J Am Oil Chem Soc (2014) 91:1583–1594
DOI 10.1007/s11746-014-2509-z

ORIGINAL PAPER

Antioxidant Activity of Amaranth Protein Hydrolysate Against Thermal Oxidation of Vegetable Oils

Valeria A. Tironi · María C. Añón

Received: 30 December 2013 / Revised: 23 June 2014 / Accepted: 27 June 2014 / Published online: 10 July 2014

Abstract The antioxidant capacity of amaranth protein hydrolysate (AH) during the thermally induced oxidation of two different vegetable oils, sunflower oil (SO) and canola oil (CO), was assessed by differential scanning calorimetry by means of isothermal and non-isothermal assays. Interactions between AH and tocopherols were also analyzed. In both oils, AH (10 % w/w) produced an increase in the induction period in isothermal assays, presenting a synergistic effect with tocopherols. In the case of non-isothermal assays, thermograms of oils with AH showed a significant diminution in the second exothermic peak, suggesting an inhibition of the decomposition of primary to secondary oxidation products. In addition, AH produced an increase in the oxidation rate constant (k) value at low temperatures being more evident in the case of canola oil. However, this effect decreased as a function of the temperature increase, which suggests that AH would have a pro-oxidant effect at low temperatures but a heat stabilizing effect at high temperatures (above 210 °C for SO and 190 °C for CO).

Keywords Amaranth · Protein hydrolysates · Peptides · Antioxidant activity · Thermal oxidation · Differential scanning calorimetry (DSC)

V. A. Tironi (✉) · M. C. Añón
Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CCT-La Plata-CONICET, UNLP, 47 y 116, 1900 La Plata, Argentina
e-mail: valeriatironi@hotmail.com

Introduction

Amaranth (*Amaranthaceae*) seeds, an ancestral American pseudocereal, present an interesting nutritional value due to—among other properties—their high protein content (15–17 %) and their excellent amino acidic balance. Various biological activities of amaranth proteins or peptides are being studied in our laboratory and the antioxidant activity is one of them. Thus, we have demonstrated the presence of naturally-occurring peptides and polypeptides with free radical scavenging activity in *Amaranthus mantegazzianus* seeds, which were distributed into different protein fractions (albumins, globulins and glutenins) [1]. In addition, alcalase hydrolysis improved the scavenging activity of both the isolate and the protein fractions by producing the release of small peptides and/or free amino acids with such activity [1]. Amaranth peptides and polypeptides also presented the capacity to inhibit the linoleic acid oxidation in an emulsion model system [1]. Physiological relevance of these peptides is being studied as well. Results obtained so far have portrayed amaranth proteins as a potential source of antioxidant peptides which could be released into the human body after gastrointestinal digestion. This way, both the amaranth protein isolate and the alcalase-hydrolysate have shown a potential capacity to scavenge free radicals after gastrointestinal digestion [2]. Another aspect that has been evaluated is the capacity of amaranth proteins and peptides to inhibit the lipid oxidation when they are incorporated into a food matrix. In this sense, the amaranth protein isolate as well as its alcalase-hydrolysate have demonstrated the ability to partially inhibit the lipid oxidation process in restructured fish products, preventing the formation of secondary products (García Filleria and Tironi, unpublished work). In this regard, antioxidant activity of peptides from different

sources added to meat products has been evaluated [3–7]. Also, antioxidant activity of peptides has been established in emulsion systems [8, 9].

The present work proposes to evaluate the antioxidant capacity of amaranth protein ingredients during the thermally induced oxidation of vegetable oils, due to the great importance of temperature to accelerate oxidation processes. Given that the oxidative process occurs by exothermal reactions, it can be evaluated by differential scanning calorimetry (DSC), being possible to use isothermal and non-isothermal DSC techniques to calculate oxidation kinetic parameters [10, 11]. Due to their relative simplicity, accuracy and rapidity [11], these were the methodologies selected for this work. They have been previously used to assess the antioxidant capacity of other kind of substances such as BHA, BHT and propyl gallate in rapeseed and sunflower oil [12]; ascorbyl palmitate, α- and δ-tocopherols and propyl gallate in sunflower and palm oils [13]; methanolic extracts of defatted rice brand in soybean oil [14]; and microencapsulated red chili oleoresin in canola oil [15], among others. Two important vegetable edible oils presenting different fatty acids profiles were selected for this work: sunflower oil (mean fatty acid composition: 7 % 16:0, 5.5 % 18:0, 20 % ω9-18:1, 66 % ω6-18:2), and canola oil (mean fatty acid composition: 4 % 16:0, 1.8 % 18:0, 63 % ω9-18:1, 20 % ω6-18:2, 8.6 % ω3-18:3) (Food and Agriculture Organization of the United Nations (FAO) (accessed May 2013) Fatty acid composition canola oil ftp://fao.org/es/esn/food/bio-10t.pdf). In this way, canola oil contains about ten times more ω3 fatty acids, about three times more ω9 fatty acids and about one-fifth of saturated fatty acids than sunflower oil, this composition being beneficial in the prevention of cardiovascular diseases.

Experimental Procedures

Chemicals

Alcalase 2.4L (protease of *Bacillus licheniformis*, Novozyme Corp) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aluminum oxide 90 active neutral (chromatography grade) was obtained from AppliChem (Darmstadt, Germany). Methanol was of chromatography grade. Other reagents were of analytical grade.

Samples

Oils

Sunflower and canola oils were obtained from the local market. Tocopherols were eliminated by means of aluminum oxide according to [16]. Briefly, two parts of oil were mixed with one part of alumina, vigorously shaken and kept in the dark at 4 °C overnight. The mixture was centrifuged at 3,000×*g* for 20 min at 20 °C to separate the sorbent. Stripped oil was collected and stored in the dark in a −20 °C nitrogen atmosphere.

The tocopherols content in the oils was determined according to [17]. The oil sample was diluted in hexane (1:10 v/v). A sample of 200 µl was mixed with 600 µl of methanol and 200 µl of the internal standard solution (300 µg/ml of α-tocopherol acetate in ethanol). The mixture was agitated in vortex, centrifuged (3000 g, 5 min, room temperature) and filtered through a 0.45 µm pore size nylon filter. Fifty microliters of *sample* were injected in a 5 µm C18 symmetry (150 × 4.4 mm ID) column and ran using a Waters 1525 Binary HPLC Pump. The following conditions were applied: mobile phase methanol:water (96:4 v/v), flux 2 ml/min, temperature 45 °C. Detection was performed at 292 nm with a Waters 2998 photodiode array detector.

Protein Isolates

Amaranthus mantegazzianus from commercial variety (Pass CV Don Juan) was grown at the Facultad de Agronomía, Universidad Nacional de La Pampa, Argentina in 2008. Flour was obtained by grinding the seeds in an Udy mill (Udy Corporation, Fort Collins, Colorado, USA), 1 mm mesh, screened by 0.092 mm mesh, and defatted by extraction with hexane (1/10 w/v) for 24 h at 4 °C. Amaranth protein isolates (***I***) were obtained from the defatted flour by extraction at pH 9, isoelectric precipitation (pH 5), neutralization and freeze-drying using the previously reported method [18]. Composition of protein isolates was: 79.1 ± 0.1 g proteins/100 g, 9.7 ± 0.4 g carbohydrates/100 g; 7.0 ± 0.3 g water/100 g, 3.2 ± 0.1 g ash/100 g, and 1.7 ± 0.2 g lipids/100 g.

