochondrial enzyme complexes. Due to its unique antioxidant properties, it has been administered in many oxidative stress related diseases and is also commercially available in the form of dietary supplements. The aim of the present research work was to develop a simple and rapid analytical method to determine α-LA content in various dietary supplements and foodstuffs using high performance liquid chromatography (HPLC) with different detection modes i.e. ultraviolet (UV), coulometric electrode array (CEAD) and electrospray mass ionization (ESI-MS). α-Lipoic acid was extracted in 0.1 M acetic acid in methanol using the simple ultra-sound assisted extraction method followed by separation from other compounds using HPLC with different detection modes. The developed chromatographic method offered a very short run time (12 min) with good sensitivity and was validated over the linear range of 0.003-1 µg/ml ($r^2 > 0.999$) with an inter- and intra-day precision in the range of 0.48-4.19% relative standard deviation (RSD), respectively. The limit of detection was measured in both standard solution (0.01 µg/ml) and samples (0.2 to 0.9 µg/g). Bisphenol A (BPA) was used as an injection standard to measure instrument stability. Stability experiments were performed for both α-LA and internal standard bisphenol A.

α-Lipoic acid content was successfully determined in six different dietary supplements (0.4 to 33.9 mg/g), chicken eggs, dried chicken egg powder, mayonnaise, potatoes and fine peas (0.5 to 4.2 µg/g) using the developed analytical method. The recoveries were between 67 and 98% with less than 10% RSD. This newly developed method can be further used to determine α-LA content in other biological systems and foodstuffs.

ZUSAMMENFASSUNG

Alpha Liponsäure, 1,2 Dithiolan-3-pentansäure, ist ein universelles Antioxidans, das natürlich in eukaryontischen und porkaryontischen Zellen vorkommt. Endogen erzeugte α-Liponsäure fungiert als Cofaktor für verschiedene wichtige mitochondriale Enzymkomplexe. Aufgrund ihrer besonderen antioxidativen Eigenschaften wird sie bei Krankheiten, die auf oxidativem Stress beruhen, eingenommen und ist auch in kommerziell erhältlichen Nahrungsergänzungsmitteln in höherer Konzentration enthalten. Das Ziel der vorliegenden Arbeit war, eine einfache als auch schnelle analytische Methode basierend auf der Hochleistungs-flüssig-chromatographie (HPLC) in Kombination mit Ultraviolett- (UV), coulometrischer Elektrodenarray (CEAD) und massenpektrometrischer (ESI-MS) Detektion zu entwickeln. α-Liponsäure wurde mit 0.1 M Essigsäure in Methanol im Ultraschall aus den Proben extrahiert, von weiteren Substanzen mittels HPLC getrennt und anschließend detektiert. Die neu entwickelte chromatographische Methode zeichnet sich durch eine sehr kurze Laufzeit (12 min) mit verbesserter Sensitivität aus und wurde über einen linearen Bereich von 0,003 bis 1 µg α–Liponsäure/ml (r^2>0,999) mit einer „Inter-day bzw. Intra-day“ Reproduzierbarkeit von 0,48 bis 4,19% relativer Standardabweichung (RSD) validiert. Die Nachweisgrenzen (S/N=3) wurden in einer Standardlösung (1 ng/mL) als auch in Proben (0,2 - 0,9 µg/g) ermittelt. Bisphenol A (BPA) wurde als interner Standard zur Bewertung der Stabilität des Messinstrumentes herangezogen.

Der Gehalt an α-Liponsäure wurde sowohl in sechs verschiedenen Nahrungs-ergänzungsmitteln als auch in Hühnereiern, Trockeneipulver, Mayonnaise, Kartoffeln und Erbsen erfolgreich mittels der neu entwickelten Analysemethode gemessen und lag im Bereich von 0,4 bis 33,9 mg/g in Nahrungsergänzungsmitteln und 0,5 bis 4,2 µg/g in Nahrungsmitteln. Die Wiederfindung varierte abhängig von der Probenmatrix zwischen 67 bis 98% mit einer RSD < 10%. Diese neu entwickelte Methode eignet sich für die Quantifizierung von α-Liponsäure in Nahrungsmitteln als auch in anderen biologischen Proben.

1 INTRODUCTION

First chapter is based upon a brief introduction of each following chapter to give an overview of present research work. This summarized detail will help to better understand the basis and importance of this research project.

1.1 Background

In addition to other essential nutritional components, plants contain antioxidants. These natural occurring antioxidants have received considerable interest because of their potential safety, nutritional and therapeutic effects. Alpha lipoic acid (α-LA), or 1,2-dithiolane-3-pentanoic acid, is a natural antioxidant present in prokaryotic and eukaryotic cells. In the human body, it is linked to lysine residues and acts as a cofactor in different multi-enzyme complexes. It has been characterized as a universal antioxidant as it functions both in membranes and in aqueous phases and plays an important role in the synergism of antioxidants by directly recycling vitamin C, glutathione and coenzyme Q10 and indirectly recycling vitamin E.

Alpha lipoic acid is mainly present in free or protein-bound form and is of great interest in the field of food chemistry with reference to its precise determination in different matrices. Different analytical methods have been developed so far to determine α-LA content in biological samples (tissue and plasma) and various foodstuffs both from animal and plant sources. These methods include the use of different extraction methods to improve the recovery and of different analytical methods to improve the sensitivity of measurements.

Second chapter starts with a brief discussion on the structure of α-LA, its physical and chemical properties and then leads to a comprehensive literature review which consists of following two main parts:

1. Biological and therapeutic functions of α-LA
2. Previously reported analytical/chromatographic methods for the quantitation of α-LA and their applications in various samples

Here, it is important to note that α-LA is present in two forms, free α-LA and protein-bound α-LA. Alpha lipoic acid is capable of performing different functions in

both forms. Different extraction methods have been developed and validated to determine α-LA in protein-bound and total (free + protein-bound), however there is no specific method to determine free α-LA in supplements and foodstuffs.

1.2 Research questions

According to the literature survey, different analytical methods were used to determine α-LA in different sample matrices; however most of these methods faced limitations either in terms of recoveries or sensitivities. This required the development of a rapid and reliable method to determine α-LA in different matrices with better sensitivity. Among the different available forms, free α-LA is of more interest due to its more pharmacological and therapeutic function. Unfortunately, very few analytical data are available for the quantitative determination of free α-LA in different sample matrices such as pharmaceutical preparates, dietary supplements and also in foodstuffs.

High performance liquid chromatography (HPLC) is one of the most widely used analytical techniques in the determination of biologically active compounds in different sample matrices and hence can be used and further developed to determine α-LA.

Despite the fact that α-LA is a natural compound and is present in most foodstuffs, it is also commercially available in the forms of pharmaceutical preparates and dietary supplements. In past, few analytical methods were developed to analyze α-LA in above matrices using HPLC with different detection modes. However, these methods require destructive extraction methods and/or tedious derivatization steps for ultra violet (UV) or fluorescence (FL) detection. Intensive extraction conditions usually result in the loss of free α-LA and hence give only a rough estimate of bound α-LA content. High performance liquid chromatography coupled with mass spectrometry (HPLC-MS) was also used to determine α-LA in different matrices, however there was still a need to improve the conditions for better sensitivities.

1.3 Aim of the research

The aim of the present research work was to develop a non-destructive method to extract free α-LA from different matrices followed by precise determination using high performance liquid chromatography with different detection modes.

Third chapter gives a brief sketch of the experimental work to be followed in the next pages of this thesis and can be easily divided into following two main parts:

1. Development and validation of a rapid and reliable analytical method to determine free α-LA content with better sensitivity
2. Determination of free α-LA in various dietary supplements and foodstuffs by successfully applying the developed analytical method

1.4 Theoretical background

Different instruments were used in the experimental work and are briefly discussed in the following chapter (chapter: 4). It includes a basic introduction to high performance liquid chromatography with an insight to three different detectors used in the experiments i.e. ultraviolet (UV), coulometric electrode array (CEAD) and electrospray mass ionization (ESI-MS). It also gives a basic introduction to three different set-ups used in the sample preparation namely solid-phase extraction, accelerated solvent extraction and ultra-sound assisted extraction.

1.5 Method development

In chapter 5, an analytical method was developed to determine α-LA content using high performance liquid chromatography (HPLC) with different detection modes i.e. ultraviolet (UV), coulometric electrode array detection (CEAD) and mass spectrometric detection with electrospray ionization (ESI-MS). Newly developed method required short and simple sample preparation step with a new approach and gave short run time with better sensitivity and selectivity. For HPLC-UV, different wavelengths were checked for better sensitivity despite the fact α-LA is a weak UV absorbing compound. We were able to determine α-LA at 215 nm with better sensitivity then the one cited in the literature i.e. 208 nm and 332 nm.

In HPLC-ESI-MS, electrospray ionization mode gave more possibilities than the conventional single quadruple/ ion trap detection mode. A more selective and sensitive detection method was developed using electrospray mass ionization in the multiple reaction monitoring mode (MRM) by first fragmenting the deprotonated molecular ion (*m*/*z* 205) without being isolated, and then isolating the fragment ion (*m*/*z* 171). Hence, both the original fragment ion (the one created in the source) and the fragment ion created from the deprotonated molecular ion in the trap contributed to the total ion current and the corresponding peak area was used for quantification. The approach gave higher sensitivity then all the other mass spectrometric methods cited in the literature.

In HPLC-CEAD, half wave potential was used as cell potential to determine α-LA content which is again a new approach for the determination of α-LA. This new approach discarded the extensive cleaning of the cells as was previously done with the conventional electrochemical cells while determining α-LA content and also provided a decreased noise of the detector and a lower detection limit.

1.6 Optimization of sample preparation

Sample preparation is one of the most crucial steps in any analytical method. Chapter 6 deals with the selection and optimization of this step for the quantitative determination of α-LA in various samples of complex matrices.

α-Lipoic acid is mainly present in two forms i.e. free and protein-bound form. The main emphasis of this thesis is to determine free α-LA content in different sample matrices. Most analytical methods cited in the literature do not differentiate between these two forms of α-LA and hence do not give a clear overview of its distribution in the sample matrix. The use of drastic hydrolytic conditions may lead to the destruction of free α-LA form and hence give merely the information about the bound form which is also not so stable under the applied conditions and hence the recoveries are very low.

In this research work, both hydrolytic and non-hydrolytic methods were used to extract α-LA from the samples under investigation. Acid, base and enzymatic hydrolytic methods were checked for the recoveries at different conditions. Non-

hydrolytic methods such as accelerated solvent extraction and ultra-sound assisted extractions were also applied at different conditions. More detailed information is given in the chapter 6. Among these methods, ultra-sound assisted extraction was selected due to its non-destructive nature and less complex extracts. This extraction method can only extract free α-LA and not the protein-bound form and hence gives a clear investigation of only one form of α-LA. The results were verified using MS and the method was further applied to determine the free α-LA in various foodstuffs and dietary supplements.

1.7 Brief overview of results

In chapter 7, six different dietary supplements, purchased in Austria and United States, were analyzed for the α-LA content. Only in one supplement, the experimentally calculated value matched with that of the claimed one. While in rest, the experimentally calculated values were less than the claimed ones. Standard addition was performed to eliminate the influence of matrix compounds on the measurement of α-LA and gave good recovery 96 ± 2.1% (n = 3). The data also confirmed the results obtained by the external calibration method. The deviation between the claimed values and the data found could be due to improper handling during the production of the supplements or degradation of α-LA during storage.

In addition, free α-LA was determined in different foodstuffs using high performance liquid chromatography with coulometric electrode array detection (chapter 8). Foodstuffs analyzed included fresh egg yolk, fresh egg white, dried whole egg powder, boiled egg yolk, boiled egg white, mayonnaise, fresh potatoes and canned peas. The recoveries were good in all the experiments (70-94%) with good intra and inter-day repeatabilities. Best source of free α-LA was fresh potatoes (1.5-4.2 μg/g; 94 ± 4.7% RSD). It was also found in good quantities in dried egg powder (1.3 μg/g; 92 ±3.8% RSD), fresh egg yolk (0.5-0.9 μg/g; 89 ±4.5% RSD) and also in young peas (0.5-1 μg/g; 93 ± 5.3% RSD). However α-LA was found to be in low amount i.e. 0.6 μg/g (recovery: 67 ±7.2% RSD) in mayonnaise and below limit of detection in other food samples under investigation.

1.8 Suggestions and recommendations

The developed method was successfully utilized to determine free α-LA content in different dietary supplements and foodstuffs. The rapidity of the ultra-sound assisted extraction can be used as such or with some modifications to determine free α-LA in other foodstuffs and pharmaceutical preparates. The use of coulometric electrode array detection offers addition advantages being more sensitive and selective than other conventional detection methods.

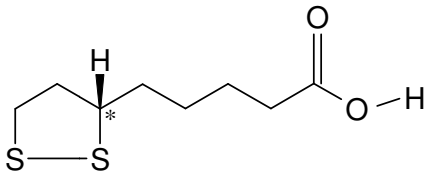
Following are some suggestions or recommendations to further extend the present research work:

1. This method can be used to determine free α-LA in egg and egg-based products.
2. This method can be used to determine free α-LA content in different vegetables and vegetable-based foodstuffs.
3. This method can be used to compare the free α-LA content in food obtained from different regions and also in cooked and raw foodstuffs.
4. Different pharmaceutical preparates and supplements can be investigated for the available α-LA content.
5. This method when coupled with a specific enzymatic hydrolysis can give information about both free and protein-bound α-LA content in different samples.

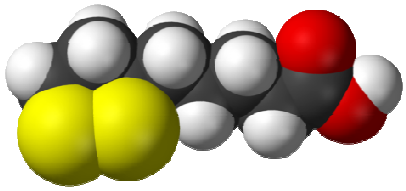
2 LITERATURE SURVEY

Alpha lipoic acid, α-LA, [$C_8H_{14}O_2S_2$] is a disulphide compound found naturally in a diverse group of micro-organisms and in a variety of plant and animal tissues (HERBERT and GUEST; 1975). It is mainly present in mitochondria and plays a pivotal role in energy metabolism.

α-Lipoic acid is an eight-carbon fatty acid containing two sulphur atoms attached at carbons 6 and 8 (Figure 1). Carbon atom 6 is asymmetric and α-LA exists as two enantiomers designated R (d) and S (l). The naturally occurring form is R (d). Most commercial preparations of α-LA consist of the racemic mixture, i.e. a 50/50 mixture of the R- and S-enantiomers.



(a)



(b)

Figure 1: Alpha lipoic acid (a) Chemical structure; (b) Space filling model

2.1 Brief history

In the 1930s, it was found that a certain "potato growth factor" was necessary for growth of certain bacteria and was named pyruvate oxidation factor (POF) later on (GUNSALUS; 1953). It was first isolated in 1951 and the synthetic compound designated α-LA proved to be the correct molecule (REED, DE, GUNSALUS and HORNBERGER; 1951; INAM-UL-HAQUE and AMIN; 2007). α-LA was tentatively

classified as a vitamin after its isolation, but it was later found to be synthesized by animal and humans (CARREAU; 1979).

2.2 Chemical names

5-(1,2-dithiolan-3-yl) valeric acid; acide 5-(1,2-dithiolanne-3-yl) valerique (French); 5-(1,2-Dithiolan-3-yl)valeriansäure (German); acido 5-(1,2-ditiolan-3-il)valerico (Spanish) (EINECS); Thioctic acid; 5-(1', 2'-Dithiolan-3'-yl)-valerianic acid; 1,2-Dithiolane-3-pentanoic acid; (±)-Lipoic acid; α-Liponic acid; 1,2-Dithiolane-3-valeric acid; 5-(1,2-Dithiolan-3-yl) pentanoic acid; 6,8-Thioctic acid; 6-Thioctic acid; Lipoic acid; Liposan ; Lipothion; Protogen A; Pyruvate oxidation factor; Thioctsan; Tioctacid; Tioctidasi; Tioctidasi acetate replacing factor

2.3 Biological functions

α-Lipoic acid is present in both free and protein bound form and plays significant role in the biological system. It is essential as an acyl carrier in the oxidative decarboxylation of the α-keto acids i.e. pyruvate and α-ketoglutarate (REED, FERNANDEZ-MORAN, KOIKE and WILLMS; 1964; SCHMIDT, GRAFEN, ALTLAND and GOEDDE; 1969) and it is an aminomethyl carrier in the glycine-cleavage enzyme systems (FUJIWARA, OKAMURA-IKEDA, PACKER and MOTOKAWA; 1997). General names and characteristics of α-LA are summarized in Table 1.

The stereo-chemical form of α-LA confers most of the characteristics that give its chemical and biochemical activities. Sulphur atoms of α-LA are easily reduced and oxidized. The reduced form of α-LA is a vicinal dithiol called dihydrolipoic acid, DHLA. The LA/DHLA redox couple can interact with a wide range of reactive oxygen species, other antioxidants and thiol compounds.

Lipoate is biosynthesized by involving a non-heme iron-containing synthase capable of inserting S atoms into positions 6 and 8 of octanoate for the dithiolane ring system (JORDAN and CRONAN; 1997). Lipoate is fairly absorbed *in vivo* even at the therapeutic levels.

Table 1: Physical and chemical properties of α-LA

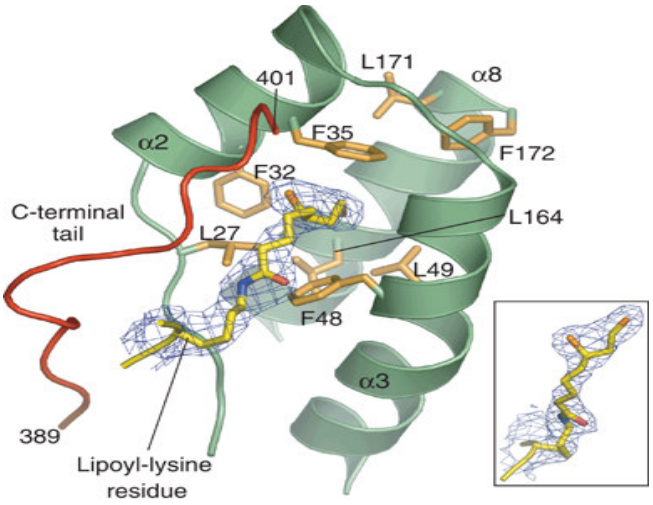
Alpha lipoic acid (α-LA)	
Registry number:	1077-28-7
Formula:	$C_8H_{14}O_2S_2$
Molecular weight (g/mol)	206.35 Da
CA index name:	1,2-Dithiolane-3-pentanoic acid
CAS registry number:	62-46-4
Accession number:	97320 CHEMLIST
Appearance	Slightly yellow crystal powder almost odourless
Particle size	100% through 40 mesh
Melting point	60-62 °C; Press: 760 Torr
Boiling point	160-165 °C
Density	1.218±0.06 g/cm^3; Temp: 20 °C; Press: 760 Torr
Freely rotatable bonds	5
Molecular weight	206.33
pKa	5.3*/4.75±0.10; Most Acidic; Temp: 25 °C *Different values reported
Polar surface area	87.9 A^2

Following is a brief description of biological roles played by α-LA as a prosthetic group bound to proteins and also as a free molecule (BUSTAMANTE, LODGE, MARCOCCI, TRITSCHLER et al.; 1998).

2.3.1 Protein-bound alpha lipoic acid

α-Lipoic acid when coupled to its different complexes via an amide linkage to a lysine is referred to as a lipoyl group (KOIKE and REED; 1960; KOCHI and

KIKUCHI; 1976), the structure is shown in Figure 2. It acts as a cofactor in *α-keto-acid dehydrogenases* and in the glycine cleavage system. These enzyme complexes are involved in the metabolic pathways of pyruvate oxidation, the citric acid cycle, and amino acid degradation and biosynthesis.



Source: http://www.nature.com/emboj/journal/v24/n10/full/7600663a.html

Figure 2: Structure of alpha lipoic acid linked with the lysine residues

2.3.1.1 Alpha keto acid dehydrogenase complexes

The *α-keto acid dehydrogenase* complexes, i.e., the pyruvate *dehydrogenase complex* (PDC), the *α-ketoglutarate dehydrogenase* complex (α-KGDC), and the branched chain α-ketoacid dehydrogenase complex (BCKADC) constitute an almost ubiquitous family of enzymes (PATEL, ROCHE and HARRIS; 1996). Following is a schematic representation of the reactions conducted by three enzymes, E_1, E_2 and E_3 of *pyruvate dehydrogenase* complex (Figure 3).

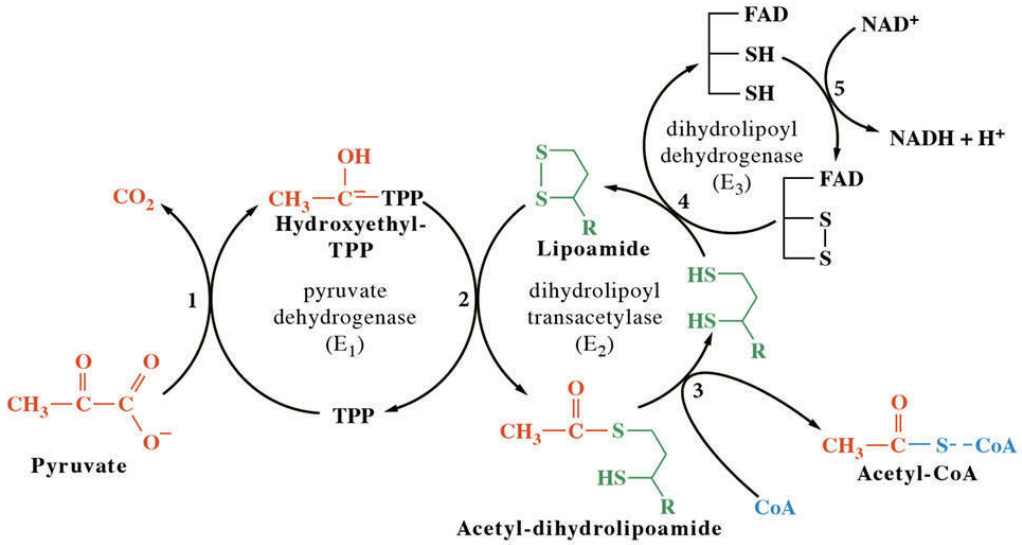


Figure 3: Schematic representation of the reactions conducted by alpha lipoic acid working as a cofactor in pyruvate dehydrogenase complex

The lipoyl domains, whose number depends on the species, contain the α-LA attached in an amide linkage to the ε-amino group of a lysine residue and this acts as a "swinging arm" ferrying the substrate between the active sites of the complex. It binds acyl groups and transfers them from one part of the enzyme complex to another one. Some examples are shown in Table 2; taken from Bustamante et al. (BUSTAMANTE et al.; 1998). In this process, α-LA is reduced to DHLA, which is subsequently re-oxidized by *lipoamide dehydrogenase* with the formation of NADH.

Overall, α-LA and DHLA act as a redox couple, carrying electrons from the substrate of the *dehydrogenase* to NAD^+ (REED; 1996; RECHE and PERHAM; 1999; GUEGUEN, MACHEREL, JAQUINOD, DOUCE and BOURGUIGNON; 2000).

Table 2: Enzymes with lipoyl or biotinyl-bounded lysine

Enzyme	**Substrate**	**Product**	**Lysine Positions (Accession Number)**	**Moiety-Bound to Lysine**
E_2 component of pyruvated dehydrogenase (human)	Pyruvate	Acetyl-CoA	K[1] 100(P10515)	Lipoyl
E_2 component of pyruvated dehydrogenase (human)	α-ketoglutarate	Succinyl-CoA	K[1] 110(P36957)	Lipoyl
E_2 component of pyruvated dehydrogenase (human)	Leucine, isoleucine and valine oxoacids	Isovaleryl-CoA	K[1] 105(P111182)	Lipoyl
E_3 component of pyruvated dehydrogenase (human)	E_3	Supramolecular complex	K[1] 97	Lipoyl
Glycine cleavage system (H protein, human)	Glycine	CO_2 and NH_4^+	K[1] 107(P23434)	Lipoyl
α-Propionyl-CoA[2] carboxylase (rat)	Propionyl-Co-A	(D,L)-methyl-malonyl-CoA	K[1] 668(P05165)	Biotinyl

[1]As defined in the SWISS-PROT database.

[2]Interestingly a G-X15-K-X10-G structural motif is highly conserved in various mitochondrial enzymes such as *α-propionyl-CoA carboxylase*, *pyruvate carboxylase*, *acetyl-CoA carboxylase*, *methyl crotonyl-CoA carboxylase*, and the E_2 component of the branched chain *α-ketoacid dehydrogenase* complex (BROWNER, TARONI, SZTUL and ROSENBERG; 1989). These enzymes share the common property to possess a biotinylated or a lipoylated lysine residue, respectively, which play a central role for enzyme activity.

