Dietary secoisolariciresinol diglucoside and its oligomers with 3-hydroxy-3-methyl glutaric acid decrease vitamin E levels in rats

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(Received 20 October 2003 – Revised 6 February 2004 – Accepted 1 March 2004)

Secoisolariciresinol diglucoside (SDG) is an important dietary lignan that is found at very high levels in flaxseed (1–4 %, w/w). Flaxseed lignans have received much research interest in recent years because of reported phyto-oestrogenic, anticarcinogenic, and anti-atherogenic effects. Previously, flaxseed feeding has been shown to decrease vitamin E concentrations in rats despite the antioxidant potential of SDG in vitro. Sesamin, a sesame lignan, on the other hand has been shown to increase vitamin E concentrations in rats. The aim of the present study was to investigate the effect of dietary SDG and its oligomers on vitamin E and cholesterol concentrations in rats. SDG was extracted from defatted flaxseed flour with a dioxane–ethanol mixture and purified by silica column chromatography. The major oligomers with 3-hydroxy-3-methyl glutaric acid, containing a high ratio of SDG to p-coumaric and ferulic acid glucosides, were purified from the extracts by reversed-phase liquid chromatography. When fed to rats at 0.1 % in the diet for 27 d, both SDG and its oligomers had no effect on animal performance but caused an increase in liver cholesterol and a 2-fold reduction in the levels of α- and γ-tocopherols in rat plasma and liver. It is notable that a phenolic antioxidant, such as SDG, causes a vitamin E-lowering effect in rats. This cannot be explained at present, but warrants further investigations with respect to the magnitude, mechanism, and significance of the observed effect for human nutrition.

Flaxseed: Secoisolariciresinol diglucoside: Enterodiol: Vitamin E: Cholesterol

Secoisolariciresinol diglucoside (SDG) is an important dietary lignan that is found at low-to-moderate levels in many plant foods and at very high levels in flaxseed (Linum usitatissimum L.; Axelson et al. 1982; Mazur & Adlercreutz, 1998). The level of SDG in flaxseed, 1–4 % (w/w) (Johnsson et al. 2000; Eliasson et al. 2003), is 60–700 times higher than that in other edible plant parts (Ford et al. 2001). SDG was isolated from flaxseed for the first time by Bakke & Klosterman (1956) and was noted to exist in a tan polymeric material that includes among other things 3-hydroxy-3-methyl glutaric acid (HMGa) (Klosterman & Smith, 1954) and 4-O-β-D-glucopyranosyl-p-coumaric acid (linocinnamarin) (Klosterman et al. 1955). 4-O-β-D-Glucopyranosyl ferulic acid was isolated and identified as one of the main components of the ‘flaxseed polymer’ (Johnsson et al. 2001). A straight-chain oligomeric structure, composed of SDG residues inter-linked by HMGa residues with an average molecular weight of 4000 was assigned to the main lignan of flaxseed (Ford et al. 2001; Kamal-Eldin et al. 2001). Other lignans reported to be present in flaxseed in much lower quantities include an SDG isomer (Bambagliotti-Alberti et al. 1994a,b), isolariciresinol (Meagher et al. 1999), matairesinol (Mazur & Adlercreutz, 1998; Meagher et al. 1999; Liggins et al. 2000), and pinoresinol (Meagher et al. 1999; Qui et al. 1999; Sicilia et al. 2003).

Flaxseed lignans have received much research interest in recent years because of reported phyto-oestrogenic, anticarcinogenic, and anti-atherogenic effects (for a review, see Westcott & Muir, 2000). Ingested SDG is converted by enzymes of the gut microflora into the aglycone, secoisolariciresinol, which is subsequently transformed to the mammalian lignans enterodiol (END) and enterolactone (ENL) (Axelson & Setchell, 1981; Setchell et al. 1981; Axelson et al. 1982; Borriello et al. 1985). Excretion of these mammalian lignans was found to correlate negatively with the incidence of breast cancer (Adlercreutz et al. 1982, 1986) and SDG has been described as protective

Abbreviations: DFF, defatted flaxseed flour; END, enterodiol; ENL, enterolactone; HMGa, 3-hydroxy-3-methyl glutaric acid; SDG, secoisolariciresinol diglucoside.

