



Effect of extreme heat processing on the Moroccan Zantaz' honey antioxidant activities

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Abstract The effect of an extreme heat processing on Zantaz honey samples was studied using a panel of physicochemical parameters, antioxidant activities and FTIR-ATR spectroscopy. Honey samples were heated at 121 °C for 30 min and the heat processing effect was confirmed indirectly through the assessment of hydroxymethylfurfural content, for which the values increased significantly ($p < 0.01$), and diastase activity, which was totally absent after the thermal processing. Besides, the effects of the heat on the antioxidant activities were diverse. Indeed, while the ability to scavenge 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid cation radicals (ABTS^{•+}) and 2,2-diphenyl-1-picrylhydrazyl radicals was

enhanced ($p < 0.05$ only for ABTS^{•+}), after the heat processing, nitric oxide radicals scavenging activity was reduced drastically ($p < 0.01$). Regarding the chelating power, it was totally abolished following heating. Other activities showed no significant alteration. The initial values of antioxidant activities seem to be determinant in the changes occurring after the heat processing. Amongst the analysed parameters, following the heat processing, the honey colour was the variable where the influence of baseline values was the greatest. The spectral analyses confirmed that FTIR-ATR is a useful technique to discriminate the chemical differences occurring in honey after heat processing.

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Introduction

The most important factors influencing honey marketing are those that are easily evaluated by customers, including rheology (the ability of honey to crystallize), colour and shelf-lifetime. Having those characteristics into consideration, honey heating used to be a common practice to reach the desired rheological properties thereby avoiding the crystallization process as well as to increase the shelf-lifetime by elimination possible contaminant microorganisms (Abu-Jdayil et al. 2002; Tosi et al. 2002).

In spite of the theoretical benefits of heating, it is important to mention that during thermal processing the composition of honey undergoes a series of changes that are linked to the interactions between its major (sugars) and minor (amino acids) compounds. Those reactions include the conversion of organic acids to alcohols or that of polyphenolic compounds into volatiles or even degradation of compounds like vitamins (da Silva et al. 2016). One of the reactions is the non-enzymatic browning, Maillard reaction, which in the ultimate stages, the degradation of Amadori products occur and generate Strecker aldehydes, pyrazines, pyrroles and furans, amongst others (Turkmen et al. 2006). The condensation of the generated active molecules with amino-acids form high molecular weight brown polymers known as melanoidins (Brudzynski and Miotto 2011a). Hydroxymethylfurfural (HMF) is considered to be the most important product and is therefore used for the confirmation of honey freshness when its values do not exceed 40 mg/kg (Turhan et al. 2008). In addition, in a recent review made by Shapla et al. (2018) about the influence of HMF levels present in honey on human health, the authors reported that it remains inconclusive because this compound has been described as exerting pernicious effects, such as mutagenic, genotoxic, organotoxic and enzyme inhibitory activities; but also beneficial effects including antioxidant, anti-allergic, anti-inflammatory, anti-hyperuricemic, and anti-hypoxic activities.

Several studies analysed the thermal effects on honey by evaluating the antioxidant activities and proved that the content of melanoidins correlated positively with honey antioxidant activity. The formation of melanoidins also explained the acquired activity after the heat processing (Brudzynski and Miotto 2011b, c). The observed increase depends on heat processing degree, and showed different behaviours towards different temperatures and heating times (Turkmen et al. 2006). Another influencing factor is honey botanic and geographical origin (Escuredo et al.

2019). In their studies about the effect of melanoidins formation on honey antioxidant activity after heat processing, Brudzynski and Miotto (2011c) observed that this phenomenon was more pronounced in light-coloured honey than in the dark-coloured samples.

While the increase in melanoidins' content is shown to increase the ability of honey to scavenge the free radicals used in the ORAC (Oxygen Radical Absorbance Capacity) assay (Brudzynski and Miotto 2011c), the degradation of other bioactive molecules may affect negatively the antioxidant ability toward other assays. Nagai et al. (2001) reported that the antioxidant activity of honey samples, determined using a linoleic acid-based lipid peroxidation assay, was drastically decreased following thermal treatment. The same authors linked the observed decrease to the decomposition of vitamins B1, B2, and C, and the destruction of catalase and peroxidase.

With the aim of better understanding heat effects on honey, we studied eight Zantaz honey samples produced in Morocco, mainly from *Bupleurum spinosum*. This honey was characterized in two previous contributions by the same group (Elamine et al. 2018, 2019). The collected samples have been characterized regarding their physico-chemical properties and antioxidant activities, before and after the heat processing at 121 °C during 30 min. To estimate the antioxidant activity it were used some of the most commonly employed assays like radical scavengers, metal-chelators and hydrogen donating agents (Brudzynski and Miotto 2011a). The physicochemical characteristics were studied using spectroscopic methods among which Fourier-transform infrared-attenuated total reflection, which has the advantage of being a non-destructive technique that only needs very small amounts of sample (Salvador et al. 2019).

