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Phytosterol and α-Lipoic Acid Conjugates: Synthesis, Free Radical Scavenging Capacity and RP-LC-MS-APCI Analysis

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Plant sterols (PS) are bioactive compounds effective in reducing plasma cholesterol. Fatty acid esters of PS have improved solubility and blending properties when utilized in various food products. Naturally occurring α-lipoic acid (LA) and its reduced form dihydrolipoic acid (DHLA) are known for their antioxidant activity. In addition, they have shown an array of health beneficial properties against obesity, diabetes, cancer, cardiovascular and inflammatory diseases *etc*. Different LA conjugates have been reported to have increased bioactivity compared to the parent compounds. The objective of this study was to synthesize PS esters of LA (PSLA) and DHLA (PSDHLA) in order to increase their cholesterol lowering effect and reducing the risk of atherosclerosis with additional health benefits *e.g.* against oxidative stress.

Synthesis of PSLA and PSDHLA was performed with a pure PS mixture of β-sitosterol, stigmasterol, campesterol and brassicasterol. The free radical scavenging capacity of the conjugates was assessed by the DPPH method. Remaining percentage of DPPH free radicals was measured at the steady state for different concentrations of PSLA and PSDHLA. High free radical scavenging capacity was observed for PSDHLA compared with PSLA. Efficient concentration EC_{50} as a molar ratio for PSDHLA was 0.43. The derivatives were analyzed by RP-HPLC-MS-APCI. The order of the elution times of the compounds observed in HPLC-MS analysis was PS< PSDHLA< PSLA. Baseline separation was not achieved between campesterol and stigmasterol and their derivatives. These compounds could be identified by their characteristic fragment ions from the mass spectral data.

INTRODUCTION

Phytosterols (PS) are bioactive compounds associated with plant lipids. When taken in sufficiently high (0.8–2.5 g) amounts daily, they have shown to be effective in lowering total cholesterol (TC) and 5–14% of low density lipoprotein (LDL) cholesterol [Normén *et al*., 2004; Berger *et al*., 2004; Plat & Mensink, 2005; AbuMweis *et al*., 2008]. Based on studies on the cholesterol lowering effect of PS (reduction of 10% LDL-cholesterol), it has been estimated that the occurrence of coronary heart disease (CHD) can be reduced by up to 20% [Miettinen & Gylling, 2004; Demonty *et al*., 2009]. Pure PS commercially available in powder form, are hydrophobic and difficult to dissolve in food and food products such as vegetable oil, margarine *etc.* and hence it is difficult to include them in food formulation. Esterification of PS with fatty acids improves solubility and dispersion while optimizing uniform blending in foods and food products. In addition to the above effects, PS are believed to possess other health benefits such as anti-inflammatory and antioxidative properties *etc.* [Berger *et al*., 2004; Rudowska, 2010]. PS esters are hydrolysed after ingestion, resulting in free PS and free fatty acids which are absorbed in the small intestine [Platt & Mensink, 2005]. Attempts have been made to combine the beneficial health effects of PS with esterification of compounds other than fatty acids [Kutney *et al*., 2001; Milanova *et al*., 2001; Condo *et al*., 2001; Jia *et al*., 2006].

Naturally occurring α-lipoic acid (LA) is present in many foods but in very small amounts. In living organisms, LA occurs as lipoamide and acts as a co-enzyme in multienzyme complexes involved in energy metabolism. Dihydrolipoic acid (DHLA) is the reduced form of LA and together they act as a redox couple and have shown to perform an important role as biological antioxidants by scavenging reactive oxygen species (ROS), chelating metal ions, restoring intracellular glutathione level *etc*. Further, DHLA has shown capability in regenerating reduced levels of exogeneous antioxidants such as tocopherols and vitamin C [Packer *et al*., 1995; Biewenga *et al*., 1997; Shay *et al*., 2009]. Increased oxidative stress is believed to be associated with several health risk factors such as obesity, hypertension, diabetes mellitus and inflammation

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interconnected as metabolic syndrome [Hopps *et al*., 2010]. Dietary administration (0.5%, w/w) of LA for 14 weeks gave a decrease in body weight and visceral fat mass in genetically obese rats by enhancing peripheral energy expenditure *via* suppressed hypothalamic adenosine monophosphate-activated protein kinase (AMPK) activity [Kim *et al*., 2004]. In a study with LA supplementation for 20 weeks, it has been shown to prevent the increase of plasma total cholesterol and atherosclerotic lesions induced by diabetes in apolipoprotein E-deficient mice fed with high fat-low cholesterol diet [Yi & Maeda, 2006]. It has been further demonstrated that LA reduced atherosclerotic plaques in the abdominal aorta in hyperlipidemic rabbits with established atherosclerosis [Ying *et al*., 2010]. Therapeutic doses of free LA are much higher than the dietary intake [Biewenga *et al*., 1997]. Today LA is used as a dietary supplement in varying amounts, from 50–600 mg/day, and no upper limit has yet been established. Bioavailability of LA can vary depending on the form it is ingested. A sodium salt of LA showed increased human plasma maximum concentration (C_{max}) and total amount absorbed compared to free LA supplementation. According to previous studies, one of the limitations in the application of oral ingestion of LA is its short half-life [Carlson *et al.*, 2007; Shay *et al.*, 2009].

Preparation of hybrid molecules is a common approach to create efficient and/or synergistic effects either on single or dual targets. It was also suggested that multifunctional LA conjugates may bring their therapeutic effects at lower concentrations compared to the LA as a single molecule [Koufaki *et al*., 2009; Melagraki *et al*., 2009]. The main objectives of this study were to synthesize LA and DHLA conjugates of PS in a mixture (β-sito-, stigma-, campe-, and brassicasterol), yielding phytosterol lipoate (PSLA) and phytosterol dihydrolipoate (PSDHLA), respectively, and to verify them by NMR and HPLC-MS. To our knowledge, no research paper has been published on these conjugates [Milanova *et al*., 2001]. The conjugates PSLA and PSDHLA are contemplated to possess multiple health benefits such as weight reducing properties and anti-oxidative properties *etc*., in addition to the cholesterol lowering effect. These potential synergistic effects might be due to enhanced solubility, dispersibility and stability of the conjugates in foods and food products. Further, the potential free radical scavenging capacity of the conjugates was evaluated using the DPPH method and characterized by RP-HPLC-APCI-MS.

MATERIALS AND METHOD

Materials

Since pure individual PS are very expensive, four different PS in a mixture were esterified with LA and DHLA to prepare PSLA and PSDHLA in a laboratory scale. A mixture of PS, racemic LA, DHLA, 2, 2-diphenyl-1–1picrylhydrazyl (DPPH) and 4-dimethyl amino pyridine (DMAP) were purchased from Sigma-Aldrich Sweden AB (Stockholm, Sweden). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDCI) was purchased from TCI Europe N.V. (Zwijndrecht, Belgium). Analytical grade solvents were used in all synthesis and purification steps while HPLC grade solvents were used in LC-MS analysis. All the solvents were purchased from VWR International AB, Stockholm, Sweden except ethanol which was obtained from Solveco AB (Rosersberg, Sweden). Milli-Q water purified through a Millipore system (Millipore, USA) was used for HPLC-MS analysis.

