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Oxidative stability of virgin olive oil enriched with carnosic acid

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ABSTRACT

The aim of this study was to evaluate the effect of the addition of carnosic acid on the oxidative stability of virgin olive oil. Two different amounts of carnosic acid (0.01 and 0.1 g/100 g oil) and two different temperatures (accelerated aging temperature, 60 °C; deep frying temperature, 180 °C) were considered. The influence of carnosic acid and heating time on the stability of the oils was studied by experimental design. The results obtained at 60 °C showed a dose dependent inhibition in the formation of primary and secondary oxidation products and a dose dependent enhancement of radical scavenging activity, which was only less significantly influenced by heating time. On the contrary, at 180 °C no protective effect against lipid oxidation was observed and the radical scavenging activity was practically zeroed by heating, probably as a consequence of a fast decomposition of carnosic acid.

1. Introduction

The Mediterranean coastal areas have a mild, warm climate that fully meets the climatic requirements of *Olea europaea* L., and they are thus considered an ideal habitat for its growth and development. Virgin olive oil consumption is growing all over the world for its pleasant and tasteful aroma. Moreover, its balanced fatty acid composition and its phenolic antioxidant content contribute to its high nutritional quality. Though virgin olive oils have an appreciable oxidative stability, Yanishlieva and Marinova (2001) and Wagner and Elmadfa (2000) have studied the possibility to further increase it by the addition of phospholipids and tocopherols, respectively. Natural antioxidants have recently gained popularity because consumers think that natural food ingredients are better and safer than synthetic ones. The interest in natural antioxidants is further increased by the suggestions that many of these compounds, such as plant phenolics and diterpenoids, often display anticarcinogenic, antiatherogenic and antitumor activity (Duthie, 1991; Kinsella, Frankel, German, & Kanner, 1993). Moreover, since oxygen reactive species and free radicals are the major causes of aging processes, antioxidative nutraceuticals can slow these processes by reducing the levels of reactive oxygen species and free radicals. Thus, incorporation of such plant secondary metabolites into food might significantly contribute both to the health benefit of consumers and to the stabilisation of the product itself.

In order to enhance olive oil nutritional quality and in sight of functional food formulation, the addition of carnosic acid to virgin olive oils could be particularly interesting, since it could increase the radical scavenging activity of their phenolic antioxidants, particularly tocopherols (100–400 mg/kg) and hydrophilic phenols (40–800 mg/kg), mainly derived from olive glucosides. Carnosic acid (CA) is an abietane diterpene found in various Lamiaceae such as sage and rosemary (Chang, Ostric-Matijasevic, Hsieh, & Huang, 1977; Curvelier, Richard, & Berset, 1996). It is considered a precursor of other diterpenoids constituents in the herbs (Tada, 2000). Thus, the interesting activities of carnosic acid and of its parent terpenoids support the hypothesis of the employment of rosemary extracts as food additives (EFSA, 2008).

CA is the most powerful antioxidant among diterpenes and its antioxidant mechanism was studied by Masuda, Inaba, and Takeda (2001). The antioxidant activity of purified carnosic acid was also studied in bulk and emulsified lipid systems (Hopia, Huang, Schwarz, German, & Frankel, 1996; Huang, Frankel, Schwarz, Aeschbach, & German, 1996), rapeseed (Trojakova, Reblova, & Pokorny, 2000) and fish oils (Frankel, Huang, Prior, & Aeschbach, 1996) and in model systems (Miura, Kikuzaki, & Nakatani, 2002). Moreover, Hopia et al. (1996) reported that in corn oil carnosic acid exhibited a significant protective effect toward α-tocopherol during oxidation. A possible mechanism for this effect may be similar to that of ascorbic acid, i.e. carnosic acid could reduce tocopheroxyl radical to active tocopherol. Therefore, carnosic acid may protect α-tocopherol from oxidation by a sparing effect (Hopia et al., 1996).

