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Influence of rosemary (*Rosmarinus officinalis*, L.) on plant sterol oxidation in extra virgin olive oil

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The objective of this study was to investigate the potential role of rosemary compounds in inhibiting the plant sterol oxidation in extra virgin olive oil during heating. The stability of plant sterols was measured by quantification of plant sterol and sterol oxide formation upon 6 h of heating in both the extra virgin olive oil and its respective oil at 10% rosemary concentration (ROE). The total sterol and sitosterol oxide contents were determined by GC-flame ionization detection (GC-FID) and GC-MS techniques, respectively. Heating experiments were carried out at 180°C for 0, 1, 3 and 6 h. The total sterol content in the extra virgin olive oil was 255 mg/100 g and that in the ROE 270 mg/100 g. Sitosterol was the most abundant sterol in both samples (50% of total sterol). The ROE showed a lower content of sitosterol oxides with respect to the extra virgin olive oil during heating: after 6 h of heating only 6.1% of sitosterol oxides were formed, while up to 11.5% of sitosterol oxidized in the extra virgin olive oil. Our findings suggested that rosemary compounds were able to counteract the oxidation of plant sterols in the extra virgin olive oil during heating, preventing formation of potentially harmful compounds to human health.

Keywords: Heating / Olive oil / Oxidation / Rosemary / Sterol

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1 Introduction

Plant sterols (phytosterols) are compounds belonging to the unsaponifiable lipids and are mainly located in the cell membranes of plants, where they play important functional roles: they regulate the fluidity of membranes and are involved in cellular differentiation and proliferation. Plant sterols are regarded to have health-promoting properties. Studies highlight a possible role of plant sterols in the prevention of cardiovascular diseases by inhibiting the intestinal absorption of cholesterol [1, 2]; consumption of 2 g/day of plant sterols could reduce the risk of heart diseases by about 25% [3]. Recently it has been suggested that plant sterols may possess antitumour properties: consumption of high levels of plant sterol rich food is associated to a decreased risk for colon cancer [4 5]. Generally the human diet contains around 200–300 mg/day of plant sterols [6]. The most important dietary source of plant sterols is vegetable oil followed by cereal grains, fruits, berries and vegetables [7].

Among vegetable oils, olive oil is a good source of plant sterols; its total plant sterol content generally ranges from 144 mg/100 g to 209 mg/100 g [8–10]. Heating procedures can lead to changes in the sterol composition of oil through formation of oxides potentially harmful to human health [11, 12]. However, scarce data on formation of plant sterol oxides during heating are available. Lampi *et al.* [13] found that during pan-frying of rapeseed oil at 180°C for 10 min, less than 2% of sterols was oxidized, but during heating in tripalmitin at 180°C for 3 h, more than 20% of sterols were oxidized.

Herbs and spices, largely used in the Mediterranean diet, are known to keep the nutritional quality of food products and increase their shelf life by retarding the oxidative degradation of lipids [14, 15]. Among aromatic herbs rosemary is used in the manufacture of meat products and in edible oils for its antioxidant properties [16–19]. Phenolics such as rosmarinic and carnosic acids, rosmanol and carnosol have been identified to contribute to the antioxi-

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Abbreviations: FID, flame ionization detection; **ISTD**, internal standards; **ROE**, rosemary extra virgin olive oil; **TMS ether**, trimethylsilyl ether

dant activity in rosemary [20]. To our knowledge, no data on the potential inhibiting effect of herbs on sterol oxidation in vegetable oils is available.

This study was designed to investigate the potential role of rosemary in inhibiting phytosterol oxidation in extra virgin olive oil during heating. An Italian extra virgin olive oil and its respective rosemary extra virgin olive oil (ROE), obtained by properly macerating the herb in extra virgin olive oil, were analysed for their plant sterol and sterol oxide contents during heating at 180°C.