2.3.1.2 Glycine cleavage system (GCS)

The glycine cleavage system is a multi-enzyme complex that is located only in the hepatic mitochondrial matrix and catalyzes the oxidation of glycine to carbon dioxide and ammonia, forming NADH and (N^5, N^{10})-methylenetetrahydrofolate. The complex consists of four proteins termed P-, T-, L-, and H-protein. α-Lipoic acid is covalently attached to a lysine in the H-protein.

During the enzymatic catalysis P-protein, a pyridoxal phosphate-dependent *decarboxylase,* catalyzes the release of carbon dioxide from glycine and transfers the methylamine moiety to the lipoyl prosthetic group of H-protein. The α-LA group is reduced during the transfer. T-protein catalyzes the formation of ammonia and the transfer of one-carbon group from the lipoyl residue of H-protein to tetrahydrofolate. L-protein is a lipoamide dehydrogenase that catalyzes the oxidation of the dihydrolipoyl residue of H-protein and the reduction of NAD^+. Thus, the α-LA prosthetic group of the H-protein interacts with the active sites of three different enzymes in a similar manner as was found in the *α-keto-acid dehydrogenase complexes* (KADC).

2.3.2 Free alpha lipoic acid

Free α-LA (non-protein bound) is a water and lipid soluble fatty acid. Its solubility in water is controlled by its carboxylate moiety. The octanoic backbone is hydrophobic. Limited water solubility and hydrophobicity of α-LA affects its stability and utility in cell culture systems. Factors that affect the availability of α-LA for use by cells in culture include; i) its possible loss upon storage at low temperatures, ii) its filterability, and iii) the complexes it forms.

Interaction of α-LA with various protein systems has been analyzed and α-LA has been observed to act at various levels in biochemical pathways, as a substrate, an inhibitor, or an effector (PACKER, WITT and TRITSCHLER; 1995).

2.3.2.1 Universal antioxidant

α-Lipoic acid has gained considerable attention as an antioxidant. Lipoate, or its reduced form, dihydrolipoate, reacts with reactive oxygen species such as

superoxide radicals, hydroxyl radicals, hypochlorous acid, peroxyl radicals, and singlet oxygen (BIEWENGA, HAENEN and BAST; 1997). It also protects membranes by interacting with vitamin C and glutathione, which may in turn recycle vitamin E (PODDA, TRITSCHLER, ULRICH and PACKER; 1994). In addition to its antioxidant activities, dihydrolipoate may exert pro-oxidant actions through reduction of iron.

Administration of α-LA has been shown to be beneficial in a number of oxidative stress models such as ischemia-reperfusion injury, diabetes, cataract formation, HIV activation, neurodegeneration, and radiation injury (PACKER, WITT and TRITSCHLER; 1995).

2.3.2.2 Alpha lipoic acid as an enzyme substrate

An effect of free α-LA on the enzyme activity of the glycine cleavage system has also been reported (HIRAGA and KIKUCHI; 1980). Free α-LA was able to replace the entire H-protein of this complex as an acceptor of the methylamine carbanion (the intermediate that is formed during the conversion of glycine to CO_2, NH_4^+, and N^5, N^{10}-methylenetetrahydrofolic acid) although with an efficacy 1000-fold lower than that of H-protein containing α-LA as a prosthetic group.

It has also been observed that *serine hydroxyl-methyltransferase*, the enzyme responsible for the transformation of a large number of amino acids to glycine, can catalyze in the presence of free α-LA resulting in the direct decarboxylation of glycine with subsequent formation of methylamine-lipoate (ZIESKE and DAVIS; 1983).

In addition, α-LA has been reported to be a substrate for a lipoamide dehydrogenase detected in human serum. In this case, the efficacy of α-LA as substrate was eightfold lower than that of lipoamide (PELLEY, LITTLE, LINN and HALL; 1976).

2.3.2.3 Alpha lipoic acid as an enzyme inhibitor

An inhibitory effect of α-LA on the activity of various enzymes has also been observed. α-Lipoic acid (at a dose ranging between 50 and 150 mg/kg) prevents the conversion of *xanthine dehydrogenase* to *xanthine oxidase* in rat intestine

homogenates (at a level ranging from 40% to 52%) after 20 min of incubation at 10°C.

2.3.2.4 Redox regulation of proteins and influence on protein folding

Thiolation of proteins has been reported to be a protective mechanism against oxidative stress, as well as affecting the function of some thiol-containing proteins. Glutathione is the most abundant thiol in mammalian cells and perhaps a primary agent involved in redox regulation of protein thiols under normal conditions (MEISTER; 1989). α-Lipoic acid may influence intracellular function not only through antioxidant actions but also through affecting the redox status of thiol-containing proteins, such as thioredoxin, enzymes, and transport proteins (THOMAS, CHAI and JUNG; 1994).

2.3.2.5 Effects on gene expression

There has recently been a great deal of interest in the effects of oxidants and antioxidants on signal transduction and gene expression in both normal and abnormal conditions. In this regard α-LA and DHLA have been investigated in terms of their effect on the transcription factors NF-kB, which regulates the expression of genes such as human immunodeficiency virus type 1 and those involved in inflammatory responses (BAEUERLE; 1991). The effects of both α-LA and DHLA, on the expression of the growth-regulating gene c-Fos, have also been investigated (MIZUNO and PACKER; 1994). Although it appears that DHLA and/or α-LA may influence gene expression at one or more levels, the exact mechanisms and significance have yet to be elucidated.

Administration of α-LA has been shown to be effective in preventing pathology in various experimental models in which reactive oxygen species have been implicated. Before considering these studies, however, it is necessary to discuss how α-LA is absorbed as a dietary supplement, to what degree it is taken up by tissues, is it reduced to DHLA?, or is it metabolized to shorter chain homologues?

2.4 Absorption, uptake, intracellular reduction, and metabolism of α-LA

Various studies on the distribution of radioactivity in rat tissues after intraperitoneal or oral administration of *dl*-[^{14}C]- or [^{35}S]-α-LA led to the observation that α-LA is rapidly absorbed in the gut, taken up into various tissues where it is metabolically altered, and then excreted. After supplementation with α-LA for 5 weeks, free α-LA was found in various tissues, the highest being the heart (PODDA, TRITSCHLER, ULRICH and PACKER; 1994). When given to cells *in vitro*, α-LA is rapidly taken up by the cells and reduced to dihydrolipoic acid, which is released by the cell (HANDELMAN, HAN, TRITSCHLER and PACKER; 1994).

2.4.1 Diabetes

α-Lipoic acid has potential applications for many aspects of the pathology of diabetes. In Type I (insulin-dependent) diabetes, destruction of pancreatic β-cells causes loss of insulin secretion, whereas in Type II (noninsulin-dependent) diabetes insulin resistance of peripheral tissues is the major problem (WOLFF, JIANG and HUNT; 1991). α-Lipoic acid has potential preventive or ameliorative effects in both Type I and Type II diabetes. It has been shown to have a number of beneficial effects, both in prevention and treatment of diabetes, α-LA acts in a number of ways that are especially protective in diabetes. It prevents β-cell destruction leading to Type I diabetes. It enhances glucose uptake in Type II diabetes. It prevents glycation reactions in some proteins. Its antioxidant effects may be particularly useful in slowing the development of diabetic neuropathy and cataractogenesis, and this may be especially significant in alleviating diabetes-induced reduction in intracellular vitamin C levels (GANDHI, WAGH, NATRAJ and MENON; 1985; WAGH, NATRAJ and MENON; 1987).

2.4.2 Liver diseases

α-Lipoic acid is often used for therapy in conditions that involve liver pathology. The basis for such treatment is the metabolic role of α-LA, not its antioxidant properties. The two most extensively studied conditions are mushroom

poisoning and alcoholic liver degeneration (MARSHALL, GRAUL, MORGAN and SHERLOCK; 1982; BUSTAMANTE et al.; 1998).

2.4.3 Neurodegenerative disorders

Tissues of the central nervous system can be vulnerable to oxidative stress because of their constant high rate of oxygen consumption and high mitochondrial density. Mitochondria inevitably produce free radicals as "by-products" of normal oxidative metabolism, and these free radicals damage the mitochondrial DNA (RICHTER, PARK and AMES; 1988). The defective proteins coded by the damaged DNA can lead to synthesis of mitochondria in which components of the electron transport chain preceding the damaged protein become reduced, leading to greater free radical production and more mitochondrial damage, in a vicious cycle. Such a vicious cycle may be responsible, in part for neurodegenerative diseases. Logical therapy or prevention would therefore involve antioxidant treatment.

α-Lipoic acid is a good candidate as an antioxidant agent in neurodegenerative diseases. It can interrupt the chain at several points; by competing for free transition metals as a chelator (DEVASAGAYAM, SUBRAMANIAN, PRADHAN and SIES; 1993), by scavenging hydroxyl or superoxide radicals (REED; 1957), and by scavenging peroxyl radicals (KAGAN, SHVEDOVA, SERBINOVA, KHAN et al.; 1992). Few other antioxidants possess this kind of versatility.

2.4.4 Radiation injury

Irradiation is known to produce a cascade of free radicals and antioxidant compounds have long been used to treat irradiation injury. α-Lipoic acid, but not DHLA, protected against radiation injury to haematopoietic tissues in mice, and increased the LD_{50} from 8.67 to 10.93 Gy (RAMAKRISHNAN, WOLFE and CATRAVAS; 1992). α-LA administration to murine neroblastoma cells (BUSSE, ZIMMER, SCHOPOHL and KORNHUBER; 1992) increased cell survival after irradiation from 2% to about 10%, and the effect correlated with an increase of the intracellular GSH/ GSSG ratio induced by α-lipoate. These results extended to mice irradiated with 8 Gy; administration of α-lipoic acid (16 mg/kg) increased survival rates from 35% in untreated animals to 90% in α-LA treated animals.

α-Lipoic acid, alone or together with vitamin E, is an effective treatment for radiation exposure, lessening indices of oxidative damage and normalizing organ function (KORKINA, AFANAS'EF and DIPLOCK; 1993).

2.4.5 Heavy metal poisoning

The possible chelating effects of α-LA, together with its antioxidant effects, make it a good candidate for the treatment of heavy metal poisoning. It can be especially effective against arsenite, cadmium, and mercury. Grunert demonstrated that α-LA administration completely protected mice and dogs from arsenite poisoning, if the ratio of α-LA to arsenite was at least 8:1. This was true even if the α-LA was administered when severe symptoms of poisoning were already seen (GRUNERT; 1960).

2.4.6 Anti-ageing effects

In various effects, α-LA appears to help restore a cellular “signaling” process that tends to break down in older blood vessels. It reduces mitochondrial decay in cells, which is closely linked to the symptoms of aging. With age, glutathione levels naturally decline, making older animals more susceptible to both free radicals and other environmental toxins but α-LA can restore glutathione function to almost normal. The expression and function of other genes seem to come back to life (ATAMNA; 2004; KUMARAN, PANNEERSELVAM, SHILA, SIVARAJAN and PANNEERSELVAM; 2005; SAVITHA and PANNEERSELVAM; 2007).

2.5 Sources

Good food sources of α-LA include spinach, broccoli, beef, yeast (particularly Brewer's yeast), liver, kidney, heart, potatoes and skeletal muscle (ENSMINGER, ENSMINGER, KONLANDE and ROBSON; 1994; LEY; 1996; MURRAY; 1996). However, quantitative information on the α-LA or lipoyllysine content of food is limited and published databases lack this information.

2.6 Side effects

Neither animal nor human studies, to date, have shown serious side effects with administration of α-LA. The LD_{50} is approximately 400-500 mg/kg following intravenous administration in rats and 400-500 mg/kg after oral dosing in dogs. In long-term oral supplementation at doses sufficient to reduce body weight gain, no functional or laboratory adverse effects were seen in animals. No evidence suggests carcinogenic or teratogenic effects, but it is recommended that pregnant women avoid taking supplemental α-LA until more data is available. In humans, side effects include, allergic skin reactions and possible hypoglycaemia in diabetic patients as a consequence of improved glucose utilization, with high doses of α-LA (FOSTER; 2007).

It is less clear whether or not supplement use for otherwise healthy individuals confers any benefit, including delay of aging. More carefully controlled scientific studies with larger numbers of subjects over longer periods will be needed to clarify the possibility that the sale of this compound is justified on a nutritional basis (MCCORMICK; 2005).

2.7 Analytical methods for the determination of alpha lipoic acid

The analysis of α-LA and related compounds, such as its reduced form DHLA, its amide form lipoamide (LAM) and other analogues, in biological and food samples is important in biochemistry, nutritional and clinical chemistry. Unfortunately, due to the lack of simple and reliable quantitative methods of determination, limited data on their contents in animal and plant samples are available. The reason of this depends on the fact that both molecules do not possess chromospheres strong enough to permit the use of conventional high-performance liquid chromatography (HPLC) with UV or fluorescence detectors (NAVARI-IZZO, QUARTACCI and SGHERRI; 2002).

Various analytical methods have been developed for the quantitative determination of α-LA in biological material, drugs and food samples (KATAOKA; 1998). This section is divided into the following two main parts:

1. General aspects of the analytical methods for the determination of α-LA and related compounds according to the instrument type
2. Applications of these methods to the various samples according to the matrix type

Techniques for the determination of α-LA rely on extraction of free and/or protein-bound. Different chromatographic methods were used to analyze α-LA and related compounds. Important features of these analyses are summarized in Table 3.

2.7.1 Gas chromatography

Gas chromatography (GC) has been widely used for the analysis of volatile compounds due to its inherent advantages of simplicity, high resolving power, high sensitivity and low cost. Therefore, GC with different detection modes such as flame ionization detection (FID), flame photometric detection (FPD) and mass spectrometry (MS) are among the most frequently applied techniques for α-LA and related compounds, the results are summarized in Table 3. However, the necessity of different tedious derivatization steps is a considerable drawback.

2.7.1.1 GC-FID and GC-FPD

In 1966, it was reported that a mixture containing α-LA and LAM could be separated and determined directly without derivatization, using a 15% diethyleneglycol succinate polyester packed column (75 cm×3 mm I.D), by GC with FID (IGUCHI, YAMAMOTO and AOYAMA; 1966). However, these polar compounds tended to elute as broad and tailing peaks, due to strong adsorption to the column and injector during GC analysis. Therefore, they could not be detected in low concentrations without derivatization. Thus, several derivatives, such as methyl ester (SHIH and STEINSBERGER; 1981; KOZMA-KOVACS, HALASZ, HAJOS, SASS and BOROSS; 1991; VIANEY-LIAUD, KOBREHEL, SAUVAIRE, WONG and BUCHANAN; 1994), *S*,*S*-dibenzyl (DBZ) methyl ester (WHITE; 1981), *S*,*S*-diethoxycarbonyl (DEOC) and methyl ester (KATAOKA, HIRABAYASHI and MAKITA; 1993; KATAOKA, HIRABAYASHI and MAKITA; 1997) have been tested.

Alpha lipoic acid in standard solutions could be easily determined using gas chromatography as its methyl ester derivative, but it was difficult to detect the α-LA in biological and food samples as its derivatives because a part of α-LA forms mixed disulphides (REED; 1953) and disulphide polymers (THOMAS, CHAI and JUNG; 1994) in these samples. Therefore, these sulphides must be reduced to thiols and then derivatized with appropriate reagents for GC analysis. α-LA was determined as its *S*,*S*-DBZ methyl ester derivative (WHITE; 1981) after reduction to DHLA using sodium borohydride ($NaBH_4$) by GC–FID. Calibration curves were constructed using C_7 and C_9 homologues as internal standards and were linear in the range 0–20 μg/ml. Furthermore, increased sensitivity should be possible by using an electron capture detector and/or using pentafluorobenzyl chloride in the derivatization step instead of benzyl chloride (BZ-Cl).

A selective and sensitive method for the determination of α-LA by GC with flame photometric detection (FPD) was developed (KATAOKA, HIRABAYASHI and MAKITA; 1997) in which α-LA was converted into its *S*,*S*-DEOC methyl ester derivative after reduction with $NaBH_4$. α-Lipoic acid was eluted as a single and symmetrical peak within 6 min using a DB-210 capillary column (15 m×0.53 mm I.D., 1.0 μm film thickness). The observed calibration curve was linear in the range 20–500 ng/μl.

2.7.1.2 GC-MS

GC–MS can be usually operated in two modes, total ion monitoring and selected ion monitoring (SIM). For SIM, only the base peaks are chosen to obtain the highest possible sensitivity. A magnetic sector instrument is used for positive ion electron ionization (EI)–MS, which yields excellent fragmentation patterns, with further confirmation being achieved using chemical ionization (CI) of the sample with a quadruple instrument. Since CI–MS is a much softer ionization method, it has the advantage of producing far less fragmentation of the compound and so allows a greater chance of the molecular ion being present, which can aid interpretation. Thus, GC–MS is one of the best on-line identification systems because of selectivity and sensitivity, but it requires conversion into volatile derivatives before analysis.

Table 3: Overview of chromatographic methods used for the quantitative determination of alpha lipoic acid

Method	Derivatization reagent	Detection limit	Reference
GC-FID			(IGUCHI, YAMAMOTO and AOYAMA; 1966)
FID	CH_2N_2		(SHIH and STEINSBERGER; 1981; KOZMA-KOVACS, HALASZ, HAJOS, SASS and BOROSS; 1991)
FID	CH_2N_2		(KÖNIG, L. and KNABE; 1990)
FID	HCl-MeOH	20 ng/µl	(NATRAJ, GANDHI and MENON; 1984)
FID	BF_3-MeOH	50 ng/µl	(VIANEY-LIAUD, KOBREHEL, SAUVAIRE, WONG and BUCHANAN; 1994)
FID	BZ-Cl- CH_2N_2	50 ng/µl	(WHITE; 1981)
FPD	ECF-HCl-MeOH		(KATAOKA, HIRABAYASHI and MAKITA; 1993; KATAOKA, HIRABAYASHI and MAKITA; 1997)
GC-MS-EI	CH_2N_2		(PRATT, CARLES, CARNE, DANSON and STEVENSON; 1989; JACKMAN, HOUGH, DANSON, STEVENSON and OPPERDOES; 1990)
EI	BZ-Cl- CH_2N_2		(WHITE; 1980)
CI	MTBSTFA	10 pg/µl	(MATTULAT and BALTES; 1992)
EI	BSTFA-TMCS		(BIEWENGA and BAST; 1995)

(Continued)

Table 3: Overview of chromatographic methods used for the quantitative determination of alpha lipoic acid (Continued)

Method	Derivatization reagent	Detection limit	References
HPLC-UV		15 nmol	(GATTI, BOUSQUET, BONAZZI and CAVRINI; 1996)
FL	Br-AMN		
FL	OPA-D-Phe	3 ng/ml	(NIEBCH, BUCHELE, BLOME, GRIEB et al.; 1997)
EC		0.05 ng/µl	(KAMATA and AKIYAMA; 1990)
EC		1 ng/ml	(TEICHERT and PREISS; 1992; TEICHERT and PREISS; 1995; TEICHERT and PREISS; 1997)
EC		0.05 nmol	(HAN, TRITSCHLER and PACKER; 1995)
CE-UV			(PANAK, RUIZ, GIORGIERI and DIAZ; 1996)

Few GC–MS data on the analysis of α-LA and related compounds using EI and CI after derivatization are available. Methyl ester of lipoic acid was identified by monitoring the presence of the fragment ion (*m*/*z* 123) and the molecular ion (*m*/*z* 220) (PRATT, CARLES, CARNE, DANSON and STEVENSON; 1989; JACKMAN, HOUGH, DANSON, STEVENSON and OPPERDOES; 1990).

White developed a method for the identification of α-LA as its *S*,*S*-DBZ methyl ester derivative by EI–MS (WHITE; 1981). This derivative gave a strong mass ion at *m*/*z* 311 (M^+–91) and a weak ion at *m*/*z* 137, which resulted from cleavage of the C_7 and C_8 bond of α-LA. He also tested some reagents, such as dinitrofluorobenzene, iodoacetate and 2,2-dimethoxypropane, for the derivatization of reduced α-LA, but the derivatives prepared by these reagents proved unacceptable for the GC–MS analysis of α-LA due to either non-volatility, instability or difficulty in preparation.

Another method was developed by converting α-LA into its *tert.*-butyl-dimethylsilyl (*tert.*-BDMS) derivative with *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) followed by CI–MS (MATTULAT and BALTES; 1992). The detection limit was about 10 pg/µl of LA derivative and the calibration curve was linear from 10 pg to 20 ng/µl using the base peak *m*/*z* 189 for quantification.

2.7.2 High performance liquid chromatography

High performance liquid chromatography (HPLC) is one of the most popular analytical techniques and can be used in combination with various detection systems such as ultraviolet (UV), fluorescence (FL) and electrochemical (EC) detectors. However, little work has been done on the determination of α-LA and related compounds by HPLC.

2.7.2.1 HPLC-UV and HPLC-FL

α-Lipoic acid and some of its analogues were separated by reversed-phase HPLC on a µBondapak C_{18} column (30 cm×3.9 mm, I.D. 10 µm) using UV detection at 330 nm (absorption maximum of the dithiolane ring) with a gradient elution system (HOWARD and MCCORMICK; 1981). Eight compounds could be separated within

16 min, but the sensitivities of these compounds were very low, due to the lack of a strong chromophore (SCHMIDT, GRAFEN, ALTLAND and GOEDDE; 1969; FURR, SHIH, HARRISON, CHANG et al.; 1979), which is needed for conventional UV or FL detection.

Few derivatization reagents have been used for the determination of α-LA by FL detection. 2-Bromoacetyl-6-methoxynaphthalene (Br-AMN) was used as a fluorogenic labelling reagent and α-LA was separated using a Hypersil 5 ODS column (25 cm×4.5 mm; I.D. 5 μm) under isocratic conditions and detected at an excitation wavelength of 300 nm and an emission wavelength of 460 nm (GATTI, BOUSQUET, BONAZZI and CAVRINI; 1996).

A stereoselective and sensitive method for the high performance liquid chromatographic analysis of α-LA enantiomers (+)*R*- and (−)*S*-LA was developed by derivatization with *o*-phthalaldehyde (OPA) in the presence of D-phenylalanine (D-Phe) after reduction to the dithiol enantiomers and the two diastereomeric derivatives were separated by reversed-phase HPLC on a LiChrospher 60 SelectB column (25 cm×4 mm, I.D. 5 μm particle size) using FL detection with an excitation wavelength of 230 nm and an emission filter of >418 nm (NIEBCH et al.; 1997). The limit of detection was 3 ng/ml and the working range of the assay was 15–1000 ng/ml for either enantiomer.

In another method, α-lipoic acid in plasma and tissues was determined by reversed-phase HPLC-FL. After extraction with diethyl ether, the dithiolane ring of α-LA was opened by reduction with $NaBH_4$ before the free thiols could react with the fluorescent label monobromobimane. The concentration-response curve was linear from 20 to 3000 nM in plasma. The recovery as determined with [^{3}H]LA was 60.9% from plasma and 61.4% from rat heart tissue (WITT and RUSTOW; 1998).

α-Lipoic acid and DHLA contents in human plasma and urine were determined using simple isocratic elution with acetonitrile-water (80:20) and detected fluorimetrically (excitation: 343, emission: 423 nm). After extraction, the free carboxylic function of α-LA and the SH-protected DHLA were converted into their amide derivatives with the strong fluorophore, 2-(4-aminophenyl)-6-methylbenzothiazole, in the presence of a coupling agent and a base catalyst (HAJ-

YEHIA, ASSAF, NASSAR and KATZHENDLER; 2000). The resulting fluorescent amides of both LA and DHLA were separated on a reversed-phase Ultrasphere C8 column (25 cm×4.6 mm, I.D. 5 μm, Beckman, USA).

2.7.2.2 HPLC-EC

HPLC with electrochemical detection usually offers a sensitive quantification of organic compounds. α-Lipoic acid and DHLA can be readily inter-converted by application of an electric potential and, therefore, these compounds are suitable for measurement by an EC detector. The choice of instruments and electrode materials, and the composition of the mobile phase, are factors that influence both the sensitivity and selectivity of the EC analysis. Isocratic elution is usually used in the combination of HPLC and electrochemistry, because gradient elution cannot be used in a high sensitivity range with this detector. In another experimental work, α-LA and LAM were simultaneously analyzed by HPLC on a Shim-pack CLC ODS column (15 cm×6 mm I.D. 5 μm particle size) using a Ag, Ag–AgCl electrochemical detector and 50% acetonitrile in 0.05 *M* potassium dihydrogen phosphate (pH 2) as the mobile phase (KAMATA and AKIYAMA; 1990). The detection limits of α-LA and LAM were 50 and 100 pg/μl, respectively, and the calibration curves for these compounds were linear over the range 0.5–5 ng/μl.

