The heating and storage of structured acylglycerols with succinyl-linked stigmasterol residue does not affect their chemical and biological negative changes

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Abstract

Four structured acylglycerols with stigmasterol bonded by succinyl linker were investigated and their stability were analyzed. Samples were heated at 60°C which simulated storage test and at 180°C simulated a frying conditions. The degradation of synthesized compounds and formed derivatives were determined. Also their cytotoxicity and genotoxicity on normal human cells from digestive system were analyzed.

Heating at 180°C resulted in greater degradation of the tested compounds than at 60°C. In all the tested samples, compounds containing oleic acid in the structure were the most stable. At 60°C this was DO2SSt, and at 180°C it was DO3SSt. The results showed that the type of fatty acid residue in the molecule is more important than its position in the glycerol structure.

All analyzed DASStGs, before and after heating, did not exhibit the cytotoxic and genotoxic potential of the analyzed compounds to the small intestine and colon mucosa cells.

Introduction

Plant sterols, named also as phytosterols (PSs), are the natural steroids widely founds in different parts of plants where they play an important role in their cell membranes. They exist in plants in different forms, such as free compounds, esters with fatty acids, steryl glycosides and acylated glycosides. The chemical structure of phytosterols is similar to cholesterol and they can lower total and low density lipoprotein cholesterol (LDL-C) in human plasma. Additionally, they have been described as substances that increase insulin resistance and lipid metabolism, reduce the risk of cancer, Alzheimer's disease and cardiovascular diseases. Their main sources in human diet are vegetable oils, nuts and cereals and their consumption is 100–500 mg/day. But the content of plant sterols in natural food is too low to meet the supplementary recommendation of 2–3 g/day for adults. Phytosterols are more frequently added as functional compounds to different food products. Since they are used as fatty acids esters, their daily intake in Europe is much lower than recommended 2 g/day. On the other hand, dietary exposure varied from 3.4 to 10.9 mg/(kg x day) from baked food to 13.2 mg/day from potatoes fries on PS-enriched margarines.

The US Department of Agriculture in report 2015–2020 recommended intake of cholesterol < 300 mg/day but some data showed that it could be even 530–750 mg/day in Finland and USA. The bioavailability of plant sterols is very low and their absorption in digestive system is less than 5% while cholesterol can be absorbed at 50–60%. Because of that, the intake of plant sterols must be much higher than cholesterol. But this high consumption increase the content of their derivatives in human diet. The cholesterol and phytosterols are not stable during food processing and storage. They can formed a wide group of derivatives with negative effect on food quality and human organisms. These included oxidation products, volatile compounds, polar compounds, oligomers and fragmented molecules. Sterol oxidation can occur due to autoxidation, photosensitized and enzymatic oxidation. It can be
affected by degree of unsaturation, temperature, light, water activity, presence of prooxidants, antioxidants, metals and photosensitizers. The bioaccessibility of phytosterol oxidation products (POPs) is much higher than free sterols and for triol, α-epoxysitosterol and 7β-hydroxysitosterol was as high as 60%. The diversity of biological influences of POPs were described as pro-inflammatory, cytotoxic, affecting cholesterol metabolism.

The stability and safety of triacylglycerols (TAGs) depend on the length carbon chain of esterified fatty acids, their unsaturation level and position in the glycerol backbone. TAGs with unsaturated fatty acids linked at the sn-2 position were more stable towards oxidation than that linked at the position sn-1 or sn-3. Studies conducted by Camacho Paez et al. have shown that the introduction unsaturated fatty acids to the sn-2 position of glycerol by transesterification improve the absorption rate of the essential fatty acids.

Structured acylglycerols are a class of glycerides with specific molecular structure or function that chemically or enzymatically change composition or position distribution of the glycerol skeleton.

In these studies four modified acylglycerols containing one molecule of stigmasterol were analyzed for oxidative stability and biological properties. The stigmasterol molecule was attached to the glycerol skeleton at sn-2 or sn-3 position by succinyl linker. The distigmasterol-modified acylglycerols bonded by a succinyl linker and products formed during their thermo-oxidation showed no cytotoxic or genotoxic activity to normal human cells and were more stable than compounds with stigmasterol bonded with glycerol backbone by carbonate linker.

The aim of this work was to analyze the thermal and oxidative stability of acylglycerols containing a phytosterol molecule in the sn-2 or sn-3 position, to investigate the influence of temperature and the position of stigmasterol in the glycerol skeleton on their degradation and toxicity.

**Results and discussion**

The structured acylglycerols with two residues of fatty acids – palmitic or oleic – at the external positions of glycerol and the residue of stigmasterol linked to the internal position (compounds DP2SSSt and DO2SSSt) as well as their asymmetric counterparts containing stigmasterol residue linked to sn-3 position and fatty acid residues at sn-1 and sn-3 positions (compounds DP3SSSt and DO3SSSt) were synthesized by. The stigmasterol was attached to a glycerol skeleton by succinyl linker (Fig. 1) The stability and safety of these compounds after heating at temperatures simulated storage (Schaal test) and frying was determined.

