Talanta 130 (2014) 347–355

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Chemical derivatization combined with capillary LC or MALDI-TOF MS for trace determination of lipoic acid in cosmetics and integrated protein expression profiling in human keratinocytes

Chia-Ju Tsai [a], Ying-Chi Lin [b], Yen-Ling Chen [a], Chia-Hsien Feng [a,*]

[a] Department of Fragrance and Cosmetic Science, College of Pharmacy, Kaohsiung Medical University, 100, Shih-Chuan 1st Road, Kaohsiung 80708, Taiwan
[b] School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

ARTICLE INFO

Article history:
Received 14 April 2014
Received in revised form
3 July 2014
Accepted 7 July 2014
Available online 14 July 2014

Keywords:
Lipoic acid
Cosmetics
Dietary supplements
Cellular proteome
Human keratinocytes

ABSTRACT

Lipoic acid (LA) is an essential cofactor in mitochondrial enzymes and an ideal antioxidant in prokaryotic and eukaryotic cells. Capillary liquid chromatography coupled with ultraviolet detection (CapLC–UV) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) are two environmentally friendly methods for determining LA. In this study, a pre-column microwave-assisted derivatization with 4-bromomethyl-6,7-dimethoxycoumarin enhanced the UV absorbance of LA and was monitored at 345 nm by CapLC–UV. Gradient separation was performed using a reversed-phase C18 column with a mobile phase consisting of acetonitrile–0.1% formic acid solution. The ionization of LA was increased, and the LA derivative was detected by MALDI-TOF MS at *m/z* 683 with an α-cyano-4-hydroxycinnamic acid matrix. The linear response ranged from 0.1 to 40 μM with a correlation coefficient of 0.999. The CapLC–UV and MALDI-TOF MS had detection limits of 5 and 4 fmol, respectively. These methods effectively detected LA in dietary supplements and cosmetics. Cellular proteomes of a human keratinocyte cell line (HaCaT) irradiated with UV radiation were also compared with and without LA treatment. The cellular proteomes were identified by nanoultra performance LC with LTQ Orbitrap system after trypsin digestion. Protein identification was performed by simultaneous peptide sequencing and MASCOT search. The analysis revealed changes in several proteins, including CDC42, TPI1, HNRPA2B1, PRDX1, PTGES3 and MYL6.

1. Introduction

The lipophilicity and hydrophilicity of lipoic acid (LA) makes it an ideal or universal antioxidant due to its functions in the membrane and in aqueous phase [1]. LA stabilizes the antioxidant capacity of the body by directly scavenging reactive oxygen species (ROS), which indirectly helps to regenerate endogenous antioxidants such as ascorbic acid, tocopherol, ubiquinone and glutathione [2,3]. Other reported functions of LA include chelating transition metals such as Cu^{2+} and Pb^{2+}, stimulating normal gene expression, and controlling regulatory proteins [4,5]. Because of its powerful antioxidant activity, LA has been used in recent decades for prevention and therapeutic treatment of various diseases and disorders associated with oxidative stress, including diabetes, inflammation, cardiovascular diseases, neurodegenerative disorders, and cancer [6–9]. LA also acts as a redox regulator of proteins such as prostaglandin E2, phospholipase A2, NF-kappaB transcription factor, prolyl hydroxylase, microphthalmia-associated transcription factor and cAMP-activated protein kinase. Therefore, its *in vivo* and *in vitro* effects include anti-inflammatory effects, anti-aging effects on the skin, and anti-obesity effects [10–14]. In addition to the use of LA in drug treatments, improved understanding of the diverse pharmacological characteristics of LA has increased its use in dietary supplements and cosmetics.

The analytical methods conventionally used to analyze LA in pharmaceutical preparations, biological materials, and food samples include gas chromatography (GC) [15–17], high performance liquid chromatography (HPLC) [18–31] and capillary electrophoresis (CE) [32–34]. In GC, the low volatility of LA requires a derivatization step to obtain a symmetrical peak within a reasonable retention time. The HPLC methods must be combined with ultraviolet (UV) detection, fluorescence detection or electrochemical detection (ECD). Whereas UV detection and fluorescence detection require a derivatization step, ECD enables direct analysis of LA. However, ECD requires regeneration of the electrode to maintain sensitivity and is not user friendly. In contrast, CE is

* Corresponding author. Tel.: +886 7 3121101 2805; fax: +886 7 321 0683.
E-mail address: chfeng@kmu.edu.tw (C.-H. Feng).

http://dx.doi.org/10.1016/j.talanta.2014.07.014
0039-9140/

environmental friendly, but LA adsorption on the inner walls of capillaries causes poor sensitivity.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a rapidly performed, sensitive, easily operated, high throughput analytical tool [35]. However, a positive MALDI-TOF MS does not provide the sensitivity required for trace analysis of LA because LA is difficult to ionize when detected in positive mode. Therefore, chemical derivatization is needed to enhance detectability and selectivity.

In capillary liquid chromatography coupled with UV detection (CapLC–UV), derivatization provides the high detection sensitivity and hydrophobicity needed for extraction by attached chromophores. In MALDI-TOF MS, an appropriate structure facilitates the analyte in absorbing energy transferred from the laser through the matrix material. Moreover, the derivatization can increase the analyte mass, which reduces interference and improves specificity in MALDI-TOF MS. Generally, the derivatization reaction is performed by conventional heating, which requires a reaction time ranging from 30 min to several hours. Microwave energy, which causes molecular motion by dipole rotation and ionic conduction, is more rapid and efficient compared to conventional methods of heating by conduction and convection [36–38]. These limitations motivated this study to develop a fast microwave-assisted derivatization (MAD) method for microanalysis of LA by CapLC–UV and MALDI-TOF MS.

The ROS produced by UV irradiation causes an imbalance between ROS and the endogenous antioxidant protection system [39,40]. Thus, ROS can cause inflammation, oxidative stress and photo-aging [41]. Many studies of LA have confirmed its antioxidant and anti-aging properties [3]. Therefore, this study used shotgun proteomic techniques to screen differentially expressed proteins in human keratinocytes with or without UV exposure.

The aim of this study was to develop the fast, reliable and eco-friendly methods of using CapLC–UV and MALDI-TOF MS to quantify LA in dietary supplements and cosmetics. Although LA is a major constituent in many dietary supplements, LA is trace component in most cosmetic products due to no regulations for label. This study developed a rapid MAD procedure with coumarin tag for modifying the UV absorbance and ionization of LA. The MAD conditions and the extraction conditions for the LA derivative were optimized, and the method was validated. This study also obtained the protein expression profile for UV-exposed human skin keratinocytes with and without LA treatment.

