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ORIGINAL PAPER

Amaranth Lemon Sorbet, Elaboration of a Potential Functional Food

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Abstract

In the present work, amaranth proteins were used as a functional ingredient of formulated food. An amaranth lemon sorbet, a healthy alternative dessert for celiac, vegan and any consumer that chooses to follow a conscious diet, was elaborated and characterized. The sorbet base mixture behaved like a pseudoplastic and thixotropic fluid, with a Kokini viscosity of 0.42 ± 0.07 Pa.s, a suitable value for elaborating products with good thickness characteristics. Aeration rate of the sorbet was 36.15 ± 3.48, an overrun value within the expected range of 30–50%, indicating that amaranth proteins presented adequate foaming properties in low pH elaboration conditions. Moreover, the stability study showed that the sorbet did not suffer significant changes in its structure over time during the first two months of storage at −20 °C. Sensory evaluation evidenced panelists accepted the product (8 ± 1, acceptability assay) and related it with airy, creamy and healthy attributes (CATA assay). Finally, simulated gastrointestinal digestion was able to release peptides capable of inhibiting fibrin clot formation (IC_{50} = 3.4 ± 0.1 mg protein/mL), demonstrating that amaranth proteins could be used as a good foaming ingredient in the elaboration of potential functional foods with antithrombotic activity.

Keywords Amaranth proteins · Lemon sorbet · Bioactive peptides · Antithrombotic activity · Functional food

Introduction

Ice cream is a very popular dessert around the world. The main countries that consume ice cream are USA, China, Germany, Italy and Japan, 3798, 2088, 756, 733 and 729 million liters *per* year, respectively (Constanza Business & amp Research Bulletin, 2016). In Argentina, ice creams are widely consumed with an average annual ingest *per* capita of 6.9 L, and a marked deseasonalization tendency is evidenced (Artisanal and related ice cream manufacturers association, 2017).

Sorbets are a dairy- and fat-free frozen dessert containing a mixture of fruit juice or puree, sugar, and water, and it could include a protein source to make more easily the existence of bubble interfaces after incorporating air in the whipping process [1]. The structure of sorbets is different and less complex than that of ice creams. While in products made with dairy ingredients air bubbles, fat globules, ice crystals, and a concentrated non-frozen liquid phase coexist [2], in sorbets, air bubbles are surrounded by a layer of proteins and ice crystals would be dispersed in a concentrated liquid phase [3]. Sorbet's quality is linked to the size of ice crystals [3], the amount of air incorporated and the viscosity of the sorbets base mixture [4]. Moreover, the whipping process promotes small and discrete ice crystals formation that produce a smooth texture and better palatability of sorbets. The amount of air incorporated should be limited but sufficient, to avoid perceiving the product as a foam or, on the contrary, as a heavy dessert.

Amaranth, a Mesoamerican pseudocereal, presents gluten-free seeds that contain a high concentration of proteins (13 to 19% *w*/w wb) with excellent nutritional quality [5]. This ancestral crop has been revalued in recent years, promoting its study as a food ingredient focusing on its health benefits, through the biological activity of natural components contained in the seeds [6] or through bioactive compounds released after performing gastrointestinal digestion. In this sense, amaranth proteins exerted several biological activities when submitted to gastrointestinal digestion [7–9]. Furthermore, foaming properties of amaranth proteins have been previously studied, describing that a pH 2 treatment significantly improved the proteins surface

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behavior not only due to dissociation and denaturation effect of acid medium but also because of the presence of an endogenous aspartic protease [10–12], active in a limited pH range (optimum pH 2). The protease partially hydrolyzes amaranth proteins and these hydrolytic modifications produce positive changes in their foaming properties [12].

At present, producing and consuming foods that contribute to health is a food scientists target, since it is known that there is a direct relationship between diet and many prevalent diseases in the world. In this sense, a healthy diet in the near future will necessarily imply the consumption of functional foods [13], which would benefit a limited number of functions in the body, providing welfare and health. Cardiovascular diseases can be affected by diet. These pathologies are very diverse and represent the main cause of death in Argentina due to non-communicable diseases. The presence of antithrombotic substances in food could decrease thrombosis incidence, representing an innovative strategy to promote healthful eating to address these public health crises.

The aim of this work was to elaborate and characterize a new product using amaranth proteins as a potential functional ingredient protein source, combining its good foaming properties and the presence of potentially bioactive peptides encrypted in amaranth proteins with antithrombotic activity.

Materials and Methods

Plant Material and Samples Preparation

Amaranth protein isolate (API) obtaining: *Amaranthus hypochondriacus* seeds (var. Antorcha) were cultivated at Río Cuarto, Córdoba, Argentina. Flour preparation consisted in grounding the seeds with a cyclone mill (1 mm mesh) and defatting with *n*-hexane (10 g flour/100 mL *n*-hexane) for 24 h. API was obtained from amaranth defatted flour suspended in distilled water (10 g defatted flour/100 mL) at pH 9 and room temperature, 1 h stirring. The dispersion was centrifuged at 9000×*g*, 10 °C, 20 min, and the supernatant was adjusted to pH 5 to precipitate the proteins at their isoelectric point. After another centrifugation step (9000×*g*, 4 °C, 20 min) the precipitate was suspended in water (1:3 relation), neutralized and lyophilized [14].