α-Lipoic acid and DHLA were determined using a glassy-carbon working electrode and a Ag/AgCl-reference electrode at the high oxidation potential of 1.1 V. HPLC separation was achieved on a Nucleosil 120 C 18 column (25 cm×4 mm I.D. 5 μm particle size) and 28.5% acetonitrile in 0.05 *M* potassium dihydrogen phosphate (pH 2.5) as the mobile phase (TEICHERT and PREISS; 1992; TEICHERT and PREISS; 1995; TEICHERT and PREISS; 1997). The detection limit for α-LA was 1 ng/ml and the calibration curve was non-linear in the range 0.01–50 μg/ml, but could be described by a power function. The method suffered from an extreme loss in sensitivity due to poisoning of the electrode. Later the same authors overcame this drawback by applying pulsed amperometric detection (TEICHERT and PREISS; 2002).

An alternative EC system with a dual Hg–Au electrode was used for the determination of α-LA and DHLA (HANDELMAN, HAN, TRITSCHLER and

PACKER; 1994; HAN, HANDELMAN and PACKER; 1995). In this dual-electrode system, one electrode acts as the generator and the second electrode acts as a detector. α-Lipoic acid was reduced to DHLA on the high negative potential generator electrode (Hg) and then detected as DHLA on the downstream electrode (Au). α-LA and DHLA were separated within 8 min using a Microsorb C 18 column (10 cm×4.6 mm I.D. 3 μm particle size) and 0.2 *M* monochloroacetic acid (pH 2.9)/methanol/acetonitrile (50/30/20; v/v/v) as the mobile phase. The detection limits for α-LA and DHLA were 10 and 2 ng/μl, respectively. But the electrodes lost sensitivity after 30–50 injections and had to be reconditioned. Sen et al. reported previously a HPLC method based on coulometric detection. Using this method, detection limits for α-LA and dihydrolipoate (DHLA) were 1-5 pmol which is at least 10 times more sensitive for the detection of α-LA compared to the Au/Hg electrode-based detection reported previously (SEN, ROY, KHANNA and PACKER; 1999).

The mercury surface-specific reaction is much more specific than the reaction on the glassy-carbon surface and, therefore, the former is commonly used for the measurement of disulphides and thiols (FURR et al.; 1979; ALLISON and SHOUP; 1983). Although both α-LA and DHLA can be measured in their free form by these EC methods, bound forms of these compounds could not be detected.

Another method based upon voltammetry and coulometric titration with electro-generated halogens was used to determine α-LA with RSD of 1-2% in the model solutions. The analytical range of α-LA found by voltammetry at a glassy-carbon electrode was 1.15×10^{-5} to 1.73×10^{-4} M and the detection limit for α-LA was 5.75×10^{-6} M (ZIYATDINOVA, BUDNIKOV and POGOREL'TSEV; 2004).

2.7.2.3 HPLC-MS

A simple, sensitive, and specific LC-MS/MS method for the determination of α-LA was developed and validated over the linearity range 5-1000 ng/ml ($r^2 > 0.99$). α-Lipoic acid was determined in 200 μl rat plasma using rosigliatzone as an internal standard (IS). The API-3000 LC-MS/MS was operated under the multiple reaction monitoring mode (MRM) using the electrospray ionization technique. The recovery of α-LA and IS was >70% and 97%, respectively. The limit of quantification (LOQ) of α-LA was 5.0 ng/ml. The inter- and intraday precision in the measurement of quality

control (QC) samples 5, 15, 400, and 800 ng/ml were in the range 2.18-5.99% RSD and 0.93-13.77% RSD, respectively. Accuracy in the measurement of QC samples was in the range 87.40-114.40% of the nominal values (TRIVEDI, KALLEM, MAMIDI, MULLANGI and SRINIVAS; 2004).

2.7.3 Capillary electrophoresis

Capillary electrophoresis (CE) is capable of achieving higher separation efficiency, uses less organic solvents and requires small amounts of samples in comparison with HPLC. The migration behaviour of ionized compounds is dependent on various factors, such as buffer pH, organic modifier, concentration of buffer solution, temperature of the capillary tubing and the electric field strength.

A CE method for the determination of α-LA was developed (PANAK, RUIZ, GIORGIERI and DIAZ; 1996) using an uncoated capillary column (30 cm effective length, 50 mm I.D.) with 50 m*M* tris(hydroxymethyl) amino-methane–hydrochloric acid (Tris–HCl)–30 m*M* sodium dodecyl sulphate (pH 7.0) as the running buffer, at 20 kV. A detection limit in the femtomolar range was achieved by monitoring the UV absorbance at 214 nm.

Another rapid capillary electrophoresis method was developed to determine α-LA in dietary supplement preparates. α-Lipoic acid was extracted three times with methanol at ambient temperature and was determined using UV detection at 208 nm with recovery of 98.3% and a precision of 2.8% RSD (SITTON, SCHMID, GUBITZ and ABOUL-ENEIN; 2004).

2.7.3.1 General techniques for sample preparation

Majority of the previous work is based on the analysis of protein-bound α-LA in the biological and food samples and hence used the hydrolytic methods for the sample preparation. A brief description of these methods is given in the next sections.

2.7.3.1.1 Hydrolytic method

Different acid, base and enzymatic hydrolysis methods were adopted to analyse α-LA in different matrices.

2.7.3.1.1.1 Acid hydrolysis

Samples containing α-LA were hydrolyzed using 6 *M* HCl at 120 °C for 2–4 h followed by extraction of hydrolyzed α-LA into dichloromethane (WHITE; 1980); (WHITE; 1981), (PRATT, CARLES, CARNE, DANSON and STEVENSON; 1989) and (VIANEY-LIAUD, KOBREHEL, SAUVAIRE, WONG and BUCHANAN; 1994). The release of α-LA from its protein bound form under these hydrolysis conditions was greater than 95%, but α-LA tended to become oxidized to its thiosulphinate or thiosulphonate form during hydrolysis. The recovery of α-LA from plasma by hydrolysis in 6 *M* HCl at 120 °C for 6 h was about 30% (TEICHERT and PREISS; 1997). Moreover, the recoveries of α-LA from the synthesized model compound ε-lipoyllysine (LLys) by hydrolysis with 1 and 6 *M* HCl at 100 °C for 3 h were about 80 and 25%, respectively (MATTULAT and BALTES; 1992).

The addition of several thiol protecting agents, such as mercaptoacetic acid, mercaptoethanol or ethanethiol, to the hydrolysis did not effect the oxidation of α-LA (WHITE; 1980). Therefore, [^{3}H] and [^{13}C] homologues of α-LA were added to the sample as internal standards before hydrolysis (WHITE, BLEILE and REED; 1980).

As an alternative acid hydrolysis method, samples were hydrolyzed using 6 M sulphuric acid at 125 °C for 6 h, and α-LA in hydrolysate was extracted into benzene (SHIH and STEINSBERGER; 1981). By using [^{13}C-α-LA], the recovery during hydrolysis and extraction was found to be 34%. Moreover, the recoveries of α-LA from lipoyllysine by hydrolysis with 1 and 4 M sulphuric acid at 100 °C for 3 h were 80–85% (MATTULAT and BALTES; 1992).

For food samples, optimum hydrolysis conditions were heating at 120 °C for 7 h in 2 M sulphuric acid and the recoveries of α-LA from samples were about 60–70%. In addition, the use of organic acids, such as p-toluenesulphonic acid (KOCHI and KIKUCHI; 1976) and methanesulphonic acids [(WHITE; 1980; WHITE, BLEILE and REED; 1980), have also been described for the release of α-LA from the protein-bound form.

2.7.3.1.1.2 Base hydrolysis

Base hydrolysis was used to release α-LA from samples, because its oxidation during hydrolysis was far more resistant to base hydrolysis than to acid hydrolysis (KATAOKA, HIRABAYASHI and MAKITA; 1993; KATAOKA, HIRABAYASHI and MAKITA; 1997). Optimum hydrolysis conditions were heating at 110 °C for 3 h in 2 M potassium hydroxide containing 4% bovine serum albumin. The degradation of α-LA during hydrolysis could be reduced by bovine serum albumin. The released lipoic acid could be easily extracted into dichloromethane and directly derivatized to its S, S-DEOC methyl ester. The recoveries of lipoic acid from biological samples during hydrolysis and extraction were 50-60%.

2.7.3.1.1.3 Enzymatic hydrolysis

A specific enzyme *lipoamidase* was used to release α-LA from the ε-amino group of a lysine residue (SUZUKI and REED; 1963), but it has never been applied directly to tissue homogenates. A mild enzymatic hydrolysis, using several proteases, was also reported to release α-LA from plasma sample (TEICHERT and PREISS; 1992; TEICHERT and PREISS; 1995; TEICHERT and PREISS; 1997). The recoveries of α-LA from plasma by hydrolysis with thermophilic *protease*, *alcalase* and *subtilisin A* were about 70%, 80% and 82%, respectively. The released α-LA in hydrolysate was extracted by a solid-phase extraction cartridge. In this method, if all preparations were carried out under inert gas, DHLA could also be detected in some cases.

In food samples, an enzymatic recycling method was developed to determine tissue level of α-LA. Bound lipoyl groups were liberated in the form of lipoyllysine by protease digestion and were assayed by *lipoamide dehydrogenase*-mediated NADH oxidation. NADH oxidation was coupled to reduction of the lipoyl disulfide group. The method was applied to the protease lysates of bovine, rat and rabbit tissues to determine lipoyllysine levels. Kidney and liver were found to have the highest content of α-LA in the range of 3.9 to 4.6 nmol/g wet tissue of rat or rabbit or 11.6 - 13.1 nmol/g bovine acetone powder (KONISHI and PACKER; 1999).

Direct determination of lipoyllysine was carried out using several enzymes such as *pronase E* and *subtilisin A* followed by labelling of the reduced LLys with

ammonium 4-fluoro-2,1,3-benzoxadiazole-7-sulfonate (SBD-F) at 50 °C for 1h. The resulted fluorophore, SBD-LLys, was quantified by reversed-phase HPLC-FL detection (SATOH, TOYO'OKA, FUKUSHIMA and INAGAKI; 2007). The assay values of LLys per 1 g wet tissues were 3.67 µg (kidney), 1.97 µg (liver), 2.09 µg (heart), 0.59 µg (brain), 0.30 µg (pancreas), and 0.20 µg (spleen).

2.7.3.1.2 Non-hydrolytic methods

α-Lipoic acid was extracted in methanol from pharmaceutical dosage forms using simple solid phase extraction. Statistical analysis proved that the method was reproducible (SITTON, SCHMID, GUBITZ and ABOUL-ENEIN; 2004).

α-Lipoic acid was extracted from health food by ultrasonication in methanol, filtered and quantified by HPLC. The average recovery was 98.89%, and RSD was 2.26% (SUN and CHEN; 2006).

2.7.3.2 Bacteria

α-Lipoic acid has been identified in *Escherichia coli* (*E. coli*) by 6 *M* HCl hydrolysis, extraction into dichloromethane, subsequent derivatization with BZ-Cl and GC–MS analysis (WHITE; 1980). Pratt et al. detected α-LA in *E. coli* and *Halobacterium halobium* by a modified GC–MS method (PRATT, CARLES, CARNE, DANSON and STEVENSON; 1989). They identified the released α-LA from the protein-bound form as its methyl ester. In addition, (WHITE; 1981) determined the α-LA content in acid hydrolysates of some bacterial samples by GC–FID analysis, based on preparation of the *S*,*S*-DBZ methyl ester derivative, and showed that the α-LA content of *E. coli* depended on the carbon source used for its growth. The contents of bacterial α-LA are summarized in Table 4. The values are in good agreement with previously reported data (NAKATA; 1962).

Table 4: α-LA content in different bacteria

Bacteria	Method	Content (µg/g)	Reference
Escherichia coli B	GC-FID	2.3-11.8	[1]
Escherichia coli	GC-FPD	23.45	[2]
Bacteroides fragilis	GC-FID	2.4	[1]
Aerobacter aerogenes	GC-FPD	10.62	[2]
Aerobacter vinelandii	GC-FPD	34.96	[2]
Bacillus subtilis	GC-FPD	17.65	[2]
Clostridium perfringens	GC-FPD	Not detected	[2]
Micrococcus lysodeikticus	GC-FPD	4.34	[2]
Pseudomonas fluorescens	GC-FPD	16.25	[2]
Saccharomyces cerevisiae	GC-FPD	1.54	[2]
Saccharomyces cerevisiae	GC-FID	3.3-8.3	[3]

Where:

[1] (WHITE; 1981)

[2] (KATAOKA, HIRABAYASHI and MAKITA; 1993)

[3] (KÖNIG, L. and KNABE; 1990)

α-Lipoic acid content in *Saccharomyces cerevisiae* was determined by GC–FID analysis of its methyl ester. The recovery of α-LA from sample during sulphuric acid hydrolysis and dichloromethane extraction was about 30% (KOZMA-KOVACS, HALASZ, HAJOS, SASS and BOROSS; 1991). In addition, α-LA content in several bacterial cells was also determined by GC–FPD, based on the preparation of its *S*,*S*-DEOC methyl ester derivative. Base hydrolysis was also used to release α-LA from different bacterial cells. It was difficult to determine the content of α-LA by GC–FID due to the interfering peaks and low sensitivity, but α-LA could be analyzed by GC–FPD without any such interference from matrix substances (KATAOKA,

HIRABAYASHI and MAKITA; 1993). The analytical data in Table 4 represent the total content of free and bound forms of LA and DHLA in these samples, as the samples were hydrolyzed with potassium hydroxide and reduced with $NaBH_4$. The detection limit of α-LA in these samples was 10 ng/g.

2.7.3.3 Animal tissues

α-Lipoic acid content was determined in rat tissues and other biological materials by GC–FID analysis based on de-lipidation, sulphuric acid hydrolysis, benzene extraction and derivatization with diazomethane (SHIH and STEINSBERGER; 1981). It was found that α-LA level in the chicken egg increases with incubation time or during embryo development.

α-Lipoic acid content in the rat tissues was determined by GC–FID as its *S*,*S*-DBZ methyl ester and methyl ester derivatives, respectively (WHITE; 1981; NATRAJ, GANDHI and MENON; 1984).

In another study, α-LA content in several tissues of mouse was determined as its *S*,*S*-DEOC methyl ester derivative by GC–FPD (KATAOKA, HIRABAYASHI and MAKITA; 1993). Han et al. determined free α-LA and DHLA in Jurkat cells and fibroblasts by HPLC–EC analysis (HAN, HANDELMAN and PACKER; 1995). Moreover, Teichert et al. developed a selective and sensitive method for the determination of α-LA and DHLA in human plasma (TEICHERT and PREISS; 1992; TEICHERT and PREISS; 1995; TEICHERT and PREISS; 1997). This method consisted of enzymatic hydrolysis to release the protein-bound α-LA, solid-phase extraction, and HPLC–EC analysis. The detection limit of α-LA in plasma for this method was 1 ng/ml, and the basic levels of α-LA and DHLA in plasma from six healthy volunteers were 1–25 and 33–145 ng/ml, respectively.

2.7.3.4 Drug

Pharmacokinetics of *R*- and *S*- α-LA has been under investigation due to their different *in vitro* and *in vivo* biological activities. Kamata et al. developed a HPLC–EC method for the determination of α-LA and lipoamide (LAM) and reported that these compounds could be quantitatively recovered from a spiked placebo formulation (KAMATA and AKIYAMA; 1990).

α-Lipoic acid and LAM were determined in a commercial formulation of the cited acidic drug by a HPLC–FL method, using pre-column derivatization with Br-AMN (GATTI, BOUSQUET, BONAZZI and CAVRINI; 1996). Another method was developed for the determination of α-LA enantiomers and applied to pharmacokinetic studies (NIEBCH et al.; 1997).

A new spectrophotometric method was published to assay α-LA (thioctic acid) in pharmaceutical formulations using an absorption maximum at 365 nm. The method is simple, sensitive and reproducible (KORICANAC, CAKAR, TANASKOVIC and JOVANOVIC; 2007).

A potentiometric lipoate-selective sensor was developed, based on mercuric lipoate ion-pair as a membrane carrier, to determine α-LA in pharmaceutical preparations and urine (ABBAS and RADWAN; 2008). The sensor exhibited a significantly enhanced response towards the lipoate ions over the concentration range of 1×10^{-7} mol/l to 1×10^{-2} mol/l with a limit of detection of 9×10^{-8} mol/l and a slope of −29.4 mV/decade, with standard deviation of the slope is 0.214 mV. The sensor has a response time of ≤ 12s and can be used for at least 6 weeks without any considerable divergence in its potential response.

2.7.3.5 Dietary supplements

Supplementation of α-LA under various oxidative stress related conditions has been extensively studied, however very few analytical data are available for the quantification of α-LA content in different dietary supplements.

Sitton et al. developed an analytical method for the quantitative determination of α-LA in a dietary supplement preparation by a rapid capillary electrophoretic method, using UV detection at 208 nm (SITTON, SCHMID, GUBITZ and ABOUL-ENEIN; 2004). Although α-LA is only weakly UV-absorbing, at this wavelength it could be detected with sufficient sensitivity with LOD and LOQ of 0.8 and 2.5 μg/ml, respectively. The compound was extracted from tablets using methanol with a recovery of 98.3% and a precision of 2.8% RSD.

α-Lipoic acid content was determined in two dietary supplements, syrup and capsules by differential pulse voltammetry at a glassy carbon electrode. The limits of

detection (LOD) and quantification (LOQ) were 1.8 and 6.1 μM at pH 6.9, respectively (CORDUNEANU, GARNETT and BRETT; 2007).

2.7.3.6 Food

α-Lipoic acid was determined in meat of commercial quality by a sensitive GC–CI-MS method based on sulphuric acid hydrolysis, diethyl ether extraction, derivatization with MTBSTFA and SIM, using the base peak *m/z* 189 for quantification (MATTULAT and BALTES; 1992). The recoveries of α-LA in meat with this method were about 60–70%. α-LA was also determined in vegetables by GC-FID and was found to be present at low levels in wheat germ (ca. 0.1 ppm), but was not detected in flour or semolina (VIANEY-LIAUD, KOBREHEL, SAUVAIRE, WONG and BUCHANAN; 1994). In addition, it was found that α-LA content was higher in food derived from animal sources than in vegetables (KATAOKA, HIRABAYASHI and MAKITA; 1997).

The values of α-LA content in tissues and food samples obtained from different experiments are summarized in Table 5.

2.7.4 Summary

Most of the analytical methods deal with quantitation either in foodstuffs or only for the pharmaceutical preparates. Even there is no clear differentiation between free or total lipoic acid content in most methods. The lack of a standard and specific method to extract α-LA has lead towards the basis of this research work.

Table 5: Alpha lipoic acid content in tissue and food samples

No.	Sample	Method	Content (µg/g)	Reference	No.	Sample	Methods	Content (µg/g)	Reference
1.	Human plasma	HPLC-EC	1-25[b]	[1]	8.	Pig heart	GC-MS	1.1-1.6	
2.	Cow's milk	GC-FPD	ND[a]	[7]		Liver	GC-MS	0.6-0.8	[5]
3.	Beef	GC-FPD	2.36	[7]		Kidney	GC-MS	0.4-0.7	
						Muscle	GC-MS	0.15-0.3	
4.	Rat liver	GC-FID	8.-13.1	[2]	9.	Calf heart	GC-MS	0.5-0.7	
	Liver	GC-FID	1.2	[3]		Liver	GC-MS	0.07	[5]
	Kidney	GC-FID	1.2	[3]		Kidney	GC-MS	0.15	
						Muscle	GC-MS	0.07-0.15	
5.	Mouse brain	GC-FPD	0.83		10.	Pork	GC-FPD	1.07	[7]
	Heart	GC-FPD	2.03		11.	Yellowtail	GC-FPD	0.75	[7]
	Lung	GC-FPD	0.8						
	Liver	GC-FPD	1.23	[4]	12.	Lamb heart	GC-MS	0.5-0.7	
	Spleen	GC-FPD	0.52			Liver	GC-MS	0.7-0.8	[5]
	Pancreas	GC-FPD	0.84						

(Continued)

Table 5: Alpha lipoic acid content in tissue and food samples (Continued)

No.	Sample	Method	Content (µg/g)	Reference	No.	Sample	Method	Content (µg/g)	Reference
5. (cont'd)	Mouse muscle Kidney Testis	GC-FPD GC-FPD GC-FPD	0.7 1.54 1.07	[4]	13.	Lamb Kidney Muscle	GC-MS GC-MS	0.5-0.7 0.2-0.4	[5]
6.	Cuttlefish	GC-FPD	0.55	[7]	14.	Chicken liver	GC-FPD GC-FID	0.91 5-10	[7] [6]
7.	Wheat grain	GC-FID	0.1	[8]	15.	Egg white Egg yolk	GC-FPD GC-FPD	ND 1.24	[7]

[a]Not detectable

[b](ng/ml)

[1] (TEICHERT and PREISS; 1992)

[2] (NATRAJ, GANDHI and MENON; 1984)

[3] (WHITE; 1981)

[4] (KATAOKA, HIRABAYASHI and MAKITA; 1993)

[5] (MATTULAT and BALTES; 1992)

[6] (SHIH and STEINSBERGER; 1981)

[7] (KATAOKA, HIRABAYASHI and MAKITA; 1997)

3 AIM OF THE PRESENT STUDY

One of the main drawbacks of previous experiments performed to determine α-LA content was the use of drastic sample preparation steps which resulted merely in the destruction of free α-LA. In addition, the chromatographic methods used also required derivatization of α-LA prior to analysis. Due to these facts, very few analytical data were available to account for the α-LA content in food samples especially the free α-LA content.

The aim of the present work was to develop an analytical method to determine free α-LA content in different dietary supplements and food samples using high performance liquid chromatography with different detection modes such UV, CEAD and MS. Main theme of this project was to develop a rapid and reliable analytical method based upon simple and non-destructive sample preparation method.

First the chromatographic method was developed to obtain well shaped peaks of α-LA in a shorter run time. Bisphenol A was used as an internal standard and three chromatographic systems were optimized using standard solutions i.e. HPLC-UV, HPLC-CEAD and HPLC-ESI-MS. Then, different extraction methods were investigated to obtain free α-LA content from different dietary supplements and foodstuffs. The structure of the experimental work is summarized in Figure 4.

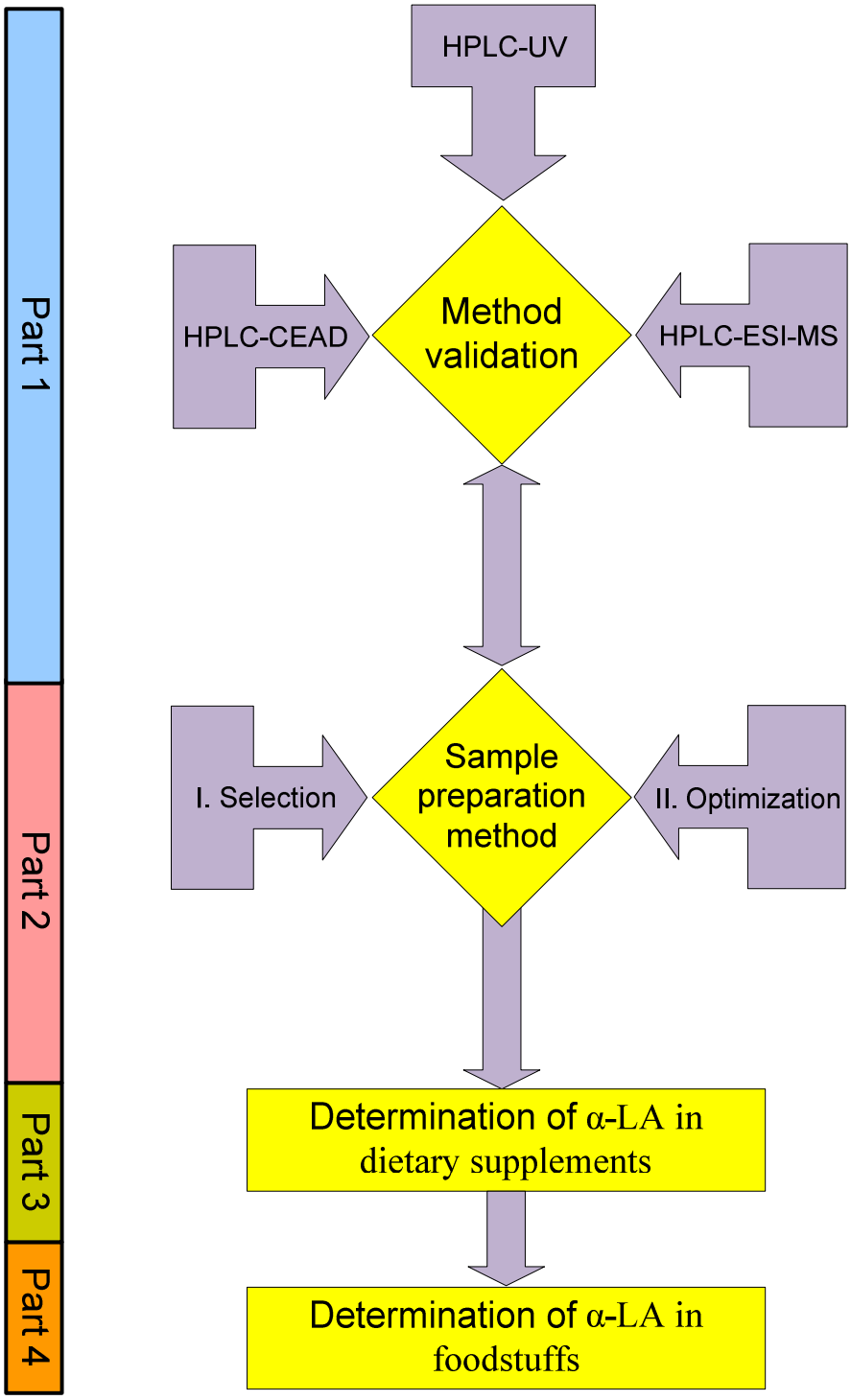


Figure 4: Schematic representation of research work

4 INSTRUMENTAL SET-UP

This chapter gives a brief description of instrumental set-up used in this research work with special emphasis on the theoretical background (SNYDER, KIRKLAND and GLAJCH; 1997).