2. Materials and methods

2.1. Reagents and materials

Lipoic acid, 4-bromomethyl-6,7-dimethoxycoumarin (Br-DMC) as derivatizing reagent, sodium borohydride ($NaBH_4$), dithiothreitol (DTT), *tris*(2-carboxyethyl)phosphine (TCEP), heptafluorobutyric acid (HFBA), sodium hydroxide (NaOH), potassium hydroxide (KOH), ammonium bicarbonate (NH_4HCO_3), α-cyano-4-hydroxycinnamic acid (CHCA), and D-tubocurarine chloride hydrate (DTC) used as internal standard (IS) in MALDI-TOF MS were purchased from Sigma-Aldrich (St. Louis, MO, USA). The methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), acetone, *n*-hexane, toluene, ethyl acetate (EtOAc), dichloromethane (DCM), chloroform (TCM), dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), hydrochloric acid (HCl), formic acid (FA), acetic acid (AA) and trifluoroacetic acid (TFA) were supplied by Merck (Darmstadt, Germany). The 1-(Methylamino)anthraquinone (MAAQ) used as IS in CapLC–UV was obtained from TCI (Tokyo, Japan). HyClone Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and fetal bovine serum (FBS) were purchased from Thermo Scientific (MA, USA). Sequence-grade modified trypsin was obtained from Promega (Madison, WI, USA).

The NP-40 cell lysis buffer was supplied by Life Technologies (Carlsbad, CA, USA). The deionized water used as the aqueous solutions in all experiments was produced with a Milli-Q Lab system (Bedford, MA, USA). Tablets of LA (300 mg per tablet) were purchased from a local pharmacy in Tampa (USA). Samples of six commercially available cosmetic products (samples A–F) were purchased from local retail stores in Kaohsiung (Taiwan).

2.2. Preparation of standard solutions

Stock solutions of LA (200 μM) and Br-DMC (12 mM) were dissolved in deionized water and ACN, respectively. Internal standard stock solutions of MAAQ (700 μM) and DTC (100 μM) were prepared in MeOH. The aqueous stock solutions of NaOH, KOH, NH_4HCO_3, FA, AA, TFA and HFBA (1 M) were prepared in deionized water. The stock solution of HCl (5 M) was also prepared in deionized water. The stock solutions of $NaBH_4$, DTT and TCEP (1 M) were prepared by adding appropriate amounts of these compounds to 50 mM NaOH aqueous solution. The standard solutions of LA, MAAQ and DTC were stored at 4 °C when not in use, and other solutions were stored at room temperature. The Br-DMC standard solution was stored in a dark room until use. Sequence-grade modified trypsin (100 μg mL^{-1}) was dissolved in the buffer solution in the kit provided by the manufacturer and stored at −20 °C until use.

2.3. Microwave-assisted derivatization and extraction procedures

The MAD was performed with a standard consumer-grade microwave oven. After placing the sample solution (300 μL) or cosmetic product (300 mg) in a 1.5 mL eppendorf tube, 3 μL of 1 M $NaBH_4$ solution and 9 μL of 10 mM Br-DMC were successively added. After thorough mixing, the tube was placed in the microwave oven. Derivatization was performed under 200 W irradiation power for 5 min. After reaction, 200 μL ethyl acetate was added to the tube, vortexed for 2 min, then centrifuged at 14,000 rpm for 2 min. The organic layer was removed. Next, 6 μL of 1 M HCl solution and 20 μL of ethyl acetate were added to the tube, vortexed for 2 min, then centrifuged at 14,000 rpm for 2 min. A 0.5-μL quantity of the EtOAc layer was then drawn from the supernatant into another eppendorf tube, and 0.5 μL of IS (DTC, 2 μM) solution was added. The solution mixture was then spotted on the target plate for MALDI-TOF MS analysis. Additionally, 15 μL of the EtOAc layer was drawn from the supernatant into another eppendorf tube and then evaporated to dryness in a vacuum evaporator centrifuge. The residue was redissolved with 15 μL of the IS (MAAQ, 700 μM) solution. Finally, 0.5 μL solution was injected into the CapLC system for analysis.

2.4. Analysis of LA dietary supplement

For content analysis of the LA tablets, ten tablets were weighed, vigorously ground, and mixed well. A 0.5-mg portion was precisely weighed and transferred to a 20-mL volumetric flask. Deionized water was added and diluted to the mark of volumetric flask. The extraction solution was ultrasonicated for 10 min then centrifuged at 14,000 rpm for 2 min. The supernatant (300 μL) was then subjected to derivatization.

In each tablet, the variation in LA was measured by content uniformity test. Ten tablets were individually weighed and finely ground. A 0.5-mg portion of each resulting powder was precisely weighed and then transferred to a 20-mL volumetric flask, which was then completely filled to the volume with deionized water. The supplement solution was ultrasonicated for 10 min and

centrifuged at 14,000 rpm for 2 min. The clean 300 μL supernatant was subjected to derivatization.

For the relative recovery study of the LA tablets, one tablet was weighed and finely ground. A 0.5-mg portion of the resulting powder was precisely weighed, transferred to the 20-ml volumetric flask, diluted with deionized water to the volume of the flask, ultrasonicated for 10 min, and centrifuged at 14,000 rpm for 2 min. The supernatant (150 μL) was spiked with LA aqueous solution (0, 2, 10 and 25 μM). Finally, the mixed solution was subjected to derivatization.

2.5. Cell culture and treatment

Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific, MA, USA) containing 100 IU/mL penicillin, 100 μg/mL streptomycin, 10% fetal bovine serum and 2.5 μg/mL fungizone (Gibco, NY, USA) was used to culture the human keratinocyte cell line HaCaT. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. The HaCaT cells were cultured in 10 cm dishes and maintained until they reached 70–80% confluence. The medium was then removed, and the cells were washed with phosphate-buffered saline (PBS) before further experiments. The PBS was then removed, and the cells were treated with 250 μM LA in DMSO (experimental group) or with DMSO alone (control group) for 24 h at 37 °C in antibiotics-free culture medium. In each group, three dishes received different treatments: irradiation with UV-B (302 nm) for 2 h, irradiation with UV-A (365 nm) for 2 h, and no irradiation with UV. The three dishes were then maintained under identical incubation conditions.

2.6. Preparation of protein samples from cell cultures

For all treatments, the medium was removed after 2 h UV irradiation. The cells were then washed with PBS twice, harvested and transferred to an eppendorf tube. Transfer of the cell pellets was followed by addition of 50 μL aqueous lysis buffer containing 1% NP-40, 10 mM β-glycerol phosphate, 50 mM Tris–HCl (pH 8.0), 1 mM Na_3VO_4, 50 mM NaF, and 150 mM NaCl. After 30 min lysis in an ice bath, the tube was centrifuged at 12,000 rpm for 10 min. The supernatant (10 μL) was collected, and 90 μL acetone was added to precipitate protein. The supernatant was removed, and the residue was evaporated to dryness in a vacuum evaporator centrifuge. The residues were diluted with 200 mM NH_4HCO_3 to obtain final protein concentrations of 1 mg mL^{-1}. Sequence-grade trypsin was then added, and the mixture was heated in a 37 °C dry bath for 16 h. After digestion, peptide solution with 10 μL of 0.1% formic acid aqueous solution was injected into the nanoultra performance LC (UPLC) with LTQ Orbitrap system for protein identification.