### 2.1 Changes of DASStGs during heating at 60°C

#### 2.1.1 Stability of DASStGs
The level of DASStGs degradation during heating at 60°C for 8 hours is presented in Fig. 2A. The degradation of DASStGs ranged from 11% in DO2SSt to 25% in DO3SSt. After heating of DP2SSt and DP3SSt their amounts decreased by 20 and 24%, respectively. Obtained results showed that new structured acylglycerol with stigmasterol at position sn-2 and two oleic acid residues at sn-1 and sn-3 positions was the most stable.

During heating of distigmasterol-modified acylglycerols at 60°C for 8h the lowest degradation was observed for compound with oleic acid and amounted 5% and when oleic acid was replaced by palmitic acid, degradation increased to 8%.

The chemical structure of triacylglycerols (TAGs) has a significant impact on their stability and bioavailability in the gastrointestinal tract. The oxidation induction period of soybean oil was delayed by 2–3 days because of the addition of monoacylglycerol with DHA at sn-2 position. Studies conducted by Camacho Paez et al., (2003) have shown that location of essential fatty acids at the sn-2 position can improve their absorption during digestion.

When diacylstigmasterylcarbonoyl-sn-glycerols with the stigmasterol linked to the sn-2 position was heated to 60 °C, their degradation was not observed, but for compounds with the stigmasterol at sn-3 position the degradation was 18%. Barriuso et al. demonstrated that the presence of unsaturated fatty acids in lipid matrices show protective effect on phytosterols during heating.

### 2.1.2 Degradation of fatty acids

The effect of temperature, which simulated storage test, on the oxidative stability of fatty acids in DASStGs is presented in Fig. 2B. When palmitic or oleic acids were linked at sn-3 position of glycerol their degradation ranged 8–11%. When fatty acids were placed at sn-2 only 1–3% of palmitic and oleic acids content decreased. The position of fatty acids in glycerol backbone was more significant than the unsaturation level and chain length. Similar results were obtained by X. Y. Wang et al., (2015), where soybean oil, which contained a higher amount of linoleic acid at the sn-2 position, was more resistant to autoxidation than structured soybean oil, which had a similar total fatty acid composition but a different positional distribution of linoleic acid. The position of fatty acids in glycerol backbone has also influence on the nutritional properties of oils. The location of γ-linolenic acid at sn-2 position is important in determining the biological and clinical efficacy of borage oil. Fatty acids at sn-2 position appeared to influence the cholesterol levels rather than total fatty acids of the triglycerides.

### 2.1.3 Degradation of stigmasterol part

The degradation of stigmasterol as a component of DASStGs during heating at 60°C for 8 hours is presented in Fig. 2C. The lowest decrease of stigmasterol content was determined for DO2SSSt and amounted to 13%, whereas the degradation of other DASStGs ranged from 22 to 27%. The results showed that the position of stigmasterol in the glycerol backbone has a significant effect on its degradation and the sn-2 position is preferred. The degree of saturation of fatty acids had less effect on the loss of sterol than its position in the structure of the glycerol molecule.
When free stigmasterol and stigmasteryl palmitate were heated at 60°C, about 3% and 6% of stigmasterol was degraded \(^{21}\). After heating of distigmasterol-modified acylglycerols at the same temperature, a decrease of sterol ranged from 3 to 14% and when a saturated fatty acid was a part of the these compounds, the degradation of sterol was 2-fold lower than in compounds containing oleic acid \(^{21}\).

When diacylstigmasterylcarbonoyl-\(sn\)-glycerols were heated to 60°C and the stigmasterol was at the \(sn\)-2 position, its degradation was not observed but acylglycerols containing stigmasterol at the \(sn\)-3 position degradation of from 16 to 21% was observed depending on the fatty acids in molecule \(^{30}\).

### 2.1.4 Oxystigmasterols

Stigmasterol oxidation products (StOPs) were detected in all samples heated at 60°C and obtained results are presented in Fig. 2D. The lowest total content of these compounds was 0.11 and 0.14 mg/g for DP2SSSt and DO2SSSt, respectively. When the stigmasterol was linked to \(sn\)-3 position, the content of StOPs was higher and amounted to 0.36 mg/g for DP3SSSt and 1.39 mg/g for DO3SSSt.

Four oxidized derivatives of stigmasterol were identified in the heated samples – 7\(\alpha\)- and 7\(\beta\)OHSt, \(\beta\)EpSt and 7ketoSt. The 7\(\beta\)OHSt and 7ketoSt were detected in all samples, but 7\(\alpha\)OHSt was only in DASStGs with the stigmasterol at \(sn\)-3 position, while the \(\beta\)EpSt was determined only in DO3SSSt. The most toxic oxidized derivative, triolSt was not identified in these samples.

The monitoring of phytosterol oxidation products (POPs) formed during storage of food products is more important than daily intake of phytosterol-enriched diets \(^{10}\). Animal studies showed that absorption of POPs is much higher than non-oxidized sterols and 7hydroxy-derivatives were more relevant in plasma than 7keto-sterols which are typical major oxyphytosterols in food products \(^{14,35}\).