High performance liquid chromatographic method was selected to determine α-LA content in the samples under investigation using different detection modes. In addition, different extraction techniques used in this research work are also briefly described.

4.1 High performance liquid chromatography

High-performance liquid chromatography (HPLC), also sometimes referred as high-pressure liquid chromatography, is a form of column chromatography and is one of the most widely used analytical separation method. HPLC is used to separate components of a mixture by using a variety of chemical interactions between the substance being analyzed (analyte) and the chromatography column. It depends on the interaction of sample analytes with the stationary phase (packing) and the mobile phase to effect a separation.

In reversed phase (RP) chromatography the mobile phase is more polar than the stationary phase and is commonly used for non-polar or weakly polar substances. Stationary phases may be either coated to a support, or they may be chemically bonded to the surface.

4.1.1 Theoretical background

The goal of any HPLC experiment is to achieve the desired separation in the shortest possible time. Optimization of the experiment usually involves manipulation of column and mobile phase parameters to alter the relative migration rates of the components in the mixture and to reduce zone broadening. These can generally be optimized fairly independently. Some important parameters of a HPLC system are briefly discussed in the following pages.

The quality of a separation is measured by the resolution *Rs* of adjacent bands. Two bands that overlap badly have a small value of *Rs*:

$$Rs = \frac{2(t_2 - t_1)}{W_1 + W_2} \qquad (1)$$

Here t_1 and t_2 are the retention times of the first and second adjacent bands and W_1 and W_2 are their baseline bandwidths. The resolution of two adjacent bands with $R = 1$ is shown in Figure 5.

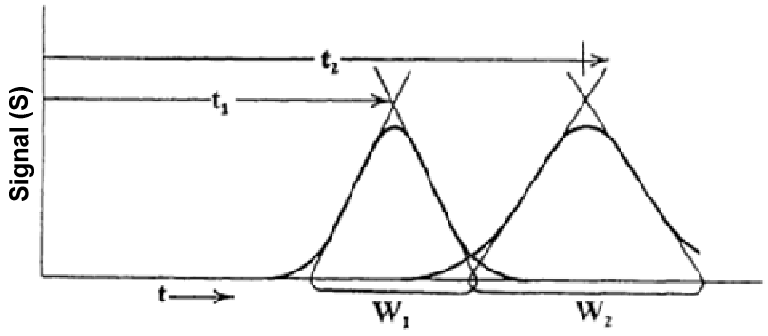


Figure 5: Illustration of resolution*s* of two adjacent bands 1 and 2

Resolutions *Rs*, is equal to the distance between the peak centres divided by the average bandwidth. To increase resolution, either the two bands must be moved farther apart, or bandwidth must be reduced. Resolution can be estimated or measured in three different ways:

1. Calculations based on equation 1
2. Comparison with standard resolution curves
3. Calculations based on the valley between the two bands

Separation of two bands in the chromatogram can be varied systematically by changing experimental conditions. Resolution *Rs* can be expressed in term of three parameters (*k*, α and *N*) which are directly related to experimental conditions as shown in equation 2.

$$Rs = \frac{1}{4}(\alpha - 1) \qquad N^{1/2} \qquad \frac{k}{1+k} \qquad (2)$$

(*selectivity*) (*efficiency*) (*retention*)

Here k is the average retention factor for the two bands (also called the capacity factor, k'), N is the column plate number, and α is the separation factor and can be calculated using the following formulae:

$$\alpha = \frac{k_2}{k_1} \qquad (3)$$

Here k_1 and k_2 are values of k for adjacent bands 1 and 2. This equation is very important in method development because it classifies the dozen or so experimental variables into three categories: retention (k), column efficiency (N), and selectivity (α). This simplifies the systematic variations of conditions to achieve some desired separation. It is convenient to regard k, N and α as independent to each other, so that changes can be made in each variable without affecting the other two. However, this is only a rough approximation, especially in the case of k and α.

The retention factor, k, is given as follows:

$$k = \frac{t_R - t_0}{t_0} \qquad (4)$$

Here t_R is the band retention time and t_0 is the column dead time. The column dead time (t_0) is related to the column dead volume Vm (Volume of the mobile phase inside the column) and flow rate F.

$$t_0 = \frac{V_m}{F} \qquad (5)$$

The parameters k and α are determined by those conditions that affect retention or the equilibrium distribution of the sample between the mobile phase and the column packing:

1. Composition of the mobile phase
2. Composition of the stationary phase (column)
3. Temperature

Changes in the mobile phase or stationary phase will generally affect both k and α, but will have less effect on N. The column plate number N is primarily dependent on column quality and can be varied by changing column conditions:

1. Flow rate
2. Column length
3. Particle size

A change in these conditions will not affect k or α as long as the mobile phase and stationary phase type are not changed

Column quality/performance can be defined in terms of values of N and asymmetry (band shape) for a test substance run under 'favourable' conditions. The column plate number, N, is defined as follows:

$$N = 16\left(\frac{t_R}{W}\right)^2 \qquad (6)$$

As manual measurement of the baseline bandwidth W may be subjected to error. Therefore, a more practical equation for N is

$$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2 \qquad (7)$$

Here t_R is band retention time and $W_{1/2}$ is the bandwidth at half-height. Another relationship that is used to measure N is

$$N = 2\pi\left(\frac{t_R h'}{A}\right)^2 \qquad (8)$$

Here h' is the peak height and A is the peak area. Equation 8 is often is used in HPLC data systems to determine a value of N.

4.1.2 Instrumentation

HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector (Figure 6). Compounds are separated by injecting a plug of the sample mixture onto the column. The different components in the mixture

pass through the column at different rates due to differences in their partitioning behaviour between the mobile liquid phase and the stationary phase.

The pumps provide a steady high pressure with no pulsation, and can be programmed to vary the composition of the solvent during the course of the separation. Solvents must be degassed to eliminate formation of bubbles. An autosampler equipped with sample loop of certain volume (20 µl in this case) is used to introduce solvent containing analyte into the system. A direct injection into the column, as used in GC, is impossible because of the high pressure in the system (up to 300 bars). A detector continuously monitors the effluent from the column, so that the separated components of a mixture may be observed as soon as they emerge from the column.

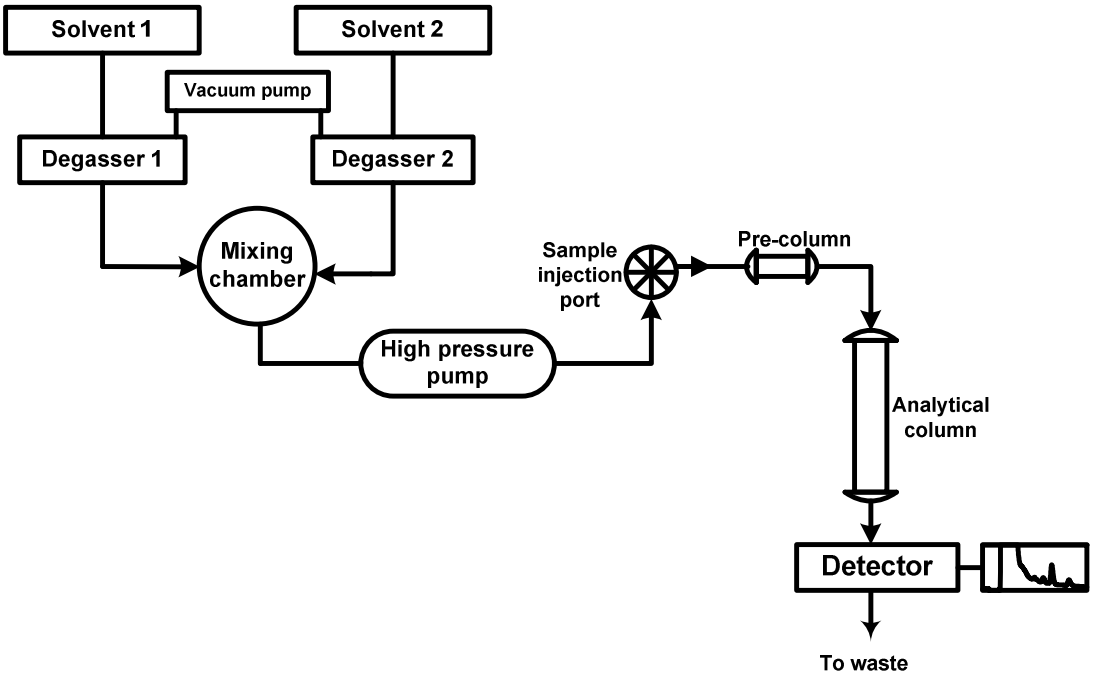


Figure 6: Schematic representation of a typical HPLC system

4.1.3 The column

The column is the heart of HPLC separation processes. The availability of a stable, high-performance column is essential in developing a rugged, reproducible method. Commercial columns can differ widely among suppliers, and even between supposedly identical columns from a single source. Such differences can have a

serious impact on developing the desired HPLC method. Specifically, different columns can vary in plate number, band symmetry, retention, band spacing, and lifetime.

Most common packings used for HPLC separations make use of a silica particle (support). Column based on porous-polymer supports or other materials also are commercially available for use in certain separations.

Stationary phases are usually bonded silanes. Silica-based reversed-phase packings typically are made by covalently bonding an organosilane or by depositing a polymeric organic layer on the support surface. Most widely used packings are with surface-reacted organosilane shown in Figure 7. Many bonded-phase packings are made with mono-functional reagents and some use a polymerized surface layer resulting from the reaction of tri-functional (also sometimes bi-functional) silanes with the silica surface.

The stability of bonded-phase packing is especially important in method development. Once the desired separation is obtained, column characteristics should remain unchanged for as long as possible. Good column stability minimizes the need for further adjustment of separation conditions or replacement of the column. When used under the same conditions, longer-chain alkyl-bonded-phase packings (e.g., C_{18} and C_8) are more stable than short-chain bonded-phases.

The stability (and lifetime) of silica-based bonded-phase columns is directly related to the types of silica supports and bonded phases. Column stability also is strongly dependent on mobile-phase pH and the type of buffer and organic modifier used. Loss of silane bonded-phases results from hydrolysis of the Si-O-Si bond that binds the silane to the support. This degradation is accentuated at higher temperature, low pH, and highly aqueous mobile phases, which are preferred conditions for many separations.

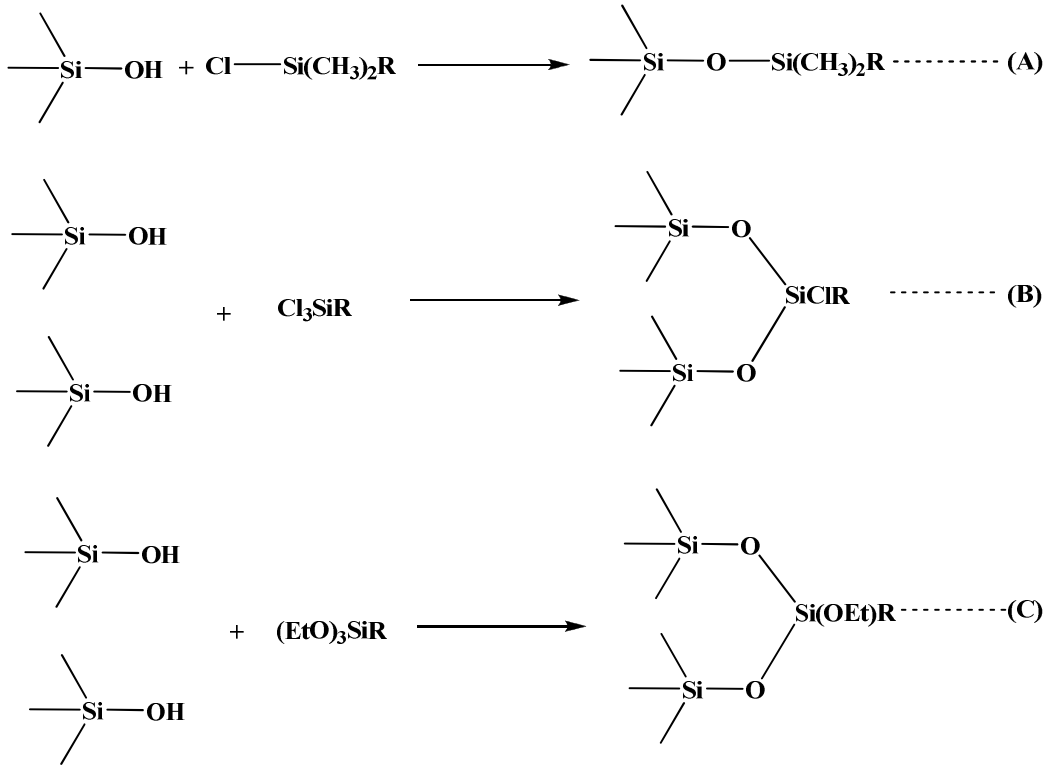


Figure 7: Chemistry of bonded-phase packings. [A] Reaction of surface silanol with chlorodimethylsilane; [B] Reaction of surface silanols with trifunctional silane; [C] Reaction of surface silanols with trifuncitonal alkoxysilane

4.1.4 Detectors in HPLC

Within the last 25 year high performance liquid chromatography (HPLC) in combination with different detectors has become an integral part of modern food laboratories.

Three different detectors were used to analyze α-LA content in different samples and brief description of each detector is given in the following sections.

4.1.4.1 UV detection

In most cases HPLC method development is carried out with ultraviolet (UV) detection using either a variable-wavelength (spectrophotometric) or a diode-array detector (DAD). A light source, typically a deuterium lamp, provides acceptable light intensity from 190 to 400 nm. When measurements at visible wavelength (400 to 700

nm) are required, a higher-energy tungsten-halide lamp is often used. However, most HPLC applications are carried out using wavelengths below 400 nm.

Schematic illustration of a typical UV detector is show in Figure 8. Light from the lamp passes through a UV-transmitting flow cell, connected to the column and impinges on a diode that measures the light intensity *I*. The detector electronics then convert the signal from the two diodes into the absorbance A, which is transmitted to the data system:

$$A = \log \frac{I_0}{I} \qquad (9)$$

Here, I is the light intensity before the flow cell and I_0 is the light intensity after the flow cell.

Analyte concentration C in the flow cell is related to absorbance A, analyte molar absorptivity ε, and flow-cell length L_{fc} by Beer's law:

$$A = C\varepsilon L_{fc} \qquad (10)$$

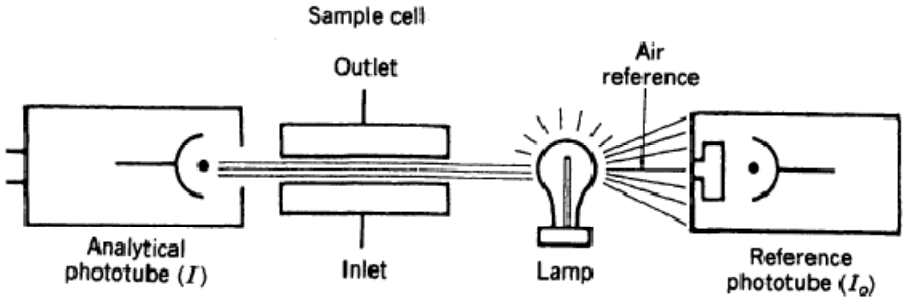


Figure 8: Schematic presentation of a typical UV detector

A general goal in selecting experimental conditions that affect detection is to maximize the signal S (equal to A at peak maximum) of sample components of interest. For many samples, good analytical results will be obtained only by careful selection of the wavelength used for detection. This choice requires knowledge of the UV spectra of individual sample components.

4.1.4.2 Coulometric electrode array detection

Coulometric electrode array is a type of electrochemical (EC) detector used whenever greater sensitivity is required. The detection can be performed in either the oxidative or reductive mode, depending, in part, on the analyte. All EC detectors have three electrodes:

1. Working or test electrode
2. Reference electrode
3. Auxiliary or counter electrode

A typical coulometric electrode cell is shown in Figure 9. Overall reaction taking place in the coulometric cells can be divided into following three steps:

1. The molecule is transported, by convective diffusion, to the electrode surface. At any liquid/solid interface there is always a stationary or stagnant layer or liquid.
2. The transfer of electrons takes place and the chemical reaction has occurred.
3. The products of reaction desorb and diffuse back through the diffusion layer.

The electrochemical reaction occurs at the surface of the working/test electrode. The potential required to drive the reaction is applied between the working and reference electrodes. The current produced by the reaction is called limiting current, i_L, and is measured between the test and counter electrodes.

$$i_L = zFcU \qquad (11)$$

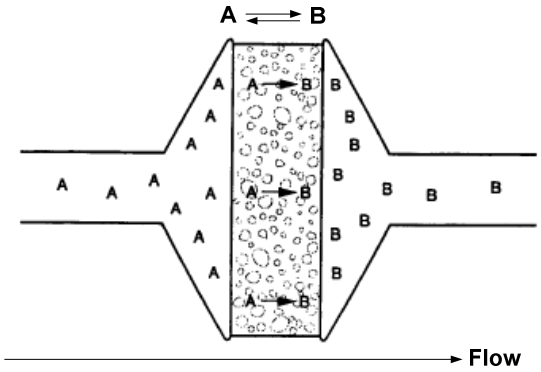
Here i_L is the limiting current, z is the number of electrons converted/analyte molecule, F is the Faraday constant (96485 C/mol), c is the concentration of the electrochemically active substances (mol/ml) and U is the flow rate (ml/s). The peak area is the integral of the current over time:

$$Q = zFn = \int_{t_1}^{t_2} Idt \qquad (12)$$

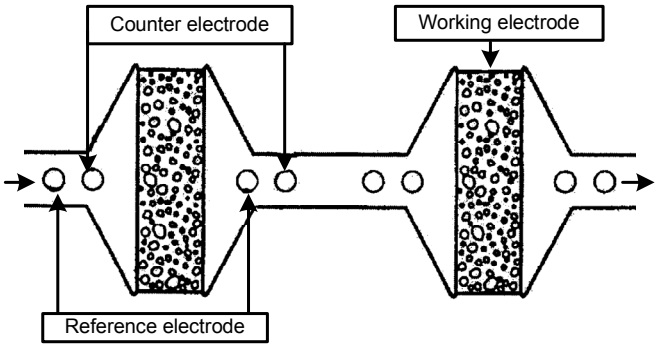
Here, Q is the charge (C) and n is the number of moles (mol). Both peak area and peak height can be used for the quantitative purpose.

Electrochemical compounds are typically aromatic and contain hydroxyl, methoxyl or amine groups. Aliphatic compounds such as thiols and amines are electroactive, too. Electrochemically inactive compounds can not be detected by this type of detector.

The surface area of the test electrode in the coulometric sensor is larger than in an amperometric detector; most reactions go to completion, yielding a maximum signal. The reference and counter electrodes are positioned very close to the test electrode making it possible to use the cell with any mobile phase: 0 to 100% organic modifier. Two reference and counter electrodes/test electrodes lead to greater stability of the cell.



(a)

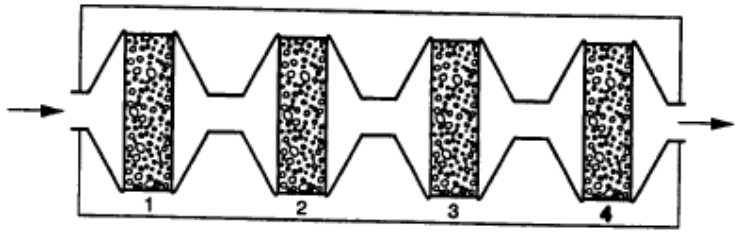


(b)

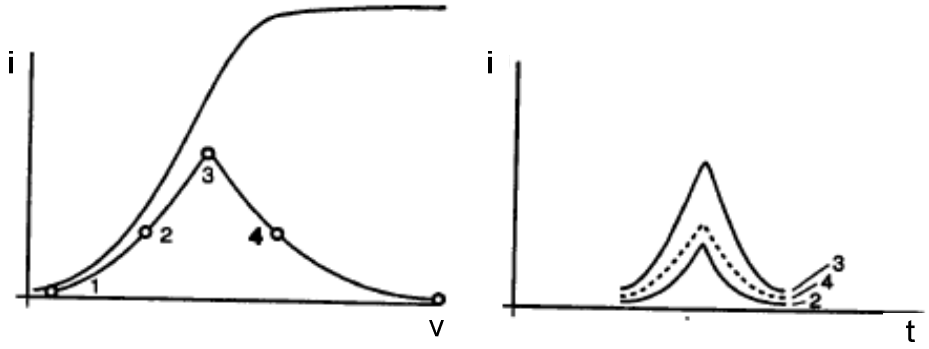
Figure 9: Schematic representation of a typical coulometric electrode array cell. (a) Cross section of cell showing efficiency with close to 100% of the analyte being measured; (b) Cross section through a dual coulometric electrode, taken from Progress in HPLC-HPCE, vol 6, 1997 p 18

The coulometric array cell is built upon the dual serial flow-through graphite cell design described in Figure 9. There are several characteristics, inherent in the electrode design, that make the porous graphite electrode attractive for arrays. First, they are coulometrically efficient; that is, a compound will present a maximum at one electrode and then drop to zero at subsequent sensors. Second, the band spreading, dead-volume and pressure characteristics of the cells are such that there is no significant degradation of the chromatographic separation at the last sensor in the array and no significant difference in registration time between the first and last electrode (MATSON, LANGLAIS, VOLICER, GAMACHE et al.; 1984). Third, hydrodynamic voltammograms (HDVs) from the flow-through coulometric electrodes are sharper than those from amperometric electrodes for many compounds. Fourth, analytes can be measured at higher potentials with sensitivity, as noise will be diminished by the preceding sensors.

A typical coulometric electrode array system consists of 4 to 16 coulometric flow-through cells, also called 'channels' as shown in Figure 10. Signal from a single compound is measured on three consecutive electrodes (preceding - 2, dominant - 3 and following – 4) with applied potentials placed along the compound's potential axis (Figure 10). The response ratios of 2/3 and 4/3 (along with retention time) are used qualitatively to identify the compound. A plot of the cumulative detector response against applied voltage produces the compound's HDV. These channels have the benefits of eliminating the calibration drift, have extended cell shelf-life due to very slow loss of signal through cell poisoning and their response is independent of flow rate.



(a)



(b)

Figure 10: Coulometric electrode array cell. (a) Four serially placed flow-through graphite electrodes; (b) Resolution of a compound across the array at its elution time and construction of hydrodynamic voltammograms (HDV); taken from Progress in HPLC-HPCE, vol 6, 1997 p 30

4.1.5 Liquid chromatography-mass spectrometry

Liquid chromatography-mass spectrometry (LC/MS) is an analytical technique that combines the separation power of HPLC with the detection particular in mass spectrometry. HPLC separates the sample components and introduces them into a mass spectrometer. This instrument forms and detects ions for analysis. LC/MS data provides information about the molecular weight, structure of specific sample components, and quantitative proportion.

A mass spectrometer can facilitate HPLC method development and avoid common problems by:

1. Tracking and identifying individual peaks in the chromatogram between experiments

2. Distinguishing compounds of interest from minor compounds or interferences
3. Recognizing unexpected and overlapping interference peaks to avoid a premature finish to method development

Mass spectrometers have three distinct features: (1) the source, (2) the analyzer, and (3) the detector, and differences in these three components differentiate the types of MS techniques that are useful with HPLC. For all MS techniques, an analyte is first ionized in the source, since the MS can only detect charged species. Ions of discrete mass/charge ratios (*m/z*) are then separated and focused in the mass analyzer. The final focused beam impinges on a detector that determines the intensity of the beam. The analyzer is thus comparable to the prism or monochromator for spectrophotometric techniques, except that ions of discrete *m/z* ratios are separated and focused rather than photons of discrete wavelengths.

Selection of the ion source differentiates among different MS techniques for HPLC applications. Different techniques are often based solely on the source utilized. Samples are run by electron ionization (EI), chemical ionization (CI), fast-atom bombardment (FAB), electrospray ionization (ESI), thermo spray (TSP), laser desorption (LD), and so on.

