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Determination of stigmasterol primary oxidation products by high-performance liquid chromatography

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Abstract

A new specific chromatographic method for the determination of plant sterol primary oxidation products, hydroperoxides, was developed. Separation was performed by normal-phase high-performance liquid chromatography (NP-HPLC) using two types of detectors. A UV detector was used as a general detector and a fluorescence detector after post column diphenyl-1-pyrenylphosphine reagent addition (DPPP-FL detection) as a hydroperoxide-specific detector. Stigmasterol was used as a model compound. Hydroperoxides were obtained by photo-oxidation in dichloromethane in the presence of methylene blue as a sensitizer. Specificity of the DPPP-FL detection was confirmed by establishing the structures of the main hydroperoxides by gas chromatography mass spectrometry. 5α-Hydroperoxide, 7α-hydroperoxide and 6α- and 6β-hydroperoxides were identified by comparing mass spectral data and retention times with those of the corresponding hydroxy derivatives of cholesterol. Moreover, the suitability of the method for monitoring the formation of hydroperoxides during photo-oxidation was demonstrated. The results showed that the new NP-HPLC method with DPPP-FL detection provides information about the structures and amounts of different plant sterol hydroperoxides in a sample without prior derivatization, complex sample pre-treatment or heating.

Keywords: Stigmasterol; Photo-oxidation; Hydroperoxides; Normal-phase high-performance liquid chromatography; Diphenyl-1-pyrenylphosphine; Gas chromatography mass spectrometry; Peroxide values

1. Introduction

The beneficial health effects of plant sterols have been demonstrated in several studies. Results have shown that plant sterols reduce serum total cholesterol and LDL cholesterol levels (Hallikainen, Sarkkinen, & Uusitupa, 2000; Hendriks, Weststrate, van Vliet, & Meijer, 1999; Jones et al., 2000; Miettinen, Puska, Gylling, Vanhanen, & Vartiainen, 1995). In vitro studies have shown that cholesterol oxides have cytotoxic, mutagenic and atherogenic activities and thus they may have harmful effects on human health (Adcox, Boyd, Oehrl, Allen, & Fenner, 2001; Schroepher, 2000). Due to their structural similarity (Fig. 1), plant sterol oxidation products may have similar health implications and therefore oxidation might be a problem, especially in plant sterol-enriched functional foods.

Plant sterols and cholesterol, like other unsaturated lipids, are subjected to oxidation when exposed to air. Oxidation is enhanced by heat, ionising radiation, exposure to light or by chemical catalysts (Csallany, Kindom, Addis, & Lee, 1989). In general, oxidation of sterols is a free radical chain reaction called autoxidation. Oxidation may also begin by an attack of reactive singlet oxygen, as in photo-oxidation. Photo-oxidation provides an important way to produce hydroperoxides from unsaturated fatty acids and esters in the presence of oxygen, light energy and photosensitizer (Frankel, 1998). Photosensitizers absorb visible or near-UV light to become electronically excited. Sensitizer in the triplet state interacts with oxygen by energy transfer to give singlet oxygen ($^1O_2$), which reacts further with unsaturated lipids. Singlet oxygen is highly electrophilic and reacts rapidly with the double bonds of the sterol by a cyclic

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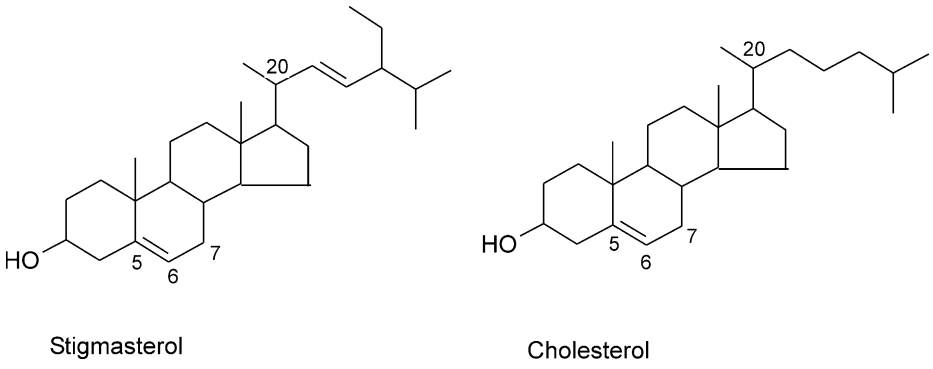

Fig. 1. Structures of stigmasterol and cholesterol.

ene mechanism which results in oxygen insertion at one of the carbon atoms of a double bond (Frankel, 1998; Kulig & Smith, 1973). In all cases, unstable neutral primary oxidation products, i.e. hydroperoxides, are formed. Hydroperoxides give rise to secondary oxidation products, which have multiple chemical functional groups (hydroxy, keto and epoxy compounds).

