Determination of non-polar and mid-polar monomeric oxidation products of stigmasterol during thermo-oxidation.

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Abstract

Oxidation products of stigmasterol were characterized by their polarity and molecular size using solid phase extraction (SPE) and high performance size exclusion (HPSEC) methods. Monomeric oxides were studied further by GC-MS and GC-FID. The focus was on identifying and quantificating non-polar and mid-polar monomeric oxides after SPE fractionation. Commercial stigmasterol was subjected to 180 ºC up to 3 hours. Six oxidation products were identified by GC-MS in the non-polar and mid-polar monomeric fractions, and they appeared already after the first hour of heating. Quantification by GC-FID showed an increase in the non-polar and mid-polar oxidation products during heating time, and their amounts reached values of 6.1 and 47.0 μg/mg of commercial stigmasterol, respectively. Polar oxidation products commonly measured reached a value of 193 μg/mg after 1 hour of heating, while after 3-hour heating, the amount was only 164 μg/mg. Since as much as 550 μg/mg of stigmasterol was decomposed, the monomeric products explained only partly the stigmasterol loss. Dimeric and polymeric products contributed to 165 μg/g of gap showing the importance of polymerization reactions at 180 ºC.

1. Introduction

Phytosterols (plant sterols) are natural constituents of vegetable foods and oils and their consumption at certain levels has been demonstrated to be useful for the treatment of hypercholesterolemia (Katan, Grundy, Jones, Law, Miettinen & Paoletti, 2003). For this reason, several functional foods formulated with esterified or free phytosterols and phytostanols have appeared on the market, increasing the interest in researching on different aspects related to their stability and safety.

Chemical structure of phytosterols is closely related to that of cholesterol and, therefore, their oxidation leads to similar type of oxidation products (Lercker & Rodríguez-Estrada, 2002; Cercaci, Rodríguez-Estrada, Lercker, & Decker, 2006, Zhang et al., 2006). The main oxidation products are hydroxyl, ketone, epoxyl and triol derivatives, all of which are polar products (Smith, 1981). Although several studies on phytosterol oxides content in different types of foods have been performed, the number of research papers studying their formation is still scarce comparing to those studying cholesterol oxides formation (Conchillo, Cercaci, Ansorena, Rodríguez-Estrada, Lercker & Astiasarán, 2005; Dutta, & Appelqvist, 1997; Lampi, Juntunen, Toivo & Piironen, 2002). Moreover, the products derived from phytosterol oxidation seem to be more complex in chemical structures due to presence of e.g. additional double bonds than those of cholesterol. Menéndez-Carreño, Ansorena and Astiasarán (2008), analyzing the stability of sterols in phytosterol-enriched milk, found that drastic heating conditions gave rise to a significant decrease in sterol contents, without a quantitative corresponding increase of polar sterol oxides. Thus, sterol losses detected are not only explained by the formation of polar oxidation products (Soupas, Huikko, Lampi & Piironen, 2005).

There are only a few studies where less polar oxidation products have been studied. Yanishlieva, Schiller and Marinova (1980) studied β-sitosterol oxidation after one hour of heating at 150 ºC, identifying sterones (i.e. steranes with a ketone group) and steradienes as non-polar products and also two oxidation products in the mid-polar region, namely stigmastan-5,24-dien-3-β-ol and 6-hydroxystigmast-4-en-3-one. Blekas and Boskou (1989) found four non-polar oxidation products: stigmastan-3,5,22-trien-7-one, stigmastan-3,5,22-triene, stigmastan-4,22-dien-3-one and Δ^5-pregnen-3β-ol-2-one, and also more polar products, hydroxyl and epoxyl derivatives, when they analyzed the oxidation products in a triacylglycerol mixture containing 5% stigmasterol by column chromatography and preparative thin–layer chromatography (TLC). Bortolomeazzi, De Zan, Pizzale and Lanfranco (2000) also found sterenes with double bonds located at C2, C4, and C6 positions in refined vegetable oil. The sterenes were synthesized from the dehydration of hydroxysterols during the refining. Some authors pointed out that polymers and/or other products of relatively high molecular weight are also formed during sterol oxidation at high temperatures (Kim & Nawar, 1993; Chien, Wang & Chen, 1998; Soupas, Huikko, Lampi & Piironen, 2006). Finally, Johnsson and Dutta (2003) defined mid-polar oxidation products as those compounds that presented retention factor *(Rf)* values in a TLC plate run with ether/cyclohexane (9:1) between epoxyl and unoxidized sterols, and isolated 6α- and 6β- epimers of 6-hydroxystigmastan-4-en-3-one in this fraction. Nevertheless, all these studies on non-polar and mid-polar sterol oxidation products implied complicated procedures including enrichment of non-polar and mid-polar oxidation products by preparative TLCs and use of various techniques to separate the oxidation products based on their polarity.

The main purpose of this study was to investigate formation of non-polar and mid-polar monomeric oxidation products of phytosterols during thermo-oxidation at 180 ºC for different time periods. Stigmasterol was used as a phytosterol model compound. Analysis of non-polar and mid-polar oxidation products was based on separation of thermo-oxidation products by solid phase extraction (SPE) (Lampi et al, 2009). The non-polar and mid-polar fractions were further analyzed by high performance size exclusion chromatography (HPSEC) to separate the products based on their molecular sizes and to collect the monomeric products. The identification of chemical structures of the non-polar and mid-polar monomeric oxidation products was performed by GC-MS, and the quantification was done using GC-FID. Sterols degradation and formation of polar oxidation products were also analyzed in order to study the evolution of thermo-oxidation products during the heating experiment.

2. Material and Methods

Stigmasterol and trimethyl-chlorosilane (TMCS) were purchased from Fluka Chemie (Buchs, Switzerland); 19-hydroxycholesterol was from Steraloids (Wilton, NH, U.S.A.). Dihydrocholesterol, 5α-cholestane, analytical grade pyridine (>99%) and butylated hydroxytoluene (BHT) were obtained from Sigma (St. Louis, MO, USA). Tetrahydrofuran (THF), heptane, dichloromethane and acetone were purchased from Rathburn (Walkerburn, Scotland). Diethyl ether was obtained from J.T. Baker (Deventer, Netherlands) and ethanol was from Altia (Finland). Bis(trimethylsilyl)-trifluoracetamide (BSTFA) from Merck & Co., Inc (Whitehouse Station, NJ, U.S.A.). Si-1 Silica Strata cartridges (55 μm, 70A; 500 mg/3 mL) were purchased from Phenomenex (Torrance, CA, USA).

2.1 Thermo-oxidation of stigmasterol

Aliquots of 25 mg of stigmasterol were weighted into glass vials (22 x 46 mm, i.d. 19 mm) and heated at 180 °C during 0, 1, 2 and 3 hours in a Termaks oven (Bergen, Norway). At each sampling point, vials were taken from the oven and cooled in a dessicator before further analysis. All heating experiments were carried out two times, and each sample was analyzed for non-polar and mid-polar monomeric products in triplicate and for residual sterols and polar oxidation products in duplicate or triplicate.

