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Determination of stigmasterol and cholesterol oxides using atmospheric pressure chemical ionization liquid chromatography/mass spectrometry

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Abstract

A new sensitive HPLC–MS method for the detection of sterol oxidation products was developed. The method was developed and optimized with commercially available cholesterol oxidation products. MS detection was carried out with an ion-trap mass spectrometer using atmospheric pressure chemical ionization in positive ion mode. With this new method, both primary and secondary oxidation products of cholesterol and stigmasterol, including epoxides, could be detected. Applicability of the method was investigated using thermo-oxidized (180 °C) stigmasterol as a model compound. Formation and degradation of two hydroperoxides were observed and formation of eight secondary oxidation products quantitatively determined. In conclusion, this method proved to be a powerful tool for investigating oxidation mechanisms of sterols.

Keywords: Stigmasterol; Cholesterol; Oxidation; APCI; HPLC–MS

1. Introduction

Plant sterols and cholesterol, like other unsaturated lipids, are subject to oxidation when exposed to air. In general, oxidation of sterols is a free radical chain reaction, involving initiation, propagation and termination phases. The rate of oxidation is enhanced by exposure to heat, light, ionizing radiation or a chemical catalyst (Frankel, 1998; Porter, Caidwell, & Mills, 1995). After formation of initiating free radicals, sterol hydroperoxides are formed as primary oxidation products, which decompose to secondary oxidation products with different chemical functional groups, such as hydroxy, epoxy and keto groups. In vitro studies have shown that cholesterol oxides have cytotoxic, mutagenic and atherogenic activities, and thus they may have harmful effects on human health (Schroepher, 2000). Harmful effects, similar to those of cholesterol oxidation products, have also been reported in recent scientific literature for plant sterol oxides (Adcox, Boyd, Oehrl, Allen, & Fenner, 2001; Maguire, Konoplyannikov, Ford, Maguire, & O'Brien, 2003). The toxicity of oxidation products varies depending on their chemical structure (Brown & Jessup, 1999; Schroepher, 2000).

Methods used to analyse plant sterol oxidation products are based on those developed for cholesterol oxides. Their final separation and quantification are usually performed by using gas chromatography methods (Dutta, 1997a, 1997b; Johnsson & Dutta, 2003a, 2003b; Lampi, Juntunen, Toivo, & Piironen, 2002; Soupas, Juntunen, Säynäjoki, Lampi, & Piironen, 2004) or high performance liquid chromatography methods (Caboni, Costa, Rodriguez-Estrada, & Lercker, 1997; Kemmo, Soupas, Lampi, & Piironen, 2005; Säynäjoki, Sundberg, Soupas, Lampi, & Piironen, 2003) with different detection techniques. The major advantage offered by HPLC, compared with GC, is operation at room temperature, making HPLC a suitable method for detection of thermolabile molecules, such as hydroperoxides. Another advantage is that no time-consuming sample pre-treatment or derivatization is needed. Ultra-violet detection is commonly used for cholesterol

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(Caboni et al., 1997; Csallany, Kindom, Addis, & Lee, 1989) and plant sterol (Kemmo et al., 2005; Säynäjoki et al., 2003) oxidation products. However, many researchers have noted that some oxidation products of cholesterol, such as epoxy and dihydroxy sterols (i.e. triols), do not possess adequate UV absorption characteristics (Caboni et al., 1997; Careri, Ferretti, Manini, & Musci, 1998; Manini, Andreoli, Careri, Elviri, & Musci, 1998). We have also observed in our previous study that 5α,6α-epoxystigmasterol and 5β,6β-epoxystigmasterol had lower UV absorptivity than the other oxidation products of stigmasterol (Kemmo et al., 2005). Recently, HPLC–mass spectrometry has become more common for the analysis of cholesterol oxidation products (Careri et al., 1998; Giuffrida, Destaillats, Robert, Skibsted, & Dionisi, 2004; Manini et al., 1998; Razzazi-Fazeli, Kleineisen, & Luf, 2000). Careri et al. (1998) analysed cholesterol, 5α,6α-epoxycholesterol, 25-hydroxycholesterol, 7-ketocholesterol, 7β-hydroxycholesterol and dihydroxycholesterol by using LC–MS with a particle beam interface. Recently HPLC–MS with atmospheric pressure chemical ionisation interface was used for the analysis of cholesterol oxidation products in biological matrices. Manini et al. (1998) analysed five cholesterol oxidation products (5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol, 7-ketocholesterol, 7β-hydroxycholesterol and 7α-hydroxycholesterol) in lyophilized beef, and Razzazi-Fazeli et al. (2000) seven cholesterol oxides (25-hydroxycholesterol 5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol, 7-ketocholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol and dihydroxycholesterol) in different food stuffs. We have previously published an HPLC–MS method for the analysis of stigmasterol hydroperoxides (Kemmo, Ollilainen, Lampi, & Piironen, 2004). Giuffrida et al. (2004) analysed plant sterol epoxides by using HPLC–MS with positive electrospray ionization (ESI). To our knowledge, plant sterol secondary oxidation products other than epoxides have not been analysed previously by HPLC–MS.