Cholesterol and plant sterol hydroperoxides have mainly been isolated and separated on the basis of their thin-layer chromatographic (TLC) mobilities and specific colour reactions (Bortolomeazzi, De Zan, Pizzale, & Conte, 1999; Kulig & Smith, 1973; Smith & Hill, 1972). Final identification is usually made after reduction to the corresponding hydroxides by gas chromatography mass spectrometry (GC–MS) (Bortolomeazzi et al., 1999) or hydrogen nuclear magnetic spectroscopy (NMR) (Beckwith, 1989). TLC is a very rapid sample isolation and separation method but it allows exposure of the samples to potential oxidation as a result of its large surface area and it also suffers from low loading capacity (Csallany et al., 1989).

A method commonly applied in the measurement of lipid oxidation is the peroxide value (PV). With PV, the total amount of hydroperoxides may be determined easily and rapidly with no expensive equipment. However, in order to obtain more information than the total amount of hydroperoxides, chromatographic methods are needed (Hartvigsen, Hansen, Lund, Bukhave, & Hølmer, 2000). Recently, several new chromatographic methods have been developed for the determination of lipid hydroperoxides. In addition to quantitative data, these methods provide information on the molecular structures and the amounts of specific hydroperoxides present in a certain sample. In particular, liquid chromatography (HPLC) has become a popular technique for the analysis of hydroperoxides. Its major advantage compared with GC is operation at room temperature, which decreases the risk of artefact formation. Moreover, derivatization before analysis is not needed (Csallany et al., 1989; Manini, Andreoli, Careri, Elviri, & Musci, 1998).

One interesting approach to increase the sensitivity and specificity of hydroperoxide HPLC analysis has been the use of a post-column reaction. Mäkinen, Piironen, and Hopia (1996) and Yamamoto and Ames (1987) introduced a method for determining lipid hydroperoxides by post column chemiluminescence detection. Other examples of post column detection of lipid hydroperoxides include fluorescence detection after post column diphenyl-1-pyrenylphosphine (DPPP) addition (DPPP-FL detection) (Akasaka, Ohrui, & Meguro, 1988; Akasaka, Suzuki, Ohrui, & Meguro, 1987a, 1987b). This new method can be used with many solvents with different polarities and therefore it is suitable for both reversed-phase (RP) and normal-phase (NP) HPLC separation modes. The method is sensitive, simple and selective. Some applications of this method have already been reported for analyses of hydroperoxides of triacylglycerol, cholesterol esters (Akasaka, Ohrui, & Meguro, 1994; Akasaka, Ohrui, & Meguro, 1993a, 1993b, 1993c; Akasaka, Ohta, Hanada, & Ohrui, 1999), methyl linoleate (Ohshima, Hopia, German, & Frankel, 1996) and phosphatidylcholine (Akasaka et al., 1988, 1993c, 1999). However, plant sterol hydroperoxides have not been analyzed with this method. The method is a suitable detection method for hydroperoxides, but final identification must always be made by other techniques such as GC–MS.

The aim of this study was to develop an NP-HPLC method with DPPP-FL detection to specifically detect and investigate the formation of plant sterol hydroperoxides. Stigmasterol was used as a model compoud and its hydroperoxides were obtained by photo-oxidation. The specificity of the detection was confirmed by identification of the main hydroperoxides as hydroxides by GC–MS. The method was needed in order to facilitate studies of the oxidation mechanisms of plant sterols.