2.2 Characterization of non-polar and mid-polar monomeric products

2.2.1 Solid phase extraction (SPE)

Each stigmasterol sample (25 mg) was dissolved in 5 mL of dichloromethane, and 1 mL of the sample solution was taken to be fractionated by SPE. SPE was used to separate non-polar and mid-polar products for GC-FID quantification and as a fractionation method before HPSEC.

When non-polar and mid-polar products were quantitatively measured by GC-FID (see section 2.2.3), 1 mL of the sample solution was first transferred to a tube containing 1 mL of 5α-cholestane (520 μg/mL) as an internal standard for non-polar products and 1 mL of dihydrocholesterol (400 μg/mL) as an internal standard for mid-polar products. The solvents were evaporated under stream of nitrogen using a Reacti-Vap 18780 evaporating unit coupled to a reacti-term 18790 heating module at 30 °C

(Pierce, Rockford, IL, USA) and the residues were redissolved in 1 mL dichloromethane.

The 1 mL sample solutions were applied to a SPE silica cartridge previously conditioned with 5 mL of dichloromethane. The non-polar fraction was first eluted from the cartridge with 5 mL of dichloromethane:heptane (1:1, v/v), and the mid-polar fraction was eluted with 5 mL of heptane:diethyl ether (1:1, v/v). The solvents were evaporated under stream of nitrogen at 30 ºC. The residues were redissolved in 1 mL of HPLC-grade THF with 0.025% BHT before HPSEC analysis or in 200 μL of heptane before gas chromatographic analysis.

2.2.2 High performance size exclusion chromatography (HPSEC)

HPSEC was used to characterize the profile of oxidized stigmasterol by molecular size. In addition, HPSEC was used to separate monomeric non-polar and mid-polar oxidation products of stigmasterol from dimeric and polymeric products after SPE fractionation when the products were identified. An instrument including a binary pump, an autosampler, a column compartment and a HP10474 refractive index (RI) detector was used (Agilent Technologies International, Morges, Switzerland). The mobile phase was HPLC-grade THF with 0.025% BHT at a flow rate of 0.6 mL min^{-1} and the injection volume of stigmasterol sample was 30 μL. Separation was performed by using one PLGel 100 Å and two PLGel 50 Å columns (300 x 7.5 mm each, highly crosslinked styrene divinylbenzene, particle size 5 Å; Polymer Laboratories Inc., Amherst, MA) at 35 ºC.

The stability of the HPSEC-RI method was verified in every sample set by analyzing a rapeseed oil sample (10 mg/mL, 50 μL) and collecting the triacylglycerol

peak areas from them. The response was stable during the experiment, because the coefficient of variation of the triacylglycerol peak areas was < 5% (n = 9). For quantification of the HPSEC-RI method, stigmasterol was used as an external standard. The calibration curve consisted of five levels and ranged from 0.1 to 400 μg/injection. Calibration curves showed excellent linearity with determination coefficients (R^2) of 1.0 for each calibration curve. Calibration samples were analyzed after ca. 20 injections. Results of a set of samples were calculated based on the calibration samples analyzed immediately before and after each sample set.

2.2.3 Gas Chromatographic Analysis

Gas chromatography with mass spectrometric detection (GC-MS) was used in confirming the identities of the non-polar and mid-polar monomeric oxidation products of stigmasterol, and GC with flame ionization detection (GC-FID) for quantification of the compounds of the two fractions.

GC-MS analysis was performed on a GC 6890N Hewlett Packard (Wilmington, DE, USA) coupled to a 5973 Mass Selective Detector (Palo Alto, CA, USA) using a Rtx-5MS w/Integra Guard (Crossbond 5% diphenyl- 95% dimethyl polysiloxane; 60 m x 0.25 mm i.d., 0.10 μm) capillary column (Restek, Bellefonte, PA, USA) and an on-column injection as described earlier (Soupas, Juntunen, Lampi & Piironen, 2004). Characterization of the peaks was done in full scan method (m/z 50-600): Characterization was performed with Agilent G1701DA GC/MSD ChemStation (Agilent Technologies, Inc., CA, USA). GC-FID analyses were performed on an automated instrument equipped with an on-column injector and a flame ionization detector (Hewlett-Packard 5890N series II, Karlsruhe, Germany) and using a similar Rtx-5MS w/Integra Guard with a 0.32 mm i.d. as described earlier (Soupas, Juntunen,

Lampi, Piironen, 2004). The performance of the GC-FID and the GC-MS was assured daily by analyzing a sterol standard mixture and was shown to be good and stable.

2.3 Analysis of polar stigmasterol oxidation products (POPs) and residual sterols

After thermo-oxidation and cooling in the desiccators, stigmasterol samples (25 mg) were subjected to analysis of polar oxidation products and residual sterols. For polar oxidation products, 1 mL of the internal standard (19-hydroxycholesterol) in ethanol was added to a stigmasterol sample. The concentration of the standard depended on the heating time, being 7.62 μg/mL for the non-heated stigmasterol and 76.22 μg/mL for the stigmasterol heated for 1, 2 and 3 hours. Ethanol was evaporated under a stream of nitrogen at 30 ºC and each sample was redissolved in 5 mL of a mixture heptane:diethyl ether (9:1, v/v). Then, they were sonicated in a Branson 5510 sonicator (Wethersfield, CT, USA) to ensure the total dissolution of the heated stigmasterol. Polar oxidation products were separated by SPE using silica cartridges as previously described (Lampi, Juntunen, Toivo & Piironen, 2002). Solvent of the acetone eluted polar compounds was evaporated to dryness under stream of nitrogen at 30 °C, and 100 μL of silylation reagent (BSTFA:TMCS, 99:1, v/v) and 100 μL of anhydrous pyridine were added and the samples were left to silylate overnight at room temperature in a desiccator. The following day, the excess of silylation reagent on the samples was evaporated under stream of nitrogen at 30 °C and the residues were redissolved in 200 μL of heptane before gas chromatographic analysis.

For analysis of residual sterols, the stigmasterol samples were dissolved in 50 mL of ethanol and 1 mL of dihydrocholesterol (0.2 mg/mL) were added as an internal

standard. Then, 100 μL of each sample were evaporated to dryness under stream of nitrogen at 30 °C and silylated and finally redissolved in 200 μL of heptane before gas chromatographic analysis as described above. GC-MS was used to confirm the identities of the phytosterols and their oxidation products, and GC-FID was used for quantification. The same GC methods were used as for analysis of non-polar and mid-polar monomeric products (see 2.2.3).

2.4 Statistical analysis

All results are expressed as mean ± standard deviation of the mean. The differences between the groups were evaluated by one-way ANOVA, and Tukey post-hoc test was applied when suitable. A Pearson correlation test was performed between sterols and non-polar, mid-polar and polar oxidation products content in the commercial standard of stigmasterol subjected to 0, 1, 2 and 3 heating treatment at 180 ºC (SPSS 15.0 packages for Windows, Chicago, IL, USA). A level of probability at $p<0.05$ was set up as statistically significant.