Materials and methods

Reagents and instruments

Sodium phosphate, sodium hydroxide, and sodium carbonate were purchased from Pronalab, Madalena, Portugal; hydrochloric acid was provided by Fisher Scientific UK Ltd, Loughborough, UK; Acetic acid, Ascorbic acid, Folin–Ciocalteu, fuchsin, potassium hydroxide, methanol, and 2-propanol were provided by Merck KGaA products, Darmstadt, Germany; 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ferrozine, ferrous sulfate, gallic acid, nitroblue tetrazolium (NBT), ninhydrine, phenazine methosulfate (PMS), and proline were purchased from Acros Organics, New Jersey, USA; aluminium chloride and acetic anhydride provided by Panreac Química, MontcadaReixac, Barcelona, Spain; catechin was

purchased from Fluka. Biochemika. Sigma-Aldrich. Steinheim, Germany; sulfuric acid, sodium acetate, starch, potassium iodide, iodine, and sodium nitroprusside were obtained from Riedel-de-Haen, Germany; microplate reader (Tecan Infinite M200, Tecan, Austria); analytical balance (Shimadzu, Aux 220, Philippines); electric furnace (Cassel, Portugal); Abbe Refractometer (HANNA, HI968601, Romania); High Performance Liquid Chromatography (Hitachi, LaChrom Elite, Japan); Optic microscope (Leitz Messtechnik GmbH, Wetzlar, Germany); Alpha FTIR-ATR spectrometer (Bruker, Germany); Shimadzu 160-UV spectrophotometer (Naab, Tamame, & Caccavari).

Sample preparation

In this study, eight monofloral honey samples (classified as monofloral according the methodology proposed by Elamine et al. (2019) purchased from local Moroccan beekeepers were used. Each honey sample was divided into two aliquots: half was kept at room temperature and studied within 1 week ($n = 8$); and half were heat processed at 121 °C for 30 min ($n = 8$). Both raw and heat-treated samples were prepared in distilled water (50%, w/v) and centrifuged at 5000g, 20 min. The temperature, the heating period as well as preparation of samples were selected based on a previous work claiming that the conditions were sufficient to increase the degree of browning in both light- and medium-coloured honeys (Brudzynski and Miotto 2011b). All measurements were performed in triplicate and the results were presented as mean \pm SD (standard deviation).

Pollen analysis

The qualitative and quantitative analysis of pollen spectrum was accomplished following the International Commission for Bee Botany (ICBB) method, as described previously (Louveau et al. 1978). An optic microscope with 400 \times and 1000 \times objectives, was used for pollen identification and count. One thousand pollen grains were counted for each sample, and the frequent classes were determined twice. A pollen species was considered dominant, when it represents more than 45% from the pollen spectrum, secondary with percentage between 16 and 45%, important minor pollen from 3 to 15% and minor pollen when it is less than 3%.

Physicochemical parameters

Preliminary characterization

Moisture, diastase activity, HMF and proline content were assessed following the Harmonized Methods of the International Honey Commission (Bogdanov et al. 2009).

Colour, and melanoidins estimation

Colour and melanoidins estimations were determined by measuring the absorbance of aqueous solutions (10 g of honey in 20 mL of distilled water) at three wavelengths by using Shimadzu 160-UV spectrophotometer. The mm Pfund values of honey samples were obtained using the following algorithm: mm Pfund = $-38.7 + 371.39 \times A_{635}$ (Naab et al. 2008). Melanoidins content was estimated based on the browning index (net absorbance = $A_{450} - A_{720}$) (Brudzynski and Miotto 2011c). Spectrophotometric measurements were performed in a 1 cm quartz cell; results were expressed as absorbance units (AU).

Polyphenols, flavonoids and antioxidant activities

Polyphenols

The total polyphenolic content (TPC) was determined in honey solutions using the Folin–Ciocalteu method (Singleton and Rossi 1965). To eliminate a possible interference of sugars in the assay, sugar solutions were made considering the sugar profile of each 50% honey sample solution (w/v), used for the estimation of polyphenols content. The assay was made for both honey and sugar solutions, and the results were expressed as mean of gallic acid equivalent (GAE) mg \pm SD (standard deviation)/100 g honey, after the elimination of sugar interference. This interference was eliminated as follows: Abs = Abs (H) – Abs (S), where Abs is the absorbance considered for the calculation of the polyphenolic content; Abs (H) is the absorbance of the honey solution and the Abs (S) is the absorbance of the sugar solution.

Flavonoids

The total flavonoid content (TFC) of different honey samples was determined using the aluminium chloride assay according to Samatha et al. (2012). To study a possible interference of sugars in this assay the sugar solution made for polyphenols content estimation was also used. To avoid possible interaction of sample colour with the reaction's reagents, the blank was constituted by water (substituting the other components of the reaction) plus the sample as reported by Sancho et al. (2016). The resulting

absorbance was subtracted from the whole reaction absorbance before TFC estimation. The TFC was expressed in mg of catechin equivalents (CE) per 100 g of honey as the mean of three triplicate \pm SD.