2 Materials and methods

2.1 Chemicals and materials

5-Cholesten-3β,19-ol (19-hydroxycholesterol) and 3β-hydroxy-5α-cholestane (dihydrocholesterol) were used as internal standards (ISTD) and were purchased from Steraloids (Newport, RI, USA) and Sigma (St. Louis, MO, USA), respectively. Rapeseed oil (Raisio Group, Raisio, Finland) was used as an in-house reference sample. Reagents and materials used in saponification, extraction, SPE and derivatization steps of the samples are described in detail in our previous publications [21 – 23]. Filters of 5 µm Versapor® Membrane (Acrodisc® 25-mm syringe filter) used for rosemary oil (ROE) preparation were purchased from Pall Gelman Laboratory (Ann Arbor, MI, USA).

2.2 Samples

The ROE was prepared using rosemary leaves (*Rosmarinus officinalis* L.) dried at 40°C in ventilated oven; the herb was ground and macerated in extra virgin olive oil (purchased from Italy, brand Monini) at four concentrations (0.1, 1, 5, 10% w/w) in a closed Erlenmeyer flask and mixed thoroughly using of a magnetic stirrer in the dark for 72 h. The oils were then centrifugated and the supernatant was filtered by a 5 µm filter. The ROEs were kept in closed flasks in the dark until used. The ROEs prepared at these different concentrations were tested for antioxidant activity by the ABTS method [24]. Based on these results, the 10% ROE was chosen for the heating experiments.

2.3 Characterization of the extra virgin olive oil and ROE

The extra virgin olive oil and the ROE used for the heating experiments (10% ROE) were analysed for their plant sterol and sterol oxide contents. The extra virgin olive oil was also characterized for its fatty acid and tocopherol contents. For tocopherol analysis, the oil samples were dissolved in heptane and analysed by a normal phase HPLC method [25]. Fatty acids were determined as methyl ester derivatives by GC-flame ionization detection (GC-FID) [26].

2.4 Thermooxidation experiments

The extra virgin olive oil and the ROE (1.0 g) were heated in glass vials in an electric oven as reported earlier [22]. Samples were heated at 180°C for 0, 1, 3 and 6 h and the heating experiment was repeated. Samples of each time point were analysed in duplicate.

2.5 Procedure for total sterol analysis

Total sterols were determined following the method described earlier [22, 27]. Dihydrocholesterol (80 µg) used as an ISTD was added to 0.5 g of native and heated oil before hot saponification. The unsaponifiable lipids were extracted by diethyl ether-heptane (1 : 1 v/v). An aliquot of the extract was silylated and the trimethylsilyl ether (TMS ether) derivatives were determined by an Agilent Technologies 6890 N GC-FID system equipped with an Rtx-5 w/ Integra Guard capillary column (crossbond 5% diphenyl-95% dimethyl polysiloxane; film thickness 0.10 µm, 60 m × 0.32 mm i.d.; Restek, Bellefonte, PA, USA), an autosampler and an on-column injection system.

An in-house reference sample (rapeseed oil) was analysed in each sample batch to check the daily repeatability of the method and a sterol standard mixture (cholesterol, dihydrocholesterol, stigmasterol) was also analysed to evaluate the GC-FID performance.

2.6 Procedure for sterol oxides analysis

Sterol oxides were determined according to the method described by Lampi *et al.* [21] and Soupas *et al.* [22]. Artifact formation and losses were avoided by working in the dark and at room temperature. Native and heated samples (0.25 g) were gently cold saponified overnight after the addition of 19-hydroxycholesterol (0.9 – 7.5 µg) used as an ISTD. The unsaponifiable lipids were extracted by diethyl ether. Sterol oxides were purified from the extract by silica SPE (SiOH-SPE). A secondary ISTD (dihydrocholesterol) was added to the samples after SPE elution to calculate the recovery of the ISTD. Oxides were silylated and the TMS ether derivatives were injected into a GC-MS system composed of a Hewlett Packard 6890 Series GC (Wilminton, DE, USA) coupled to an Agilent 5973 MS (Palo Alto, CA, USA). On-column injection technique and an Rtx-5MS w/ Integra Guard capillary column, 60 m × 0.25 mm id (cross-

bond 5% diphenyl-95% dimethyl polysiloxane; Restek), and film thickness 0.10 μm, were used.