Protein Hydrolysate

The 1 % w/v isolate suspensions in 1 mm NaOH were prepared by adjusting to pH 10. Suspensions were agitated for 1 h at 37 °C, maintaining the pH = 10 by adding 0.1 M NaOH. After that, alcalase (≥2.4 U/g, Anson units) was added in a ratio of 8 µl/100 mg sample. The reaction mixture was incubated at 37 °C for 4 h. The enzyme activity was stopped by heating at 85 °C for 10 min and the suspension was freeze-dried, obtaining the corresponding hydrolysates (***IH***). The hydrolysis grade (HD) was measured by reaction of free amino groups with 2,4,6-trinitrobenzenesulfonic acid (TNBS) [19], and calculated using the equation: $HD = ([-NH_2]h - [-NH_2]_0)/([-NH_2]_\infty - [-NH_2]_0 \times 100$; where $[-NH_2]$ indicates the concentration of free amino groups in the non-hydrolyzed (0) or the hydrolyzed sample (h). The

parameter [–NH2]$_\infty$ was estimated according to: $[-NH_2]_\infty = 1/M_{aa} \times (1 + f_{Lys}) \times C_{prot}$, where M_{aa} is the average of the amino acid molecular weight in the amaranth proteins (130 g/mol), f_{Lys} is the rate of lysine in these proteins (1/15) [20] and C_{prot} is the protein concentration (1 g/L for this assay). The HD of the alcalase hydrolysate used in the present work was 30 %. Composition of ***IH*** was: 78.5 ± 0.6 g proteins/100 g, 11 ± 3 g carbohydrates/100 g; 9.2 ± 0.3 g water/100 g, 5.2 ± 0.3 g ash/100 g, and 1.06 ± 0.01 g lipids/100 g. ***IH*** contained a very high proportion of peptides with mass lower than 3 kDa and, according to chromatographic analysis (molecular exclusion FPLC), mostly of the molecules were lower than 6.5 kDa, with a very high proportion of peptides with molecular mass <0.5 kDa (about a 47 % of the total protein mass), including a significant presence (2.6 %) of molecules of very low molecular mass (<0.25 kDa) [1, 2].

Thermal Oxidation Analysis

Oils (sunflower and canola, each one with and without tocopherols) and their dispersions of amaranth protein hydrolysate (10 % w/w) in oil were subjected to thermal oxidation processes in a DSC instrument (Q100, TA Instruments, New Castle, USA) driven by an Advantage Software (TA instruments) and using Universal Analysis 2000 (TA instruments) for data analysis. The equipment was calibrated at a heating rate of 10 °C/min using indium as a standard for enthalpy and temperature, and sapphire for heat capacity. To obtain dispersions, the protein ingredient and the oil were mixed in a Thermomixer Eppendorf (800 rpm, 1 h, 10 °C). Immediately before the analysis, they were vigorously agitated in a vortex mixer and placed in an open capsule (4–6 mg). Analyses were carried out in an oxygen atmosphere (100 ml/min). Two kinds of processes were performed:

Isothermal Analysis

Samples were subjected to constant temperatures (120 and 130 °C). Oxidative stability was represented by the induction period (IP), which corresponds to the intersection of the extrapolated baseline and the tangent line of the exotherm in the heat flux *versus* time curve. From IP values a protection factor f was calculated as:

$$f = IP_{(\text{with antioxidant})} / IP_{(\text{without antioxidant})}$$

Non-Isothermal Analysis

Linear temperature increases from 20 to 350 °C at different heating rates (β = 5, 10, 15 and 20 °C/min) were applied and heat flux *versus* temperature curves were obtained. Onset temperature (T_{on}) was determined as the temperature at which the heat flow significantly deviates from the baseline. In addition, oxidation kinetic parameters of each sample were calculated. For that, activation energies of oxidation (E) and pre-exponential factors (Z) were calculated using the Ozawa–Flynn–Wall method [11]. According to this method, the temperature of the start or temperature of the maximum heat flow obtained from several DSC scans (each run with different β from 1 to 20 °C/min) can be used for determining kinetic parameters from the equation:

$$\log \beta = a \frac{1}{T} + b \quad (1)$$

where: $a = 0.4567$ E/R and $b = 2.315 + \log$ (ZE/R). So, plotting log β *versus* 1/*T*, E and Z can be obtained, and these values can be used to calculate the rate constant of reaction presented by the Arrhenius equation:

$$k = Z.e^{-(E/RT)} \quad (2)$$

Statistical Analysis

Data was analyzed by means of Analysis of Variance (ANOVA), using Microsoft Office Excel 97–2003 software. When significant differences ($\alpha = 0.05$) were detected, mean value differences were analyzed carrying out the Tukey test. At least two replicates of each assay were performed, using different oil batches for each one, and the results of them were averaged.

Results and Discussion

Sunflower Oil

Different batches of commercial sunflower oils were used. Their tocopherol contents were 0.17 and 0.27 mg/ml, with a high proportion of α-tocopherol (83–91 %), according to the RP-HPLC analyses performed. Although tocopherol contents were not identical, results from each replicate were comparable and they were averaged. After treating oils with aluminum oxide, tocopherols were not detectable (stated detection limit for the method is DL = 11.5 ng α-tocopherol that is 0.57 μg/ml of oil [17]) by RP-HPLC analysis.

Preliminary studies have proved that the addition of amaranth protein isolate (1 and 10 % w/w) as well as alcalase-hydrolysate (1 % w/w) did not produce any protective effect against thermal oxidation of sunflower oil in the presence of tocopherols (SO_t). However, antioxidant effects were registered when alcalase-hydrolysate was added at a ratio of 10 % w/w. Thus, this condition was selected for the present work.

Isothermal Analysis

Isothermal thermograms for sunflower oil with (SO_t) and without tocopherols (SO) can be observed in Fig. 1. As expected, IP at 120 °C (Fig. 1a, c, black lines) as well as at 130 °C (Fig. 1b, d, black lines) was increased by the presence of tocopherols, giving these antioxidants the following protection factors: $f = 3.2 \pm 0.4$ at 120 °C and $f = 2.27 \pm 0.06$ at 130 °C. The effectiveness of antioxidants in this kind of assays is related to their capacity to inhibit the radical chain by interacting with the peroxyl radicals, which are responsible for the duration of the IP [21]. This is the antioxidation mechanism exhibited by tocopherols, which inhibit or retard lipid oxidation by interfering with radical chain initiation and propagation by donating hydrogen atoms to lipid peroxyl and alkoxyl radicals [22]. Obtained results also suggest a negative effect of the temperature increase in the activity of the tocopherols due to the diminution of f at 130 °C in respect to 120 °C. Giuffrida et al. [13] have studied the thermal degradation of α- and δ-tocopherols via non-isothermal DSC assays, observing that α-tocopherol was more susceptible to thermooxidative degradation than δ-tocopherol, although the temperature at which degradation starts was above 130 °C for both. Taking into account that α-tocopherol is the main antioxidant in SO_t and the results obtained, it would be possible to postulate a partial degradation of this compound because of a combination of temperature (130 °C) and time of exposure.

When the oil without tocopherols (SO) was added with 10 % w/w amaranth protein hydrolysate AH (SO_h), a slight increase in the IP was obtained at 120 °C (Fig. 1a) and at 130 °C (Fig. 1b). The corresponding protection factors were $f = 1.2 \pm 0.2$ at 120 °C and $f = 1.2 \pm 0.2$ at 130 °C. These assays prove the presence of some components in AH with the ability to moderately extend the oxidative stability of the sunflower oil. This capacity was not influenced by the temperature increase between 120 and 130 °C. In order to evaluate if the antioxidant activity observed was due to oil soluble or/and insoluble components of AH, the dispersion SO_h was centrifuged (10,400×*g*, 10 min, room temperature) and the oil in the supernatant was newly analyzed at both temperatures. Thermograms obtained were similar to those corresponding to the sunflower oil without any addition (data not shown).