Thus, the aim of this study was to evaluate the effects of the addition of different amounts of carnosic acid to extra-virgin olive oil. Both accelerated aging of oils at 60 °C and deep frying temperature conditions (180 °C) were considered. The oil oxidation trends were estimated by the amounts of both primary and secondary oxidation products. The DPPH test was used in order to evaluate the radical scavenging activity of the heated oils.

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2. Experimental

2.1. Materials

Carnosic acid was extracted by *Salvia officinalis* L. and *Rosmarinus officinalis* L. leaves and purified by column chromatography (Marrero, Andrés, & Luis, 2002). Extra-virgin olive oil was purchased from the local market.

p-Anisidine and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH**.**) were supplied by SIGMA Chemie (Steinheim, Germany), syringic acid was purchased from Fluka Chemie GmbH (Buchs, Switzerland). The employed solvents were analytical, HPLC or spectroscopic grade and were supplied by Merck (Darmstadt, Germany). Eighteen microohm deionised water from a Millipore (Billerica, Massachusetts, USA) Milli-Q water purification system was used to prepare the chromatographic mobile phase.

2.2. Samples

CA was added to a commercial virgin olive oil at a 0.1% w/w concentration. A further dilution allowed to obtain the sample with a 0.01% w/w carnosic acid concentration.

2.3. Oxidation experiments

Two temperature conditions (60 °C and 180 °C) and three CA concentrations (0%, 0.01% and 0.1% w/w; in this case a logarithmic scale was applied) were considered.

As far as the frying experiments at 180 °C are concerned, 90 g aliquots of the samples were heated in open 250 mL beakers at 180 °C for 10 h, with a gentle sample stirring by glass road at 1 h intervals. The area exposed to air was 1.71 cm^2/g. In order to explore a large experimental domain with a limited number of experiments, a face centred experimental design with two independent variables (heating time and CA concentration) was used (Fig. 1). At 0, 5 and 10 h heating times 15 g test samples were transferred from each beaker into 20 mL amber glass bottles, which were then stored at −20 °C up to the analysis time. Three independent replicates of the center point (0.01% CA and 5 heating hours) were performed, for a total of 11 experiments.

As far as the accelerated aging test (60 °C) is concerned (AOCS Recommended Practice Cg 5–97, 1998), 110 g aliquots of the three samples containing 0, 0.01 and 0.1% w/w CA, respectively, were divided in 10 g sub-samples in closed amber glass 20 mL bottles. The area exposed to air was 0.80 cm^2/g. Samples were stored at ambient temperature up to the beginning of the aging test (two months), thus simulating the storage conditions that are commonly used for extra-virgin olive oils. Then the 20 mL bottles were heated at 60 °C in a forced drafts oven, taking out one sample bottle for each CA concentration, at 2 days intervals, up to 18 days ageing.

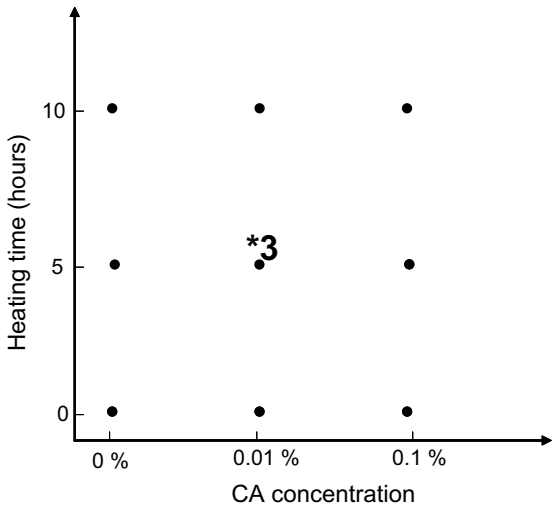

Fig. 1. The central composite design at 180 °C. Three replicates were performed at the central point. CA, Carnosic acid.

2.4. Analytical determinations

2.4.1. Fatty acid composition, free acidity and analysis of minor polar compounds (MPC) of the non-heated oils

Fatty acid composition and free acidity of the crude extra-virgin olive oil were determined following the analytical methods described in the European Regulation (EC) no. 2568/91 (1991) and later amendments. MPC (biophenols and secoiridoid acids) were extracted from 1 g of crude oil by a mixture of water and methanol 20:80 vol/vol after the addition of the internal standard (syringic acid). Then, the identification and quantification of MPC was carried out by reverse phase HPLC (Cortesi, Rovellini, & Fusari, 2002).

