

Stabilization of Fat Fraction in Walnut-based Freeze Dried Products

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Aim of this work was to create an innovative walnut product able to increase the shelf life of the walnuts themselves and consequently facilitate their industrial use. Main purpose was to protect the walnuts against rancidity, making the fat fraction less exposed to oxidizing agents and preserving all the well-known positive compounds of the fruit (polyunsaturated fatty acids, vitamins, fiber and antioxidants).

To this end an encapsulation process was developed consisting of three different stages: roasting and milling of walnut kernels for paste production, adding of different coating materials for emulsion formation, drying of emulsified samples by lyophilization. Different encapsulation agents (maltodextrin DE12, tragacanth gum, starches, and whey protein concentrates with 80% and 50% proteins on dry basis) were tested, either individually or in combination, in order to assess the effect exerted by different formulations and technological treatments on the product stability. Preliminary tests were carried out to select type, concentration, and combination of coating materials. The fittest samples were therefore examined in terms of residual moisture, pH, total acidity, color, peroxide value, conjugated dienes and trienes. Phenolics and compounds resulting from the oxidation process were detected using an ultra-high performance liquid chromatograph coupled to quadrupole-time-of-flight mass spectrometer (UHPLC/Q-TOF). Analysis were carried out immediately after freeze-drying, and on freeze-dried products after 15 day 60°C storage. Chemicals found in the fresh lyophilized and in the stored samples were identified and compared through the loading plot: flavonoids (flavanones and isoflavones) and phenolic acids, proved to be the most detected differential compounds.

The formulation consisting in walnut paste added with 0.2% tragacanth gum, 15% whey protein concentrates with 50% protein/d.m., and 18.3% maltodextrin DE12 allowed to obtain a freeze-dried product with good texture, dry (with less than 0.8% moisture), and with good stability of the fat phase.

1. Introduction

Due to the important nutrients and sensory properties, interest in the use of walnuts in the food industry is broad and growing. However, some compositional characteristics can adversely affect the walnut quality, making valuable the development of more versatile and stable derived products (Dordoni et al., 2015). Encapsulation is a technique already widely applied for treatment of walnut oil (rich in polyunsaturated fatty acids and tocopherols) (Calvo et al., 2011); nevertheless, the remaining part of nut is a source of bioactive compounds (phenolic compounds, fiber, and minerals) that can be enhanced by encapsulating the pasta obtained by roasting and milling of the whole kernels. During the encapsulation process, carbohydrates and proteins of walnuts (about 10 and 16% w/w, respectively) (Dordoni et al., 2015) can contribute to the formation of the network for the protection of the lipid phase, limiting the addition of others coating materials. The aim of this study was to explore oxidative processes that occur in walnut-based freeze dried products obtained by different formulations.

2. Materials and methods

2.1 Materials

Shelled Chile's walnuts (*Juglans regia* L.), harvest 2014, were purchased from the local market (I frutti del Convento, Alfano F.lli, Italy). Cold-water swelling starches, Gelcream CL (CLS) and Gelcream CS (CSS), and whey protein concentrates with 80% (WPC80) and 50% (WPC50) proteins on dry basis were provided by SCAsrl (Italy). Maltodextrin DE 12 (Glucidex® 12) was supplied from Roquette Italia (Italy), Tragacanth gum powder (CEROTRAG 888) was procured from Roeper (Germany). All chemicals used in the analytical determinations were high-purity commercially available reagents.