Synthesis of phytosterol lipoate (PSLA)

Synthesis of PSLA was performed according to the method of Chiu *et al*. [1996] and Uyeda *et al*. [2005] with some modifications. Dry PS mixture (1.0 g, 2.44 mmol; calculated based on the average molecular weight of PS mixture) was dissolved in CH_2Cl_2 (9 mL) and DMAP (64 mg, 0.52 mmol), LA (580 mg, 2.81 mmol) and EDCI (468 mg, 2.44 mmol) were added while stirring under N_2 at 0°C. Thereafter, the reaction mixture was stirred at room temperature overnight. The mixture was acidified until *ca*. pH 2 with 1 mol/L HCl and extracted with CH_2Cl_2 (2x10 mL). The combined organic layer was washed with water (2x5 mL), aqueous saturated NaCl (2x5 mL), dried (Na_2SO_4) and evaporated under vacuum. Crude extract was purified by solid phase extraction (SPE). The SPE column (1 g silica; Sorbent AB, Stockholm, Sweden) was activated with 5 mL hexane. Approximately 200 mg of sample dissolved in 3 mL hexane was applied on the SPE column, eluted using 5 mL hexane which contained pure PSLA. The remaining crude PSLA was eluted with additional 10 mL hexane and the purification was repeated once more with a new SPE column. The yield was more than 60%. The summarized reaction with the example of β-sitosterol-LA and β-sitosterol-DHLA are shown in the scheme (Figure 1).

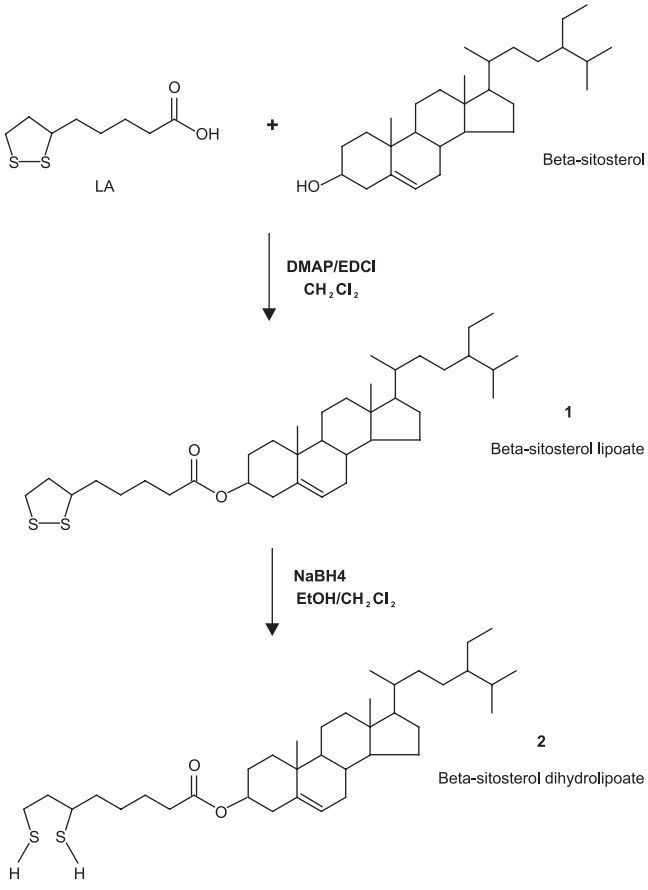


FIGURE 1. Scheme showing the synthesis of PSLA and PSDHLA with the example of β-sitosterol-lipoate (**1**) and β-sitosterol-dihydrolipoate (**2**).

Synthesis of phytosterol dihydrolipoate (PSDHLA)

Pure PSLA was reduced according to the method of Chittiboyina *et al*. [2006] with few modifications. PSLA (200 mg, 0.33 mmol) was dissolved in CH_2Cl_2/EtOH (12 mL, 1:5, v/v) and $NaBH_4$ (56 mg, 1.5 mmol) was added in portions while stirring under N_2 and the reaction mixture was stirred for 1 h at room temperature (Figure 1). The mixture was acidified with HCl (1 mol/L) until pH 2 and extracted with CH_2Cl_2 (2x25 mL). The combined organic layer was washed with water (2x10 mL), aqueous saturated NaCl (2x10 mL), dried (Na_2SO_4) and evaporated under vacuum. The product was further purified by preparative TLC as described below to obtain the pure product in 80% yield.

Thin-layer-chromatography (TLC)

Separation of PSLA and PSDHLA along with the initial compounds used in the synthesis were checked by analytical TLC (Silica gel 60, 10 cm x 20 cm x 0.25 mm, Merck, Darmstadt, Germany), developed in hexane: diethyl ether: acetic acid (75:25:0.85, v/v/v). The plate was visualized by spraying with phosphomolybdic acid solution and dried in an oven at 120°C for 15 min. For the preparative TLC, the product PSDHLA was dissolved in hexane: CH_2Cl_2 (1:1, v/v) and 250 μL was applied on a pre-coated TLC plate (Silica gel 60, 20 cm x 20 cm x 0.50 mm, Merck, Darmstadt, Germany) using a TLC applicator (CAMAG Linomat IV, Muttenz, Switzerland). The TLC plate was developed as above and the band of PSDHLA was located by comparing with the analytical TLC and scraped off, collected in a glass tube and extracted twice with hexane: diethyl ether (1:1, v/v). Then solvent was evaporated under vacuum and stored at -20°C for further analysis.

NMR

1H-, 13C-NMR and 2-D experiments (COSY, TOCSY and HSQC-dept) spectra were obtained on a Bruker 400 MHz spectrometer (Bruker DRX, Germany) and chemical shifts (δ) are given in ppm relative to TMS for both PSLA and PSDHLA (Figures 2–3). The spectra were recorded in $CDCl_3$ as the solvent at 30°C.

DPPH assay

Free radical scavenging capacity of PSLA and PSDHLA was assayed according to the methods of Brand-Williams *et al*. [1995] and Wettasinghe & Shahidi [2000]. Different concentrations, (expressed as molar ratios (MR) = No. of moles test compound/No. of moles DPPH• in the final mixture) from 1 to 12 for PS standard mixture, from 1 to 8 for PSLA and from 0.05 to 1 for PSDHLA were tested for scavenging with DPPH• radicals at the room temperature. The test compound dissolved in 0.1 mL toluene was added to 3.9 mL of 0.06 mmol/L DPPH in toluene and the absorbance was measured at 515 nm every 15 min, until steady state or maximum time of 5 h. All analyses were performed in triplicates and toluene was used as a reference. The mean % of remaining DPPH• at steady state was determined from reaction kinetics and plotted against the molar ratio between test compound and DPPH• radical. The antioxidant activity defined as efficient concentration (EC_{50}) was calculated from the plotted curve.