2. Materials and methods

2.1. Standards and reagents

Cholest-5-en-3β-diol (cholesterol), cholest-5-en-3β,25-diol (25-hydroxycholesterol), cholestan-5α,6α-epoxy-3β-

ol (5α,6α-epoxycholesterol), cholest-5-en-3β-ol-7-one (7-ketocholesterol), cholestan-3β,5α,6β-triol (cholestanetriol), 24α-ethylcholest-5,22-dien-3β-ol (stigmasterol), 2,6-di-t-butyl-p-cresol (BHT) and ammonium thiocyanate were purchased from Sigma Chemical Co (St. Louis. MO, USA), and cholest-5-en-3β,7α-diol (7α-hydroxycholesterol) from Steraloids (Wilton, NH, USA). Methylene blue, sodium chloride (NaCl) and iron(III)chloride (Titrisol) were purchased from E. Merck (Darmstadt, Germany), and sodium borohydride ($NaBH_4$) and sodium sulfate (Na_2SO_4) from Fluka (Buchs, Switzerland). Iron(II)chloride-4-hydrate was obtained from Riedel-de-Haën, Germany. N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA; >98%; E.Merck, Darmstadt, Germany) and trimethylcholosilane (TMS; 99%; Fluka Chemie AG, Buchs, Switzerland) were used as a 99:1 mixture for silylation of hydroxysterols. All organic solvents used were of HPLC-grade and were purchased from Rathburn Chemicals Ltd. (Walkerburn, UK). Diphenyl-1-pyrenylphosphine (DPPP) was kindly donated by Dr. Toshiaki Ohshima (Department of Food Science and Technology, Tokyo, Japan).

2.2. Photo-oxidation

Hydroperoxides were produced by photo-oxidation. Stigmasterol (20 mg) was dissolved in 1.95 ml of dichloromethane. The photo-oxidation was sensitized with 50 μl methylene blue (0.5 mg/ml) in dichloromethane. The solution in the flask (45 mm × 25 mm i.d.) was exposed to a 75-watt light source (2000 lux) through a 3-cm layer of water. The water layer filters out the infrared radiation. The time of photo-oxidation varied from 0 to 90 min (0, 30, 60 and 90 min) and the experiment was repeated twice. After photo-oxidation, dichloromethane was evaporated under a stream of nitrogen and the residual photo-oxidized stigmasterol was dissolved in 4 ml of *n*-heptane-isopropanol (97:3, v/v). After that, the solution was filtered through GHP membrane filters (PALL, Gelman laboratory, USA) to remove the photosensitizer methylene blue. Cholesterol hydroperoxides were also produced with this method to assist the final identification of the stigmasterol hydroperoxides.

2.3. HPLC analysis

Separation was performed by an NP-HPLC method, followed by DPPP-FL detection. The HPLC conditions were slightly modified from the previously published methods (Akasaka et al., 1993b; Ohshima et al., 1996). The mobile phase was *n*-heptane-isopropanol (97:3, v/v) and the separation was performed on a silica Supercosil column (250 mm × 2.1 mm i.d., 5 μm; Supelco, Bellefonte, PA, USA). The flow rate of the mobile phase was 0.6 ml/min. After separation, the HPLC eluent was monitored by a UV detector (Waters 486, Milford, USA) at 206 nm, prior to the post column reaction with DPPP. Both the mobile phase pump and the DPPP reagent pump were model Waters 510 (Milford, USA). The DPPP solution (3 mg DPPP/400 ml of 1-butanol-methanol, 1:1, containing 100 mg BHT) was added to the eluent at a rate of 0.4 ml/min. The mixture reacted in a stainless steel coil (10 m × 0.5 mm i.d.) at 80 °C (MGW Lauda thermometer, Germany). The non-fluorescent DPPP-reagent reacts specifically and quantitatively with various lipid hydroperoxides to produce fluorescent DPPP oxides (Fig. 2) (Akasaka et al., 1987b; Ohshima et al., 1996). After the reaction the eluent from the coil was cooled to room temperature by passing through a short stainless steel coil (0.5 m × 0.5 mm i.d.), placed in a water jacket. Detection was performed by monitoring the fluorescence intensity at 380 nm, after using excitation at 352 nm. The fluorescence detector used was a Hewlett Packard 1046 A (Waldbronn, Germany). The injection volume was 50 μl and all samples were injected twice. Data handling was performed using Millenium 2010 software (Waters, Milford, USA).