3. Results and Discussion

3.1 Effects of thermo-oxidation on stigmasterol as measured by polar oxidation products and residual sterols

Pure stigmasterol represented approximately 96% of the commercial standard of stigmasterol used as a model compound in this work. Besides stigmasterol that eluted at 20.41 min with the GC-MS method used, three other compounds were present at

low concentrations: stigmastanol (1.2%), brassicasterol (1.8%) and campesterol (0.8%).

As expected, stability of ~~sterols~~ stigmasterol was affected by the heating treatment at 180 ºC, showing progressive decreasing amounts with the heating time (figure 1). The highest degradation level was observed after 3 hours heating, finding a 61 % of the initial stigmasterol content ~~(table 1)~~. ~~The compound with the saturated ring, stigmastanol, was least affected by heating (data not shown)~~.

While the amount of ~~sterols~~ stigmasterol decreased, the amounts of polar oxidation products increased during thermo-oxidation (table ~~2~~ 1). After 1-hour heating, the total amount of oxides was 193 µg/mg, which accounted for 21% of the initial stigmasterol content. The amount of polar oxidation products began to decrease after 2 hours of heating. After the 3-hour heating experiment, 550 µg/mg of stigmasterol was decomposed and only 164 µg/mg of polar oxidation products were present, which accounted just for 29.8 % of the total decomposition products. The total amount of polar oxidation products accounted thus only for 18% of the initial stigmasterol indicating that other types of products had to be formed. The polar oxides may have been suffered further reactions, such as polymerization (Kemmo, Soupas, Lampi & Piironen, 2005; Soupas et al., 2006) or dehydration, giving rise to other types of non polar products (Bortolomeazzi, De Zan, Pizzale & Lanfranco, 2000). These results are in agreement with those obtained by Lampi, Juntunen, Toivo and Piironen (2002), with a maximum content of polar oxidation products after 1 hour heating of stigmaterol at 180 ºC

7-ketostigmasterol was the only product whose content increased statistically during the whole course of the experiment. 6β-hydroxystigmasterol also presented a concentration increase after the second hour of heating. The rest of the polar oxidation

products either maintained their concentrations, or even decreased after the first hour of heating (table ~~2~~ 1). After 3 hours of heating, 7-ketostigmasterol became the major product. The formation of 7-ketostigmasterol can be attributed to dehydration of 7-hydroperoxystigmasterol or dehydrogenation of 7-hydroxystigmasterol under dry and oxygen-rich conditions (Chien; Wang & Chen, 1998). In fact, 7β-hydroxystigmasterol and 7α-hydroxystigmasterol and also 6β-hydroxy-3-ketostigmasterol showed their greatest level after 1 hour heating, and then decreased, especially during the third hour.

3.2 Characterization of thermo-oxidation products of stigmasterol

When a thermo-oxidized (180 °C, 3 hours) commercial stigmasterol sample was subjected directly to HPSEC separation, four different peaks appeared in the chromatograms corresponding, in order of elution, to polymeric, dimeric, monomeric and minor monomeric products. The monomeric compounds were earlier shown to co-elute with cholesterol, a sterol, and the minor monomeric compounds with 3,5-cholestane, a dehydration product of cholesterol (Lampi, Kemmo, Mäkelä, Heikkinen & Piironen, 2009). In thermo-oxidized stigmasterol the amount of polymeric plus dimeric products ~~increased to~~ accounted for a 23% of total products detected (table 2). The amount of smaller products (monomeric and minor monomeric products), which included non-oxidized stigmasterol and its monomeric oxidized products, accounted only for 77%, indicating commercial stigmasterol degradation.

When the thermo-oxidized sample was subjected to SPE fractionation prior to HPSEC-RI analysis, the polymeric, dimeric and monomeric oxidation products could further be separated into non-polar, mid-polar and polar products (table ~~3~~ 2). Polar

products were the major ones in polymeric and dimeric products, while non-polar and mid-polar compounds had a greater contribution in minor monomeric products. The mid-polar monomeric products might also contain oxidation products although the peak was dominated by non-oxidized stigmasterol. Polar monomeric fraction contributed up to 31% of all monomers. Residual stigmasterol, and also the rest of the sterols of the commercial sample of stigmasterol, were supposed to be mainly included in the mid-polar monomeric fraction. The minor monomeric compounds eluting after stigmasterol probably included sterol dehydration products and sterones. The sum of non-polar and mid-polar products in the monomeric and minor monomeric fractions represented 69% and 85% of the fractions, respectively.

Results from the HPSEC-RI analysis showed that the amounts of the polymeric, dimeric and monomeric products obtained after SPE fractionation were 98.6% of those obtained in the same sample without SPE fractionation, supporting the idea that the SPE fractionation method used only caused minor losses and maintained the overall profile of the oxidation products. Thus the SPE method could be used to characterize thermo-oxidation products by polarity.

3.3 Analysis of non-polar and mid-polar monomeric products by SPE, HPSEC and GC, and by SPE and GC

When stigmasterol samples were fractionated by SPE and the collected non-polar and mid-polar products were further separated by size using HPSEC, some of the mid-polar monomeric oxidation products appeared in the minor monomeric peak whereas the quantity of products appearing in the monomeric fraction was unknown due to co-elution with non-oxidized stigmasterol. The non-polar monomeric oxidation

products gave two peaks in HPSEC chromatograms, both of which had a similar intensity. The first monomeric peak could correspond to stigmasterol which was not retained by the SPE. Thus, these results suggested that a large quantity of the non-polar and mid-polar oxidation products were smaller than stigmasterol indicating degradation of stigmasterol.

By injecting the SPE fractions directly into GC-MS, i.e. without HPSEC fractionation, possible interference caused by dimeric and polymeric oxidation products on GC analysis was studied. The results were promising, since no adverse affects of larger products were found on the identification and quantification of the monomeric non-polar and mid-polar oxidation products from the heated stigmasterol. Therefore the HPSEC fractionation prior to GC analysis could be omitted (data not shown). In general, removal of di- and polymeric products is not done when sterol oxidation products are analyzed by GC (Soupas, Juntunen, Lampi & Piironen, 2004; Menéndez-Carreño, Ansorena & Astiasarán, 2008).

To show the performance of the SPE fractionation method combined with GC-FID analysis, residual sterols of heated stigmasterol samples eluting in the two SPE fractions were quantified. Only minute amounts of sterols were found in the non-polar fractions (table ~~4~~ 3) indicating that the SPE cartridge was able to retain sterols and other mid-polar compounds. Moreover, contents of stigmasterol ~~sterols~~ found in the mid-polar fractions (table ~~5~~ 4) was comparable to that found by the direct saponification method used to measure residual sterols ~~(table 1)~~. Thus sterols and obviously other mid-polar compounds could be efficiently eluted from the SPE cartridge. Consequently, the new method could also be applied for the quantification of sterols.