The aim of this study was to develop a new sensitive HPLC–MS method for the detection of plant sterol oxidation products. With this instrument, the MS fragmentation behaviour of 20α-hydroxycholesterol, 22-hydroxycholesterol, 6-ketocholesterol and several stigmasterol oxidation products not previously examined was elucidated. Moreover, the applicability of the method for following the oxidation behaviour of sterols at 180 °C was investigated, by using stigmasterol as a model compound.

2. Materials and methods

2.1. Chemicals

5-Cholesten-3β-ol (cholesterol), 5-cholestene-3β,20α-diol (20α-hydroxycholesterol), 5-cholestene-3β,22(S)-diol (22β-hydroxycholesterol), 5-cholestene-3β,25-diol (25-hydroxycholesterol), cholesterol-5α,6α-epoxide (5α,6α-epoxycholesterol), 5α-cholestan-3β-ol-6-one (6-ketocholesterol), 5-cholesten-3β-ol-7-one (7-ketocholesterol), cholest-5-ene-3β,19-diol (19-hydroxycholesterol) and cholestane-3β, 5α,6β-triol (dihydroxycholesterol) were purchased from Sigma Chemical Co, (St. Louis, MO, USA), and 5-cholestene-3β,7α-diol (7α-hydroxycholesterol) and 5-cholestene-3β,7β-diol (7β-hydroxycholesterol) from Steraloids (Wilton, NH, USA). 3β-Hydroxy-24-ethyl-5,22-cholestadiene (stigmasterol), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were obtained from Fluka Chemie AG (Buchs, Switzerland). BSTFA and TMCS were used in a 99:1 ratio for silylation of sterol oxidation products. Reserpine was kindly donated by Dr. Tiia Kuuranne (Department of Pharmaceutical Chemistry, Helsinki, Finland). All organic solvents used were of HPLC grade and purchased from Rathburn Chemicals Ltd. (Walkerburn, UK).

2.2. Thermo-oxidation

Stigmasterol samples (10 mg) were heated in an oven at 180 °C, which corresponds to a deep-frying temperature. The heating time varied from 0 to 60 min (0, 15, 30 and 60 min), and the experiment was carried out in triplicate. The heating was done in open glass vials (22 × 46 mm, Brown Chromatography Supplies Inc., Wertheim, Germany). After thermo-oxidation, the samples were cooled in a dessicator and dissolved in 2 ml of *n*-heptane:isopropanol (98:2, v/v). The solution was filtered through GH Poly-Pro Acrodisc® membrane filters (PALL, Gelman Laboratory, Ann-Arbor, MI, USA) and stored in a freezer until HPLC–MS analysis.

2.3. HPLC–MS analysis

Determination of sterol oxidation products was carried out using HPLC–MS. The separation was performed on a silica Supelcosil column (250 mm × 2.1 mm i.d., 5 μm; Supelco, Bellefonte, PA, USA) at room temperature. The HPLC instrument used was an Agilent 1100, which included a binary pump with a degasser, an autosampler and a column compartment. The best resolution between oxidation products was achieved by using a gradient of heptane (solvent A) and isopropanol (solvent B), programmed as follows: 0–15 min 2% B, 15–35 min from 2% B to 20% B, 35–40 min from 20% B to 2% B and post-run 30 min with 2% B. The total flow rate of the mobile phase was 0.6 ml/min, and the injection volume was 1 μl. The direct online MS detection was carried out with a quadrupole ion-trap mass spectrometer (Esquire-LC, Bruker Daltonik, Bremen, Germany) using APCI in positive ion scanning mode. The performance of the APCI interface was verified using the manufacturer's standard operation procedure with reserpine. The MS parameters were optimized using 5α,6α-epoxycholesterol, 7-ketocholesterol and 7β-hydroxycholesterol because plant sterol oxidation products were not commercially available. The optimization was carried out in direct flow injection mode of standard solutions mixed with

