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Alkaloid content and taste profile assessed by electronic tongue of Lupinus albus seeds debittered by different methods

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Abstract:	Lupin seeds are rich in proteins, lipids and bioactive compounds, but before consumption they need to be debittered to remove toxic alkaloids. Traditionally, debittering is a water-intensive and protracted process, lasting up to six days. To develop a more efficient procedure, different washing solutions (0.5% and 1% NaCl or citric acid), with or without the use of ultrasound, were applied on Lupinus albus seeds and compared to two established methods, the first with water (traditional), and the second with a sodium chloride solution (reference). The sonication did not accelerate debittering, while the sodium chloride and citric acid solutions significantly shortened debittering time, reduced water consumption and decreased alkaloid content to commercial values (0.31-1.03 g/kg dry matter). Debittering with a 1% citric acid solution saved 88 h and 65 L water/kg dry lupin compared to the traditional method, and 13 h and 31 L water/kg dry lupin compared to the salt solution reference method. The electronic tongue grouped the experimental and commercial samples in well-defined clusters; bitter and umami tastes were the main factors, well correlated with alkaloid content.
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Dear Editor

We are submitting you our article “Alkaloid content and taste profile assessed by electronic tongue of Lupinus albus seeds debittered by different methods” for a possible publication in Journal of Food Composition and Analysis.

Lupin seeds are a promising food resource because they have high content of proteins, unsaturated lipids and bioactive compounds. However, they also contain bitter (and often toxic) quinolizidine alkaloids, removed before consumption by a water-intensive and time-consuming process.

In our work, we propose an experimental debittering method using different solvents with or without supplementary sonication. We analysed the alkaloid content as well as the taste profile (by electronic tongue) of white lupin seeds before and after debittering by different methods. We found that sonication did not accelerate debittering, but that the use of sodium chloride or citric acid solutions (instead of water) significantly shortened debittering time, reduced water consumption and decreased alkaloid concentration. In particular, debittering with a 1% citric acid solution saved 88 h and 65 L water/kg dry lupin compared to the traditional method with water. The electronic tongue profile grouped the experimental and control commercial samples in well-defined clusters, mainly defined by bitter and umami tastes, which were correlated with alkaloid content.

In conclusion, our best innovative method (washing with 1% citric acid solution) is a step forward in lupin debittering because significantly reduces water consumption and decreases treatment time, thus helping both the processing industry and the environment.

We hope you will find our article suitable for publication in Journal of Food Composition and Analysis.

Waiting for your kind reply in due time

Best regards

Andrea Brandolini

Highlights

Lupin seeds must be debittered before consumption to remove toxic alkaloids

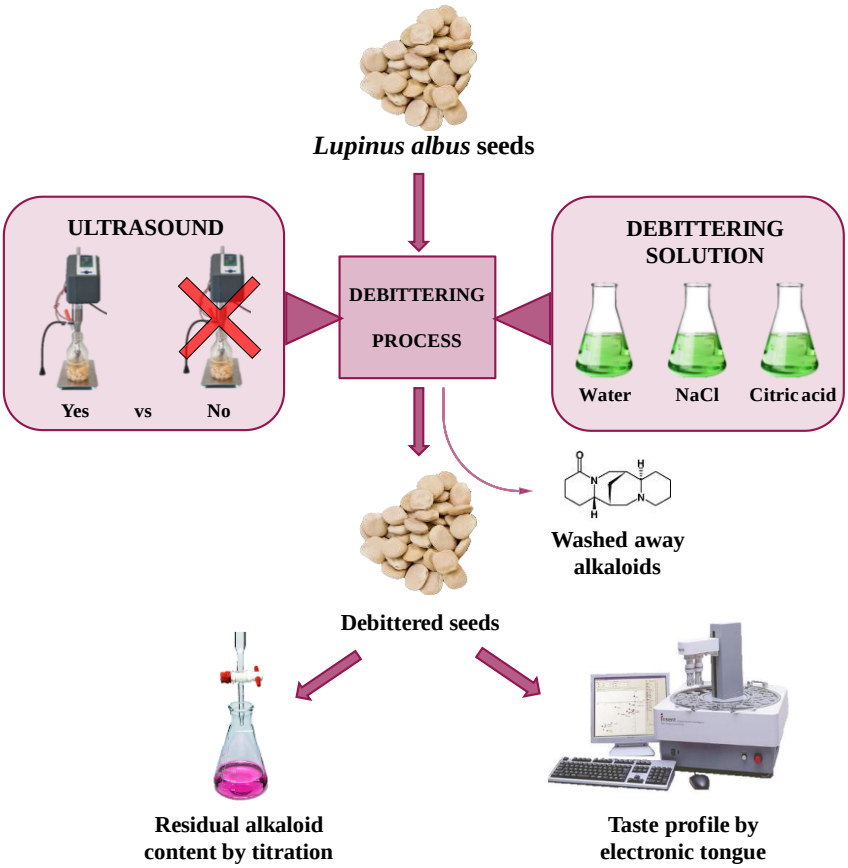
Different debittering solutions of NaCl or citric acid were tested on *Lupinus albus*

Low frequency ultrasound effect was assessed, but did not improved debittering

The citric acid solutions shortened debittering time and reduced water consumption

The e-tongue found that bitter and umami sensors were correlated to alkaloid content

Graphical Abstract



Alkaloid content and taste profile assessed by electronic tongue of *Lupinus albus* seeds debittered by different methods

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ABSTRACT

Lupin seeds are rich in proteins, lipids and bioactive compounds, but before consumption they need to be debittered to remove toxic alkaloids. Traditionally, debittering is a water-intensive and protracted process, lasting up to six days. To develop a more efficient procedure, different washing solutions (0.5% and 1% NaCl or citric acid), with or without the use of ultrasound, were applied on *Lupinus albus* seeds and compared to two established methods, the first with water (traditional), and the second with a sodium chloride solution (reference). The sonication did not accelerate debittering, while the sodium chloride and citric acid solutions significantly shortened debittering time, reduced water consumption and decreased alkaloid content to commercial values (0.31-1.03 g/kg dry matter). Debittering with a 1% citric acid solution saved 88 h and 65 L water/kg dry lupin compared to the traditional method, and 13 h and 31 L water/kg dry lupin compared to the salt solution reference method. The electronic tongue grouped the experimental and commercial samples in well-defined clusters; bitter and umami tastes were the main factors, well correlated with alkaloid content.

