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Summary

Fortification of food products with iron is a common strategy to prevent or overcome iron deficiency. However, any form of iron is a pro-oxidant and its addition will cause off-flavours and reduce a product's shelf life. A highly bioavailable heme iron ingredient was selected to fortify a chocolate cream used to fill sandwich-type cookies. Two different strategies were assessed for avoiding the heme iron catalytic effect on lipid oxidation: ascorbyl palmitate addition and co-spray-drying of heme iron with calcium caseinate. Oxidation development and sensory acceptability were monitored in the cookies over one-year of storage at room temperature in the dark. The addition of ascorbyl palmitate provided protection against oxidation and loss of tocopherols and tocotrienols during cookies preparation. In general, ascorbyl palmitate, either alone or in combination with the co-spray-dried heme iron, prevented primary oxidation and hexanal formation during storage. The combination of both strategies resulted in cookies that were acceptable from a sensory point of view after 1 year of storage.

Keywords: Heme iron / Food fortification /Co-spray-drying/ Antioxidants / Oxidative stability

List of abbreviations used: AP, ascorbyl palmitate; AUC, area under the curve; CAS, calcium caseinate; CAS 1:1, co-spray-dried heme iron ingredient with calcium caseinate in 1:1 ratio; CHP eq., cumene hydroperoxide equivalents; CIE, Commission International de L'Eclairage; FA, fatty acid; FOX, ferrous oxidation-xylenol orange; LHP, lipid hydroperoxide; MAXLHP, maximum lipid hydroperoxide; MUFA, monounsaturated fatty acids; *p*-AnV, *p*-Anisidine value; PUFA, polyunsaturated fatty acids; PV, peroxide value; SFA, saturated fatty acids; TMAX, time to reach the maximum lipid hydroperoxide value.

1. Introduction

Iron deficiency is the most prevalent health disorder in the world (WHO, 2001), and it is estimated that at least 20% of the world's population is affected by this micronutrient deficiency. Moreover, iron deficiency is prevalent in both developed and developing countries (Benoist, McLean, Cogswell, Egli, & Wojdyla, 2008).

Food fortification is generally recognised as a good strategy to counteract this nutritional deficiency; there is a large body of evidence indicating its efficacy, and it is regarded as the most cost-effective long-term strategy (Baltussen, Knai, & Sharan, 2004; Zimmermann & Hurrell, 2007). For an iron fortification programme to be effective, it is essential that the form of iron selected be highly bioavailable. At the same time, organoleptic modifications of the food vehicle should be minimal. This presents a challenge for the food industry, as many iron forms are potent pro-oxidants (Lotfi, Mannar, Merx, Naber-van, & Heuvel, 1996). In general, the more bioavailable non-heme iron compounds are also the most reactive ones (Allen, De Benoist, Dary, & Hurrel, 2006).

There are two main forms of dietary iron, namely heme and non-heme iron. Heme iron is known to be the most bioavailable iron form, since its absorption process is different from that of non-heme iron and it is not affected by food ligands (WHO/FAO, 2004).

Selection of the food vehicle should take the target population's diet into account (Allen et al., 2006). If the entire population presents iron deficiency, staple foods such as wheat flour or rice would be suitable food vehicles for iron fortification. However, such mass fortification may be insufficient for some women of childbearing age and children (Lotfi et al., 1996).

Those population groups have increased iron needs, mainly due to growth or menstruation, but their total food intake is lower than other population groups and hence the intake of iron-fortified staple foods may not meet their iron requirements. Thus, targeted fortification seems an optimum approach for these population groups.

Although the pro-oxidant effect of heme and non-heme iron in foods is well known, the oxidative stability of iron fortified foods during storage has been studied in a limited number of works (Asenjo et al., 1985; Bovell-Benjamin, Allen, Frankel, & Guinard, 1999; Hurrell, 1997; Mohammadi, Abedi, Azizi, Ahmadian, & Pouraram, 2011). In addition, to our knowledge, only Bovell-Benjamin, Allen, & Guinard, (1999) studied how lipid oxidation is affected by antioxidant addition in iron fortified foods. These authors observed that the addition of butylated hydroxyanisole in maize meal porridges fortified with non-heme iron prevented hexanal formation and improved sensory quality (Bovell-Benjamin, Allen, Frankel, et al., 1999; Bovell-Benjamin, Allen, & Guinard, 1999). The encapsulation of iron forms in order to decrease their reactivity is another approach to prevent oxidation in fortified foods (Zimmermann & Windhab, 2010). The addition of non-heme iron forms encapsulated with different coating materials has been studied in several foods and in some cases a decrease in lipid oxidation, compared to the non-encapsulated iron forms, has been observed (Abbasi & Azari, 2011; Gupta, Chawla, Arora, Tomar, & Singh, 2015; Jayalalitha, Balasundaram, Palanidoral, & Naresh Kumar, 2012; Kwak, Ju, Ahn, Ahn, & Lee, 2003; Kwak, Yang, & Ahn, 2003). However, to our knowledge, apart from or previous work (Aleman et al., 2015) there are no studies in the literature dealing with the use of encapsulated heme iron in fortified foods.

In previous studies (Aleman et al., 2010, 2014, 2015) , different strategies were tested to avoid the oxidation of a palm oil fortified with heme iron as a model for bakery product

fortification. The strategies studied were: the addition of antioxidants and the encapsulation of heme iron by co-spray-drying it with different coatings. The effectiveness of several antioxidants at different concentrations was assessed in that model and ascorbyl palmitate (AP) at 400 mg/kg palm oil was found to be the most effective (Aleman et al., 2010, 2014). Subsequently, the same model was used to assess the additional advantages of co-spray-drying heme iron with calcium caseinate either at a 2:1 or a 1:1 ratio (heme iron concentrate:caseinate, w/w, dry weights). The authors reported that the combination of co-spray-dried heme iron with calcium caseinate and AP was the most effective strategy to prevent oxidation during storage (Alemán et al., 2015).

The aim of the present study is to assess the oxidative stability and overall acceptability of sandwich-type cookies filled with a chocolate cream fortified with heme iron. Not only would this food product be easy to introduce into children's diets but also the chocolate could help disguise the dark colour of the heme iron ingredients. In order to avoid oxidation, the previous strategies that proved effective in a model for iron fortification of bakery products were combined: the addition of AP at 400 mg/kg of palm oil in the cookie filling, and the co-spray-drying of heme iron with calcium caseinate at a 1:1 ratio (CAS 1:1). The evolution of oxidation and the overall acceptability of these cookies were monitored over a one-year period of storage at room temperature in the dark.