A sterol standard mixture was also analysed to evaluate the GC performance. Also, the mass spectrum of stigmasterol in the mixture was monitored everyday. Sitosterol oxides were identified by GC-MS in full scan mode (*m/z* 100–600) and quantified in SIM mode. As commercial standards of sitosterol oxides were not available, the calibration curves for sitosterol oxides were constructed indirectly *via* GC-FID, as described by Soupas *et al.* [28]. The main sitosterol oxide TMS ether derivatives were quantified by SIM acquisition of the following target and qualifier ions: *m/z* 353.3 and 366.4 for 19-hydroxycholesterol (ISTD), *m/z* 484.5 and 485.5 for 7α- and 7β-hydroxysitosterol) *m/z* 431.4 and 574.5 for 6β-hydroxysitosterol, *m/z* 412.4 and 502.5 for 5,6α- and 5,6β-epoxysitosterol, *m/z* 473.4 and 502.5 for 6-ketositostanol and *m/z* 500.5 and 395.3 for 7-ketositosterol.

The limits of determination for sitosterol oxides were the lowest level of the calibration curves: 0.5 μg/g for 7α-hydroxysitosterol, 0.2 μg/g for 6β-hydroxysitosterol, 0.7 μg/g for 7β-hydroxysitosterol, 0.7 μg/g for 5,6β-epoxysitosterol, 0.5 μg/g for 5,6a-epoxysitosterol, 0.1 μg/g for 6-ketositostanol and 1.4 μg/g for 7-ketositosterol.

Although the GC-MS was the main technique used for the determination of sitosterol oxides in this study, samples were also analysed by GC-FID technique to support the GC-MS quantification.

2.7 Statistical analysis

Statistical analysis was performed using Student's *t*-test. Differences were considered significant at $p < 0.05$.

3 Results and discussion

The extra virgin olive oil used in this study was characterized for its fatty acid profile and antioxidant vitamin content.

Oleic acid was the main fatty acid contributing to 73.4% of total fatty acids. The other unsaturated fatty acid content was 23.2% of the total fatty acids (13.5% MUFA + 9.7% PUFA). In contrast, the saturated fatty acid content was only 3.4%.

The extra virgin olive oil was also a rich source of α-tocopherol, a vitamin exhibiting antioxidant activity. The contents of α- and γ-tocopherols were 225 ± 1 μg/g and 11 ± 0 μg/g, respectively.

*TAA, Total Antioxidant Activity

Figure 1. Antioxidant activity expressed by ROE at different rosemary leaves concentrations (w/w).

3.1 Choice of the ROE

In Fig. 1 the antioxidant activity of the ROEs prepared at four different concentrations of leaves is reported. The antioxidant activity of the ROEs was measured as a function of the antioxidant activity of the extra virgin olive oil, used as a control sample. As shown, the antioxidant activity increased with the concentration of the rosemary leaves, the sample at 10% showing the highest activity (Fig. 1). This led us to select the oil at the highest rosemary concentration (ROE) for the plant sterol oxidation experiments; furthermore this ROE, even if not subjected to a panel test, did not show unpleasant flavour and taste.

3.2 Effect of heating on plant sterol contents

Table 1 shows the amounts of individual sterols and their relative abundances in both the extra virgin olive oil and ROE samples before heating. The initial total sterol content of the extra virgin olive oil was 255 mg/100 g and that of the ROE 270 mg/100 g, showing that rosemary had only a minor effect on the sterol contents. The most abundant sterol found in both types of samples was sitosterol which accounted for about 50% of the total sterols, followed by 24-methylcycloartanol and cycloartenol. As a minor interesting finding, the amount of sitostanol increased three-fold during rosemary leave extraction being 8.5 mg/100 g in ROE.