the mobile phase (0.6 ml/min). The Esquire HPLC–MS was tuned for each of the three compounds by using the automatic optimization mode included in the Controlling Software program (version 4.5); dry gas and APCI interface temperatures were optimized manually by using temperatures between 200 and 350 °C at 25 °C intervals. Collision-induced dissociation was produced with helium (99.96% AGA, Finland) at a pressure of 0.6 mPa in the ion trap. The optimized temperature and flow rate of the dry gas (nitrogen) were 300 °C and 7.00 l/min, respectively, and the APCI interface temperature was 300 °C. The pressure of the nebulizer gas (nitrogen) was 206.9 kPa, and the voltage of the corona discharge needle was 3500 V. Capillary voltage was −3600 V, end-plate offset −750 V, skimmer (I) 27.5 V and skimmer (II) 6.5 V. The octopole was at 2.7 V, octopole Δ 2.3 and octopole RF 160.7 (Vpp). The trap ion current control value was set at 35,000 units, and maximal accumulation time was 200 ms. APCI mass spectra were recorded using a scan range of 150–700 *m/z* and summation of eight spectra.

The optimized capillary exit offset, capillary exit and trap drive values were different for 5α,6α-epoxycholesterol, 7-ketocholesterol and 7β-hydroxycholesterol. For 5α,6α-epoxycholesterol, these were 38 V, 65.5 V and 44; for 7-ketocholesterol, 85 V, 112.5 V and 44; for 7β-hydroxycholesterol, 38 V, 65.5 V and 44.6, respectively. Because certain optimized MS values were different for each of the cholesterol oxidation products, three time segments were made: from 0 to 9.5 min the parameters optimized for 5α,6α-epoxycholesterol were applied; from 9.5 to 15.5 min the parameters optimized for 7-ketocholesterol were applied; and from 15.5 to 40 min the parameters optimized for 7β-hydroxycholesterol were applied.

Stigmasterol hydroperoxides and secondary oxidation products formed during thermo-oxidation were identified by GC–MS, as previously published by us (Kemmo et al., 2005; Säynäjoki et al., 2003). The quantitative determination of stigmasterol oxidation products by HPLC-APCI was made using 5α,6α-epoxycholesterol, 7-ketocholesterol and 7β-hydroxycholesterol as external standards. Since the in-source fragmentation could not be completely avoided in spite of careful optimization procedure of APCI parameters, the quantification was based on the summation of certain ions of the analytes: 5α,6α-epoxycholesterol was quantified by using the sum of ions *m/z* 403.3 $[M+H]^+$, 385.3 $[M-H_2O+H]^+$and 367.3 $[M-2H_2O+H]^+$, 7-ketocholesterol by using the sum of ions *m/z* 401.3 $[M+H]^+$, 383.3 $[M-H_2O+H]^+$ and 365.3 $[M-2H_2O+H]^+$ and 7β-hydroxycholesterol by using the sum of ions *m/z* 403.3 $[M+H]^+$, 385.3 $[M-H_2O+H]^+$ and 367.3 $[M-2H_2O+H]^+$. The concentrations of 5α,6α-epoxycholesterol, 7-ketocholesterol and 7β-hydroxycholesterol calibration solutions were measured by GC-(flame ionization detection) (Lampi et al., 2002). Five concentration levels in an external standard calibration curve were used with ranges of 7.9–370.3 μg/ml for 5α,6α-epoxycholesterol, 8.6–404.7 μg/ml for 7-ketocholesterol and 11.0–525.1 μg/ml for 7β-hydroxycholesterol. The calibration curve was measured at the beginning of each of the three oxidation experiments. Calculation of the detection limit for the compounds studied was based on a signal-to-noise ratio of 3. Linearity of the calibration graphs was evaluated using regression analysis and comparison of regression lines with Statgraphics Plus 4.0 software (Statistical Graphics Corp., USA).

Epoxides, 5α,6α-epoxystigmasterol and 5β,6β-epoxystigmasterol were quantified with 5α,6α-epoxycholesterol by using an extracted ion chromatogram obtained by the summation of ion *m/z* values of 429.4 $[M+H]^+$, 411.4 $[M-H_2O+H]^+$ and 393.3 $[M-2H_2O+H]^+$. Keto compounds, 24-ethylcholest-4,22-diene-6α-ol-3-one (6α-hydroxy-3-ketostigmasterol), 24-ethylcholest-4,22-diene-6β-ol-3-one (6β-hydroxy-3-ketostigmasterol) and 7-ketostigmasterol were determined with 7-ketocholesterol by using ion *m/z* values of 427.4 $[M+H]^+$, 409.4 $[M-H_2O+H]^+$ and 391.4 $[M-2H_2O+H]^+$. Quantification of hydroxy derivatives, 6β-hydroxystigmasterol, 7β-hydroxystigmasterol and 7α-hydroxystigmasterol was done with 7β-hydroxycholesterol, using ion *m/z* values of 411.4 $[M-H_2O+H]^+$ and 393.3 $[M-2H_2O+H]^+$. The hydroperoxides formed during thermo-oxidation were not quantified in this study because neither plant sterol nor cholesterol hydroperoxides were commercially available.