2.4.2. Peroxide values of heated and not-heated oils

Peroxide values were determined following the European Regulation (EC) no. 2568/91 (1991). Two replicates were performed for each test sample.

2.4.3. p-Anisidine value of heated and non-heated oils

p-Anisidine values were measured by AOCS Official Method Cd 18–90 (1998). Commercial *p*-anisidine was carefully purified by crystallization before use and its solution was prepared daily. Two replicates were performed for each test sample.

2.4.4. Radical scavenging activity (RSA) of the heated and non-heated oils

The heated and non-heated oils were analysed in order to detect their aptitude to scavenge the stable DPPH**.** radical. The procedure reported by Kalantzakis, Blekas, Pegklidou, and Boskou (1998) was slightly changed: 1 g oil (exactly weighed) was dissolved in ethyl acetate in a 10 mL volumetric flask; then, depending on the antiradical activity of the sample, exactly 1 or 2 mL of this solution were transferred in a second 10 mL volumetric flask where a daily prepared DPPH**.** mother solution (approximately 10^{-4} M in ethyl acetate) was added to the mark. The reaction flask was shaken for 10 s in a Vortex apparatus and it was allowed to stand in the dark for 30 min. The residual absorbance was then measured at 515 nm against a blank solution (without radical). The initial DPPH**.** concentration was measured by control samples (without oils), obtained by the dilution of 1 or 2 mL of ethyl acetate, respectively, to 10 mL by the DPPH**.** mother solution.

The Radical scavenging activity (RSA) of the samples was expressed as the% reduction of DPPH**.** concentration in a DPPH**.** solution exactly 10^{-4} M and was not dependent on the concentration of DPPH**.** solutions.

$$RSA = ([DPPH.]_{control} - [DPPH.]_{sample})/10^{-4} \times 100$$

Three replicate analysis were performed for each sample.

2.4.5. Experimental design and statistical analysis

Experimental design and statistical analysis were performed by using Matlab (Matlab 4.2, 1994) routines written by one of the authors. The response surface methodology (RSM) was used to study

the effects of the two experimental variables (CA concentration and heating time) on the oxidation of the oils, which was evaluated by peroxide value, RSA and *p*-anisidine value as response variables.

3. Results and discussion

At the beginning of the experimental activity, the commercial extra-virgin olive oil was analysed in order to confirm its quality and to investigate its natural content of phenolic antioxidants (MPC). The oxidative and hydrolytic integrity of the oil was confirmed by the 11.0 peroxide value and by the low contents of free fatty acids (free acidity, 0.26 $g_{oleic\ acid}$/100 g oil) and free hydroxytyrosol and tyrosol. The total content of MPC determined by HPLC was about 180 mg/kg (expressed as tyrosol): tyrosol (4-hydroxyphenylethanol, 11.4 mg/kg) and hydroxytyrosol (2,4-dihydroxyphenylethanol, 10.8 mg/kg) were the major free phenolic compounds. Two secoiridoids precursor of hydroxytyrosol, i.e. an isomer of the oleoeuropeine aglycone (40.0 mg/kg) and the diadheyidic form of elenolic acid linked to 3,4-dihydroxyphenylethanol (41.1 mg/kg) (Montedoro et al., 1993) were also detected, together with the two lignans pinoresinol (8.7 mg/kg). and 1-acetoxypinoresinol (19.8 mg/kg) (Brenes et al., 2000), the dialdheyidic form of elenolic acid linked to 4-hydroxyphenylethanol (30.9 mg/kg) (Montedoro et al., 1993) and other minor secoiridod derivatives. The preliminary analysis were completed by the determination of the fatty acid composition of the crude oil, which showed a high content of oleic acid (77.65%) coupled with a 6.15% linoleic and 0.60% linolenic acids amounts. Among saturated fatty acids, palmitic was 10.77% and lower amounts of stearic (2.87%) and eisosanoic (0.39%) acids were found. Lower amounts of minor saturated and unsaturated fatty acids were also detected.