Electron-ionization or electron impact mass spectrometry (EI-MS) is the most familiar and commonly utilized form of MS today and is a staple of all environmental labs. The EI-MS may provide both quantitative and qualitative information. The EI process requires that volatile compounds be introduced into the MS, however many analytes in HPLC are polar and non-volatile, this technique is not useful for online LC-MS. Fortunately, other ionization methods exist that are based on the desorption of non-volatile and thermally labile compounds directly from solutions or solid surfaces.

4.1.5.1 Ion trap electrospray mass spectrometry

Electrospray ionization (ESI) is one of the most commonly used ionization methods and has revolutionized the field of MS. It is best suited for ionizing highly polar and thermally little stable compounds that are intrinsically charged in solution or

may be protonated or deprotonated under the solution conditions employed and for non-polar compounds that undergo oxidation or reduction at the electrospray capillary tip. The schematic presentation of a typical ESI-MS system is shown in Figure 11.

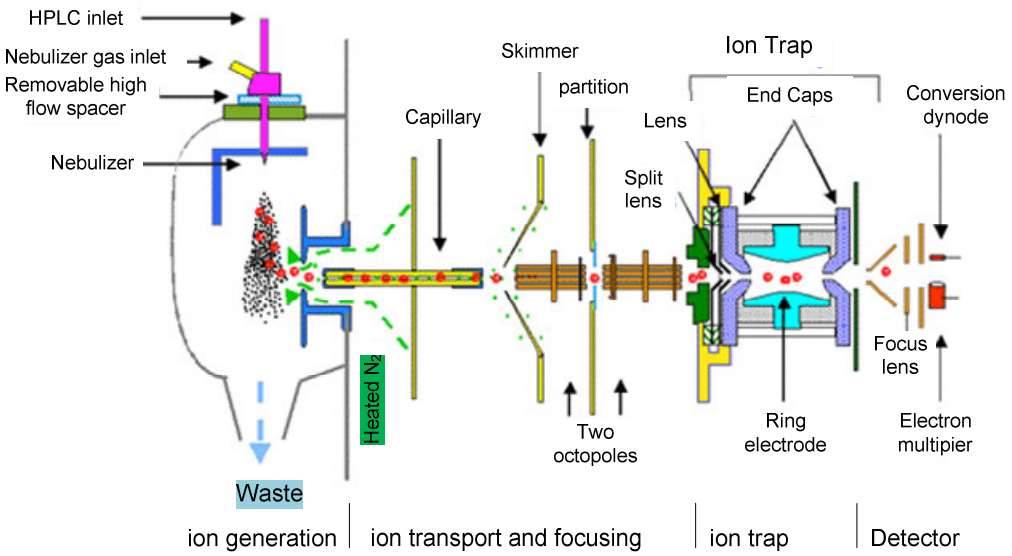


Figure 11: Schematic representation of Atmospheric Pressure Interface-Electrospray Ionization (API-ESI) mass spectrometric system; modified from HCT/esquire Series User Manual, Version 1.1

A spray is generated at ambient pressure and a high voltage is supplied to the eluting solvent. There are some variations on the original electrospray (ESP) technique, most notably the use of a sheath or supporting gas, which has often been termed ion spray. As the eluent is sprayed at ambient pressure, an organic sheath liquid is commonly mixed with an eluting aqueous solvent to reduce surface tensions and enhance evaporation of the charged droplets. Analyte molecules that are generated via electrospray contain various charged states (varying amounts of adducted sodium ions or protons).

This multi-charging produces a nearly Gaussian distribution of peaks (often referred to as an envelope) corresponding to the different *m/z* ratios of the multiplied charged ions. Only those analytes capable of sustaining such multiple charges, such as proteins, peptides, and nucleic acids, generally are amenable to this type of MS analysis.

The process of ESI can be summarized into following four steps:

1. Formation of ions
2. Nebulisation
3. Desolvation
4. Ion evaporation

Solution chemistry plays an important role in enhancing sensitivity for both positive and negative electrospray ionization. Many compounds can be analyzed as neutral molecules in a neutral environment. Other compounds, however, can be analyzed with much greater sensitivity if the chemical environment is one that favours ion formation.

More base analytes are generally analyzed in positive ion mode as the sample molecule (base) picks up a proton from the more acidic solvent solution.

$$M^0 + HA \Leftrightarrow \left[M + H\right]^+ + A^- \qquad (13)$$

Acidic analytes are generally analyzed in negative ion mode. The sample molecule (acid) loses a proton to a base (pH > 7) in solution and becomes negatively charged.

$$M^0 + B \Leftrightarrow \left[M - H\right]^- + HB^+ \qquad (14)$$

Optimum range of flow rate in ESI is ≤ 0.25 ml/min and hence requires columns with reduced internal diameter (≤ 2.1 mm).

4.1.5.2 Mode of analysis

Ion traps are three-dimensional quadruples and consist of two end caps and a ring electrode. Ions enter and exit the ion trap through small perforations in the centre of the end cap. In order to efficiently trap the incoming ions, a collision gas (helium or argon) is present in the trap. Ions are held at complex stable trajectories in a quadrupolar field created by application of a high voltage radio frequency potential to the ring electrode while the end caps are held at ground.

Depending on the intensity of the ion signal, ions can be accumulated for 10 µs to 1s. During accumulation, all voltages in the ion transport and focussing region are set for transmission.

In the scan mode, ions are progressively ejected by increasing the quadrupolar and dipolar fields. When all ions have been removed from the trap, the initial accumulation conditions are restored and a new accumulation and ejection scan cycle starts.

In the MS(n) mode, all ions except one target ion are ejected. The selected ion is isolated and can now be fragmented by increasing the ion's energy by resonance excitation. The produced fragment ions can now all be sent to the detector (whereupon a new accumulation cycle starts) or a daughter ion can again be isolated (by ejecting all ions but the target fragment ion) and subjected to fragmentation. This whole process can theoretically be repeated for 10 times.

In the multiple reaction monitoring mode (MRM), ten compounds can be selected for consecutive isolation and/or fragmentation experiments. Each compound is isolated and/or fragmented individually. A special feature of MRM is that ions can be fragmented without being isolated. This results in an increase in the sensitivity of quantification.

The most important ion trap settings can be done by the trap drive which depends mainly on the *m/z* ratio of the analytes, the scan range and ICC (ion charge control) settings which are important for automatic adjustment of the accumulation time of ions in the ion trap during LC-MS runs with time spans of low (background) and high ion intensities (peaks).

The great advantage of ion trap mass analyzers are high sensitivity in the scan mode due to high scan rates up to 13 000 *m/z* per second at normal resolution (in contrast to single- and triple quadrupole mass analyzers) and the possibility of sequential MS(n) analyses. The greatest limitation is low linearity of the detector response due to overloading the ion trap.

Optimum settings for ion source, ion transfer and focussing region and ion trap are determined experimentally by software controlled optimization. For that, a solution containing the analyte of interest is continuously introduced into the mass

spectrometer and a range of values are tested for each parameter. This can also help in optimizing the condition for well separated peaks in case of co-eluting compounds.

4.1.6 Sample preparation techniques

Sample preparation is an essential part of any solid-liquid extraction. A sample must be in a liquid state prior to HPLC analysis. Some insoluble solids contain soluble analytes such as additives in a solid polymer, fats in food, and poly-aromatic hydrocarbons in soil. Contacting the sample with solvent allows the extraction of analytes, following which the solvent is separated from the solid residue by decanting, filtration, or centrifugation. The filtrate is further treated, prior to HPLC analysis.

Different sample preparation techniques were used during the research work (Table 7) and are briefly discussed as follows:

1. Solid Phase Extraction (SPE)
2. Accelerated Solvent Extraction (ASE)
3. Ultrasound-assisted Extraction (UAE)

4.1.6.1 Solid phase extraction (SPE)

Solid phase extraction (SPE) is an extraction method that uses a solid phase and a liquid phase to isolate one, or one type, of analyte from a solution. It is usually used to clean up a sample before using a chromatographic or other analytical method to quantify the amount of analyte(s) in the sample.

The general procedure is to load a solution onto the SPE phase, wash away undesired components, and then wash off the desired analytes with another solvent into a collection tube. Solid phase extractions use the same type of stationary phases as are used in liquid chromatography columns. The stationary phase is contained in a glass or plastic column above a frit or glass wool. The column might have a frit on top of the stationary phase and might also have a stopcock to control the flow of solvent through the column (Figure 12). Commercial SPE cartridges have 1-10 ml capacities and are discarded after use.

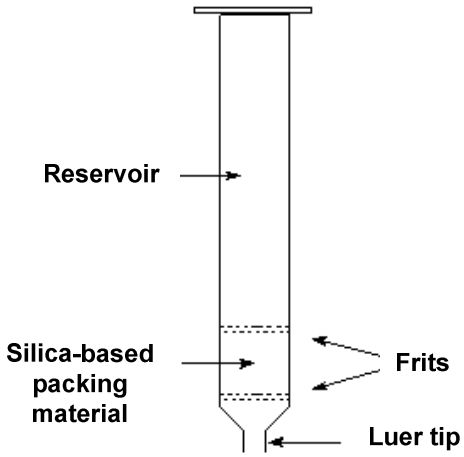


Figure 12: Schematic presentation of a solid phase-extraction column

With SPE, it is also possible to obtain a more complete removal of interferences from the analyte fraction. Reversed-phase SPE techniques are most popular, as only small amounts of organic solvents are required for elution, maintaining a high concentration of analyte. Some disadvantages of SPE include:

- Variability of SPE cartridges
- Irreversible adsorption of some analytes on SPE cartridges

4.1.6.2 Accelerated solvent extraction (ASE)

Accelerated solvent extraction (ASE) is a technique for extracting solid and semisolid samples with liquid solvents. ASE, also known as enhanced solvent extraction, in closed extraction vessels uses conventional organic solvents at elevated temperatures (50 to 200 °C) and pressures (150 to 2000 psi) to extract soluble analytes from solid samples. Analyte recovery is enhanced and accelerated by the higher temperatures, and solvent volume can be reduced because of the high solute capacity in the heated solvents.

The experimental apparatus used in ASE includes a pump for transporting solvent into and out of the extraction vessel, extraction vessels with an automated sealing mechanism to withstand high pressures, an oven for heating the sample compartment, and collection vials to hold the collected extracts. Increased

temperature accelerates the extraction kinetics, while elevated pressure keeps the solvent below its boiling point, thus enabling safe and rapid extraction (Figure 13).

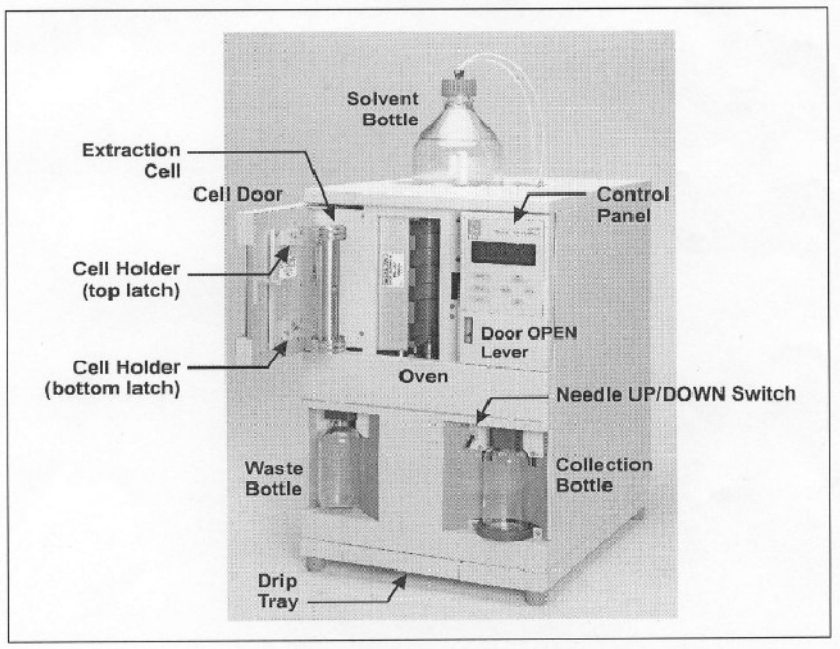


Figure 13: Key operating features of ASE 100; taken Dionex operator manual of ASE 100

A typical accelerated solvent extraction system consists of following steps:

1. Sample cell loading (typical sample sized 5 to 20 g)
2. Solvent introduction and pressurization
3. Sample cell heating (under constant pressure)
4. Static extraction
5. Transfer of extract to sealed vial with fresh vent wash of solid sample
6. Nitrogen purge of cell
7. Loading of sample
8. Once the sample is loaded into the extraction cell, the entire process is automated and time programmable. ASE provides unattended preparation for up to 24 samples serially and extractions that normally take hours can be done in minutes. Compared to techniques like soxhlet and sonication,

ASE generates results in a fraction of the time. In addition to speed, ASE offers a lower cost per sample than other techniques by reducing solvent consumption by up to 95%.

A method is hence loaded which defines all of the timed events that occur during a sample extraction. Important parameters of the method control are summarized in Table 6.

Table 6: Method parameters used in ASE 100

Parameter	Function	Value range
Temperature	Temperature at which to heat the extraction cell.	Off, 40 to 200 °C (default = 100)
Static time	Static solvent extraction time	0 to 99 min (default = 5)
Flush volume	Amount of solvent to flush the extraction cell after the static heating step. The flush volume is expected as percentage of the cell volume; for example, if the flush volume is set to 50%, 5 ml is flushed through a 10 ml cell, 17 ml is flushed through a 34 ml cell, and so on.	0 to 150% volume in 5% increments (default = 60)
Purge time	Amount of time the cell is purged with nitrogen	20 to 900 sec (default = 1)
Static cycle	Number of times the static heating and flushing steps are performed. When more than one cycle is specified, the flush volume is divided among the cycles.	1 to 5 (default = 5)

4.1.6.3 Ultrasound-assisted extraction (UAE)

Ultrasonic agitation (sonication) often allows more effective solid-liquid contact. Sonication is a procedure recommended for the pre-treatment of many solid environmental samples, such as U.S. EPA Method 3550 for extracting non-volatile and semi-volatile organic compounds from solids such as soils, sludge, and wastes. In this method, different extraction solvents and sonication conditions are recommended, depending on the type of pollutants and their concentration in the solid matrix.

High intensity (20 KHz range) ultrasonic generation is sufficiently powerful to achieve useful liquid processing in a wide variety of applications with rapidity and reproducibility. The ultrasonic generator produces an electrical signal at a particular frequency. The converter/transducer transforms the electrical signal into mechanical vibration. The mechanical vibration is transmitted down the length of the horn/probe. The tip of the horn/probe expands and contracts at the same frequency as the electrical signal through a prescribed amplitude (distance). In liquid, the rapid (i.e., 20 KHz) vibration of the horn/probe tip causes cavitation, the formation and violent collapse of microscopic bubbles. The collapse of the thousands of cavitations bubbles releases tremendous energy in the cavitation field. Objects and surfaces that are within the cavitation field are 'processed' by the released energy. The choices of a generator (and transducer) and horns/probes are matched to the volume, viscosity and other parameters of the particular application. A typical ultrasonic bath, or commonly called sonicator, is show in Figure 14.

Figure 14: An ultrasonic bath (sonicator)

A brief comparative description of these three extraction methods is summarized in Table 7.

Table 7: Sample pre-treatment methods

Method of sample pre-treatment	Principles of technique	Comments
Solid-phase extraction	Liquid is passed through solid phase, which selectively removes analyte (or interferences); analyte can be eluted with strong solvent; in some cases interferences are retained and analytes allowed to pass through solid phase un-retained; same mechanisms as HPLC.	Wide variety of stationary phases available for selective removal of desired inorganic, organic, and biological analytes; specially phases for drugs of abuse, carbohydrates, catechol amines, and many other classes of compounds, trace enrichment from water.
Accelerated solvent extraction	Sample is placed in a sealed container and heated to above its boiling point, causing pressure in vessel to rise; extracted sample is removed automatically and transferred to vial for further treatment.	Greatly increases speed of liquid-solid extraction process; automated; vessel must withstand high pressure; extracted sample is diluted and requires further concentration; safety provisions are required because of over-pressured, high-temperature solvents.
Ultra-sound assisted extraction	Sample is immersed in ultrasonic bath with solvent and subject to ultrasonic radiation. An ultrasonic probe or ultrasonic cell disrupter can also be used.	Dissolution is aided by ultrasonic process; heat can be added to increase rate of extraction; safe; rapid; best for coarse, granular materials; multiple samples handled simultaneously; efficient contact with solvent.

5 METHOD DEVELOPMENT

High performance liquid chromatography is an excellent quantitative analytical technique. A properly designed, validated and executed analytical method should show high levels of both accuracy and precision for a main component analysis (±1 to 2% precision and accuracies within 2% of actual values).

Main goals of HPLC method development are summarized in Table 8 where the goal is roughly in order of decreasing importance but may vary with analysis requirements. The final procedure should meet all the goals that were defined at the beginning of method development. The method should also be robust in routine operation and usable by all laboratories and personnel for which it is intended.

Table 8: Separation goals in HPLC method development

Goal	Comments
Resolution	Precise and rugged quantitative analysis requires that resolution be greater than 1.5
Separation time	< 5-10 min is desirable for routine procedures
Quantitation	≤2% for assays; ≤5% for less-demanding analyses; ≤15% for trace analyses
Pressure	<150 bar is desirable, <200 bar is usually essential (new column assumed)
Peak height	Narrow peaks are desired for large signal/noise ratios
Solvent consumption	Minimum mobile-phase use per run is desirable

This chapter is based upon the development and validation of HPLC method for the rapid and sensitive determination of free α-LA. The method was developed and validated using three different detection modes.

5.1.1 Reagents and chemicals

Alpha lipoic acid [DL-α-lipoic acid; purity ≥ 99.0%], bisphenol A (99%) and naproxen (≥ 98%) were purchased from Sigma-Aldrich (Steinheim, Germany).

Sodium acetate p.A, potassium dihydrogen phosphate p.A, *ortho*-phosphoric acid (85%), glacial acetic acid, HPLC-gradient grade acetonitrile and methanol were delivered from Merck (Darmstadt, Germany).

Deionized water, prepared using a Barnstead Easypur LF (Dubuque, Iowa, USA) water purification system, was used in all experiments.

5.1.2 Analysis of standard solution

5.1.2.1 Internal standard

Two different internal standards bisphenol A and naproxen were selected and standard solution of each (0.5 µg/ml) was run separately and also with standard α-LA solution (0.5 µg/ml). Bisphenol A was selected as an internal standard because it eluted soon after α-LA when injected into the HPLC system and also showed similar behaviour in the UV and CEAD detection system. The behaviour and optimized condition of internal standard bisphenol A (BPA) is discussed in detail in the following sections.

5.1.2.2 Stationary phase

Stationary phase consisted of ASE 3 C 18 column with two different dimensions. A longer column (150 mm × 3.0 mm, particle size 3 µm) for UV and CEAD detection systems while a smaller one (150 × 2.1 mm, particle size 3 µm) for the MS was used. These columns were equipped with pre-columns of the same stationary phase material.

5.1.2.3 Mobile phase

In the course of HPLC method development, several mobile phases containing different proportions of acetonitrile and acetic acid/sodium acetate buffer were tested for better resolution and shorter run time.

1. Acetonitrile/methanol/50 mM sodium acetate (pH 3, adjusted with glacial acetic acid), 290/150/560, (v/v/v)
2. Acetonitrile/methanol/50 mM sodium acetate (pH 3, adjusted with glacial acetic acid), 350/65/585, (v/v/v)

3. Acetonitrile/methanol/50 mM sodium acetate (pH 3, adjusted with glacial acetic acid), 410/45/615, (v/v/v)
4. Acetonitrile/methanol/50 mM sodium acetate (pH 3, adjusted with glacial acetic acid), 440/20/540, (v/v/v)
5. Acetonitrile/methanol/50 mM sodium acetate (pH 3, adjusted with glacial acetic acid), 470/10/520, (v/v/v)
6. Acetonitrile/methanol/50 mM potassium dihydrogen phosphate (pH 3, adjusted with phosphoric acid), 350/65/585, (v/v/v)

With the increase in the concentration of acetonitrile, α-LA and BPA eluted earlier due to strong coordination with the mobile phase. However, analysis of a complex sample extract often requires a reduction in the percentage of the organic modifier in order to achieve better resolution of the early eluting compounds. The concentration of acetonitrile was decreased to a certain extent to improve the elution time without increasing the run time to a greater extent.

Initially, mobile phase 2 was used for the method development. However, the appearance of a ghost peak after some injections was a problem. A possible reason of this ghost peak could be the degradation of the buffer system after some time. Change of buffer system solved this problem and also gave better resolution. Potassium dihydrogen phosphate appeared to be a good choice with reference to stability of the mobile phase and resolution of the peaks.

Column efficiency N was determined for the optimized mobile phase (no. 6) on the basis of the retention time of α-LA and the baseline width at the optimum conditions using equation 6 as shown in Table 9.

Table 9: Column efficiency (N) at the optimized condition

Retention time t_R (min)	**Baseline width (min)**	**Number of theoretical plates (N)**
8.2	0.53	3830

5.1.3 UV detection

5.1.3.1 Instrumentation

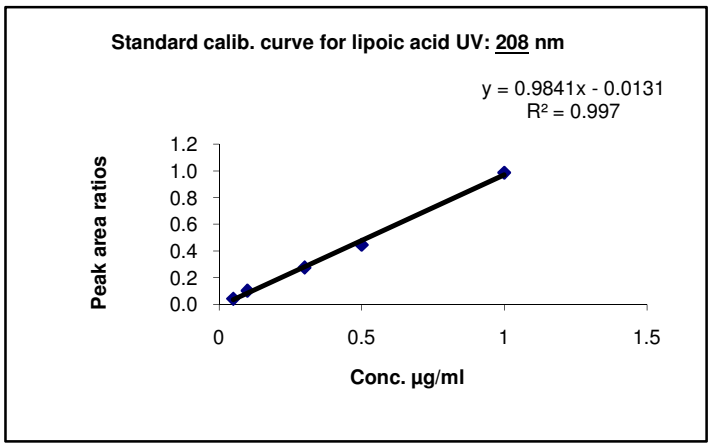
The HPLC system consisted of a Merck Hitachi pump L-6200, a Basic Marathon autosampler (Spark, Holland B.V., Emmen, The Netherlands), and an ACE 3 C 18 (150 mm × 3.0 mm, particle size 3 µm) column (Advanced Chromatography Technologies, Aberdeen, Scotland) equipped with a pre-column of same stationary phase.

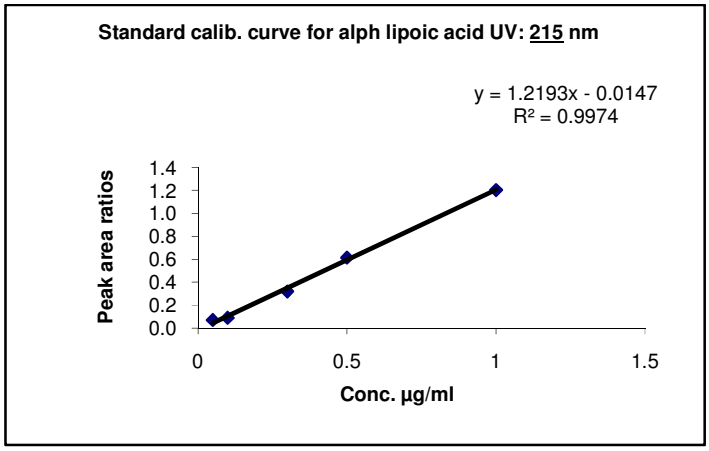
A Merck Hitachi L-4250 UV/Vis detector was used together with D-6000 Interface (Merck Hitachi). Data were obtained using chromatography data station software, HPLC manager version 2.

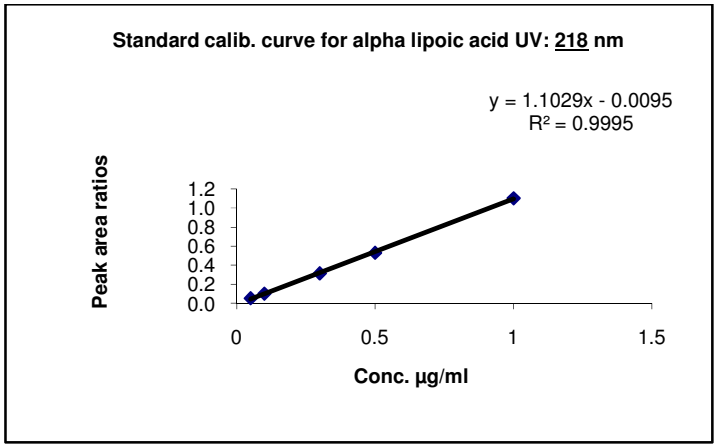
5.1.3.2 Selection of optimum wavelength

Standard solutions or the samples were injected (20 µl) into the HPLC system and chromatographic separations were achieved isocratically using mobile phase containing acetonitrile/methanol/50 mM potassium dihydrogen phosphate (pH 3, adjusted with phosphoric acid), 350/65/585, (v/v/v) at a flow rate of 0.45 ml/min.