The total sterol content found in the present study was higher than that reported in our previous study [7] carried out on natural sources of plant sterols. In that study, the total sterol content of olive oil was less than 200 mg/100 g and rapeseed oil had the highest sterol content (about 700 mg/100 g) among analysed vegetable oils [7]. Phillips *et al.* [29] reported a total sterol content up to 166 mg/100 g in

Table 1. Plant sterol contents and their relative abundances in extra virgin olive oil and ROE (mg/100 g)

	Campe-sterol	Stigma-sterol	Sito-sterol	Sito-stanol	Δ5-Avena-sterol	Cyclo-artenol	Δ7-Avena-sterol	24-Methyl cycloartanol	Citro-stadienol	Total plant sterol
Extra virgin olive oil										
Mean	5.5	1.3	131.2	2.4	20.0	33.9	0.8	47.9	12.5	255.3
S.D.	0.1	0.0	1.1	0.1	0.2	0.3	0.0	0.3	0.1	1.5
C.V.%	1.0	1.7	0.8	3.9	0.9	0.9	3.3	0.6	0.8	0.6
Sterol%	2.1	0.5	51.4	0.9	7.8	13.3	0.3	18.7	4.9	100.0
ROE										
Mean	5.8	n.q.*	134.7	8.5	24.9	36.2	0.7	46.7	12.0	269.5
SD	0.1	–	2.3	0.1	0.4	0.4	0.0	0.5	0.2	3.8
CV%	2.1	–	1.7	1.5	1.7	1.1	6.7	1.1	1.6	1.4
Sterol%	2.2	–	50.0	3.1	9.2	13.4	0.3	17.3	4.4	100.0

Values are means of four determinations; * n.q., not quantified due to impurities coeluting with stigmasterol.

Table 2. Total sterol content (mg/100 g) in extra virgin olive oil and ROE during heating

Heating time (h)	Extra virgin olive oil	ROE
0	255 ± 2^a	270 ± 4^b
1	245 ± 1^a	268 ± 4^b
3	212 ± 1^a	259 ± 3^b
6	172 ± 3^a	228 ± 4^b

Values are mean ± SD of four determinations.
Values with different letters, at the same heating time, are significantly different (a *vs.* b, $p < 0.001$).

extra virgin olive oil and a range from 716 to 785 mg/100 g in rapeseed oil [6].

It should be noted that sitosterol generally accounts for more than 50% of total sterols and therefore it is the main sterol not only in extra virgin olive oil but also in other edible oils. The sterol content found in extra virgin olive oil in this study was higher compared to those reported in other studies [29], probably due to cultivars and production techniques.

Table 2 shows the influence of heating on the total sterol contents at different time points of the heating experiments. The total sterol content decreased in both samples during heating. However, the degree of deterioration was significantly lower ($p < 0.001$) in the ROE than in the extra virgin olive oil sample for each heating point (Table 2). After 6 h of heating, the sterol losses were 32% in the extra virgin olive oil and only 15% in the ROE; this finding further supports that the addition of rosemary leaves to olive oil improves the stability of its sterols. These results were confirmed by the GC-MS analysis which monitored the formation of the sitosterol oxides during heating.

3.3 Formation of sitosterol oxides during heating

As sitosterol was the main plant sterol in extra virgin olive oil, its oxides predominated among sterol oxidation products. Sterol oxides from other sterols were also formed but their contents were much lower. Therefore, only sitosterol oxide contents are reported in this study. Sitosterol oxides in both the extra virgin olive oil and the ROE samples were determined both by the GC-FID and GC-MS techniques. As results derived from GC-FID were found comparable to those from GC-MS, only GC-MS data are presented. The concentration of sitosterol oxides in both oil samples at the different heating times is reported in Table 3. 7β-hydroxy-, 5,6β-epoxy-, 5,6α-epoxy- and 7-ketositosterol were the main oxidation products in both the extra virgin olive oil and ROE after heating for 6 h. During heating the individual oxide contents increased in both extra virgin olive oil and ROE. However, formation of sterol oxides for all heating times was significantly higher ($p < 0.001$) in the extra virgin olive oil compared with the ROE (Table 3). The only exception to this behaviour was 6-ketositostanol that was already present at a reasonable level in the nonheated samples (2.60 μg/g in extra virgin olive oil and 2.98 μg/g in ROE), and its contents did not increase markedly during heating. This result revealed that 6-ketositostanol content was not affected by heating as observed for the other oxides.

