Environmental friendly cold-mechanical/sonic enzymatic assisted extraction of genipin from genipap (Genipa americana)

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Short Communication

Environmental friendly cold-mechanical/sonic enzymatic assisted extraction of genipin from genipap (*Genipa americana*)

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A B S T R A C T

An efficient cold-mechanical/sonic-assisted extraction technique was developed for extraction of genipin from genipap (*Genipa americana*) peel. Ultrasound assisted extraction (285 W, 24 kHz) was performed at 5, 10 and 15 °C for 5, 10 and 15 min. After cold-extraction, genipin was separated from pectin and proteins by aid of fungal pectinesterase. The maximum yield of non-cross-linked genipin was 7.85 ± 0.33 mg/g, at 10 °C for 15 min by means of ultrasound extraction. The protein amount in extracts decreased in all samples. If mechanical process is combined with ultrasound assisted extraction the yield is increased by 8 times after the pectinesterase-assisted polyelectrolyte complex formation between pectic polysaccharides and proteins, avoiding the typical cross-linking of genipin. This novel process is viable to obtain non-cross-linked genipin, to be used as a natural colorant and cross-linker in the food and biotechnological industries.

Keywords:
Ultrasound
Pectin
Pectinesterase
Protein
Polyelectrolyte complex

1. Introduction

Genipa americana L. is a tree that is widely distributed from Mexico to the Caribbean region and South America [1]. It is also known as jagua, juito, huito and genipa (Spanish areas in Latin America), genipap and genipa (English), bois de fer (French) and genipapo (Portuguese) [2]. *G. americana* and *Gardenia jasminoides Ellis* fruits contain genipin, an iridoid cross-linking compound [3,4] that is able to react spontaneously with primary amino acids groups, peptides or proteins to form dark blue pigments. It is soluble in water, alcohol and propylene-glycol and it presents stability in the pH range from 4 to 9.

Genipin cytotoxicity is lower than other chemical cross-linking reagents. It has been reported that it is approximately 5000–10,000 times less cytotoxic than glutaraldehyde. Due to its healthy attributes and to its ability to develop blue color, genipin has been used as a natural food colorant in some regions of Asia [5]. Genipin also has been used to cross-link molecules such as chitosan or proteins [6]. In the cross-linking reaction, a bimolecular substitution occurs, that includes a substitution of an ester group in the genipin molecule by a secondary amide. The second reaction is a nuclephilic substitution of ester clustering. The cross-linking reactions allow the formation of blue pigments that include other reactions with a higher level of complexity [7].

The blue color plays an important role in the food development industry. There is a need of natural blue colorants in the beverage, ready-meals, soups, breakfast cereal production, as well as in pet food, confectionery and baby food industries [2]. The food industry has taken advantage of genipin characteristics in order to use it as a natural colorant in beverages [8], juices, nectars, desserts and gels [9]. Moreover, genipin has also been used in the biomedical area as biomaterials cross-linking agent [10], in the forensic science as fingerprint developer [11], and in the textile industry, when cotton, wool and leathers are dyed [12,13]. In addition, genipin has been included in analytical purposes, such as aminoacid quantification [14], where studies have been carried out to elucidate the mechanism and kinetics of the cross-linking reaction between biopolymers containing primary amine groups and genipin [15]. Ramos-Ponce et al. (2010) have demonstrated that D-glucosamine is an amino sugar that can be cross-linked with genipin to determine the amount of genipin that is present in a sample [4]. Fig. 1 shows the chemical structure of genipin.