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against breast, prostate, and colon cancers (Jenab & Thompson, 1996; Thompson et al. 1996). Flaxseed low in α-linolenic acid (<3%) was found to reduce athero-
sclerotic plaques in rabbits (Prasad et al. 1998), and this effect was attributed to the antioxidant and hypocholesterol-
olaemic properties of SDG (Prasad, 1997, 1999). Because of these effects, processes were developed for the extrac-
tion and purification of flaxseed SDG (Westcott & Muir, 1996, 1998) to be incorporated in consumer products
(Westcott & Muir, 2000).

It was previously reported that sesamin, a major lipophi-
lignan in sesame seed, lowers cholesterol and elevates γ-tocopherol levels in rats (Kamal-Eldin et al. 2000).
Since this observation, we have screened the effects of various antioxidants including butylated hydroxy toluene,
curcumin, (+)-catechin, cyanidin-3-glucoside, ferulic, caffeic and chlorogenic acids for their effects on the levels of cholesterol and tocopherols in rats (Kamal-Eldin et al. 2000; Frank et al. 2002, 2003a,b). The different phenolic compounds showed variable effects ranging from no effect to significant elevating effects on either or both parameters. Despite the beneficial effects of SDG (see earlier), the consumption of flaxseed was found to cause a significant lowering of α- and γ-tocopherol levels in rats (Ratnayake et al. 1992). In the present study, we isolated SDG and its major oligomers from flaxseed. We then tested their effects on tocopherol and cholesterol levels in a Sprague–Dawley rat model in order to elucidate whether these compounds are responsible for the pre-
viously observed effects (Ratnayake et al. 1992) or whether they would spare vitamin E as was previously reported for sesame lignans by our group (Kamal-Eldin et al. 2000).

Materials and methods

Chemicals and reagents

SDG and its oligomers (SDG-oligomers) were extracted (as described later) from a flaxseed cake (Alternativ Forödlings AB, Glanshammar, Sweden). α-Coumaric acid, used as an internal standard in the HPLC analysis of SDG, was pur-
chased from Sigma-Aldrich Chemie (Taufkirchen,
Germany). Ethanol was purchased from Kemethyl AB (Hanginge, Sweden) and 2H-labelled methanol and 2H2O,
used for NMR, were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). The rapeseed oil used in the rat diets was purchased from a local grocery store and the cholesterol, added to these rat diets, was pur-
chased from Sigma Chemical Co. (St Louis, MO, USA).
All other solvents and reagents were purchased from E. Merck (Darmstadt, Germany) and were used without further purification.

Extraction and fractionation of flaxseed oligomeric phenols

Pellets of flaxseed cake were mixed roughly using a kitchen mixer and defatted at room temperature twice
with n-hexane (1:3, v/v), first for 5 h and then overnight.
After drying, the powder was milled to obtain finer particles in a micro-mill (sieve 0.5 mm, speed 1, Retsch type ZM 1; Haan, Germany). This defatted flaxseed flour (DFF) was extracted at 60°C by continuous stirring over-
night with 50% 1,4 dioxane–ethanol (1:4, v/v). The sus-
pension was filtered and the filtrate was evaporated to dryness in vacuo at 40°C to provide the extracts that were used for the purification of SDG-oligomers as described later.