2.7. Instrumentation and analysis conditions

2.7.1. CapLC system

The Agilent 1200 series LC system (Santa Clara, CA, USA) used for analysis consisted of a capillary pump, a standard autosampler, a micro vacuum degasser and a multiple wavelength detector. Separation was performed on a Jupiter 4 μm Proteo 90 Å column (150 × 0.3 mm, 4 μm; Phenomenex, Torrance, CA, USA), and the injection volume for LA analysis was 0.5 μL. The detection wavelengths for the LA derivative and IS were set to 345 nm and 500 nm, respectively. The mobile phase of solvents A and B were 0.1% formic acid aqueous solution and ACN, respectively. The samples were eluted in gradient mode at a flow rate of 13 μL min^{-1}. The gradient program used for separation was as follows: start at 25% B; ramp to 100% B at 1.5 min and maintain until 10 min. The mobile phase was returned to initial condition before the next injection.

2.7.2. MALDI-TOF MS system

The MALDI-TOF system used for mass spectral analysis was an Autoflex III Smartbeam (Bruker Daltonics; Billerica, MA, USA) equipped with a Nd:YAG laser radiating at 355 nm. Mass spectra were recorded by summing 1000 laser shots. The MALDI-TOF MS system obtained the signals in positive ion reflector mode. Ions of interest were monitored by performing the ion trap in full scan mode with a mass range of m/z 50–1000. For LA analysis by MALDI-TOF MS, 0.5 μL of a matrix (CHCA) solution was spotted on a ground target plate and allowed to dry. Then, 1.0 μL of the extraction phase and IS mixture solution was added and dried in an air stream at room temperature. The CHCA (10 mg mL^{-1}) solution was dissolved in ACN–0.1% TFA aqueous solution (50:50, v/v). Data processing was performed with FlexAnalysis software (Bruker Daltonics).

2.7.3. NanoUPLC–MS/MS system

The proteins were identified by NanoUPLC–MS/MS system. The nanoUPLC system (nanoACQUITY UPLC, Waters, Milford, MA, USA), which included a sample cooler, an autosampler, and a column, was connected to the nanospray ion source. Peptide enrichment and separation were performed on Symmetry C18 (180 μm × 20 mm, 5 μm; Waters, Milford, MA, USA) and BEH C18 (75 μm × 100 mm, 1.7 μm; Waters, Milford, MA, USA), respectively. The mobile phase of solvents A and B were water containing 0.1% FA and ACN containing 0.1% FA, respectively. The samples were trapped and desalted by 0.1% FA for 3 min at a flow rate of 5 μL min^{-1} and then separated in gradient mode at a flow rate of 400 nL min^{-1}. The gradient elution conditions were as follows: start at 1% B and hold at 1 min; ramp to 45% B at 20 min; ramp to 85% B at 30 min; maintain until 35 min. The mobile phase was returned to initial condition before the next injection.

The LC-eluting peptides were analyzed with an LTQ Orbitrap Discovery hybrid Fourier Transform Mass Spectrometer (FTMS) (Thermo Fisher Scientific, Inc. Bremen, Germany) with a nanospray source at a resolution of 30,000. Voltages at the source, capillary and tube lens were set to 2.1 kV, 28 V and 80 V, respectively. Spray capillary temperature was maintained at 200 °C. The MS system was operated in positive ionization mode. In profile mode, the precursor ion trap was performed in full scan mode. The mass scan range was 400–2000 m/z. The MS/MS spectra were acquired in data-dependent mode. Precursor ion fragments were obtained by using helium as collision gas with collision energy at 35 eV in linear ion trap. Depending on the data-dependent scan results, multiple-charged ions were chosen for collision-induced dissociation (CID). A single-charge ion filter was used to minimize undesired data in dynamic exclusion mode.

All resulting peptide raw spectra were processed with Mascot Distiller software (Matrix Science Inc., Boston, MA). For protein identification, the peak lists were converted to mgf format and analyzed with an in-house MASCOT™ server (Version 2.2, Matrix Science Inc., Boston, MA).

2.8. Validation of the methods

To obtain the validation parameters, evaluation of the peak area ratio of analyte to IS was used to quantify the LA level. For quantitative analysis of LA, five calibration solutions with concentrations ranging from 0.1 to 40 μM were prepared in triplicate by dilution of the stock solution and derivatization as described above. The calibration curve was constructed by linear least-square regression analysis. The correlation coefficient (r^2), slope

and intercept data were evaluated. Intra-day and inter-day assays performed using the proposed methods, and the standard solutions used in all analytical steps were prepared at low (1 μM), medium (10 μM) and high (30 μM) concentrations. Intra-day and inter-day analyses of precision and accuracy were performed by six replicate analyses performed on the same day and on six consecutive days, respectively. The limit of detection (LOD) was defined as consecutive dilution of LA until the concentration showed a signal-to-noise ratio ≥ 3. Stability was examined by analyzing the standard solution in triplicate at 40 μM. The solutions were tested at 0.5, 2, 4, 6, 12 and 24 h after optimum derivatization. All solutions were stored at room temperature throughout the analysis.

3. Results and discussion

The LA was almost transparent in the UV–vis wavelengths, and derivatization with Br-DMC enhanced detectability in UV. Depending on the LA structure, the dithiolane ring and carboxyl group made poor ionization in positive-ion mode MS. The Br-DMC was used to solve the problems of LA absorptivity and ionization. The Br-DMC induced nucleophilic substitution in the dithiol groups of LA in alkali solution. Microwave-assisted derivatization was performed, and all parameters for optimizing derivatization were discussed. The various advantages of the Br-DMC reagent are (1) commercial availability, (2) a maximum absorption wavelength (λ_{max}=345 nm) approximating that of the the Nd:YAG laser radiating (355 nm) in MALDI MS, and (3) more stable derivatives compared to other coumarin derivatizing agents. Fig. 1 shows the derivatization reaction of LA with Br-DMC.

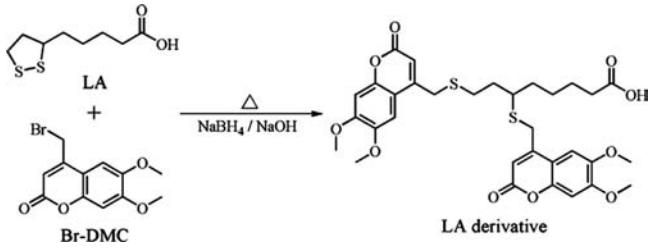

Fig. 1. The derivatization reaction scheme for Br-DMC with thiol groups of LA.

3.1. Detection conditions of CapLC–UV and MALDI-TOF MS

The optimum detection wavelength was identified by recording the UV spectrum of the LA derivative and displaying the three highest absorption wavelengths: 205, 230 and 345 nm. At 205 and 230 nm, UV detection sensitivity was high. However, interference from the sample matrix and a large baseline drift during gradient elution resulted in poor specificity. Therefore, an absorption wavelength of 345 nm was considered the optimum in the following experiments (Fig. S1A). The chemical formula for the LA derivative is $C_{32}H_{36}O_{10}S_2$, and its molecular weight is 644. The MALDI-TOF mass spectra (Fig. 2A) showed that the three highest mass-to-charge ratios were, in ascending order, $[M+H]^+$ at *m/z* 645, $[M+Na]^+$ at *m/z* 667 and $[M+K]^+$ at *m/z* 683. The highest intensity peak corresponded with the adduct of LA derivative with potassium. Therefore, $[M+K]^+$ was selected for LA quantitation in the following experiments. To ensure a highly accurate analysis, MAAQ and DTC were chosen as the IS for CapLC–UV (detected at 500 nm) and for MALDI-TOF MS (detected at *m/z* 609), respectively.