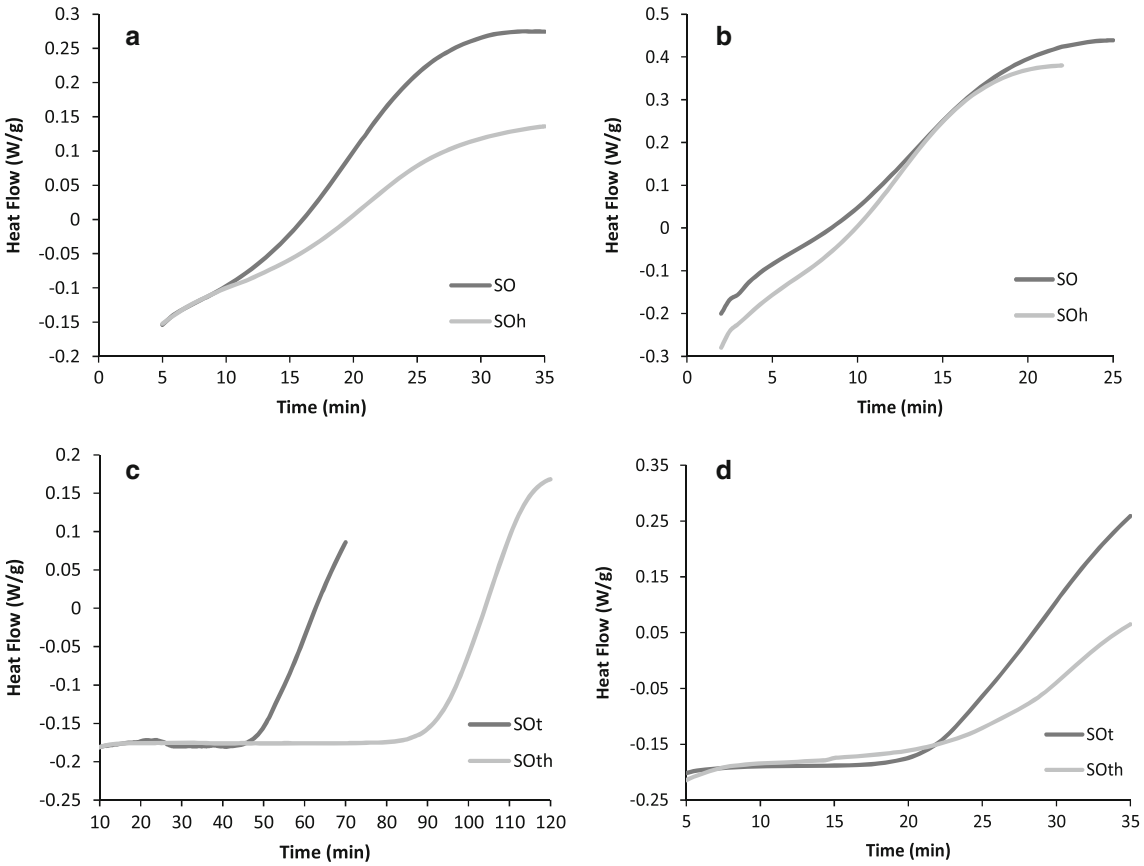

Fig. 1 Oxidative stability by DSC isothermal analysis of sunflower oil with and without the addition of 10 % amaranth hydrolysate (AH): **a** SO, 120 °C; **b** SO, 130 °C; **c** SO_t, 120 °C; **d** SO_t, 130 °C. Letters *t* and *h* next to *SO* note tocopherols and AH, respectively

These results suggest that antioxidant components of AH were only dispersed—not solubilized—in the oil.

In the case of the sunflower oil with tocopherols (SO_t), the addition of 10 % AH (SO_{th}) produced a significant increase in the oxidative stability at 120 °C (Fig. 1c) with an f value (calculated in respect to SO_t) of 1.8 ± 0.1; whereas, at 130 °C the increase was lower (Fig. 1d) with an f value of 1.3 ± 0.1.

According to the aforementioned results, the addition of 10 % AH could increase the induction time at 120 °C by about 20 % in the absence of tocopherols; while the sole presence of tocopherols (mainly α-tocopherol) produced an increment of about 320 % on IP. However, the increase in IP when both—tocopherols and AH—were present together was about 650 % (SO_{th} in respect to SO). These facts suggest a synergistic effect of the AH antioxidant components and the tocopherols at 120 °C. This effect was not evident at 130 °C due to the fact that the increase in IP of SO_{th} in respect to SO was close to the sum of increases caused by each antioxidant separately. This fact could be associated with the diminution of activity of tocopherols at this temperature, as was previously mentioned.

Non-Isothermal Analysis

Figure 2 presents typical non-isothermal lipid autoxidation thermograms, which show two exothermal processes. According to the Litwinienko studies [11], the first exothermal peak corresponds to the hydroperoxides formation while the second one is caused by further oxidation of peroxides. In addition, the increase in the heating rate produced a rise in the temperature at which the oxidation starts (T_{on}) [17], as can be seen in Fig. 2. The main difference registered between the sunflower oil without (SO) and with tocopherols (SO_t) was a significant ($p < 0.05$) shift of T_{on} at higher temperatures in the presence of these antioxidants at all the heating rates assayed (Table 1, Fig. 2a, c). These results are in agreement with the fact that tocopherols interfere with radical chain propagation [15].

The dispersion SO_h has shown changes in the thermograms in respect to SO, mainly a significant decrease in the