Total antioxidant activity by ammonium molybdate reduction method

Total antioxidant activity (TAA) was measured in different concentrations of samples according to the described by Prieto et al. (1999). Honey solution was mixed with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). After 90 min incubation at 95 °C, the samples were cooled at room temperature and their absorbance values were measured at 695 nm in Shimadzu 160-UV spectrophotometer. The antioxidant activity was expressed as the number of ascorbic acid equivalents (AAE)/100 g of honey.

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity

Fifty microliters of each sample at different concentrations was placed in a cuvette, and 2 mL of 60 μ M methanolic solution of DPPH was added (Brand-Williams et al. 1995). After 60 min incubation at room temperature, absorbance measurements were made at 517 nm using Shimadzu 160-UV spectrophotometer (Tokyo, Japan). The percentage of inhibition of free radicals was calculated following the formula: $[(A_0 - A_1/A_0) \times 100]$. A_0 is the absorbance of the negative control (blank sample containing the same amount of water and DPPH solution); A_1 is the absorbance of the sample. The IC_{50} was then determined from the resulting concentration-inhibition percentage plot, as honey concentration inhibiting 50% of the generated free radicals and was expressed as mg/mL.

ABTS⁺ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) free radical scavenging activity

The determination of ABTS⁺ radical scavenging capacity was carried out as reported previously (Miguel et al. 2010). The reaction of ABTS⁺ aqueous solution (7 mM) with potassium persulfate ($K_2S_2O_8$) (2.45 mM) was done in the dark, for 16 h to generate ABTS⁺ free radical. The absorbance of the resulted solution was adjusted to 0.7 at 734 nm. Twenty-five μ L of honey dilutions were added to 275 μ L ABTS⁺ and the absorbance was read at 734 nm, after 6 min incubation. The IC_{50} values were calculated from three replications and the results were expressed as mean \pm SD (mg/mL).

Reducing power

The reducing power of honey samples was evaluated as described previously (Chu et al. 2000). In brief, honey solution in phosphate buffer (0.2 M, pH 6.6) was added to 0.5 mL of potassium ferricyanide (1%), and the mixture was incubated at 50 °C for 20 min. After the incubation trichloroacetic acid solution (TCA) (10%) was added to the mixture. A centrifugation at 600g was applied for 10 min, and the supernatant was mixed with H₂O and ferric chloride solution (0.1%) for 10 min. The absorbance was measured at 700 nm. Results were expressed as the absorbance of the reaction at the highest honey concentration.

Chelating metal ions

The chelating ability of honey samples against ferrous ions was evaluated as previously described in detail (Miguel et al. 2010). Briefly, honey solutions [different dilutions starting from 50% (w/v) solution] were incubated with 50 μ L of iron(II) chloride, $FeCl_2$ (2 mM). The reaction was initiated by the addition of 200 μ L ferrozine 5 mM, and after 10 min, the absorbance at 562 nm was measured. The IC_{50} values were calculated and expressed as mean \pm SD (mg/mL) of three replicates.

Nitric oxide (NO) scavenging activity

The protocol was initiated by generating NO radicals from sodium nitroprusside (SNP) ($Na_2[Fe(CN)_5NO]$) and was measured by using the Griess reagent (*N*-(1-Naphthyl) ethylenediamine) (Ho et al. 2010). Briefly, 50 μ L of serially diluted honey solutions were added to 50 μ L of 10 mM SNP in phosphate buffer saline (PBS) into a 96-well plate, and incubated at room temperature for 90 min. Finally, the remaining free fraction of NO radicals was revealed by adding 50 μ L Griess reagent and absorbance was measured at 546 nm. The IC_{50} was calculated with slight different of what was explained in DPPH section: $[1 - (A_{\text{sample}} - A_{\text{sample blank}})/(A_{\text{control}} - A_{\text{control blank}})] \times 100$, where $(A_{\text{sample}} - A_{\text{sample blank}})$ is the difference in the absorbance of a sample, with or without 10 mM SNP, and $(A_{\text{control}} - A_{\text{control blank}})$ is the difference in the absorbance of the PBS control, with or without 10 mM SNP.

Superoxide anion radical ($O_2^{\cdot-}$) scavenging activity (non-enzymatic method)

Superoxide anion radical $O_2^{\cdot-}$ ions were generated in a non-enzymatic phenazine methosulfatenicotinamide adenine dinucleotide (PMS-NADH) system based on the oxidation

of NADH and assayed by reduction of nitroblue tetrazolium (NBT) (Albano and Miguel 2011). Honey solutions (25 μ L) were mixed with 25 μ L nitroblue tetrazolium (0.42 mg/mL) and 25 μ L NADH (1.32 mg/mL) and H₂O (125 μ L) followed by 25 μ L phenazine methosulfate (PMS) (0.25 mg/mL). The percentage inhibition was calculated by the formula given below: $[(A_0 - A_1/A_0) \times 100]$ in which A_1 and A_0 are absorbances of solvent with and without sample, respectively. The results were expressed as the mean \pm SD (mg/mL) of three IC₅₀ values (replicates).