RP-HPLC-MS-APCI analysis

Pure PS, LA, DHLA, PSLA and PSDHLA were analyzed by HPLC-MS (HP 1100 Series, Agilent Technologies Inc., Palo Alto, CA) equipped with an autosampler, quaternary gradient pump, thermostated column compartment and single quadrapole mass analyzer (G 1946D) controlled by Chemstation Rev.B.04.01 software. Chromatographic conditions and parameters in MS were based on the methods of Careri *et al*. [2001]; Bedner *et al*. [2008]; Carretero *et al*. [2008] and optimized with several modifications. Several C_{18} columns (HP GOLD column 150 x 4.6 mm i.d., 3.5 μm; YMC-Pack Pro C18, 250 x 4.6 mm i.d., 5 μm), a FluroPhase PFP column (150 x 4.6 mm i.d., 5 μm) and a C_8 column (Agilent Zorbax SB, 150 x 4.6 mm i.d, 5 μm) were tried to separate test compounds. Finally the C_8 column was selected for further HPLC-MS analysis. An isocratic elution with acetonitrile and 17 mmol/L aqueous acetic acid (92.5: 7.5, v/v) was used over a period of 32 min, at 1.0 mL/min flow rate and at 50°C column temperature. The analyte was dissolved in CH_2Cl_2 (0.5–1.0 μg/μL) and 1 μL was injected. APCI-MS analyses was performed at the optimized settings; vaporizer temperature 350°C, drying gas temperature 350°C at a flow rate of 9 L/min, nebulizer pressure at 60 psi, corona current 8μA, capillary voltage at 3000 V and fragmentor voltage at 70 V. Total ion current of mass spectra were recorded in the mass range *m/z* 100–1000.

RESULTS

In our study, a commercially available mixture of the four most common PS was used in the synthesis. Composition of this PS mixture was 53% β-sitosterol, 29% campesterol, 15% stigmasterol, 1% brassicasterol and 2% other PS. The initial compounds, PSLA and PSDHLA were primarily identified and the purity was controlled by analytical TLC. Among different solvent systems tested, hexane: diethyl ether: acetic acid (75:25:0.85, v/v/v) showed best separation between PSLA and PSDHLA. The crude product was purified by SPE or preparative TLC and confirmed by NMR spectroscopy. In 1H NMR, integrals corresponded to lipoic acid and phytosterols as well as for DHLA and PS, indicating full esterification. 2-D NMR (cosy and tocsy) confirmed an intact ring structure of LA (Figure 2), and open thiol groups of DHLA (Figure 3).

In vitro free radical scavenging capacity measured by % reduction of DPPH• at the steady state of different MR of PSLA and PSDHLA are shown in Figure 4(a) and 4(b). Interaction between DPPH• and PSLA was observed as very low but linearly increased with increasing MR tested in this study. The time taken to reach the steady status varied from 30 min to 3 h for PSLA, when MR in the final reaction mixture increased from 1 to 8. The highest free radical scavenging capacity recorded at steady state for PSLA was at MR = 8. In contrast, PSDHLA showed a much higher free radical scavenging activity at relatively low MR. The remaining DPPH• was 17.8% for PSDHLA at MR = 1 compared to 99.2% in PSLA at the same MR. The time taken to reach the steady state in PSDHLA varied from 20 min to 6 h for the concentrations tested from MR 0.05

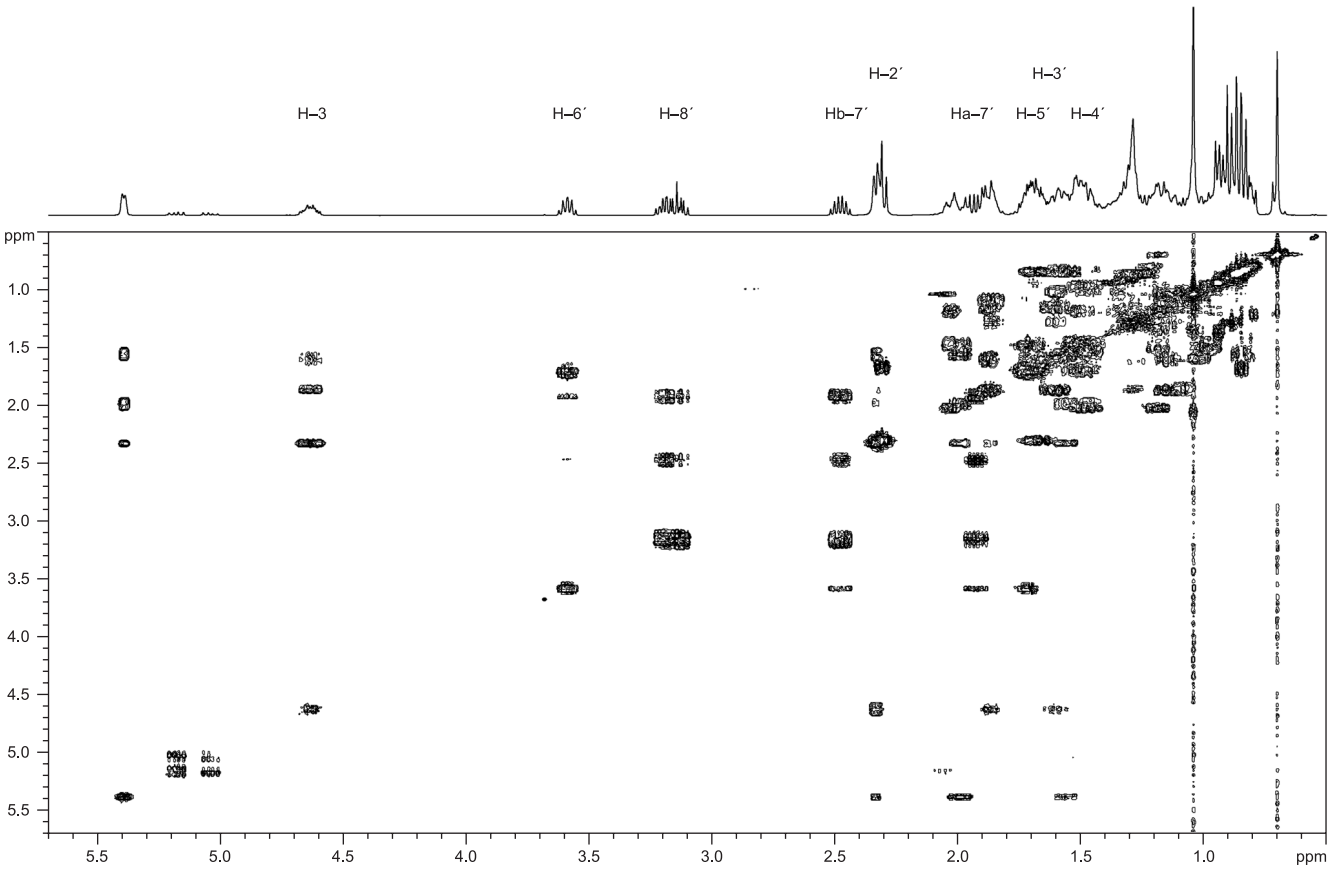


FIGURE 2. 2-D NMR (COSY). Crosspeaks in cosy and tocsy clearly showing that esterified lipoic acid in PSLA is intact.