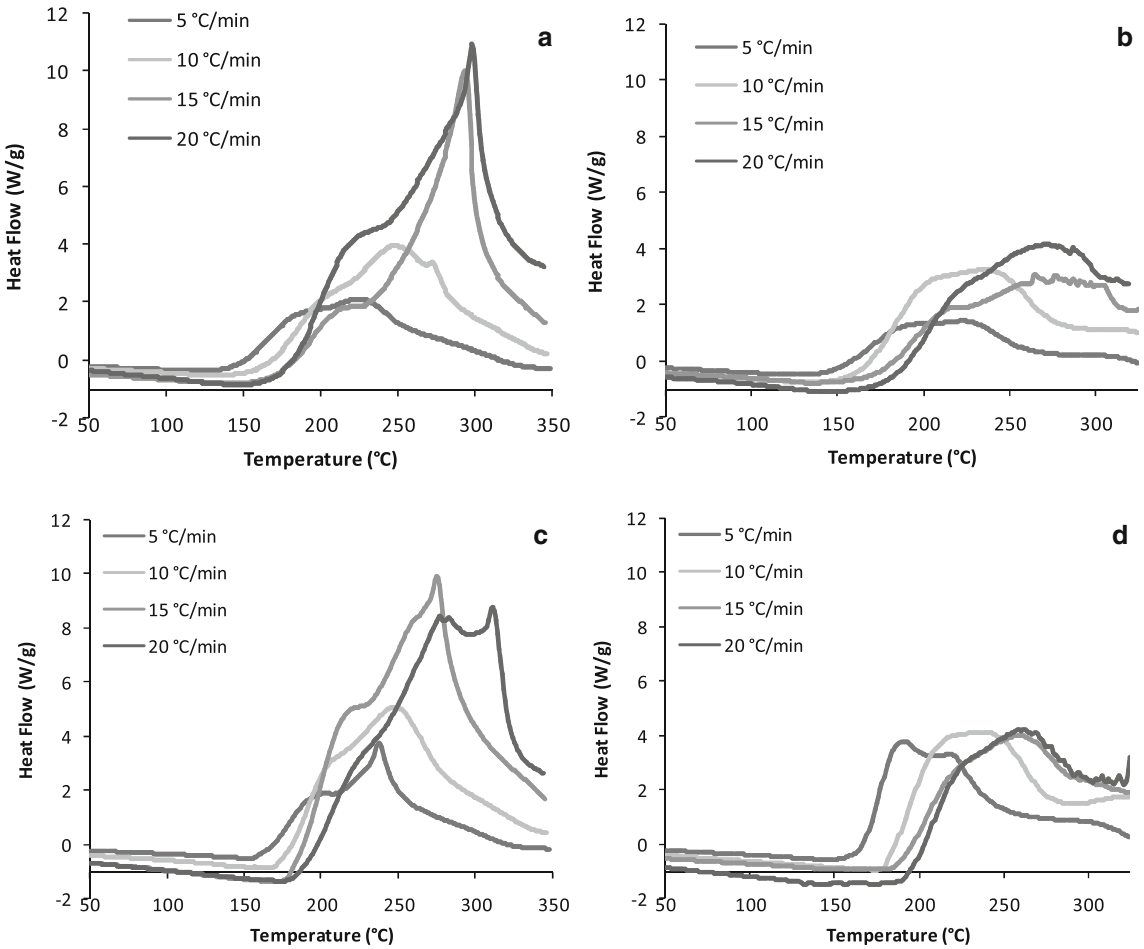

Fig. 2 Non-isothermal DSC thermal oxidation of sunflower oil at different heating rates: **a** SO; **b** SO_h; **c** SO_t; **d** SO_{th}. Letters *t* and *h* next to *SO* note tocopherols and AH, respectively

Table 1 Onset temperatures (T_{on}) and kinetic parameters for sunflower oil

Sample	T_{on} (5 °C/min)	T_{on} (10 °C/min)	T_{on} (15 °C/min)	T_{on} (20 °C/min)	E (kJ/mol)	Z (min^{-1})
SO	145.4 ± 6.5[a]	161.2 ± 4.3[a]	170.0 ± 2.4[a]	176.6 ± 2.2[a]	61.7	1.8×10^7
SO_h	144.7 ± 3.9[a]	159.1 ± 3.9[a]	170.7 ± 3.0[a]	177.4 ± 1.6[a]	60.1	1.2×10^7
SO_t	161.0 ± 0.5[b]	173.1 ± 0.3[b]	178.7 ± 0.6[b]	186.67 ± 0.01[b]	87.0	1.0×10^{10}
SO_{th}	166.4 ± 0.5[b]	180.0 ± 1.6[b]	187.4 ± 0.7[c]	190.9 ± 2.1[b]	87.8	9.1×10^9

Letters t and h next to SO note tocopherols and AH, respectively

Results are presented as the means ± SD of values obtained from two oil batches

Different superscript letters in the same column indicate significant differences between values ($p < 0.05$)

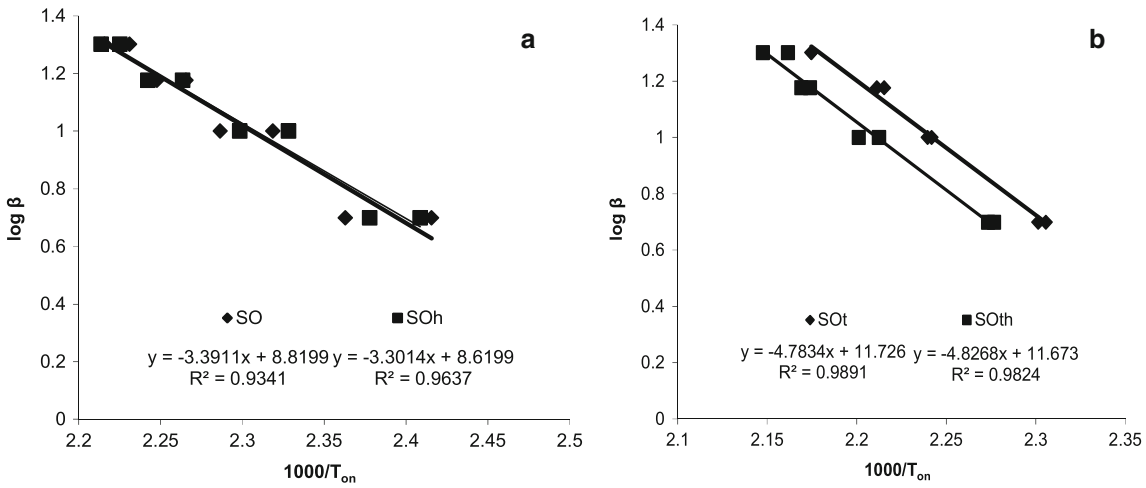

Fig. 3 Plot corresponding to the Ozawa–Flynn–Wall equation: **a** SO and SO_h; **b** SO_t and SO_{th}. Letters *t* and *h* next to *SO* note tocopherols and AH, respectively

size of the second peak (Fig. 2b); whereas the T_{on} did not present significant changes at any heating rate (Table 1). A possible explanation to these results is that, different to tocopherols, the AH components could act in this system inhibiting the formation of secondary oxidation products, without any action on the start of the lipid oxidation and the formation of the peroxides.

The SO_{th} dispersion has shown a similar behavior, with a decrease in the size of the second peaks in respect to SO_t (Fig. 2d, c). However, in this case, the T_{on} values showed an increasing tendency at all heating rates, the difference was significant only in the case of $\beta = 15$ °C/min ($p < 0.05$) (Table 1).

Activation energies of oxidation (E) and pre-exponential factors (Z) were calculated according to Ozawa–Flynn–Wall by plotting log β *vs* $1/T_{on}$ (Eq. 1) as it can be seen in Fig. 3 and Table 1. Both parameters increased due to the presence of tocopherols. From these results, the values of *k* in a range of temperatures between 50 and 250 °C were obtained. Figure 4 shows the ratio between the *k* values in the presence of antioxidants with respect to its corresponding control oil as a function of temperature. The presence of tocopherols (SO_t) rendered very small *k* values at low temperatures with a continuous increase as a function of temperature, achieving a similar value as SO at 210 °C and higher values above this temperature (Fig. 4a). These facts newly suggest a loss of heat stabilizing effectiveness of tocopherols as temperature increased and even a pro-oxidant effect at temperatures higher than 210 °C. A possible explanation to this last fact could be the formation of thermooxidative decomposition products from tocopherols having pro-oxidant effect.