For the separation of SDG-oligomers, 50 g C18 Bondesil silica (40 μm; Varian Inc., Palo Alto, CA, USA) was packed in Buchner funnels (50 mm × 70 mm internal diam-
er). Two different separations were performed on the same type of column for each of seventeen portions, each corresponding to an extract of 50 g DFF. First, a pre-washing was performed where a part of the extract was dissolved in methanol (70%, 100 ml), loaded on the column, and eluted with methanol (70%, 260 ml) to collect the whole oligomeric material. The filtrate was then evapor-
ated in vacuo at 40°C, re-dissolved in methanol (50%, 100 ml) and loaded on a new column for further fraction-
ation. The first eluate was collected as fraction 50A (100 ml) followed by nine other fractions collected separately by elution with: (i) methanol (50%, 3 × 100 ml) to yield fractions 50B, 50C and 50D; (ii) methanol (60%, 3 × 100 ml) to yield fractions 60A, 60B, and 60C; (iii) methanol (70%, 3 × 100 ml) to yield fractions 70A, 70B, and 70C. Similar fractions were mixed together depending on their HPLC chromatograms before and after base hydrolysis. For all seventeen separations, the follow-
ing fractions were mixed together: (i) 50B and 50C forming the mixed fraction F50; (ii) 60B and 60C forming the mixed fraction F60; (iii) 70A, 70B and 70C forming the mixed fraction F70. The mixed fractions F50, F60 and F70 were analysed by HPLC as whole or in hydrolysed forms. By mixing the F60 and F70 fractions, 7·6 g SDG in the form of oligomeric extracts were available for the rat experiment.

Purification of secoisolariciresinol diglucoside

DFF (3 × 250 g) was used for the separation of pure SDG. Each of the three portions was hydrolysed using aqueous sodium hydroxide (2 m, 1 litre) at room temperature for 19 h. The phenolic glucosides were separated from salt using 40 g C18 gel (AA125S0; YMC Co., Kyoto, Japan) packed in a Buchner funnel (35 mm × 70 mm internal diameter). After conditioning with methanol and thereafter with water, an extract corresponding to 75 g DFF was loaded on the column, and the salts were washed with water (50 ml) before the phenolic compounds were eluted with methanol. The filtrate was evaporated in vacuo at 40°C to dryness, and re-dissolved in chloroform–metha-

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High-performance liquid chromatographic analysis of phenolic oligomers and aglycones

HPLC analysis of the oligomeric extracts was performed directly after filtration, while HPLC analysis of the aglycones was performed after aqueous base hydrolysis. Base hydrolysis of the oligomers, using o-coumaric acid as an internal standard, was performed essentially as described by Eliasson et al. (2003). The internal standard (0·8 mg in 1 ml methanol) was mixed with the diluted fractions corresponding to 0·1 g DFF before hydrolysis with 2·8 sodium hydroxide (10 ml) under continuous shaking at 20°C for 1 h. After hydrolysis, samples were acidified using H2SO4 (2·m, to pH 3) to prevent the ionisation of the carboxylic and phenolic groups. Ethanol (95 %) was added up to 60 % concentration to precipitate unwanted water-soluble materials, i.e. polysaccharides, proteins and the salts formed during the acidification. Those materials were removed by centrifugation (5 min; 11 000 g) and the supernatant fraction was analysed by HPLC after filtration.

HPLC analyses were performed on Dionex equipment (Dionex, Sunnyvale, CD, USA) at 25°C using an Econosil C18 column (250 × 4·6 mm, 5 μm particles; Alltech, Deerfield, IL, USA). The mobile phase was a gradient of acetonitrile (solvent B) and phosphate buffer containing 5 % acetonitrile (solvent A) and the flow rate was 1 ml/min. The following gradient was used for the analysis of oligomeric fractions: 0 min (0 % B), 30 min (50 % B), 33–48 min (70 % B), 56–80 min (0 % B). A different gradient was used for the analysis of the hydrolysed extracts: 0 min (0 % B), 30 min (30 % B), 33–51 min (70 % B), 55–80 min (0 % B). Peaks were detected with a diode array detector between 215 and 400 nm and chromatograms were recorded at 280 nm using Chromleon™ software (Dionex Chromatography Data System, version 6.20; Soft- ron GmbH, Germering, Germany). The amounts of aglycones in each fraction were calculated using the response factors established before (Eliasson et al. 2003).

Experimental animals, diets and study design

Twenty-four 21-d-old Sprague–Dawley rats (mean body weight 60 g; B&K Universal AB, Sollentuna, Sweden) were housed individually in Macrolon IV cages (Ehret GmbH & Co., Emmendingen, Germany) with aspen wood bedding (Beekay bedding; B&K Universal AB). They were kept in a conditioned room at 23°C and 60 % relative humidity with 12 h light (07:00–19:00 hours) and 12 h darkness. The rats had free access to feed and water throughout the experiment, which was carried out in accordance with the guidelines and approval of the Ethical Committee for Animal Experiments in the Uppsala region.