3.2. Optimal derivatization conditions

Because of the antioxidant character of LA, oxidative state of LA presented disulfide bond. In the case of a nucleophilic reaction, the dithiolane ring is converted to dithiol groups. Three reducing

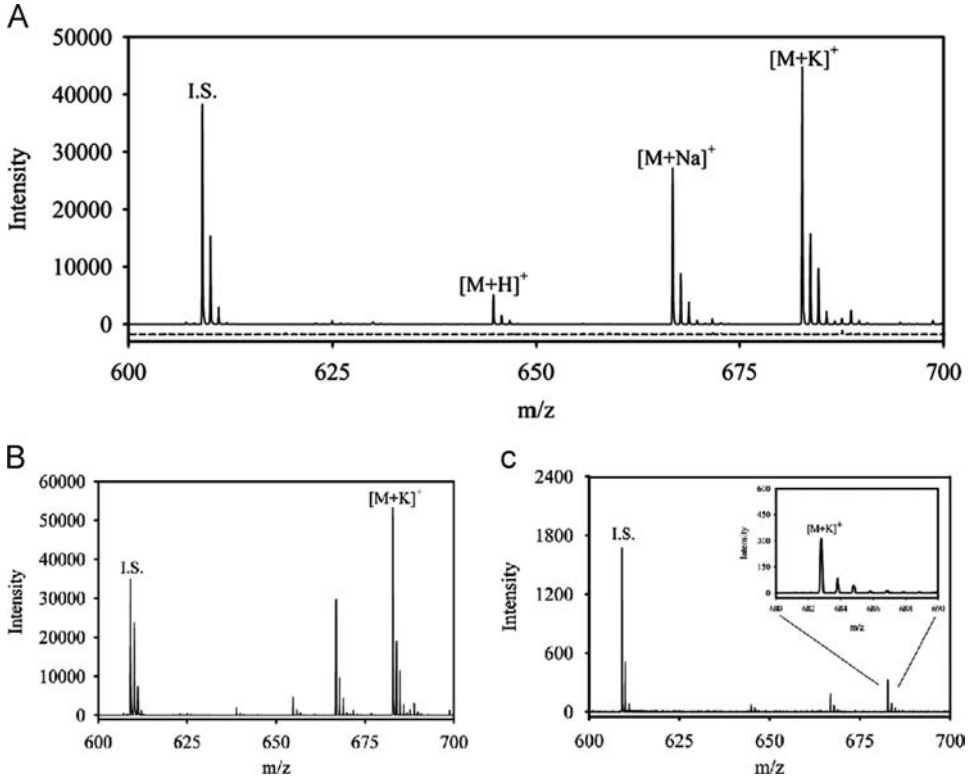

Fig. 2. The MALDI-TOF MS spectra obtained for LA derivative in positive mode. (A) Spectra for blank (dashed line) and for 20 μM LA standard solution after derivatization (solid line); (B) spectrum for LA derivative in dietary supplement tablet; (C) spectrum for LA derivative in cosmetic sample E. Peak: $[M+H]^+$=LA derivative (*m/z* 645); Peak: $[M+Na]^+$=LA derivative (*m/z* 667); Peak: $[M+K]^+$=LA derivative (*m/z* 683); I.S.=DTC (*m/z* 609).

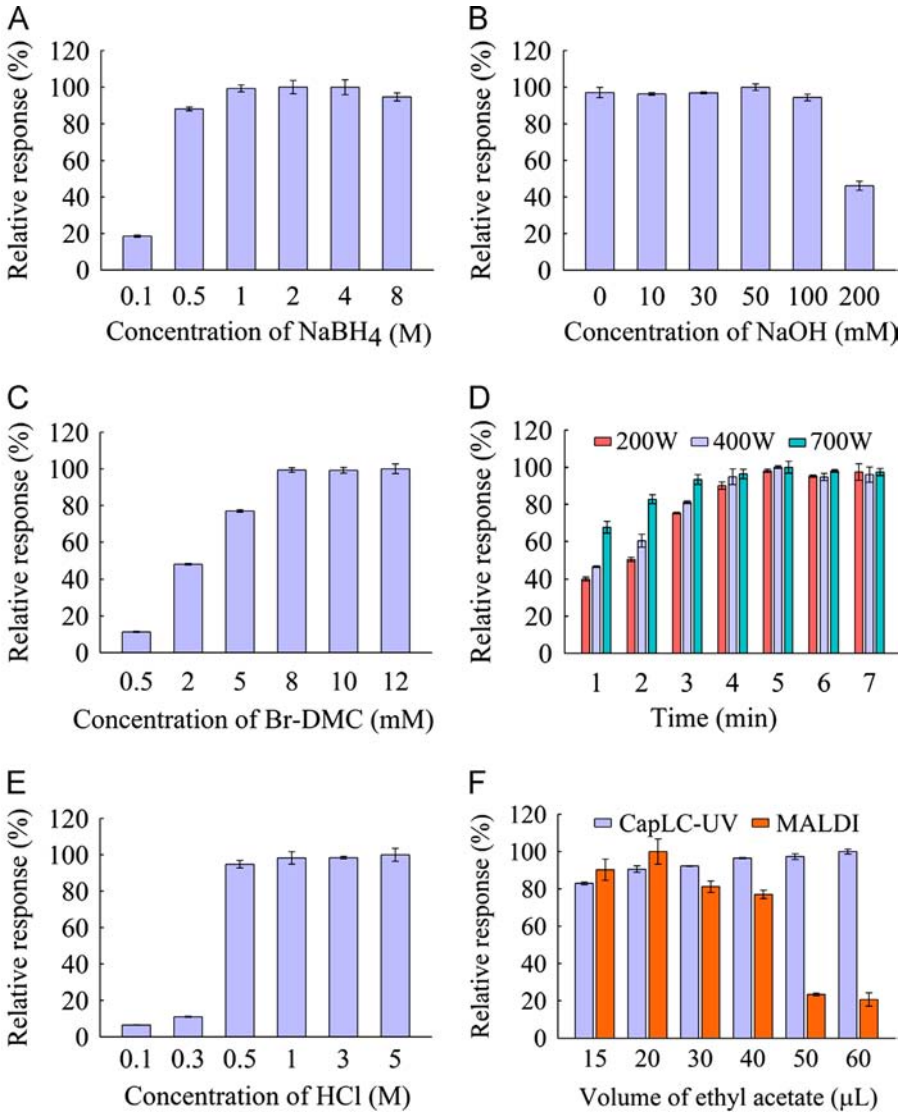

Fig. 3. Optimized conditions for derivatization and extraction of LA. (A) $NaBH_4$ concentration, (B) NaOH concentration, (C) Br-DMC concentration, and (D) the microwave energy and reaction time for formation of the LA derivative, (E) HCl concentration and (F) ethyl acetate volume used for extraction of the LA derivative. The CapLC–UV and MALDI-TOF MS obtained similar results for A–E, but not for F.

agents were tested, including DTT, TCEP, and $NaBH_4$. The DTT consumed the following Br-DMC, which resulted in poor derivatization results. The TCEP required a strictly controlled reaction condition because it autoxidized rapidly at a high pH value [42] and because it reacted with the derivatization reagent when the concentration of TCEP exceeded that of the derivatization reagent [43]. Finally, the $NaBH_4$ produced a higher relative response compared to DTT and TCEP. The quantity of $NaBH_4$ was optimized for concentrations ranging from 0.1 to 8 M with 3 μL. Fig. 3A shows that the relative response plateaued when $NaBH_4$ exceeded 1 M. Therefore, 1 M $NaBH_4$ was prepared in NaOH solution.