Keywords: bitterness, citric acid, electronic tongue, sodium chloride, ultrasound, white lupin.

1. Introduction

Lupin seeds are a promising food resource for their high content of proteins, unsaturated lipids and bioactive compounds (Briceño Berru et al., 2021; Brandolini et al., 2022). They can be consumed as snack or side dish; otherwise, lupin flour may be used to prepare sauces, vegetable milk, cheese and meat, fermented products (tofu, tempeh, etc.), pasta and noodles, baked goods (cakes, biscuits, tortillas, muffins and bread, etc.), etc. (Güémes-Vera et al., 2008; Jayasena et al., 2010a; Jayasena et al., 2010b; Albuja-Vaca et al., 2019; Al-Saedi et al., 2020; Özcan et al., 2021). Furthermore, lupin seeds are a good source of oil (Fontanari et al., 2017; Pascual-Chagman et al., 2019) as well as of protein isolates (Lo et al., 2021) that, besides the intrinsic nutritional value, have physical and functional properties comparable to soybean and boast interesting technological properties like water and oil absorption, emulsifying, foaming, gelling and stabilizing capacity (Pollard et al., 2002; Lampart-Szczapa et al., 2006; Duranti et al., 2008; Paraskevopoulou et al., 2010; Alu'datt et al., 2017; Lo et al., 2021). Lupin flours lack gluten-forming proteins hence they can be used in gluten-free foods for people suffering from celiac disease.

The greatest obstacle to lupin utilisation in human and animal nutrition is the high content of bitter (and often toxic) quinolizidine alkaloids, which grant the plant protection against pathogens and pests. To reduce alkaloid content and make the seeds suitable for consumption, appropriate technological processes have been developed. In parallel, varieties with low alkaloid levels have been selected (Muzquiz et al., 1994; Uauy et al., 1995; Kroc et al., 2019). This last strategy, however, is still of limited effectiveness since the so-called “sweet” varieties are less productive, more susceptible to pests and diseases, and tend to reacquire their bitterness over time (Uauy et al., 1995).

The most widespread debittering methods rely on extracting the alkaloids from whole seeds using water as solvent. However, debittering triggers changes in the chemical composition of

the seeds because many water-soluble molecules are washed away. Compared to non-debittered seeds, the contents in minerals, fibre, carbohydrates and oligosaccharides decrease during the process; conversely, protein and lipid levels increase for a concentration effect due to the removal of the other compounds (Erbaş, 2010; Carvajal-Larenas et al., 2016; Córdova-Ramos et al., 2020).

The traditional debittering method starts with preliminary hydration in water, followed by cooking and repeated washings for several days (Erbaş, 2010; Carvajal-Larenas et al., 2014). Besides disrupting cell walls and facilitating alkaloids removal, cooking blocks germination, coagulates proteins, inactivates enzymes and sanitises the product (Gross et al., 1983; Carvajal-Larenas et al., 2013).

To shorten processing times and save water, several improved debittering methods have been proposed. Generally, they include longer boiling times (Jiménez-Martínez et al., 2001), post-cooking washing with warm water (Fontanari et al., 2012), different rinsing solutions (Jiménez-Martínez et al., 2001; Mohammed et al., 2016; Villacrés et al., 2020), germination (Cortes Sánchez et al., 2005; Mohammed et al., 2016), fermentation (Jiménez-Martínez et al., 2007) or their combination (Erbaş, 2010; Jiménez-Martínez et al., 2010). Recently, low frequency ultrasound (US) has been tested with promising results on lupin seeds hydration and debittering (Miano et al., 2019; Yaver & Bilgiçli, 2021). The US technology is already employed by the food processing industry to accelerate many operations such as compounds extraction and products homogenization, filtration, dehydration, freezing, thawing and sanitization. (Schmidt et al., 2019; Bhargava et al., 2021; Mohammadi et al., 2021). Furthermore, US was very effective in improving the yield and reducing the duration of micro- and macromolecules extraction (Estivi et al., 2022).

The aim of this work was therefore to improve the debittering process of *Lupinus albus* seeds using different solvents (water and solutions with 0.5% or 1.0% of NaCl or citric acid),

either with or without ultrasound assistance; an additional aim was to evaluate the main factors related to the taste of debittered samples. To achieve these goals, the alkaloid content of lupin seeds debittered for varying soaking times with the different solvents and ultrasound was determined, and their taste profile was assessed by the electronic tongue (e-tongue).

2. Materials and methods

2.1. Materials

Two different lots (Lot 1 and Lot 2) of *Lupinus albus* seeds from Chile, purchased from Colombo Legumi e Frutta Secca (Modica, Italy), were analysed. Four commercial debittered *L. albus* snacks (C1 and C2, without liquid in sealed plastic boxes; C3, in brine in sealed plastic bag; C4, without liquid under vacuum bag), were collected from Milan (Italy) supermarkets. All the reagents were purchased from Sigma-Aldrich (Burlington, USA).

2.2. Methods

2.1. Seeds debittering

Two trials were performed to assess:

1) Influence of sonication (with or without) and solvent (water, NaCl 1% or citric acid 1%) on alkaloid content: the seeds of Lot 1 and Lot 2 were debittered applying the experimental method for two different soaking times (28.5 h and 45 h, respectively);

2) Effect of solvent (water, NaCl 0.5%, NaCl 1%, citric acid 0.5% or citric acid 1%) and soaking time (45, 57 or 69 h) on alkaloid content and taste profile: the seeds of Lot 2 were debittered by the experimental method, without sonication. Seeds debittered by the traditional (water) and the reference (sodium chloride solution) methods, as well as four commercial samples, were also considered.

Traditional method, with water - 100 g of seeds were debittered according to Erbaş (2010), with the minor modifications made by Córdova-Ramos et al. (2020). After a hydration phase (1:6 w/v seeds:water ratio) for 12 h at room temperature, the seeds were cooked in boiling water (hydrated seeds:water 1:3 w/v) for 1 h, changing the water after 30 min, and finally washed by water soaking (cooked seeds:water 1:3 w/v) for 5 days at room temperature, replacing the water every 12 h.