2. Material and methods

2.1. Materials

Refined palm oil was donated by Lípidos Santiga S.A. (Santa Perpètua de Mogoda, Spain), cocoa powder (11% fat, w/w) by Nutrexpa (Barcelona, Spain), lecithin by Cargill (Martorell,

Spain) and food grade AP (purity >98%, w/w) by Induxtra (Banyoles, Spain). Skim dry milk (1.5% fat, w/w) and vanilla extract were a gift from BDN S.L. (Barcelona, Spain). Calcium caseinate was purchased from Ferrer Alimentación S.A. (Barcelona, Spain). Commercial Marie biscuits and icing sugar were purchased in a supermarket. The aluminium-coated plastic ziplock bags used for packing the sandwich-type cookies were of very low permeability to water and oxygen (<0.01g of water/m^2/24 h with 90% of relative humidity at 33.7ºC and 0.1 cm^3 of oxygen/m^2/24 h/bar with 0% of relative humidity at 23ºC, respectively) and were purchased from Flexico (Barcelona, Spain). More details about the composition of the main materials used are provided in Supplementary data.

FA methyl ester standards (purity >98%, w/w), cumene hydroperoxide (80%, w/w) and hexanal (98%, w/w) were purchased from Sigma-Aldrich (Madrid, Spain), dimethyl sulphoxide (DMSO) was purchased from Scharlab S.L. (Barcelona, Spain) and tocopherol standards (>95%, w/w) were purchased from Calbiochem (San Diego, CA).

All chemicals used were of ACS grade, with the exception of the solvents used in the ferrous oxidation-xylenol orange (FOX) method, in the tocopherol and tocotrienol determination and in the heme iron determination, which were of HPLC grade. The iron standard solution was purchased from High-Purity standards (Charleston, SC) and was traceable to the Standard Reference Materials of NIST.

2.2. Manufacture and characterisation of heme iron ingredients

The heme iron ingredient used as control was the commercial product AproFER 1000™ obtained from APC Europe S.A. (Granollers, Spain). The heme iron co-spray-dried ingredient, namely CAS 1:1 (heme iron concentrate:calcium caseinate, 1:1, w/w, dry weights) was

produced by APC Europe (as described in the Supplementary data). Colour evolution of heme iron ingredients during storage was measured using a Konica Minolta Chroma-meter (model CR-410; Konica Minolta Sensing Inc., Osaka, Japan) based on the CIE L*a*b* colour space. Water absorption and water solubility indexes were determined in the heme iron ingredients used in this study as described elsewhere (Zhang et al., 2012). The methods used for characterisation of the heme ingredients are provided in the Supplementary data.

2.3. Manufacture of sandwich-type cookies

Different chocolate creams with and without heme iron fortification were used to fill sandwich-type cookies according to the treatments described in Table 1. A manufacturer of this kind of cookies kindly provided a common formulation for chocolate creams, consisting of 54.3% icing sugar, 23.6% palm oil, 16.3% cocoa powder (11% fat), 5.6% skim dry milk (1.5% fat), 0.1% vanilla extract and 0.1% lecithin (percentages expressed as w/w).

The total iron content of the control heme iron ingredient (AproFER 1000™) was 1.74% (w/w) whereas the content of the co-spray-dried heme iron ingredient (CAS 1:1) was 0.88% (w/w). In order to maintain the quantity of iron in samples constant (0.31 mg Fe/g cream or 0.11 mg Fe/g cookie), different amounts of both heme iron ingredients were added to the chocolate cream fillings. These creams were prepared in our laboratory and then used to sandwich two commercial Marie biscuits together. The complete procedure for preparing the heme iron-fortified sandwich-type cookies is given in the Supplementary data.

The resulting sandwich-type cookies were stored at room temperature in the dark for 0, 90, 180 and 360 days. At the end of each storage period, the corresponding cookies were vacuum packed and stored at -20°C until performing analyses.

Our aim was for each chocolate-filled biscuit weighing 20 g with 7 g of chocolate filling to provide more than 15% of the recommended daily allowance of iron (i.e. 14 mg Fe/day) (European Comunities Commission, 2008). When the samples were analysed, the total iron was found on average to be 0.12 mg Fe/g cookie. Therefore, one of these cookies would provide 17% of the recommended daily allowance of iron.

2.4. Lipid extraction from cookies

The lipid fraction of cookies was extracted in order to characterise its tocopherol and tocotrienol content and to assess oxidation status at the different storage periods. The extraction procedure employed was adapted from Rose & Oklander (1965). Briefly, cookies were grinded and the lipids were extracted first with isopropanol/chloroform (3:2, v/v) and then with methanol/chloroform (1:2, v/v). This double extraction was used in order to avoid the presence of heme pigments in the lipid extract, since the pigments colour interferes in some of the subsequent analytical determinations carried out on the lipid extract. Further details of the lipid extraction procedure are given in the Supplementary data.

2.5. Palm oil characterisation and tocopherol and tocotrienol content of cookies prior to storage

Fatty acid (FA) methyl esters were prepared from 100 mg of palm oil as described elsewhere (Guardiola, Codony, Rafecas, Boatella, & López, 1994) and the chromatographic conditions were those described by Aleman et al., (2010). In addition, the peroxide value (PV) (European Communities Commission, 1991), lipid hydroperoxide (LHP) content (by non-induced FOX method, measured after exactly 30 min of incubation) (Navas et al., 2004), *p*-AnV (AOCS,

1998) and hexanal content (Aleman et al., 2014) were assessed in the palm oil used to prepare the chocolate filling. All the analyses were replicated five times.

Tocopherol and tocotrienol content was determined in 200 mg of fresh palm oil and in the same amount of lipids extracted from cookies prior to storage by normal-phase HPLC as described by Aleman et al., (2010). This analysis was conducted in triplicate. In the case of the cookies, three lipid extractions were conducted in order to replicate the analysis.

2.6. Heme and total iron content

The heme iron content of the heme iron ingredients and cookies prior to storage was determined after extraction (40 mg of ingredients or 1.5 g of cookies) with acidified acetone (acetone/hydrochloric acid/water, 80:2:18, v/v/v) according to the colourimetric method described by Hornsey (1956).