Alkaline medium was used to assist the Br-DMC (bromomethyl group) reaction with thiol groups of LA. The $NaBH_4$ aqueous solution (1 M) had a pH value of 10.2 [44], which was sufficient basicity for the following derivatization. Therefore, derivatization efficacy did not substantially differ among the different bases (*i.e.*, NaOH, KOH and NH_4HCO_3). However, an increase in pH resulted in a more stable $NaBH_4$ solution due to the decreased hydrolysis of $NaBH_4$ to $NaB(OH)_4$ with the release of hydrogen gas. Addition of NaOH stabilized the $NaBH_4$ and increased the storage period. As shown in Fig. 3B, the reaction yield was similar in the range of 0–50 mM NaOH whereas NaOH exceeding 50 mM decreased the derivative production. When NaOH exceeded 50 mM, the chromatograms revealed other peaks, and the LA derivative intensity was reduced. Therefore, 50 mM NaOH was chosen as the preparation medium for $NaBH_4$.

The Br-DMC is a well-known reagent for UV or fluorescence labeling. Three aprotic solvents, DMSO, THF and ACN, were used for S_N2 reaction. The DMSO produced interfering peaks and a complicated chromatogram whereas the THF reaction medium obtained a lower reaction yield. Therefore, ACN was the selected solvent for this application. The effect of Br-DMC concentration (range, 0.5–12 mM) on LA derivative formation was examined. Fig. 3C shows that the derivative yield plateaued at concentrations exceeding 8 mM. However, to prevent consumption of the reagent by water or sample matrix, 10 mM Br-DMC was considered optimal.

In past studies, derivatization reactions with Br-DMC were performed by conventional heating, which requires a long reaction time (30–60 min). Therefore, microwave energy was used to improve reaction efficiency. The effect of microwave energy on reaction time was assessed from 1 to 7 min at 200, 400 and 700 W (Fig. 3D). Regardless of the irradiation energy, the maximum

derivative yield could be obtained within 5 min. Therefore, an irradiation time of 5 min and an irradiation power of 200 W were applied in further experiments.

3.3. Optimization of extraction conditions

Fig. 3C shows that increasing the amount of derivatizing reagent increased derivatization efficiency. However, a surplus of reagent produced a poor peak resolution and interfered with quantitation of the LA derivative (Fig. S1B). A large amount of derivatizing reagent also adversely affected the stationary phase and reduced the column lifetime. Thus, excess derivatizing reagent was removed with ethyl acetate before performing the procedure for extracting the LA derivative.

After the labeling reaction, the LA derivative tended to ionize in alkaline media because LA has a carboxyl group. For good extraction efficiency, the pH should be adjusted to an acidic value so that the LA derivative is in its molecular state. Comparison of HCl, FA, AA, TFA and HFBA at 1.0 M showed that HCl had the best efficiency in extracting the LA derivative. Comparisons of the effect of HCl on extraction efficiency within a range of 0.1–5.0 M showed that the relative response plateaued at 1.0 M (Fig. 3E). Therefore, 1.0 M HCl was applied in further experiments.

Five organic solvents were compared in terms of efficiency in extracting LA derivative: ethyl acetate, chloroform, dichloromethane, toluene and *n*-hexane. In CapLC–UV analysis, ethyl acetate and toluene showed similarly high extraction efficiency. In MALDI, however, co-crystallization of the matrix (CHCA) and LA derivative was poor when toluene was used as the extraction solvent. Thus, only ethyl acetate conformed to the requirements of both CapLC and MALDI. Efficiency in extracting LA derivative was compared in ethyl acetate in varying volumes (15–60 μL) (Fig. 3F). In CapLC–UV, the response increased with the amount of extraction solvent. The MALDI-TOF MS obtained the best response at an extractant volume of 20 μL because the increase in ethyl acetate had a diluting effect that resulted in an appropriate molar ratio of CHCA to LA derivative. Under the optimal derivatization and extraction conditions, it significantly improved the selectivity and detectability of LA. Comparisons showed that the signal for 2 mM of LA without derivatization approximated that for 1 μM of LA derivative in CapLC–UV and MALDI-TOF MS. Thus, the developed method enhanced the LA detection sensitivity of both CapLC–UV and MALDI-TOF MS.

3.4. Method validation

Each method was precisely validated in terms of linearity, precision, accuracy, detection limit and stability. The peak area ratio of the LA derivative to IS was used for quantitation. Both methods showed good linearity ($r^2=0.999$) throughout the calibration range of 0.1–40 μM. The between-day regression equations for CapLC–UV and MALDI-TOF MS were $y=(0.0525\pm0.0008)x-(0.0344\pm0.0024)$ and $y=(0.0604\pm0.0014)x-(0.0116\pm0.0152)$, respectively. The LOD for the CapLC–UV method was 5 fmol, which was slightly higher than the 4 fmol LOD obtained for the MALDI-TOF MS method. Regarding within-day precision and accuracy, the RSD and RE were lower for CapLC–UV (2.33–3.22% and 0.45–4.92%, respectively) compared to MALDI-TOF MS (3.92–5.88% and 3.04–6.62%, respectively). Regarding between-day precision and accuracy, the RSD and RE were also lower for CapLC–UV (2.89–5.74% and 0.60–1.93%, respectively) compared to MALDI-TOF MS (4.86–8.85% and 4.75–7.22%, respectively). The LA derivative revealed no significant change in IS peak area ratio after 24 h at room temperature. The proposed methods revealed good linearity, precision, accuracy and stability of the LA derivative.

As described above, both of the proposed methods of LA analysis were compared with conventional methods in terms of LOD, linear range, reaction time, analytical runtime, and sample volume (Table 1). The comparisons showed that the proposed

Table 2
Assay results for samples of commercially available LA dietary supplement tablets.

Sample[a]	Amount found (mg)	R.S.D.[b] (%)	Percentage of claimed content (%)
CapLC–UV			
1	327.86	2.96	109.29
2	314.51	2.72	104.84
3	327.48	2.28	109.16
4	324.07	1.47	108.02
5	331.68	0.66	110.56
MALDI-TOF MS			
1	325.42	3.56	108.47
2	301.47	4.20	100.49
3	321.52	5.62	107.17
4	328.32	7.81	109.44
5	328.78	8.17	109.59

[a] Labeled concentration of LA in each tablet is 300 mg.
[b] R.S.D. of triplicate analysis.