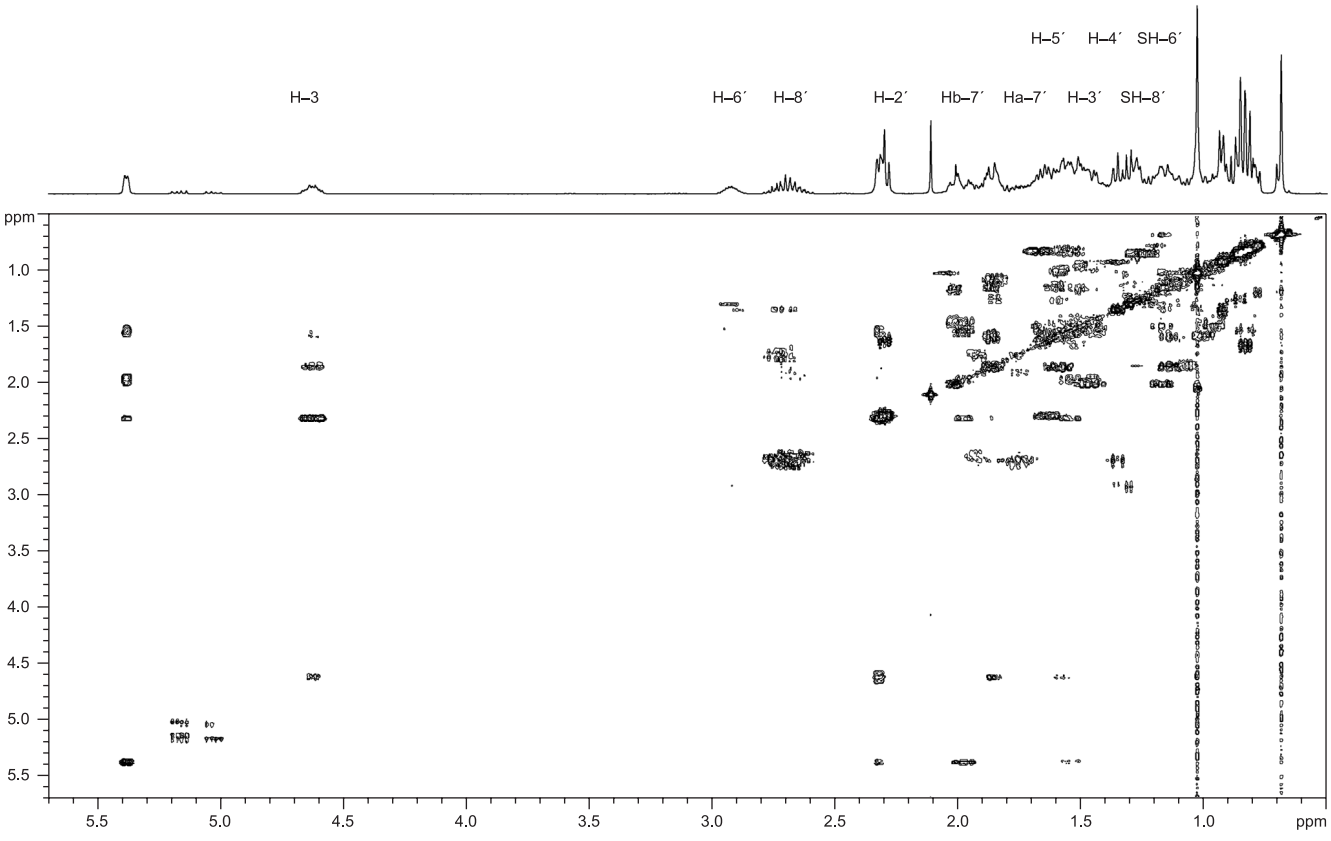


FIGURE 3. 2-D NMR (COSY). Crosspeaks in cosy and tocsy clearly showing that esterified dihydrolipoic acid in (PSDHLA) is intact with open thiol groups.

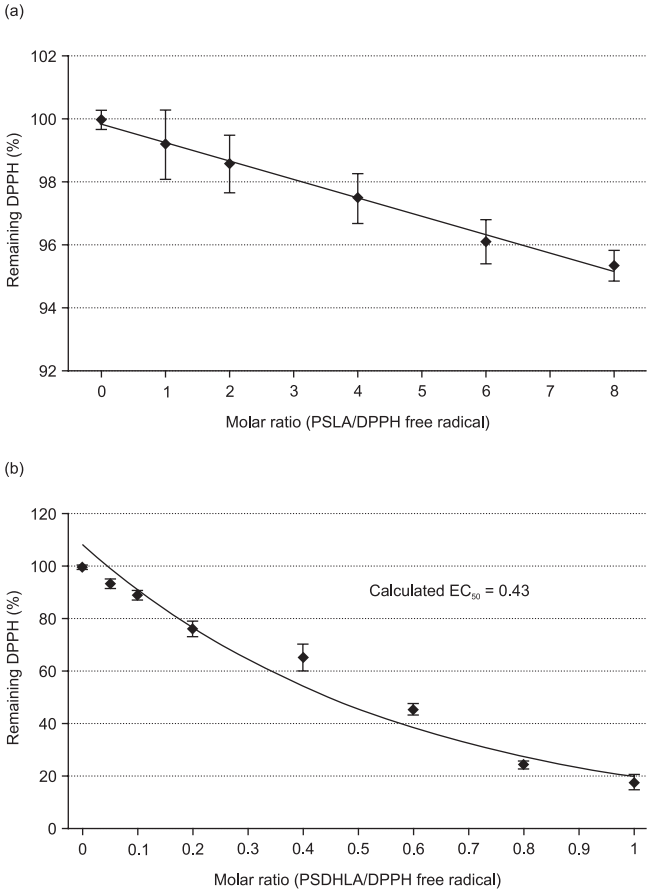


FIGURE 4. Scavenging of DPPH free radical (%) as a function of molar ratio of (a) PSLA/DPPH free radical (b) PSDHLA/DPPH free radical. The EC_{50} value was calculated from the curves.