Then, the 180 °C experiments were performed, followed by the experiments at 60 °C that were performed two months later.

The development of oxidation in the test samples in the two different experimental conditions was then evaluated by peroxide and *p*-anisidine values (primary oxidation products and secondary oxidation products, respectively), whilst DPPH test was used in order to measure the RSA. As reported above, peroxide and *p*-anisidine analysis were performed twice and RSA determination three times for each test sample.

In order to evaluate oil oxidation under deep frying conditions (180 °C), 11 test samples were analysed. To estimate the analytical variability, pooled standard deviations were calculated for the three responses in the 11 test samples. To estimate the process variability, the standard deviation of the averages responses of the three replicates of the test sample at the central point was calculated (Table 1). Since, for all the responses, the two standard deviations were not significantly different, it can be concluded that the process variability was comparable to the analytical variability. Therefore, it is an acceptable approximation to consider the replicate analyses as individual samples, so that 22 individual experiments were taken into account for the statistical evaluation of the responses peroxide and *p*-anisidine and 33 for the response RSA.

Table 1
Calculated standard deviations for the three response variables.

	Peroxide value	RSA[a]	*p*-Anisidine value
Pooled SD[b] of responses (11 test samples)	0.4	0.9	1.2
SD[b] of average responses (three replicates of the central test sample)	0.8	0.4	1.2

[a] RSA = radical scavenging activity.
[b] SD = standard deviation.

The two independent variables were then coded in the range [−1, +1], as follows:

- for CA amount (variable X_1): −1 (0%), 0 (0.01%) and +1 (0.1%) on a log scale;
- for heating time (variable X_2): −1 (0 h), 0 (5 h) and +1 (10 h) on a linear scale.

For all the responses the postulated model contained the linear terms, the interaction and the quadratic terms. The following models were obtained:

$$\text{Peroxide value} = 24.4 + 4.7X_1(***) + 6.5X_2(***) + 3.1X_1X_2(*) + 0.6X_1^2 - 7.1X_2^2(***)$$

$$\text{RSA} = 3.5 + 7.1X_1(***) - 27.7X_2(***) - 6.7X_1X_2(***) + 3.1X_1^2(*) + 29.3X_2^2(***)$$

$$p-\text{anisidine value} = 73.7 + 4.8X_1(***) + 45.7X_2(***) + 4.6X_1X_2(***) + 0.9X_1^2 - 21.3X_2^2(***)$$

where $^{*} = p < 0.05$, $^{**} = p < 0.01$, $^{***} = p < 0.001$.

The same coefficients are reported in a graphical way in Fig. 2, graphs a, c and e.

As reported above, the initial peroxide value of the non-heated processed extra-virgin olive oil was 11, and this response is increased by both heating times and CA amounts. The strong quadratic effect of the heating time is clearly visualized in Fig. 2 (graph b) that shows that after about seven hours a plateau is reached. From the same plot the significant interaction can also be interpreted, since it can be seen how the effect of an increase of CA amount is greater the longer the heating time and that the effect of the heating time is greater the greater the amount of CA.

A strong and quadratic effect of the heating time was observed also for *p*-anisidine values (Fig. 2, graph e), which started from 6 in the crude oil. The effect of CA concentration and the interaction between the two variables, though highly statistically significant, were much lower and practically negligible, as shown by the isoresponse plot (Fig. 2, graph f), this meaning that CA was not very effective in preventing the formation of secondary oxidation products.

As far as the RSA is concerned (Fig. 2, graph c and d), once more the heating time showed the stronger effect in the 180 °C heated oils. Looking at the isoresponse surface it can be seen a fast decrease of RSA with time, with values around zero after about five hours. The positive effect of CA gave practical problems, since the RSA of the non-heated oil containing 0.1% CA was so high that the 2 mL oil solution completely discoloured the DPPH solution. Therefore, the amount of oil was reduced to 1 mL, this producing a detectable 81.6% RSA.