In the case of sunflower oil without tocopherols, the addition of AH produced a small increase in the *k* value at low temperatures, decreasing as the temperature increased (Fig. 4b). However, in the presence of tocopherols and AH (SO_{th}), *k* presented lower values in respect to SO_t with no modifications in the calculated temperature range, indicating a diminution of the oxidation rate because of the amaranth peptides (Fig. 4c). Finally, comparing SO_{th} to SO (Fig. 4d), behavior was similar to that obtained in the case of SO_t *vs* SO (Fig. 4a), but with a reduction of the *k* values

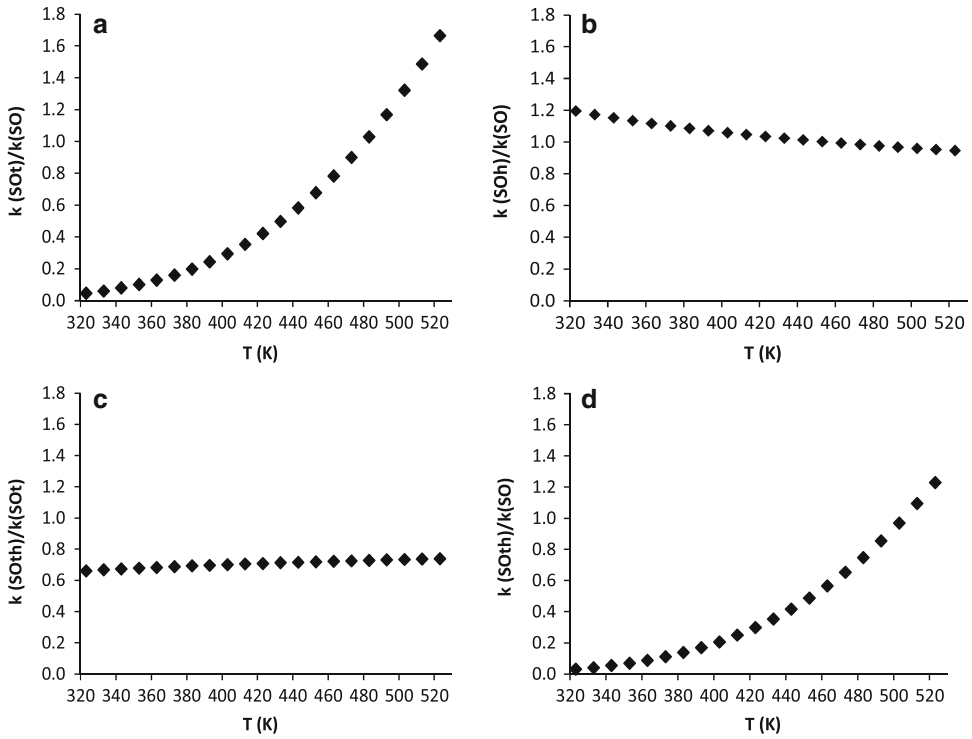

Fig. 4 Oxidation rate constant (k) as a function of temperature for sunflower oils. Letters t and h next to SO note tocopherols and AH, respectively

due to the AH addition. Results newly suggested a synergistic effect of AH and tocopherols, being the effect of AH especially important when temperature increased.

Canola Oil

Different batches of commercial canola oil were used and data obtained were averaged. According to the RP-HPLC analyses, tocopherol contents were 0.22 and 0.29 mg/ml, being the composition different from those present in the sunflower oil, with a high proportion of γ and β-tocopherols (73–75 %) followed by α-tocopherol (25 %). After the aluminum oxide treatment of the oils, tocopherols were not detectable (stated detection limit for the method is DL = 11.5 ng α-tocopherol that is 0.57 μg/ml of oil [17]) by RP-HPLC analysis.

Isothermal Analysis

Figure 5 shows thermograms obtained for canola oil without (CO) (Fig. 5a, b, 120 and 130 °C, respectively) and with tocopherols (CO_t) (Fig. 3c, d, 120 and 130 °C, respectively). Tocopherols increased the induction period at both temperatures, giving protection factors of $f = 1.5 \pm 0.6$ at 120 °C and $f = 2.1 \pm 0.6$ at 130 °C. IP values were quite different between canola oil batches, especially in the case of CO_t. Differently to sunflower oil, tocopherols activity was not diminished by the temperature increment, probably due to the different tocopherols composition present in each oil.

When CO was added with 10 % amaranth hydrolysate (CO_h), an increase in the induction times was observed at both temperatures (Fig. 5a, b), presenting the dispersions the following f values in respect to CO: 1.4 ± 0.1 at 120 °C, and 1.29 ± 0.03 at 130 °C. The effect of AH was more important in the oil with tocopherols CO_t (Fig. 5c, d). The protection factors for CO_{th} in respect to CO_t were $f = 1.9 \pm 0.5$ at 120 °C and $f = 2.4 \pm 0.1$ at 130 °C. Similar to sunflower oil, a synergistic effect of tocopherols and AH could be observed, but in this case the synergy was more evident at 130 °C (IP increment between CO and CO_{th} was about 600 %) than at 120 °C (IP increment between CO and CO_{th} was about 240 %).

Non-Isothermal Analysis

Tocopherols in the canola oil produced a T_{on} shift to higher values, although differences were significant ($p < 0.05$) only at $\beta = 5$ °C/min (Fig. 6a, c, Table 2). Kinetic parameters were also modified by these antioxidants, showing CO_t an increment of E and Z in respect to CO (Table 2).

Dispersions of 10 % AH in the oil without tocopherols (CO_h) did not show significant changes in the T_{on} values at any heating rate (Fig. 6a, b, Table 2), with a small change in the kinetic behavior (Fig. 7, Table 2). However, as it

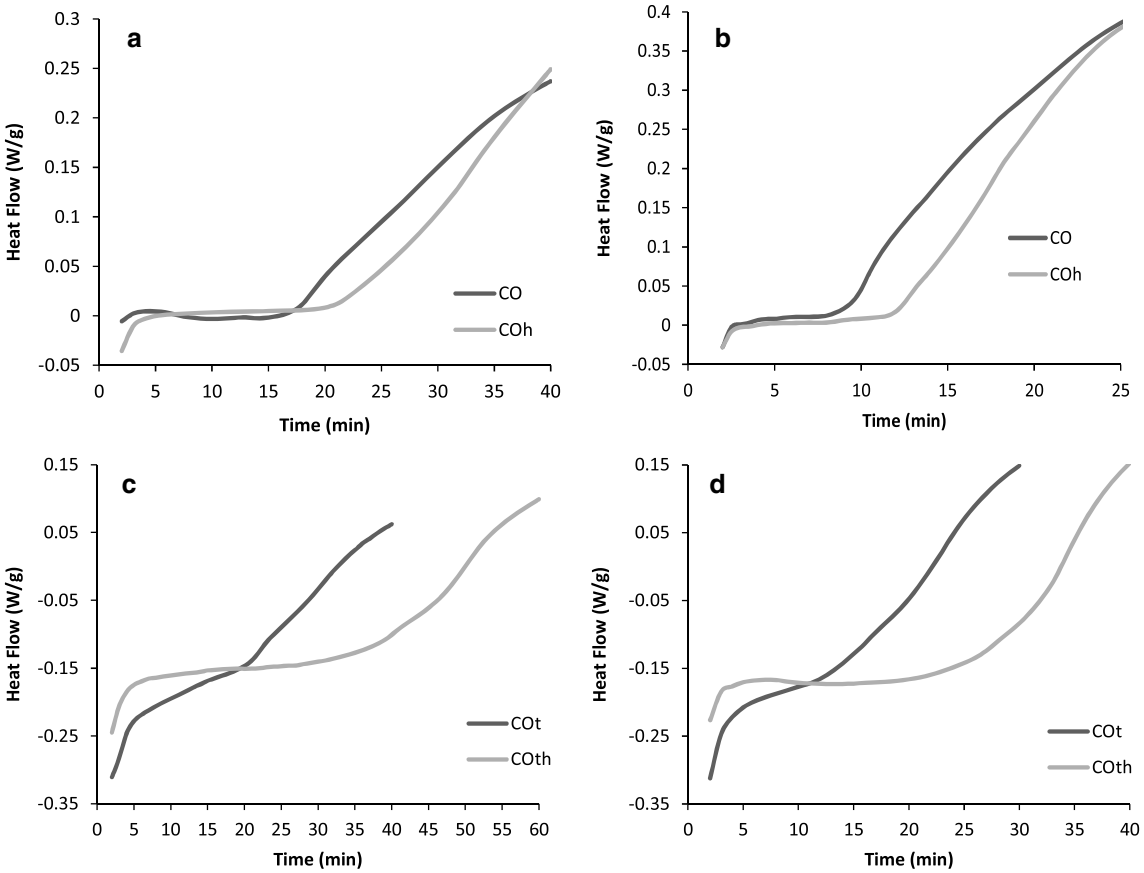

Fig. 5 Oxidative stability by DSC isothermal analysis of canola oil with and without the addition of 10 % amaranth hydrolysate (AH): **a** CO, 120 °C; **b** CO, 130 °C; **c** CO_t, 120 °C; **d** CO_t, 130 °C. Letters *t* and *h* next to *CO* note tocopherols and AH, respectively

was observed in sunflower oil, comparison of the thermograms showed a significant reduction in the size of the second peak, suggesting an inhibition on the formation of oxidation secondary products by AH.