3.4 Identification and quantification of non-polar and mid-polar monomeric products of thermo-oxidized stigmasterol

To identify the monomeric non-polar and mid-polar oxidation products, heated stigmasterol samples were analyzed after SPE fractionation by GC-MS. Table 5 shows the specific mass spectral ions and retention times of non-polar and mid-polar oxidation products, and figure 2 chemical structures of selected products. Tables 3 and 4 show the quantification data for the non-polar and mid-polar oxidation products from stigmasterol, respectively, obtained by the new SPE fractionation methodology and CG-FID analysis. During the heating treatment, three different monomeric products were detected in the non-polar fraction and their amounts increased with the heating time. After 3 hours of heating, the total amount of these non-polar products accounted for 0.6% of the initial stigmasterol content. Four different oxidation products appeared in the mid-polar fraction which accounted for 5.2% of the initial stigmasterol content after 3 hours of heating.

Non-polar monomeric products

The non-polar fraction of heated stigmasterol was composed mainly by sterenes. The first product had a retention time of 15.71 min with the GC-MS method used and was identified as stigmastan-2,4,6,22-tetraene. The molecular ion corresponded to the peak with the m/z 392 and the peaks at m/z 253 and 211 were due to loss of side chain and of the chain plus ring D, supporting the presence of three double bonds in the ring system (Bortolomeazzi, De Zan, Pizzale & Lanfranco, 2000). The formation of these double bonds, being located at the 2, 4 and 6 positions, can be explained by the loss of two molecules of water from a hydroxyl derivative of sterol (Bortolomeazzi Pizzale,

Novelli & Conte, 1996). The ion at m/z 251 originated from the loss of the side chain together with two hydrogen atoms from the D ring, which is characteristic of a sterol with a double bond in the side chain (Willie & Djerassi, 1968). Ion at m/z 349 was derived from the loss of an isopropyl group at the end of the side chain, also characteristic of Δ^{22}-sterols (Pizzoferrato, Nicoli & Lintas, 1993).

The product characterized as stigmastan-3,5,22-triene eluted at 21.99 min using GC-MS, in both non-polar and mid-polar fractions. It showed a molecular ion at m/z 394 and ions at m/z 379, which corresponded to the loss of a methyl group, and at m/z 255 due to loss of side chain. The lack of an M^+-H_2O ion confirmed the absence of oxygen in the molecule. Similar dehydration products were also identified in heated stigmasterol (Blekas & Boskou, 1989) and in heated β-sitosterol (Yanishlieva, Schiller & Marinova, 1980).

The presence of an additional double bond in stigmastan-2,4,6,22-tetraene made the molecule more non-polar than the corresponding triene, stigmasta-3,5,22-triene. These two sterene molecules were the major products formed from dehydration of stigmasterol during the heating treatment. These products are usually present in refined vegetable oils being formed during the bleaching and deodorization process (Crews, Calvet-Sarret, & Brereton, 1999; Verleyen, Szulczewska, Verhe, Dewettinck, Huyghebaert, & De Greyt, 2002). In fact, sterenes have been selected as markers for detecting adulteration of non-refined oils and fats (AOCS Official Method Cd 26–96, 1990; AOCS Official Method Cd 27-96, 1997; Dobarganes, Cert, & Dieffenbacher, 1999). In the Official Methods, n-hexane is used to extract these products from oil samples, to run them onto a fractionating silica gel column and to dissolve the residues before GC-FID analysis. However, the use of n-hexane gives rise to a probable over estimation of steradiene content due to peak overlap with other

hydrocarbons (Verleyen, Szulczewska, Verhe, Dewettinck, Huyghebaert, & De Greyt, 2002). In the present work, two solvent mixtures with different polarities, i.e. dichloromethane-heptane (1:1, v/v) and heptane-diethyl ether (1:1, v/v), respectively, were used to get a better separation of the non-polar and mid-polar products.

Both sterenes increased their concentration during the heating experiment reaching values of 2.37 μg/mg of stigmastan-2,4,6,22-tetraene and 5.61 μg/mg of stigmasta-3,5,22-triene. The latter product appeared in both fractions with an approximate proportion 1:3 between the non-polar and mid-polar fractions.

Mid-polar monomeric products

The products corresponding to sterones appeared at significant amounts mainly in the mid-polar fraction. The mass spectrum of the product described as stigmastan-4,22-dien-3-one showed a molecular ion at m/z 410, indicating that stigmasterol had lost two mass units. The peak at m/z 271 corresponded to the elimination of the side chain with the transfer of two protons and there was an intensive peak at 298 corresponding to cleavage of a C20-C22 bond with transfer of one hydrogen. The base peak at m/z 55 could represent the removal of ring A (C1 to C4) plus transfer of one hydrogen, due to cleavage of a C4-C5. Sheikh and Djerassi (1974) found stigmastan-4,22-dien-3-one when describing steroids from sponges showing similar mass spectrum as the product formed by heating treatment in this study. Smith (1987) observed that direct oxidation of the hydroxyl group in C3 position gave rise to cholest-5-en-3-one, which immediately rearranged to cholestan-4-en-3-one, generating a structure that was more stable due to a conjugated double bond. Cholestan-4-en-3-one reached values of 4.76 μg/mg after 1-hour heating and after which it only slightly increased its level.

The product identified as stigma-3,5,22-triene-7-one had a molecular ion at m/z 408. Other ions appeared at m/z 187, 174 and 161. These represent a cleavage of the most heavily substituted bond C8-14 followed by breakages of the C12-C13, C11-C12 and C9-C11 bonds, respectively, which is characteristic of $\Delta^{3,5}$-dien-7-ones (Budjikieweicz & Djerassi, 1962). Dehydration of 7-ketostigmasterol led to the formation of the conjugated diene, stigmastan-3,5,22-triene and the subtraction of the hydroxyl group from position 3, favoured by the presence of a double bond in position C5-C6, gave a rise to stigmastan-3,5-diene-7-one (Lercker & Rodriguez-Estrada, 2002). The amount of this product increased during the heating treatment, reaching its maximum level after 3-hour heating (13.20 μg/mg) appearing mainly in the mid-polar fraction. An isomer of stigmastan-3,5-diene-7-one appeared only in the non-polar fraction reaching a value of 1.60 μg/mg.

The latest eluting identified product of the mid-polar fraction, stigmastan-4,22-dien-3,6-dione, showed a molecular ion at m/z 424, which implied the addition of one oxygen to stigmasterol and the presence of one double bond in the ring structure besides the double bond in the side chain. The ion at m/z 285 corresponded to the elimination of side chain corroborating the presence of 2 atoms of oxygen in the ring structure. Stigmastan-4,22-dien-3,6-dione showed the highest amount in every analyzed sample, reaching values of 24.87 μg/mg during the first hour of heating. Then, this product showed a significant 1.3-fold increase after every hour of heating treatment.