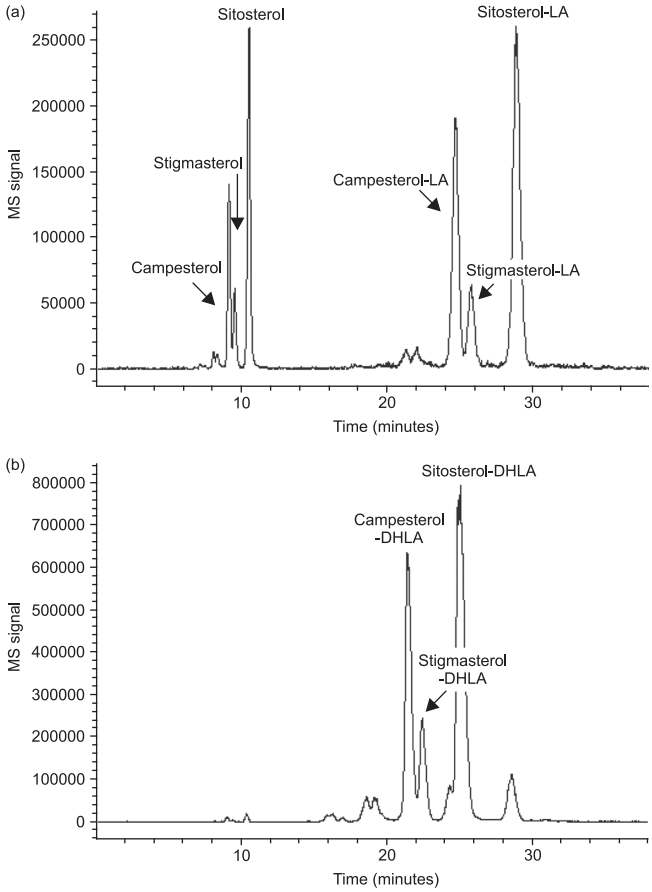


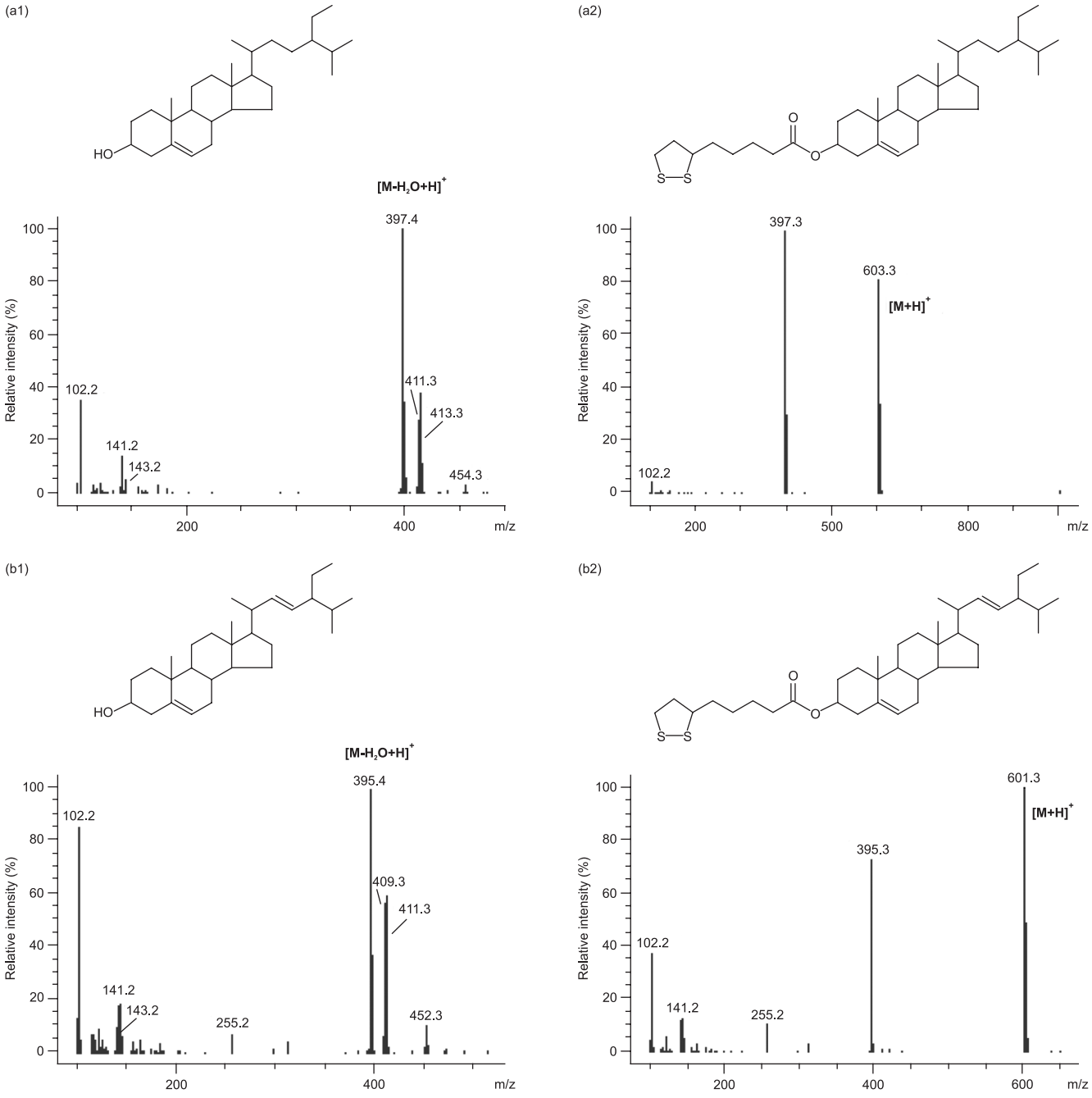
FIGURE 5. Total ion chromatograms (TIC) showing the separation and retention time of β-sitosterol, campesterol, stigmasterol and brassicasterol in (a) initial PS mixture and their derivatives in PSLA mixture (b) PSDHLA mixture, using Zorbax-SB C_8 column in RP-HPLC-MS--APCI.

to 1. When MR≥1, DPPH• scavenging activity of PSDHLA seemed to be constant. The initial PS mixture did not show any interaction with DPPH• even at MR = 12, the highest concentration tested in our study (results are not shown for PS mixture).

In order to improve separation of the individual components in PS, PSLA and PSDHLA, we have tested different RP columns (see methods section). Better separation was observed between campesterol and stigmasterol when the C_8 column was used compared with different C_{18} columns tested in this study. However, baseline separation between campesterol and stigmasterol could not be achieved by any of the columns tested. The RP-HPLC-MS-APCI total ion chromatogram (TIC) of PS and PSLA in a single mixture is shown in Figure 5a while PSDHLA is shown in Figure 5b. The individual derivatives of PS in the PSDHLA mixture eluted relatively earlier than those of PSLA but their elution order remained the same. The resolution between campesterol-LA and stigmasterol-LA was slightly better than separation between free campesterol and free stigmasterol. Mass spectra of β-sitosterol, stigmasterol, campesterol and brassicasterol and their corresponding LA derivatives recorded in the positive ion mode are shown in Figure 6 (A1-D2). Molecular ions of the PSDHLA derivatives could not be observed in positive ion mode. Mass spectra of PSLA and PSDHLA, observed in negative ion mode are shown in Figure 7 (A1-D2).

DISCUSSION

Both PS and LA are minor bioactive compounds naturally occurring in foods. They have shown health promoting effects when they are provided as a supplement in higher amounts than naturally present in food. The most interesting health benefit of PS is their cholesterol lowering effect in animals [Normén *et al*., 2004]. The mechanisms of cholesterol lowering effect of PS, are not yet fully established and different mechanisms have been proposed. Cholesterol and PS have very similar structures and thereby they compete with each other for incorporation into micelles in the intestinal lumen. It is believed that high concentration of PS from dietary sources in the gut, occupy more in the micelle and reduce cholesterol absorption [Plat & Mensink, 2005]. The other mechanism is that interaction of PS with enterocyte ATP-binding cassette transport proteins (ABCG5/ABCG8) cause increased excretion of both dietary cholesterol and sterols from pancreatic and biliary excretions [Harding *et al*., 2010; Nijjar *et al*., 2010; Davidson, 2011]. Esterification of PS with fatty acids is useful in the food industry to enhance the solubility and create more uniform dispersion in preparing functional foods such as oil blends, margarine, yoghurt *etc*. Improved solubility promotes more uniform formulation which is important for consistent cholesterol reducing effects, even though free PS and PS es-



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ters are considered to have similar cholesterol reducing properties [Plat & Mensink, 2005].

Conjugates of LA have been synthesised for their anticipated dual and/or synergistic health benefits. In addition, it has been suggested that LA conjugates may possess its therapeutic effect at lower concentrations than required for LA alone [Koufaki *et al*., 2009].