Similar results were obtained in the case of the addition of AH to the oil with tocopherols (CO_{th}) (Fig. 6c, d, Fig. 7 and Table 2). Also, thermograms presented a very important reduction on the second peak in the presence of AH.

Rate constant values were calculated for all samples and the ratios with respect to oils without antioxidants were plotted as a function of the temperature (Fig. 8). Some differences with sunflower oil could be detected. Although tocopherols were not so efficient to diminish oxidation rate at low temperatures in this case with respect to sunflower oil, the effect of the increment of temperature was notably lower, evidencing a lower effectiveness loss of tocopherols (Fig. 8). Newly, this fact could be associated with the different tocopherols composition present in the canola oil and the lower susceptibility of γ-tocopherol to thermooxidative degradation in respect to α-tocopherol [13]. In this sense, it is known that the oxidation products of γ-tocopherol are still effective as antioxidants, while the oxidation products of α-tocopherol do not show antioxidant activity [23].

The addition of AH to CO (Fig. 8b) produced an important increase in the k value at low temperatures, this effect was less evidently observed in SO. Temperature increase rendered a diminution of the ratio, and above 190 °C, *k* value for CO_h was lower than for CO. Similarly to SO, these results suggest that AH would have a prooxidant effect at low temperatures but an heat stabilizing effect at temperatures above 190 °C. Although it is difficult to explain these facts, some possible hypotheses are the solubilization of some AH components at high temperatures or that some modifications in AH components rendered products with higher antioxidant capacity. In this sense, it is known that lipid oxidation-derived aldehydes react with amino acids in a Maillard-type reaction, yielding browning products of low and high molecular weight with free radical scavenging activity and the ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions, both indicating the antioxidative activity *in vitro* [24].

When AH was added to CO_t (Fig. 8c), behavior was similar as in the case of CO but with a lower increase in the

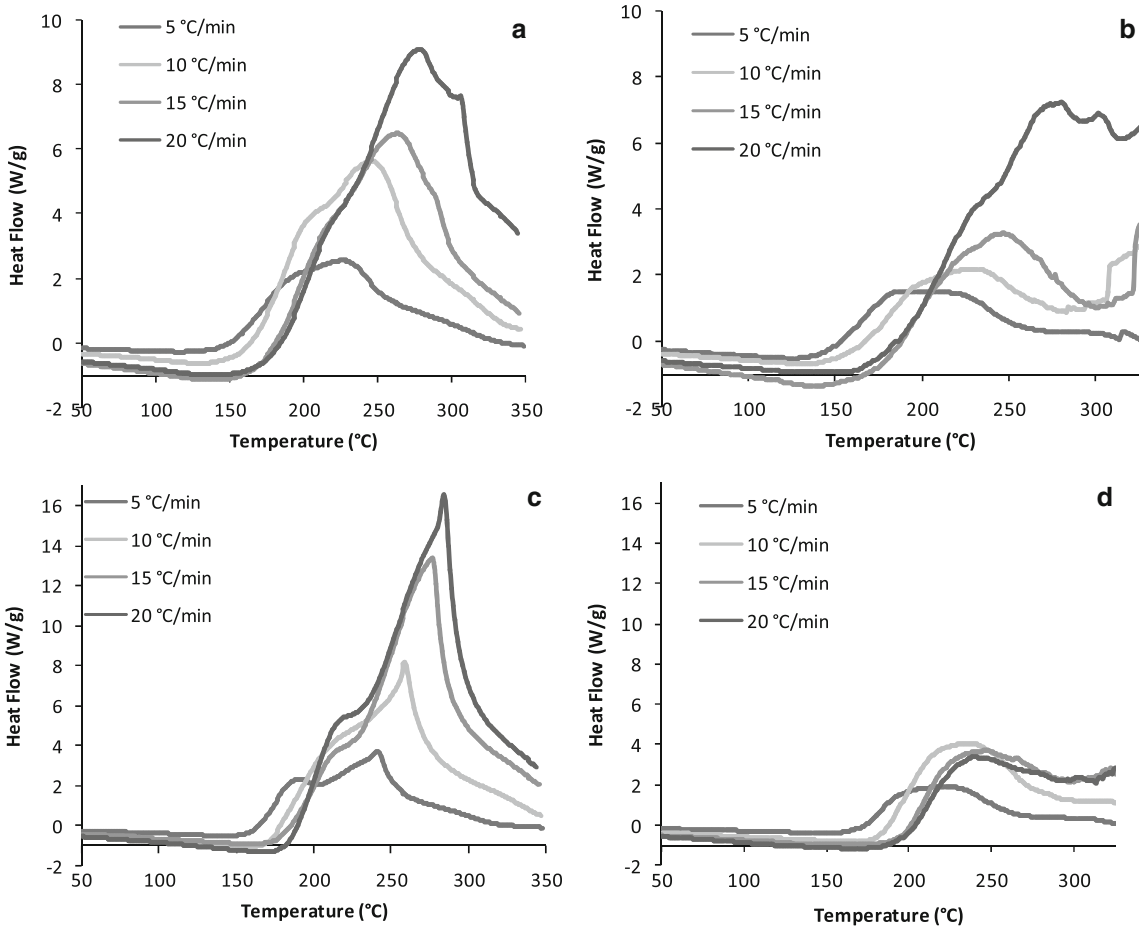

Fig. 6 Plot corresponding to the Ozawa–Flynn–Wall equation **a** CO and CO_h; **b** CO_t and CO_{th}. Letters *t* and *h* next to *SO* note tocopherols and AH, respectively

Table 2 Onset temperatures (T_{on}) and kinetic parameters for canola oil samples

Sample	T_{on} (5 °C/min)	T_{on} (10 °C/min)	T_{on} (15 °C/min)	T_{on} (20 °C/min)	E (kJ/mol)	Z (min^{-1})
CO	147.4 ± 4.5^{a}	$161.7 \pm 3.6^{a,b}$	171.3 ± 4.9^{a}	177.8 ± 3.6^{a}	64.6	3.9×10^{7}
CO_h	144.2 ± 2.1^{a}	155.6 ± 5.7^{a}	168.3 ± 0.5^{a}	178.2 ± 3.8^{a}	57.2	5.8×10^{6}
CO_t	164.0 ± 5.3^{b}	$174.6 \pm 4.9^{a,b}$	$184.7 \pm 6.1^{a,b}$	188.3 ± 4.9^{a}	75.3	3.8×10^{8}
CO_{th}	165.8 ± 3.9^{b}	178.3 ± 6.1^{b}	191.0 ± 4.9^{b}	192.9 ± 3.1^{a}	70.1	7.9×10^{7}

Letters t and h next to CO note tocopherols and AH, respectively

Results are presented as the means ± SD of values obtained in two oil batches

Different superscript letters in the same column indicate significant differences between values ($p < 0.05$)

oxidation rate at low temperatures, probably due to tocopherols antioxidant capacity. Finally, the combination of tocopherols (mainly γ and β-tocopherols) and AH (Fig. 8d) produced a decrease in the oxidation rate at all temperatures, with a slight effectiveness loss because of the temperaturés increase.