When free stigmasterol and its esters with oleic and linoleic acids were heated at 60°C for 12 hours, the total content of oxophytosterols amounted 1.1, 0.2 and 9.7 mg/g, respectively \(^{15}\). The content of StOPs in distigmasterylmodified acylglycerols heated at this temperature for 8 hours was 1.4 mg/g for derivatives with stigmasterol attached by a carbonate linker and 0.4–0.5 mg/g for derivatives with stigmasterol attached by a succinyl linker \(^{21}\).

### 2.1.5 Fragmented molecules and oligomers

The heating of DASStGs at 60°C for 8 hours did not cause the formation of fragmented molecules and oligomers. Absence of fragmented molecules and oligomers in analyzed samples indicates their stability during storage. This could have a significant impact when using these compounds as functional food additives.

Fragmented molecules and oligomers were determined after heating at 60°C of free sterols, their esters and acylglycerols modified with stigmasterol \(^{13,15,21}\), but it was rather compounds with a lower molecular weight than monomers. Among them volatile compounds and stigmasteryl formate or hemisuccinate were detected \(^{13,21}\).
2.2 Changes of DASStGs during heating at 180°C

2.2.1 Stability of DASStGs

The degradation of DASStGs after heating at 180°C for 8 hours ranged from 39% for DO3SSt to 91% for DO2SSt (Fig. 3A). When two palmitic acid molecules were bonded with glycerol backbone the degradation was 57% for DP2SSt and 76% for DP3SSt. The differences in thermo-oxidative stability of analyzed compounds were significant and they can be caused by position of fatty acids and stigmasterol in glycerol skeleton as well as saturation level of fatty acids. However, this requires further research.

When 2,3-distigmasterylcarbonyl-1-oleoyl-sn-glycerol was heated at 180°C for 8 hours almost 85% was degraded, but only 20% of 2,3-distigmasterylsuccinoyl-1-oleoyl-sn-glycerol content decreased.

Relative thermo-oxidative stability of triacylglycerols depend on the unsaturation level of fatty acids and their position in glycerol backbone but did not depend on the carbon chain length. Our results showed that it also depends on the linker used for bonded the stigmasterol to glycerol skeleton and compounds with succinyl linker were more stable than those with carbonate one.

2.2.2 Degradation of fatty acids

The degree of degradation of the fatty acid part was correlated neither with the number of double bonds in the acid structure nor with its position in the triacylglycerol. The lowest decrease of fatty acid part was detected for DO3SSt and amounted to 25% (Fig. 3B). When palmitic acid was linked to sn-1 and sn-2 positions of DASStGs, the degradation was much higher and reached 65%. The opposite relationship was observed when the fatty acid residue was located at sn-1 and sn-3 positions. During heating at 180°C for 8h 53% of DP2SSt and 71% of DO2SSt was degraded. During heating at this temperature, the fatty acid residues undergo a number of reactions due to their oxidation, polymerization and decomposition.

Phytosterols chemically acts as antioxidants and antipolymerizers, and their presence in the structure of triacylglycerol may have different effects on the transformation of the whole molecule, which requires further research.

There is lack of information about the influence of acyl group position in glycerol backbone on the oxidation and degradation during long term heating at the frying temperature. Previous studies have mainly compared the stability of oils and fats which are characterized by different composition of fatty acids and their position in glycerol. The structure of triacylglycerols could be one of the most important factors determining their thermal stability. The mixture of saturated (PPP) and unsaturated (LLL) triacylglycerols was more susceptible to thermal oxidation at 150 and 180°C than PPP.PLL and PPL. The oxidation rates of LnLnL and LLLn were faster than LnLLn and LLnLn. When 1,2-distigmasterylsuccinoyl-3-palmitoyl-sn-glycerol and 2,3- distigmasterylsuccinoyl-1-oleoyl-sn-glycerol were heated at 180°C for 8 h, the degradation of fatty acids amounted 40 and 6%, respectively.
data showed that the chemical structure of triacylglycerols has a crucial influence on their stability. New structured acylglycerols can play an important role due to their technological and nutritional value.

### 2.2.3 Degradation of stigmasterol part

The degradation of stigmasterol during heating at 180°C was different than at 60°C and ranged from 16% for DO3SSt to 73% for both acylglycerols with palmitic acid – DP2SSt and DP3SSt (Fig. 3C). For DO2SSt 65% of stigmasterol was degraded.