The currently methods used for genipin recovery involve the use of organic solvents such as chloroform and very specific and

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complex steps for purification (concentration under reduced pressure, chromatography, crystallization, etc.) [16], that makes the final product very expensive and difficult to obtain. It is necessary to find new and feasible alternatives for genipin recovery promoting the environmental preservation using emerging technologies and moderate temperatures which avoid the blue color formation during the extraction process. Recovery of genipin is certainly a critical technological step because aqueous extracts release simultaneously pectic substances and proteins. Recently, an enzymatic approach for phytochemical separation after the extraction process has been reported, where exogenous pectinesterase and calcium promote precipitation of water soluble pectin into calcium pectate [17]. Ultrasound assisted technology has gained great interest nowadays due to industries can be provided with practical and reliable ultrasound equipment. Its emergence as green novel technology has attracted the attention because it is concerned to the environmental sustainability [18]. This technology has demonstrated to enhance the mass transfer by acoustic-induced cavitation in la liquid medium and the mechanical effects induced by cavitation bubbles that include microjet impatcs and shockwave-induced damage [19] in this case, of the plant cell wall of *G. americana* L. fruit. Ultrasound is an emerging technology that enhances the acquisition of clean products after extraction [20].

To date, however there has been no report on eco-friendly assisted-extraction of genipin from genipap under mild conditions by means of ultrasound. The aim of this work is to investigate the genipin recovery from genipap fruit after mechanical and mechanical/sonic enzymatic assisted extraction at cold temperatures in order to obtain non-cross-linked genipin extract free from pectin and proteins. This purified extract can be used as a natural colorant in the food industry, in dairies, savory/bakery, soft drinks, confectionary and beverages or as a raw material for preparation of highly purified genipin.

2. Materials and methods

2.1. Materials

Genipap fruit was obtained from Cintalapa city, Chiapas state, Mexico. Pectinesterase (9347.72 U/mL; Lot number KENOO510; Novoshape) from *Aspergillus aculeatus* was purchased from Novozymes® (Krogshøjvej, Bagsværd, Denmark. Isopropyl alcohol (98%) was procured from Coyotefoods Biopolymer and Biotechnology Co. (Saltillo City, Coahuila State, Mexico). D-glucosamine was obtained from Cargill (Indianapolis, Minnesota, USA). Genipin was procured from Chengdu King-tiger, Pharm-chem, Tech. Co., Ltd. (Dayi, Chengdu, China). All other reagents were analytical grade.

2.2. Methods

2.2.1. Cold-mechanical extraction and enzymatic assisted treatment

Genipap fruit was peeled and the flesh was frozen until use. Peel was weighted and 50 g were taken for each experiment. Peel was cut with a stainless steel knife into cubes. Water (200 mL) at different temperatures (5, 15 and 30 °C) was added to the genipap peel pieces. Temperature was selected in order to explore the genipin recovery between 5 and 30 °C, to promote the use of moderate temperatures to avoid the genipin cross-linking when temperature is higher than 30 °C. The mixture was blended for 1 min and the extract and solid phase were recovered separately through filtration with cheese cloth. The solid phase was weighed and dried at 60 °C for 12 h. The extract was incubated for 1 h after the addition of 500 U of PE and 50 mM CaCl2 in order to achieve the enzyme activation, avoiding the enzyme trapping by carboxyl groups on pectin. This enhances the interaction between salt ions with negatively charged groups, allowing the enzyme to interact with the ester bonds to be cleaved [26]. Extracts without PE enzyme were referred as blanks. After incubation, extracts were centrifuged at 8873*g* for 20 min in a Biofuge primo R Sorvall® (Hanau, Hesse, Germany) and the supernatant was removed from the pellet after filtration through cheese cloth. Pellet was obtained with 10 mL of isopropyl alcohol and weight was recorded in wet and dry basis before and after drying at 60 °C for 12 h. Centrifuged extracts were subjected to pH and chemical analysis.

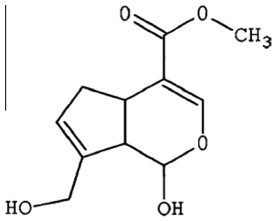

Fig. 1. Chemical structure of genipin [11].