The functioning of the instrument was checked daily with a methyl linoleate hydroperoxide solution (0.765

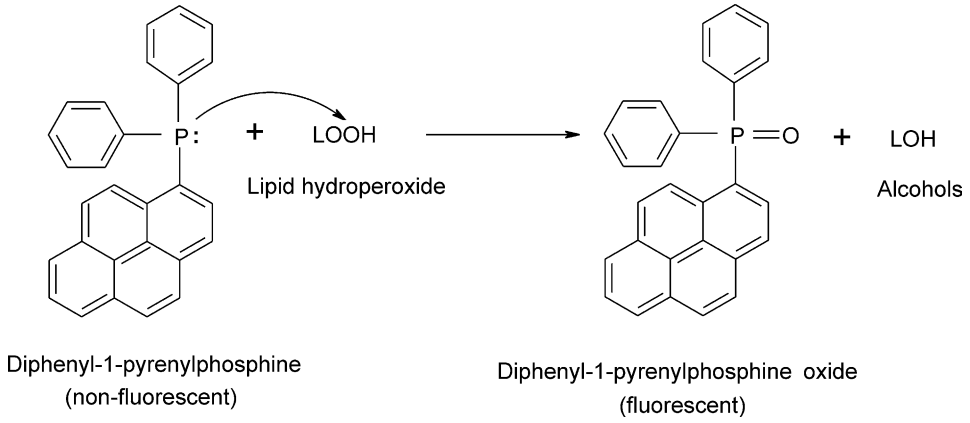

Fig. 2. Reaction of diphenyl-1-pyrenylphosphine (DPPP) with hydroperoxides (Akasaka & Ohrui, 2000).

mg/ml) (MeLo) by monitoring the retention time and detector response. The MeLo hydroperoxide fraction was extracted from autoxidized MeLo and diluted in heptane. The amounts of different stigmasterol hydroperoxide isomers were studied by measuring the peak areas of the fluorescence detection. Because no hydroperoxide standard was available, the total amount of hydroperoxides was estimated by measuring PV, which was determined in each sample spectrophotometrically by the ferric thiocyanate method. The method used was slightly modified from that presented by Ueda, Hayashi, and Namiki (1986). The specificity of the post column detection was investigated with cholesterol secondary oxidation products (concentrations varied from 0.6 mg/ml to 1.4 mg/ml). Cholesterol oxidation products were used because no plant sterol oxide standards are commercially available.

2.4. Confirmation of the hydroperoxide structures

To further confirm the specifity of the DPPP-FL detection, GC–MS was applied. Stigmasterol and cholesterol hydroperoxides (20 mg/4 ml), produced by the photo-oxidation method described above, were fractionated using the method presented in Section 2.3. but omitting the post column reaction system of the instrument. The injection volume was 150 μl and the injection was repeated six times. The separated fraction of each peak was evaporated to dryness under nitrogen flow without heating, dissolved in diethyl ether/methanol (1:1), and immediately reduced to hydroxides with an excess of $NaBH_4$ (Bortolomeazzi et al., 1999). After this, the reaction mixture was stirred at 0 °C for 1 h, and quenched with water (2.5 ml) (Hämäläinen et al., 2001). The excess of the reduction reagent was destroyed with aqueous HCl (3.7%, w/w). Saturated aqueous solution of NaCl (5 ml) was added and the solution was extracted with diethyl ether (4 × 5 ml). The combined organic layers were washed with water (3 × 5 ml) and dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness under nitrogen flow at 30 °C. Samples were dissolved in *n*-heptane-isopropanol (97:3, v/v) (1 ml). The solution was analysed by NP-HPLC followed by DPPP-FL detection, to ensure that all hydroperoxides were completely reduced.

The residual samples (800 μl) were dried under nitrogen flow and subjected to silylation by BSTFA/TMCS (99:1, v/v) reagent in pyridine (Toivo, Piironen, Kalo, & Varo, 1998). After silylation overnight at room temperature, the reagent was evaporated and the hydroxy TMS ethers were dissolved in 200 μl hexane before GC–MS analysis.