Stigmastan-4,22-dien-3-one, stigmastan-3,5,22-triene-7-one and stigmastan-4,22-dien-3,6-dione were the main mid-polar oxidation products from stigmasterol heated. Yanishlieva, Marinova, Schiller and Seher (1983) defined stigmastan-4-en-3-one, stigmastan-3,5-diene-7-one and stigmastan-4-en-3,6-dione as three of the main

oxidation products when free sitosterol was heated at 100 ºC, showing similar behaviour than heated stigmasterol. The presence of two ketone groups in stigmastan-4-en-3,6-dione made the molecule more polar than stigmastan-4,22-dien-3-one with only one ketone group. However, the dione product showed less polarity than the polar oxidation products, and appeared in the mid-polar fraction. On the other hand, stigmastan-3,5,22-triene-7-one presented some affinity for the non-polar fraction due to the formation of 2 double bonds in the ring structure.

Finally, an unidentified product with a high molecular weight eluted in the non-polar and mid-polar fractions at 32.30 min using GC-MS. The presence of that peak suggests that drastic heating conditions leads to other reactions giving rise to dimeric and polymeric products; thus it is necessary in the future to develop new methodologies to study the structures and formation of these products in more detail.

3.5 Overall evaluation of different types of thermo-oxidation products of stigmasterol

Changes in the contents of non-polar, mid-polar and polar oxidation products during the heating experiments show that sterols are partially destroyed giving rise to the formation of a great variety of compounds with different polarities. As shown in ~~table~~ figure 1, the amount of stigmasterol decreased steadily and only 39% of it was left at the end of the 3-hour heating. Polar oxidation products reached their maximum content already after the first hour of heating (table 1), while the amounts of six identified products in the non-polar and mid polar fractions increased with the heating time and reached total ~~values~~ sum of 6.1 and 47.0 μg/mg, respectively, after 3 hours of heating (tables 3 and 4). Regarding non-polar oxidation products, a

continuous increase was observed for all products detected. Among mid-polar oxidation products, the content of stigmastan-4,22-dien-3,6,-dione increased with the heating time (24.9 µg/mg after 3 hours of heating), and the rest tended to maintain their concentrations stable.

The sum of non-polar (6.1 µg/mg), mid-polar (47 µg/mg) and polar (164µg/mg) monomeric products derived from stigmasterol oxidation was 217 µg/mg after 3 hours of heating (data from tables 1, 3 and 4). This amount contributed to 39% of stigmasterol loss, indicating that further reactions have to be taken into account to explain sterol losses during thermo-oxidation. HPSEC-RI analysis revealed that dimeric and polymeric oxidation products formed (165 µg/g; table 2) contributed to 30% of stigmasterol degradation after 3 hours of heating and explained part of the other types of reactions.

Significant negative Pearson correlation coefficients between total sterols and non-polar and mid-polar oxidation products (-0.83 and -0.99, respectively; $p < 0.001$) were found. On the contrary, significant positive correlation coefficients between polar oxidation products and non-polar and mid-polar oxidation products (0.62 and 0.89, respectively; $p < 0.001$) were detected. These analyses show that there are strong relationships among the non-polar and mid-polar oxidation products and the other indicators of oxidation reactions.

In summary, thermo-oxidation of stigmasterol gave rise to a diverse group of oxidation products because of stigmasterol degradation. Besides usually measured polar oxidation products, several non-polar and mid-polar oxidation products were synthesized through oxidative and thermal reactions of sterols and polar oxidation products. The new SPE fractionation method combined with GC analyses permitted a

better separation, identification and quantification of sterol oxidation products based on their different polarities.

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Results

Table 1. Formation of polar oxidation products in ~~commercial standard of~~ stigmasterol when heated at 180 ºC.

Polar oxidation products (µg/mg commercial stigmasterol)	**0 h**	**1 h**	**2 h**	**3 h**
7α-hydroxystigmasterol	n.d.	23.58±1.32^{c}	18.06±2.59^{b}	7.88±1.80^{a}
6β-hydroxystigmasterol	n.d.	6.75±0.67^{a}	9.72±0.84^{b}	10.02±1.45^{b}
7β-hydroxystigmasterol	n.d.	34.84±1.46^{c}	32.03±0.55^{b}	20.14±0.73^{a}
6α-hydroxystigmasterol	n.d.	3.32±0.35^{b}	2.23±0.43^{a}	2.08±0.14^{a}
5,6β-epoxystigmasterol + 6β-hydroxy-3-ketostigmasterol	n.d.	48.06±2.08^{c}	45.56±1.55^{b}	35.43±0.41^{a}
5,6α-epoxystigmasterol	n.d.	24.95±1.06^{b}	26.79±0.90^{b}	22.71±1.95^{a}
stigmastanetriol	n.d.	1.71±0.25^{a}	1.79±0.28^{a}	2.08±0.19^{a}
6α-hydroxy-3-ketostigmasterol	n.d.	11.54±0.82^{b}	11.29±0.59^{ab}	10.30±0.60^{a}
7-ketostigmasterol	n.d.	37.78±4.00^{a}	45.08±5.13^{b}	53.25±0.77^{c}
Total products	n.d.	192.51±7.31^{a}	192.54±3.91^{a}	163.87±4,97^{b}

Total compounds are the sum of all individual polar oxidation products detected. Results are expressed as mean ± standard deviations. Values in the same row bearing different letters are significantly different ($p< 0.05$). n. d. means non detected products

Table 2. Distribution of monomeric, dimeric and polymeric products by HPSEC-RI in SPE fractionated commercial stigmasterol and non SPE fractionated stigmasterol when heated at 180 ºC for 3 hours.

	Polymeric products (µg/mg)	**Dimeric products (µg/mg)**	**Monomeric products (µg/mg)**	**Minor Monomeric products (µg/mg)**
Non-polar fraction	6.24 ± 0.27	18.60 ± 0.93	11.30 ± 0.87	11.47 ± 0.35
Mid-polar fraction	8.87 ± 0.48	31.09 ± 1.65	361.40 ± 19.60	16.32 ± 1.45
Polar fraction	40.23 ± 2.12	59.91 ± 1.49	167.24 ± 8.19	4.88 ± 0.72
Sum of SPE fractions	55.34 ± 2.32	109.50 ± 3.48	539.94 ± 26.23	32.67 ± 1.79
Non SPE fractionated stigmasterol	56.69 ± 3.01	115.19 ± 1.07	542.28 ± 0.80	33.31 ± 1.79

Table 3. Formation of non-polar oxidation products ~~in commercial standard~~ of stigmasterol when heated at 180 ºC.