2.2.2. Cold-mechanical/sonic enzymatic assisted treatment

This extraction process was similar to the mechanical procedure, except that a smaller scale was used, also temperatures and times of treatment were different. Genipap fruit peel was cut with a stainless steel knife into cubes of regular size (1 × 1 × 1 cm) and 25 g were mixed for 1 min in a blender which contained water (100 mL) at 5, 10 and 15 °C. After blending, 7 mL of solid–liquid sample were taken and placed into a glass test tube with screw cap. The rest of the sample was discarded. The sample was subjected to ultrasound treatment in a cup horn (Qsonica, LLC, Newton, Connecticut, USA) at 5, 10 and 15 °C for 5, 10 and 15 min (285 W, 24 kHz). Finished the ultrasound assisted extraction, 500 U of PE and 720 mM $CaCl_2$ were added to the samples and they were incubated for 1 h at the corresponding temperature. After incubation, samples were filtered through cheese cloth and through filter paper (Whatman TM, GE Healthcare, Little Chalfont, Buckinghamshire, UK). The liquid phase was stored at 9 °C until use. The solid phase was dried at 60 °C for 12 h, then weight was registered. Blanks were defined as samples treated mechanically but without being subjected to ultrasound assisted extraction. Fig. 3 shows the flow diagram of the cold-mechanical/sonic enzymatic assisted extraction.

2.2.3. Chemical and statistical analysis

Samples of 5 mL were taken from extracts to determine the cross-linked genipin [4]. A calibration curve (Fig. 2a) was prepared with genipin (0–500 mg L^{-1}) and the spectra of cross-linked genipin is showed in Fig. 2b. Samples were mixed with D-glucosamine (2000 mg L^{-1}) and heated for 1 h in a boiling water bath. After cooling, absorbance of samples was determined at 589 nm in a Shimadzu UV–vis Photodiode Array Spectrophotometer MultiSpec-1501 (Greater Toronto area, Ontario, Canada). Protein content was also determined [21]. Pectinesterase activity of the commercial preparation was determined (pH 4.5/40 °C; Pectin: 1%, w/v) [22]. Each experiment was done in quintuplicate. Analysis of variance (ANOVA) was done using the Statistica (StatSoft Inc., Tulsa, OK, USA) software.

3. Results and discussion

3.1. Cold-mechanical extraction and enzymatic assisted treatment

Fig. 4 shows the effect of temperature on extracts pH without PE and when enzyme was added. In both cases pH decreased from

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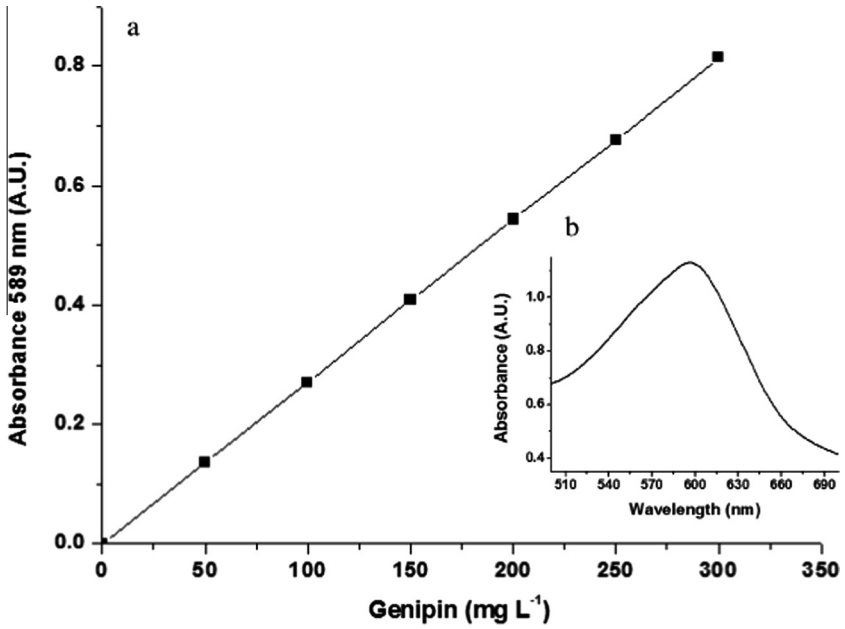

Fig. 2. Calibration curve for genipin with D-glucosamine (a) and spectra of genipin cross-linked with D-glucosamine (b).