LA given by dietary means is safe in moderate use and has shown to be effective, bringing multiple metabolic and therapeutic effects. The mechanisms by which LA performs its functions are explained by cellular actions as an antioxidant, metal ion chelator and a mediator in cell signalling pathways [Shay *et al*., 2009]. Chemical properties of LA are highlighted by a dithiolane ring with an intra-molecular disulphide bond which shows a high reducing tendency due to high electron density resulting from the special position of the two sulphur atoms in the 1, 2-dithiolane ring. Previous studies have shown that LA can scavenge reactive oxygen species (ROS) such as hydroxyl radicals, peroxyl radicals, singlet oxygen, hypochlorous and peroxynitrite. High antioxidant capacity of DHLA is explained by its thiol groups which can be easily oxidized to 1, 2-thiolanes. Similar to LA, it is a strong scavenger of ROS except singlet oxygen. LA/DHLA acts as a redox couple and DHLA is considered to possess higher antioxidant potential. In addition, DHLA has displayed effective in regenerating other antioxidants such as tocopherols and ascorbic

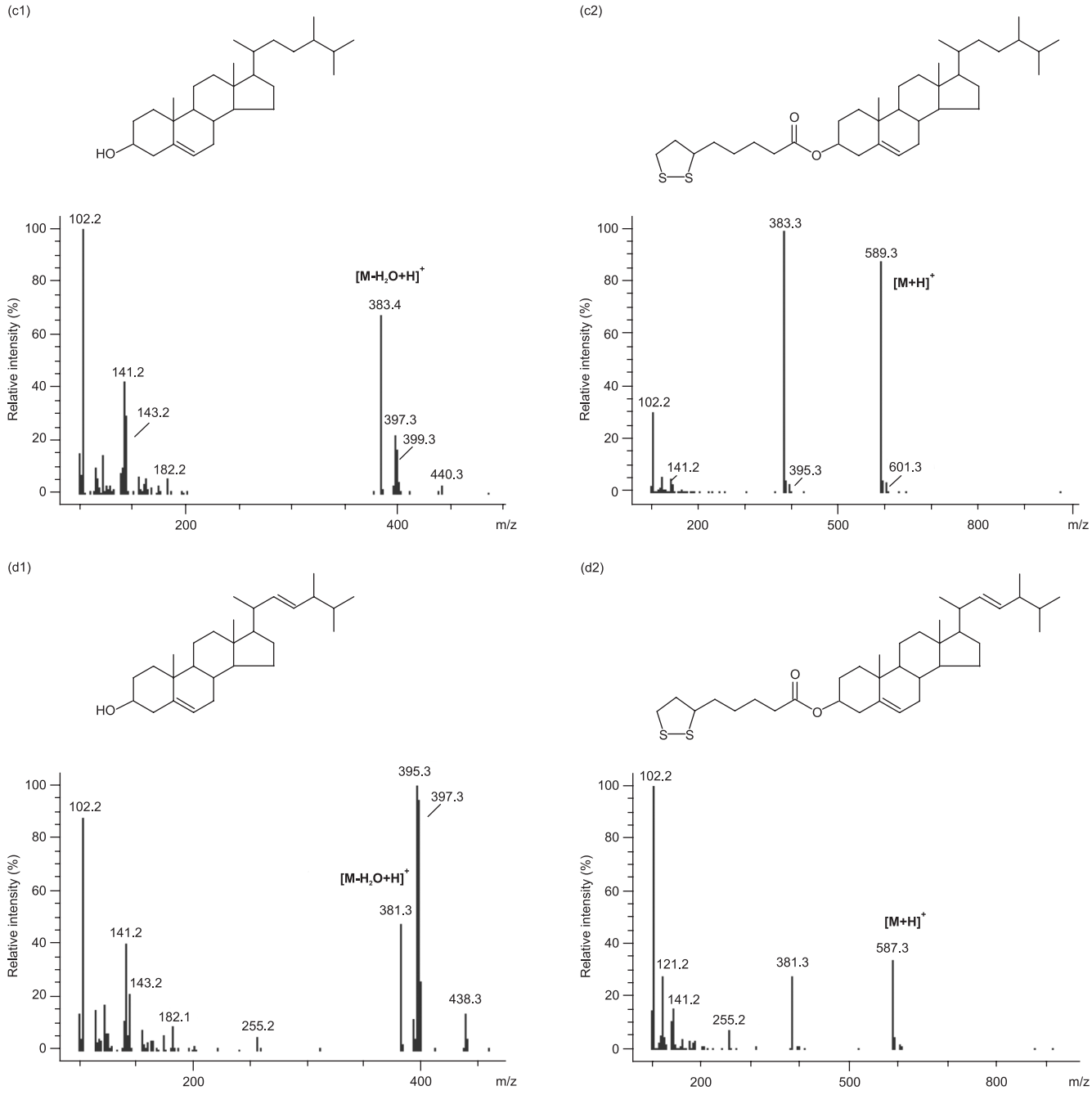
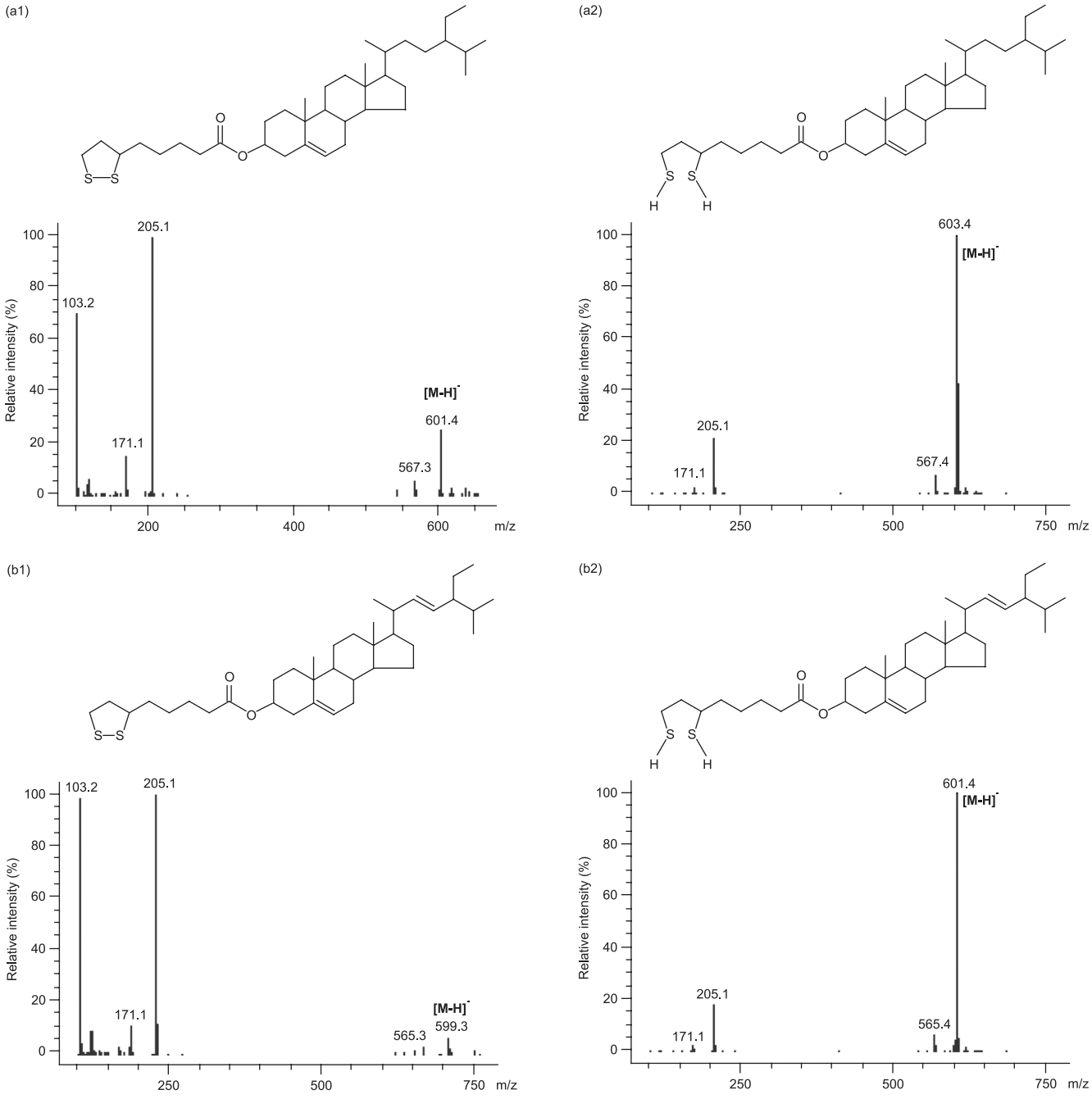