During heating of free stigmasterol at 180°C for 8h the degradation was about 90% and during heating of stigmasterol esters with palmitic and oleic acids, the degradation of stigmasterol moiety was in the range of 50–53% \(^{21}\). Heating of the stigmasteryl stearate, oleate, linoleate and linolenate at the same temperature for 12h caused their degradation from 55 to 97% \(^{26}\). Higher unsaturation caused faster degradation, indicating that free radicals stimulated the degradation of sterol part of the molecule and affect the decomposition of sterol \(^{26}\). Distigmasterol-modified acylglycerols with palmitic acid residue degraded slower than stigmasteryl esters and their degradation amounted to 38% for 1,2-distigmasterylsuccinoyl-3-palmitoyl-sn-glycerol and 46% for 2,3-distigmasterylsuccinoyl-1-oleoyl-sn-glycerol \(^{21}\). When stigmasterol has been incorporated into a glycerol molecule, its degradation is affected not only by the degree of saturation of the fatty acids, but also by its position in the glycerol backbone. The influence of fatty acid position in triglycerides on the thermal oxidative stability and dietary properties were determined \(^{40}\) but incorporated sterol in glycerol was analyzed only for asymmetric distigmasterol-modified acylglycerols \(^{21}\). The important role in stability of stigmasterol in distigmasterol-modified acylglycerols played the linker and compounds with succinyl linker showed higher stability than those with carbonate linker.

### 2.2.4 Oxystigmasterols

The total content of stigmasterol oxidation products (StOPs) formed during heating of DASSStGs at 180°C for 8 hours ranged from 0.1 to 31.0 mg/g. The lowest amount of StOPs was detected in DP2SSt and the highest content was in DO2SSt (Fig. 3D). When 2,3-distigmasterylsuccinoyl-1-oleoyl-sn-glycerol and 1,2 distigmasterylsuccinoyl-3-palmitoyl-sn-glycerol were heated at 180°C for 8 hours, the total content of StOPs was 0.8 and 4.1 mg/g, respectively \(^{21}\). Analysis of total oxysterols will be incomplete without checking the composition of this fraction, with particular attention to the triol derivative, which is considered the most toxic. In our samples triolSt was not determined. In DP2SSt only 7αOHSt was identified, but in DP3SSt 7βEpSt and 7ketoSt were detected. Samples with oleic acid were characterized by four or five oxidation derivatives such as 7αOHSt, 7βOHSt, 7βOHSt in DO2SSt and additional 7βOHSt in DO3SSt.

The oxidation of sterols, as a free radical chain reaction, starts with the formation of hydroperoxides. Double bonds easily undergo radical attack followed by hydrogen atom abstraction at the carbon atoms in the α-positions to the double bonds. Such allylic type of hydrogen atoms can be easily abstracted due to the relatively low C-H bond dissociation enthalpy. The final oxidation products of sterols are hydroxy,
keto and epoxy compounds. The cytotoxicity, pro-inflammatory and pro-atherogenic effects of phytosterol oxidation products were determined. When stigmasteryl esters with different fatty acids were heated at 180°C for 12 hours, the content of oxyphytosterols increased at the beginning then decreased and it depend on the unsaturation level of fatty acids bonded with sterol. The sterol oxidation products are formed as a ring and side chain free radical reactions and they can interact among themselves. The low amount of oxyphytosterols can be caused by the formation of dimers, oligomers and low molecular compounds.

2.2.5 Fragmented molecules and oligomers

The formation of low weight molecules, dimers and oligomers of phytosterols after heating was determined first time by Rudzińska et al. These compounds were also detected in esters of plant sterols after their thermo-oxidation. They have influence on the nutritional quality and biological properties of food products containing them.

After heating of DASStGs, the samples were divided into polar, mid-polar and non-polar fractions and dimers, oligomers and fragmented molecules in all fractions were separated using HPLC.

DO2SSSt consist of 3% of polar dimers, 36% of mid-polar dimers and 29% of mid-polar fragmented molecules and was the most unstable compound. From other side in DO3SSSt non dimers and fragmented molecules were detected and it was the most stable sample. The composition of DP2SSSt and DP3SSSt was similar and the share of mid-polar dimers was 15 and 16%, and mid-polar fragmented molecules was 28 and 22%, respectively. Trimers and other oligomers were not detected in analyzed samples.

When free sitosterol was heated at 120 and 180°C for 24 hours trimers and tetramers were determined. After heating of stigmasteryl oleate and stigmasteryl linoleate at 180°C for 8 h polar trimers, dimers and non-polar dimers were detected and their share was 13, 21, 2% and 14, 63, 2%, respectively. During heating of triacylglycerols with palmitic (P) and linoleic (L) acids at 150°C for 8 hours the highest amount of polymers was determined for PPP/LLL (2:1) followed by PPP/PLL (1:1) and it was concluded that thermal stability of triacylglycerols such as edible oils depends on their fatty acid composition. Our results showed that also position of fatty acids influence on their stability and sterols can have protection effect on unsaturated fatty acids.