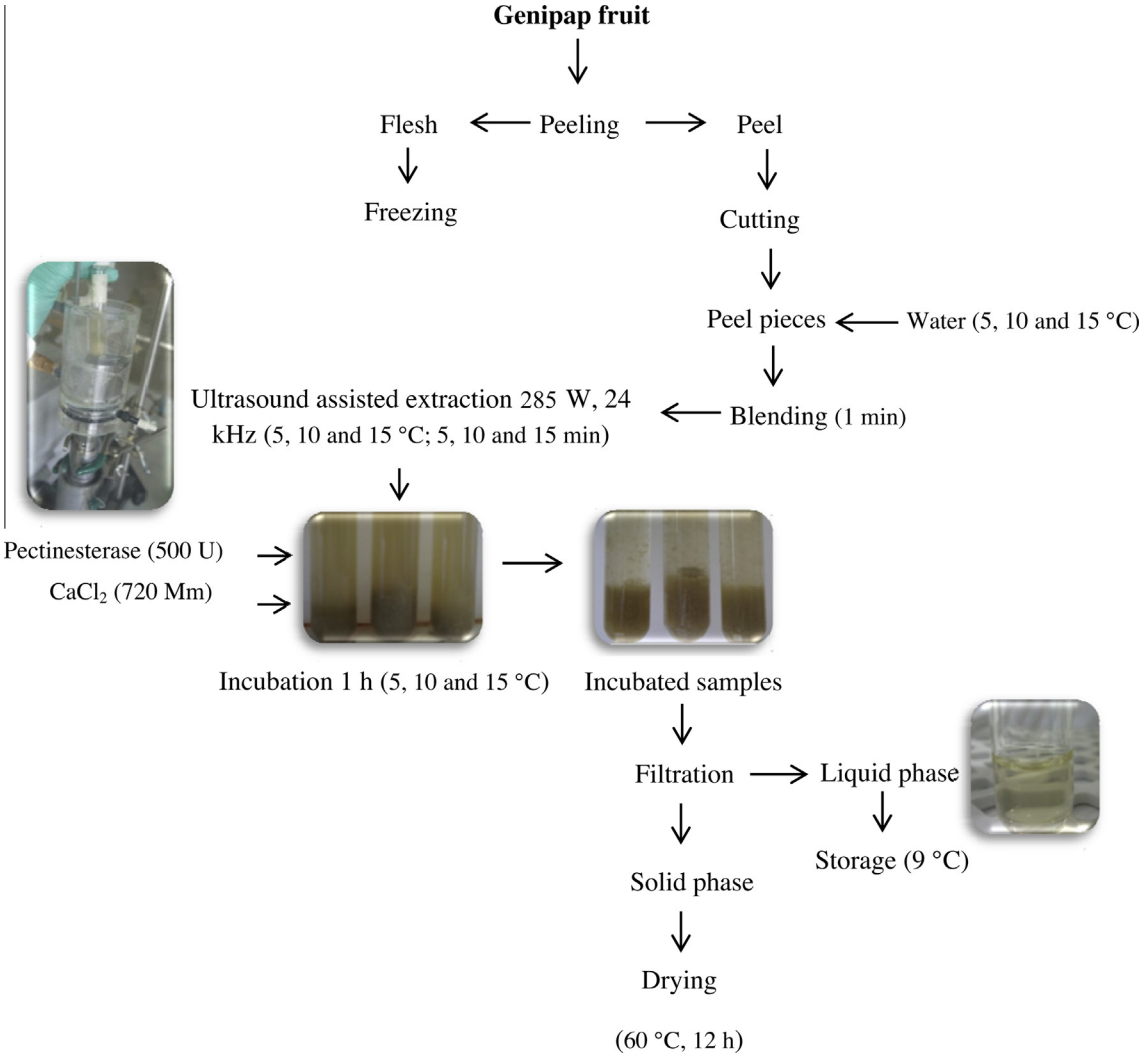

Fig. 3. Cold-mechanical/sonic enzymatic assisted extraction.