Amaranth lemon sorbet (ALS) elaboration: ALS recipe (1.1 kg product): 4.4 g of amaranth protein isolate, 100 ml of freshly squeezed lemon juice, 250 g of sugar (Ledesma, Jujuy, Argentina), 30 g of cornstarch (Maizena, Unilever, Buenos Aires, Argentina), and 750 mL of water. Amaranth protein isolate was dissolved in the lemon juice and stirred for 1 h, and syrup was prepared boiling for 5 min in a saucepan sugar, cornstarch, and water. The API and the lemon juice were whipped using a domestic hand blender (12,500 rpm, Atma, Buenos Aires, Argentina) for 5 min until a white and firm foam was obtained. The cooled syrup was then incorporated over the foam and stirred with the hand blender until obtaining a homogeneous mixture. Finally, the mixture obtained, called amaranth base mix, was placed in an ice cream maker (Antártida, CABA, Argentina) and frozen at −20 °C for 4 h at slow and constant agitation. ALS was then transferred to styrofoam containers and stored at −20 °C.

Traditional lemon sorbet (TLS) elaboration: TLS recipe (1.1 kg product): 30 g white egg, 100 mL of freshly squeezed lemon juice, 250 g of sugar, 30 g of cornstarch, and 750 mL of water. The syrup was prepared boiling for 5 min in a saucepan sugar, corn starch, and water. Lemon juice was incorporated in the cooled syrup and finally, a whipped white egg was added. The mixture obtained, called traditional base mix, was processed to obtain the TLS using the same conditions employed for the ALS.

Lemon Sorbets Physicochemical and Sensorial Characterization

Centesimal composition of API, ALS and TLS was determined using Kjeldahl, f=5.85 and 6.25 for amaranth and egg protein content respectively; Anthrone [15] for carbohydrates content; AOAC official methods of analysis for water and ashes content [16].

Lemon sorbet base mixes rheology: Viscosity of base mixes was measured at 20 °C using a Haake RS 600 rheometer (Thermo Science, Germany). Share stress vs share rate curves were obtained using a sequential increment program of share rate ranging from 0.1 to 600 s^{-1} in a period of 120 s, followed by a period of 60 s at maximum speed and a decrease sequence of 120 s. The apparent viscosity of the sorbet base mixes at a shear rate of 50 s^{-1} (Kokini viscosity) was calculated. The Herschel-Bulkley model was used to relate the shear rate and the shear stress (adjusted coefficient, r* > 0,9993 ± 0,0005). Assays were performed in triplicate.

Superficial color was measured on different zones of the sorbet surface using a Chromameter CR 300 (Minolta Optics Inc., Osaka, Japan). Results were expressed with L*, a*, and b* parameters, for lightness, redness, and yellowness, respectively. Values were the average of at least 15 measures. The total color difference (ΔE*) between ALS and TLS was calculated using the following equation:

$$\Delta E^* = \sqrt{\left(L^*_{ALS} - L^*_{TLS}\right)^2 + \left(a^*_{ALS} - a^*_{TLS}\right)^2 + \left(b^*_{ALS} - b^*_{TLS}\right)^2}$$

Overrun: Aeration properties of the sorbet were measured in terms of foaming capacity, calculated as percentage of overrun using the following equation:

$$\%\text{Overrun} = \frac{V_{sorbet} - V_{base\ mix}}{V_{base\ mix}} \times 100$$

Overrun implies the amount of air incorporated into the base mix, expressed as % increase volume of sorbet compared with the amount of base mix used to elaborate it. Assays were performed in triplicate.

Melting test at different storage times: 20 g of freshly made TLS, ALS, a 2-month and a 5-month storage ALS sorbet were placed on top of a plastic funnel attached to a test tube in a controlled temperature room (25 °C). Dripped volume was measured at different times until reaching 10 mL. Assays were performed in triplicate.

Sensory evaluation: Sensory evaluation of ALS and TLS was made with an untrained panel of 60 consumers. Acceptability test was performed using a hedonic scale from 1 to 10 and CATA test (check all that apply) was performed as described by Cardinal et al. [17] using 14 different attributes associated with lemon sorbets.

Lemon Sorbet Functional Characterization

Simulated gastrointestinal digestion: Simulated gastrointestinal digestion (SGD) of ALS sorbet was performed according to Minekus et al. [18]

Tricine SDS-PAGE gel electrophoresis: API and ALS proteins before and after SGD were analyzed by tricine-SDS-PAGE [19]. Low molecular weight (LMW) standards from GE Healthcare (Boston, MA, USA) and very low molecular weight (VLMW) standards from Bio-Rad (Hercules, CA, USA) were used. Gels were fixed in a methanol-acetic acid solution and stained with Coomassie Brilliant Blue R-250 (Anedra, San Fernando, Argentina). Assays were performed in duplicate.