Reference method, with sodium chloride solution - 100 g of seeds were debittered as described by Villacrés et al. (2020). After an 8 h hydration at 80 °C (seeds:water with 0.5% NaCl, ratio 1:3 w/v), the solution was replaced and the seeds were cooked for 1 h at 91 °C (1:3 w/v), renewing the solvent halfway through cooking. Five washes were then carried out using water with 0.5% NaCl at 35 °C up to 28 h (first and second wash: 1:15 w/v, 3 h each; third wash: 1:5 w/v, 16 h; fourth wash: 1:7.5 w/v, 3 h; fifth wash: 1:5 w/v, 3 h). Finally, two washes with water at 18 °C (seeds:water 1:5 w/v), the first lasting 18 h and the second 3 h, were performed to remove excess salinity.

Experimental method - The experimental debittering method proposed in this study (Figure 1) was developed and fine-tuned after several preliminary trials (not shown). Five different debittering solutions were tested: distilled water, NaCl 0.5% and 1%, citric acid 0.5% and 1%. Additionally, two different treatments were tested: without and with ultrasound. The UP400St US homogeneiser (Hielscher Ultrasonics GmbH, Teltow, Germany), operating at 24 kHz and mounting a 14 mm diameter probe, was used to treat the seeds during hydration (60% amplitude), cooking (100% amplitude) and washing (60% amplitude). The net power of the probe, determined by the calorimetric method as reported by Margulis and Margulis (2003) sonicating distilled water at the tested combinations of temperature and amplitude, was equal to 65.0 ± 3.3 W at 50 °C and 60% amplitude, and to 26.9 ± 1.7 W at 95 °C and 100% amplitude.

After an initial hydration phase of 30 min at 50 °C (Figure 1), performed either without or with US, 100 g seeds were soaked for 1 h and then cooked for 30 min at 95 °C, without or with US. Soaking and cooking steps were repeated after replacing the solvent. Afterwards, the solvent was changed again and a series of soaking at room temperature/washing for 30 min at 50 °C (without or with US) was carried out, renewing the solvent according to the timeline in Figure 1. After the last washing (i.e., t_0 = 28.5 h), from 0 to 3 soakings with distilled water were carried out for 12 h, to complete the debittering and remove salt or citric acid from the seeds. A dry seeds:solvent 1:5 (w/v) ratio was always employed.

For the Lot 1 seeds the final rinsing was done at 25.5 h, to interrupt the process after 28.5 h. For Lot 2, samples were taken at the end of the first (t_1 = 45 h), second (t_2 = 57 h) and third (t_3 = 69 h) final soaking.

The debittered seeds debittered with/by the aforementioned methods were dried as follows: the seeds of trial 1 were dried 8 h at 60 °C in a Venticell 55 ventilated oven (MMM-Group, Planegg/München, Germany); the seeds of trial 2 were lyophilised under vacuum (10^{-2} mbar) in an Edwards 304 freeze dryer (Atlas Copco, Stockholm, Sweden). The dried seeds were ground with an MDI 204 disc mill (Buhler, Uzvil, Switzerland) and stored in the freezer until the time of analysis.

2.2. Analyses

2.2.1. Seeds characteristics

The 100-seeds weight was determined by weighing three independent replicates on an E154 analytical balance (Gibertini Elettronica S.R.L., Novate Milanese, Italy). Seed size (larger size, smaller size and thickness) was measured on 100-seeds using a CD-15DC calliper (Mitutoyo Italiana S.R.L., Lainate, Italy). The moisture was determined gravimetrically according to the official method AOAC 925.10 (AOAC, 2000).

140

141 *2.2.2 Alkaloid content*

142 The alkaloids quantification was performed in triplicate by colorimetric titration as
143 described by von Baer, Reimerdes, and Feldheim (1979), with some modifications. Briefly,
144 exactly 0.6 g of sample was weighed in a mortar, 0.6 mL of 15% potassium hydroxide and 1.8
145 g of basic aluminium oxide were added, and the mix was homogenized manually. The sample
146 was then transferred to a 25 mL glass tube with a screw cap, 10 mL of chloroform were added,
147 and the contents were shaken with a TX4 Digital IR Vortex Mixer (VELP Scientifica, Usmate
148 Velate, Italy) for 1 min. After sedimentation, the supernatant containing the alkaloids was
149 filtered through paper and collected in a 100 mL flat-bottomed flask; the extraction was
150 repeated several times, until 1 mL of the most recent extract, dried with nitrogen and
151 resuspended in 5 drops of 0.01 N sulfuric acid, had a negative reaction with 4 drops of
152 Dragendorff's reagent. The extracts were joined and evaporated under vacuum using a Laborota
153 4000 Efficient rotavapor (Heidolph, Schwabach, Germany) at 35 °C for about 8 min. For
154 titration, 5 mL of 0.01 N sulfuric acid were added to the flask and the flask was gently mixed.
155 The excess acid was titrated in the presence of 4 drops of methyl red with 0.01 N sodium
156 hydroxide by means of a 10 mL glass burette. The volume (mL) of sodium hydroxide
157 consumed during the titration (V_{NaOH}) was measured and the alkaloid content, expressed as
158 g/kg dry matter (DM), was calculated using the following formula, considering that 1 mL of
159 0.01 N sulfuric acid equals 2.48 mg of lupanine (INEN, 2005):

160 $$\text{Alkaloids (g/kg DM)} = \frac{2.48 \cdot [5 - \text{V}_{\text{NaOH}}\ \text{(mL)}]}{\text{sample weight (g)} \cdot [1 - \text{moisture (\%)}]}$$

161 An empirical evaluation of the residual bitterness was also performed in all the trials by
162 tasting the seeds.

163

164 *2.2.3. Electronic tongue*

E-tongue measurements were carried out by Taste-Sensing System SA 402B (Intelligent Sensor Technology Co., Atsugi City, Japan). The system consists of sensors whose surface is attached with artificial lipid membranes having different response properties to chemical compounds on the basis of their taste. In this work five detecting sensors (CA0; CT0; C00; AAE; AE1) and two reference electrodes were used, separated in two arrays according to membrane charge.

Samples preparation was performed according to Marengo et al. (2016), with some modifications. Exactly 3 g sample were weighed into a 500 mL centrifuge bottle, and 100 mL of a 10 mM potassium chloride (KCl) solution was added and stirred with a digital Ultra-Turrax T25 homogenizer (IKA, Germany) at 15000 rpm for 2 min. The homogenizer probe was washed with 20 mL of 10 mM KCl and collected in the bottle to obtain a sample/solvent ratio of 1:40 (w/v). The tube was centrifuged with a RC5B Plus centrifuge (Sorvall, USA) at 6000 rpm (6085 *g*) for 5 min at room temperature. The supernatant, containing the extract, was filtered on paper filters and analysed by e-tongue.