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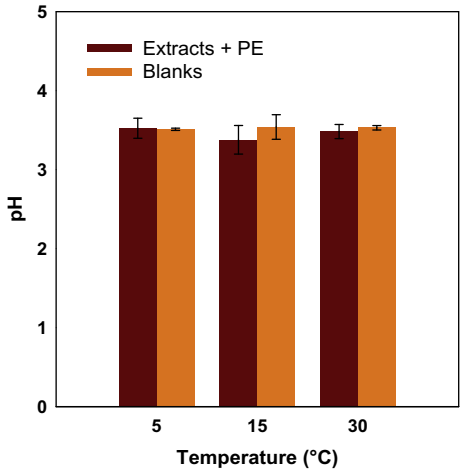

Fig. 4. Effect of temperature on pH in extracts without PE and with enzyme.

4.5 to ~3.5. The pH decrease is associated with the liberation of water soluble pectin after the vegetable tissue disintegration [23] and with the formation of acetic acid and methanol upon the demethylation of pectin due to the pectinesterase activity [24]. There was no statistical difference when temperature varied according to ANOVA.

Cross-linked and non-cross-linked genipin liberation is reported in Table 1. The effect of temperature (5, 15 and 30 °C) on the genipin content can be seen in blanks and in extracts with PE. The cross-linked genipin content is higher than non-cross-linked due to genipin reacted with genipap proteins and also with D-glucosamine. In this case the total content of genipin (including free genipin) was cross-linked and quantified. The highest content of cross-linked genipin (2.41 ± 0.73) mg/g in blanks and with PE (2.02 ± 0.39) mg/g was obtained at 15 °C. The amount of cross-linked and non-cross-linked genipin was higher in blanks than in extracts with enzyme, this probably due to genipn could be trapped in the complex formed between pectin–proteins and calcium due to the intermolecular binding after the $CaCl_2$ addition [17,25,26]. Protein content and pellet weight in wet basis was estimated and Fig. 5 describes the behavior of protein liberation (Fig. 5a) and pellet yield (Fig. 5b) in blanks and in extract with PE when temperature was raised. The protein content increased as temperature was higher in blanks and when PE was added. This in agreement with solubilization of cell wall components when heat is increased. However, proteins in blanks were ~2 times higher than in samples with enzyme. Yields of pellets were higher in the extracts with PE and they increased as temperature did. It has been reported that fungal PE has high specific activity at pH 3.5 [27] which indicates that PE demethoxyled pectin and a complex was formed with $CaCl_2$, where proteins were also included in the complex [28].

Absorbance at 589 nm of all samples (extracts with PE and without enzyme) was recorded in order to study the change in color of genipap extracts during processing according temperature changes. They are showed in Fig. 6. Blanks presented minimal values of absorbance at 30 °C (0.12 ± 0.02) probably due to genipap fruit used for 5 and 15 °C treatments had genipin previously cross-linked.

There was no statistical difference between temperatures in extracts with enzyme according to ANOVA. Absorbance values in both cases were lower than 0.3, which indicates that using moderate temperatures (between 5 and 30 °C) makes feasible the genipin recovery without developing the dark blue color which is typical after genipin cross-linking under oxygen presence [1].

3.2. Cold-mechanical/sonic enzymatic assisted treatment

Due to statistical difference was not found in the recovery of genipin when temperatures between 5 and 30 °C were used in the mechanical extraction, the interval of temperatures for

Table 1
Cross-linked (with D-glucosamine) and non-cross-linked (only heated) genipin yield found in extracts + PE and in extracts where enzyme was not added (blanks) after mechanical extraction.