The total iron content of these samples was determined by inductively coupled plasma - atomic emission spectrometry (PerkinElmer, Optima 3200 RL model) following mineralisation. This was achieved as follows: first, 250 mg of either heme-iron ingredient or ground cookie was weighed into quartz digestion tubes. Second, 25 mL of HNO_3 was added and tubes were placed in a digestion block and left at 60°C overnight. Thereafter, the temperature was raised to 120°C and left to stand for 1 hour. Then, nitric acid was evaporated at 160°C until approximately 5 mL was left. Next, 5 mL of $HClO_4$ was poured into the quartz tube and the temperature was raised to 180°C. After one hour, $HClO_4$ was evaporated at 210°C until 1 mL was left. Finally, the solution was transferred to a 50 mL volumetric flask and filled up with HNO_3 1% (v/v). Two wavelengths were measured for Fe (238.204 and 259.939 nm). Instrumental measurement conditions for inductively coupled plasma - atomic emission

spectrometry are described in Supplementary data. Aqueous (1% HNO_3) calibration curves (intercept equal to 0) were used for total iron quantification and the selected wavelength was 259.939.

The heme and total iron were determined in quintuplicate (in five different samples, see Supplementary data).

2.7. Susceptibility to oxidation

Prior to storage of cookies, the induced version of the FOX method was performed to assess sample susceptibility to oxidation (Bou, Codony, Tres, Decker, & Guardiola, 2008; Grau, Codony, Rafecas, Barroeta, & Guardiola, 2000). The reaction conditions were those described in Navas et al. (2004). This assay measures the formation of LHP in the fat extracted from cookies during an incubation period that was set at 191 hours. By using a standard curve prepared with cumene hydroperoxide (CHP), the content of the LHP formed during incubation was expressed as millimoles of CHP eq/kg in each sample. The following parameters were determined as described elsewhere (Tres, Nuchi, Bou, Codony, & Guardiola, 2009) to better describe the differences observed in the susceptibility to oxidation of the samples: Initial LHP (the initial LHP value was measured after 30 minutes of incubation), MAXLHP (the maximum LHP value), TMAX (the time until MAXLHP was achieved), Final LHP (the final LHP value measured after 191 hours of incubation) and AUC (area under the curve of LHP formation). Susceptibility to oxidation was assessed in 5 different lipid extracts.

2.8. Evolution of oxidation during storage

The formation of primary oxidation compounds in cookies stored for different periods was assessed in the fat extracted using the PV (European Communities Commission, 1991) and by measuring the LHP content by means of the non-induced FOX method version (Navas et al., 2004). The formation of secondary oxidation compounds in samples was assessed by the *p*-anisidine value (*p*-AnV) (AOCS, 1998) and the hexanal content determined by static headspace analysis. This method was set up following a similar approach to that described in Aleman et al., (2014).

The extraction times and temperatures (30 minutes at 70°C) were the same as in previous studies (Alemán et al. 2014). However, to identify the best extraction conditions for the determination of hexanal content, we assessed different sample amounts (0.5-1.0-1.5 g) and the addition of water (0–0.5–1mL) using a Combi PAL autosampler (CTC Analytics, Switzerland). The final extraction conditions selected were as follows: 500 mg of ground cookie was weighed into 10-mL vials to which 1 mL of water was then added. The vials were sealed with screw caps. Extraction of the volatile compounds was carried out over 30 minutes at 70°C. Following this, one millilitre from the vial headspace was injected into an Agilent 4890D model (Waldbronn, Germany) gas chromatograph equipped with a flame ionisation detector and a split-splitless injector. The injector was set for 2 minutes in splitless mode; the split ratio was 1:10.

Chromatographic separation of volatile compounds was performed in a fused-silica capillary column (30 m × 0.20 mm i.d.) coated with 0.2 μm of a stationary phase of 5% diphenyl- plus 95% dimethyl-polysiloxane (Equity TM-5 from Supelco, USA). Helium, at 20 psi, was used as a carrier gas. The injector and detector temperatures were 200°C and 325°C, respectively. The

283 oven programme was as follows: 1 minute at a temperature of 40°C, which was then
284 increased by 10°C/min to 300°C and then kept at this temperature for 5 minutes.
285
286 Hexanal was identified by comparing the retention time with a standard and was quantified
287 using the external standard method. To prepare the standard curves, fresh ground cookies
288 (control, control heme and CAS 1:1) were used as matrixes. 100 µL of solutions with different
289 concentrations of hexanal in DMSO was added to the vials containing the ground cookies
290 (concentration range, 1.5-200 µg hexanal/kg sample) and sealed with screw caps. To ensure
291 that the samples and the vials of the standard curves had the same matrix, 100 µL of DMSO
292 was added to all treatment samples.
293
294 This method displayed a good linearity (R^2=0.991-0.994), a good precision (RSD = 3.04%, 4
295 determinations within the same day in a sample containing 32 µg hexanal/kg) and a good
296 recovery (99%). The recovery was assessed in 2 samples, which were each injected four
297 times. Sample hexanal concentrations were 32 µg/kg and 16 µg/kg. The hexanal standard
298 added was approximately one third of the initial concentration (final hexanal concentration
299 43 µg/kg and 22 µg/kg, respectively). Given the analyte content of samples (between not
300 detected - 180 µg/kg), these values comply with AOAC recommendations for validation of
301 methods (AOAC international, 1998). Furthermore, the limit of detection and quantification of
302 the method were 0.86 and 1.12 µg/kg of sample, respectively. Both limits were calculated as
303 3 and 10 times the standard deviation of the base line noise, respectively.
304
305 PV, LHP content and *p*-AnV were determined in the lipid extracts from cookies, whereas the
306 hexanal content was assessed in ground cookies. All the oxidation parameters were
307 determined in quintuplicate (in five different samples, see Supplementary data).
308

2.9. Chocolate creams filling colours

The colour of the chocolate cream fillings was measured prior to storage using a Konica Minolta Chroma-meter (model CR-410; Konica Minolta Sensing Inc., Osaka, Japan) based on the CIE L*a*b* colour space. To this end, sandwich -type cookies were heated for 5 seconds in a microwave. Then, biscuits were separated and the chocolate filling was carefully removed and transferred to a capsule.

The colour was determined from four different random surfaces of the chocolate creams. The instrument was set for illuminant D-65 and at a 2°-observer angle, and standardised using a standard white plate. In addition, the colour of the chocolate cream fillings was compared with commercial chocolate cream fillings and chocolate bars with different percentages of cocoa (33%, 72% and 85%, w/w).

2.10. Sensory analysis

Sensory analysis of the sandwich-type cookies was performed after 0, 180 and 360 days of storage. A panel of 32 volunteers assessed the overall acceptability of fortified cookies at each storage time using a 9-point hedonic scale, where 9 = excellent and 1 = very bad. Samples were served at room temperature and presented to panellists on coded white plastic plates. Each panellist tasted all the different cookies in each session. Spring water was provided for participants to rinse their palates between samples. Only volunteers who consumed chocolate bakery products at least 12 times per year were considered for participation. The selected panel was familiar with sensory analysis and included men and women aged between 18 and 63 years.