Non-polar oxidation products (µg/mg commercial stigmasterol)	**0 h**	**1 h**	**2 h**	**3 h**
Stigmastan-2,4,6,22-tetraene	n.d.	0.30±0.03[a]	1.05±0.03[b]	2.37±0.18[c]
Stigmasterol	n.d.	0.22±0.02[a]	0.22±0.02[a]	0.22±0.02[a]
Stigmastan-3,5,22-triene	n.d.	0.68±0.03[a]	1.52±0.14[b]	1.86±0.15[c]
Stigmastan-3,5,22-triene-7-one (isomer)	n.d.	0.33±0.02[a]	0.97±0.07[b]	1.60±0.12[c]
Stigmastan-4,22-dien-3-one	n.d.	n.d	n.d	n.d
Stigmastan-3,5,22-trien-7-one	n.d.	n.d.	0.15±0.01[a]	0.22±0.01[b]
Stigmastan-4,22-dien-3,6-dione	n.d.	n.d.	n.d.	n.d.
Unknown product	1.69±0.18[a]	1.54±0.46[a]	1.17±0.40[a]	1.28±0.64[a]
Total products	1.69±0.18[a]	3.38±0.56[b] 3.07 ¿?	4.27±0.40[c] 5.08 ¿?	7.94±0.30[d] 7.56 ¿?

Total compounds are the sum of all individual non-polar oxidation products detected. Results are expressed as mean ± standard deviations. Values in the same row bearing different letters are significantly different ($p < 0.05$). n. d. means non detected product.

Table 4. Formation of mid-polar oxidation products in ~~commercial standard of~~ stigmasterol when heated at 180 ºC.

Mid-polar oxidation products (µg/mg commercial stigmasterol)	**0 h**	**1 h**	**2 h**	**3 h**
Stigmastan-2,4,6,22-tetraene	n.d.	n.d.	n.d.	n.d.
Stigmasterol	859.57±18.10^{b}	639.80±5.55^{b}	425.14±11.30^{ab}	357.05±11.30^{a}
Stigmastan-3,5,22-triene	n.d.	3.19±0.23^{a}	3.31±0.14^{a}	3.75±0.20^{b}
Stigmastan-3,5,22-trien-7-one (isomer)	n.d.	n.d.	n.d.	n.d.
Stigmastan-4,22-dien-3-one	n.d.	4.76±0.32^{a}	5.13±0.60^{a}	5.35±0.34^{a}
Stigmastan-3,5,22-trien-7-one	n.d.	9.01±0.58^{a}	12.39±0.57^{b}	12.98±0.87^{b}
Stigmastan-4,22-dien-3,6-dione	n.d.	12.53±1.03^{a}	19,36±1.67^{b}	24.87±0.52^{c}
Unknown product	n.d.	1.38±0.28^{a}	1.36±0.62^{a}	1.32±0.50^{a}
Total products	859.57±18.10^{d}	670.60±1.86^{c}	464.01±3.85^{b}	405.31±3.88^{a}

Total compounds are the sum of all individual mid-polar oxidation products detected. Results are expressed as mean ± standard deviations. Values in the same row bearing different letters are significantly different ($p < 0.05$). n. d. means non detected product.

Table 5. Specific mass spectral ions and retention times of non-polar and mid-polar oxidation products.

	Specific ions m/z	Retention time (min)	
		GC-MS	GC-FID
Stigmastan-2,4,6,22-tetraene	392(100) 377(4) 349(3) 273(5) 253(15) 251(8) 211(7)	15.71	17.84
Stigmastan-3,5,22-triene	394 (91) 379(26) 255(74) 253(17) 213(19) 159 (100)	21.99	24.23
Stigmastan-3,5,22-trien-7-one (isomer)	408(69) 269(100) 267(39) 187(26) 174(42) 161(29)	23.08	25.29
Stigmastan-4,22-dien-3-one	410(49), 367(33), 298 (38), 271(55), 269(50), 245(30), 145 (26), 55 (100)	24.08	26.41
Stigmastan-3,5,22-trien-7-one	408(100) 269(52) 267(18) 187(23) 174(40) 161(19)	25.05	27.36
Stigmastan-4,22-dien-3,6-dione	424(24), 381(23), 311(26), 285(39), 243(16), 137(46), 55(100)	30.02	32.35

Figure 1. Total stigmasterol content after the different heating times (µg/mg)