Several mechanisms have been postulated for the antioxidant properties of peptides, including metal ion chelation, free radical scavenging, and aldehyde adduction; being the amino acid composition, sequence, structure and size important in determining their activity. Different amino acid residues and peptide sequences are responsible for the inhibition of oxidative reactions that are initiated by different types of free radicals or pro-oxidants such as metal ions, as well as in different molecular environments (aqueous, lipid, emulsion systems; different pH conditions;

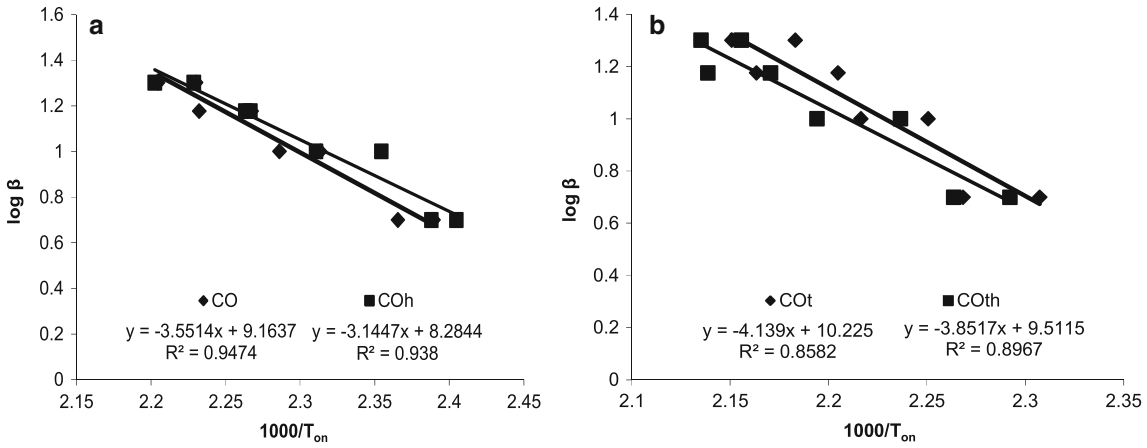

Fig. 7 Oxidation rate constant (k) as a function of temperature for canola oils. Letters t and h next to SO note tocopherols and AH, respectively

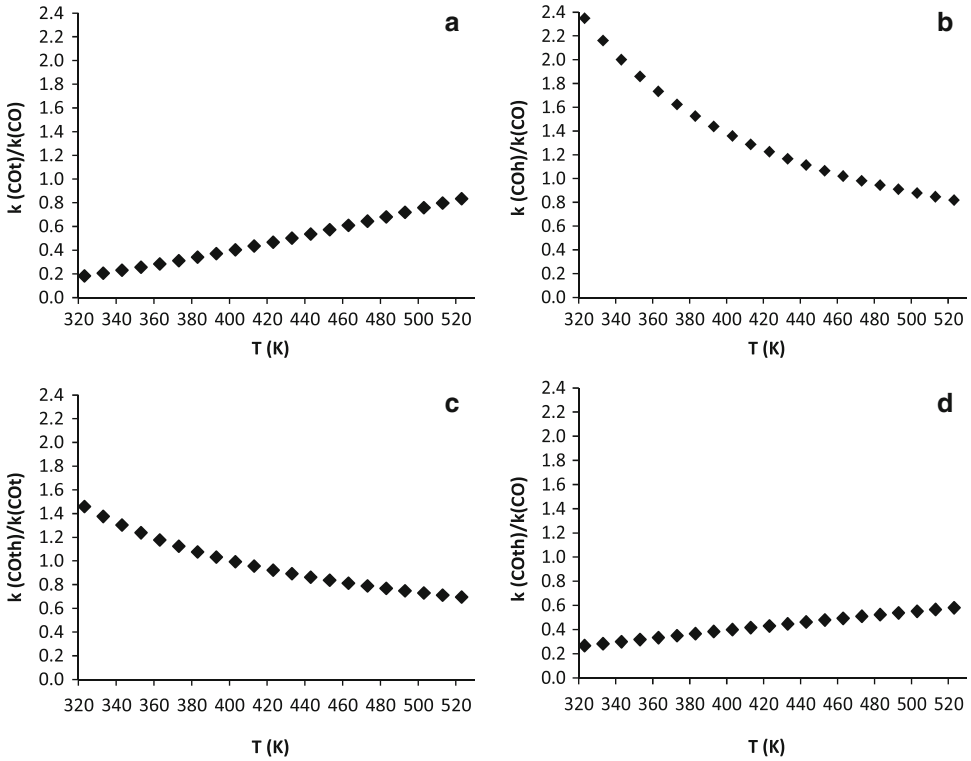

Fig. 8 Non-isothermal thermal oxidation of canola oil at different heating rates: **a** CO; **b** CO_h; **c** CO_t; **d** CO_{th}. Letters t and h next to SO note tocopherols and AH, respectively

the presence of other compounds in the food matrices or biological systems) [25]. According to this, a protein hydrolysate as the one used in the present work had the potential of containing peptides with different antioxidant actions. Antioxidant activity of amaranth hydrolysate in oil could not be related to the inhibition of the formation or propagation of the free radical chain or primary oxidation products, as in the case of tocopherols. Furthermore, it could act inhibiting the degradation of primary products to secondary oxidation products. Comparable results have been obtained by adding 2 % w/w of amaranth protein ingredients (isolate and alcalase hydrolysate) to fish restructured products, where these ingredients were able to retard the formation of secondary lipid oxidation products without any effect on primary products (García Filleria and Tironi, in revision). Elías et al. [8] assessed the antioxidant activities of β-lactoglobulin and its chymotryptic hydrolysates in a bulk oil system. For that, various concentrations of samples (5, 50, or 100 μg/g of oil) were dissolved directly in menhaden oil, and the capacity to inhibit lipid oxidation (lipid hydroperoxides production) was measured at 37 °C. However, in contrast to the present work,

hydrolysates did not significantly inhibit bulk menhaden oil oxidation. The authors proposed that the physicochemical properties of these compounds may not be appropriate for such a system. According to the present results, other hypotheses could be proposed: (1) the concentration of hydrolysates in the oil was not enough; at this point it is necessary to take into account that hydrolysates contain active and inactive peptides and the concentration of the active ones could be low; (2) the methodology of measurement of the lipid oxidation—lipid hydroperoxides detection—could not be the ideal one due to what we have demonstrated in the present work that the main effect of peptides in a bulk oil was on the decomposition of primary to secondary oxidation products.

In addition to the action mechanism, tocopherols and amaranth hydrolysate have also presented different behaviors regarding temperature, the last one being more effective at high temperatures at which tocopherols showed a loss in effectiveness. These facts would justify a very interesting increase in the antioxidant effectiveness when both kinds of antioxidants are present in the oils. Synergistic effects of some antioxidant peptides with tocopherols in food and model systems have been previously reported. Gelatin hydrolysates from Alaska pollack skin exhibited synergistic effects on combined use with α-tocopherol in the inhibition of the linoleic acid oxidation in a water/alcohol system [26]. Chen et al. [27] have analyzed the synergistic effects against the oxidation—evaluated by the ferric thiocyanate method—of emulsified linoleic acid, of synthetic peptides with BHA, BHT, and δ-γ-tocopherols; in the case of tocopherol, some of the studied peptides (HPLH, HHLP, PLHH, and HPHL) were synergistic, while others (LLPH, LLPHH, and LLPHHH) were not. Also, hydrolysates derived from soy β-conglycinin, glycinin, and basic 7S globulin exhibited synergistic effects with the tocopherols.