Molecular exclusion chromatography: ALS proteins before and after SGD and API proteins were analyzed in a Gel Filtration Liquid Chromatography FPLC system, AKTA Purifier equipment (GE-Healthcare), using a Superdex Peptide 10/300 GL column (exclusion limit = 10 kDa, optimal separation range 0.1–7 kDa) at room temperature. 500 μL of soluble samples in buffer 50 mmol/L Tris-HCl, pH 7.2, NaCl 0.1 mol/L were loaded and eluted at 0.5 mL/min. The elution profile detected at 210 nm was analyzed with GraphPad Prism 5 program. Calibration was performed using Blue Dextran for exclusion volume (V_0 = 7.6 mL), aprotinin (6.5 kDa), vitamin B12 (1.85 kDa) and hippuric acid (0.179 kDa), obtaining an expression for the molecular weight (MW) of proteins and peptides:

$\log MW = 4.84–3.30 \times K_{av}$ ($R^2 = 0.9398$), where $K_{av} = (V_e\text{-}V_0)/(V_t\text{-}V_0)$ and V_e = elution volume.

ASSAYS WERE PERFORMED IN DUPLICATE.

Antithrombotic activity: The ability to inhibit the coagulation of fibrinogen (Sigma-Aldrich) was measured using the microplate method according to Zhang et al. [20]. Antithrombotic activity of digested amaranth lemon sorbet was studied and calculations of inhibitory effects were performed according to Sabbione et al. [21]. The IC_{50} value to produce a clotting inhibition of 50% was determined. Biological assay was performed at least in quadruplicate. The soluble protein content was measured by the Lowry colorimetric method [22].

Statistical Analysis

Results were evaluated using analysis of variance (ANOVA). Student test comparison of means was performed ($p < 0.05$). IC_{50} values were calculated from % Inhibition clot formation vs Protein concentration curves fitted (linear or nonlinear regression-sigmoidal dose-response variable slope) with GraphPad Prism 5 program (San Diego, CA, USA). CATA sensory analysis results were processed using multivariate statistical technique principal component analysis (PCA) with Infostat statistical software (Córdoba, Argentina).

Results and Discussion

In order to define the amount of amaranth isolate used in the ALS formulation, the concentration of proteins in egg white was considered as a reference value (126.8 mg protein/mL). Four different dispersions of API in lemon juice (123.2, 82.8, 35.2 and 18.5 mg protein/mL) were used to obtain foams and compared with egg white ones. The amaranth protein dispersion selected to formulate ALS was 35.2 mg/mL of API since the foam obtained was similar to that obtained with whipped egg white, with an adequate consistency of the foam (results not shown).

Lemon Sorbets Physicochemical and Sensorial Characterization

Composition of API (% *w*/w), 79 ± 1 proteins, 9.8 ± 0.3 carbohydrates, 3.5 ± 0.1 H_2O and 3.3 ± 0.5 ashes, was similar to that informed by Condés et al. [23]. Total amount of 4.2% w/w of fiber was estimated by difference. ALS and TLS sorbets showed similar centesimal composition (Table 1), with a significant difference in the protein content ($p < 0.05$), as these two sorbets differ in their protein source, one elaborated with API and the other one with egg white.

Lemon sorbet base mixes rheology: Fig. 1 shows shear stress (**a**) and apparent viscosity (**b**) as a function of base mixes share rate. In Fig. 1a it is observed that the flow behavior of base mixes corresponds to pseudoplastic type fluids. The base mix of ALS presented a typical behavior of this type of fluids, with a decrease in apparent viscosity as the shear rate increases (Fig. 1b). In addition, the flow behavior of this base mix shows a large area of hysteresis (Table 1), with a higher apparent viscosity in the curve in which the share rate increases (Fig. 1b), indicating that ALS mix base behaves like

Table 1 Centesimal composition (% *w*/w), base mixes rheology parameters, superficial color parameters and overrun values of amaranth lemon sorbet (ALS) and traditional lemon sorbet (TLS)

		ALS	TLS
Centesimal composition	Proteins	0.40 ± 0.03^{b}	0.21 ± 0.01^{a}
	Carbohydrates	27.4 ± 1.1^{b}	31.0 ± 1.0^{b}
	H_2O	71.6 ± 0.2^{b}	72.6 ± 0.5^{b}
	Ashes	0.04 ± 0.01^{a}	0.05 ± 0.02^{a}
Base mixes rheology	η app (Pa.s) at $\dot{\gamma}$ 50 1/s	0.42 ± 0.07^{a}	0.11 ± 0.02^{b}
	Hysteresis Area (Pa/s)	7100 ± 700^{b}	0^{a}
Sorbet superficial color	L*	73.91 ± 2.05^{b}	71.57 ± 2.41^{a}
	a*	-0.88 ± 0.02^{a}	-0.84 ± 0.05^{a}
	b*	4.5 ± 0.34^{b}	2.22 ± 0.21^{a}
Sorbet overrun		36.15 ± 3.48^{a}	14.08 ± 3.43^{b}