2.3 Cytotoxicity and genotoxicity

The effects of DP2SSSt, DP3SSSt, DO2SSSt, and DO3SSSt on the proliferation, viability, and metabolic activity of human cells derived from normal tissues of the digestive system are shown in Fig. 4. The experiments performed did not show the cytotoxic potential of the analyzed compounds to the small intestine and colon mucosa cells (Figs. 4A and 4B). Also, thermo-oxidative treatment of the stigmasterol derivatives at 60 and 180°C did not alter their cytotoxic potential to intestinal cells (Figs. 4D, 4E, 4G, and 4H). Moreover, the DO3SSSt compound at 10 µg/mL, 50 µg/mL, and 100 µg/mL stimulated the proliferation of the small intestine cells (up to 34%) and colon mucosa cells (up to 23%) (Figs. 4A and 4B). Thermo-oxidation
products of DO3SSt also enhanced the growth of intestinal FHS 74Int (up to 28%) and CCD 481CoN cells (up to 26%) (Figs. 4D, 4E, 4G, and 4H); however, the proliferation of FHS 74Int cells was significantly increased only under treatment with heated DO3SSt at doses of 50 µg/mL and 100 µg/mL (Figs. 4D and 4G).

It's worth noting that DO3SSt heated at 180°C showed a higher capacity for FHS 74Int cell growth stimulation than DO3SSt heated at 60°C. DO3SSt subjected to thermo-oxidative process at 60 and 180°C and introduced to the FHS 74Int cell cultures at maximum concentration tested (100 µg/mL) increased cell proliferation by 14% and 25%, respectively (Figs. 4D and 4G). In the culture of colon CCD 481CoN cells treated with DO3SSt, cell proliferation was increased independently on the thermo-oxidative treatment (Figs. 4B, 4E, 4H).

The analyzed non-heated stigmasterol derivatives did not affect THLE-2 liver cell proliferation and viability, except DP3SSt, which decreased hepatocyte proliferation and viability when applied at 50 µg/mL (↓14%) and 100 µg/mL (↓15%) doses. DP3SSt at a lower dose of 10 µg/mL did not inhibit THLE-2 cell growth (Fig. 4C). A similar cytotoxic potential was observed for DP3SSt heated at 180°C under oxidative conditions (Fig. 4I). In contrast, DP3SSt heating at 60°C reduced its cytotoxic potential. No cytotoxic effects were noted in THLE-2 hepatocyte cultures treated with DP3SSt heated at 60°C (Fig. 4F).

The small intestinal FHS 74Int cells, colon CCD 481CoN cells, and liver THLE-2 cells were analyzed by an alkaline comet assay to detect DNA strand breaks induced in single cells by treatment with the stigmasterol compounds. Figure 5 presents DNA damage in the FHS 74Int (Fig. 5A), CCD 481CoN (Fig. 5B), and THLE-2 (Fig. 5C) cells treated for 48 h with the compounds at the maximum dose tested (100 µg/mL). Data in Fig. 5 (A, B, C) documents that the level of DNA strand breaks in the cells non-treated and treated with the compounds tested did not differ significantly. Similarly, the thermally processed (60 and 180°C) stigmasterol compounds did not increase DNA damage in the cells treated. In comparison, the levels of DNA strand breaks in the non-treated cells and in the cells treated with an oxidant (100 µM H$_2$O$_2$) were shown in Figs. 5D and 5E, respectively.

Additionally, microscopic documentation in Fig. 5 images of the small intestine cells, colon cells, and hepatocytes with non-induced and H$_2$O$_2$-induced DNA damage. Based on the data obtained, it can be concluded that the analyzed stigmasterol compounds do not show genotoxic effects on the normal human cells of the intestinal tract and liver.

**Conclusions**

The new structured acylglycerols with stigmasterol bonded by succinyl linker to the glycerol backbone were synthesized and their stability during heating at 60°C and 180°C which simulated storage and frying, were analyzed.

Heating at 180°C resulted in greater degradation of the tested compounds than at 60°C. Only in the case of DO3SSt was the loss of sterol greater at the lower temperature (26%) than at the higher temperature.
(16%). This is probably related to the fact that rather oxidation reactions took place at 60°C, whereas at 180°C the compounds underwent rapid thermal degradation. In all the tested samples, compounds containing oleic acid in the structure were the most stable. At 60°C this was DO2SSt, and at 180°C it was DO3SSt.

When heated at 60°C for 8 hours, the degradation of DO2SSt was 11%, of oleic acid 3% and of stigmasterol 13%. After heating at 180°C, DO3SSt content decreased by 39%, oleic acid by 25% and stigmasterol by 16%.

The results showed that the type of fatty acid in the molecule is more important than its position in the glycerol structure.

All analyzed DASStGs, before and after heating, did not exhibit the cytotoxic potential of the analyzed compounds to the small intestine and colon mucosa cells. Moreover, the DO3SSt compound can stimulate the proliferation of the small intestine cells and colon mucosa cells. It can be also concluded that the analyzed compounds do not show genotoxic effects on the normal human cells of the intestinal tract and liver.

The synthesized new stigmasterol derivatives could be a very safe and stable source of phytosterols for humans diet. However, their use requires additional studies related to their absorption in the human body and bioavailability.