2.11. Statistical analyses

Pearson's correlation coefficients were used to study the relationships between PV, LHP content, *p*-AnV and hexanal content. Spearman's correlation coefficients were used to study the relationships between the parameters obtained by the induced FOX method and the oxidation values measured in cookies after 360 days of storage. One-way ANOVA was used to determine the effect of the different treatments on tocopherol and tocotrienol content and FOX-induced parameters. Multifactorial ANOVA was used to determine whether the studied factors had a significant effect on PV, LHP content, *p*-AnV and hexanal content of cookies. The main factors studied were the treatments applied (Table 1) and the storage time (0, 90, 180 and 360 days). In addition, a series of one-way ANOVAs was applied at each storage time to determine any significant effect of the treatments on PV, LHP content, *p*-AnV and hexanal content. In all cases, $P \leq 0.05$ was considered significant. When significant differences were produced by the main factors, the least-squares means or means were separated using Scheffé's test ($\alpha=0.05$).

3. Results and discussion

3.1. Characterisation of palm oil and heme iron ingredients

The FA composition of the palm oil used to prepare the chocolate cream filling was typical of this kind of oil (Tres, van der Veer, Alewijn, Kok, & van Ruth, 2011): 49.95% SFA, 39.74% MUFA, 10.51% PUFA and 0.08% *trans* FA (the complete FA composition is given in the Supplementary data). Palm oil is characterised by its high content of both tocopherol and tocotrienol, especially alpha- and gamma-tocotrienol (Sambanthamurthi, Sundram, & Tan,

2000; Tres et al., 2011). In this case, the total tocopherol and tocotrienol content was 673.9 mg/kg (total tocopherols: 218.6 mg/kg, total tocotrienols: 455.3 mg/kg) (Table 2).

Regarding oxidation status, the fresh palm oil had a PV and LHP content of 0.06±0.0001 meq O_2/kg oil and 0.17±0.005 mmol CHP eq/kg oil, respectively (n=5). Hexanal was not detected whereas the *p*-AnV was 2.30±0.36 (n=5).

The heme iron ingredients could be described as dark. Nevertheless, we found differences between the colour of the co-spray-dried heme iron and the control heme iron (see Supplementary data). In addition, both water absorption and solubility indexes of the co-spray-dried heme iron were higher than those of the control heme iron (see Supplementary data).

3.2. Tocopherol and tocotrienol content of fat extracted from cookies prior to storage

Table 2 shows the tocopherol and tocotrienol content of the lipids extracted from the different cookies prior to storage. The tocopherol and tocotrienol content of control cookies with heme iron was much lower than that of the control cookies without heme iron. Thus, heme iron addition induces a higher tocopherol and tocotrienol degradation during preparation of the chocolate fillings.

When comparing cookies, those fortified with heme iron without added AP (control heme iron and CAS 1:1) presented a lower tocopherol and tocotrienol content than the control cookies without heme iron (48% and 53%, respectively) and also than that of the samples with AP (AP and CAS 1:1 + AP cookies). Indeed, the tocopherol and tocotrienol content of cookies with AP did not differ from control cookies (without added heme iron). Therefore, the addition of

AP protected against the degradation of tocopherols and tocotrienols induced by the addition of heme iron during preparation of the chocolate fillings, which involves heating to 40°C for approximately 40 minutes. This result is in accordance with previous results in palm oil samples fortified with heme iron, where the addition of AP reduced tocopherol and tocotrienol loss during heating treatments (Aleman et al., 2015). However, it should be noted that samples from this previous study were used as a model for bakery products and were thus subjected to higher temperatures (220°C for 10 minutes) to mimic typical baking conditions.

The protective effect of the AP against tocopherol and tocotrienol oxidation has been previously reported in some foods and lipid models (Beddows, Jagait, & Kelly, 2001; Gordon & Kourimskb, 1995; Kancheva et al., 2014). However, the mechanism of AP to preserve and/or regenerate the tocopherols is not clear, and two hypotheses have been pointed out for explaining this behaviour (Beddows et al., 2001; Kancheva et al., 2014; Masson et al., 2002): (i) AP is more sensitive than α-tocopherol to radical attack; and (ii) AP has the ability of regenerating α-tocopherol by donating a hydrogen to the tocopheroxyl radicals formed during the oxidation. In addition, other antioxidant mechanisms ascribed to AP (Let, Jacobsen, & Meyer, 2007; Madhavi, Singhal, & Kulkarni, 1996; Márquez-Ruiz, Ruiz-Méndez, & Velasco, 2014), such as the oxygen scavenging and metal chelating, could contribute to tocopherol preservation.

3.3. Heme and total iron content

The control heme iron ingredient (AproFER 1000™) contained 1.74% (w/w) of iron, of which 97.3% was heme iron. The heme iron ingredient encapsulated by co-spray-drying (CAS 1:1) contained 0.88% (w/w) of iron, of which 91.8% was heme iron.

412

413 The heme and total iron content of samples was also assessed in cookies prior to storage. On
414 average, the fortified heme iron cookies contained 0.10 ± <0.01 mg heme iron/g of cookie and
415 0.12 ±<0.01 mg total iron/g cookie. Therefore, two cookies, which could be considered a
416 normal serving size, would provide on average 4.63±0.16 mg of total iron/serving (33% of the
417 recommended daily allowance of iron) (European Comunities Commission, 2008), with heme
418 iron as the main source (84-88%). Given that the total iron content of the non-fortified cookie
419 was 0.03 ±<0.01 mg iron/g cookie, negligible amounts of iron were liberated from the heme
420 moiety of the heme iron ingredient during the preparation of the chocolate cream filling.

421

422 **3.4. Susceptibility to oxidation of fortified cookies**

423

424 Susceptibility to oxidation of the lipids extracted from the different cookies was determined
425 by means of the induced FOX method prior to storage. Table 3 shows the different parameters
426 calculated according to (Tres et al., 2009) to better describe the time course of LHP evolution
427 during incubation.