Different superscript letter in the same line corresponded to different values (LSD Fisher, $p < 0.05$). η app: apparent viscosity. Surface color parameters L*: lightness, a*: redness, b* yellowness

a thixotropic fluid. In contrast, the TLS base mix differentiates less from the newtonian behavior, without dependence with time, since it shows no hysteresis area. According to Marshall et al., base mixes exhibiting a pseudoplastic and thixotropic behavior are better for sorbet elaboration [4] suggesting that amaranth base mixes present adequate rheological characteristics. Table 1 shows Kokini's apparent viscosity of sorbet base mixes (shear rate 50 1/s), representing the feeling of low-viscosity melting foods in the mouth [24] which correlates with thickness characteristics in these products [25]. ALS presented a significantly higher value of Kokini viscosity (0.42 ± 0.07 Pa.s, Table 1) compared with TLS. Base mix viscosity value provides relevant information since it was believed that a high viscosity was essential for sorbets elaboration, but for fast freezing- rapid whipping- in modern equipment a lower viscosity seems desirable. However, a certain level of viscosity is essential for proper whipping and retention of air, and good body and texture in sorbets [26].

Superficial color: ALS and TLS lightness, redness, and yellowness were measured to evaluate color differences. Table 1 shows no significant difference of a* parameter ($p > 0.05$) between the sorbets but exhibited a significant difference in lightness and yellowness, L* and b*. Results indicate that ALS color was similar to TLS with a slightly yellowish hue. Both sorbets presented medium to high lightness. Color difference between sorbets, ΔE* value, was 3.16. The color difference between the sorbets was slightly perceptible since a $\Delta E^* < 3$ is undetectable by the human eye.

Overrun: Aeration is a very important characteristic in sorbets elaboration because it is directly related to the achieved texture. If the amount of air is excessive it can cause a strong detriment of the final quality of the product, since sorbets with high amount of air can give a low consistency sensation with no thickness that melts in the mouth. On the contrary, a sorbet with little air incorporated gives a heavy sensation in the mouth. Good quality sorbets should have aeration rates in the range of 30 to 50% (Ice cream elaboration guide, Di Bartolo Eduardo 2005). Table 1 shows overrun values of ALS 2.5 times higher compared with TLS, indicating that amaranth proteins incorporated in acid conditions during sorbet elaboration showed outstanding foaming properties [12].

Melting test at different storage times: A manifestation of the structure of the sorbet is the measure of their stability. Melt-down rate study was performed in freshly made ALS

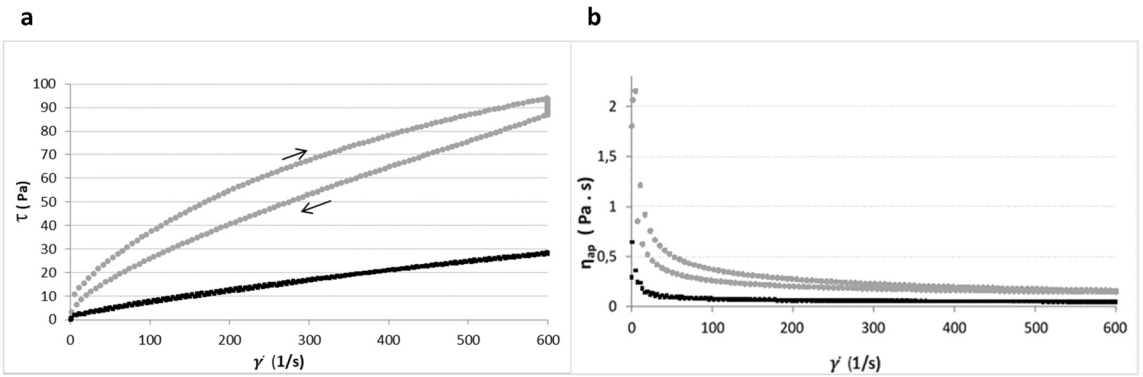

Fig. 1 **a** Shear stress as a function of share rate of amaranth lemon sorbet (ALS, ●) and traditional lemon sorbet (TLS, ■) base mixes. **b** Apparent viscosity as a function of share rate of ALS (●) and TLS (■) base mixes