2.2 Analytical determinations

Moisture content was determined according to the method AOAC 931.04, pH and total acidity were performed following the methods recommended by the Office International du Cacao, du Chocolat et de la Confiserie. Color measurements were taken on a Konica-Minolta CR-310 reflectance colorimeter in the CIE L*a*b* color system. Peroxide values and conjugated dienes and trienes were determined as described by the Regulation (Commission Regulation 2568/91, III and IX annexes), on the oil fraction extracted according to Calvo et al. (2011) (Dordoni et al., 2015). Phenolics and oxidation compounds were screened through a hybrid quadrupole-time-of-flight mass spectrometer coupled to an UHPLC chromatographic system (UHPLC/Q-TOF) (all from Agilent Technologies, Santa Clara, CA, USA). The instrument was operated in the positive scan mode and set to acquire MS-only spectra (Lucini et al., 2015). Samples were extracted in 20 vol of 80% methanol added to 5 mM formic acid, using an Ultra-Turrax, centrifuged (5000 rpm for 15 min at 4°C), filtered through a 0.22 µm cellulose membrane, and transferred to an amber vial for analysis. A 1290 liquid chromatograph system, equipped with a binary pump and a Dual Electrospray Jet Stream ionization system, coupled to a G6550 mass spectrometer detector (all from Agilent technologies Santa Clara, CA, USA) was used for the screening. The mass spectrometer was operated to acquire positive ions in MS-only mode, in the 100–1200 m/z range. Chromatographic separation was carried out using an Agilent Zorbax eclipse plus C18 column (50 × 2.1 mm, 1.8 µm) under a water-methanol gradient elution. The mobile phase temperature was set to 35 °C. The injection volume was 2 µL, and the flow rate was 220 µL/min. Raw data were processed by the Mass Hunter Qualitative Analysis B.06 software (Agilent Technologies). Compound identification was performed using the database exported from Phenol-Explorer 3.0 (Rothwell et al., 2013).

2.3 Experimental plan

2.3.1 Encapsulation process

Walnut paste was obtained by roasting kernels in a forced convection oven at 160 °C for 15 min (Vaidya and Eun, 2013) and by grinding them in a planetary micro mill (Pulverisette, Fritsch, Germany) at 800 rpm for 3 min. Two volumes of water and different combinations of biopolymers - starches (S), whey protein concentrates (WPC), maltodextrin (MD), and/or tragacanth gum (TG) - were added to the walnut paste and homogenized for 5 min at 25 °C. Resulting emulsions were placed into silicone molds and frozen at -18 °C for 24 h. The emulsions were dried for 72h by using aChrist Alpha 1-2 LD freeze dryer. During the process, the ice condenser was set at lower than -50 °C, and the pressure was around 0.120 mbar (Dordoni et al., 2015).

2.3.2. Preliminary test

Preliminary tests were carried out first to select starch (CS gelcream or Gelcream CL) and whey protein concentrate (WPC50 or WPC80) types and amounts (5% starch, 15%, 25% or 30% WPC50, and 15%, 25% or 30% WPC80). Based on the obtained results, different combinations of coating materials (WPC-TG; WPC-MD; WPC-TG-MD; WPC-TG-MD-S) were evaluated. All samples arising from the above tests were examined in terms of moisture content, total acidity and pH.

2.3.3 Shelf life test

According to the preliminary outcomes, different formulations were arranged to perform the accelerated storage tests. Aliquots (50 g) of each sample were placed in open glass vessels and stored at 60 °C for 15 days in the absence of light (Vaidya et al., 2013). Residual moisture, pH, total acidity, color, peroxide value, dienes and trienes were measured on samples immediately after lyophilization and after storage. Phenolics and compounds resulting from the oxidation process were detected by UHPLC / Q-TOF.

2.4 Statistical analysis

Data represent mean values (n=6) ± standard error. A factorial analysis of variance (ANOVA) with at $p \leq 0.05$ was used to measure the significance among the experiments. Within the tables, different letters indicate

statistically different values. The statistics package IBM SPSS Statistics 21 (IBM Corporation, New York, USA) was used. The metabolomic data were interpreted using Mass Profiler Professional B.12.05 (from Agilent technologies). The abundance value for each compound in the dataset was normalized at 75th percentile and baselined to the median. Interpretation and statistics were then carried out on the latter dataset; ANOVA analysis ($p < 0.01$, Bonferroni multiple testing correction) and fold-change analysis (cut-off = 5) were combined into Volcano plots. Unsupervised hierarchical cluster analysis on both features and treatments (Euclidean similarity measure and Wards linkage rule) and multivariate Partial Least Square Discriminant Analysis (PLS-DA) was carried out to integrate Volcano and cluster outputs. The PLS-DA loadings used to build the class prediction model were plotted according to their weight within the latent vectors, and the most differential compounds exported.