Likewise, we cannot disregard the presence of non-peptide components in the amaranth protein hydrolysate, which could produce some antioxidant effect in oils. The used hydrolysate has been analyzed in its content of phenolic components, observing that it conserved some of the phenolics present in the flour and the protein isolate. The amount of these detected compounds was low (about 50 μg/g of hydrolysate), being rutin and 4-hydroxybenzoic acid the most important quantitative components (unpublished data). Antioxidant potential of amaranth phenolics has been reported [28], though using techniques different from the DSC and extracts obtained from amaranth products.

Conclusions

The present work constitutes new scientific evidence of the presence of peptide components with antioxidant activity in the amaranth alcalase-hydrolysate, being the first one—as far as we know—in which the antioxidant activity of peptides is proved in a not-emulsified oil system. In conclusion, amaranth hydrolysate seems to be a potential natural antioxidant against thermal oxidation to be used dispersed in oil phases of food formulations, especially in combination with tocopherols.

Acknowledgments This work was supported by a Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina) project (PIP 1330). The authors would like to thank Javier Lecot and Daniel Russo for their technical assistance in DSC analysis, and to Claudio Reyes for his technical assistance in RP-HPLC runs. The authors are members of CONICET.

References

1. Tironi V, Añon MC (2010) Amaranth as a source of antioxidant peptides. Effect of proteolysis. Food Res Int 43:315–322
2. Orsini Delgado MC, Tironi V, Añón MC (2011) Antioxidant activity of amaranth protein or their hydrolysates under simulated gastrointestinal digestion. LWT-Food Sci and Tech 44:1752–1760
3. Peña-Ramos E, Xiong Y (2003) Whey and soy protein hydrolysates inhibit lipid oxidation in cooked pork patties. Meat Sci 64:259–263
4. Sakanaba S, Tachibana Y (2006) Active oxygen scavenging activity of egg-yolk protein hydrolysates and their effects on lipid oxidation in beef and tuna homogenates. Food Chem 95:243–249
5. Nieto G, Castillo M, Xiong Y, Álvarez D, Payne F, Garrido M (2009) Antioxidant and emulsifying properties of alcalase-hydrolyzed potato proteins in meat emulsions presenting different fat concentrations. Meat Sci 83:24–30
6. Rossini K, Noren C, Cladera-Olivera F, Brandelli A (2009) Casein peptides with inhibitory activity on lipid oxidation in beef homogenates and mechanically deboned poultry meat. LWT - Food Sci and Tech 42:862–867
7. Hogan S, Zhang L, Li J, Wang H, Zhou K (2009) Development of antioxidant rich peptides from milk protein by microbial proteases and analysis of their effects on lipid peroxidation in cooked beef. Food Chem 117:438–443
8. Elias R, Bridgewater J, Vachet R, Waraho T, McClements J, Decker E (2006) Antioxidant mechanisms of enzymatic hydrolysates of β-lactoglobulin in food lipid dispersions. J Agric and Food Chem 54:9565–9572
9. Cheng Y, Xiong Y, Chen J (2010) Antioxidant and emulsifying properties of potato protein hydrolysate in soybean oil-in-water emulsions. Food Chem 120:101–108
10. Thurgood J, Ward R, Martini S (2007) Oxidation kinetics of soybean oil/anhydrous milk fat blends: a differential scanning calorimetry study. Food Res Int 40:1030–1037
11. Litwinienko G (2001) Autooxidation of unsaturated fatty acids and their esters. J Thermal Anal and Calorim 65:639–646
12. Kowalski B (1991) Thermal-oxidative decomposition of edible oils and fats. DSC studies. Thermoch Acta 184:49–57
13. Giuffrida F, Destaillats F, Herrero Egart M, Hug B, Golay P, Skibsted L, Dionisi F (2007) Activity and thermal stability of antioxidants by differential scanning calorimetry and electron spin resonance spectroscopy. Food Chem 101:1108–1114
14. Renuka Devi R, Jayalekshmy A, Arumughan C (2007) Antioxidant efficacy of phytochemical extracts from defatted rice bran in the bulk oil system. Food Chem 104:658–664

15. Pérez-Alonso C, Cruz-Olivares J, Barrera-Pichardo J, Rodríguez-Huezo M, Báez-González J, Vernon-Carter E (2008) DSC thermo-oxidative stability of red chili oleoresin microencapsulated in blended biopolymers matrices. J Food Eng 85:613–624
16. Berton C, Genot C, Ropers M (2011) Quantification of unadsorbed protein and surfactant emulsifiers in oil-in-water emulsions. J Colloid and Interface Sci 354(2):739–748
17. Gimeno E, Castellote A, Lamuela-Raventós R, de la Torre M, López-Sabater M (2000) Rapid determination of vitamin E in vegetable oils by reversed phase high-performance liquid chromatography. J Chromat A 881:251–254
18. Martinez NE, Añón MC (1996) Composition and structural characterization of amaranth protein isolates. An electrophoretic and calorimetric study. J Agric Food Chem 44:2523–2530
19. Adler-Nissen J (1979) Determination of degree of hydrolysis of food proteins hydrolysates by trinitrobenzenesulfonic acid. J Agric Food Chem 27:1256–1262
20. Bressani R (1994) Composition and nutritional properties of amaranth. In: Paredes-López O (ed) Amaranth: biology, chemistry and technology. CRC Press, Boca Raton, pp 185–206
21. Yanishilieva N, Kamal-Eldin A, Marinova E, Toneva A (2002) Kinetics of antioxidant action of α- and γ-tocopherols in sunflower and soybean triacylglycerols. Eur J Lipid Sci and Tech 104:262–270
22. Frankel EN (1996) Antioxidants in lipid foods and their impact on food quality. Food Chem 57:51–55
23. Huang S, Frankel E, German J (1994) Antioxidant activity of α- and γ-tocopherols in bulk oils and in oil-in-water emulsions. J Agric Food Chem 42:2108–2114
24. Kitryté V, Adams A, Venskutonis PR, De Kimpea N (2012) Impact of lipid oxidation-derived aldehydes and ascorbic acid on the antioxidant activity of model melanoidins. Food Chem 135:1273–1283
25. Samaranayaka A, Li-Chan E (2011) Food-derived peptidic antioxidants: a review of their production, assessment, and potential applications. J Funct Foods 3:229–254
26. Kim S, Kim Y, Byun H, Nam K, Joo D, Shahidi F (2001) Isolation and characterization of antioxidative peptides from gelatin hydrolysate of Alaska pollack skin. J Agric Food Chem 49:1984–1989
27. Chen H, Muramoto K, Yamauchi F, Nokihara K (1996) Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. J Agric Food Chem 44:2619–2623
28. Barba de la Rosa A, Fomsgaard I, Laursen B, Mortensen A, Olvera-Martínez L, Silva-Sánchez C, Mendoza-Herrera A, González-Castañeda J, De León-Rodríguez A (2009) Amaranth (*Amaranthus hypochondriacus*) as an alternative crop for sustainable food production: phenolic acids and flavonoids with potential impact on its nutraceutical quality. J Cereal Sci 49:117–121