The twenty-four rats were divided into groups of eight animals with similar mean body weights, kept on the basal diet for a 4-d-accommodation period and then fed their respective diets for 27 d. The composition of the control diet is given in Table 1 and the extracted SDG and SDG-oligomers (see earlier) were added to the control diet at concentrations corresponding to 1 g SDG/kg to make two test diets. Body weights were measured weekly and at the time of killing. At the end of the experiment, the rats were fasted for 12 h before intraperitoneal injection of an overdose of sodium pentobarbital and killing by exsanguination. Blood samples were withdrawn from the heart and collected in tubes containing EDTA as an anticoagulant and centrifuged (1000 g, 10 min). The plasma was transferred to test tubes with screw caps and stored at −20°C until analysed. Liver and lung tissues were excised, weighed, and stored in 2-propanol at −80°C until further analysis.

Analysis of cholesterol and tocopherols in rat tissues

Triacylglycerols and cholesterol were quantified in plasma and isolated lipoprotein fractions (Seigler & Wu, 1981) and tocopherols were analysed in blood plasma as described previously (Frank et al. 2003a). Liver lipids were extracted by the method of Hara & Radin (1978) and were analysed for cholesterol and tocopherols as previously described (Frank et al. 2003a).

Statistical analyses

Statistical analysis of the registered variables was performed using the statistical software StatView (version 4.51; Abacus Concepts, Inc., Berkeley, CA, USA). Least significant differences from the t test function of StatView were used to make statistical comparisons between the different groups and effects were considered significant at P<0·05.

Results and discussion

The DFF used for the extraction of SDG and its oligomers was analysed by HPLC after direct alkaline hydrolysis (Eliasson et al. 2003) and was found to contain SDG, p-coumaric acid-4-O-glucoside, and ferulic acid-4-O-glucoside (Fig. 1) at levels of 4·58, 0·95, and 0·67 g/100 g DFF, respectively. The hydroxycinnamic acid derivatives,
p-coumaric and ferulic acid glucosides, also seem to exist in esterified forms of as yet unidentified structure(s). SDG exists in flaxseed in the form of straight-chain oligomers with HMGA (Ford et al. 2001; Kamal-Eldin et al. 2001). When analysed by HPLC, three fractions (F50, F60, and F70) eluted with peak maxima at 19.6, 22.2 and 23.6 min compared with the peak maximum at 22.3 min for the whole oligomeric extract (Fig. 2). The relative ratios of oligomers in the different fractions, calculated using the relative peak areas, were about 64% (60% fraction), about 21% (70% fraction), and about 15% (50% fraction). The chromatogram of F60 was the most similar to that of the whole extract in agreement with the fact that it is the largest fraction (Fig. 2). The $^1$H NMR spectra of F50, F60, and F70 (not shown) were similar to those obtained previously (Kamal-Eldin et al. 2001) showing that F60 and F70 have a rather similar composition, while F50 has proportionally less SDG and more hydroxycinnamate derivatives. These findings were confirmed by HPLC analysis of the hydrolysed fractions (Fig. 3). Peaks at retention times 13.1, 15.0, and 20.9 min were assigned

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**Fig. 1.** Structures of phenolic compounds in flaxseed. (a) p-Coumaric acid glucoside, where R represents H, and ferulic acid glucoside, where R represents OCH$_3$; (b) secoisolariciresinol diglucoside (SDG); (c) SDG-oligomers (for average oligomers, n is 3).
Table 2. Total and relative amounts of secoisolariciresinol diglucoside (SDG), p-coumaric acid glucoside, and ferulic acid glucoside in the mixed oligomeric fractions*