The detecting sensors and the reference electrodes were first dipped into the reference solution (30 mM potassium chloride and 0.3 mM tartaric acid); the electric potential was measured for each sensor and defined as Vr. Then the sensors were dipped for 30 s into the sample solution and for each sensor the measured potential was defined as Vs. For each sensor the "relative value" (Rv) is the difference between the potential of the sample and the reference solution (Vs − Vr). Sensors were rinsed for 6 s and then dipped again into the reference solution and the measured potentials were defined as Vr'. From the difference between the potential of the reference solution after and before sample measurement (Vr' – Vr), the "CPA value" (CPAv), where CPA stands for "Change of membrane Potential caused by Absorption", was detected. Before starting a new measurement cycle, the sensors were rinsed for 90 s with a washing solution and then for 180 s with the reference solution.

Each sample was evaluated in triplicate and the "taste values" were calculated multiplying sensor outputs by appropriate coefficients based on Weber-Fechner's law, which provides the intensity of sensation considering the sensor properties for tastes (Kobayashi et al., 2010).

In particular, the "taste values" were estimated as:

Sourness = 0.3316 Rv (CA0)

Saltiness = − 0.252 Rv (CT0)

Bitterness = − 0.140 Rv (C00) + 0.084 Rv (CT0)

Aftertaste-bitterness = − 0.210 CPAv (C00)

Astringency = 0.1575 Rv (AE1) + 0.1575 Rv (CT0)

Aftertaste-astringency = − 0.252 CPAv (AE1)

Umami = − 0.1575 Rv (AAE)

2.3. Statistical analysis

To compare the morphological characteristics of the two lots of lupin seeds, the data were processed by means of a t-test. To compare the samples within each test the data underwent one-way analysis of variance (ANOVA), while to evaluate the effect of treatment (T), solvent (S) and time (t) or the effect of time and solvent, the data were processed by three- and two-way ANOVA. The normal distribution of the data was always verified; the inverse transformation of the square root was only necessary for the alkaloid content in the test about the different debittering methods. When significant differences ($p \leq 0.05$) were detected, Fisher's LSD test at 95% significance level was applied. All analyses were performed with the Statgraphics Centurion XVI statistical program (Statgraphics Technologies Inc., The Plains, USA). Means, standard deviations and standard errors were computed using the Excel® software (Microsoft, Redmond, WA, USA). The electronic tongue data were processed using the XLSTAT statistical and data analysis software (Addinsoft, New York, USA). Principal

Component Analysis (PCA) was applied as an exploratory tool to uncover in a reduced space the data structure and the relationships between objects and variables, thanks to graphical outputs (i.e., score plot, loading plot and bi-plot) (Wold et al., 1987).

3. Results and discussion

3.1 Seeds characteristics

The morphological characteristics of the two *Lupinus albus* lots are presented in Table 1. The Lot 2 seeds were significantly bigger and heavier ($p \leq 0.05$) than those of Lot 1. The seed weights of both batches were higher than those (0.151-0.479 g) reported by several authors (Julier et al., 1993; Julier et al., 1995; Mera et al., 2006; Annicchiarico et al., 2014), but still fell within the range (0.100 to 1.000 g) described by Huyghe (1997), who stressed the existence of extreme intraspecific variability both for weight and size. Additionally, cropping year and climatic conditions influence these parameters even within the same variety (Annicchiarico et al., 2014).

The alkaloid content in the untreated seeds of Lot 1 and Lot 2, 20.35 ± 0.17 and 20.92 ± 0.01 g/kg DM respectively, was low but significantly different ($p \leq 0.05$). These results are coherent with those of Muzquiz et al. (1994), that recorded a range between 17.0 and 26.9 g/kg alkaloids among 28 bitter *L. albus*.

3.2. Effect of debittering method on alkaloid content

The results of trial 1 are summarised in Figure 2, that shows the residual alkaloid concentration after debittering with deionised water, 1% NaCl or 1% citric acid solutions, with or without US and after two different soaking times. The ANOVA (Supplementary Table 1) evidenced a highly significant effect ($p \leq 0.001$) of solvent and time, but the sonication did not significantly reduce alkaloid content; the interaction between solvent and time was also

significant, while those including the US treatment were not. Hence, we can conclude that US did not improve at all the debittering process. The LSD test demonstrated that citric acid produced a stronger debittering effect (residual alkaloids: 0.80 ± 0.09 g/kg DM) than NaCl (1.37 ± 0.15 g/kg DM), and that water was the worst solvent (5.42 ± 0.24 g/kg DM).

Sonication did not improve the removal of alkaloids, contrary to the report of Miano et al. (2019), probably because the seeds were cooked before the US treatment: cooking denatures cellular membranes and breaks cell compartmentalization of the seed, performing the same function of sonication. A positive US effect may occur at the very beginning of the treatment, fostering seed hydration and formation of pores and canals, but the relatively long times required for debittering probably masked its effect. However, our preliminary trials demonstrated that US treatment was not enough to replace the cooking step. In fact, Miano et al. (2019) did not reach a satisfactory debittering (their residual alkaloids score was 15.1 g/kg DM) and we have demonstrated that, even if US accelerates alkaloids removal during the early debittering stages, it does not constitute a process improvement for practical purposes.

The positive debittering effect of sodium chloride is attributable to the salt increasing the microporous structure of the seeds and, consequently, facilitating the solvent penetration and the alkaloids leakage (Sievwright & Shipe, 1986). Furthermore, the osmotic effect of sodium chloride may favour the disorganization of phospholipid membranes and cellular compartments (Hameed et al., 2021) as well as foster counter-current mass transport from seed tissues to the saline solution (Casp Vanaclocha & Abril Requena, 2003). The better debittering capacity of saline solutions compared to water, evident also from the results of the solutions with sodium chloride, was likewise reported by Villacrés et al. (2020). Furthermore, these results agree with Jiménez-Martínez et al. (2010) and Karara (1987), who demonstrated greater debittering efficiency using, respectively, 0.1 M acetic acid and 0.1% citric acid solutions instead of pure water.