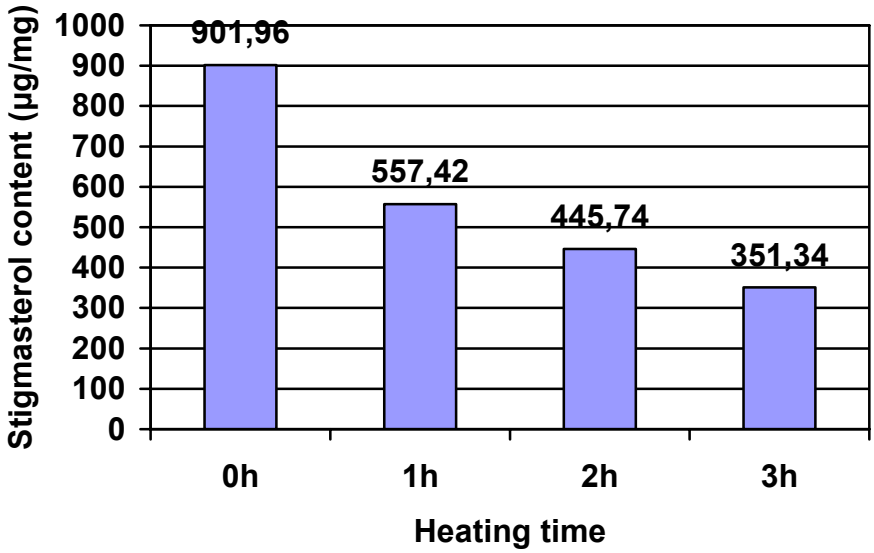
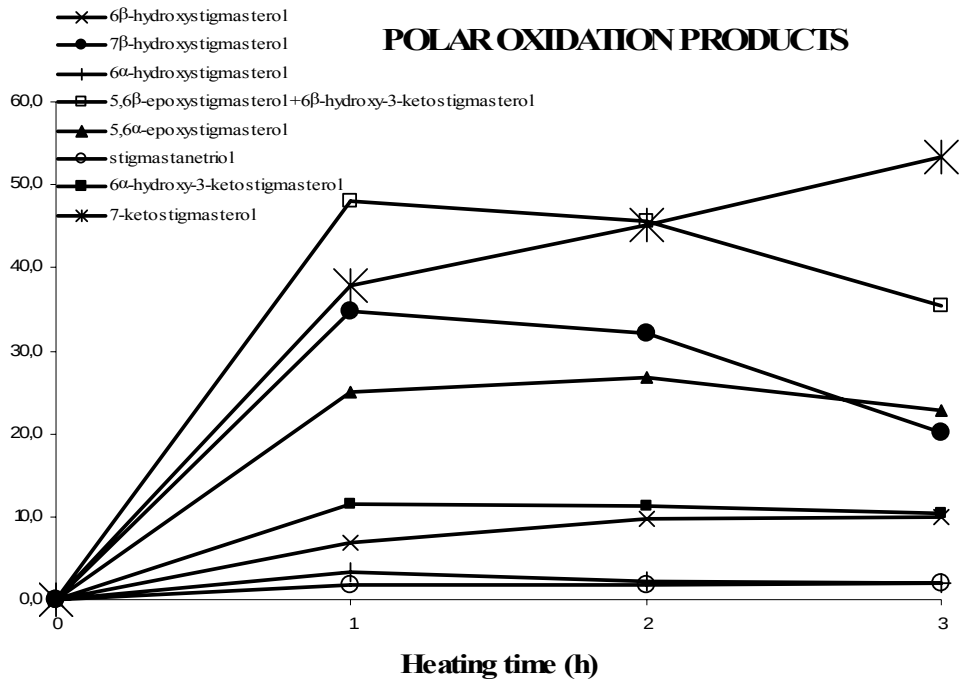
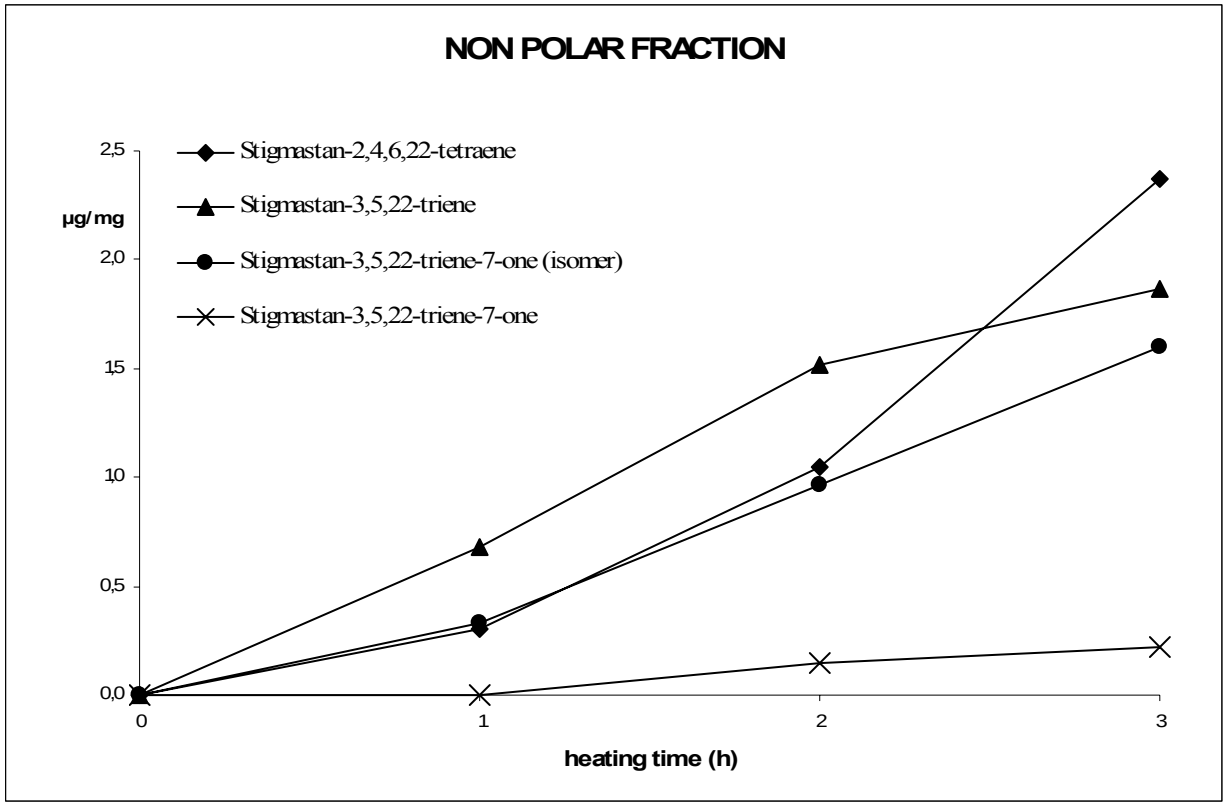


Figure 2. Polar oxidation products content along the three-hour heating time.