Table 1
Comparison of the proposed method with previously reported methods of analyzing LA.

Chromatographic method	Derivatization reagent	Required time[a] (min)	Linear range (μM)	LOD (pg)	Sample volume (μL)	Sample	Ref.
GC–MS	MTBSTFA	30+25	0.05–96.93	10	500	Meat	[17]
HPLC–UV	–	0+10	48.46–2423.3	88,000	20	Dietary supplement	[20]
HPLC–FLD	mBBr	90+20	0.02–3	200	250	Human plasma and rat tissue	[21]
	ABD-F	10+40	0.12–12	70	300	Human plasma and dietary supplement	[22]
	SBD-F	60+40					
	PIAA	15+60	0.75–120	0.7	40	Human urine and dietary supplement	[23]
	PMIA	15+60					
HPLC–ECD	–	0+10	0.05–290.80	60	20	Dietary supplement	[28]
HPLC–MS/MS	–	0+5	0.048–48.47 nM	0.03	50	Rat tissue	[31]
CapLC-UV	Br-DMC	5+15	0.1–40	1.03	300	Dietary supplement and cosmetic	This work
MALDI-TOF MS	Br-DMC	5+2	0.1–40	0.83	300	Dietary supplement	This work

MTBSTFA, N-methyl-N-(tert.-butyldimethylsilyl)-trifluoroacetamide; mBBr, monobromobimane; ABD-F, 4-(Aminosulfonyl)-7-fluoro- 2,1,3-benzoxadiazole; SBD-F, ammonium 4-fluoro-2,1,3-benzoxadiazole-7-sulfate; PIAA, N-1-Pyreneiodoacetamide; PMIA, N-1-Pyrenemethyliodoacetamide; Br-DMC, 4-bromomethyl-6,7-dimethoxycoumarin.
[a] Required time=derivatization reaction time+analytical runtime.

method is superior in terms of reaction time [17,21–23] and LOD [17,20–22,28]. In addition to its good performance, the newly developed methods of LA analysis can be performed quickly and easily with high reliability and robustness.

3.5. Application to analysis of dietary supplements and cosmetics

The effectiveness of the methods was verified by analyses of one dietary supplement and six cosmetics. Tables 2–4 show the content assay results, the content uniformity and the relative recovery, respectively, for the LA tablets. For determining LA content, both methods were highly consistent with the labels. The two methods showed comparable accuracy and precision in measuring the LA content of commercial products. *T* test and *F* test were used to compare the means and variances of the measures obtained by the two methods. A two-tailed *P* value less than 0.05 was considered statistically significant. GraphPad Prism Version 5.01 was used for all statistical analyses.

Table 3
Assay results for content uniformity of LA tablets.

Sample[a]	Amount found (mg)	R.S.D.[b] (%)	Percentage of claimed content[c] (%)
CapLC–UV			
1	305.60	4.08	101.87
2	301.49	2.08	100.50
3	337.17	0.87	112.39
4	306.00	2.93	102.00
5	275.07	6.04	91.69
6	356.74	3.50	118.91
7	281.45	6.25	93.82
8	283.75	6.82	94.58
9	308.03	5.95	102.68
10	326.43	2.89	108.81
		Mean (%)	102.72
		R.S.D.	8.38
MALDI-TOF MS			
1	307.73	5.65	102.58
2	297.02	5.19	99.01
3	348.86	3.45	116.29
4	307.69	3.08	102.56
5	278.92	4.69	92.97
6	325.10	2.87	108.37
7	277.82	7.89	92.61
8	287.19	9.89	95.73
9	311.09	5.31	103.70
10	310.47	4.54	103.49
		Mean (%)	101.73
		R.S.D.	7.09

[a] Labeled concentration of LA in each tablet is 300 mg.
[b] R.S.D. of triplicate analysis.
[c] Content uniformity test is used to check the variation of LA in each tablet.

Table 4
Relative recoveries of LA tablets.

Amount spiked (μM)	Amount found (μM)	R.S.D. [a](%)	Recovery (%)
CapLC–UV			
0	12.80	3.71	–
2	14.66	2.39	93.08
10	23.89	2.54	110.86
25	35.32	2.64	90.08
MALDI-TOF MS			
0	14.19	5.55	–
2	15.97	10.02	88.83
10	24.02	3.37	98.29
25	39.26	1.11	100.24

[a] R.S.D. of triplicate analysis.

Table 5
The CapLC–UV assay results for samples of commercially available cosmetic products.

Sample	Formulation	Amount found (μg/g)	R.S.D. [a](%)
A	Lotion	7.21	4.06
B	Lotion	0.37	5.42
C	Essence	3.71	1.88
D	Essence	N.D.	–
E	Mask	0.88	3.01
F	Emulsion	4.15	4.24

N.D.: LA has been labeled on the product packaging, but no detection signal appeared.
[a] R.S.D. of triplicate analysis.

Table 2 shows that variations in the sample measurements were smaller in the CapLC–UV method than in the MALDI-TOF MS method. However, the two methods did not significantly differ in means or variances ($p=0.51$ and $p=0.31$ for *t*-test and *F* test, respectively). Table 3 further shows that the two methods did not significantly differ in measurements of content uniformity ($p=0.45$). Table 4 shows that the two methods did not significantly differ in relative recoveries of LA tablets ($p=0.253$ and $p=0.052$ for slopes and intercepts, respectively). The results also confirmed the high accuracy and precision of these techniques. Figs. S1C and 2B show the chromatogram and mass spectrum of the LA tablets, respectively. The experimental procedure was also performed in the six cosmetics samples. Figs. S1D and 2C show the chromatogram and mass spectrum of cosmetic sample E, respectively. The MALDI-TOF MS did not obtain satisfactory results when used to determine LA concentration in cosmetics because the complex matrix decreased the ionization ability in both the target analyte and the IS. Table 5 presents the CapLC–UV results for six cosmetic samples. Since LA content is not regulated in cosmetic products, they may only contain trace amounts of LA.

3.6. Biological relevance of identified proteins

Fig. 4 shows the results obtained by the PANTHER system (http://www.pantherdb.org), which was used to categorize the proteins expressed in the HaCaT cell lysate in terms of biological process, molecular function and protein class. In terms of biological processes, the proteins were predominantly associated with metabolic processes (25.9–28.5%), cellular processes (14.5–16.6%), cell communication (8.3–11.5%), transport (7.3–9.5%) and immune system processes (6.7–9.4%). In terms of molecular functions, the proteins were predominantly associated with binding (33.3–36.9%), catalytic activity (27.6–30.1%) and structural molecule activity (9.2–16.1%). The predominant protein classes were nucleic acid binding (13.3–18.4%) and enzyme modulator (5.2–11.0%).