and TLS, and in ALS stored at −20 °C for 2 and 5 months to study the effects of sorbet storage over time on melting properties. Figure 2 shows sorbet dripped volume as a function of time. ALS and TLS freshly made melt-down curves exhibited different behavior, presenting ALS a better stability behavior. The ALS with the slowest thawing was the fresh one, followed by the 2-month storage ALS. Melting kinetics of these two samples was similar, but in fresh ALS 10 mL were drained in a significantly longer time compared to 2-month storage ALS, 43.1 and 38.1 min, respectively ($p < 0.05$, Fig. 2). This indicates that changes occurred in the structure of ALS during storage, which are reflected only after 30 min of study and do not result in significant losses of stability. On the contrary, 5-month storage ALS presented a higher melting rate, with a dripping volume of 5 mL at 25 min, a similar melting kinetic to fresh TLS. In this sense, 5-month storage of ALS implies significant structural changes that affect melting resistance and consequently, the stability of the product. ALS is a sorbet without lipids, and changes on melting properties could be related to the disproportionation of air cells during long-term storage leading to interconnections between air cells and collapse. Chang and Hartel [26] described this phenomenon in vanilla ice creams at different storage times and showed the disproportionation of air cells growing until they contacted other air cells in the vicinity. Larger storage times implied individual air cells losing boundaries as channeling began to take place with the increasing irregularity of the collapsed air cells. In addition, Muse and Hartel [27] found that ice creams with low overrun melted quickly, whereas ice creams with high overrun began to melt slowly and had a good melting resistance. This slower melting rate in sorbets with high overrun could be attributed to a reduced rate of heat transfer due to a larger volume of air but it could also be due to the more tortuous path through which the melting fluid must flow [4].

Sensory study: Acceptability and CATA test were performed to study sensory characteristics of ALS and TLS. An untrained panel of 60 habitual consumers of lemon sorbets, adults between 20 and 60 years old, 25% women, was provided with 25 g of lemon sorbet placed in a 60 ml lidded styrofoam container in a controlled temperature room (25 °C). Acceptability test was performed using a hedonic scale from 1 to 10 to globally evaluate ALS and TLS. Mean values of the acceptability levels were 8 ± 1 for both sorbets, showing no significant difference between them, both with good overall acceptability. Figure 3a shows consumers' frequency of 60 panelists in the 1–10 hedonic scale for overall acceptability in ALS and TLS. Ratings on the hedonic scale did not vary between the samples, and a similar distribution of frequency was observed in both sorbets (Fig. 3a).

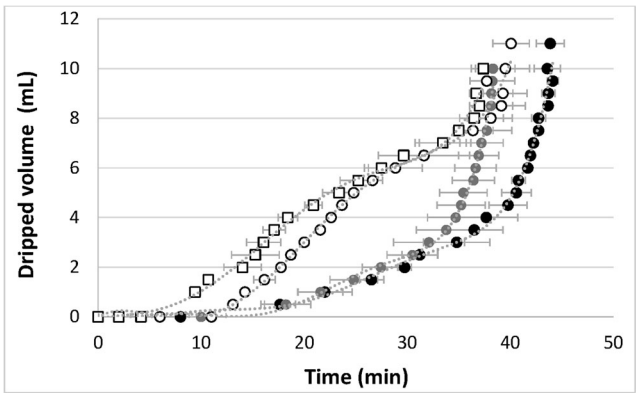

Fig. 2 Melting kinetics of traditional (TLS, □) and amaranth lemon sorbets (ALS) at different storage times: fresh ALS (●), 2-month storage ALS (●), 5-month storage ALS (○) 25 °C

CATA sensory evaluation methodology is a technique with great potential that has been initially designed and used in the marketing field to obtain rapid product profiles from consumers. CATA assay implied panelists identification and assessment of specific characteristics of ALS and TLS by choosing from the provided list everything that applies to these products. 14 possible attributes were selected: sensory (lemon taste, sweetness, acidity, metallic flavor, refreshing capacity, presence of crystals, aeration), non-sensory (healthy), and hedonic terms (delicious, unpleasant). CATA results are shown in Fig. 3b and c. The most selected attribute in both sorbets was refreshing, followed by delicious. Consumers showed a marked preference for ALS in attributes associated with sorbets quality: 32% selected ALS creamy attribute, while only 8% rated the TLS in the same way, and 17% considered ALS was airy while only 3% selected this characteristic for TLS (Fig. 3b). ALS base mix presented a higher Kokini viscosity (Table 1), representing thickness characteristics, and through CATA sensory test it was reflected how this rheology parameter correlates with the "creamy sensation" perceived by consumers. Moreover, ALS higher overrun would explain why 17% of the panel chose airy as an attribute associated with this sorbet. In this sense, better foaming properties of amaranth proteins summited to low pHs [12] are reflected in the panelist's creamy perception of ALS. Regarding healthy attributes, 45% considered ALS as a healthy sorbet, while only 7% chose this characteristic for TLS (Fig. 3b) due to the presence of bioactive peptides within amaranth proteins [9]. The presence of crystals is a negative characteristic associated with the texture of sorbets, as it does not produce a pleasant sensation to the palate [28]. 32% of the panelists considered that ALS had this defect, and a higher percentage, 52%, perceived it when consuming TLS (Fig. 3b). Since the homemade ice cream maker took approximately 4 h to freeze base mixes, a slow formation of crystals may have occurred, producing large size crystals associated with this attribute.