1 Figure 3. Non -polar oxidation products content along the three-hour heating time.



1 Figure 4. Mid polar oxidation products content along the three-hour heating time.

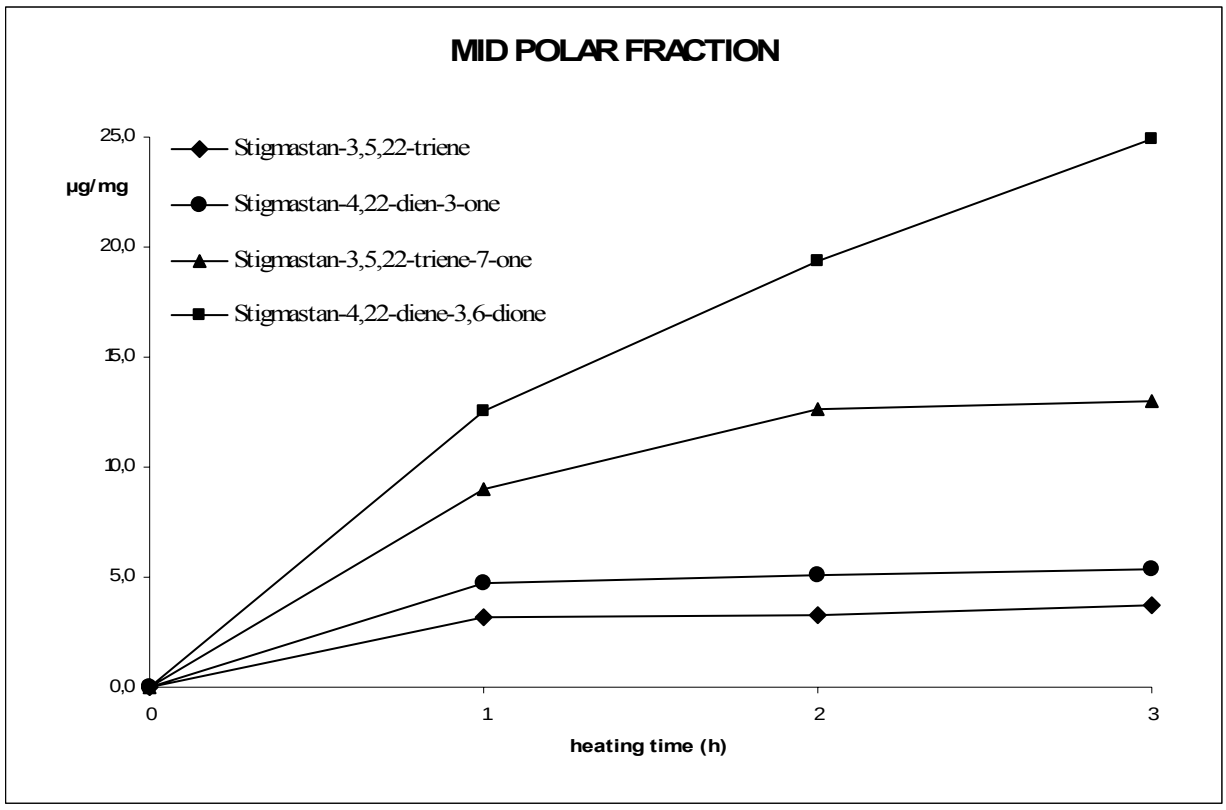
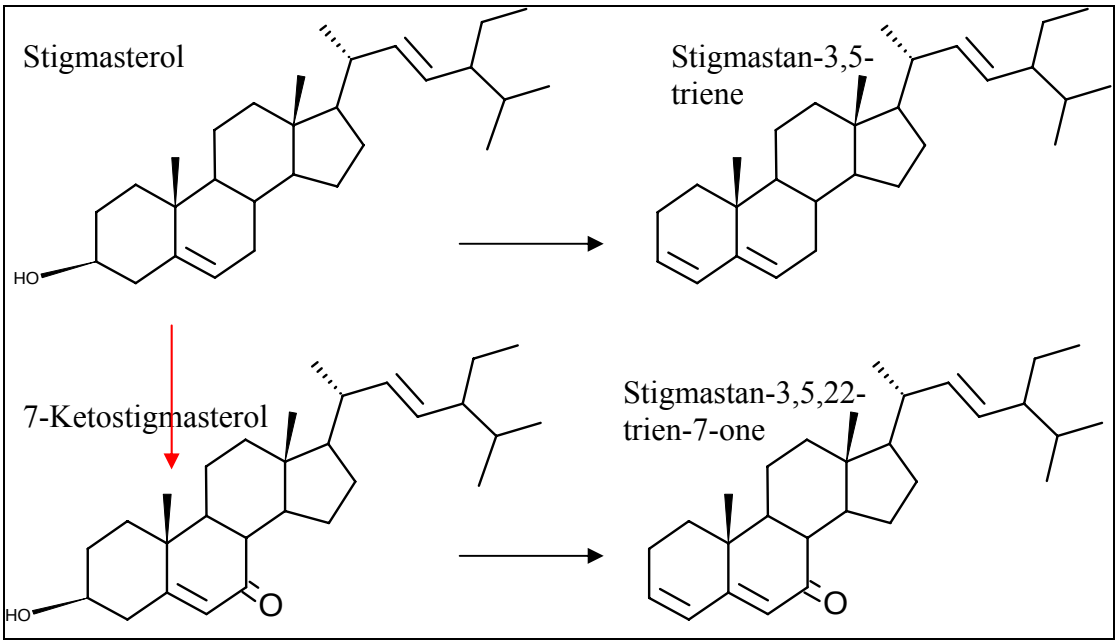
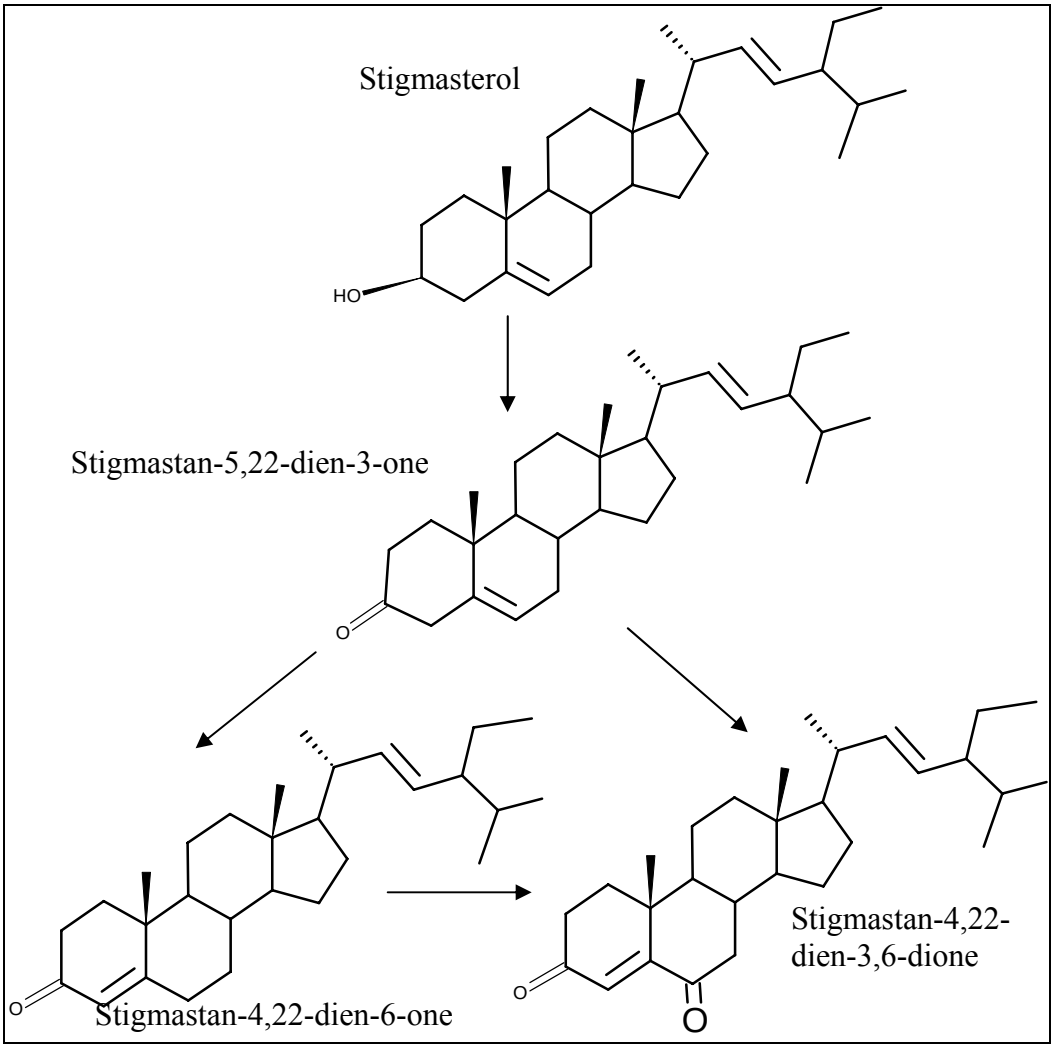


Figure 5. Scheme reactions for the degradation of stigmasterol and products obtained.




Stigmasterol
HO
Stigmastan-5,22-dien-3-one
O
Stigmastan-4,22-dien-6-one
O
Stigmastan-4,22-
dien-3,6-dione
O
O

1
2

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AOCS Official Method Cd 27-96. (1997). Steroidal hydrocarbons in vegetable oils. *The Official Methods and Recommended Practices of the American Oil Chemists' Society,* Champaign, IL.

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