3. Results and discussion

We developed a sensitive HPLC–MS method for the determination of sterol oxidation products. With this method, both hydroperoxides and secondary oxidation products could be detected directly after dissolving the sample, with no time-consuming sample pre-treatment or derivatization. The method proved to be a useful tool in investigating oxidation mechanisms of sterols.

3.1. Optimization of HPLC–MS parameters

3.1.1. HPLC separation

The results obtained for oxidation products of cholesterol have shown that epimeric isomers have similar MS fragmentation patterns (Razzazi-Fazeli et al., 2000). Thus, proper separation of epimeric isomers is necessary. Normal-phase chromatography was chosen because of its ability to separate both positional and epimeric isomers, which cannot be done on size and alkyl group specific reverse-phase columns. Full resolution over the entire polarity range of sterol oxidation products has not been achieved with isocratic solvent systems (Kemmo et al., 2005; Maerker, Nungesser, & Zulak, 1988). The best separation between nine commercially available cholesterol oxidation products (20α-hydroxycholesterol, 22-hydroxycholesterol, 25-hydroxycholesterol, 5α,6α-epoxycholesterol, 6-ketocholesterol, 7-ketocholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol and dihydroxycholesterol) was achieved by using a gradient of heptane and isopropanol (Fig. 1). Separation of oxidation products was achieved

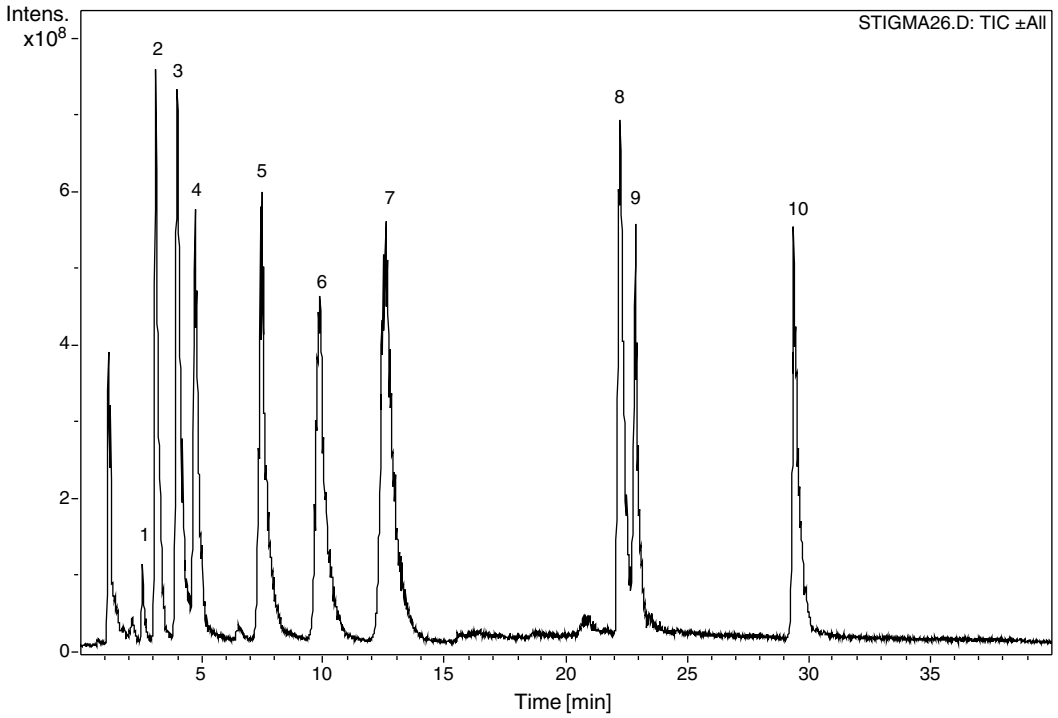

Fig. 1. Total ion monitoring of cholesterol oxidation products: 1 = cholesterol, 2 = 20α-hydroxycholesterol, 3 = 22-hydroxycholesterol, 4 = 25-hydroxycholesterol, 5 = 5α,6α-epoxycholesterol, 6 = 6-ketocholesterol, 7 = 7-ketocholesterol, 8 = 7β-hydroxycholesterol, 9 = 7α-hydroxycholesterol, 10 = dihydroxycholesterol.

within 30 min, and retention times were reproducible between chromatographic runs. The chromatographic separation was generally good; the resolution value of 1.3 was obtained for the critical peak pair (peaks 8 and 9). With this gradient elution we could separate nine stigmasterol secondary oxidation products and two hydroperoxides and the peaks eluted in the same order as the corresponding cholesterol oxidation products.