Genipin	Cross-linked[a]			Only heated[a]		
Temperature (°C)	5	15	30	5	15	30
Extracts + PE (mg/g)	1.62 ± 0.30	2.02 ± 0.39	1.66 ± 0.57	0.92 ± 0.16	1.15 ± 0.29	0.49 ± 0.24
Blanks (mg/g)	1.70 ± 0.02	2.41 ± 0.73	1.81 ± 0.08	0.96 ± 0.13	0.97 ± 0.17	1.21 ± 0.01

[a] Average and standard deviation on: 5 samples.

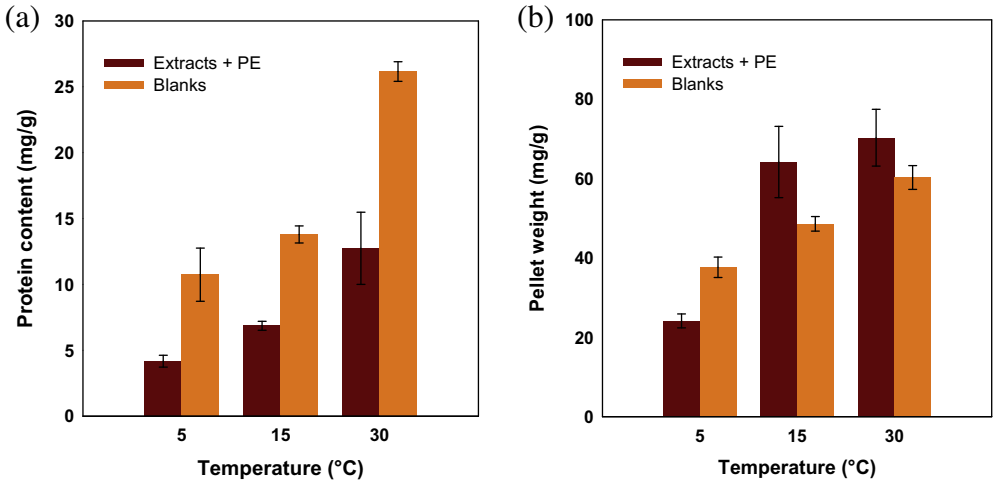

Fig. 5. Effect of temperature on protein content and pellet weight in extracts without PE and with enzyme.

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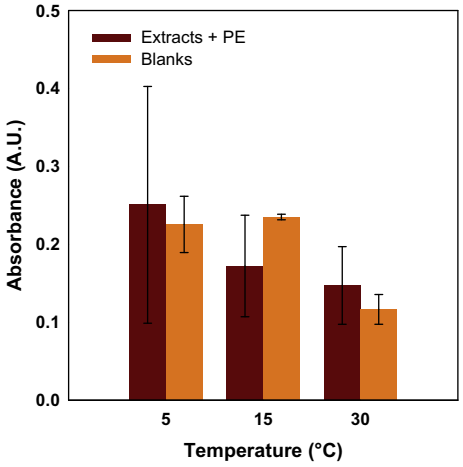

Fig. 6. Effect of temperature on absorbance of extracts without PE and with enzyme.

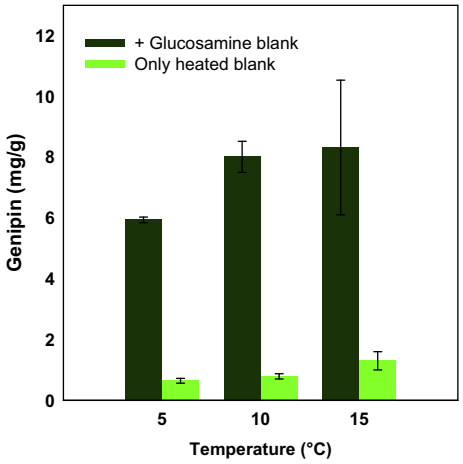

Fig. 7. Effect of temperature on genipin liberation after mechanical extraction (blanks).