As far as the accelerated aging test is concerned, 30 samples were analysed. The 60 °C temperature was chosen since the mechanism of oxidation at 60–80 °C is the same as oxidation at ambient temperature (Pike, 2003, Chap. 14). The experiment was conducted in the dark in a forced draft oven. The mean values of the replicate analysis were used for the multivariate analysis.

The two independent variables, were coded in the range [−1, +1] as follows

- for CA amount (variable X_1) −1 (0%), 0 (0.01%) and +1 (0.1%) on a log scale;
- for heating days (variable X_2) −1 (0 days) to +1 (18 days) on a linear scale.

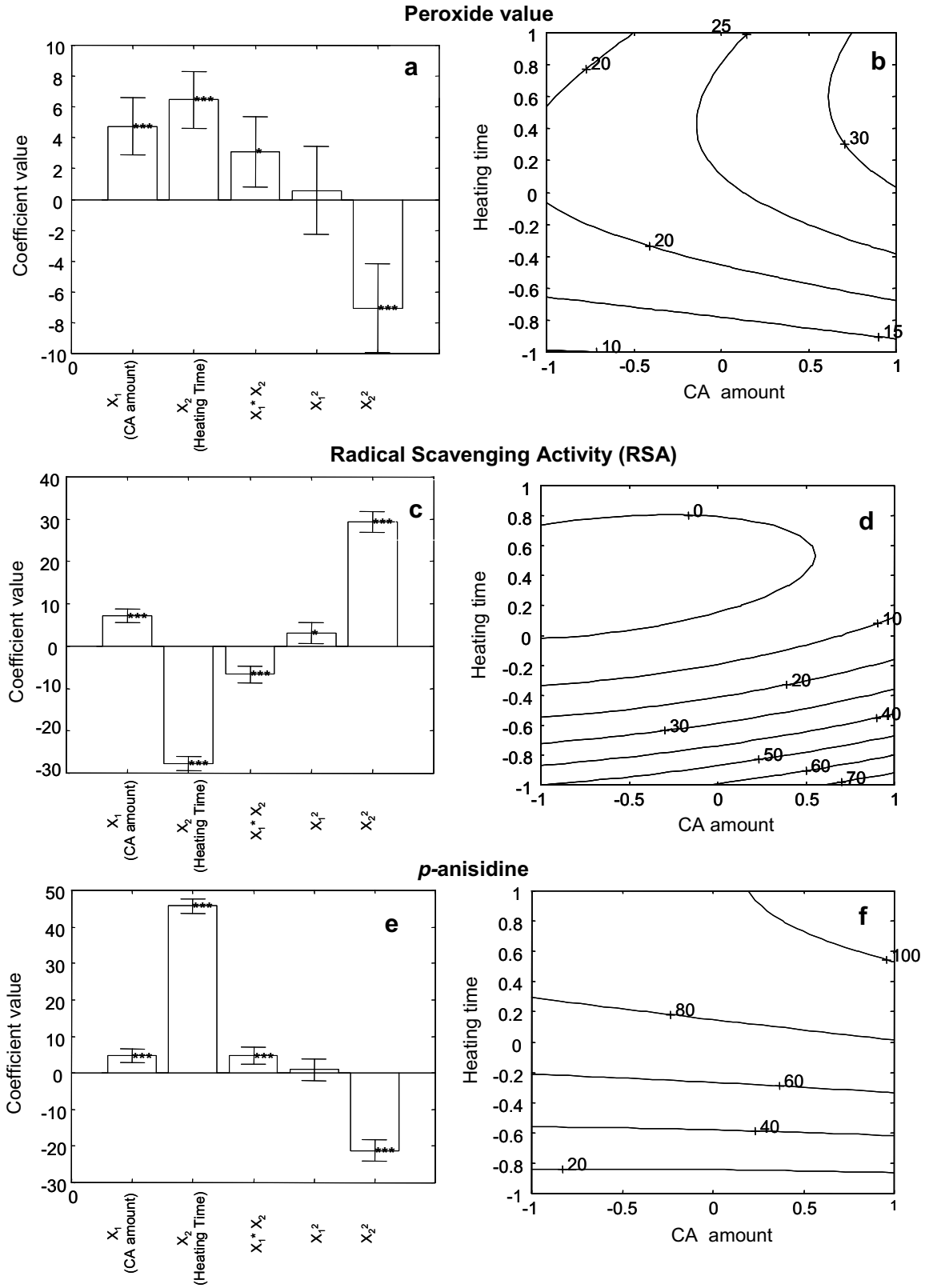

Fig. 2. Plots of the coefficient of the models (a, c, e) and of the response surfaces (b, d, f) of peroxide, RSA and *p*-anisidine values for oils heated at 180 °C (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

The following models were obtained for peroxide value and RSA, respectively

$$\text{Peroxide value} = 21.5 - 5.0X_1(***) + 1.7X_2 - 3.2X_1X_2(*) + 2.9X_1^2 - 3.2X_2^2$$