GC–MS was used for identification of the stigmasterol and cholesterol hydroxides. The analysis was performed on a Hewlett Packard 6890 gas chromatograph (Wilmington, PA, USA) connected to an Agilent 5973 mass spectrometer (Palo Alto, CA, USA). The GC was equipped with an Rtx-5MS w/Integra-Guard fused silica capillary column (crossbond 5% diphenyl–95% dimethyl polysiloxane, 60 m × 0.25 mm i.d., 0.1 μm film; Restek corporation, Bellefonte, PA, USA). Samples (0.1 μl) were injected using on-column injection. The column temperature programme used was: 70 °C (1 min), 40 °C min^{-1} to 280 °C (35 min). Helium was used as the carrier gas at a constant flow of 1.2 ml/min. Spectra were scanned within the mass range of m/z 100–600 using the electron impact mode (70 eV). The ion source and interface temperatures were 230 and 280 °C, respectively.

2.5. Analysis of the results

NP-HPLC results and PVs obtained at different photo-oxidation time-points (0, 30, 60 and 90 min) were compared by simple regression testing using Statgraphics 3.0 software (STCC Inc., Rockville, ML).

3. Results and discussion

3.1. General

Stigmasterol hydroperoxides, produced by photo-oxidation, could be separated by NP-HPLC and detected specifically by the DPPP-FL method. This method provides information about the structures and amounts of different hydroperoxides in a sample without derivatization, complex sample pretreatment or heating.

3.2. Separation of the hydroperoxides

Photo-oxidized stigmasterol resulted in four different hydroperoxide peaks, as shown in the UV and fluorescence chromatograms (Fig. 3A and B). Both the primary and secondary oxidation products and unoxidized stigmasterol could be detected with the UV-detector at 206 nm (Kermasha, Kubow, & Goetghebeur, 1994). However in Fig. 3A no secondary oxidation products were detected, because the oxidation had not proceeded far enough. On the other hand, the hydroperoxides could be specifically detected by the DPPP-FL detection. Different mobile phase systems (96:4, 98:2 and 97:3 heptane:isopropanol) were examined but the best separation was achieved with the 97:3 heptane:isopropanol (v/v) mobile phase. This NP-HPLC method was rapid, and separation of the hydroperoxides of sterols was achieved within 15 min. With this mobile phase the elution order 6α- and 6β-hydroperoxides (6α- and 6β-OOH), 5α-hydroperoxide (5α-OOH) and 7α-hydroperoxide (7α-OOH) was obtained (for structure identification see Section 3.3). Our findings were similar to those of Ohshima et al. (1996), who reported that the

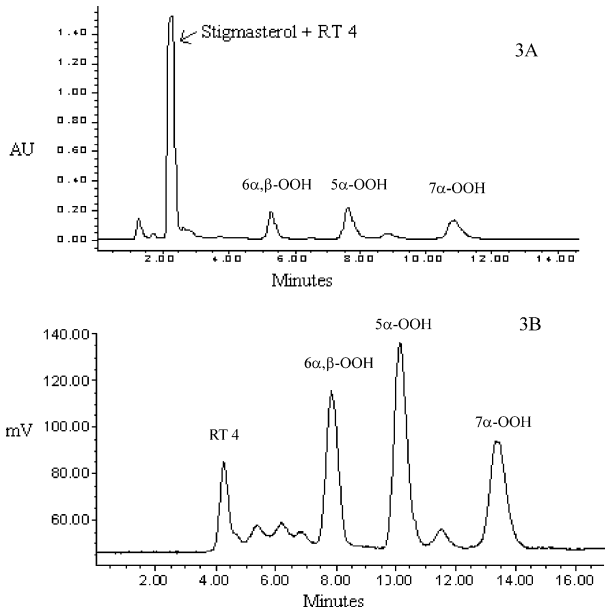

Fig. 3. NP-HPLC chromatogram of photo-oxidized (90 min) stigmasterol with (A) UV detection at 206 nm and (B) DPPP-FL detection. RT 4, unidentified compound.

different positional isomers of hydroperoxides of fatty acid esters could be successfully separated by the NP-HPLC-method. Resolution between hydroperoxides was good, only 6α- and 6β-OOH coeluted. This coelution was noticed when the collected hydroperoxide fractions were analysed by GC–MS. 6α- and 6β-OH of stigmasterol have different fragmentation patterns and relative ion abundances. The retention times were reproducible between the chromatographic runs. The minor peaks between RT 4 and 6α- and 6β-OOH (retention times 5–7 min) and the peak with retention time about 11 min, were formed during photo-oxidation or were present in the non-oxidized sample. The minor peaks were not identified in this study.