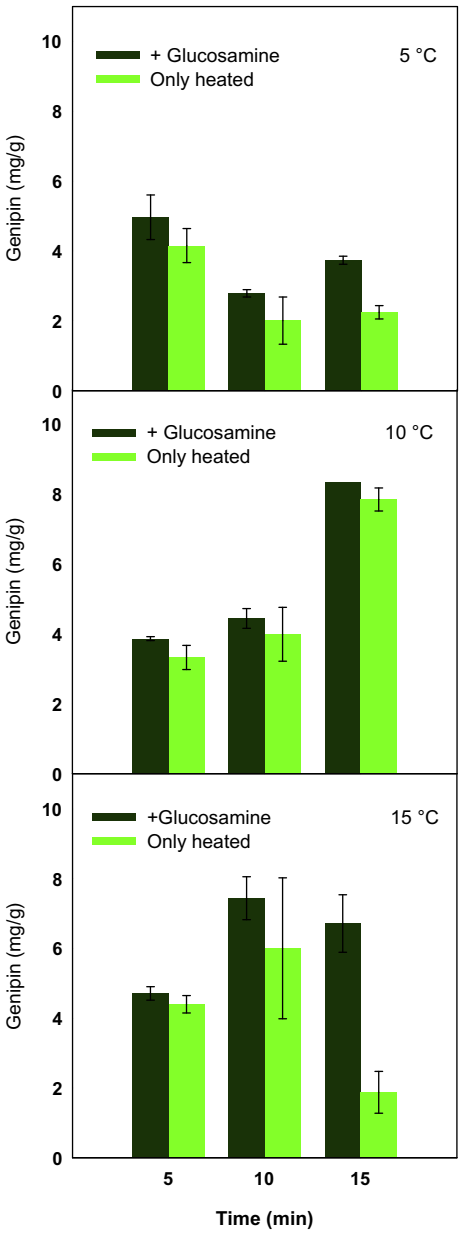

Fig. 8. Effect of time and temperature on the genipin liberation after the ultrasound assisted extraction.

ultrasonic treatment was reduced to 5–15 °C in order to keep avoiding the cross-linking of genipin and to find the adequate temperature for the environmental friendly recovery of genipin. Time of sonication was also determined in order to achieve the highest yield of genipin in the shortest period of time (5–15 min).

Genipin was quantified after mechanical (blanks) and ultrasound assisted extraction, Fig. 7 shows the effect of temperature on genipin liberation in blanks. Genipin was measured after being cross-linked with D-glucosamine and after heating and without the addition of D-glucosamine. It can be seen that the genipin content in samples with D-glucosamine was 8 times higher than in samples which were only heated. There was not statistical difference between treatments of each response. The amount of cross-linked genipin with D-glucosamine also was higher (~4 times) than the reported in the previous mechanical process described above. This difference is attributed to the PE activity, that led to the formation of the polyelectrolyte complex, that included proteins in the matrix that avoided the cross-linking of genipin [26].

Fig. 8 indicates the amount of genipin cross-linked with D-glucosamine subjected to heat treatment and the detected only after heating when ultrasound assisted extraction took place at 5, 10 and 15 °C for 5, 10 and 15 min. According to ANOVA, there was not statistical difference between treatments when time was considered, instead of, different temperatures caused the statistical difference in this process. In this case, the amount of genipin cross-linked with D-glucosamine and heat was slightly higher than the genipin that only was heated. When the extraction was carried out at 10 °C, the highest genipin yield detected after only heating was 7.85 ± 0.33 mg/g, then it decreased when temperature was 15 °C to 6.00 ± 2.02 mg/g and the lowest yield was detected at 5 °C, being 4.15 ± 0.49 mg/g. Ultrasound assisted extraction of genipin led to the production of the major content of available genipin at 10 °C when treated for 15 min. This only heated genipin represents the genipin that reacted with the primary amino groups from endogenous proteins of *Genipa americana* fruit after heating. Before heating no blue coloration was observed for this available genipin extracted with ultrasound, meanwhile genipin obtained from mechanical extraction (blank) only, turned its color to blue even without heating. Fig. 9 shows this fact in blanks (b) and the

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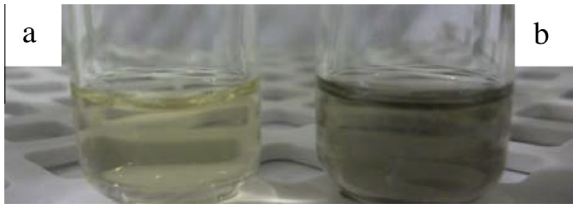

Fig. 9. Extracts from peel of genipap fruit after ultrasound assisted extraction (a) and mechanical extraction (b) after 65 days of storage at 9 °C.