In summary, both the extra virgin olive oil and the ROE had a very low total sitosterol oxide content before heating, 0.25 and 0.28% of sitosterol, expressed as a percentage of the original sitosterol content, respectively (Table 3). Amounts of 7β-hydroxy-, 5,6β-epoxy-, 5,6α-epoxy- and 7-ketositosterol in the nonheated samples are not reported because their concentrations were under their limits of determination. During heating the individual oxide contents increased in both extra virgin olive oil and ROE (Table 3). After 6 h of heating, 11.5% of sitosterol was oxidized in the extra virgin olive oil. This is more than found in our earlier study, where oxidation of sitosterol and campesterol in rapeseed oil in similar conditions was measured yielding in 3–4% of oxidized sterols [13]. As the PUFA amount was rather small (<10%) in olive oil compared to rapeseed oil, sterols were oxidized more. We have shown that in a more unsaturated oil plant sterols are oxidized less than in a less unsaturated

Table 3. Formation of sitosterol oxides (μg/g) in extra virgin olive oil and ROE at 180°C

Heating time (h)	7α-OH	6β-OH	7β-OH	5,6β-epo	5,6α-epo	6-keto	7–keto	Total	Oxidized sitosterol* (%)
Extra virgin olive oil									
0	0.5 ± 0.0[a]	0.2 ± 0.0[a]	n.d.	n.d.	n.d.	2.6 ± 0.0[a]	n.d.	3.3 ± 0.0	0.25
1	3.6 ± 0.4[a]	0.80 ± 0.0[a]	4.6 ± 0.2[a]	3.2 ± 0.6[a]	2.10 ± 0.0[a]	3.1 ± 0.2[a]	2.1 ± 0.1[a]	19.5 ± 0.8[a]	1.49
3	15.2 ± 0.1[a]	3.2 ± 0.5[a]	19.5 ± 1.1[a]	15.5 ± 2.6[a]	9.8 ± 0.6[a]	4.7 ± 0.6[a]	6.9 ± 0.5[a]	74.8 ± 0.6[a]	5.70
6	28.9 ± 1.0[a]	5.7 ± 0.2[a]	32.7 ± 0.7[a]	36.8 ± 1.1[a]	20.6 ± 0.9[a]	5.9 ± 0.1[a]	20.5 ± 1.0[a]	151.2 ± 5.0[a]	11.52
ROE									
0	0.6 ± 0.0[b]	0.2 ± 0.0[a]	n.d.	n.d.	n.d.	3.0 ± 0.0[b]	n.d.	3.8 ± 0.0[b]	0.28
1	1.6 ± 0.1[b]	0.4 ± 0.0[b]	1.8 ± 0.1[b]	1.0 ± 0.1[b]	0.9 ± 0.0[b]	3.1 ± 0.1[a]	1.3 ± 0.1[b]	10.0 ± 0.2[b]	0.74
3	4.2 ± 0.2[b]	0.9 ± 0.0[b]	5.4 ± 0.1[b]	3.2 ± 0.4[b]	2.6 ± 0.1[b]	3.2 ± 0.1[c]	3.0 ± 0.0[b]	22.6 ± 0.5[b]	1.68
6	14.3 ± 0.5[b]	3.6 ± 0.0[b]	19.4 ± 0.7[b]	17.5 ± 0.5[b]	10.6 ± 0.1[b]	4.5 ± 0.1[b]	12.6 ± 0.2[b]	82.6 ± 2.1[b]	6.13

Values are mean ±SD of four determinations; n.d., not detected.
Values with different letters, for individual oxide, at same heating time, are significantly different (a *vs.* b, $p < 0.001$; a *vs.* c, $p < 0.01$).
* Oxidized sitosterol indicates the sum of analysed sitosterol oxides as a percent of the original sitosterol content.
7α-OH = 7α-OH-sitosterol; 6β-OH = 6β-OH-sitosterol; 5,6β-epo = 5,6β-epoxysitosterol; 5,6α-epo = 5,6α-epoxysitosterol; 6-keto = 6-keto-sitostanol; 7-keto = 7-ketositosterol.