Materials and methods

3.1 Materials

N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% TMCS, tert-butyl methyl ether (MTBE), ethyl acetate, methanol, n-hexane, dichloromethane, diisopropyl ether, toluene, anhydrous pyridine, trifluoroacetic acid, sodium methanolate, and silica gel (70–230 mesh, high purity) and standards of stigmasterol (95%), 5α-cholestan and ethyl heptadecanoate were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Glyceryl heptadecanoate was obtained from Larodan (Sweden).

3.2 Methods

3.2.1 Synthesis of diacylstigmasterylsuccinoylglycerols (DASStGs)

1,3-Dipalmitoyl-2-stigmasteryl succinoyl glycerol (DP2SSt) and 1,3-dioleoyl-2-stigmasteryl succinoyl glycerol (DO2SSt) were synthesized from 2-benzyloxy-1,3-propanediol and dihydroxyacetone, respectively. 1,2-Dipalmitoyl-3-stigmasteryl succinoyl sn-glycerol (DP3SSt) and 1,2-dioleoyl-3-stigmasteryl succinoyl sn-glycerol (DO3SSt) were synthesized from 1,2-O-isopropylidene sn-glycerol. The
details of synthetic protocols were described by Gladkowski et al.\textsuperscript{22}. In Fig. 1 the chemical structures of synthesized compounds are presented.

### 3.2.2 Heating of samples

The synthesized compounds were placed into glass vials filled with oxygen to ensure the oxygen atmosphere during thermal treatment. Samples were heated for 8 hours at 60°C, the temperature which simulates accelerated storage test\textsuperscript{23} and at 180°C to simulate frying process. The experiment was performed in two replicates.

### 3.2.3 Degradation of heated DASStGs

The level of DASStGs degradation depended on the stability of fatty acids and stigmasterol. Because of that decrease of DASStGS, stigmasterol and fatty acids were determined separately.

The level of DASStGs degradation was performed according to Rudzińska et al.\textsuperscript{21} Briefly, after dissolving samples in DCM, they were separated on an HPLC-ELSD Agilent 1260 Infinity II (Agilent, Santa Clara, CA, USA) equipped in EC-C18 InfinityLab Poroshell 120 column worked in isotherm at 44°C. The mobile phase consist of acetonitrile (A) and DCM (B) with flow 0.5 mL min\textsuperscript{-1}, programmed as follows: 0 min 80% A and 20% B for 30 min, changed to 55% A and 45% B; changing the phase composition to initial parameters after 80 min and maintaining this composition for 10 min. The temperature of evaporator and nebuliser was 30°C, gas flow rate 1.6 L min\textsuperscript{-1} (SLM) and photomultiplier tube (PMT) Gain 1.0. As the internal standard glyceryl heptadecanoate was used. All analyses were performed in three replications.

For determination of sterol part the AOCS Official Method Ch 6–91, (2009) was used. Briefly, samples were saponified and unsaponifiables were silylated by BSTFA + 1% TMCS. Derivatives were separated on gas chromatograph an HP 6890 equipped with a DB-35MS capillary column heated at programmed temperature from 100°C for 5 min, increased to 250°C at 25°C min\textsuperscript{-1}, held for 1 min, then to 290°C at 3°C min\textsuperscript{-1} and held for 20 min. The temperature of FID was set at 300°C. The hydrogen was used as carrier gas with flow rate of 1.5 mL min\textsuperscript{-1}. Internal standard 5α-cholestane was added to samples before analysis. Stigmasterol was identified by a comparison with retention time of the standard. Samples were analyzed in triplicate.

The degradation of fatty acids content was determined according to AOCS Official Method Ce 1k-07, (2007). Briefly, samples were hydrolyzed and then methylated with boron trifluoride in methanol. To separate of fatty acid methyl esters, a Trace 1300 gas chromatograph equipped with an SP-2560 capillary column and a FID was used. Hydrogen was the carrier gas, supplied at a rate of 1.5 mL min\textsuperscript{-1}. The oven temperature program increased from 160°C to 220°C at a rate of 12°C min\textsuperscript{-1}, and held there for 20 min. The injector and detector temperatures were both set to 240°C. Fatty acid methyl esters were identified in comparison with the standard retention times. As the internal standard, methyl heptadecanoate was used. All analyses were performed in duplicate.

### 3.2.4 Stigmasterol oxidation products (StOPs)
The content of stigmasterol oxidation products (StOPs) was determined according to the procedure described by Raczyk et al. 26. Briefly, samples of DASStGs before and after heating were treated with sodium methoxide. After 2h at room temperature, the organic fraction was extracted with chloroform and fractionated with SEP-PAK NH₂ using hexane and hexane:MTBE. Finally, the StOPs were removed with 7 mL of acetone, derivatized by BSTFA + 1% TMCS and analyzed on a Hewlett-Packard 6890 gas chromatograph equipped with an DB-5MS column (50 m x 0.2 mm x 0.33 µm; J&W, Folsom, CA). The column temperature was programmed from initial 160 °C held for 1 min, then increased at 40 °C min⁻¹ to 270 °C and held for 1 min; further programmed at 4 °C min⁻¹ to 280 °C, with the final temperature held for 25 min. The injector worked in a splitless mode. Hydrogen as a carrier gas was used at a flow rate of 1 mL min⁻¹.