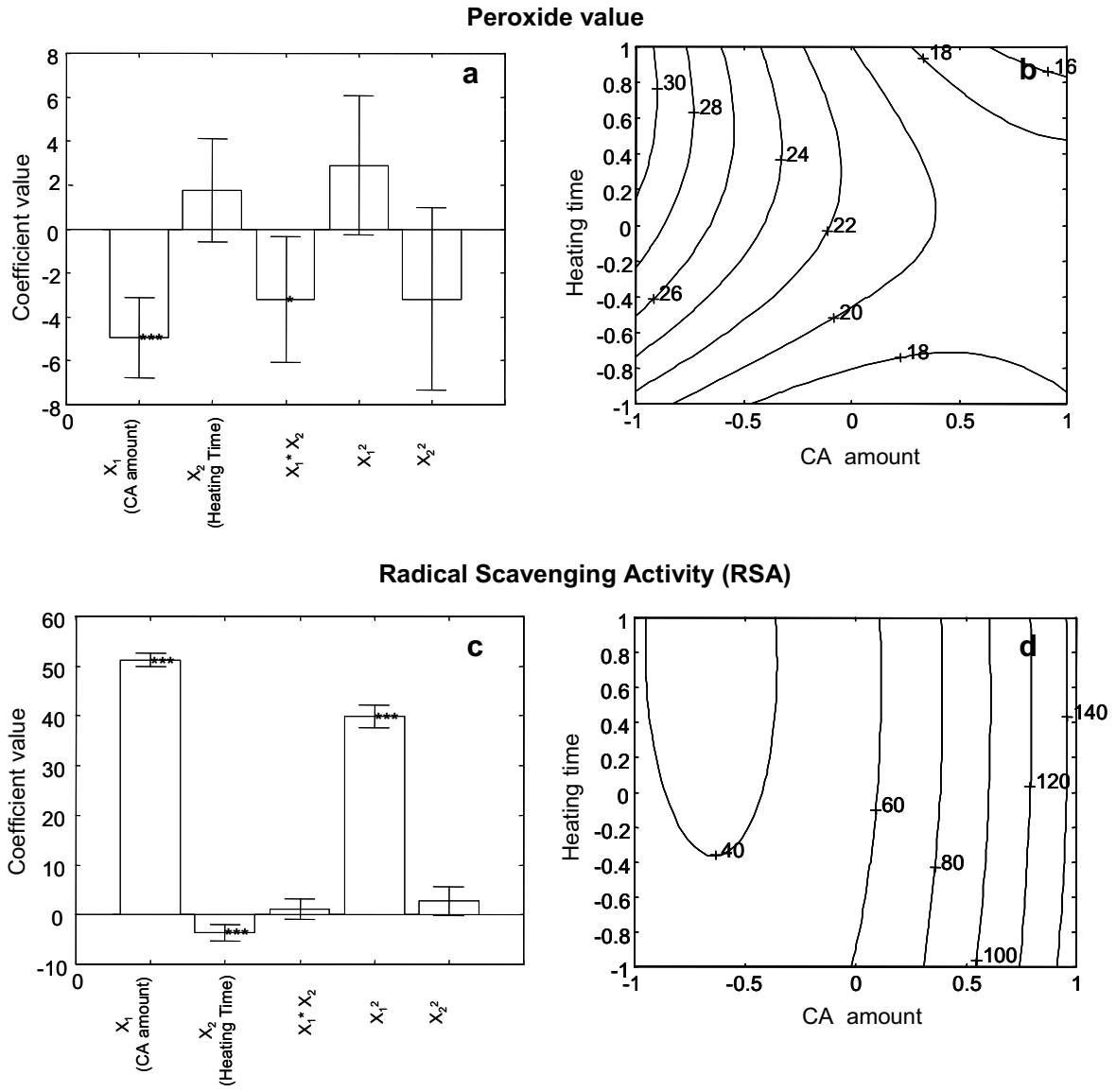

Fig. 3. Plots of the coefficient of the models (a, c) and of the response surfaces (b, d) of peroxide and RSA values for oils heated at 60 °C ($^{*} = p < 0.05$, $^{**} = p < 0.01$, $^{***} = p < 0.001$).

$$\mathrm{RSA} = 54.5 + 51.2X_1(***) - 3.5X_2(***) + 1.0X_1X_2 + 39.9X_1^2(***) + 2.7X_2^2$$

For the response *p*-anisidine it was not possible to build a reliable model, since its response did not vary significantly throughout the whole aging period.

The response surface for peroxide shows (Fig. 3b) the strong effect of CA and its interaction with heating time. Though higher amounts of added CA always correspond to lower hydroperoxides, it has to be noticed that this effect is greater the greater the heating time. It can also be seen that the greater the amount of CA the lower the effect of heating time, and that at the highest concentrations of CA even the longest heating time did not produce any increase of hydroperoxides.

The response surface for RSA (Fig. 3d) shows a strong quadratic effect for CA, this indicating that additions <0.01% do not have any effect, while at higher concentrations the effect increases very fast. The effect of heating time, though statistically significant, was not practically relevant.

In conclusion, the obtained results highlighted that in samples heated at 180 °C the addition of CA did not succeed in inhibiting oil oxidation and even enhanced both primary and secondary oxidation products. Moreover, the fast decrease of RSA in enriched samples stood for a possible fast CA degradation at 180 °C, and confirmed the useless, if not negative, effect of CA addition in deep frying conditions.