FIGURE 6. RP-HPLC-MS-APCI mass spectra recorded in positive ion mode for free PS showing dehydrated and protonated molecular ions at $[M-H_2O+H]^+$ and molecular ions at $[M + H]^+$ and their corresponding lipoates showing molecular ions at $[M+H]^+$. A1, β-sitosterol; A2, β-sitosterol-LA; B1, stigmasterol; and B2, stigmasterol-LA; C1, campesterol; C2, campesterol-LA; D1, brassicasterol; D2, brassicasterol-LA.

acid [Packer *et al*., 1995; Biewenga *et al*., 1997; Moini *et al*., 2002; Shay *et al*., 2009].

Since the test compounds PS, PSLA and PSDHLA were not completely soluble in either methanol or ethanol, toluene was used as the solvent in the DPPH test [Wettasinghe & Shahidi, 2000]. Further, PSLA and PSDHLA have not been tested previously using DPPH method. Therefore, reaction kinetics at different concentrations was measured and % DPPH remaining at stable state, was plotted against MR showing the interaction with DPPH$^{\bullet}$ at different concentrations [Brand-Williams *et al*., 1995]. This provided a better illustration compared to measuring DPPH activity at 20 or 30 min using one or two selected concentrations of a test compound. According to the results from the DPPH test, PSDHLA showed very high free radical scavenging capacity (Figure 4b) compared with PSLA (Figure 4a). Similar interaction and kinetic behaviour was observed for pure DHLA and 1, 3-diolein derivative of DHLA by the DPPH test [Madawala *et al*., 2011]. In that study LA was also included in the test and showed very low free radical scavenging activity compared with DHLA. Relatively low interaction between PSLA and DPPH$^{\bullet}$ in this study is in agreement as it was observed in LA derivatives of 1,3-diolein [Madawala *et al*., 2011]. In a previous study, LA and several coumarin derivatives of LA dissolved in ethanol at 0.5 mmol/L concentration of the final mixture measured after 20 and 60 min have also



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shown low interaction with DPPH• [Melagraki *et al*., 2009]. Reaction kinetics of PSLA and PSDHLA both showed a slow rate of interaction with DPPH• dependent on concentration. A relatively long time was taken to achieve steady state, especially in PSDHLA, which suggests a slow but continuous interaction. This highlights the importance of measuring free radical activity until the steady state, unlike for some antioxidant compounds which show rapid interaction with DPPH• and achieve steady status within 30–60 min as it was highlighted by Brand-Williams *et al*. [1995]. It has been suggested that PS may also possess antioxidant properties based on some *in vitro* as well as *in vivo* studies [Berger *et al*., 2004; Rudowska, 2009]. However, in this study we could not observe any interaction between DPPH• and pure PS dissolved in toluene for the concentrations (0.06–0.72 mmol/L). The concentrations tested in our study might be too low compared to naturally available levels of PS or enriched diets, which contain PS in mg/g levels. No published result is available on the antioxidant activity of PS using DPPH• test except that in stability of oils [Wang *et al*., 2002].

The effective concentration (EC_{50}) value of an antioxidant compound is considered as a comparative measurement of its antioxidant capacity [Brand-Williams *et al*., 1995]. The EC_{50} value calculated for PSDHLA was 0.43. Since PSLA showed very low free radical scavenging capacity and never reacted with more than 10% of the initial DPPH• even after 5 h,