StOPs were identified using an Agilent Technologies 7890A GC system coupled to a quadrupole 7000 QQQ-MSD. The same column and conditions as described above were used. All mass spectra were recorded in the electron impact ionisation mode using an energy of 70 eV and masses were scanned from 100 to 700 Da. The ion source was held at 200°C, while the injector was held at 300°C. The combination of the NIST Mass Spectra Library, authors’ own laboratory library of collected sterol data and retention data of standards were used to identify the compounds. Samples from an autonomous series were analyzed in triplicate.

### 3.2.5 Oligomers

Samples were divided into the polar, mid-polar and nonpolar fractions using silica gel (Sigma-Aldrich, silica gel 60, 63–200 µm) 27. Briefly, DASStGs after heating were dissolved in toluene and applied to a silica gel column, the nonpolar fraction was eluted with a mixture of hexane and diisopropyl ether (82:18, v/v), the mid-polar fraction with diisopropyl ether, then polar fraction with chloroform-methanol (2:1, v/v). The purity of all fractions and separation accuracy was verified using the thin layer chromatography method. A silica gel TLC plate was developed with hexane – diisopropyl ether (82:18, v/v), sprayed with a copper sulphate-phosphoric acid-methanol solution and heated at 120°C.

The oligomer composition was determined by HPLC on two Phenogel columns (100 Å i500 Å, 5 µL, 300 x 7.8 mm; Phenomenex, Torrance, CA, USA) connected in series. The column temperature was at 30°C, light scattering detector at 30°C, detector pressure 2.5 bars, and injection volume 1 µL. The liquid phase was dichloromethane (DCM) at the flow rate of 1 mL min⁻¹.

### 3.2.6 Cytotoxicity and genotoxicity experiments

The cytotoxicity and genotoxicity of stigmasterol derivatives (DP2SSt, DP3SSt, DO2SSt, and DO3SSt) and their thermo-oxidative degradation products and oxidized derivatives formed under treatment at 60 °C and 180 °C at oxygen atmosphere, was determined in normal human cells from digestive system, including the small intestine FHs 74 Int (ATCC® CCL241™), colon mucosa CCD 841CoN (ATCC® CRL-179™) and liver epithelial THLE-2 (ATCC® CRL-2706™) cells obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines were cultured according to ATCC recommendations. In
the cytotoxicity experiments, the cells were seeded in 96-well plates at a density of 1.5 × 10^4 cells/cm^2 (CCD 841CoN and FHs 74 Int cell lines) and 2.0 × 10^4 cells/cm^2 (THLE-2 cell line). 24-hour cell cultures were treated for 48 h with non-heated and heated stigmasterol derivatives at concentrations of 10 µg/mL, 50 µg/mL and 100 µg/mL. The MTT assay was applied to determine cell viability and metabolic activity in the cell cultures exposed to the compounds analyzed. The detailed procedure for the MTT test was described in the previous cytotoxicity studies.28

The genotoxicity of stigmasterol compounds was analyzed using the single cell gel electrophoresis (SCGE), named the comet assay. This method detected the DNA strand breaks in normal human cells induced by treatment with non-heated and heated stigmasterol derivatives. The cells were grown at 6-well plates at the established density and standard culture conditions and exposed to the tested compounds for 48 h. The non-treated cells and the cells treated with H_2O_2 (100 µM, 30 min) to induce oxidative DNA damage were analyzed as a negative and positive control, respectively. The comet assay was performed according to the previously described protocol.29 Briefly, the harvested cells were suspended in low melting point agarose and placed on microscope slides pre-coated with normal melting point agarose. Cell lysis at 4°C, alkaline electrophoresis (pH > 13), and neutralization (pH 10) were performed sequentially. The slides were stained with SYBRGold (Molecular Probes) and viewed under a fluorescence microscope (Axiovert 200, Zeiss, Carl Zeiss, Gottingen, Germany). Cells were analyzed for DNA damage using CometScore™ software (TriTek Corp., Sumerduck, VA, USA). Data on DNA strand breaks were expressed as the mean percentage of DNA content in the comet tail. At least 100 cells were analyzed on a microscope slide; 300 cells were considered for DNA damage detection in one sample.

3.2.7 Statistical analysis

The experiments and analysis were performed in three independent replicates and the data presented are the mean values with standard deviation (± SD). Statistical analysis was performed using the STATISTICA version 13.3 software (Statsoft, Inc., Tulsa, OK, USA). The significance of the main effects was determined by One-way analysis of variance (ANOVA). The equality of variances assumption was verified with Levene's test. Parametric Tukey's post hoc test was employed to analyze differences between the mean values of multiple groups. Statistical significance was considered at p < 0.05. RStudio (version 2022.07.01 + 554 with packages FactoMineR v.2.4 and factoextra v.1.0.7) was the software used for principal components analysis (PCA).