3.1.2. Optimization of MS parameters

Atmospheric pressure chemical ionization is considered to be a soft ionization technique and should be therefore a suitable procedure for labile compounds such as hydroperoxides. Regardless of optimization procedure, the in-source fragmentation of certain pseudomolecule ions $[M+H]^+$ occur in our instrument. The MS parameters were optimized by using 5α,6α-epoxycholesterol, 7-ketocholesterol and 7β-hydroxycholesterol and direct flow injection mode of standards to the MS detector. The results showed that the signal intensities of two main ions ($[M+H]^+$, $[M-H_2O+H]^+$), recorded in full scan mode at *m/z* 150–700, varied between the dry gas and APCI interface temperatures tested. An optimum for 5α,6α-epoxycholesterol and 7-ketocholesterol was reached at 300 °C, whereas for 7β-hydroxycholesterol the best signal was recorded at 350 °C. As temperatures could not be changed during the run, 300 °C was chosen. No changes in fragmentation pattern of the tested compounds were observed over the applied temperature range of 200–350 °C which is consistent with the findings of Razzazi-Fazeli et al. (2000). For other optimized MS parameters, the values yielding the highest ion signal intensities were chosen.

3.2. Mass spectra and APCI fragmentation of cholesterol and stigmasterol oxides

The specific fragmentation behaviour and relative ion abundances of cholesterol oxidation products are presented in Table 1A. The spectra are characterized by few fragment ions, due to loss of a variable number of water molecules (up to three), dependent upon the number of hydroxyl groups in the molecule. The protonated molecular ion $[M+H]^+$ was the main peak only in the case of 7-ketocholesterol, which corresponds to the results of other researchers for 7-ketocholesterol (Manini et al., 1998; Razzazi-Fazeli et al., 2000). The protonated molecule ion was also detectable in the spectra of 5α,6α-epoxycholesterol and 6-ketocholesterol, althought in low abundances, 15% and 8%, respectively. The fragmentation behaviours of cholesterol, 25-hydroxycholesterol, 5α,6α-epoxycholesterol, 7-ketocholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol and dihydroxycholesterol were similar to earlier reports; only the relative ion abundances were slightly different (Manini et al., 1998; Razzazi-Fazeli et al., 2000). The mass spectra of two new side-chain oxidation products 20α-hydroxycholesterol and 22-hydroxycholesterol and also 6-ketocholesterol were obtained. The main fragments in the mass spectra of 20α-hydroxycholesterol and 22-hydroxycholesterol were *m/z* 385.3 $[M-H_2O+H]^+$ and 367.3 $[M-2H_2O+H]^+$. The main fragments of

Table 1A
Characteristic mass spectral ions of cholesterol oxidation products and their relative ion abundances (%)

Cholesterol oxides	RT (min)	k'	Characteristic ions m/z			
			$M+H^+$	$M-H_2O+H^+$	$M-2H_2O+H^+$	$M-3H_2O+H^+$
Cholesterol	2.5	2.6	387.3 (–)	369.3 (100)		
20α-Hydroxy	3.1	3.4	403.3 (–)	385.3 (46)	367.3 (100)	
22-Hydroxy	4.0	4.7	403.3 (–)	385.3 (100)	367.3 (87)	
25-Hydroxy	4.7	5.7	403.3 (–)	385.3 (25)	367.3 (100)	
5α,6α-Epoxy	7.5	9.7	403.3 (15)	385.3 (100)	367.3 (29)	
6-Keto	9.8	13.0	403.3 (8)	385.3 (100)	367.3 (61)	
7-Keto	12.6	17.0	401.3 (100)	383.3 (53)	365.3 (–)	
7β-Hydroxy	22.2	30.7	403.3 (–)	385.3 (100)	367.3 (79)	
7α-Hydroxy	22.8	31.6	403.3 (–)	385.3 (100)	367.3 (88)	
Dihydroxy	29.5	41.1	421.3 (–)	403.3 (3)	385.3 (100)	367.3 (21)

A dash indicates that ions were not observed.
RT = retention time.
k' = capacity factor ($t_0 = 0.7$ min).