Two different wavelengths are cited in literature i.e. 208 nm and 330 nm. Initially, 208 nm was selected for the optimization of mobile phase. Later, other wavelengths such as 208, 215, 218, 220, and 332 nm were checked. Calibration curves of standard α-LA solutions (0.01 to 1 µg/ml) were obtained at each wavelength and the results were compared (Figure 15). Among these various wavelengths, 215 nm was found to be optimum and was selected for the purpose of quantitation.


Standard calib. curve for lipoic acid UV: 208 nm
y = 0.9841x - 0.0131
R² = 0.997
Peak area ratios
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
0.5
1
1.5
Conc. µg/ml


Standard calib. curve for alph lipoic acid UV: 215 nm
y = 1.2193x - 0.0147
R² = 0.9974
Peak area ratios
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
0.5
1
1.5
Conc. µg/ml


Standard calib. curve for alpha lipoic acid UV: 218 nm
y = 1.1029x - 0.0095
R² = 0.9995
Peak area ratios
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
0.5
1
1.5
Conc. µg/ml

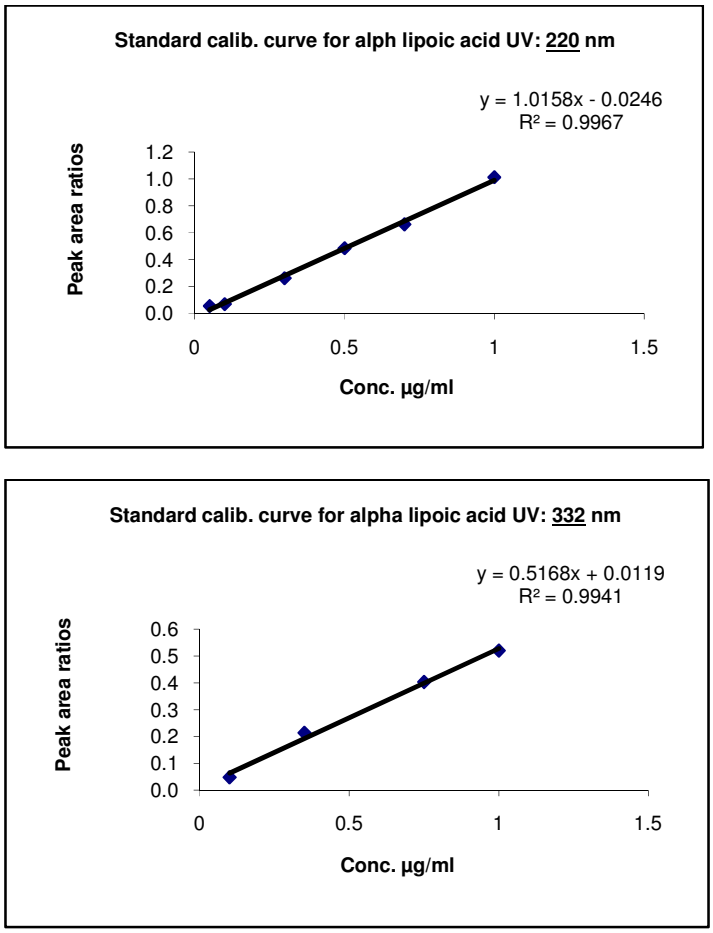


Figure 15: Calibration curves for alpha lipoic acid measured at different wavelengths

5.1.4 CEAD

5.1.4.1 Instrumentation

The HPLC system consisted of a Merck Hitachi pump L-6200, a Basic Marathon autosampler (Spark, Holland B.V., Emmen, The Netherlands), and an ACE 3 C 18 (150 mm × 3.0 mm, particle size 3 µm) column (Advanced Chromatography Technologies, Aberdeen, Scotland) equipped with a pre-column of same stationary phase.

A coulometric electrode array detector was controlled by Coul Array Win/Software (ESA, Chelmsford, USA).

5.1.4.2 Selection of optimum cell potential

20 µl of the standard solutions or the samples were injected into the HPLC system. Chromatographic separations were achieved isocratically using mobile phase containing acetonitrile/methanol/50 mM potassium dihydrogen phosphate (pH 3, adjusted with phosphoric acid), 350/65/585, (v/v/v) at a flow rate of 0.45 ml/min.

The potentials of the electrodes were set at +300, +400, +450, +500, +550, +600, +650, and +700 mV against palladium reference electrodes. By plotting the peak height versus the potential of the working electrodes, the current/voltage curves for α-LA and bisphenol A were obtained (Figure 16) and on its basis, further evaluation was made in adjacent channels (+500 and +550 mV).

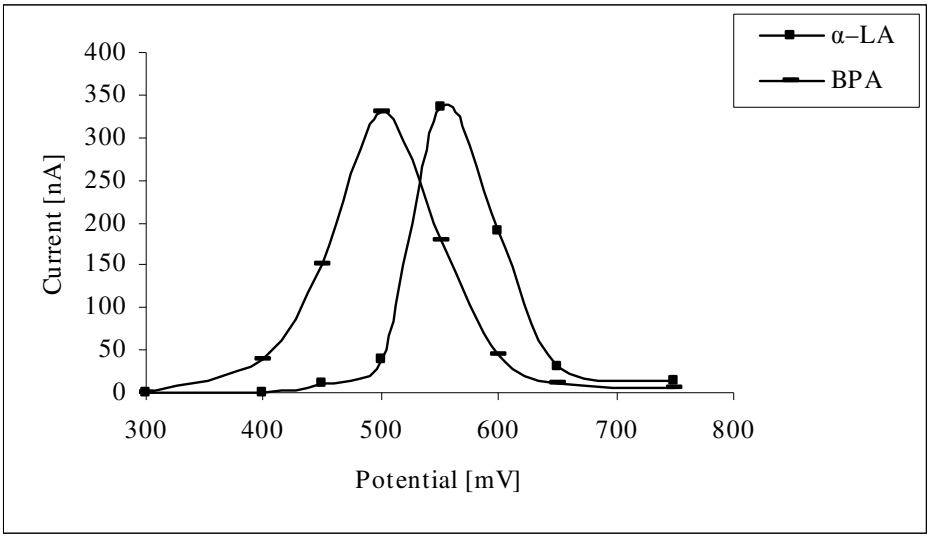


Figure 16: Current/voltage curves of alpha lipoic acid and bisphenol A

5.1.5 ESI-MS

5.1.5.1 Instrumentation

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, a ACE 3 C 18 (150×2.1 mm, particle size 3 µm) column (Advanced Chromatography Technologies, Aberdeen, Scotland) and an ion trap mass spectrometric (MS) detector (HCT plus, Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source.

Hystar 3.1, software for chromatography and hyphenated techniques, was used to acquire the data. Data were then integrated using the software, Data analysis™ version 3.3 (Bruker Daltonics).

5.1.5.2 Optimization of instrumental parameters

20 µl of the standard solution (0.4 µg/ml) was injected and the separation was made using isocratic elution with 0.1% acetic acid in water and acetonitrile (55/45, v/v).

MS detection was performed using electrospray ionization. First standard α-LA solution was directly injected into the MS system and negative mode was selected due to better sensitivity. Then, ion source, ion transfer region and ion trap were further optimized during continuous infusion of pure standard solution (c ~ 40 µg/ml in 30% ACN) by means of a syringe pump. The optimized parameters in the negative mode setting are summarized in Table 10.

Table 10: Optimized MS parameters

MS parameters	**MRM (negative mode)**
Capillary voltage	+4500 V
Nebulizer pressure	40 psi
Dry gas flow	10 l/min
Dry temperature	300°C
Skimmer	-40 V
Capillary exit	-110 V
Octapole ½ DC	-14 V
Octapole RF	40 Vpp
Lens 1-2	5-60 V
Trap drive	30
ICC smart target	250 000
Scan range	70 – 240 *m/z*

5.1.5.3 Optimization of MRM mode

Direct infusion of a standard solution of pure α-LA into the ESI-MS apparatus revealed that the deprotonated molecular ion (*m/z* 205) was partially fragmented to *m/z* 171 $[M\text{-}H\text{-}H_2S]^-$. This fact was also observed by Chen (CHEN, JIANG, CAI, TAO et al.; 2005) but due to the limited possibilities with a single quadruple instrument, the deprotonated molecular ion was favoured for quantitative determination of α-LA.

One advantage of the ion trap instrument is the possibility to use the multiple reaction monitoring mode (MRM) for quantitative analysis. Isolation and fragmentation experiments were conducted to determine the best suited settings for MRM analysis. Following settings were checked in the negative MRM mode for better sensitivity:

1. Isolation of α-LA, without fragmentation

2. Fragmentation of α-LA without isolation and fragmenting the daughter ion

In this mode the deprotonated molecular ion (*m/z* 205) can first be fragmented without being isolated, then the fragment ion (*m/z* 171) can be isolated and the corresponding peak area can be used for quantification. This approach offers both higher selectivity due to measurement of the fragment ion and higher sensitivity because both the original fragment ion (the one created in the source) and the fragment ion created from the deprotonated molecular ion in the trap contribute to the total ion current (Figure 17).

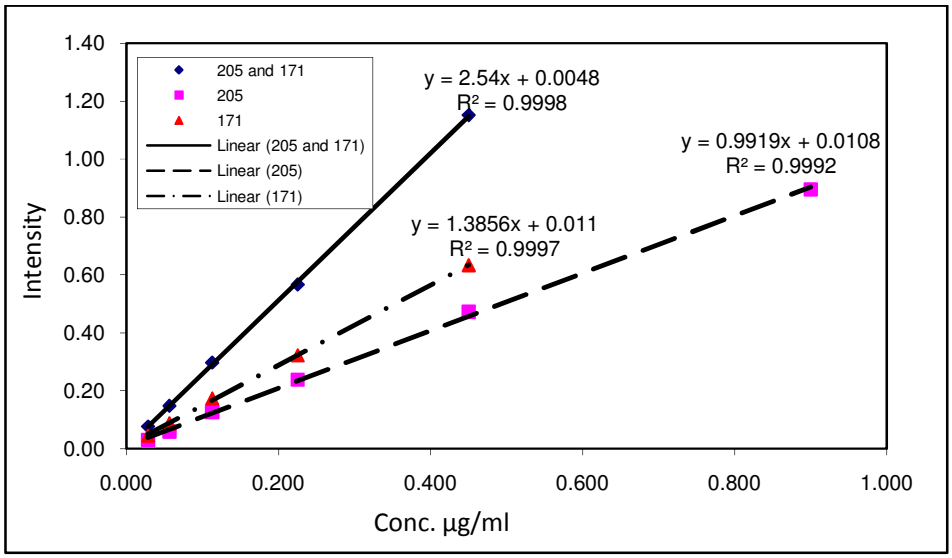


Figure 17: Optimization of MRM mode for sensitive quantitation of alpha lipoic acid

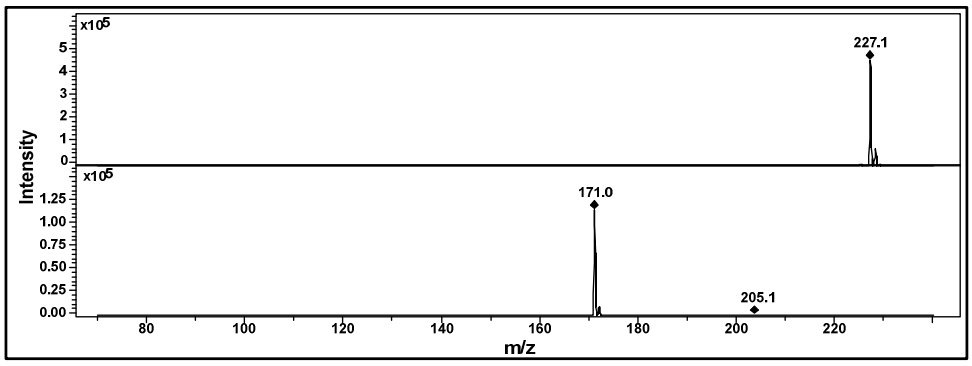


Figure 18: Mass spectrum of alpha lipoic acid fragment ion at *m/z* 171 ($[\alpha\text{-LA-H-H}_2\text{S}]^-$) and for internal standard BPA at *m/z* 227 ($[\text{BPA-H}]^-$ using MRM mode.

ESI-MS measurements applying the negative MRM mode offers, in the case of α-LA, the possibility of complete fragmentation of the $[M-H]^-$ ion into the fragment ion *m/z* 171 which is responsible for the sensitive measurement of this compound.

Before calibration functions were established, the influence of the ICC (ion charge control) value on the signal intensity was tested. A solution containing about 1 mg/l of standard α-LA was analyzed by the methods with different ICC target values such as 50.000, 100.000 and 250.000. In the negative mode, no differences were observed.

5.1.6 Calibration curves, repeatability, linearity and LOD

For all the three HPLC systems, calibration functions were recorded for daily quantitation in a concentration range of 0.003 to 2 µg/ml (N = 6) and established by linear regression of the peak area ratios of α-LA to the internal standard bisphenol A (BPA). The limit of detection was determined by measuring successive dilutions of the lowest concentrated calibration standard until a signal to noise ratio of 3 was reached. The linear range was determined by establishing calibration functions from 0.025 to 1 µg/ml, by calculating the 'experimental' concentrations (x) by putting the experimentally obtained peak areas (y) into the calibration functions $y = kx + d$ and by comparing the calculated concentrations with the nominal concentrations.

The limit of quantitation (lower end of the linear range) was experimentally calculated by successive dilutions of the standard solutions until a signal to noise ratio of 10 was obtained.

The intra-day repeatability (N = 5) of the chromatographic method was determined by injecting of a standard solution containing 0.19 µg/ml α-LA and calculation of the relative standard deviations of the retention times and the peak areas. The inter-day repeatability (N = 15) was measured by three injections (20 µl) of a standard solution containing 0.19 µg/ml over the period of five days. Stability of the standard solutions was checked both at 4 °C and at room temperature.

Table 11: Method validation parameters for the HPLC systems used

Method validation parameters		**Chromatographic methods**		
		HPLC-UV	**HPLC-CEAD**	**HPLC-MS**
Repeata-bility (% R.S.D)	Intra-day (n=5)	2.61	2.56	3.03
	Inter-day (n=15)	2.85	2.73	4.23
Limit of detection (LOD)		0.015	0.003	0.001
Linear Regression Data	Regression equation	y=1.3381x+0.002	y=3.2089x+0.073	y=10.402x+0.038
	Linear range (µg/ml)	0.045-2	0.01-2	0.005-2
Regression coefficient (R^2)		0.9989	0.9997	0.9999

The methods were compared concerning the characteristic analytical parameters for α-LA. The calibration curves were linear in the investigated concentration range. The limit of detection for the HPLC-ESI-MS turned out to be slightly lower than for HPLC-CEAD and HPLC-UV system. On the other hand, the intra- and inter-day repeatability of the coulometric electrode array was better than the other two systems. Calibration solutions were stable for more than 1 week when stored at 4 °C and were unstable at room temperature (25 °C or more) after 2 days.

6 SAMPLE PREPARATION

This chapter is based upon the study of different sample work-up methods to determine free α-LA. The best suited method was used to determine free α-LA content in different dietary supplements and foodstuffs (see chapter 7 and 8 for detail).

6.1 Experimental

6.1.1 Reagents, standards and solutions

Diatomaceous earth (suitable for most routine filtrations, ≥95% SiO_2 basis, powder); diethyl ether (anhydrous, ≥99.7%), sodium bicarbonate (powder, ≥99.5%), sodium chloride (≥98%); potassium chloride (≥99%); potassium hydroxide (≥85% KOH basis, pellets); sulphuric acid (99%), disodium phosphate dibasic hydrate (≥99%); sodium phosphate monobasic monohydrate (≥99.5%); bovine serum albumin (BSA; 30% in 0.85% sodium chloride), *alcalase* solution (BioChemika, ≥ 5 U/g) and polyamide 6 (for column chromatography) were purchased from Sigma-Aldrich (Steinheim, Germany). Deionized water, prepared using a Barnstead Easypur LF (Dubuque, Iowa, USA) water purification system, was used in all experiments.

Buffer solution for the enzymatic hydrolysis was prepared by taking sodium chloride (8.6 g), potassium chloride (0.42 g), disodium phosphate dibasic hydrate (0.97 g), sodium phosphate monobasic monohydrate (0.04 g) in 1 l volumetric flask and making the volume up to the mark with distilled water. Enzyme solution was then prepared by taking 1 ml of *alcalase* solution (≥ 5 U/g) in a 12.5 ml of the buffer in a volumetric flask and making the volume up to the mark with distilled water.

6.1.2 Validation of instrumental method

HPLC-UV, HPLC-CEAD and HPLC-MS: Standard calibration solutions were run thrice a week to obtain/compare the calibration functions in the observed concentration range. Inter-day and intra-day repeatabilities were checked by injecting 20 µl of a standard solution containing 0.19 µg/ml of α-LA in acetonitrile/water (30/70; v/v).

6.1.3 Sample preparation methods

Different extraction and hydrolysis procedures were selected and/or developed to determine the best suited method for the analysis of α-LA in different foodstuffs and dietary supplements. The optimum conditions determined for each work-up were then compared for recoveries. An overview of these sample preparation methods is shown in Figure 19.

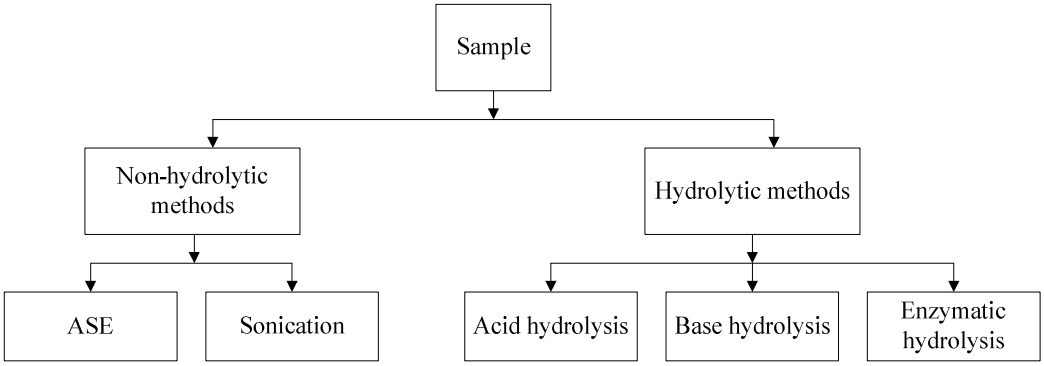


Figure 19: Schematic representation of different sample preparation methods

6.1.3.1 Hydrolytic extraction methods

Hydrolytic methods are usually aggressive methods, except the enzymatic hydrolysis methods, and can destroy the free lipoic acid present in the sample while extracting the protein-bound lipoic acid content. This hypothesis was checked by doing hydrolysis of a standard α-LA solution under the conditions as cited in the literature. Different acid, base and enzymatic hydrolysis methods were adopted in this regards and are briefly described in the following sections.

6.1.3.1.1 Acid hydrolysis

Acid hydrolysis was done by heating a known amount of standard α-LA in 2 M sulphuric acid for 7 h. After tissue hydrolysis, the α-LA in the hydrolysate was separated by a diethyl ether/sodium bicarbonate/diethyl ether extraction [Scheme adopted from Teichert et al. (TEICHERT and PREISS; 1997)]. The schematics are shown in Figure 20.

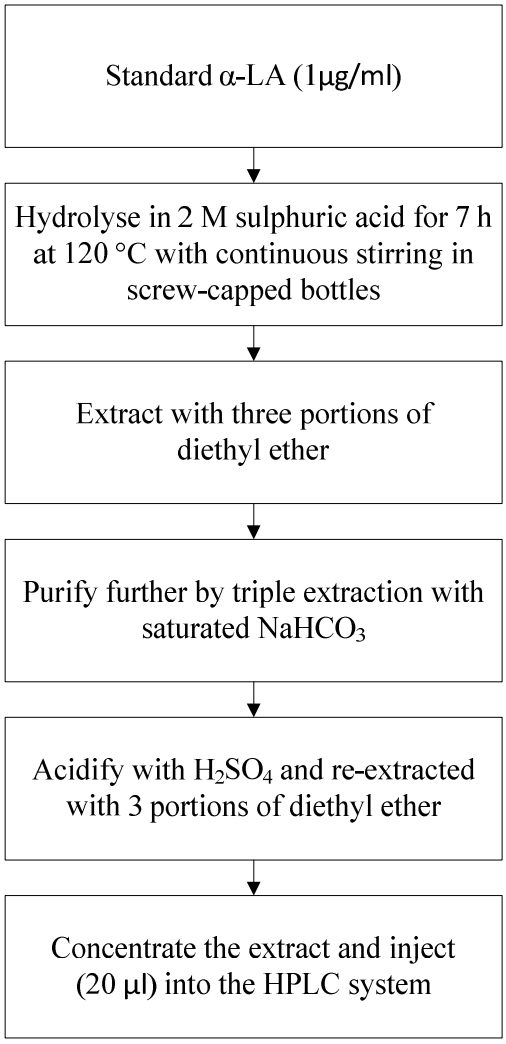


Figure 20: Schematic representation of acid hydrolysis

6.1.3.1.2 Base hydrolysis

Base hydrolysis was done following the same method as cited in the literature (KATAOKA, HIRABAYASHI and MAKITA; 1993; KATAOKA, HIRABAYASHI and MAKITA; 1997) and schematics are shown in Figure 21.

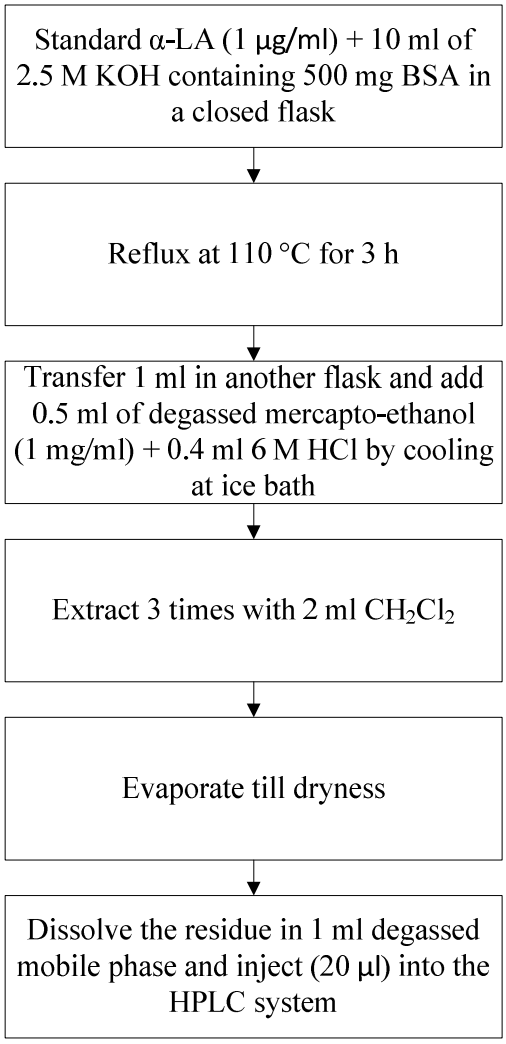


Figure 21: Schematic presentation of base hydrolysis

6.1.3.1.3 Enzymatic hydrolysis

Enzymatic hydrolysis was done by following the scheme given by Teichert and Preiss (TEICHERT and PREISS; 1992; TEICHERT and PREISS; 1995; TEICHERT and PREISS; 1997). Enzyme *alcalase* was used for the enzymatic process, a buffer was prepared and enzymatic hydrolysis was done according to the following scheme in Figure 22.

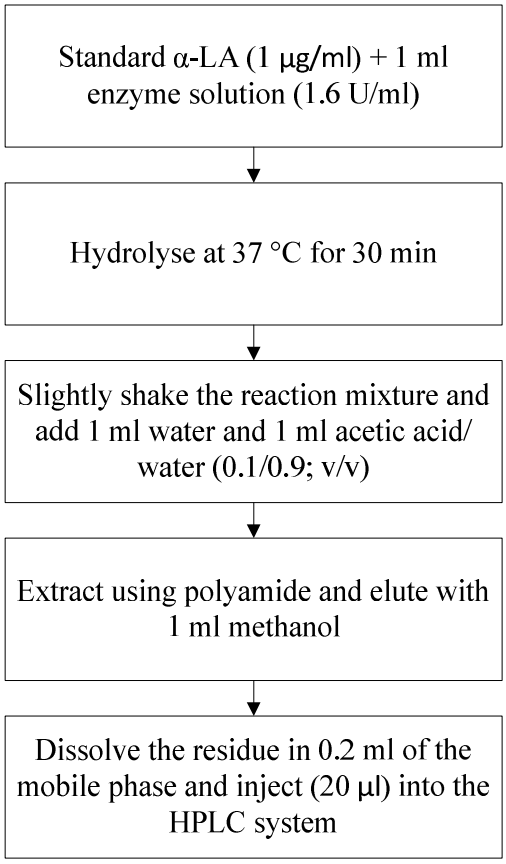


Figure 22: Schematic representation of enzymatic hydrolysis

6.1.3.2 Non-hydrolytic extraction methods

6.1.3.2.1 Accelerated solvent extraction

Accelerated solvent extraction (ASE) was used to extract α-LA from a standard solution using different extraction solvents such as distilled water, 0.1% acetic acid in water, 70% ethanol in water, pure methanol, 0.1% acetic acid in methanol and 0.5% acetic acid in methanol. The general scheme adopted for the accelerated solvent extraction method is illustrated in Figure 23.