With the notable exception of caffeine and ephedrine, alkaloids are soluble in non-polar solvents only when they are in the form of conjugated bases, while the affinity is inverted for the corresponding conjugated acids and their salts (Kukula-Koch & Widelski, 2017; Ortiz & Mukherjee, 1982). This explains both the positive removal effect of citric acid solutions and the limited capacity of water alone. Lupanine, the main alkaloid in the seeds of *Lupinus* spp. (Boschin et al., 2008; Otterbach et al., 2019), having a pKa of 9.2 (Wink & Mende, 1987), exhibits higher solubility in acidic aqueous solutions than in water, being present in its protonated form (lupanine 1+). Furthermore, the formation of salts with the carboxylic acids is commonly used as a carrier for water-insoluble conjugated bases with pharmaceutical activity (Bharate, 2021). An alternative explanation could come from Giel-Pietraszuk et al., (2007), who observed that a slight excess of hydrogen ions (pH 6.2) is sufficient to hydrolyse the C-N bond of the δ-lactam, converting part of the lupanine into the corresponding acyclic molecule (lupanic acid), whose fate during debittering is however unknown. In our case, the pH of the citric acid solutions (2.2-2.4), far lower than 6.2, might have shifted the equilibrium towards lupanic acid, richer in polar groups and hence probably more water-soluble and easier to wash away than lupanine.

The results of trial 2, assessing the alkaloid content of the Lot 2 seeds, untreated and treated with distilled water, NaCl 0.5% and 1%, citric acid 0.5% and 1%, and applying three debittering times (45 h; 57 h; 67 h), are shown in Table 2. Additionally, the results obtained with the traditional method or with the reference method, as well as the concentration in four commercial ready-to-eat lupin snacks (C1, C2, C3 and C4) are reported. Among the samples debittered according to the experimental method, those treated with the citric acid solutions gave the best results in terms of operating times, because in only 45 h the sample with 1% citric acid reached an alkaloid level (0.56 ± 0.09 g/kg DM) not significantly different ($p \leq 0.05$) from those of the C1 and C2 commercial samples. When the treatment time was extended,

performing additional soakings, the value further decreased to 0.31 ± 0.09 g/kg DM, comparable to the C3 commercial snack. It must be stressed that lupin snacks are generally stored in 6% NaCl brine until packaging (Erbaş, 2010), and sometimes even after (e.g. the C3 sample), leading to a prolongation of debittering.

The 1% solutions were significantly more efficient in removing the alkaloids than the 0.5% solutions. In fact, with 1% NaCl a residual amount of 0.72 ± 0.15 mg/kg DM in the seeds was reached after 57 h, while with 0.5% NaCl the concentration was 1.16 ± 0.12 g/kg DM. Similarly, after 45 h the sample debittered with citric acid 1% achieved 0.56 ± 0.09 g/kg DM, while that treated with 0.5% citric acid still showed 1.11 ± 0.06 g/kg DM. At equal solute concentration, citric acid had a significantly greater debittering power. Water was the solvent with the lowest performance, as already shown in the previous analyses: it is noteworthy that not even after three final soakings (69 h of treatment) the seeds reached alkaloid content suitable for consumption. Based on these results, we tried to further fine-tune the experimental method, when using only water, by increasing the seed:solvent ratio and/or soaking time; however, no satisfactory results were achieved.

The above-detailed results are further validated by the two-way ANOVA computed only on the data of the seeds treated with the experimental method, considering the time and the solvent as factors. The two-way ANOVA (Supplementary Table 2) showed that both factors and their interaction were significant: the LSD test highlighted significant differences among all soaking times as well as among solvents, except for 1% NaCl and 0.5% citric acid, equally effective.

The seeds debittered according to the traditional method achieved a residual alkaloid value of 0.95 ± 0.12 g/kg DM only after 133 h and 100 L water/kg dry seed. The reference method reduced the alkaloid content to 0.66 ± 0.00 g/kg DM after 58 h, consuming 66 L of water/kg dry seed. This result is similar to those of the samples treated with 1% NaCl and 0.5% citric acid for 57 h and 40 L water/kg dry seed, as well as with 1% citric acid for 45 h and 35 L

water/kg dry seed; however, the experimental method saved time and water, thus reducing the volume of toxic effluents to be disposed of, or treated to recover lupanine, a building block in the synthesis of pharmaceutical molecules (Esteves et al., 2020). Furthermore, the seeds debittered with 1% citric acid for 57 h and 40 L/kg of water attained an even lower alkaloid concentration (0.37 ± 0.06 g/kg DM). It must be remembered that, according to the scores recorded in commercial snacks (0.35-0.99 g/kg DM) and in seeds debittered with the control procedures, methods that reduce the alkaloid concentration below 1.00 g/kg DM are considered suitable for consumption. Interestingly, this value was also the lowest threshold of bitterness perception when tasting the seeds.

3.3. Effect of debittering method on electronic tongue profile

Figure 3A displays the PCA bi-plot of e-tongue data in combination with alkaloid content collected on the four commercial ready-to-eat lupin snacks and on the seeds untreated and treated with traditional, reference and experimental method. The sample distribution in the plane defined by the first two principal components (PC1 and PC2), explaining 72.23% of the total variance (49.05% for PC1 and 23.18% for PC2), shows that the untreated control samples (CTR), located in the positive part of PC1, were characterized by the alkaloid content and their taste was perceived more bitter and umami than the samples treated with water (W) or debittered by the traditional method (TRAD), the reference method (SREF), and the 0.5% and 1% NaCl solutions. On the opposite side of PC1, the seeds treated with 0.5% or 1% citric acid (CA) solutions were characterized by sourness and perceived less bitter than the other seeds. All the commercial samples (C1-C4), clustered in the positive part of PC2, were characterized by saltiness: although they came from several companies using different processes, their sodium chloride content was high and ranged between 6.3 and 9.9 g/100 g DM, as declared on their nutritional labels. It is noteworthy that lupins debittered with NaCl solutions (reference

method and experimental NaCl method) were located far from the commercial samples, and close to the samples treated with water, confirming the effectiveness of the final washing and soaking steps in salt removal.

Overall, the e-tongue showed a good ability to discriminate samples according to their debittering treatments and to cluster samples treated with the same solvent; on the other hand, there was no clear trend in the positioning of samples prepared with the same method and solvent, but for different times (t_1; t_2; t_3).

Considering the distribution of the variables in the plane defined by PC1 and PC2, the alkaloid content, placed to the right of PC1, was highly correlated to bitter and umami tastes, which were consequently identified as the main e-tongue variables, useful to discriminate samples on the basis of their debittering treatments.