6-ketocholesterol were m/z 403.3 $[M+H]^+$, 385.3 $[M-H_2O+H]^+$ and 367.3 $[M-2H_2O+H]^+$.

Stigmasterol oxidation products identified in our previous studies using LC fractionation and GC–MS (Kemmo et al., 2004; Kemmo et al., 2005; Säynäjoki et al., 2003) were seen to have similar fragmentation behaviour to cholesterol oxidation products (Table 1B). Only compounds with conjugated diene structure, i.e. 6α-hydroxy-3-ketostigmasterol, 6β-hydroxy-3-ketostigmasterol and 7-ketostigmasterol, had $[M+H]^+$ at the highest abundance, similar to 7-ketocholesterol. The molecular ion $[M+H]^+$ was also detectable in both 5α,6α-epoxystigmasterol and 5β,6β-epoxystigmasterol spectra, but in lower abundances, 27% and 8%, respectively. Molecular ions were not observed for any other stigmasterol oxides. In general, fragments indicating a loss of one to three water molecules, $[M-H_2O+H]^+$, $[M-2H_2O+H]^+$and $[M-3H_2O+H]^+$, were observed. In addition to loss of water molecules, the fragments representing loss of hydrogen peroxide, $[M-H_2O_2+H]^+$, or hydrogen peroxide and water, $[M-H_2O_2-H_2O+H]^+$, were present in the mass spectra of 7α-hydroperoxy and 7β-hydroperoxystigmasterol (Kemmo et al., 2004). Our findings correspond to the results of Kusaka and Ikeda (1993), who reported that fatty acid hydroperoxides formed no molecular ions, but instead fragments representing loss of water or loss of hydrogen peroxide. Mobile phase adducts were not observed in this study.

To the best of our knowledge, these are the first results of plant sterol secondary oxidation products using the APCI-MS technique. Fragmentation behaviour of sterol epoxides, using HPLC–MS with positive ESI, was recently published by Giuffrida et al. (2004). The fragment ion of stigmasterol epoxides in our study was different from theirs, due to the different ionization technique used. They used ammonium formate to enhance the ionization efficiency in electrospray ionization, therefore observing an intense molecular ion with an ammonium adduct ion $[M+NH_4]^+$ at m/z 446.4, in addition to the ions $[M+H]^+$, $[M-H_2O+H]^+$ and $[M-2H_2O+H]^+$.

Table 1B
Characteristic mass spectral ions of stigmasterol oxidation products formed in thermo-oxidation at 180 °C for 60 min and their relative ion abundances (%)

Stigmasterol oxides	RT (min)	k'	Characteristic ions m/z					
			$M+H^+$	$M-H_2O+H^+$	$M-2H_2O+H^+$	$M-3H_2O+H^+$	$M-H_2O_2+H^+$	$M-H_2O_2-H_2O+H^+$
Stigmasterol	2.8	2.5	413.4 (–)	395.4 (100)				
6β-Hydroxy-3-keto	5.0	5.3	427.4 (100)	409.4 (3)	391.4 (–)			
6α-Hydroxy-3-keto	6.3	6.9	427.4 (100)	409.4 (1)	391.4 (–)			
5α,6α-Epoxy	7.4	8.3	429.4 (27)	411.4 (100)	393.4 (25)			
5β,6β-Epoxy	8.5	9.6	429.4 (8)	411.4 (100)	393.4 (9)			
7-Keto	12.5	14.3	427.4 (100)	409.4 (58)	391.4 (–)			
7β-Hydroperoxy	14.5	17.1	445.4 (–)	427.4 (42)	409.4 (100)		411.4 (29)	393.4 (13)
7α-Hydroperoxy	17.5	20.9	445.4 (–)	427.4 (25)	409.4 (100)		411.4 (41)	393.4 (12)
6β-Hydroxy	20.1	24.1	429.4 (–)	411.4 (100)	393.4 (15)			
7β-Hydroxy	22.2	26.8	429.4 (–)	411.4 (100)	393.4 (96)			
7α-Hydroxy	22.8	27.5	429.4 (–)	411.4 (100)	393.4 (94)			
Dihydroxy	29.0	35.3	447.4 (–)	429.4 (13)	411.4 (100)	393.4 (27)		

A dash indicates that ions were not observed.
RT = retention time.
k' = capacity factor ($t_0 = 0.8$ min).