PCA analysis was performed to have a global sight of CATA assay results and a good representation of sensory characteristics. PCA determines a projection of the data into orthogonal dimensions, thus they sequentially represent as much of the variation in the data as possible. Figure 3c

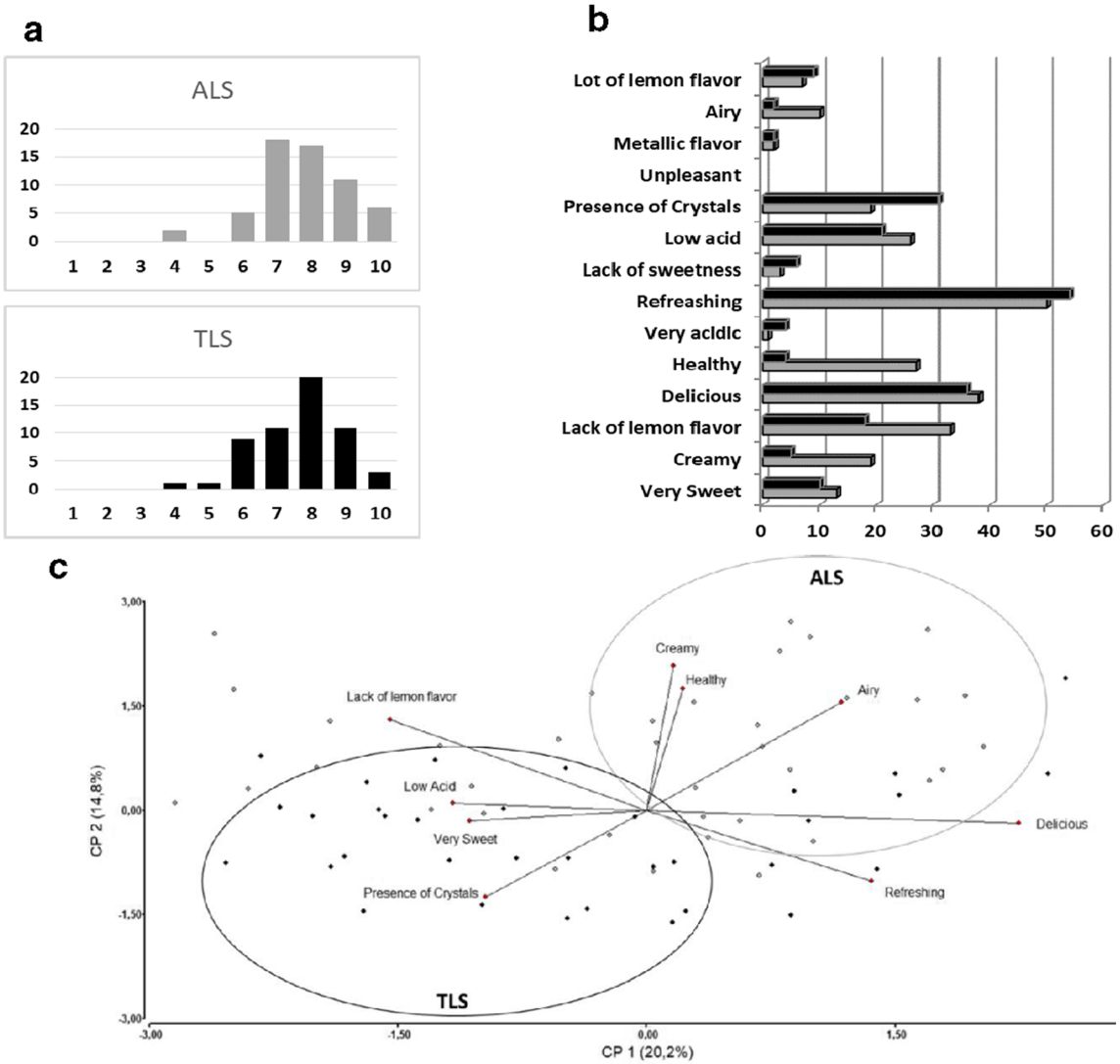

Fig. 3 Sensory studies. **a** Consumer frequency of 60 panelists in the 1–10 hedonic scale for overall acceptability in amaranth (ALS, ●) and traditional lemon sorbets (TLS, ●). **b** Consumer frequency in each attribute category of CATA test in ALS (●) and TLS (●). **c** PCA-biplot of sensory analysis CATA test of ALS (●) and TLS (●)

presents PCA-biplot of CATA sensory analysis test of ALS and TLS. In order to eliminate noise, only those attributes found in more than 15% in sorbets were considered in this analysis [29]. Differences between the samples according to their sensory parameters were evidenced, and it was possible to recognize which attributes characterized in a more significant way ALS and TLS. The interpretation of PCA was limited to the first two axes, which explained 35% of total variance. Figure 3c shows ALS was associated with creamy, airy and healthy attributes, while TLS was associated with the presence of crystals, low acid and very sweet. Moreover, Fig. 3c shows according to the angles between vectors, a correlation between sorbet characteristics such as lack of lemon flavor, low acid and very sweet. Alternatively, creamy and airy attributes were also related.