428

429 The initial LHP value was considered to measure the current amount of LHP present in the
430 samples. The initial LHP content of cookies with added AP (AP and CAS 1:1 + AP treatments)
431 was significantly lower than that of the samples without added antioxidants (control, control
432 heme iron and CAS 1:1). The lowest initial LHP value was observed in the AP treatment, thus
433 suggesting a protective effect of AP during preparation of the chocolate creams fortified with
434 heme iron. This finding is in agreement with previous studies carried out on palm oil fortified
435 with heme iron (Aleman et al., 2010, 2014, 2015). As discussed earlier, these results can be
436 also related to the fact that AP effectively protected tocopherols and tocotrienols during
437 sample preparation.

438

439 Similar conclusions can be drawn when assessing the other parameters (MAXLHP, TMAX and
440 Final LHP, Table 3). The lowest MAXLHP corresponded to cookies with added AP (AP and CAS
441 1:1 + AP). Moreover, the AP treatment presented the highest TMAX (time to reach the
442 MAXLHP), 29.5 hours, whereas the rest of the samples reached the MAXLHP in 0.5 hours. The
443 Final LHP content of samples also followed a similar trend, with lowest values observed for
444 the AP treatment.

445

446 Lastly, the AUC, which has been considered a good marker for susceptibility to oxidation in
447 different matrixes (Aleman et al., 2014; Tres et al., 2009), showed the lowest values for AP
448 samples, which were even lower than those of control cookies (without added heme iron) or
449 CAS 1:1 + AP samples. Thus, these results indicate that the addition of AP may reduce the
450 susceptibility to oxidation of cookies.

451

452 **3.5. Oxidation of fortified cookies during storage**

453

454 The development of primary oxidation compounds during storage was monitored by means of
455 PV and LHP content. The evolution of secondary oxidation of samples was assessed by means
456 of hexanal content and the *p*-AnV (Table 4).

457

458 3.5.1. Primary oxidation: PV and LHP content

459 Comparing the different treatments, the PV of the control heme iron cookies was higher than
460 that of the control cookie without heme iron, whereas the PV of the rest of the samples did not
461 differ from either control cookies (Table 4).

462

With respect to storage time, both the PV and LHP content of cookies increased with storage time and reached maximum values at 360 days of storage (Table 4). Prior to storage (Time 0), cookies with added AP (AP and CAS 1:1 + AP) presented lower values than the control with heme iron. Indeed, they were similar to or lower than those for the control without added heme iron. At the end of storage, the most effective strategy for delaying primary oxidation in those cookies fortified with heme iron was the AP treatment (Table 4). In between, cookies with CAS 1:1 and CAS 1:1 + AP displayed no differences in primary oxidation values and showed lower values than control heme samples.

In spite of some differences, the methods selected to determine primary oxidation compounds (PV and LHP content measured by means of the non-induced FOX method) provided similar results and thus were highly correlated ($r=0.978$; $p<0.001$, $n=100$).

3.5.2. Secondary oxidation: hexanal content and *p*-AnV

There were no differences in hexanal content of cookies when considering the different treatments (Table 4). Over the course of storage, a lag phase (no differences over time) was observed in hexanal evolution till 180 days. However, hexanal content increased markedly after 360 days of storage. At this latter storage time, and only considering the heme iron-fortified cookies, those containing AP alone or in combination with CAS 1:1 showed the lowest hexanal content. However, control samples showed by far the lowest hexanal content as they are not enriched with heme iron.

When analysing the treatment factor, we found that CAS 1:1 + AP cookies presented the lowest *p*-AnV value; however, this value was the same as that for the control heme iron samples. No differences were found between the other treatments (Table 4). When

489 considering the storage time factor, the *p*-AnV for samples increased slightly till 180 days but
490 thereafter decreased at 360 days (Table 4).
491
492 Contrary to results for the previous oxidation parameters, the *p*-AnV of control samples (with
493 no added heme iron) was not lower than that of the control heme (with added heme iron). In
494 this regard, it is important to note that the *p*-AnV was not correlated with any other oxidation
495 parameter, whereas hexanal content, PV and LHP content were all correlated (hexanal vs. PV,
496 $r=0.699$, $p<0.001$, $n=100$; hexanal vs. LHP content, $r=0.736$, $p<0.001$, $n=100$; and PV vs. LHP
497 content, $r=0.978$, $p<0.001$, $n=100$). In addition, the hexanal content, PV and LHP content found
498 in cookies after 360 days of storage were also correlated with MAXLHP (respectively,
499 Spearman's $r=0.583$, 0.515 and 0.551, $p=0.002$, $p=0.008$ and $p=0.004$, $n = 25$), Final LHP
500 (respectively, Spearman's $r=0.666$, 0.632 and 0.694, $p<0.001$, $p=0.001$ and $p<0.001$, $n = 25$)
501 and AUC (respectively, Spearman's $r=0.654$, 0.598 and 0.653, $p<0.001$, $p=0.002$, $p<0.001$, $n =$
502 25) values obtained from the induced FOX method. However, the *p*-AnV measured in cookies
503 at the end of the storage time was not correlated with MAXLHP, Final LHP and AUC values.
504 These findings indicate that the susceptibility to oxidation measured by means of the induced
505 FOX assay may be useful to predict the evolution of the oxidation in cookies during the
506 storage and that *p*-AnV, in this study, was not a suitable oxidation parameter for monitoring
507 the evolution of secondary oxidation during storage. The most probable explanation for this is
508 that (i) some of the secondary oxidation compounds measured by the *p*-AnV are volatile and
509 thus may have been lost during the lipid extraction procedure (e.g., when vacuum was applied
510 in rotary evaporation and in the desiccator) and (ii) it is also known that secondary oxidation
511 products have the capacity to form adducts with proteins (Zamora & Hidalgo, 2011), the
512 content of which was considerable in the matrix studied here.
513