3.3. Suitability of the HPLC–MS method for monitoring of oxidation behaviour of stigmasterol

The eight secondary oxidation products of stigmasterol formed during thermo-oxidation at 180 °C were quantified (Fig. 2). Formation of dihydroxystigmasterol was observed at the last time-point (60 min). The formation and degradation of 7α-hydroperoxystigmasterol and 7β-hydroperoxystigmasterol were followed; however, they were not quantified since sterol hydroperoxide standards were unavailable commercially.

The detection limit for 5α,6α-epoxycholesterol, 7-ketocholesterol and 7β-hydroxycholesterol was 0.3 ng/injection. Hence, this method proved to be about 20 times more sensitive in detecting compounds with conjugated diene structure and over 60 times more sensitive for other oxidation products than the HPLC–UV–FL method (Kemmo et al., 2005). The detection limit obtained was very similar to that (0.2–0.8 ng) reported by Manini et al. (1998) for cholesterol oxidation products, using HPLC–MS with an APCI interface. The plots of peak areas versus concentrations of standards revealed excellent linearity across the concentration ranges used. The slopes, intercepts, correlation coefficients and standard errors are presented in Table 2.

Formation of stigmasterol secondary oxidation products at 180 °C is presented in Fig. 3. Stigmasterol oxide contents were calculated from similar MS ions to the respective cholesterol derivatives as described in Section 2.3. The coefficients of variation (CV) for the triplicate experiments were typically below 20%. The CV values of some products, particularly those with concentrations below 2 mg/g, were over 20%. When evaluating the CV values obtained, it must be borne in mind that these are influenced by both the nature of the lipid oxidation and the analysis method used. CV values obtained here are similar to values yielded by the HPLC–UV–FL method (Kemmo et al., 2005).

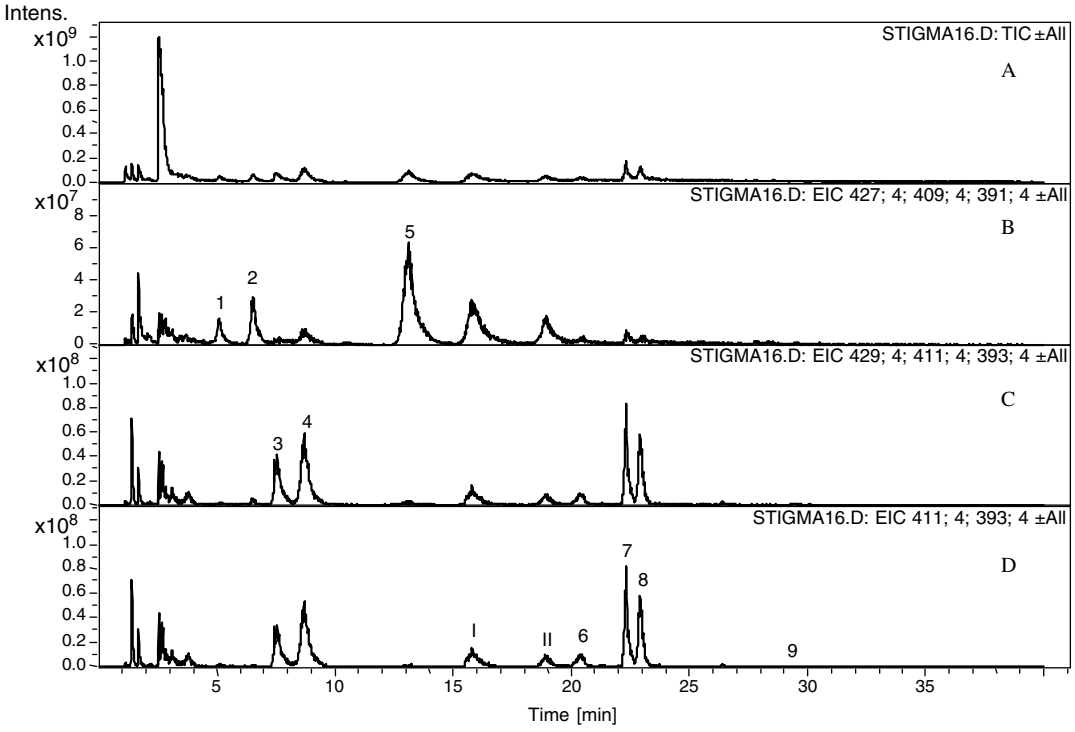

Fig. 2. Total ion chromatogram at *m/z* 150–700 (A) and extracted ion chromatograms of sums of ions *m/z* (427.4 + 409.4 + 391.4) (B), (429.4 + 411.4 + 393.4) (C), and (411.4 + 393.4) (D) of stigmasterol oxidation products formed during thermo-oxidation for 10 min at 180 °C. 1 = 6β-hydroxy-3-ketostigmasterol, 2 = 6α-hydroxy-3-ketostigmasterol, 3 = 5α,6α-epoxystigmasterol, 4 = 5β,6β-epoxystigmasterol, 5 = 7-ketostigmasterol, 6 = 6β-hydroxystigmasterol, 7 = 7β-hydroxystigmasterol, 8 = 7α-hydroxystigmasterol, 9 = dihydroxystigmasterol, I = 7β-hydroperoxystigmasterol, II = 7α-hydroperoxystigmasterol.