To better visualize the relationship between the alkaloid content and the two main e-tongue variables (i.e., bitterness and umami), the data collected from all the analysed samples (mean values) are depicted in the three-dimensional scatter plot (Figure 3B). The untreated control sample (CTR) with the maximum alkaloid content (Table 2) was characterized by high "taste values" for bitterness and umami, while the samples debittered with citric acid achieved a low alkaloid concentration and were perceived as the least bitter and umami. All the other debittered samples showed a good relationship between the three considered variables; moreover, samples debittered with the same solvent were close in the plot and were characterized by the same taste note.

The decrease in the umami taste following the debittering treatments can be explained by considering that umami is an indicator of the presence of amino acids, nucleotides and peptides (Wang et al., 2020). In lupin seeds, umami is probably linked to the presence of glutamic acid, a molecule responsible for this specific taste (Bellisle, 1999). Glutamic acid is characterized by a carboxylic group able of assuming an additional negative charge on the side chain, so it is

extremely soluble in aqueous and polar solvents and consequently it is easily solubilised and removed during debittering. Furthermore, since quinolizidine alkaloids are derived from the lysine amino acid and are characterized by a quinolizidine nucleus containing a nitrogen atom (Frick et al., 2017), they can probably be revealed by the e-tongue umami sensor (Hwang et al., 2020).

4. Conclusions

Our results clearly demonstrate that the sonication does not accelerate lupin seeds debittering, while the sodium chloride and citric acid solutions significantly shorten debittering time, limit water consumption and reduce alkaloid content to the concentrations (0.31-1.03 g/kg DM) observed in commercial snacks (0.35-0.99 g/kg DM). Debittering with a 1% citric acid solution saved 88 h and 65 L water/kg lupin compared to the traditional method, and 13 h and 31 L water/kg lupin compared to the Villacrés et al. (2020) reference method with salt. The e-tongue discriminated samples, placing those treated with the same solvent in well-defined clusters; bitter and umami tastes were the main factors characterizing samples according to their debittering treatments. Understanding the effects of debittering on the taste of lupin seeds could improve their use in several food applications.

CRediT authorship contribution statement

Lorenzo Estivi: Investigation, Formal analysis, Methodology, Software, Writing – original draft, review & editing, Susanna Buratti: Investigation, Methodology, Writing – review & editing, Davide Fusi: Formal analysis, Software, Writing – original draft, review & editing, Simona Benedetti: Formal analysis, Software, Writing – review & editing, Gilbert Rodriguez: Investigation, Writing – review & editing, Andrea Brandolini: Conceptualization,

Investigation, Writing – original draft, review & editing, Alyssa Hidalgo: Conceptualization, Investigation, Methodology, Writing – review & editing.

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622 **Figures legend**

623 **Figure 1**. Time sequence (in hours) of the different phases of the proposed debittering process.

624

625 **Figure 2**. Alkaloid content (g/kg DM) of *Lupinus albus* seeds after debittering for 28.5 h
626 (circles) or 45 h (triangles), without (filled) or with (empty) ultrasound (US), and with different
627 solvents (water, NaCl 1% solution and citric acid 1% solution). Error bars indicate the standard
628 deviations.

629

630 **Figure 3**. (A) PCA bi-plot of the results from the electronic tongue analysis of the *Lupinus*
631 *albus* samples (Lot 2) before and after debittering by traditional method (water; Erbaş, 2010),
632 reference method (NaCl solution; Villacrés et al., 2020), proposed method (different solvents),
633 and of four commercial samples (C1 - C4). Aftertaste: A astringent; B bitter. (B) 3D-plot of the
634 residual content of alkaloids and of the bitter and umami variables of seeds of *Lupinus albus*
635 based on the electronic tongue analysis results.