Lemon Sorbet Functional Characterization

Tricine SDS-PAGE gel electrophoresis: Fig. 4a shows electrophoretic profiles of API, API incubated for 1 h at 25 °C with lemon juice (API + L), ALS and ALS subjected to simulated gastrointestinal digestion (ALSd). API presents many characteristic bands of amaranth constituent polypeptides 11S globulin, globulin P and globulin 7S, previously described by other authors [14, 30]. Incubation of the isolate with lemon juice (API + L) modifies the electrophoretic profile, causing a decrease in intensity and disappearance of some bands above 30 kDa, increase in intensity or even the appearance of bands at molecular weights regions below 15 kDa (Fig. 4a). Changes in the electrophoretic profile of API + L could be a consequence of joint action of the pH and the activity of an

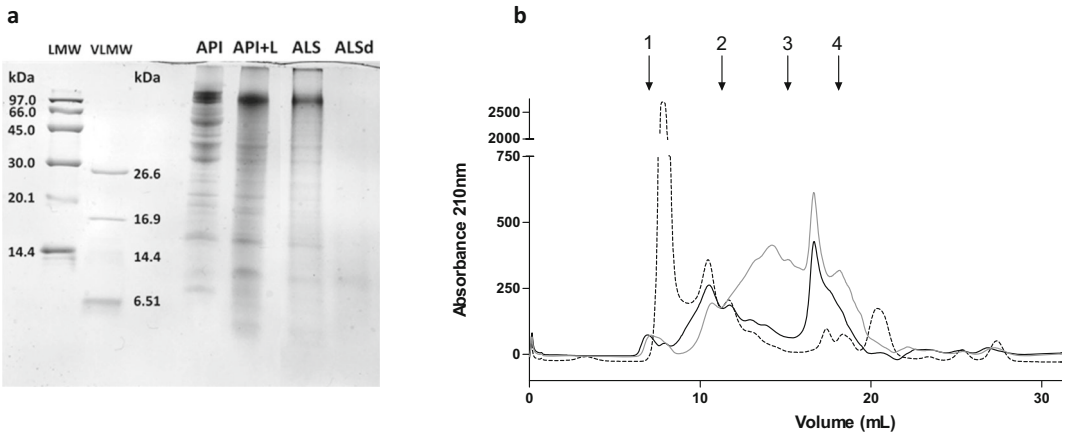

Fig. 4 **a** Tricine-SDS-PAGE. Amaranth protein isolate (API), amaranth protein isolate incubated for 1 h with lemon juice (API + L), amaranth lemon sorbet (ALS) and amaranth lemon sorbet after performing simulated gastrointestinal digestion (ALSd). LMW: low molecular weight standards. VLMW: very low molecular weight standards. **b** Gel filtration liquid chromatography of API (– –), ALS (—) and ALSd (—). Superdex Peptide 10/300GL column. Calibration standards: aprotinin (2), B12 vitamin (3), hippuric acid (4). Exclusion volume: blue dextran (1)

amaranth aspartic protease, active in a limited pH range (optimum pH = 2) that partially hydrolyzes amaranth proteins [10]. While the electrophoretic profile of the sorbet (ALS) is more tenuous, but identical to API + L, simulated gastrointestinal digestion affects sensitively sorbet proteins. ALSd electrophoresis profile gets modified: many bands disappear, except one of approximately 10 kDa, and others very faint at lower molecular weights. This indicates that the polypeptides are hydrolyzed by the digestive proteases and that the hydrolysis products have a molecular mass that is below the resolution of this electrophoretic system.