FTIR-ATR (Fourier-transform infrared-attenuated total reflection) analysis

Honey samples were also analysed with FTIR-ATR spectroscopy. The spectral information was acquired in accordance with the procedure previously reported (Anjos et al. 2015) with Bruker spectrometer (Alpha) using a diamond single reflection attenuated total reflectance device. For spectra for different aliquot of the same samples were obtained with 32 scans per spectrum at a spectral resolution of 4 cm⁻¹ in the wavenumber range from 4000 to 400 cm⁻¹. Prior analysis several pre-treated were tested namely: standard normal variate (SNV), multiplicative scatter correction (MSC), normalize, centred and scaled, first derived Savitzky–Golay (1st derived), second derived Savitzky–Golay (2nd derived), baseline and several combinations of 2 pre-treatments, to identify the better one to use in the further analysis.

Statistical analysis

To compare the means of assessed parameters before and after the heat processing, paired student t test was used. The difference was accepted to be statistically significant with $p < 0.05$. The data processing was done on IBM SPSS Statistics 25 software.

Principal component analyses (PCA) with spectral information were performed using the Unscrambler® X, version: 10.5.46461.632 (CAMO Software AS, Oslo, Norway).

Results and discussion

Effect of heat processing on the physicochemical parameters

The application of thermal treatment on honey samples has been reported to affect highly its quality and it is proven that the kinetic of this process depends on the degree and

heating time (Turhan et al. 2008). In this study, honey samples were heated at 121 °C for 30 min and the heat processing effect was confirmed indirectly through the assessment of a panel of physicochemical parameters including hydroxymethylfurfural content (HMF) and diastase activity (Table 1). The heat processing, in all the analysed samples, significantly increased ($p < 0.01$) the HMF content and the mean values changed from 7.70 ± 5.82 to 20.11 ± 11.91 mg/kg. Both values are below 40 mg/kg which is the maximum allowed by the Codex Alimentarius for the HMF content in honey (Codex Alimentarius Commission 2001). In spite of that, the HMF content increased around 55% with the thermal processing what is a very significant quick increase. (Turhan et al. 2008) reported the same pattern in floral honey submitted to a similar treatment (100 °C for 30 min) and suggested that the increase in the HMF content is more likely to be sensitive to primitive storage conditions rather than to overheating.

As expected, the use of decomposition heat processing by Tosi et al. (2008) induced a drastic decrease in the diastase activity: the recorded value changed from 21.31 ± 5.94 shade unit/g in raw samples to values below the detection limits following processing. Theoretically, a process of proteins' decomposition induces an increase in the free fraction of amino acids, including the proline. In contrast, the presence of a digestion step (sulphuric acid and thermal treatment) in the process of quantifying the proline content suppresses the ability to see this increase. On the contrary, a decrease was seen in the proline content of the analysed samples after the heat processing, yet this result was not significant (Table 1).

In the present study, HMF, proline, diastase activity, honey colour, melanoidins, polyphenols and flavonoids contents were assessed. All these parameters changed after honeys' heating, although statistical significance was not always reached. The honey colour increased 38% in mm pfund while the melanoidins' content increased by 28%. Both parameters are directly linked to the core of this study, as they provide a tool to assess the formation of the brown pigments of the Millard reaction, known to occur in honey following a thermal treatment (Wang et al. 2011). Both parameters showed a highly significant increase ($p < 0.001$), and their values increase from 60.94 ± 30.67 to 83.92 ± 29.98 pfund for the colours and from 0.98 ± 0.38 to 1.25 ± 0.44 for melanoidins content.

It has been reported that the polyphenols and flavonoids contents are involved in the antioxidant activities of honey (Brudzynski and Miotto 2011a). Therefore, these compounds were assessed as quick predictive indicators of the changes occurring in antioxidant activities', either lost or acquired. It was observed, although, that the polyphenols' contents did not vary significantly with the heat treatment.