**Abbreviations**

DASStG - diacylmonostigmasterylsuccinoyl-sn-glycerol

DP2SSt - 1,3-dipalmitoyl-2-stigmasterylsuccinoyl-sn-glycerol

D02SSt - 1,3-dioleoyl-2-stigmasterylsuccinoyl-sn-glycerol

DP3SSt - 1,2-dipalmitoyl-3-stigmasterylsuccinoyl-sn-glycerol
DO3SSt - 1,2-dioleoyl-3-stigmasteryl succinoyl-sn-glycerol

BSTFA - N,O-bis(trimethylsilyl) trifluoroacetamide

TMCS - trimethylchlorosilane

MTBE - tert-butyl methyl ether

TFA - trifluoroacetic acid

DCM – dichloromethane

StOPs – stigmasterol oxidation products

TriolSt – stigmastentriol

7ketoSt – 7-ketostigmasterol

7αOHSt - 7α-hydroxystigmasterol

7βOHSt - 7β-hydroxystigmasterol

αEpSt - α-epoxystigmasterol

βEpSt - β-epoxystigmasterol

PCA – principal component analysis

PSs – phytosterols

POPs – phytosterol oxidation products

TAGs – triacylglycerols

P – palmitic acid

O – oleic acid

L – linoleic acid

Ln – linolenic acid

Declarations

Data Availability/ Availability of data and materials
The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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References


**Figures**
Figure 1

The chemical structures of diacylstigmasterylsuccinoyl glycerols (DASStGs). DP2SS - 1,3-dipalmitoyl-2-stigmasterylsuccinoyl-sn-glycerol; DO2SS - 1,3-dioleoyl-2-stigmasterylsuccinoyl-sn-glycerol; DP3SS - 1,2-dipalmitoyl-3-stigmasterylsuccinoyl-sn-glycerol; DO3SS - 1,2-dioleoyl-3-stigmasterylsuccinoyl-sn-glycerol

Figure 2

Remaining of DAS-SStGs (A), fatty acid parts (B), stigmasterol parts (C) and the content of stigmasterol oxidation products formed after heating of DAS-SStGs at 60 °C for 8 h; 7αOHSt - 7α-hydroxystigmasterol,
7bOHSt - 7β-hydroxystigmasterol, bEpSt - β-epoxystigmasterol, 7ketoSt - 7-ketostigmasterol; means with different letters differ significantly (p < 0.05).

Figure 3

Remaining of DAS-SStGs (A), fatty acid parts (B), stigmasterol parts (C) and the content of stigmasterol oxidation products formed after heating of DAS-SStGs at 180 °C for 8 h; 7aOHSt - 7α-hydroxystigmasterol, 7bOHSt - 7β-hydroxystigmasterol, bEpSt - β-epoxystigmasterol, aEpSt - α-epoxystigmasterol, 7ketoSt - 7-ketostigmasterol; Means with different letters differ significantly (p < 0.05).
Figure 4

Cytotoxicity of 1,3-dipalmitoyl-2-stigmasterylsucinoyl-sn-glycerol (DP2SSt), 1,2-dipalmitoyl-3-stigmasterylsucinoyl-sn-glycerol (DP3SSt), 1,3-dioleoyl-2-stigmasterylsucinoyl-sn-glycerol (DO2SSt), and 1,2-dioleoyl-3-stigmasterylsucinoyl-sn-glycerol (DO3SSt) non-heated (A, B, C) and heated at 60 °C (D, E, F) and 180 °C (G, H, I) to human normal small intestinal epithelial FHS 74Int cells (A, D, G), colon mucosa CCD 481CoN cells (B, E, H) and liver THLE-2 cells (C, F, I). The cells were treated with the analyzed compounds at concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL for 48 h. The cell viability determined by the MTT test was expressed relative to untreated control cells. \(^{a}p \leq 0.05, \(^{b}p \leq 0.01, \(^{c}p \leq 0.001 \) significant differences in cell viability compared to control cells.
Figure 5

DNA strand breaks in the normal small intestinal epithelial FHS 74Int cells (A), colon mucosa CCD 481CoN cells (B), and liver THLE-2 cells (C) treated with 1,3-dipalmitoyl-2-stigmasteryl-succinoyl-sn-glycerol (DP2St), 1,2-dipalmitoyl-3-stigmasteryl-succinoyl-sn-glycerol (DP3SSt), 1,3-dioleoyl-2-stigmasteryl-succinoyl-sn-glycerol (DO2SSt), and 1,2-dioleoyl-3-stigmasteryl-succinoyl-sn-glycerol (DO3SSt) non-heated and heated at 60 °C and 180 °C for 8 h. The cells were treated with the analyzed
compounds at a 100 µg/mL concentration for 48 h. DNA damage was expressed as the percentage of DNA content in the comet tail in the comet assay performed. Non-treated cells were the negative control (D), and the cells exposed to 100 µM H₂O₂ for 30 min. to induce DNA damage were a positive control (E). 

a $p \leq 0.05$ significant differences in DNA damage compared to control cells