<table>
<thead>
<tr>
<th></th>
<th>F50</th>
<th></th>
<th>F60</th>
<th></th>
<th>F70</th>
<th></th>
<th>Total (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDG</td>
<td>0-4</td>
<td>51</td>
<td>4-9</td>
<td>74</td>
<td>2-8</td>
<td>83</td>
<td>8-1</td>
</tr>
<tr>
<td>p-Coumaric acid glucoside</td>
<td>0-3</td>
<td>34</td>
<td>1-2</td>
<td>18</td>
<td>0-4</td>
<td>11</td>
<td>1-9</td>
</tr>
<tr>
<td>Ferulic acid glucoside</td>
<td>0-1</td>
<td>15</td>
<td>0-6</td>
<td>8</td>
<td>0-2</td>
<td>6</td>
<td>0-9</td>
</tr>
<tr>
<td>Total</td>
<td>0-8</td>
<td>6-7</td>
<td>3-4</td>
<td>10-9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The F50, F60, and F70 fractions were isolated from 850 g defatted flaxseed flour (see p. 170). The relative ratios of the compounds in each fraction are shown.

Table 3. Effects of secoisolariciresinol diglucoside (SDG) and its oligomers on cholesterol levels in rat plasma and liver (Mean values and standard deviations for eight rats per group)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SDG</th>
<th>SDG-oligomers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>2-0</td>
<td>2-1</td>
<td>2-0</td>
</tr>
<tr>
<td>VLDL-LDL-cholesterol (mmol/l)</td>
<td>1-2</td>
<td>1-2</td>
<td>1-3</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>0-4</td>
<td>0-6</td>
<td>0-3</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/l)</td>
<td>1-5</td>
<td>0-4</td>
<td>1-3</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/g)</td>
<td>16-5</td>
<td>20-4</td>
<td>19-3</td>
</tr>
<tr>
<td>Percentage cholesterol in liver lipids</td>
<td>17-4</td>
<td>19-8</td>
<td>20-0</td>
</tr>
</tbody>
</table>

a, bMean values within a row with unlike superscript letters were significantly different.

Table 4 shows that the feeding of SDG and its oligomers caused a 2-fold lowering of α- and γ-tocopherols in rat plasma and liver (P < 0.0001). Previously, Ratnayake et al. (1992) reported that feeding 10, 20, or 40% of whole flaxseed in the diet caused a dose-dependent decrease in both α- and γ-tocopherols in the liver, heart, and spleen of rats. The present results are the first evidence that SDG may be at least one component responsible for this adverse effect of dietary flaxseed. The fact that SDG and its oligomers caused the same magnitude of effect indicates that the other phenolic compounds present in the oligomers, i.e. p-coumaric and ferulic acid glucosides, have negligible contribution to this effect. This is in agreement with our previous experiments showing that the feeding of ferulic acid had no effect on α- and γ-tocopherol levels in rat plasma and liver (Kamal-Eldin et al. 2000; Frank et al. 2003a). Considering its structure, SDG should behave as an antioxidant in a comparable way to ferulic acid. Both compounds contain a methoxy group and an alkyl substituent at ortho and para positions of the phenolic group, respectively, but the presence of the double-bond extending ring conjugation and the carboxylic group in ferulic acid may cause some differences in activity (Wright et al. 2001; Nenadis et al. 2003). However, questions about the physiological activity of dietary phenolic compounds in vivo should take into account aspects related to absorption, metabolism, and bioavailability. Dietary SDG is largely converted to END and ENL, by the intestinal microflora.
before absorption and only very low concentrations of secoisolariciresinol have been detected in rat and human plasma (Axelson & Setchell, 1981; Setchell et al. 1981; Axelson et al. 1982; Borriello et al. 1985). Since the phenolic rings of END and ENL are only substituted at the meta position, they would be expected to have negligible antioxidant activity (Wright et al. 2001).