514 In general, PV, LHP and hexanal content of heme iron-fortified cookies during storage was
515 lower for samples with added AP alone or in combination with CAS 1:1 than for control heme
516 iron samples. The effectiveness of AP has previously been described in palm oil fortified with
517 heme iron, used as model for iron fortification (Aleman et al., 2010, 2014). Therefore, the
518 addition of AP either alone or in combination with CAS 1:1 seems to be suitable for the
519 manufacture and storage up to 360 days of sandwich-type cookies fortified with heme iron. *p*-
520 AnV results indicated that this oxidation parameter was not a satisfactory method for
521 assessing secondary oxidation in this case.
522
523 **3.6. Colour of chocolate cream fillings prior to storage**
524
525 Figure 1 shows the different CIE L*a*b* colour space parameters for experimental chocolate
526 cream fillings, commercial chocolate cream fillings and chocolate bars with different
527 percentages of cocoa. The lightness (L*) of chocolate creams fortified with heme iron did not
528 differ from that of the control chocolate cream without heme iron (Figure 1.A). Only AP cream
529 presented less lightness than the control cream with no added heme iron. All the chocolate
530 fillings prepared for this study were less bright than the fillings of commercial cookies.
531 However, the lightness of the experimental cookies was similar to that of chocolate bars with
532 a high percentage of cocoa (72% and 85%, w/w).
533
534 In contrast to the L* values, a* values varied widely between the experimental cookie fillings.
535 The control chocolate filling without added heme iron displayed higher redness (a*) than the
536 other experimental fillings (Figure 1.B), meaning that the control filling tended towards a
537 redder colour than the samples fortified with heme iron. Samples with AP (AP and CAS 1:1 +
538 AP) presented higher a* values than the rest, thus indicating an AP effect on a* values.
539 Similarly to L* values, commercial chocolate fillings had higher a* values than the

experimental ones. Moreover, chocolate creams fortified with heme iron presented a* values similar to those of chocolate bars with a high cocoa content (72% and 85%, w/w).

The CAS 1:1 + AP chocolate filling displayed the lowest b* values of all samples (Figure 1.C). The b* value is related to a more yellowish colour when the values are positive and to a more bluish colour when the values are negative. Thus, it seems that the addition of AP had a slight effect on the colour of the chocolate cream. The differences between b* values of samples fortified with heme iron were minimal whereas the experimental chocolate cream without heme iron presented higher b* values, which were similar to those of the chocolate bars with a high cocoa content (85%, w/w). As for L* and a* values, those for the experimental chocolate creams presented b* values quite different from those found in commercial cookies.

Overall, the colour of the chocolate cream fillings fortified with heme iron differed from the control non-fortified chocolate cream and from the commercial chocolate creams. However, L* and a* values of heme iron-fortified chocolate creams were similar to those of chocolate bars with a high percentage of cocoa (72%-85%, w/w). The addition of AP had a slight effect on the colour of the cookie filling, which is difficult to explain, as the amount of AP added was very low and the colour was measured prior to storage. In spite of these differences regarding the colour of the chocolate fillings of experimental and commercial samples, sensory panellists did not dislike the overall appearance of the sandwich-type cookies fortified with heme iron. The existence of various chocolate products (e.g. chocolate bars) with different colours may explain this response.

566 ### 3.7. Sensory analysis

567

568 The acceptability scores obtained for sandwich-type cookies at each storage time (0, 180 and
569 360 days) are presented in Table 5. At the initial time, the only cookies that were not accepted
570 by the panellists were those corresponding to the CAS 1:1 + AP treatment (acceptability
571 scores < 5.0). It is difficult to explain why the combination of CAS 1:1 plus AP decreases
572 acceptability at the initial time since the panellists accepted those cookies from AP or CAS 1:1
573 treatments (acceptability scores > 5.0).

574

575 However, it should be noted that cookies from CAS 1:1 + AP treatment were accepted by
576 panellists after 180 and 360 days of storage (acceptability scores > 5.0). Moreover, after 180
577 and 360 days of storage, the acceptability of samples with CAS 1:1 or CAS 1:1 + AP did not
578 differ from that of cookies without added heme iron (i.e., control treatment). Conversely, at
579 these storage times, the control heme iron cookies (with added heme iron and without any
580 strategy to prevent oxidation) showed the lowest acceptability scores. Thus, the acceptability
581 of these cookies was clearly lower than that of the cookies without heme iron added (i.e.,
582 control treatment), which may be related to the observed prooxidant effect of the heme iron
583 after 180 and 360 days of storage (Table 4). Also, it is worth mentioning the similar overall
584 acceptability scores (always > 5) recorded at each storage time (0, 180 and 360 days) for
585 those cookies corresponding to the control (without heme iron) and to the CAS 1:1 treatment.

586

587 In general, these results seemed to be related to those regarding oxidation, but are not in
588 complete agreement as the decreased overall acceptability of cookies with AP at the end of the
589 storage period was not consistent with its higher oxidative stability (lower oxidation values at
590 360 days, Table 4). Therefore, considering the acceptability together with the oxidation
591 values, the combination of AP and CAS 1:1 heme iron ingredient seems to be the best

fortification strategy as by the end of the storage time, these biscuits were accepted by panellists and showed lower oxidation values than control heme iron cookies and lower hexanal content than cookies from CAS 1:1 treatment.

4. Conclusions

The addition of AP to heme iron-fortified chocolate creams used to fill sandwich-type cookies prevented oxidation and the loss of tocopherols and tocotrienols during preparation of the chocolate creams.

In general, during storage at room temperature of heme iron-fortified sandwich-type cookies, the formation of primary oxidation compounds and hexanal was reduced by means of the addition of AP, either alone or in combination with encapsulation of the heme iron ingredient by co-spray-drying.

The colour of freshly produced chocolate creams fortified with heme iron ingredients differed from that of commercial cookies creams. However, sensory panellists accepted the experimental cookies probably because the colour of the heme iron-fortified chocolate fillings was similar to that of chocolate bars with a high percentage of cocoa (72%-85%, w/w).

After 360 days of storage, the heme iron-fortified cookies with CAS 1:1 alone or in combination with AP were accepted by sensory panellists and, more importantly, their overall acceptability scores did not differ from those obtained for control cookies without the addition of heme iron. Therefore, a combination of added AP and co-spray-drying of heme iron with caseinate (CAS 1:1 + AP) seems to be the most suitable strategy to achieve oxidative stability and sensory acceptability of sandwich-type cookies fortified with heme iron.

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Table 1: Experimental design used to prepare the chocolate cream fillings of the sandwich-type cookies [1]

Treatments	Heme iron ingredient[2]	Ascorbyl palmitate [mg/kg palm oil]
Control	-	0
Control heme	AproFer 1000™	0
AP	AproFer 1000™	400
CAS 1:1	Heme iron concentrate : calcium caseinate at 1:1, w/w, dry weights	0
CAS 1:1 + AP	Heme iron concentrate : calcium caseinate at 1:1, w/w, dry weights	400

Abbreviations: AP, ascorbyl palmitate; CAS, calcium caseinate.

[1] All cookies were filled with chocolate creams which consisted of 54.3% icing sugar, 23.6% palm oil, 16.3% cocoa powder (11% fat), 5.6% skim dry milk (1.5% fat), 0.1% vanilla extract and 0.1% lecithin (percentages expressed as w/w). Heme iron ingredients were added to provide 0.31 mg Fe/g cream (0.11 mg Fe/g cookie).