Molecular exclusion chromatography: Molecular composition of amaranth proteins and peptides were analyzed by gel filtration chromatography. The amount of protein mass loaded was normalized in the samples to compare increments in the relative intensity of the molecules. Figure 4b shows chromatographic profiles of API, ALS, and ALSd. API profile presents a significant peak corresponding to the exclusion volume (molecular mass > 10 kDa, 7–9 mL). This sample was previously analyzed using FPLC [21], demonstrating the presence of characteristic high molecular polymers, between 280 and 360 kDa for globulin P and 11S, between 180 and 220 kDa for 7S-globulin molecules and below 100 kDa for albumins [14, 30]. Since the exclusion limit of the column used is 10 kDa, all these characteristic polymers were eluted within the exclusion volume peak observed. API chromatogram presents a peak of approximately 6.5 kDa and other smaller peaks below 2 kDa. ALS chromatogram profile (Fig. 4b) evidences that amaranth proteins suffered important modifications after 1 h lemon juice treatment during ALS elaboration: exclusion volume peak significantly decrease indicating that amaranth conformational proteins were not only dissociated and denatured due to lemon acid medium but also hydrolyzed by the endogenous aspartic protease [10]. Moreover, API and ALS chromatograms present a similar peak of approximately 6.5 kDa, but at higher volumes, between 15 and 20 mL, ALS exhibits an increase of components below 2 kDa, confirming that protein hydrolysis occurred during sorbet elaboration, in concordance with Tricine-SDS-PAGE results (Fig. 4a). ALSd chromatogram shows that high molecular mass protein species that corresponded to the exclusion volume peak (API in Fig. 4b) practically disappear, remaining only small peaks. In addition, the proportion of peptides eluting in approximately 10 mL (>6.5 kDa molecular mass) significantly decreased in comparison with API and ALS profiles, while the area of the peak in the latter zone of the chromatogram, between 12 and 20 mL, significantly increased in molecules with masses <6.5 kDa (ALSd, Fig. 4b). This behavior evidences that SGD was effective causing an important increment in protein species of molecular masses below 2 kDa. ALSd chromatography results agree with those obtained in tricine-SDS-PAGE (Fig. 4a), where bands corresponding to high molecular masses disappear and only some fainted bands of very low molecular mass were observed.

Antithrombotic activity: Potential antithrombotic activity of ALSd was evaluated using the microplates method. The assay emulates common pathway of blood coagulation, where thrombin proteolyzes fibrinogen substrate to produce fibrin monomers that subsequently polymerize. The formation of a fibrin clot is evidenced by measuring the turbidity at 405 nm, and the ability to prevent the formation of the fibrin clot is evaluated in different samples according to that diminution. Figure 5 shows ALSd curve of inhibition of fibrin clot formation as a function of soluble protein concentration. The digested sorbet exhibited inhibitory activity at 2.5 mg soluble protein/mL, and as concentrations were higher the inhibition of clot formation increased, showing a dose-response behavior until reaching a plateau at approximately 4 mg/mL. The protein concentration that inhibits 50% of fibrin clot formation (IC_{50}) was 3.4 ± 0.1 mg protein/mL, a value within the range

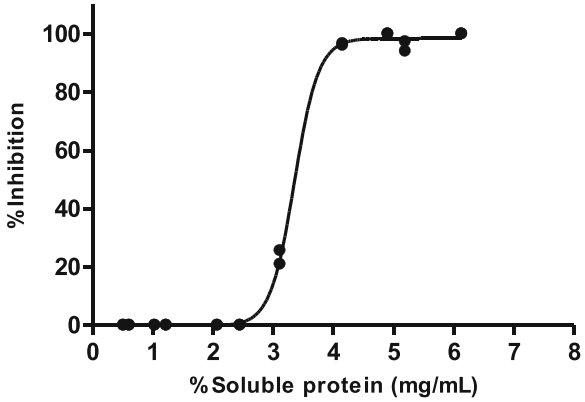

Fig. 5 Antithrombotic activity. Percentage of inhibition of clot formation as a function of soluble protein concentration of amaranth lemon sorbet after performing simulated gastrointestinal digestion (ALSd)

of IC_{50} values obtained by Kee et al. [31], who studied the antithrombotic activity of organic and aqueous extracts from a large number of South African plants and obtained IC_{50} values between 2.5 and 25 mg protein/mL. These results are also comparable to those reported by Zhang et al. [20], with IC_{50} of 30 mg protein/mL for rapeseed proteins. Moreover, digested cookies elaborated with amaranth flour exhibited antithrombotic activity, with an IC_{50} of approximately 0.25 mg protein/mL [32]. In this sense, amaranth proteins proved to be an interesting source of bioactive peptides that can be released after performing a SGD in different food matrixes, confirming its potential as a functional ingredient. The inhibitory action of amaranth peptides may be due to the union to the catalytic site or to the binding exosites of thrombin, preventing the proteolysis of the fibrinogen, or to its binding to already generated fibrin monomers preventing its polymerization [9]. In contrast, API and ALS proteins did not show a potential inhibition of coagulation in a 1–10 mg/mL soluble protein concentration. In this sense, results confirm that once submitted to SGD proteins exert *in vitro* antithrombotic activity, indicating that proteolysis releases potentially bioactive peptides that are encoded in native amaranth proteins [21]. Furthermore, it is important to highlight that these active peptides would resist the passage through the gastrointestinal tract, a desired characteristic in functional ingredients.

Conclusions

A functional lemon sorbet with suitable physicochemical characteristics was elaborated with amaranth proteins. Overrun was within desired values for sorbets and stability study evidenced that this system does not undergo major structural changes during 2-month storage. The sorbet also presented acceptable sensory characteristics, associated with creamy, airy and healthy attributes. Furthermore, simulated gastrointestinal digestion of ALS released potentially bioactive peptides able to inhibit fibrin clot formation *in vitro*, indicating that this sorbet could be an interesting alternative to improve consumer's well-being.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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