In skin cells, UV radiation is known to activate multiple signaling pathways such as NF_kB, Jun N-terminal kinase (JNK), p38 MAPK, epidermal growth factor receptor (EGFR), and extracellular signal-regulated kinase 1/2 (ERK1/2) [45]. The p38 MAPK is a major signaling cascade activated by UV in cells. In the current study, LA-treated HaCaT cells revealed down regulation of a cell division control protein 42 (CDC42) homolog in the p38 MAPK pathway. An earlier study showed that UVB radiation upregulates 28 proteins in HaCaT, including RPSA, PSMD13, C20orf77, NAPA, ESD, DNAJC14, C1QBP, CTSD, PGLS, PRDX4, PSMA6, TPI1, RHOA, HNRPA2B1, IL18, PRDX1, PTGES3, SKP1A, PPP1R14B, GMFB, CALM3, PPIA, TCEB2, MYL6, RPP2, BANF1, C19orf10 and RPL23 [46]. Additionally, the LA-treated HaCaT cells showed reduced expressions of TPI1, HNRPA2B1, PRDX1, PTGES3 and MYL6. The results showed that LA indeed has important regulatory roles in the cell cycle and in antioxidation.

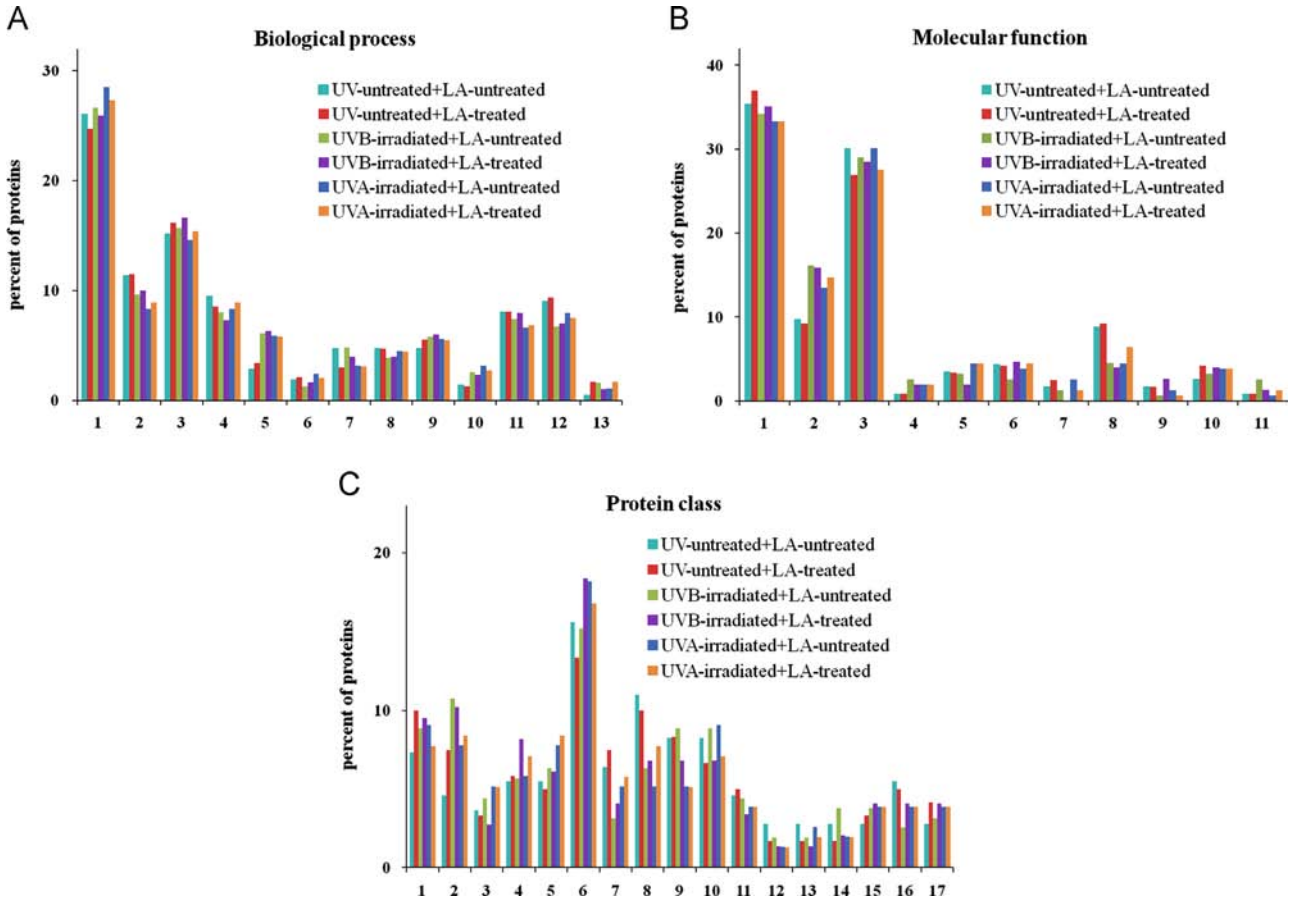

Fig. 4. The PANTHER classification for proteins expressed in HaCaT cells with/without UV treatment and with/without LA treatment according to NanoUPLC–MS/MS analysis. (A) Biological process: 1-metabolic process, 2-cell communication, 3-cellular process, 4-transport, 5-cellular component organization, 6-apoptosis, 7-system process, 8-response to stimulus, 9-developmental process, 10-generation of precursor metabolites and energy, 11-cell cycle, 12-immune system process, 13-cell adhesion. (B) Molecular function: 1-binding, 2-structural molecule activity, 3-catalytic activity, 4-ion channel activity, 5-transporter activity, 6-translation regulator activity, 7-transcription regulator activity, 8-enzyme regulator activity, 9-motor activity, 10-receptor activity, 11-antioxidant activity. (C) Protein class: 1-chaperone, 2-cytoskeletal protein, 3-transporter, 4-transferase, 5-oxidoreductase, 6-nucleic acid binding, 7-signaling molecule, 8-enzyme modulator, 9-calcium-binding protein, 10-hydrolase, 11-transfer/carrier protein, 12-membrane traffic protein, 13-phosphatase, 14-protease, 15-kinase, 16-isomerase, 17-receptor.

4. Conclusions

This study developed a rapid and simple MAD technique for enhanced detection of LA by CapLC–UV and MALDI-TOF MS. After optimum derivatization, LA detectability increased approximately two thousand times. The CapLC–UV and MALDI-TOF MS methods had LODs of 5 and 4 fmol, respectively, at a signal-to-noise ratio of 3. This study first evaluated the potential use of the two micro-scale analytical methods for routine determination of LA in cosmetic products and dietary supplements for quality control purposes. The results indicated that the LA content in sampled dietary supplement tablets was consistent with their labels. However, the LA content in cosmetic products was below the effective concentration required for anti-oxidating effects. The HaCaT cell protein profile obtained under UV exposure showed that LA can reduce the expressions of proteins such as CDC42, TPI1, HNRPA2B1, PRDX1, PTGES3 and MYL6.

Acknowledgments

The authors are grateful for financial support from the National Science Council (NSC 102-2113-M-037-012) and the Teaching and Learning Excellence Program (U19604).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.07.014.