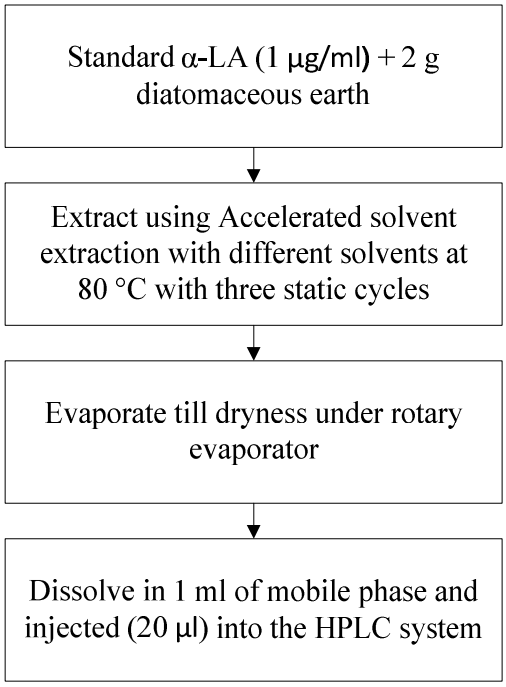


Figure 23: Schematic presentation of accelerated solvent extraction

ASE was then further investigated at different temperature conditions such as 40˚C, 60˚C, 80˚C and 100˚C.

Stability of α-LA at the evaporation step was checked with a standard α-LA solution (1 µg/ml) dissolved in 30 ml of 0.1 M acetic acid in methanol. Residue was dissolved in 1 ml of acetonitrile/water (30/70; v/v). From this solution, 0.75 ml was taken into the autosampler vial, 0.05 ml of internal standard (BPA) were added and 20 µl was injected into the system.

6.1.3.2.2 Ultra-sound assisted extraction

Ultra-sound assisted extraction was used to extract α-LA from different food samples (Schematics: Figure 24) using 0.1 M acetic acid in methanol at different incubation times i.e. 30, 60, 90 and 120 min.

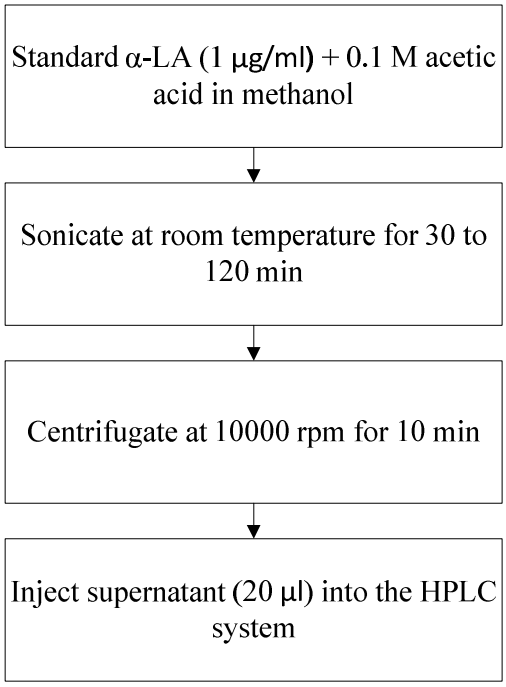


Figure 24: Schematic presentation of ultrasound assisted extraction

6.1.3.3 Qualitative analysis

Alpha lipoic acid was identified in the samples by comparison of retention time, current/voltage curves with that of authentic standards, by co-chromatography in the course of standard addition method and by MS detection in scan mode (negative ionization).

6.1.3.4 Quantitative analysis

Quantitative analyses were performed on the basis of calibration functions (peak area vs. concentration). Routine calibration was established weekly in range of 0.025-1 µg/ml. Statistical evaluation of means and standard deviations was done in Microsoft excel 2007.

6.1.4 Results and discussion

Direct injection of the centrifuged sample extract gave well resolved peaks in the HPLC-CEAD system but broad peaks were obtained by HPLC-ESI-MS. To cope with this problem, the extraction solvent was evaporated to dryness and the residue

was dissolved in 1.0 ml of acetonitrile/water (30/70, v/v). Injection of this sample extract, along with BPA, into the HPLC-ESI-MS system gave well resolved peaks

Results obtained from different extraction methods are summarized in Table 12 with respective recoveries.

Table 12: Recoveries obtained from different extraction methods

Extraction method	Recoveries (%)
Acid hydrolysis	≤30
Base hydrolysis	≤40
Enzymatic hydrolysis	≤55
Optimized ASE	≥95
Optimized ultrasonication	≥95

6.1.4.1 ASE extraction

ASE with distilled water and acidified water gave poor recoveries (below 40%). Acidified methanol (0.5% acetic acid in methanol) gave better results (above 60%) and was further improved by changing the concentration of acetic acid in methanol. In the concentration range from 0.1-1% acetic acid in methanol, 0.5% acetic acid in methanol was found to be the best choice.

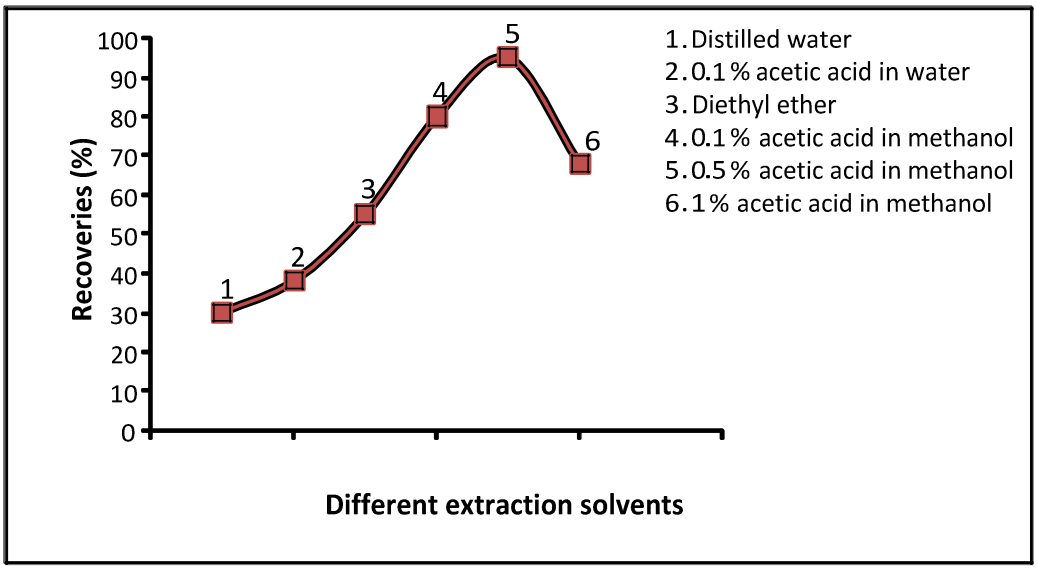


Figure 25: Comparison of different extraction solvents used in ASE

ASE was then further investigated at different temperature conditions such as 40˚C, 60˚C, 80˚C and 100˚C. With the accelerated solvent extraction, it was possible to increase the temperature up to 80˚C without destroying α-LA (≥ 95% recovery) as shown in Figure 26.

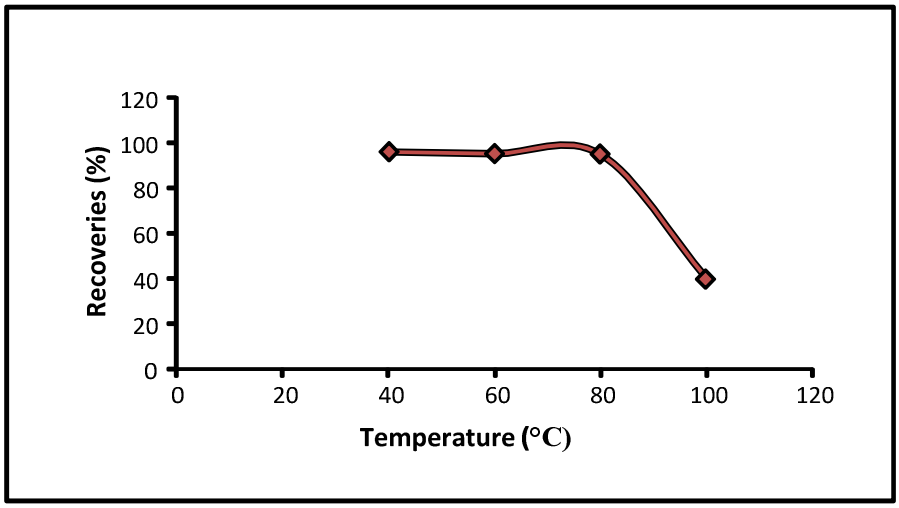


Figure 26: Effect of temperature on ASE of alpha lipoic acid

Other parameters like static cycle, flush volume and purge time were optimized and the final values are shown in Table 13.

This developed method when applied to the extraction of α-LA from fresh egg yolk resulted in a very cloudy extract. Diatomaceous earth was not sufficient to

absorb matrix compounds and the sample would require an additional cleaning step prior to the accelerated solvent extraction. In addition, ASE also required evaporation step due to a greater volume after the final extraction step.

Table 13: Optimized ASE parameters for the extraction of alpha lipoic acid

Parameter	Function	Value range
Temperature	Temperature at which to heat the extraction cell.	80 °C
Static time	Static solvent extraction time	5 min
Flush volume	Amount of solvent to flush the extraction cell after the static heating step. The flush volume is expected as percentage of the cell volume; for example, if the flush volume is set to 50%, 5 ml is flushed through a 10 ml cell, 17 ml is flushed through a 34 ml cell, and so on.	30% volume in 5% increments
Purge time	Amount of time the cell is purged with nitrogen	30 sec
Static cycle	Number of times the static heating and flushing steps are performed. When more than one cycle is specified, the flush volume is divided among the cycles.	3

6.1.4.2 Ultrasonication

Ultrasonication of egg yolk, in 0.5% acetic acid in methanol, resulted in clearer solutions which could be injected after centrifugation directly and did not require the evaporation step. Different sonication times were tested to optimize the recovery (Figure 27).

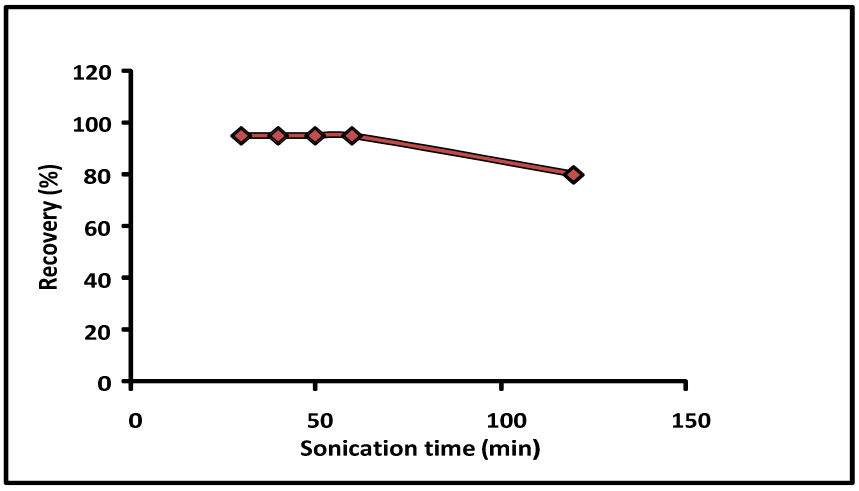


Figure 27: Optimization of sonication time

6.1.4.3 Effect of ultrasonication on extraction

To confirm that sonication will specifically extract free α-LA and does not cleave the amide bond e.g. in lipoamide, a side experiment was performed. A standard lipoamide solution (40 µg/ml) was prepared and directly injected into the MS system using syringe pump to get a scan both in negative and positive mode. Fragmentation pattern was noted and conditions were further optimized for better sensitivity. An aliquot of same standard solution was sonicated in 0.1 M acetic acid in methanol for 1 h and injected into the MS system. Both MS spectra showed the same molecular ion peak and fragmentation pattern (Figure 28). No α-LA was found in either case confirming that sonication is a mild extraction method which does not affect the protein-bound α-LA, hence giving exclusive information about the free α-LA content in the samples under investigation.

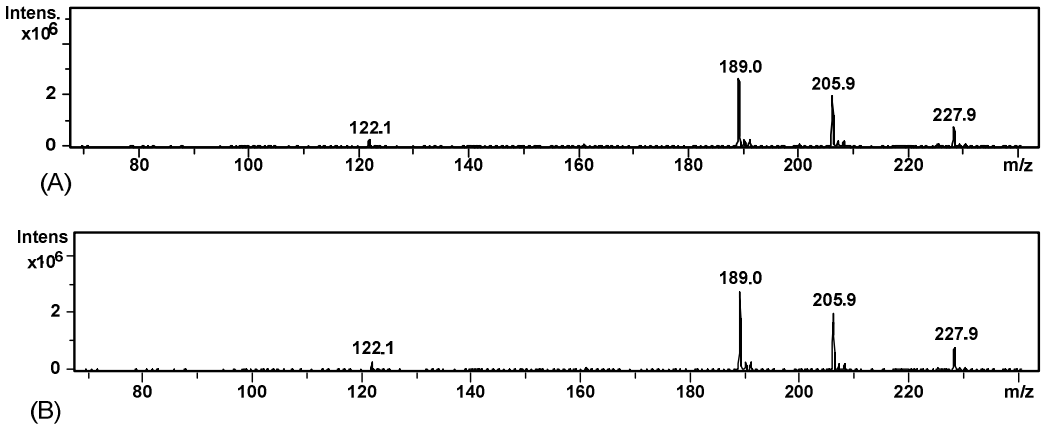


Figure 28: MS spectra of standard lipoamide in the positive scan mode: (A) direct injection, (B) after sonication for 1 h at room temperature

6.2 Conclusion

Intensive hydrolytic methods resulted in the destruction of free α-LA content. Accelerated solvent extraction is a good choice for the extraction of free α-LA. However when applied to a real sample resulted in the extraction of many other matrix compounds leading towards an additional sample preparation step to avoid complex chromatograms. Ultrasonication did not require the evaporation step and gave clear extracts and was applied to extract free α-LA from supplements and foodstuffs as described in the following chapters.

7 ALPHA LIPOIC ACID IN DIETARY SUPPLEMENTS

This part is based on the work published in the Journal of Pharmaceutical and Biomedical Analysis (DURRANI, SCHWARTZ, SCHMID and SONTAG; 2007).

The aim of the present work was to develop a rapid and reliable method for the quantitative determination of α-LA in dietary supplements using HPLC with three different detection modes i.e. UV, CEAD and ESI-MS.

Figure 29: Different dietary supplements containing alpha lipoic acid

7.1 Reagents and chemicals

Alpha lipoic acid [DL-α-lipoic acid; purity ≥ 99.0%] and bisphenol A (99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate p.A, *ortho*-phosphoric acid (85%), HPLC-gradient grade acetonitrile and methanol were delivered from Merck (Merck, Darmstadt, Germany).

Deionized water, prepared using a Barnstead Easypur LF (Dubuque, Iowa, USA) water purification system, was used in all experiments.

7.2 Preparation of solutions

7.2.1 Stock solutions

A stock solution of α-LA was prepared by dissolving 10.0 mg of α-LA in 100 ml of methanol. Calibration solutions of six different concentrations between 0.005 and 1 µg/ml were made by diluting the stock solution with acetonitrile/water (30/70,

v/v). A stock solution of Bisphenol A was prepared by dissolving 10 mg bisphenol A in 100 ml of methanol (internal standard) and was diluted with acetonitrile/water (30/70, v/v) to 2 µg/ml. All the solutions were stored in tightly sealed amber volumetric flasks at 4°C.

7.2.2 Dietary supplements

Six different dietary supplements (capsules/tablets/sachet) were purchased from local pharmacies as well as from the US market. A detailed description is given in Table 14. Ten tablets were weighed, finely ground and the average weight of one tablet was determined. Likewise, the content of 10 capsules was pooled and the average weight of one capsule was taken.

7.2.3 Mobile phase

Mobile phase I: Acetonitrile/methanol/50 mM potassium dihydrogen phosphate (pH 3, adjusted with phosphoric acid), 350/65/585, (v/v/v) were mixed, filtered through a 0.45 µm Sartolon polyamide filter (Sartorius AG, Goettingen Germany) and degassed for 20 minutes prior to use.

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

7.3 Sample preparation

Three aliquots (50 mg) of each homogenized supplement and of the sachet were dissolved in 8.0 ml of methanol and sonicated for 30 min at room temperature. The mixtures were then transferred into 25 ml volumetric flasks and the volume was made up to the mark with distilled water.

2 ml of the extracts were pipetted out into Eppendorf tubes and centrifuged for 10 min at 10,000 rpm. The supernatants were further diluted with acetonitrile/water (30/70, v/v) depending on the content of supplement. 500 µl of these solutions were then transferred to autosampler vials containing 450 µl acetonitrile/water (30/70, v/v) and 50 µl of bisphenol A solution (2 µg/ml).

Table 14: Brief description of dietary supplements

Supplement	Company	Product name	Brief description
a	Zand, United States	Cleanse today	Milk thistle extract, rosemary plant, ginger root, dandelion rot, sage leaf, oat seed bran, flax seed meal, vegetable cellulose fibre, alpha lipoic acid, broccoli head extract, kale leaves extract, nettle leaves extract, L-glutathione, cabbage leaf, maitake mushroom extract, barberry bark, bupleurum root, lonicera flowers
b	Ringana of Vienna, Austria	Wellness pack	Pineapple fruit powder, raspberry fruit powder, fructose, magnesium citrate, cherry extract, calcium citrate, potassium glaciate, blueberry fruit powder, lecithin, curcuma, green tea extract, lycopene powder, grapefruit extract, grape skin extract, black caraway oil, borage oil, natural tocopherol, iron gulconat, zinc gluconate, niacin, coenzyme Q10, sea buckthorn, lactobacillus sporogenes, pantothenic acid, black pepper extract, rosemary extract, alpha lipoic acid, palm kernel oil extract, manganese, sodium, cantaloup-melon extract, copper gluconate, chromium chloride, folic acid, biotin, vitamins
c	Pure essence labs, United States	Life essence	Ginkgo biloba extract, codonopis, lycii berries, schizandra, poria cocos, astragalus, zizyphus seed, aged citrus peel, grape seed extract, alpha lipoic acid, L-cysteine, blend of barley, kamuf, oat and wheat grains, hesperidin, querceitin, rutin, vitamins and minerals

(Continued)

Table 14: Brief description of dietary supplements (continued)

Supplement	Company	Product name	Main ingredients
d	Bio Align, United States	Renewal antioxidants	Rosemary leaf extract, ginger root, ginger root extract, milk thistle seed extract, pomegranate seed extract, red raspberry leaf extract, blueberry leaf extract, carnosine, tumeric rhizome extract, alpha lipoic acid, N-acetyl cysteine, wheat sprouts, quercetin, amla-fruit extract, grape seed extract, green tea extract, hawthorn berrry extract, gingko biloba leaf extract, coenzyme Q 10, L- glutathione, bilberry extract, tocorienol complex, myricetin, lycopene, lutein, astaxanthin
e	Natural Care, United States	OptiAll	Bilberry extract, carnosine, eyebright (herb), quercetin, rutin, alpha lipoic acid, gingko biloba leaf extract, green tea extract, zeaxanthin, riboflavin, zinc, selenium, copper, taurine, carrot powder, lutein, N-acetylcysteine, vitamins
f	Dr. Böhm, Austria	Multivitamin balance	Ginseng extract, apple pectin, spirulina powder, broccoli powder, gold millet extract, powder propolis, alpha lipoic acid, L-carnitine, lecithine, coenzyme Q10, carotenoids, polyphenols, minerals, vitamins

7.4 HPLC-CEAD

The potentials of the electrodes were set at +300, +400, +450, +500, +550, +600, +650, and +700 mV against palladium reference electrodes. 20 µl of the standard and sample solutions were injected and the separation was carried out isocratically (0.45 ml/min) with mobile phase I, using an ACE 3 C 18 (150 mm × 3.0 mm, particle size 3 µm) column (Advanced Chromatography Technologies, Aberdeen, Scotland) equipped with a pre-column of same stationary phase material.

By plotting the peak area versus the potential of the working electrodes the current/voltage curves for α-LA and bisphenol A were obtained.

7.5 HPLC-UV

UV detection was done at the wavelength of 215 nm and data were obtained using chromatography data station software, HPLC manager version 2. Twenty micro-litres of the standard or the sample solutions were injected into the HPLC system.

Chromatographic separations were achieved isocratically using mobile phase I at a flow rate of 0.45 ml/min. Column and pre-column were the same as used in the HPLC-CEAD system.

7.6 HPLC-ESI-MS

MS detection was performed with electrospray ionization using the optimized parameters as summarized in Table 10. Data were collected in the multiple reaction monitoring mode [MRM] by fragmenting the deprotonated molecular ion [*m/z* 205] without isolating it and by successively isolating the fragment ion *m/z* 171 [α-LA-H_2S-H]$^-$ and by isolating *m/z* 227 [BPA-H]$^-$.

During the run time, the divert valve was set to waste from 0–3.5 min and from 3.5–7.5 min to the source to avoid contamination from the matrix of supplements.

7.7 Validation of methods

Standard calibration curves for α-LA using HPLC-UV, HPLC-CEAD and HPLC-ESI-MS were plotted, on five successive days, using the analyte/IS (peak area ratios) versus the nominal concentrations of the analytes. The relative standard deviation of the slope was calculated.

The limits of detection (LOD) were determined at a signal-to-noise ratio of 3. The intra-day repeatability was checked by injecting α-LA standard solution (0.19 μg/ml) five times on one day. The inter-day repeatability was tested by injecting the same solution three times a day successively for 5 days. Stability of α-LA in the standard solutions was checked both at 4°C and at room temperature.

An extract of supplement 'a' was prepared and diluted (1/2, v/v) with acetonitrile/water (30/70, v/v) and stored at 4°C. 950 μl of this solution and 50 μl of bisphenol A solution (2 μg/ml) were mixed in the autosampler vial and injected three times each day to check the instrument performance.

7.8 Quantitative analysis

7.8.1 External calibration curve

The calibration curve was established by plotting the peak area ratios versus the concentrations of α-LA in the standard solutions. The content of α-LA in the supplements was determined by multiplying the obtained concentration with the appropriate dilution factor, correcting by the weighed portion and considering the recovery.

7.8.2 Standard addition

Supplement 'f' was selected for the standard addition method as it contains less amount of α-LA than other supplements. First, 50 mg of supplement (in triplicate) was treated as in section 7.3 and α-LA content was estimated with the external calibration function. Then, 50 mg aliquots of the supplement 'f' were then spiked with 50, 100, 150, and 200% of the experimentally calculated α-LA content (each in triplicate).

The un-spiked aliquots and each of the spiked samples were then treated as described above (section 7.3) and analyzed by HPLC with all the three detection modes. The analyte concentration in the supplement was determined by plotting the peak area ratios versus the added concentrations of the standard compound, linear regression and division of the y-intersection (d) by the slope of the regression line y=kx+d (MILLER and MILLER; 2002).