Figure 1

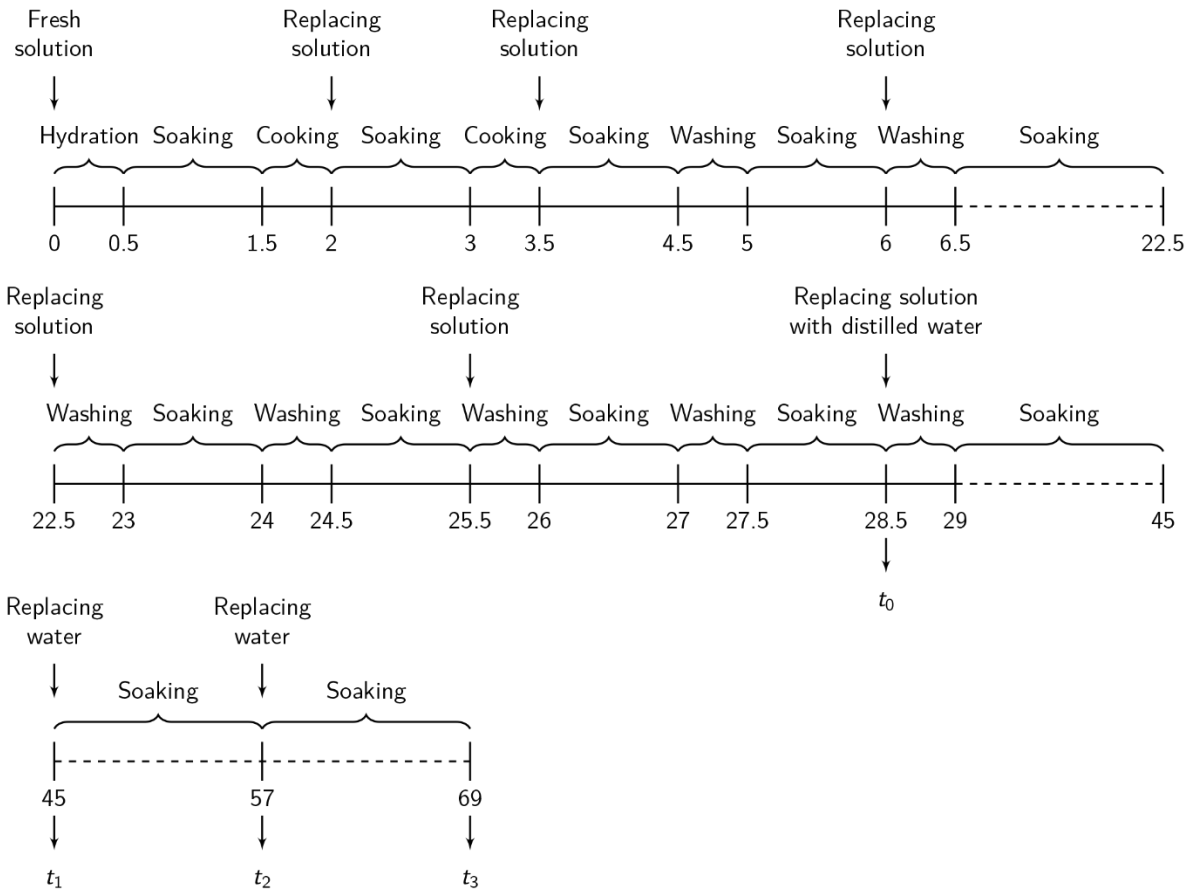


Figure 2

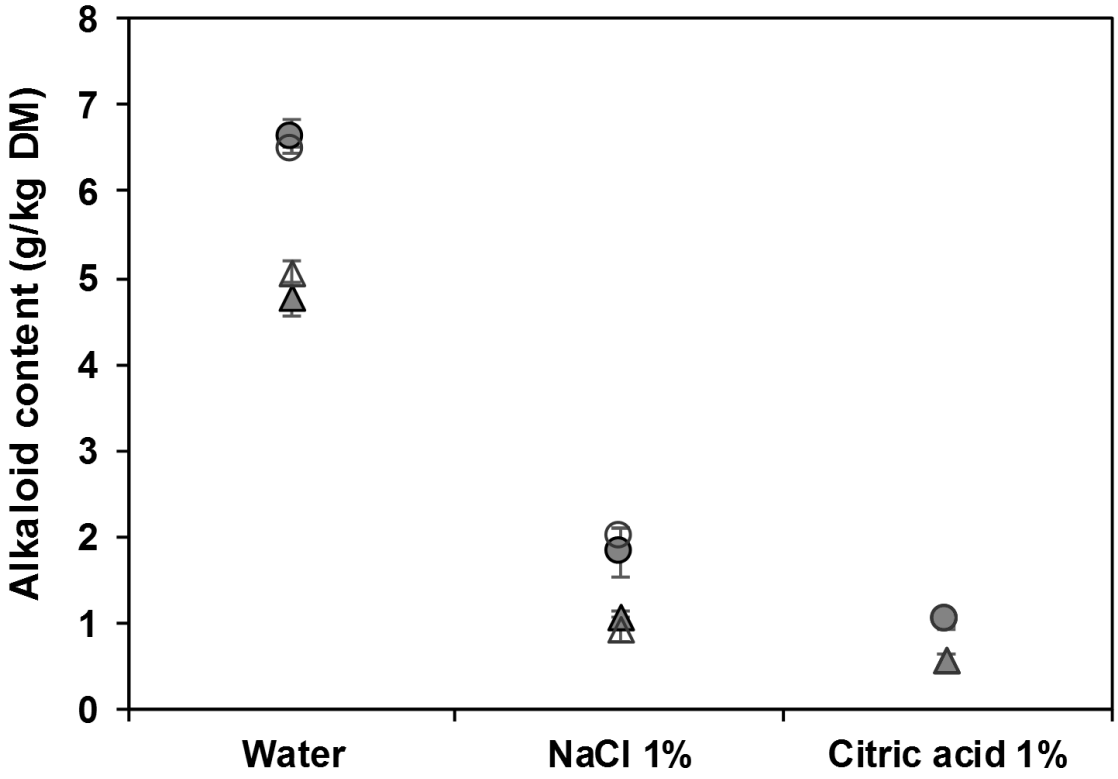
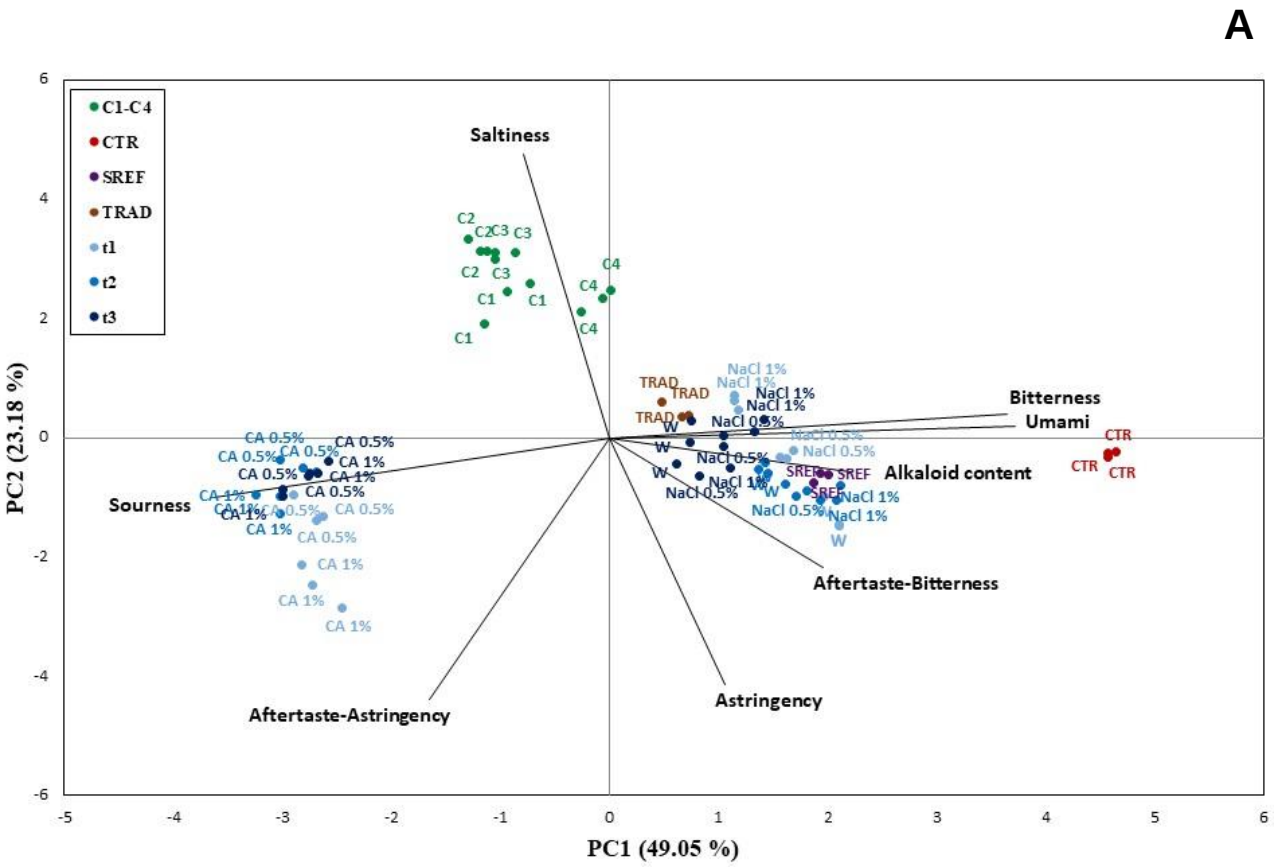