3. Results and discussion

3.1. Preliminary tests

Encapsulation is a well-known food technology in which small droplets of liquid or solid particles are packed into a wall matrix. Suitable wall materials build a barrier between the inside sensitive components and the outside environment, by protecting and controlling the spread (Mehyar et al., 2014; Hogan et al., 2001). The most widely used matrices are carbohydrates (maltodextrins, cyclodextrins, modified starches) proteins (caseins, whey proteins), gums (acacia, arabic, tragacanth), and fibers (Hogan et al., 2001). Single wall components hardly possess all the required properties (high solubility, low viscosity, good emulsifying, drying, and film-forming capabilities) (Annamalai et al., 2014; Mehyar et al., 2014; Hogan et al., 2001): this is why mixtures with different origin are usually combined (Mehyar et al., 2014). First trials were performed to screen the behaviour of some encapsulation matrices. GCS and GCL starches were off-taste, stable, and structuring agents from corn. They both were physically treated to show a slightly different optimal pH, reporting 5.50 and 6.50 pHs for 10% solutions (at 20°C), respectively. Coating only consisting of starch produced samples very friable, dusty, without consistency, separating the oily phase after breaking. The use of starch, as the sole encapsulation matrix, was not conceivable. Conversely, WPC80 and WPC50 at 30% led to very compact samples.

*Table 1: Formulation, residual moisture, total acidity, and pH of samples evaluated in the preliminary tests to evaluate type and amount, or combination of coating materials. Percentages are referred to the walnut paste content. *n.d.: not determined.*

Formulation	Moisture %	Total acidity (meq/100g d.m.)	pH
5% CSS	n.d.*	64.2 ± 8.1	6.38 ± 0.01
5% CLS	n.d.	50.0 ± 4.6	6.33 ± 0.00
15% WPC50	n.d.	55.1 ± 5.2	6.23 ± 0.00
15% WPC80	n.d.	56.1 ± 8.4	6.45 ± 0.01
25% WPC50	n.d.	60.2 ± 3.0	6.21 ± 0.00
25% WPC80	n.d.	58.5 ± 3.5	6.38 ± 0.02
30% WPC50	n.d.	62 ± 7.4	6.19 ± 0.00
30% WPC80	1.12 ± 0.10	56 ± 7.4	6.33 ± 0.01
25% WPC50+0.2%TG	0.67 ± 0.08	58 ± 3.2	6.27 ± 0.01
25% WPC50+8.3% MD	0.81 ± 0.12	56 ± 1.0	6.28 ± 0.03
25% WPC50+8.3% MD+0.2%TG	0.41 ± 0.25	45 ± 5.6	6.28 ± 0.00
25% WPC50+0.1%CSS	0.45 ± 0.16	56 ± 4.1	6.29 ± 0.01
25% WPC50+8.3%MD+0.2%TG+0.1%CSS	0.20 ± 0.18	60 ± 1.6	6.28 ± 0.00

In order to ensure right shape retention, further preliminary tests by including 25% WPC50 and different combinations of polysaccharide materials were set up. CSS starch negatively impacted on the consistency, even if used in combination and in very low percentages. Maltodextrin addition provided good results, especially with TG, performing the product with lower pH, acidity, and residual moisture.

3.2. Shelf life tests

The formulations arranged at this stage are shown in the table below (Table 2). Part of the samples were spiked with coating materials, maintaining steady and minimal (0.2%) the TG percentage (Farzi et al., 2013).

Table 2: Formulation of the samples to be subjected to freeze-drying and shelf-life tests.

Sample	Formulation
1	Nut paste
2	Nut paste + H ₂ O
3	Nut paste + 0.2% TG + 15% WPC50 + 18.3% MD + H ₂ O
4	Nut paste + 0.2% TG + 25% WPC50 + 8.3% MD + H ₂ O
5	Nut paste + 0.2% TG + 30% WPC50 + 3.3% MD + H ₂ O

The WPC50 amounts were deducted from Mehyar et al. (2014) and integrated by using MD up to obtain a constant overall wall matrix content (33.5%). Sample were evaluated immediately after freeze drying and on freeze-dried products after 15 day 60°C storage (Table 3).

Table 3: Analysis on freeze-dried samples immediately after lyophilisation and after 15 day 60°C storage. For each parameter different superscript letters indicate statistically different values within each column, and different subscript letters indicate statistically different values within each row. *n.d.: not determined.