References

[1] V.E. Kagan, A. Shvedova, E. Serbinova, S. Khan, C. Swanson, R. Powell, L. Packer, Biochem. Pharmacol. 44 (1992) 1637–1649.
[2] G.P. Biewenga, G.R. Haenen, A. Bast, Gen. Pharmacol. 29 (1997) 315–331.
[3] F. Navari-Izzo, M.F. Quartacci, C. Sgherri, Plant Physiol. Biochem. 40 (2002) 463–470.
[4] L. Packer, E.H. Witt, H.J. Tritschler, Free Radic. Biol. Med. 19 (1995) 227–250.
[5] L. Packer, E.H. Witt, H.J. Tritschler, Antioxidant properties and clinical applocations of alpha-lipoic acid and dihydrolipoic acid, in: E. Cadenas, L. Packer (Eds.), Handbook of Antioxidants, M. Dekker, Inc., New York, 1996, pp. 545–591.
[6] A.R. Smith, S.V. Shenvi, M. Widlansky, J.H. Suh, T.M. Hagen, Curr. Med. Chem. 11 (2004) 1135–1146.
[7] M.J. Stevens, I. Obrosova, X. Cao, C.V. Huysen, D.A. Greene, Diabetes 49 (2000) 1006–1015.
[8] B. Diesel, S. Kulhanek-Heinze, M. Holtje, B. Brandt, H.D. Holtje, A.M. Vollmar, A.K. Kiemer, Biochemistry 46 (2007) 2146–2155.
[9] A.I. Bush, Neurobiol. Aging 23 (2002) 1031–1038.
[10] M.S. Kim, J.Y. Park, C. Namkoong, P.G. Jang, J.W. Ryu, H.S. Song, J.Y. Yun, I.S. Namgoong, J. Ha, I.S. Park, I.K. Lee, B. Viollet, J.H. Youn, H.K. Lee, K.U. Lee, Nat. Med. 10 (2004) 727–733.
[11] N.M. Jameel, M.A. Shekhar, B.S. Vishwanath, Life Sci. 80 (2006) 146–153.
[12] Z. Ying, N. Kherada, B. Farrar, T. Kampfrath, Y. Chung, O. Simonetti, J. Deiuliis, R. Desikan, B. Khan, F. Villamena, Q. Sun, S. Parthasarathy, S. Rajagopalan, Life Sci. 86 (2010) 95–102.

[13] K. Tsuji-Naito, S. Ishikura, M. Akagawa, H. Saeki, Connect. Tissue Res. 51 (2010) 378–387.
[14] H.S. Kim, H.J. Kim, K.G. Park, Y.N. Kim, T.K. Kwon, J.Y. Park, K.U. Lee, J.G. Kim, I.K. Lee, Exp. Mol. Med. 39 (2007) 106–113.
[15] W.A. Konig, S. Lutz, P. Evers, J. Chromatogr. 503 (1990) 256–259.
[16] H. Kataoka, N. Hirabayashi, M. Makita, J. Chromatogr. 615 (1993) 197–202.
[17] A. Mattulat, W. Baltes, Z. Lebensm. Unters. Forsch. 194 (1992) 326–329.
[18] H. Kataoka, J. Chromatogr. B 717 (1998) 247–262.
[19] S.C. Howard, D.B. McCormick, J. Chromatogr. 208 (1981) 129–131.
[20] H.Y. Aboul-Enein, J. Liq. Chromatogr. Relat. Technol. 27 (2004) 3029–3038.
[21] W. Witt, B. Rustow, J. Chromatogr. B 705 (1998) 127–131.
[22] S. Satoh, T. Toyo'oka, T. Fukushima, S. Inagaki, J. Chromatogr. B 854 (2007) 109–115.
[23] T. Inoue, M. Sudo, H. Yoshid, K. Todoroki, H. Nohta, M. Yamaguchi, J. Chromatogr. A 1216 (2009) 7564–7569.
[24] A.I. Durrani, H. Schwartz, W. Schmid, G. Sontag, J. Pharm. Biomed. Anal. 45 (2007) 694–699.
[25] A.I. Durrani, H. Schwartz, M. Nagl, G. Sontag, Food Chem. 120 (2010) 1143–1148.
[26] A. Khan, Z. Iqbal, D. Watson, A. Khan, I. Khan, N. Muhammad, S. Muhammad, H. AraNasi, N. Iqbal, Faiz-ur-rehman, M. Kashi, J. Chromatogr. B 879 (2011) 1725–1731.
[27] A. Khan, M.I. Khan, Z. Iqbal, L. Ahmad, Y. Shah, D.G. Watson, J. Chromatogr. B 878 (2010) 2782–2788.
[28] W. Siangproh, P. Rattanarat, O. Chailapakul, J. Chromatogr. A 1217 (2010) 7699–7705.
[29] E. Wołyniec, J. Karpińska, S. Łosiewska, M. Turkowicz, J. Klimczuk, A. Kojło, Talanta 96 (2012) 223–229.
[30] J. Teichert, R. Preiss, J. Chromatogr. B 769 (2002) 269–281.
[31] H.T. Chng, L.S. New, A.H. Neo, C.W. Goh, E.R. Browne, E.C.Y. Chan, J. Pharm. Biomed. Anal. 51 (2010) 754–757.
[32] A. Sitton, M.G. Schmid, G. Gubitz, H.Y. Aboul-Enein, J. Biochem. Biophys. Methods 61 (2004) 119–124.
[33] K.C. Pañak, O.A. Ruiz, S.A. Giorgieri, L.E. Díaz, Electrophoresis 17 (1996) 1613–1616.
[34] M. Trentin, T. Carofiglio, R. Fornasier, U. Tonellato, Electrophoresis 23 (2002) 4117–4122.
[35] U. Bahr, M. Karas, F. Hillenkamp, Fresenius J. Anal. Chem. 66 (1994) 783–791.
[36] P. Lidström, J. Tierney, B. Wathey, J. Westman, Tetrahedron 57 (2001) 9225–9283.
[37] X. Zhang, D.O. Hayward, Inorg. Chim. Acta 359 (2006) 3421–3433.
[38] C.O. Kappe, Angew. Chem. Int. Ed. Engl. 43 (2004) 6250–6284.
[39] B. Halliwell, Biochem. J. 401 (2007) 1–11.
[40] B. D'Autreaux, M.B. Toledano, Nat. Rev. Mol. Cell Biol. 8 (2007) 813–824.
[41] Y. Ibuki, H. Naitou, N. Ohashi, R. Goto, Photochem. Photobiol. 81 (2005) 823–829.
[42] J.A. Burns, J.C. Butler, J. Moran, G.M. Whitesides, J. Org. Chem. 56 (1991) 2648–2650.
[43] K. Tyagarajan, E. Pretzer, J.E. Wiktorowicz, Electrophoresis 24 (2003) 2348–2358.
[44] M.M. Kreevoy, R.W. Jacobson, Ventron Alembic 15 (1979) 2–3.
[45] V. Muthusamy, T.J. Piva, Arch. Dermatol. Res. 302 (2010) 5–17.
[46] S. Liu, C. Guo, D. Wu, Y. Ren, M.Z. Sun, P. Xu, J. Photochem. Photobiol. B 114 (2012) 94–101.