Table 1 The physicochemical characterization and the antioxidant activities of honey sample before and after heat processing

Parameter	Raw honey samples		After heat processing	
	Mean \pm SD	Range	Mean	Range
Proline (mg/kg)	817.10 \pm 104.59	709.95–1007.49	773.65 \pm 141.68	593.63–994.15
HMF (mg/kg)	7.70 \pm 5.82	1.07–15.97	20.10 \pm 11.91	8.73–38.57
Diastase (Shade number)	21.31 \pm 5.94	11.33–30.30	–	–
Colour (mm pfund)	60.94 \pm 30.67	28.46–116.91	83.92 \pm 29.98	54.75–131.58
Melanoidins	0.98 \pm 0.38	0.56–1.55	1.25 \pm 0.44	0.74–1.88
Water content (%)	18.97 \pm 1.25	17.27–20.40	18.35 \pm 0.77	17.27–19.13
Polyphenol (mg GAE/100 g)	96.05 \pm 26.55	63.44–130.04	96.78 \pm 25.36	67.27–132.61
Flavonoids mg CE/100 g	23.70 \pm 4.89	17.12–29.32	20.44 \pm 3.41	14.52–25.59
TAA (mg EAA/g Honey)	370.44 \pm 51.97	278.53–442.68	377.25 \pm 48.54	294.60–433.12
DPPH (IC ₅₀ mg/mL)	15.34 \pm 7.55	7.94–27.13	12.70 \pm 4.61	6.45–19.69
ABTS ^{•+} IC ₅₀ (mg/mL)	7.00 \pm 2.79	4.20–12.18	5.60 \pm 2.10	3.53–9.26
Chelating Power IC ₅₀ (mg/mL)	46.11 \pm 42.39	7.98–132.19	–	–
Reducing power (Max Abs)	1.77 \pm 0.40	1.29–2.34	1.48 \pm 0.17	1.21–1.74
Superoxide (IC ₅₀ mg/mL)	50.91 \pm 28.30	15.02–88.43	50.88 \pm 22.81	24.05–85.90
Nitric oxide (IC ₅₀ mg/mL)	125.89 \pm 39.28	81.98–189.60	161.45 \pm 43.37	110.81–222.83

–, without activity

Only the flavonoids content decreased significantly ($p < 0.01$) after the thermal treatment (around 14%). A significant decrease in the content of some flavonoids and polyphenolic acids had been reported ($p < 0.05$) following the industrial pasteurization process (Escriche et al. 2014).

Effect of thermal treatment on the antioxidant activities

The effect of heat processing on the antioxidant activities is also presented in Table 1. Results are expressed as mean of all analysed samples for a given parameter, and the difference between the means was evaluated using a student *t* test.

The antioxidant activity assessed through the DPPH methodology increased after the thermal treatment, with a decrease of the IC₅₀ values from 15.34 \pm 7.55 to 12.70 \pm 4.61 mg/mL (Table 1). Even though this increase was not significant in the present study, the same behaviour was reported previously in heather honey heated at 95 °C for 5 min (Kowalski 2013). The same authors reported that the increase was more pronounced in acacia honey samples ($p < 0.05$), suggesting that the change in the antioxidant activity following thermal treatment is influenced by the botanic origin of honey samples.

Similar results were obtained for the ABTS^{•+} antioxidant assay, where an increasing of 20% in the activity was observed after the heat processing. Still, in contrast to the registered in the DPPH assay, this difference was

significant ($p < 0.05$). Both assays are used to evaluate the free radical scavenging capacity, but the ABTS^{•+} is more likely to be used for honey samples since aqueous solutions were used and the DPPH assay is strongly influenced by the solvent whereas the ABTS^{•+} method can be used in hydrophilic and lipophilic samples (Magalhães et al. 2008).

The heat processing applied in this study could abolish the ability of samples to chelate the iron ions. Indeed, following the chelating power experimental procedure the IC₅₀ of the thermally treated samples could not be determined, in opposite to what occurred with raw fresh samples where IC₅₀ was of 46.11 \pm 42.39 mg/mL. Organic acid and flavonoids are known to possess metal chelating activity (Chua et al. 2013). Therefore, the observed decrease in flavonoids content along with a possible deterioration of organic acids (not determined in the present work) may have an influence in the observed effect on iron chelating power after the thermal treatment. Previous study on Zantaz honey reported a correlation between chelating activity from one side and proline and diastase activity from the other side (Elamine et al. 2018). This relationship can be seen in the present contribution as both proline and diastase activity was seen after the heat processing.

The ability of honey samples to reduce the ferric ions was another activity that was negatively affected by the heat processing, reaching a value of 1.48 \pm 0.17 (absorbance of the highest used concentration) compared to 1.77 \pm 0.40 of the raw samples (Table 1); although, this difference did not achieve the level of significance (Table 1).

Table 2 Correlation matrix between baseline values and the percentage of change following heat processing