On the contrary the accelerated aging test showed that the protective effect of CA against lipid oxidation significantly increases with its concentration, together with a strong and long lasting enhancement of the RSA that is particularly interesting even in sight of functional food formulation, since CA could also slow the oxidation processes in the human body by reducing the levels of reactive oxygen species (ROS) and free radicals. Thus, the incorporation of this interesting phytochemical might significantly contribute both to the health benefit of consumers and to the stabilisation of crude virgin olive oils.

References

American Oil Chemists Society (1998). *Official Methods and Recommended Practices, AOCS Official Method Cd 18–90* (5th ed.). Champaign, IL: AOCS Press.
American Oil Chemists Society (1998). *Official Methods and Recommended Practices, AOCS Recommended Practice Cg 5–97* (5th ed.). Champaign, IL: AOCS Press.

Brenes, M., Hidalgo, F. J., García, A., Rios, J. J., García, P., Zamora, R., et al. (2000). Pinoresinol and 1-acetoxypinoresinol, two new phenolic compounds identified in olive oils. *Journal of the American Oil Chemists' Society, 77*, 715–720.

Chang, S. S., Ostric-Matijasevic, B., Hsieh, O. A. L., & Huang, C.-L. (1977). Natural antioxidants from rosemary and sage. *Journal of Food Science, 42*, 1102–1106.

Cortesi, N., Rovellini, P., & Fusari, P. (2002). Determination of biophenols minor polar compounds in virgin olive oil. *Riv It Sost Grasse, 79*, 145–150.