[2] The control heme iron ingredient (AproFer 1000™) is a proteinaceous material (mix of peptones, peptides and free amino acids) that contains 1.74% (w/w) of total iron, of which 97.3% was heme iron. The CAS 1:1 ingredient comes from the co-spray drying of this proteinaceous material with calcium caseinate (at 1:1 ratio, w/w, dry weights) and contains 0.88% (w/w) of total iron, of which 91.8% was heme iron. Further details about the characteristics and preparation of these heme iron ingredients are described in the Supplementary data.

Table 2: Tocopherol and tocotrienol composition of the palm oil used to prepare sandwich-type cookies and that of the fat extracted from the different treatment cookies prior to storage[1]

	Palm oil	Control	Control heme	AP	CAS 1:1	CAS 1:1+AP
TOCOPHEROL [mg/kg]						
α	214.8±9c	169.6±18b	44.3±0a	157.7±6b	36.0±0.3a	168.2±5b
β	1.2±0.3	1.0±0.0	1.3±0.2	1.6±0.2	1.0±0.2	1.5±0.0
γ	2.6±0.3	2.1±0.4	2.9±0.3	2.4±0.5	1.8±0.7	2.0±0.5
δ	ND[2]	ND	ND	ND	ND	ND
TOTAL	218.6±9c	172.7±26b	48.5±0.5a	161.74±6b	38.9±0.6a	171.7±6b
TOCOTRIENOL [mg/kg]						
α	246.4±9c	156.1±16ab	185.6±15abc	173.0±43ab	149.9±8a	215.8±4bc
β	22.5±2	22.2±2	11.9±2	24.2±6	23.5±2	28.2±19
γ	176.5±9c	159.2±10bc	20.1±2.5a	154.0±5b	30.5±2a	153.9±3b
δ	9.9±1.2c	8.8±0.1bc	1.1±0.2a	8.8±0.4bc	0.8±0.2a	8.0±0.0b
TOTAL	455.3±20c	346.3±40b	218.7±20a	359.9±48b	204.6±4a	405.9±20bc
TOCOPHEROLS + TOCOTRIENOLS [mg/kg]						
TOTAL	673.9±29c	519.0±65b	267.2±20a	521.7±49b	243.5±3a	577.6±19bc

Results are expressed per weight of lipids.

[1] Please refer to Table 1 for the interpretation of sandwich-type cookies treatments. Values given in this Table correspond to means ± standard deviation (n = 3). Values in the same row with different letters present significant differences ($P \leq 0.05$).

[2] ND: not detected

Table 3: Susceptibility to oxidation of the lipids extracted from cookies prior to storage, measured by means of the FOX-induced method [1]

Treatments	Initial LHP [mmol CHP eq/kg]	MAXLHP [mmol CHP eq/kg]	TMAX [h]	Final LHP [mmol CHP eq/kg]	AUC [(mmol CHP eq /kg) x h]
Control	0.94±0.04c	0.94±0.04c	0.50	0.14±0.02b	51.86±3.62b
Control heme	1.00±0.04cd	1.00±0.04cd	0.50	0.19±0.02c	63.80±4.47c
AP	0.03±0.01a	0.15±0.02a	29.50	0.06±<0.01a	18.23±0.67a
CAS 1:1	1.05±0.04d	1.05±0.04d	0.50	0.20±0.03c	62.66±5.08c
CAS 1:1 + AP	0.67±0.02b	0.67±0.02b	0.50	0.17±0.03bc	47.47±4.14b

[1] See Table 1 for treatment abbreviations. The parameters determined from the curve of formation of lipid hydroperoxides were: initial lipid hydroperoxide value (Initial LHP); Maximum lipid hydroperoxide value (MAXLHP); Time to reach the maximum lipid hydroperoxide value (TMAX); Final lipid hydroperoxide value (Final LHP) and the area under the curve (AUC). Results are expressed per weight of lipid extract.

Values given in this table correspond to means ± standard deviation (n=5). Means in the same column with different letters present significant differences ($P \leq 0.05$).

Table 4: Effect of treatment and storage time on primary (PV and LHP content) and secondary (hexanal content and *p*-AnV) oxidation parameters of sandwich-type cookies stored at room temperature in the dark for 360 days [1]

	PV [meq O_2/kg]	LHP content [mmol CHP eq/kg]	Hexanal content [µg/kg]	*p*-AnV
Treatment[1, 2]				
Control	8.32a	5.03	2.1	14.13b
Control heme	20.30b	11.05	48.9	13.57ab
AP	14.47ab	6.99	31.6	14.97b
CAS 1:1	18.33ab	10.00	44.5	16.10b
CAS 1:1 + AP	14.04ab	8.30	32.2	11.39a
SEM[3]	*3.33*	*1.97*	*16*	*0.85*
Storage time[2]				
0 days	1.14a	0.78a	1.3a	13.29a
90 days	12.32b	4.98b	7.0a	14.13ab
180 days	20.20c	11.46c	13.2a	15.94b
360 days	27.20d	15.86d	106.0b	12.78a
SEM[3]	*1.58*	*0.81*	*8.2*	*0.81*
Time 0[4]				
Control	1.05ab	0.94c	ND	13.75ab
Control heme	2.35b	1.00cd	1.20a	12.84ab
AP	0.28a	0.03a	TR	13.03ab
CAS 1:1	1.27ab	1.05d	1.53b	15.98b
CAS 1:1 + AP	0.73a	0.67b	1.78b	10.85a
SEM[5]	*0.42*	*0.08*	*0.1*	*1.24*
Time 90[4]				
Control	2.60a	1.35a	TR	11.64a
Control heme	19.76c	8.11c	14.84d	14.81ab
AP	14.52b	5.40b	6.43b	17.94b
CAS 1:1	19.77c	8.16c	10.75c	15.31ab
CAS 1:1 + AP	4.92a	1.88a	1.83a	10.93a
SEM[5]	*1.28*	*0.68*	*0.47*	*1.30*
Time 180[4]				
Control	9.02a	5.41a	1.14a	17.09bc
Control heme	26.23d	14.63c	20.65c	15.20b
AP	19.40b	9.49bc	13.69b	16.00b
CAS 1:1	23.52c	13.71c	15.06b	20.68c
CAS 1:1 + AP	22.82c	14.05c	15.37b	10.71a
SEM[5]	*0.78*	*0.40*	*1.02*	*1.22*
Time 360[4]				
Control	20.60a	12.42a	5.29a	14.04
Control heme	35.28d	20.39c	158.9c	11.43
AP	23.67b	13.03a	105.4b	12.90
CAS 1:1	28.76c	16.90b	150.5c	12.44

CAS 1:1 + AP	27.70c	16.58b	109.9b	13.07
SEM[5]	*0.74*	*0.44*	*8.5*	*0.94*

[1] See Table 1 for treatment abbreviations

[2] Values correspond to least-squares means obtained from multifactorial ANOVA (n=100)

[3] Standard error of the least-squares means

[4] Values correspond to means obtained from ANOVA (n=25)

[5] Standard error of the means

Other abbreviations: PV, peroxide value; LHP content, lipid hydroperoxide content measured by means of the non-induced FOX method; *p*-AnV, *p*-anisidine value. Means and least-squares means corresponding to a given factor with different letters present significant differences ($P \leq 0.05$). PV, LHP content and *p*-AnV were determined in the lipid extracts. Hexanal content was determined in the ground cookies. Results are expressed per weight of lipid extract or whole cookie, accordingly.