	Proline %	HMF %	Colour %	Melanoidins %	Moisture %	Polyphenols %	Flavonoids %	TAA %	DPPH %	ABTS ⁺ %	Reducing power %	Superoxide %	Nitric oxide %
Proline	0.531	0.22	-0.291	-0.21	-0.543	-0.08	-0.146	-0.603	0.177	-0.495	0.161	-0.24	0.452
HMF	-0.536	-0.7	0.736*	0.472	0.513	-0.013	0.498	0.688	-0.123	0.199	0.032	0.105	-0.28
Colour	0.587	0.591	-0.797*	-0.618	-0.782*	-0.02	-0.25	-0.414	0.376	0.141	0.155	-0.018	0.261
Melanoidins	0.548	0.579	-0.848**	-0.64	-0.708*	-0.215	-0.128	-0.313	0.465	0.147	0.281	0.16	0.346
Moisture	0.871**	0.398	-0.584	-0.244	-0.970**	0.286	-0.49	-0.482	0.452	-0.157	0.215	-0.481	0.331
polyphenols	0.459	0.417	-0.865**	-0.692	-0.625	-0.423	-0.076	-0.24	0.602	0.15	0.419	0.246	0.452
Flavonoids	0.818*	0.568	-0.684	-0.326	-0.867**	0.222	-0.591	-0.554	0.382	-0.113	0.094	-0.378	0.248
DPPH	-0.599	-0.43	0.907**	0.478	0.669	0.324	0.072	-0.023	-0.754*	-0.195	-0.606	-0.254	-0.493
ABTS ⁺⁺	-0.667	-0.345	0.749*	0.353	0.791*	0.105	0.156	-0.082	-0.754*	-0.288	-0.507	-0.047	-0.311
Chelating power	0.027	0.425	-0.242	-0.536	-0.286	0.073	0.138	-0.498	-0.318	0.045	-0.288	0.07	-0.012
Reducing power	-0.315	0.379	0.29	-0.072	0.337	0.298	-0.22	-0.569	-0.848**	-0.104	-0.890**	-0.107	-0.544
Superoxide	0.047	-0.423	0.794*	0.591	0.008	0.61	-0.212	-0.337	-0.455	-0.555	-0.387	-0.796*	-0.183
Nitric Oxide	-0.376	-0.445	0.769*	0.481	0.38	0.531	-0.343	0.093	-0.438	0.12	-0.614	-0.52	-0.695

*Correlation is significant at the $p < 0.05$ level; **correlation is significant at the $p < 0.01$ level

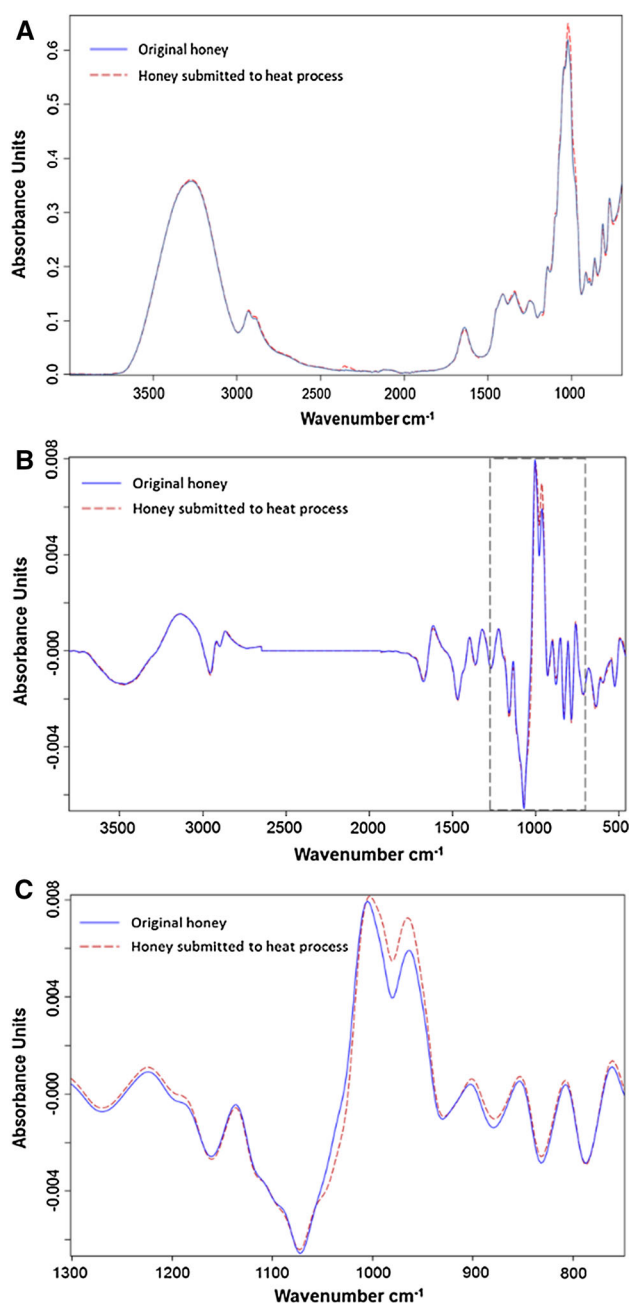


Fig. 1 FTIR-ATR spectra for honey samples with and without heating. **a** Average spectra obtained by FTIR-ATR, for honey with and without heating, **b** processed FTIR-ATR (all spectra), **c** processed FTIR-ATR (region used in the analysis)