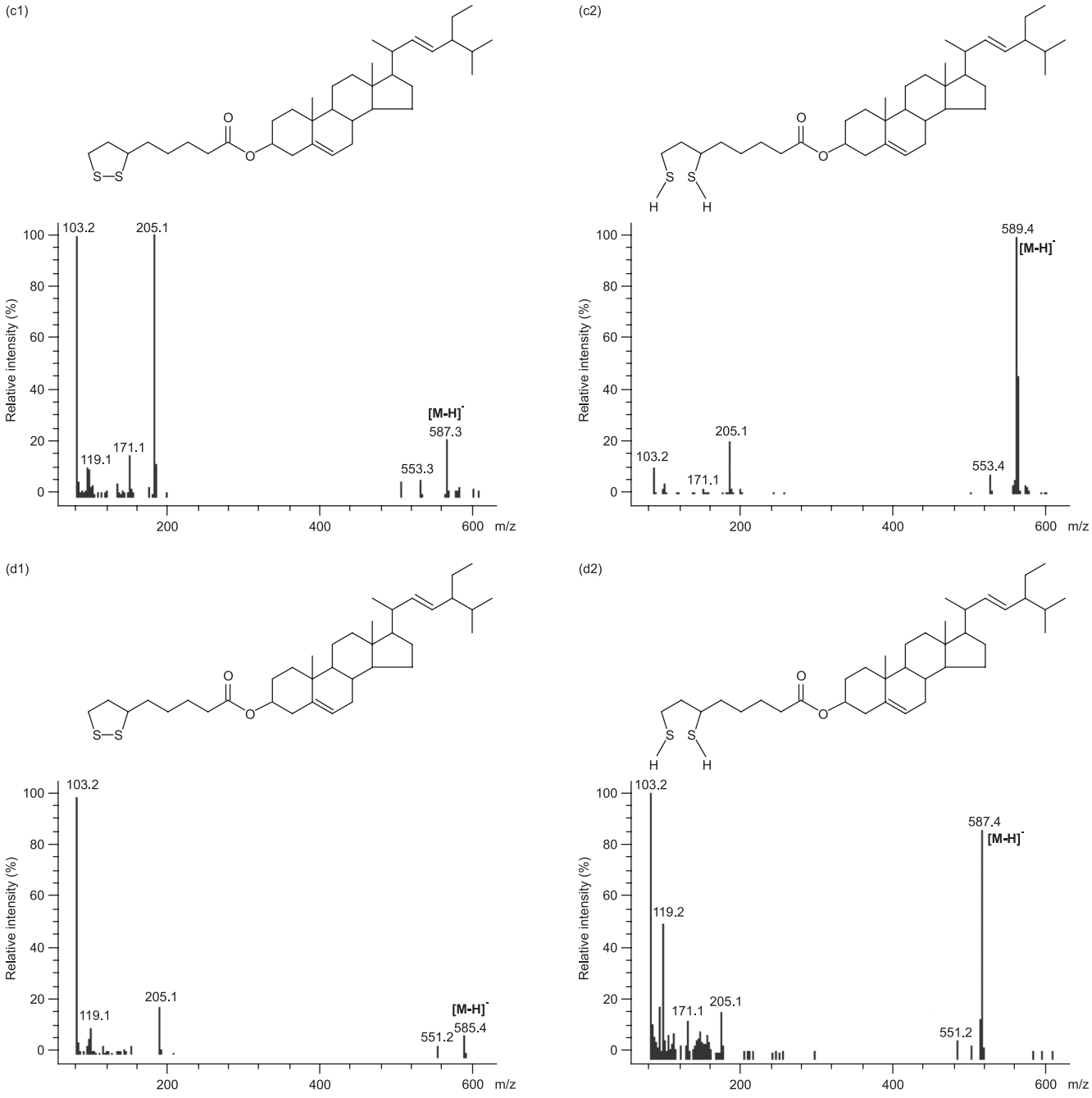

FIGURE 7. RP-HPLC-MS-APCI mass spectra recorded in negative ion mode of PSLA and PSDHLA conjugates showing 2 *m/z* units difference in of molecular ion at $[M-H]^-$. A1, β-sitosterol-LA; A2, β-sitosterol-DHLA; B1, stigmasterol-LA; B2, stigmasterol-DHLA; C1, campesterol-LA; C2, campesterol-DHLA; D1, brassicasterol-LA; D2, brassicaterol-DHLA.

at the MR tested in this study, EC_{50} was not calculated for PSLA. Compared with the anti-radical efficiency of several known antioxidant compounds reported previously by Brand-Williams *et al*. [1995], PSDHLA showed similar free radical scavenging capacity with EC_{50} value similar to ferulic acid (EC_{50} = 0.43), but lower than ascorbic acid (EC_{50} = 0.27) and α-tocopherol (EC_{50} = 0.25). It is suggested that interaction between $DPPH^{\bullet}$ and antioxidant compounds is also dependent on possible steric hindrance due to the molecular structure of the antioxidant compound.

HPLC-MS analysis of PSLA and PSDHLA derivatives were performed, since no previous MS data was available for these compounds except for the initial compounds (individual PS, LA and DHLA) used in the synthesis. Data of RP-HPLC-APCI-MS for LA and DHLA have been published recently [Madawala *et al*., 2011]. The chromatographic conditions were different in the present study but the mass spectra of these compounds were similar (MS data are not shown). The more polar compounds, LA, DHLA and PS eluted first followed by PSDHLA and PSLA in the C_8 column used in this study. The dominant and characteristic ion of all the individual PS were dehydrated molecular ions $[M-H_2O+H]^+$, which concurs with previous reports on analysis of these compounds by APCI-MS [Bedner *et al*., 2008; Rozenberg *et al*., 2003; Lembcke *et al*., 2005]. The individual PS analyzed in our study also showed $[M+H-2H]^+$ and [M+H-

4H]$^+$ fragment ions due to dehydrogenation, are in agreement with mass spectra of PS published by Rozenberg *et al.* [2003]. In addition, a possible adduct formation with acetonitrile was observed in relatively low intensity at [M+H-2H+41]$^+$as shown in Figures 6A1-D1.

The protonated molecular ions [M+H]$^+$ of β-sitosterol-LA, stigmasterol-LA, campesterol-LA and brassicasterol-LA at *m/z* 603, *m/z* 601, *m/z* 589 and *m/z* 587, respectively, were abundant. The other dominant and characteristic fragment ions of these compounds were also observed at *m/z* 397, *m/z* 395, *m/z* 383 and *m/z* 381, respectively. These fragment ions were generated after dissociation of the LA moiety and similar fragment ions were observed for the corresponding individual PS molecules (Figures 6A2-D2). The mass spectra of stigmasterol- and brassicasterol-LA showed an additional fragment ion at *m/z* 255 of relatively low abundance. This may be due to the elimination of side chains with a double bond in these two compounds.

The individual PSLA derivatives analysed in negative ion mode generated low signal under the present experimental conditions, and the deprotonated molecular ion could be observed at low intensity (Figures 7A1-D1) compared with the MS data of these conjugates in positive ion mode (Figures 6A2-D2). Mass spectra recorded in negative mode for all individual PSLA derivatives, showed the dominant fragment ion at *m/z* 205, derived from the LA moiety dissociated at the esterified position. A fragment ion at *m/z* 171 correspond to a loss at *m/z* 34, due to the elimination of H_2S from *m/z* 205 fragment concur with the previously published report [Schupke *et al*., 2001; Madawala *et al.*, 2011].

In negative ion mode, deprotonated molecular ion [M-H]$^-$ of β-sitosterol-DHLA, stigmasterol-DHLA, campesterol-DHLA and brassicasterol-DHLA at *m/z* 603, *m/z* 601, *m/z* 589 and *m/z* 587, respectively, were the most abundant ions observed (Figures 7A2-D2). Further, an abundant ion fragment at *m/z* 205 was observed in negative mode for all derivatives of PSDHLA. This fragment ion can be due to dehydrogenation of DHLA moiety while cleaving from the PSDHLA. Similar dehydrogenated fragment ion at *m/z* 205 was observed for pure DHLA and 1,3diolein dihydrolipoate in a previous study [Madawala *et al*., 2011]. We have suggested that this might be due to high vaporizer and drying gas temperatures used during the ionisation in APCI mode. The fragment ion at *m/z 567* was observed in negative ion mode analysis of both β-sitosterol-LA and β-sitosterol-DHLA. This fragment ion was possibly generated from [M-H-H_2S]$^-$ in PSLA and [M-H-2H-H_2S]$^-$ in PSDHLA. Corresponding fragment ion for -LA and -DHLA derivatives of stigmasterol, campesterol and brassicasterol were observed at *m/z* 565, *m/z* 553 and *m/z* 551, respectively.

CONCLUSIONS

The conjugates PSLA and PSDHLA were chemically synthesized in laboratory scale with the aim that these compounds may possess synergistic effects in cholesterol lowering properties and subsequent decrease in atherosclerosis. The purified PSLA and PSDHLA were stable and remained as a liquid (oil) in room temperature, suggesting effective and uniform blending properties in foods and food products. These hybrid molecules could be developed as potential functional food ingredients in varieties of food products such as oils, margarine, yoghurt *etc*. The free radical scavenging activity of PSLA and PSDHLA which is comparable with LA and DHLA indicates their potential applications as antioxidants in both *in vivo* and *in vitro* systems. The RP-HPLC-MS-APCI analysis provided relatively good separation among individual PS and their esterified derivatives with LA and DHLA. The chromatographic and mass spectral data extracted from this study would be valuable in further studies. In future, *in vivo* studies are anticipated to evaluate the contemplated health promoting effects and bioavailability of PSLA and PSDHLA.

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