Table 5: Panellists' overall acceptance of the different sandwich-type cookies after different storage times [1]

Treatments	0 days	180 days	360 days
Control	6.8 ± 2.0a	6.4 ± 1.8a	6.5 ± 2.1a
Control heme	5.3 ± 1.6ay	3.9 ± 2.3bx	3.3 ± 1.8bx
AP	5.4 ± 2.1ay	5.6 ± 2.2aby	3.8 ± 1.8bx
CAS 1:1	5.9 ± 1.7a	5.8 ± 2.3a	5.7 ± 1.7a
CAS 1:1 + AP	3.5 ± 2.4by	5.2 ± 2.0abx	5.4 ± 1.7ax

[1] See Table 1 for treatment abbreviations. Values given in this table correspond to means ± standard deviation (n=32). Means within the same column without a common letter (a-c) and means within the same row without a common letter (x-y) present significant differences ($P \leq 0.05$).

Figure 1

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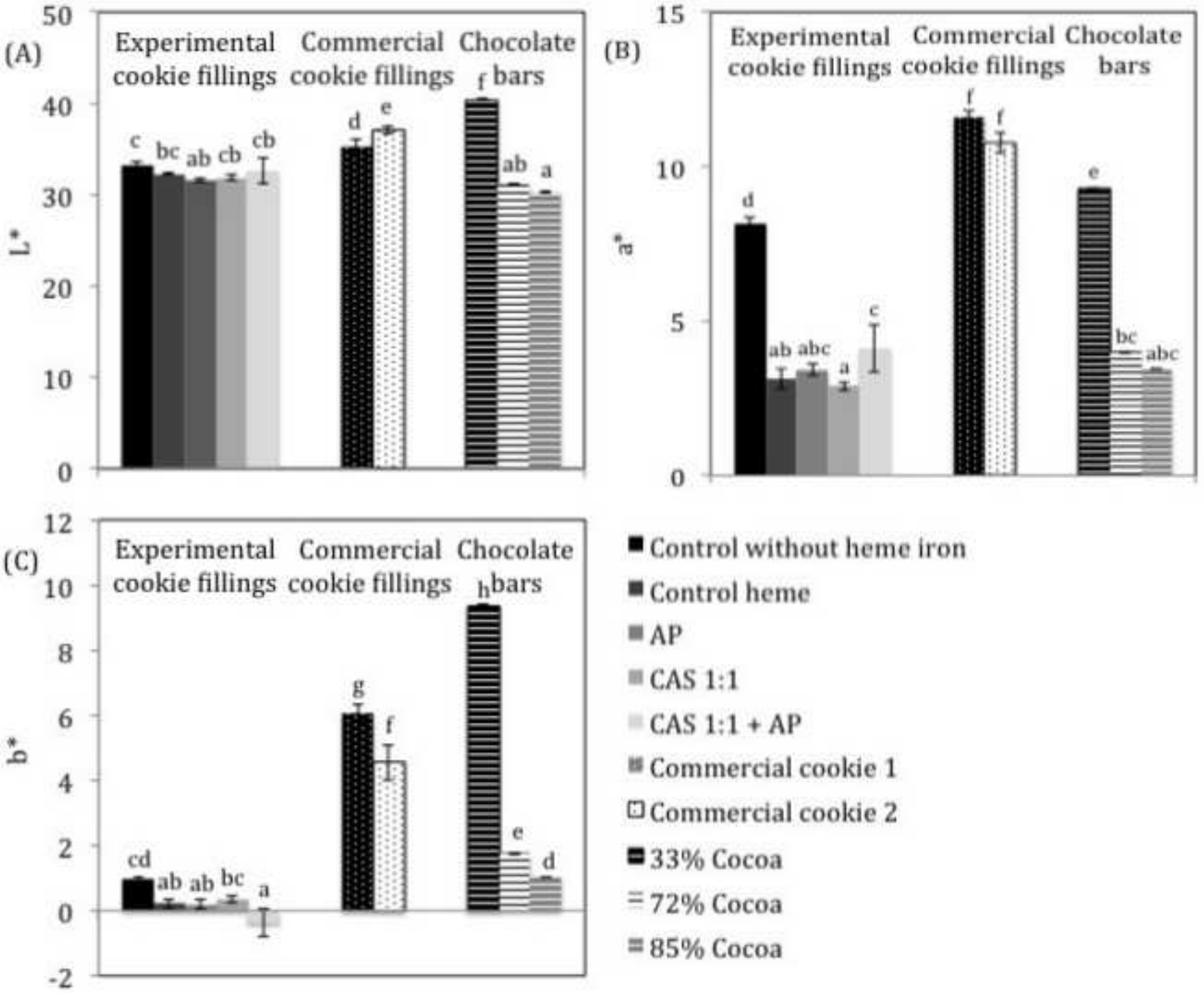

Figure 1 caption and footnote

Figure 1: Lightness (L*) (A), redness (a*) (B) and yellowness (b*) (C) values of the experimental chocolate cream fillings, commercial chocolate cream fillings and chocolate bars with different amounts of cocoa.

See Table 1 for treatment abbreviations

Supplementary Material reviewed

Click here to download Supplementary Material: FOODCHEM-D-15-01654_supplementary.doc

HIGHLIGHTS

- Fortification with heme iron was studied in chocolate sandwich-type cookies
- Antioxidant addition and heme iron encapsulation were used to minimize oxidation
- Tocopherol loss and oxidation are reduced by ascorbyl palmitate (AP) addition
- After 1 year of storage, control and Fe-encapsulated cookies are equally accepted
- AP plus encapsulation is best at limiting oxidation and maintaining acceptability