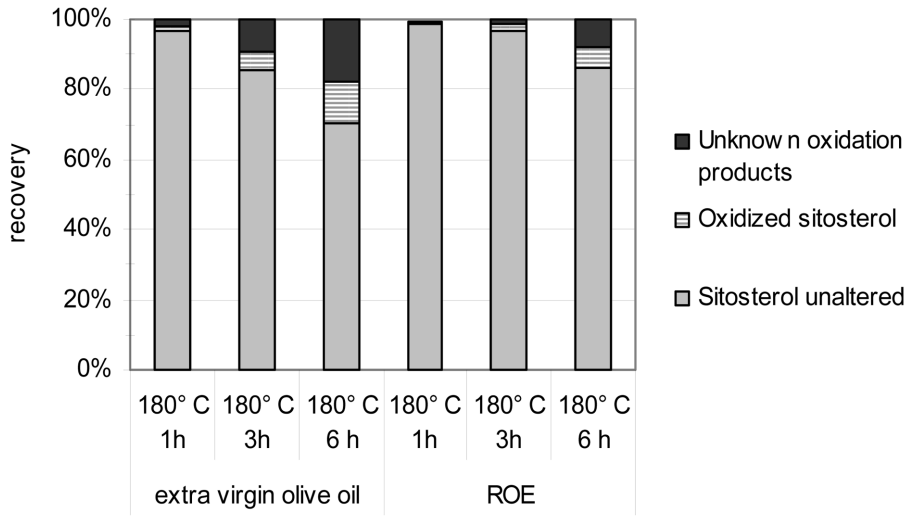

Figure 2. Amounts (%) of quantified unaltered sitosterol, its oxidation products and the unknown gap in extra virgin olive oil and ROE after heating at 180°C.

oil at 180°C [22], which is in line with these findings. By contrast after heating for 6 h, only up to 6.1% of sitosterol was oxidized in the ROE (Table 3). Therefore, compounds contained in ROE show a protective role against oxidation.

The antioxidant power of rosemary was very strong up to 3 h of heating at 180°C, when the oxide content increased only a little to 1.7%, and after which oxidation markedly increased. Thus, in the ROE sample there was a lag phase in the oxidation process. In the extra virgin olive oil, natural antioxidant present retarded sterol oxidation only for 1 h at 180°C, after which oxide formation increased notably (Table 3).

In accordance with our previous studies [28], the monomolecular secondary oxidation products, which are currently measured in sterol oxidation studies, did not account for all the sterol losses during prolonged heating at this high temperature. Figure 2 compares the sums of the total amounts of the quantified sitosterol oxides and the unaltered sitosterol with the initial sitosterol contents at the three time points. As can be noted, there was a significant gap between them after heating the extra virgin olive oil for 3 or 6 h. In the ROE, a clear gap existed only after 6 h. Thus, other unknown oxidation products were also formed and their formation could be decreased by antioxidative components of rosemary. We have tentatively identified that dimers and polymers are formed in these conditions [28].

These findings show that the effects of the rosemary components were substantial: the oxidation levels of sitosterol in heated samples were always lower in the ROE than in the extra virgin olive oil.

4 Concluding remarks

The present study evidenced that rosemary was able to counteract the oxidation of plant sterols in extra virgin olive oil during heating. The antioxidant protective effect exhibited by rosemary on sterol stability suggests that this aromatic oil could be used for increasing the shelf life of foodstuffs. Our findings suggest that this oil could be regarded as a health-promoting product in the diet, due to its ability to prevent the formation of potentially harmful compounds to human health.

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