3.3. Specificity of the DPPP-FL detection

Specificity of the DPPP-FL detection was investigated with commercially available secondary oxidation products of cholesterol (25-hydroxycholesterol, 5α,6α-epoxycholesterol, 7-ketocholesterol, cholestanetriol and 7α-hydroxycholesterol). The detection specificity was studied by direct injections into the UV detector. The results showed that the studied secondary oxidation products of cholesterol were not able to oxidize DPPP to the fluorescent DPPP oxide. No peaks from the secondary oxidation products of cholesterol (concentrations from 0.6 to 1.4 mg/ml) were observed in the fluorescence chromatograms. With the same injection volume (50 μl) a detector response of about 12 ± 1 million area units for MeLo hydroperoxide (0.765 mg/ml) was obtained. The present study demonstrated that the fluorometric method is specific for hydroperoxides. Our results were similar to those of Akasaka et al. (1987b), who reported that DPPP reacts specifically with hydroperoxides. Unoxidized lipids, hydroxy lipids and antioxidants, such as BHT, were not able to oxidize DPPP to the DPPP oxide (Akasaka, & Ohrui, 2000).

The specificity was confirmed by identifying the structures of the fractionated stigmasterol hydroperoxides by GC–MS after their reduction to the corresponding hydroxides. Hydroperoxides were identified by comparing their mass spectral data and retention times with those of the corresponding hydroxy derivatives of cholesterol (Aringer & Nordström, 1981; Lampi, Juntunen, Toivo, & Piironen, 2002). Our findings were similar to those of Bortolomeazzi et al. (1999), who reported that the mass spectra of the TMS ethers of hydroxy derivatives of the stigmasterol and of the corresponding hydroxy derivatives of cholesterol have the same fragmentation patterns and similar relative ion abundances. The major fragment ions obtained for each stigmasterol and cholesterol hydroxide are summarized in Tables 1 and 2.

Identification of the major hydroperoxides, 6α- and 6β-OOH, 5α-OOH and 7α-OOH, was successful. However, the peak eluting together with the unoxidized stigmasterol (RT 4, Fig. 3B) could not be identified. On the basis of its retention behaviour, it is less polar than hydroperoxides eluting later. RT 4 had a UV-absorption maximum at 234 nm, which indicated that this compound may contain a conjugated diene structure.

Table 1
Spesific mass spectral ions used in identification of cholesterol hydroxy TMS ethers and their relative ion abundances (%) (M^+ = molecular ion, TMSOH = trimethyl silanol group)

Sterol hydroxy TMS ethers	Retention time (min)	Specific ions m/z					
		M^+	M^+-CH_3	M^+-29	M^+ − TMSOH	M^+ − TMSOH−CH_3	Other
5α-OH	20.8	546 (0)	531 (0)	517 (0)	456 (100)	441 (4)	384 (19), 366 (10), 351 (63), 325 (39)
6α-OH	19.8	546 (14)	531 (8)	517 (4)	456 (100)	441 (27)	403 (53)
6β-OH	18.3	546 (15)	531 (24)	517 (11)	456 (63)	441 (35)	403 (100)
7α-OH	17.2	546 (2)	531 (1)	517 (0)	456 (100)	441 (2)	

Table 2
Specific mass spectral ions used in identification of stigmasterol hydroxy TMS ethers and their relative ion abundances (%) (M^+ = molecular ion, TMSOH = trimethyl silanol group)

Sterol hydroxy TMS ethers	Retention time (min)	Specific ions m/z					
		M^+	M^+-CH_3	M^+-29	M^+- TMSOH	M^+- TMSOH$-CH_3$	Other
5α-OH	24.7	572 (0)	557 (0)	543 (0)	482 (100)	467 (3)	410 (21), 392 (8), 377 (51), 351 (24)
6α-OH	23.2	572 (18)	557 (8)	543 (4)	482 (100)	467 (21)	429 (47)
6β-OH	21.3	572 (20)	557 (25)	543 (11)	482 (64)	467 (28)	429 (100)
7α-OH	19.6	572 (2)	557 (1)	543 (0)	482 (100)	467 (1)	