Figure 3



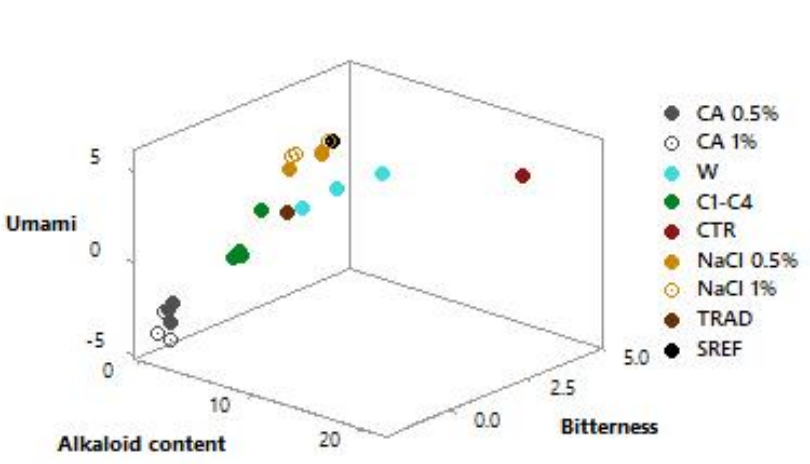


Table 1. Morphological characteristics (mean ± standard deviation) of the two lots of *Lupinus albus* seeds used in the analyses and results of the t-test.

	Lot 1	*Lot 2*
100-seeds weight (g)	$56.84^{b} \pm 0.83$	$74.75^{a} \pm 0.90$
Major diameter (mm)	$13.30^{b} \pm 0.57$	$14.28^{a} \pm 0.64$
Minor diameter (mm)	$12.37^{b} \pm 0.27$	$13.60^{a} \pm 0.51$
Thickness (mm)	$5.04^{b} \pm 0.39$	$5.32^{a} \pm 0.35$

Different letters indicate significant differences between samples according to the LSD test ($p \leq 0.05$).

Table 2. Alkaloid content (g/kg DM; mean ± standard deviation) of *Lupinus albus* seeds (Lot 2) before and after debittering by the experimental method, the traditional water method (Erbaş, 2010, modified by Córdova-Ramos et al., 2020), the NaCl solution method (Villacrés et al., 2020), and of four commercial samples (C1 - C4).

Treatment time (h)	Water consumption (L/kg)	Alkaloid content				
		Untreated	$20.92^{a} \pm 0.01$			
		Experimental methods				
		Water	0.5% NaCl	1% NaCl	0.5% citric acid	1% citric acid
45	35	$4.59^{b} \pm 0.18$	$1.75^{c} \pm 0.15$	$1.03^{def} \pm 0.06$	$1.11^{de} \pm 0.06$	$0.56^{i} \pm 0.09$
57	40	$4.48^{b} \pm 0.03$	$1.16^{cd} \pm 0.12$	$0.72^{ghi} \pm 0.15$	$0.78^{fgh} \pm 0.00$	$0.37^{j} \pm 0.06$
69	45	$3.62^{b} \pm 0.15$	$0.97^{defg} \pm 0.09$	$0.81^{efgh} \pm 0.03$	$0.62^{hi} \pm 0.00$	$0.31^{j} \pm 0.09$
		Control methods				
133	100	Traditional (water)		$0.95^{defg} \pm 0.12$		
58	66	Reference (NaCl solution)		$0.66^{hi} \pm 0.00$		
		Commercial snack samples				
		C1		$0.62^{hi} \pm 0.00$		
		C2		$0.56^{i} \pm 0.09$		
		C3		$0.35^{j} \pm 0.09$		
		C4		$0.99^{defg} \pm 0.06$		

Different letters indicate significant differences between samples according to the LSD test ($p \leq 0.05$).

Declarations of Interest: none.

Supplementary Table 1. ANOVA and LSD test (mean ± standard error) of alkaloid content (g/kg DM) in *Lupinus albus* seeds after debittering applying different treatments (without and with ultrasound),solvents and soaking times.

ANOVA		
Factor	*d.f.*	*Mean square*
Treatment (T)	1	0.006
Solvent (S)	2	57.266***
Time (t)	1	5.980***
T x S	2	0.003
T x t	1	0.004
S x t	2	0.729***
Error	26	0.11
Test LSD		
Treatment		
Without ultrasound	2.65 ± 2.35	
With ultrasound	2.68 ± 2.37	
Solvent		
Water	5.73^{a} ± 0.90	
NaCl 1%	1.45^{b} ± 0.51	
Citric acid 1%	0.80^{c} ± 0.24	
Time		
28.5 h	3.16^{a} ± 2.53	
45.0 h	2.16^{b} ± 2.04	

*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Different letters indicate significant differences between means ($p \leq 0.05$).

Supplementary Table 2. Analysis of variance (mean square) and LSD test of the alkaloid content (g/kg DM; mean ± standard error) of *Lupinus albus* seeds after debittering without ultrasound, and with different solvents.

ANOVA			
Factor	*d.f.*	*Mean square*	
Time (t)	2	0.75	***
Solvent (S)	4	14.26	***
t x S	8	0.092	***
Error	30	0.010	
Test LSD			
Time			
45 h	$1.81^{a} \pm 0.48$		
57 h	$1.50^{b} \pm 0.50$		
69 h	$1.26^{c} \pm 0.40$		
Solvent			
Water	$4.23^{a} \pm 0.20$		
0.5% NaCl	$1.29^{b} \pm 0.15$		
1% NaCl	$0.85^{c} \pm 0.07$		
0.5% citric acid	$0.84^{c} \pm 0.09$		
1% citric acid	$0.41^{d} \pm 0.05$		

d.f.: degrees of freedom; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; different letters indicate significant differences between samples according to the LSD test ($p \leq 0.05$).