Curvelier, M.-E., Richard, H., & Berset, C. (1996). Antioxidative activity and phenolic composition of pilot-plant and commercial extract of sage and rosemary. *Journal of the American Oil Chemists' Society, 73*, 645–652.

Duthie, G. G. (1991). Antioxidant hypothesis of cardiovascular disease. *Trends in Food Science & Technology, 2*, 205–207.

EFSA (2008). Scientific opinion of the panel on food additives, flavouring, processing aids and materials in contact with food on a request from the commission on the use of rosemary extracts as a food additive. *The EFSA Journal, 721*, 1–3.

European Regulation (EC) no. 2568/91 (1991). Official Journal of the European Communities, L248.

Frankel, E. N., Huang, S.-W., Prior, E., & Aeschbach, R. (1996). Evaluation of antioxidant activity of rosemary extracts, carnosol and carnosic acid in bulk vegetable oils and fish oil and their emulsions. *Journal of the Science of Food and Agriculture, 72*, 201–208.

Hopia, A. I., Huang, S.-W., Schwarz, K., German, J. B., & Frankel, E. N. (1996). Effect of different lipid systems on antioxidant activity of rosemary constituents carnosol and carnosic acid with and without α-tocopherol. *Journal of Agricultural and Food Chemistry, 44*, 2030–2036.

Huang, S.-W., Frankel, E. N., Schwarz, K., Aeschbach, R., & German, J. B. (1996). Antioxidant activity of carnosic acid and methyl carnosate in bulk oils and oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 44*, 2951–2956.

Kalantzakis, G., Blekas, G., Pegklidou, K., & Boskou, D. (1998). Stability and radical scavenging activity of heated olive oil and other vegetable oils. *European Journal of Lipid Science and Technology, 108*, 329–335.

Kinsella, J. E., Frankel, E., German, B., & Kanner, J. (1993). Possible mechanisms for the protective role of antioxidants in wine and plant food. *Food Technology, 47*, 85–89.

Marrero, J. G., Andrés, L. S., & Luis, J. G. (2002). Semisynthesis of rosmanol and its derivatives. Easy access to abietatriene diterpenes isolated form the genus Salvia with biological activities. *Journal of Natural Products, 65*, 986–989.

Masuda, T., Inaba, Y., & Takeda, Y. (2001). Antioxidant mechanism of carnosic acid: Structural identification of two oxidation products. *Journal of Agricultural and Food Chemistry, 49*, 5560–5565.

Matlab 4.2. (1994). Natick, USA: The Mathworks Inc.

Miura, K., Kikuzaki, H., & Nakatani, N. (2002). Antioxidant activity of chemical components from sage (Salvia officinalis L.) and thyme (Thymus vulgaris L.) measured by the oil stability index method. *Journal of Agricultural and Food Chemistry, 50*, 1845–1851.

Montedoro, G. F., Servili, M., Baldioli, M., Selvaggini, R., Miniati, E., & Macchioni, A. (1993). Simple and hydrolyzable phenolic compounds in virgin olive oil. 3. Spectroscopic characterization of the secoiridoid derivatives. *Journal of Agricultural and Food Chemistry, 41*, 2228–2234.

Pike, O. A. (2003). Fat characterization. In S. S. Nielsen (Ed.), *Food analysis* (pp. 227–245). New York: Plenum Publisher, Kluver Academic.

Tada, M. (2000). Biological activities of antioxidants from herbs in Labiatae. *Foods & Food Ingredients Journal of Japan, 184*, 33–39.

Trojakova, L., Reblova, Z., & Pokorny, J. (2000). Degradation of tocopherols in rapeseed oil with rosemary extract under different conditions. *Czech Journal of Food Sciences, 18*, 175–176.

Wagner, K. H., & Elmadfa, I. (2000). Effects of tocopherols and their mixtures on the oxidative stability of olive oil and linseed oil under heating. *European Journal of Lipid Science and Technology, 102*, 624–629.

Yanishlieva, N. V., & Marinova, E. M. (2001). Stabilisation of edible oils with natural antioxidant. *European Journal of Lipid Science and Technology, 103*, 752–767.