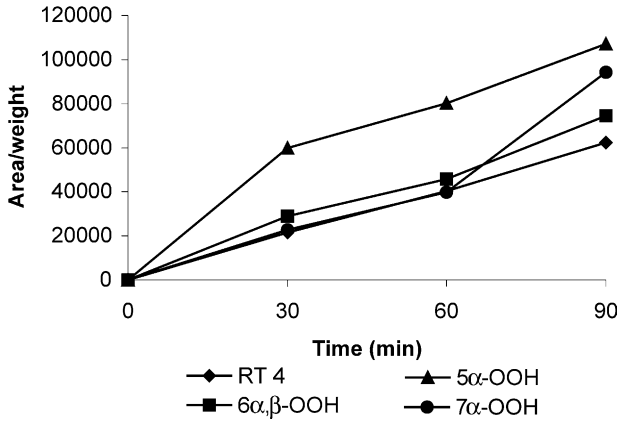

Fig. 4. Formation of stigmasterol hydroperoxides during photo-oxidation detected by DPPP-FL detection.

3.4. Suitability of the NP-HPLC method, with DPPP-FL detection, for monitoring the oxidation behaviour of stigmasterol

The suitability of the NP-HPLC method, with DPPP-FL detection, for monitoring the formation of hydroperoxide during photo-oxidation (0, 30, 60 and 90 min) was investigated by comparing the combined fluorescence detector responses of the four hydroperoxide (RT 4, 6α- and 6β-OOH, 5α-OOH and 7α-OOH) peaks to the PVs. The PVs were 6–775 (meq/kg) at the fluorescence detector responses 0–360,000, respectively. The results were from duplicate photo-oxidation experiments. The PV determination and NP-HPLC method gave similar profiles. On the basis of the simple regression testing, there was a statistically significant relationship between the NP-HPLC results and the PV at the 99% confidence level ($N = 8$). The correlation coefficient (0.997) indicated a relatively strong positive relationship between the variables ($R^2 = 99.4\%$, $P < 0.01$).

The results showed that the amount of hydroperoxides increased with increasing photo-oxidation incubation time (Fig. 4). Stigmasterol has two double bonds, one in the ring structure and one in the side chain. The ring structure double bond is at the same position as that of cholesterol. Kulig and Smith (1973) reported that 5 α-OOH is the main product in photo-oxidation of cholesterol, together with small amounts of 6α- and 6β-OOH. 5α-OOH of cholesterol can rearrange in non-polar solvents to 7α-OOH, which can in turn rearrange to 7β-OOH (Beckwith, 1989). The last rearrangement is known to be much slower than that of 5α-OOH. Our findings were similar to those of Bortolomeazzi et al. (1999), who reported that plant sterols have the same photo-oxidation behaviour as cholesterol. First, 5α-OOH and 6α- and 6β-OOH were formed, as soon as photo-oxidation was started, and the amount of 7α-OOH began to increase slightly more slowly than that of 5α-OOH, which may be due to the above mentioned rearrangement of 5α-OOH to 7α-OOH. 7β-OOH was not detected in this study. Because the NP-HPLC analysis of hydroperoxides was made immediately after photo-oxidation, it is proposed that rearrangement of 7α-OOH to 7β-OOH did not have time to take place.

The NP-HPLC method with DPPP-FL detection was sensitive compared to UV. Using the DPPP-FL detection, picomole levels of lipid hydroperoxides could be detected (Akasaka et al., 1988). In this study, sensitivity was estimated on the basis of the area of the smallest identified peak obtained in 30 min photo-oxidation. By means of PV, the concentration of this hydroperoxide in one injection was estimated. The results indicated that when 5 nmol of hydroperoxide (7α-OOH) were injected into the HPLC a signal-to-noise ratio of about 20 was obtained in the fluorescence chromatogram.

4. Conclusion

A specific chromatographic method for detection of plant sterol hydroperoxides was developed. With the NP-HPLC method, followed by DPPP-FL detection, we could detect and monitor the formation of the different positional isomers of stigmasterol hydroperoxides, without time-consuming prior derivatization and sample pretreatment. Therefore the method is a useful tool for investigation of the oxidation mechanism of sterols.

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