Phases	Parameters (m.u.)	Samples				
		1	2	3	4	5
After freeze- drying	Moisture %	0.13±0.12 _c	0.33±0.31 _{bc}	0.78±0.15 _{ab}	0.89±0.11 _a	1.13±0.14 _a
	pH	6.32±0.01 ^a _a	6.31±0.00 ^a _a	6.20±0.01 ^a _b	6.19±0.00 ^a _b	6.31±0.00 ^a _a
	Total acidity (meq/100g d.m.)	54.8±3.2 ^a _b	54.0±2.5 ^b _b	55.0±4.1 ^b _b	65.0±1.8 ^a _a	64.5±3.4 ^b _a
	Peroxide value (meqO ₂ /kg d.m.)	5.90±2.85 ^a _{ab}	0.00±0.00 ^b _b	0.00±0.00 ^b _b	0.00±0.00 ^b _b	17.50±10.85 ^a _a
	Dienes K _{232nm}	1.538±0.22 ^b _a	1.244±0.032 ^b _b	1.299±0.007 ^b _{ab}	1.382±0.027 ^b _{ab}	1.320±0.039 ^b _{ab}
	Trienes K _{270nm}	0.138±0.014 ^b _a	0.078±0.016 ^b _b	0.104±0.024 ^b _{ab}	0.104±0.011 ^b _{ab}	0.107±0.002 ^b _{ab}
	L	52.58±3.18 ^a _c	60.27±0.17 ^a _b	65.40±2.72 ^a _a	64.05±0.01 ^a _{ab}	64.48±0.16 ^a _{ab}
	a	6.40±0.05 ^a _a	5.42±0.08 ^b _b	3.51±0.08 ^b _d	4.18±0.06 ^b _c	4.03±0.05 ^a _c
	b	3.15±0.35 ^a _b	4.42±0.15 ^a _a	3.38±0.16 ^a _b	3.62±0.11 ^b _b	4.19±0.02 ^b _a
	After storage	Moisture %	n.d.	n.d.	n.d.	n.d.
pH		6.12±0.01 ^b _b	6.20±0.00 ^b _a	6.10±0.01 ^b _b	6.03±0.00 ^b _c	6.04±0.01 ^b _c
Total acidity (meq/100g d.m.)		60.0±2.5 ^a _b	65.0±4.1 ^a _b	67.0±3.2 ^a _{ab}	70.0±6.9 ^a _a	76.2±2.8 ^a _a
Peroxide value (meqO ₂ /kg d.m.)		7.66±3.25 ^a _a	9.96±6.47 ^a _a	7.68±3.24 ^a _a	9.90±6.28 ^a _a	18.60±5.52 ^a _a
Dienes K _{232nm}		2.028±0.033 ^a _d	3.028±0.128 ^a _a	2.522±0.039 ^a _b	2.324±0.056 ^a _c	2.265±0.037 ^a _c
Trienes K _{270nm}		0.170±0.012 ^a _b	0.227±0.030 ^a _a	0.177±0.026 ^a _{ab}	0.178±0.008 ^a _{ab}	0.145±0.004 ^a _b
L		47.93±1.21 ^b _d	53.88±0.70 ^b _c	62.52±0.64 ^a _a	62.47±0.45 ^b _a	59.71±0.15 ^b _b
a		6.41±0.12 ^a _a	6.14±0.19 ^a _a	4.04±0.09 ^a _a	4.44±0.03 ^a _a	3.25±2.76 ^a _a
b		18.13±0.83 ^b _c	23.36±0.14 ^b _b	23.27±0.12 ^a _b	24.91±0.10 ^a _a	25.19±0.09 ^a _a