The heat processing negatively affected ($p < 0.01$), the ability of Zantaz honey samples to scavenge the NO free radicals. The corresponding IC_{50} values increased from 125.89 ± 39.28 (in the raw unprocessed samples) to 161.45 ± 43.37 mg/mL (in honey samples submitted to heating). Such important decrease of the NO scavenging assay could be explained by the reduction of flavonoids content as a strong correlation between the two parameters

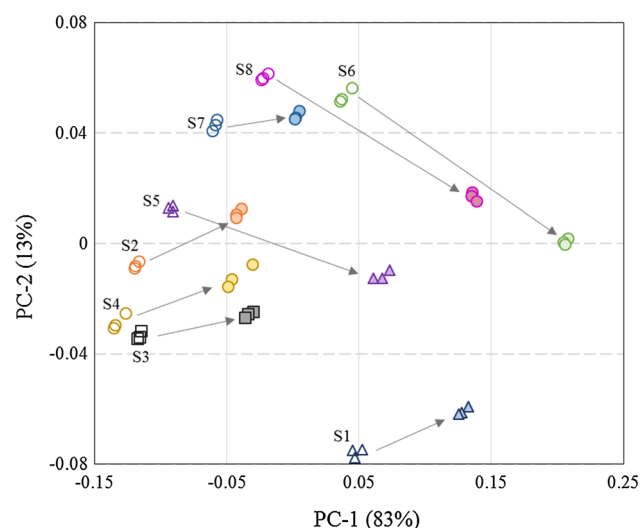


Fig. 2 Score plot of two principal components from raw spectral data acquire with FTIR-ATR data of honey based on: **a** marks without fill correspond to honey samples without heat processing, **b** marks filled belong to samples after heat processing from 800 to 1250 cm^{-1}

was already reported in honey (Dor and Mahomoodally 2014).

As for the superoxide activity, no significant changes were observed and the IC_{50} values were 50.91 ± 28.30 mg/mL before the heat processing and 50.88 ± 22.81 mg/mL after the heating process. The stability seen in this antioxidant assay suggests that the responsible compounds, did not suffer the consequences of the heat treatment. Polyphenols evaluated by the Folin–Ciocalteu method remained also constant after heat processing which may be an explanation to the behaviour of superoxide activity. It is worth mentioning that high polyphenolic contents in honey are linked to high superoxide scavenging activity (Uthurry et al. 2011). In Manuka honey, the superoxide activity was due mostly to the presence of one phenolic compound, methyl syringate (Inoue et al. 2005).

Table 2 illustrates the correlation matrix between the initial values obtained for the assessed parameters and the percentage of the changes within the same parameters following heat processing. The honey colour seems to be the most affected parameter, as a negative correlation could be seen between honey colour changes after heat processing, from one side; and the baseline values of colour ($p < 0.05$), melanoidins ($p < 0.01$), polyphenols ($p < 0.01$), and HMF ($p < 0.05$), from the other side. In addition, the same parameter had also a positive correlation with the IC_{50} obtained with DPPH ($p < 0.01$), ABTS $^{+}$ ($p < 0.05$), superoxide ($p < 0.05$) and nitric oxide ($p < 0.05$) antioxidant activities.

Other feature that could be observed in the correlation matrix is the effect of the initial antioxidant activity and the