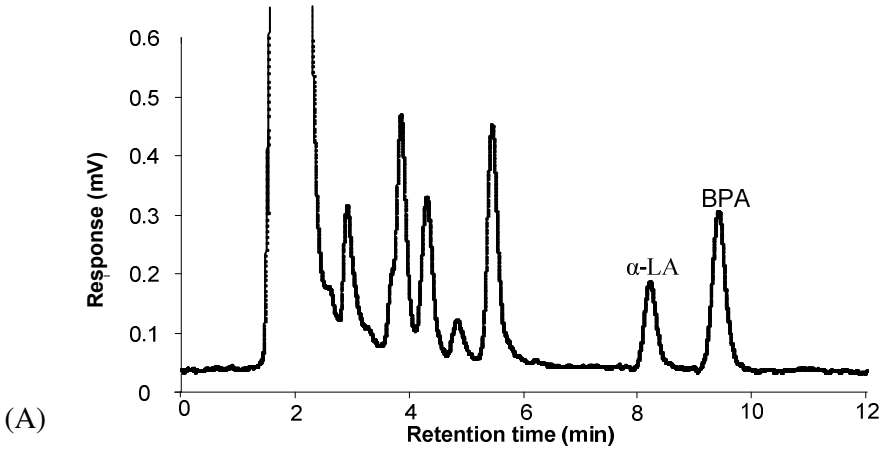
7.9 Results and discussion

7.9.1 Calibration, linearity, limit of detection and repeatability

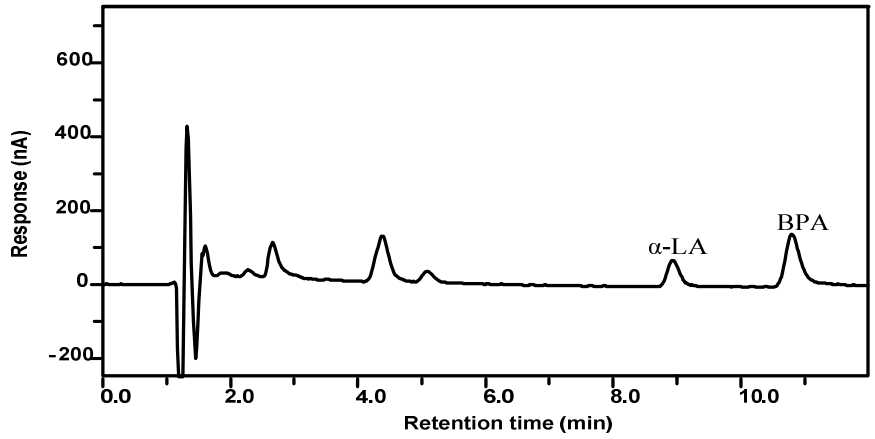
The methods were compared concerning the characteristic analytical parameters for α-LA. The calibration curves were linear in the investigated concentration range. The limit of detection (S/N = 3) for the HPLC-ESI-MS method turned out to be slightly lower than for HPLC-CEAD. On the other hand, the intra- and inter-day repeatability of the electrochemical method was better than the mass spectrometric method (Table 11).

7.9.2 Determination of α-LA in dietary supplements

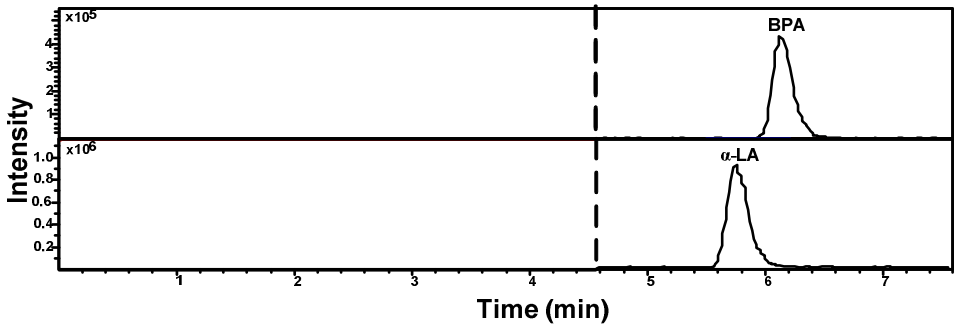
Representative chromatograms for α-LA in supplement ‘a’ are depicted in Figure 30. The relative standard deviation (N=3) for the content of α-LA in supplement ‘a’ was less than 10% for all methods.



(A)



(B)



(C)

Figure 30: Representative chromatograms of alpha lipoic acid in supplement 'a' using high performance liquid chromatography with (A) UV at 215 nm, (B) Coulometric electrode array and (C) ESI-MS detection. α-LA: alpha lipoic acid fragment ion (*m/z* 171); BPA: bisphenol A (*m/z* 227)

Quantitative evaluation by external calibration method revealed that the experimentally calculated values matched the manufacturer's claim only in case of supplement 'a' (**Table 15**). The contents of the supplements b, c, d and e determined by HPLC-CEAD and HPLC-ESI-MS showed a good correlation but were below the values claimed by the manufacturers. Additionally three supplements as representatives for a low and a high concentration (b, d and f) were investigated by HPLC-UV and corresponding results were obtained (**Table 15**).

Table 15: Content of α-LA in dietary supplements

Supplement	Claimed (µg/mg)	Found (µg/mg)		
		HPLC-UV	HPLC-CEAD	HPLC-MS
a	34.9		33.6	33.9
b	0.7	0.4	0.4	0.5
c	1.3		0.9	0.9
d	20.6	12.1	12.1	13.2
e	13.1		9.9	10.5
f	1.0	0.4	0.4	0.4

In order to check whether the matrix compounds influence the measured α-LA content or not, standard addition was performed. The recovery was determined by division of the slope of the regression lines, obtained using standard addition method, by the slope of the external calibration functions recorded in the same analysis run and multiplication by 100 and was found to be 96 ± 2.1% (N = 3). The data of the standard addition method confirm the results obtained by the external calibration method. The deviation between the claimed values and the data found could be due to improper handling during the production of the supplements or degradation of α-LA during storage.

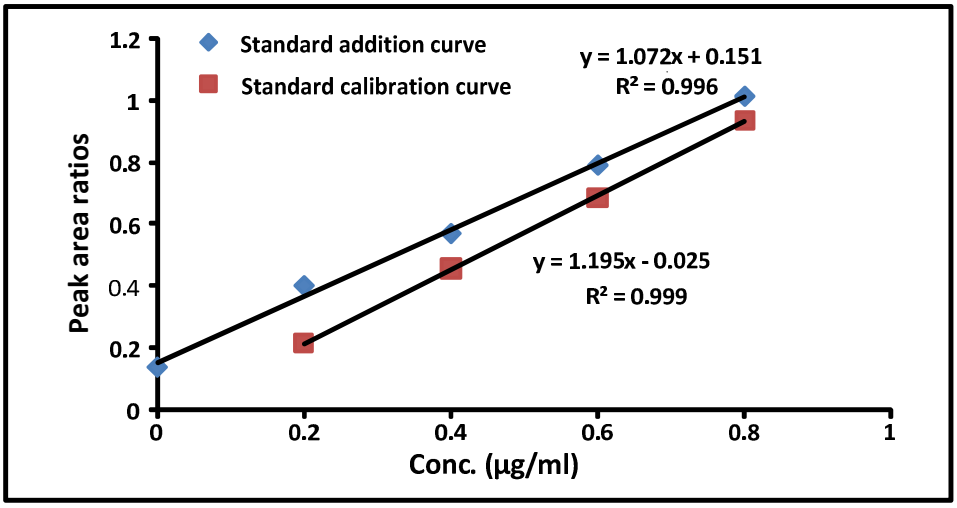


Figure 31: Standard addition and calibration curves for the determination of alpha lipoic acid in supplement 'f' using HPLC with UV detection mode

Rapid and simple sample preparation and a short run time make these methods highly useful for assaying large stocks of samples from different origin. In addition, the high sensitivity and selectivity of HPLC-CEAD and HPLC-ESI-MS methods could be of great importance in pharmacological studies where lower concentrations have to be determined.

8 ALPHA LIPOIC ACID IN FOODSTUFFS

The aim of the present work was to develop a rapid and specific extraction method for free α-LA in different foodstuffs using ultra-sound assisted extraction followed by quantitative determination using HPLC with coulometric electrode array system. In addition, HPLC-ESI-MS was used to confirm the presence of free α-LA in the foodstuffs under investigation.

8.1 Reagents and chemicals

All the reagents and chemicals used were of analytical grade. Deionized water, prepared using a Barnstead Easypur LF (Dubuque, Iowa, USA) water purification system, was used in all experiments. Alpha-lipoic acid (DL-α-lipoic acid; purity ≥ 99.0%) and bisphenol A (99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate (p.A), *ortho*-phosphoric acid (85%; w/w), HPLC-gradient grade acetonitrile and methanol were delivered from Merck (Darmstadt, Germany).

8.2 Preparation of solutions

Primary stock solution of both α–LA and internal standard (BPA) were prepared in methanol (50 µg/ml). Six calibration standards containing α–LA in the concentration range between 0.005 and 0.5 µg/ml were prepared in acetonitrile/water (30/70, v/v). All solutions were stored in tightly sealed amber glass vials at 4°C.

8.3 Samples

Different food samples of animal and plant origin were collected from supermarkets (Table 16). Fresh eggs (3 packages of six eggs each), potatoes (3 x 500 g) and canned peas (3 x 400 g) were obtained in autumn 2007. Two mayonnaise tubes (275 g each) were also obtained from the supermarkets while dried egg powder was prepared in the lab by spray drying.

Table 16: Characterization of samples purchased in Vienna, Austria, in autumn 2007

Common name	Scientific name	Manufacturer
Chicken egg	Gallus domesticus	Ja natuerlich/Organic farming
Dried egg powder	-	In lab*
Mayonnaise I	Commercial product	Clever
Mayonnaise II	Commercial product	Qualitaet aus Oesterreich (eggs from organic farming)
Potatoes	Solanum tuberosum	Fresh
Green peas	Piscum sativum	Bonduelle

*With courtesy of Ao. Univ. Prof. Dr. Emmerich Berghofer., University of Natural Resources and Applied Life Science, Vienna, Austria

In the case of eggs, egg yolk and egg white were separated and α–LA content was determined in the fresh egg yolk and egg white. In another experiment, an egg was boiled for 15 min and the α–LA content was determined separately in the boiled egg yolk and egg white. Potatoes were peeled and 50 g of the edible part from each primary sample unit were crushed and homogenized. Similarly 50 g of fine peas from each can were washed with lukewarm water, dried under the folds of tissue paper, crushed and homogenized. 10 g potions of mayonnaise were taken as such from the tube, homogenized.

8.4 Sample preparation

Three aliquots (50-150 mg) of each fresh and homogenized sample were taken into Eppendorf tubes, dissolved in 1.0 ml of acidified methanol (0.5% acetic acid in methanol, v/v) and sonicated for 1 h at room temperature. The mixtures were then centrifuged at 10000 rpm for 10 min. Two different steps were adopted to comply with the two different HPLC systems.

HPLC-CEAD analysis: 0.5 ml of the supernatant solutions were taken into autosampler vials and 0.05 ml of BPA (0.5 µg/mL) were added.

HPLC-MS analysis: The supernatants were evaporated to dryness on a rotary evaporator, the obtained residues were then dissolved in 1.0 ml of acetonitrile/water (30/70, v/v) and centrifuged at 10000 rpm for 5 min. 0.5 ml of each supernatant was then taken into autosampler vials containing 0.05 ml of BPA solution (0.5 µg/ml).

8.5 HPLC-CEAD

The HPLC system consisted of a Merck Hitachi pump L-6200, a Basic Marathon autosampler (Spark, Holland B.V., Emmen, The Netherlands), an ACE 3 C 18 (150×3.0 mm, particle size 3 µm) column (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. 20 µl of the standard or sample solutions were injected into the HPLC system.

Chromatographic separations were achieved isocratically using a mobile phase containing acetonitrile/methanol/50 mM potassium dihydrogen phosphate buffer (pH 3, adjusted with phosphoric acid), 350/65/585, v/v/v. The mobile phase was filtered through a 0.45 µm Sartolon polyamide filter (Sartorius AG, Goettingen Germany) and degassed by sonication for 20 min prior to use. The flow rate was set at 0.45 mL/min. Current/voltage curves for α-LA and bisphenol A were obtained by plotting the peak height ratios versus the potential of the working electrodes.

8.6 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 (150 × 2.1 mm, particle size 3 µm) column with an ACE 3 C 18 pre-column and an ion trap mass spectrometric (MS) detector HCT+, (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. Hystar 3.1, software for chromatography and hyphenated techniques, was used to acquire the data. Data were then integrated using the software

Data analysis™ version 3.3. Isocratic elution was carried out at a flow rate of 0.2 mL/min with a mobile phase containing 0.1% acetic acid in acetonitrile/water (45/55, v/v). The capillary voltage was set at 4.5 kV and the electrospray ionization of the column effluents was performed with nitrogen as nebulizing gas at 10 l/min, 40 psi nebulizing pressure, and 300 °C drying gas temperature.

Data were collected in MRM mode by $[M-H]^-$ to $[M-H-H_2S]^-$ without isolation and subsequent isolation of $[M-H-H_2S]^-$ formed in source and ion trap in time window 1 (LA), and isolation of $[M-H]^-$ in time window 2 (BPA); processed according to the method developed in our previous research work (DURRANI, SCHWARTZ, SCHMID and SONTAG; 2007).

8.7 Validation of methods

Standard calibration curves for α-LA were generated on five successive days by plotting the analyte/internal standard peak height ratios versus the nominal concentrations of α-LA and the relative standard deviation of the slope was calculated. The limit of detection (S/N = 3) was determined by sequentially diluting the lowest concentrated standard α-LA solution. The intra-day repeatability was checked by injecting α-LA standard solution (0.19 µg/ml) five times on one day. The inter-day repeatability was tested by injecting the same solution three times a day successively for five days.

The stability of α-LA and BPA in the standards and sample solutions was determined by leaving the solutions a) overnight in the autosampler vials at room temperature after the first injection cycle and then injecting them again the next day and b) by storing the solutions in the freezer at -18˚C for three days. Standard solutions and the spiked sample solutions were then thawed at room temperature 1 h prior to analysis.

8.8 Qualitative analysis

α-Lipoic acid was identified in the samples applying HPLC-ESI-MS and HPLC-CEAD by comparing both retention times, mass spectra and current voltage curves (CVCs) with those in the standard solutions measured during the same analysis run (Figure 32). CVCs were obtained by plotting the peak height of α-LA in each

individual channel against the applied potential. HPLC-ESI-MS was also used to confirm the presence of α-LA in the investigated food samples.

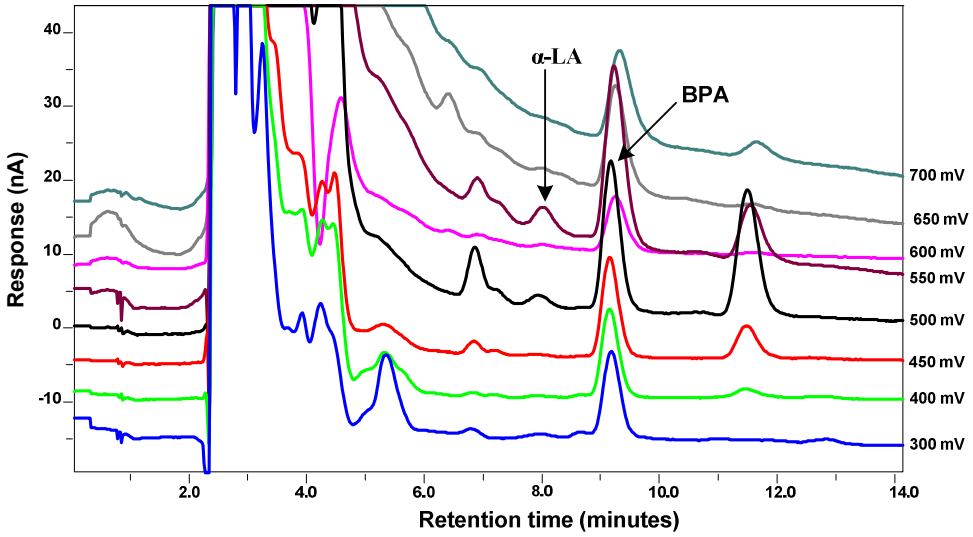


Figure 32: Coulometric electrode array chromatogram of unspiked fresh potatoes after sonication

8.9 Quantitative analysis

Unspiked samples of selected foodstuffs were analyzed as described above to get an estimation of the α-LA content on the basis of external calibration functions. Standard addition method was performed on three different days using three different primary sample units. On each day, 50-100 mg aliquots of homogenized samples at each spiking level (N=3) were spiked with 50, 100, 150, 200% of the experimentally determined α–LA content in the unspiked sample, processed as in section 8.3 and analyzed by HPLC-CEAD. The analyte concentration in the sample extracts was determined by plotting the peak height ratios versus the added concentrations of α–LA, linear regression and division of the y-intersection (d) by the slope of the regression line y=kx+d.

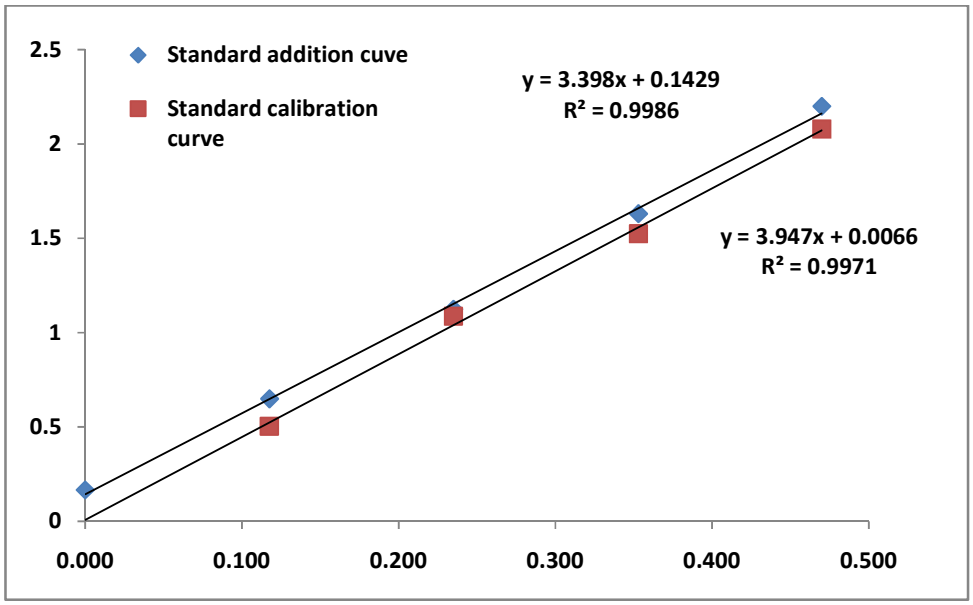


Figure 33: Standard addition curve for the determination of alpha lipoic acid in fresh egg yolk using HPLC with coulometric electrode array detection

Recoveries were measured by dividing the slope of the standard addition regression line by the slope of the external calibration function recorded during the same analysis run and multiplying by 100 (Figure 33).

8.10 Results and discussion

8.10.1 Calibration, linearity, limit of detection and precision

Calibration graphs were plotted against peak height ratios (α-LA to BPA) vs. the nominal concentrations in the range of 0.01 to 0.5 µg/ml. A linear correlation was observed in the investigated concentration range with the mean regression equation (N = 5) y = 3.947+0.0066 (r^2 >0.971) and a RSD of 4.28%. The limit of detection (S/N = 3) was 0.003 µg/ml. The intra- and inter-day reproducibility was also good with a relative standard deviation of 0.48 and 4.19%, respectively.

8.10.2 Storage stability

Stability tests showed that both α-LA and internal standard BPA are stable within the assay variability of ±15% RSD upon storage at room temperature for one day and upon freezing for two days.

8.10.3 Alpha lipoic acid in food

The developed method was applied to determine the α-LA content in different food materials, from both plant and animal sources. Representative chromatograms of spiked and unspiked dried egg powder, obtained by HPLC-CEAD, are illustrated in Figure 34. In a second experiment, an extract of unspiked dried egg powder was injected into the HPLC-ESI-MS system to verify the presence of α-LA (Figure 35).

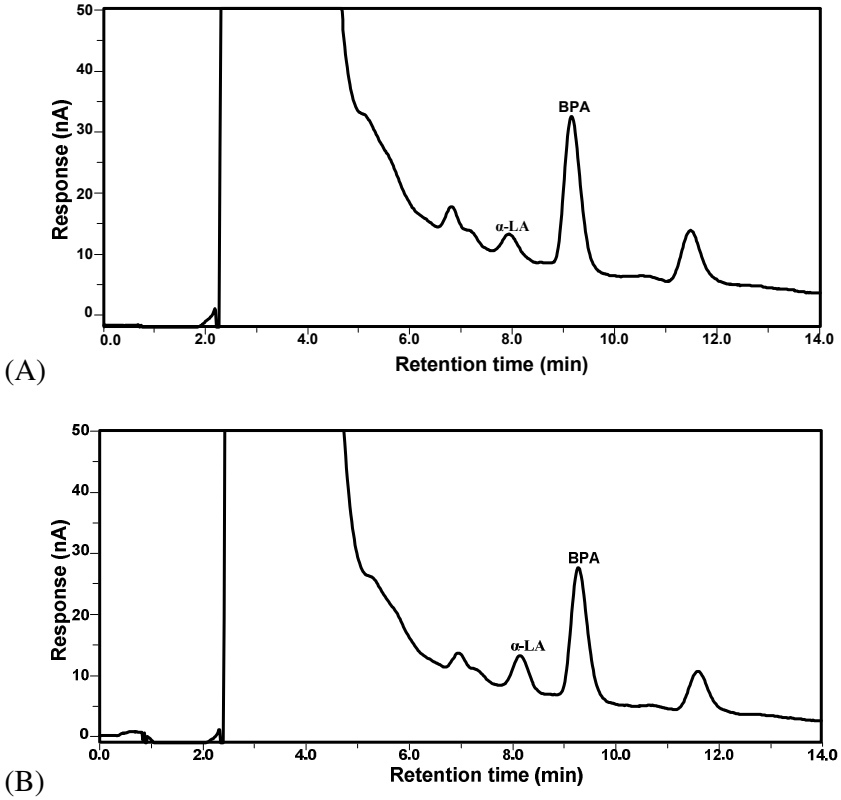


Figure 34: HPLC-CEAD chromatograms of alpha lipoic acid in dried egg powder: (A) unspiked, (B) spiked. α–LA: Alpha lipoic acid, BPA= Bisphenol A

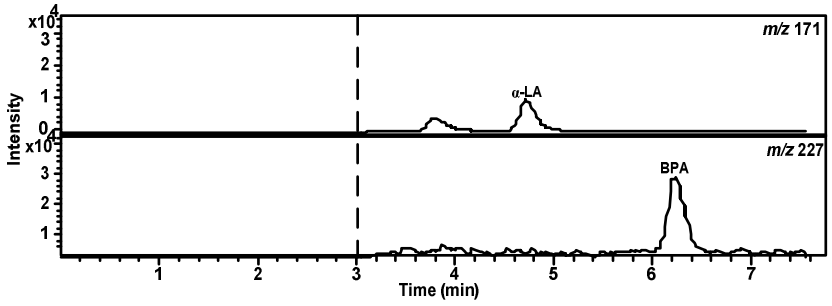


Figure 35: HPLC-MS chromatogram of unspiked dried egg showing the extracted ions of α-LA (*m/z* 171) and BPA (*m/z* 227)

Quantitative analysis was performed by standard addition method and α-LA contents and mean recoveries of three independent determinations (three standard additions starting from three different primary sample units) are summarized in Table 17. All the values obtained were within the accepted assay deviation (RSD <15%).

Table 17: α-LA content in different foodstuffs determined by three standard additions starting from three different primary sample units

Food	Content (µg/g fresh food)	Recovery (%)
Fresh egg yolk	0.5-0.9	89 ±4.5%
Boiled egg yolk	< 0.2	-
Fresh egg white	< 0.3	-
Boiled egg white	< 0.1	-
Dried egg powder	1.3	92 ±3.8%
Mayonnaise I	0.5	70 ±7.2%
Mayonnaise II	0.6	67 ±6.8%
Fresh potatoes	1.5-4.2	94 ± 4.7%
Canned peas	0.5-1	93 ± 5.3%

8.10.4 Conclusion

The α-LA content was found to be below the limit of detection in both raw and boiled egg white. Though α-LA was found in raw egg yolk, it was below the limit of detection in boiled egg yolk. One possible explanation can be the instability of α-LA at higher temperature. However, the presence of α-LA in the dried egg powder implies that α-LA is at least partially stable during the drying process. The drying process (removal of water) result in higher α-LA content then in one whole egg. The α-LA content in mayonnaise (from two different manufacturers) complies with the experimentally determined values in raw egg yolks. This shows that α-LA in egg yolk (used in preparing mayonnaise) is stable.

Previous experiments intended to obtain the α-LA content (free and/or protein-bound) in different biological and food materials were performed under drastic hydrolysis conditions and/or tedious derivatization steps prior to analysis. However, this new and rapid method is based upon mild extraction conditions with sufficient efficiency to determine the α-LA content in diverse food matrices.

Ultrasound-assisted extraction of α-LA from various samples of vegetable and animal origin has been proved as a simple, fast and reliable sample preparation method. High performance liquid chromatography coupled with coulometric electrode array detection offer more sensitivity and specificity than the chromatographic methods reported previously. Non-destructive extraction conditions and short chromatographic run-time offers additional advantages. This new method can play an important role in establishing a database for the α-LA content in different foodstuffs.

In summarizing, this method is recommendable for analyzing the free α-LA content in egg-based samples and vegetables. HPLC-ESI-MS is very useful to confirm the presence of free α-LA in complex food matrices.

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