Encapsulated samples (3, 4, and 5) showed slightly upper (but still exiguous) moisture content. Total acidity was higher in the 4 and 5 samples, as the higher the wall protein content (Waraho et al. 2011). Peroxides were found in freeze-dried walnut paste without additions (sample 1) and in sample 5, while dienes and trienes were significantly higher in the sample 1, indicating a protective effect exerted by the encapsulation (Annamalai et al. 2014) during the lyophilisation treatment. After storage, all formulations showed lower pH

and increasing total acidity (particularly in sample 5, with the greater WPC50 content). Peroxide values, dienes and trienes highlighted the negative effect of the storage conditions on baseline parameters (Ling et al., 2014). As regards the color analysis, the shift to yellow of the *b* values clearly emerged in all the samples. Predictably, during storage the samples were subjected to an oxidative stress resulting in browning and release of free fatty acids. The oxidation occurrence could be due to the barrier effect exerted by the coating materials, that can slow down the release of the naturally present antioxidant compounds (such as tocopherols and polyphenols), or, conversely, to the porosity of the freeze-dried samples allowing the permeation of oxygen, and/or to a low encapsulation efficiency (Anwar et al., 2011). Maillard reactions and lipid oxidation can concurrently occur in food exposed to heat treatment; the derived products can interact and oxidation reactions be delayed by the Maillard products with antioxidant properties. In particular, samples containing more WPC should give more Maillard reactions (Tonon et al., 2012). In respect to metabolomics data, both the not averaged unsupervised cluster analysis and the PLS-DA provided a good cluster for each formulation, clearly discriminating between freeze dried products before and after storage. Analysis of individual samples highlighted that the paste without additions (sample 1) showed net differences before and after storage, signing a noticeable change during aging. Just lyophilized and stored samples were different also in 2, 4 and 5, while this distinction was less evident in sample 3. From PLS-DA loading plot scores differential compounds discriminating between the sample before and after storage were gained. Compounds detected in the formulations and increasing during aging are summarized in table 4.

Table 4: Differential compounds discriminating between each sample before and after storage, as gained from PLS-DA loading plot scores.

Sample	Nitrogen compounds	Glucosides	Phenolics	Fatty acids	Others
1	Hystamine	Esculin	Hesperetin Rosmarinic acid p-Coumaroyl tartaric acid Caffeoyl aspartic acid	Caprilic acid	Dehydro-ascorbic acid
2	L-citrulline L-asparagine	Esculin	Petunidin 3-O-(6"-acetyl-galactoside) Pelargonidin 3-O-sophoroside Pelargonidin 3-O-galactoside Delphinidin 3-O-galactoside Apigenin 7-O-glucuronide Isoxanthohumol 6"-O-Malonyldaidzin	Caprilic acid	
3	L-asparagine L-ornithine	Esculin	Episesamin Apigenin 7-O-glucuronide Isoxanthohumol Delphinidin 3-O-galactoside 5-O-Galloylquinic acid Dihydroquercetin	Caprilic acid	
4	L-ornithine L-homoserine		Nobiletin Caffeic acid 4-O-glucoside Dihydroquercetin Stigmastanolferulate p-Coumaroyl tartaric acid		L-ascorbate
5	L-asparagine L-ornithine L-homoserine		Gingerol 3,4-Dihydroxyphenylglycol Dihydroquercetin Kaempferol 3-O-acetyl-glucoside Cyanidin 3-O-(6"-acetyl-glucoside) Caffeic acid 4-O-glucoside	Caprilic acid	

Walnut phenolic compounds are located especially in the kernel pellicle and include hydrolysable and condensed tannins, flavonoids and phenolic acids. Ellagitannins, ellagic acid and its derivatives were revealed as the most abundant. In our study, flavonoids, in particular flavanones and isoflavones, and phenolic acids, proved to be the most detected increasing compounds. As the oxidative stability of walnut oil is notoriously affected by tocopherols (Vaidya and Eun, 2013), an impact also on these phytochemicals was expected but

surprising did not occur. Presence of amino acids in all recipes came from protein degradation from roasted nuts or from WPC used as coating material in 3, 4, and 5.

6. Conclusions

This study intended to provide information on the role exerted by technological treatments and ingredient combinations on oxidative stability of freeze-dried walnut products. In particular, an encapsulation process was developed by testing different combinations of coating materials. Oxidative parameters, examined on the fat fractions after 15 day 60°C storage, increased in all samples. However, the analysis of the differentials compounds showed a greater stability of the formulation consisting in walnut paste added with 0.2% tragacanth gum, 15% whey protein concentrates with 50% protein/d.m., and 18.3% maltodextrin DE12. In particular, it showed phenolic profiles similar before and after treatment. Further studies are necessary to optimize the process in view of future industrial applications.

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