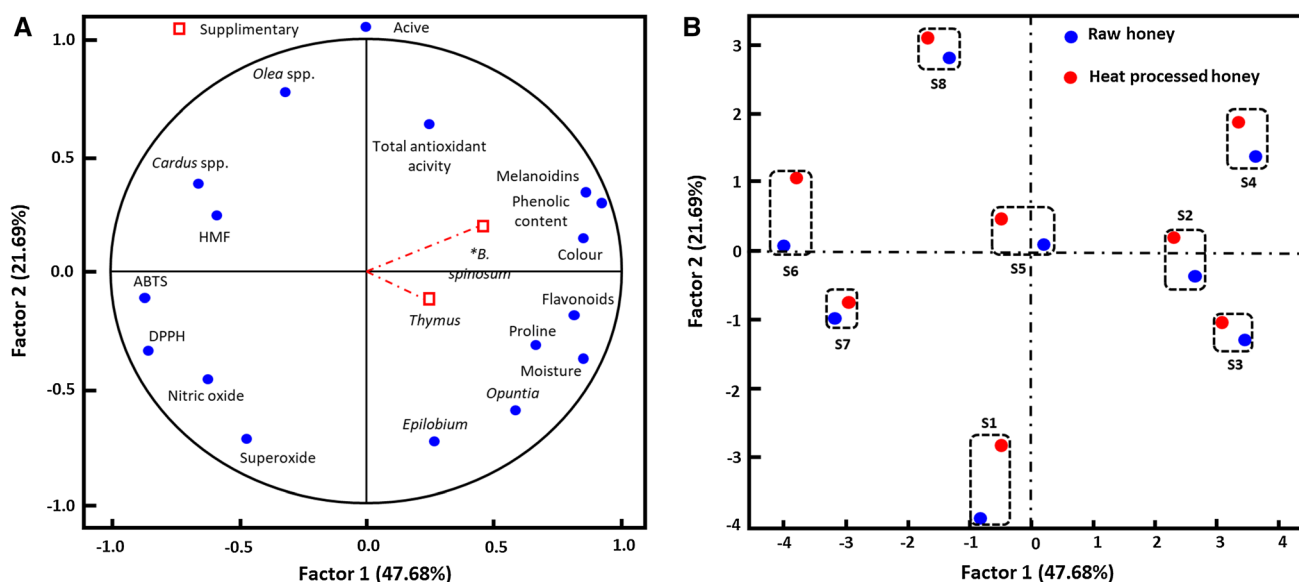


Fig. 3 Plot of two principal components from all analysed parameter (a) and scores (b) processing. Each rectangle refers to one honey sample before and after heat processing

resulting changes in such activity following heat processing. Negative correlations were obtained in the case of DPPH ($p < 0.05$), reducing power ($p < 0.01$) and superoxide ($p < 0.05$) antioxidant activities.

Higher percentages of colour changes were observed on honeys that were lighter prior heating. Similarly, higher colour changes occurred following heating in samples with baseline lower amounts of melanoidins and polyphenols, as well as lower capacity for scavenging DPPH, ABTS⁺, superoxide and NO free radicals. Changes in the chemical and antioxidant attributes of honey after heating had already been reported (Nagai et al. 2001; Brudzynski and Miotto 2011a). The results of the present work suggest that heating lighter honey, honey with lower amounts of polyphenols, or with lower antioxidant activity induces more accentuated changes than those observed in darker honeys or those already possessing higher amounts of polyphenols or higher antioxidant activity.

Effect of heating evaluated by FTIR-ATR analysis

The spectra obtained for the honey samples, represented in Fig. 1a, are similar to those obtained by other authors (de Ribeiro et al. 2014; Anjos et al. 2015).

The spectra were considered adequate for analysis when the difference between spectra for a particular sample was lower than 0.002 for 10 spectra. First derived Savitzky–Golay (1st derived) was used as pre-processed in the region between 1300 and 800 cm⁻¹ (Fig. 1b, c). The validation method used was the cross-validation method. The criteria used to classify the samples was a ratio of calibrated to validated residual variance of 0.5; ratio of validated to

calibrated residual variance of 0.75 and residual variance increase limit of 6%.

In a first screening, a PCA with all spectral regions (from 4000 to 400 cm⁻¹) was performed. However, better results were obtained when the region from 800 to 1250 cm⁻¹ was used as shown in the Fig. 2. In this case, it was observed an important spectral resolution in relation to the heating process that was explained by the PC-1 (83% of the total variation). Furthermore, in Fig. 2, it is also observed a different behaviour for the different species, that could be explain by the effect of the botanical origin as explained before. The honey samples are all classified as Zantaz honey, but the secondary nectar plays also an important influence.

In order to better understand the influence of the botanical origin and the importance of the antioxidant activities in the differentiation of honey samples, a second PCA was performed with the data collected in this work and the secondary bee pollen observed in the honey samples (between 15 and 45%) (Fig. 3).

In the Fig. 3a it is possible to understand the effect of presence of the secondary pollen grains namely *Cardus* spp., *Olea* spp. and *Opuntia* spp. in the antioxidant activity of different honey samples. The quantity of pollen of *B. spinosum* does not show to have principal role in discriminating honey samples. although samples located in the same direction as it does, showed to move to the right considering the first factor. Those samples are S2, S3, S4, S5 and S8. In contrast honey samples S1, S4 and S7 moved to the opposite direction. This suggest that *B. spinosum* pollen is important in determining a honey samples behaviour after heat processing. The scores plot (Fig. 3b) show

similar results to those observed in the Fig. 2. There are differences between the samples after and before heating process; however, this variation was explained by the Factor 2 that explained only 21.7% of the total variance. Therefore, the spectral variance observed is influenced by the degradation of the polyphenolic compounds but also related to the degradation of sugar by the Maillard reaction, also well explained by Brudzynski and Miotto (2011a).

Conclusion

The existence of different tendencies amongst the assessed antioxidant activities following the thermal treatment suggests that each one is governed by distinct molecule or group of molecules. Additionally, radicals without homologies in biological systems, namely DPPH and ABTS⁺, were increased by the heat processing. On the contrary, the capacity for scavenging NO radicals was reduced after heating honey samples; this may indicate that the ability of heated honey to scavenge this important mediator of the inflammation is reduced. Furthermore, the chelating ability of honey samples was totally removed by the heat processing. The antioxidant capacity of honey after heating is therefore also dependent on the method used for evaluating this property. Overall, it seems that heating Zantaz honey will not improve the antioxidant activity, at least when this biological property is assessed by the methodologies hereby reported. While a confirmation is needed for honeys with other floral origins, it may be important to avoid heat processing for samples destined for being applied in circumstances where the oxidative stress plays a role.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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