One study in rats (Rickard & Thompson, 1998) showed that >50% of an oral dose of SDG was excreted in the faeces and about 30% was excreted in the urine 48 h after ingestion and that the tissues with the highest concentrations of lignan metabolites are those involved in metabolism (i.e. intestine, liver, and kidney). A metabolic study in human volunteers (Nesbitt et al. 1999) showed that END was the mammalian lignan produced in greater quantity and that END:ENL varied greatly among individuals. Incubation of END and ENL with hepatic microsomes in vitro (Jacobs & Metzler, 1999; Niemeyer & Metzler, 2002) has shown that both mammalian lignans undergo oxidative metabolism by cytochrome P450 to produce metabolites carrying one additional hydroxy group at an aliphatic or an aromatic position. The major metabolic route of both lignans under the action of hepatic microsomes from rats, pigs, and especially man involves hydroxylation at aliphatic positions. It is not known to what extent this type of metabolism occurs in vivo, and whether it relates to the results obtained in the present study.

To our knowledge, a lowering effect on vitamin E levels has not been reported for a phenolic compound before. Phenolic antioxidants may increase body vitamin E levels by different mechanisms, for example, by sparing the antioxidant by synergising its scavenging effects on radicals, by recycling of its tocopheroxyl radicals, by inhibiting vitamin E metabolism, by enhancing vitamin E absorption, or by inducing antioxidant enzyme systems such as catalase or glutathione peroxidase. The sesame lignan sesamin increased γ-tocopherol levels in rats through an inhibitory mechanism, and significance of the observed effect for human nutrition before SDG can be considered for supplementation in functional foods. Moreover, investigations on the metabolism of dietary SDG might contribute to the understanding of the findings reported here.

Table 4. The effects of secoisolariciresinol diglucoside (SDG) and its oligomers on tocopherol (T) levels in rat plasma and liver (Mean values and standard deviations for eight rats per group)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SDG</th>
<th>SDG-oligomers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-T (μmol/l)</td>
<td>8.8 ± 1.64</td>
<td>5.2 ± 0.63</td>
<td>4.2 ± 0.56</td>
</tr>
<tr>
<td>γ-T (μmol/l)</td>
<td>1.2 ± 0.42</td>
<td>0.7 ± 0.25</td>
<td>0.6 ± 0.21</td>
</tr>
<tr>
<td>α-T + γ-T (μmol/l)</td>
<td>10 ± 2.01</td>
<td>5.8 ± 0.85</td>
<td>4.8 ± 0.74</td>
</tr>
<tr>
<td>γ/T/α-T</td>
<td>0.14 ± 0.03</td>
<td>0.13 ± 0.03</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-T (nmol/g)</td>
<td>16.1 ± 1.11</td>
<td>8.6 ± 0.95</td>
<td>7.8 ± 1.04</td>
</tr>
<tr>
<td>γ-T (nmol/g)</td>
<td>3.1 ± 0.62</td>
<td>1.5 ± 0.37</td>
<td>1.3 ± 0.42</td>
</tr>
<tr>
<td>α-T + γ-T (nmol/g)</td>
<td>19.2 ± 1.44</td>
<td>10.1 ± 0.87</td>
<td>9.1 ± 0.92</td>
</tr>
<tr>
<td>γ/T/α-T</td>
<td>0.19 ± 0.04</td>
<td>0.18 ± 0.06</td>
<td>0.17 ± 0.07</td>
</tr>
</tbody>
</table>

* Mean value was significantly different from control (P < 0.0001).

**References**


Axelson M & Setchell KD (1981) The excretion of lignans in rats resulting in the excessive production of reactive oxygen species (Paolini et al. 2001). The significant vitamin E-lowering effect observed for SDG in the present study was not expected, especially with regard to the anticarcinogenic and anti-atherogenic effects reported for flaxseed and its lignans, and cannot be explained at present. Reduced levels of vitamin E, and especially of γ-tocopherol, have been associated with an increased risk for cardiovascular disease (for a review, see Hensley et al. 2004). Therefore, the present results warrant further investigation with respect to the magnitude, mechanism, and significance of the observed effect for human nutrition before SDG can be considered for supplementation in functional foods. Moreover, investigations on the metabolism of dietary SDG might contribute to the understanding of the findings reported here.

**Acknowledgements**

The present study was financed by FORMAS (Swedish Research Council for the Environment, Agricultural Sciences and Spatial Planning).

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