Table 2
Linearity of calibration graphs

Standard	Slope	Slope sd	Intercept	Intercept sd	Standard error	r^2
5α,6α-Epoxycholesterol	1.700×10^{10}	5.388×10^{8}	-2.266×10^{7}	1.046×10^{8}	2.636×10^{8}	0.994
7-Ketocholesterol	2.908×10^{10}	6.753×10^{8}	2.197×10^{7}	1.432×10^{8}	3.611×10^{8}	0.997
7β-Hydroxy-cholesterol	1.360×10^{10}	3.598×10^{8}	1.429×10^{8}	9.915×10^{7}	2.498×10^{8}	0.995

Five data points with three injections ($n = 5$, $r = 3$).

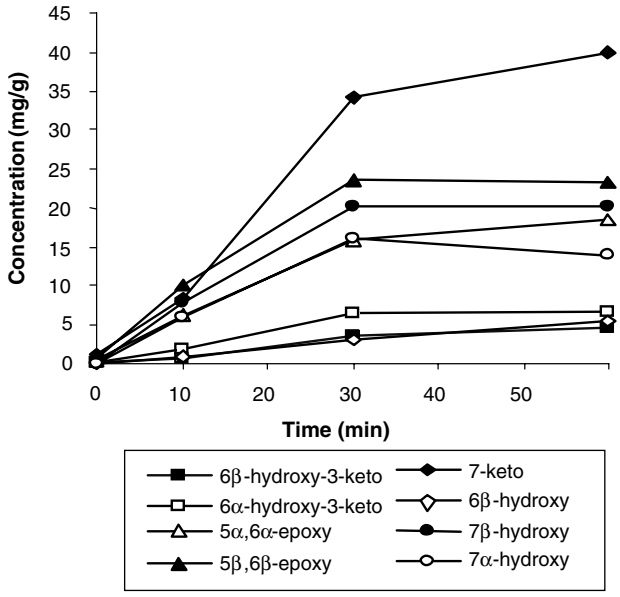

Fig. 3. Formation of stigmasterol oxidation products during heating at 180 °C. Experiments carried out in triplicate.

The amounts of secondary oxidation products increased during oxidation, reaching maximum values at 60 min. These values were 5 mg/g for 6β-hydroxy-3-ketostigmasterol, 7 mg/g for 6α-hydroxy-3-ketostigmasterol, 18 mg/g for 5α,6α-epoxystigmasterol, 23 mg/g for 5β,6β-epoxystigmasterol, 40 mg/g for 7-ketostigmasterol, 5 mg/g for 6β-hydroxystigmasterol, 20 mg/g for 7β-hydroxy-stigmasterol and 14 mg/g for 7α-OH-stigmasterol. The oxidation behaviour of stigmasterol was similar to in our previous report (Kemmo et al., 2005). 7-Ketostigmasterol was the main oxidation product after 30 min of oxidation. Before 30 min, 5β,6β-epoxystigmasterol was the main product, followed by 7β-hydroxy-stigmasterol, 5α,6α-epoxystigmasterol and 7α-hydroxy-stigmasterol. As compared with our previous results using the HPLC–UV–FL method, a larger amount of stigmasterol epoxides were observed. In our earlier studies, the amounts of stigmasterol epoxides was underestimated because of the limited UV responses of epoxides. Stigmasterol epoxides contain only one double bond in the side-chain, while other stigmasterol oxidation products contain one double bond in the ring structure and one in the side-chain.

4. Conclusions

The main advantage of this new HPLC–MS method is its ability to simultaneously detect primary and secondary oxidation products of sterols. The method is rapid and sensitive. Moreover, it enables analysis of epoxy and dihydroxy compounds, which are difficult to analyse using traditional HPLC methods. The APCI-MS results for 20α-hydroxycholesterol, 22-hydroxycholesterol, 6-ketocholesterol and many stigmasterol oxidation products, which have to our knowledge not been previously described, were now obtained. In conclusion, this method has proven to be a powerful tool for investigating the oxidation mechanisms of sterols.

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