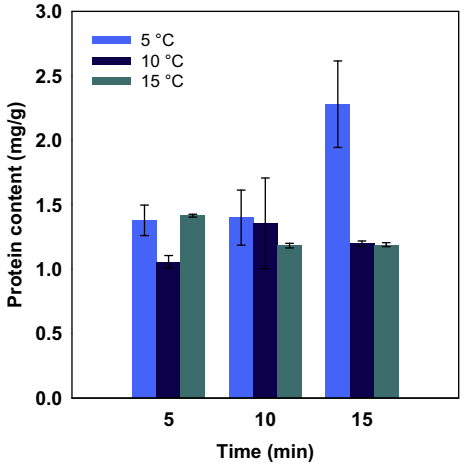

Fig. 10. Effect of time and temperature on protein liberation after ultrasound assisted extraction.

ultrasound assisted extracted genipin (a) did not change their appearance after 65 days (stored at 9 °C) of extraction. This is an advantage of the ultrasound extraction process, due to higher yield of available genipin can be recovered, without being cross-linked and without heating.

The ultrasound assisted extraction showed to be a reliable technology for genipin extraction. Mason et al., (1996) [20], indicate that mechanical effects of ultrasound enhances the penetration of solvent into cellular materials that promotes the mass transfer, and that this emerging technology cause plant cell wall disruption, which facilitates the liberation of contents. These liberated contents have been reported earlier after ultrasonic extraction of hemicellulose release from buckwheat hulls [29]. The contents included proteins, pectic polysaccharides and hemicellulose, basically. Fernandes and Rodrigues (2012) [30] studied the previous treatment to air drying process of *Genipa americana* fruit by means of ultrasound for 20 min. They reported 16.6% of reducing sugars loss during the process in ultrasonic bath, caused by the difference in sugar concentration between the fruit and the liquid medium. In our study, genipin was detected in the extract after being liberated from the cell wall.

Fig. 10 shows the protein content liberated after the ultrasound assisted extraction of genipin at 5, 10 and 15 °C for 5, 10 and 15 min. No statistical difference could be observed between treatments, except when the extraction was carried out at 5 °C for 15 min. The total content of proteins detected in blanks was 2.41 ± 0.54 mg/g. The protein content obtained after ultrasound assisted extraction was lower than those obtained in the first mechanical stage. This difference is due to the ultrasound effect that promoted the liberation of polysaccharides, such as pectin [31] and also to the activity of PE added to the extract, that led to a more efficient demethylation of the pectin molecule, enhancing the formation of a polyelectrolyte complex between $CaCl_2$ and pectin, trapping proteins in the matrix formed. When periods of time of 15 min were used, the highest amount of cross-linked and non-cross-linked genipin was obtained. Short periods of time were used according to it has been reported that ultrasound requires short times to achieve the maximum yield of extraction [20].

Wang et al. (2011) [32] obtained genipin from Gardenia fruit using methanol as solvent as well as sodium hydroxide, hydrochloric acid for solvent adjustment. They reported an average of 8.83 and 7.84 mg/g of genipin from *G. jasminoides* Ellis and *G. jasminoides* Ellis var. grandiflora Nakai fruits, respectively. These values are close to the obtained in our research and this fact indicates that average yield for genipin recovery can be achieved using emerging technologies and environmental friendly conditions.

4. Conclusions

Mechanical extraction of genipin from genipap fruit allows to recover cross-linked and non-cross-linked genipin. The presence of proteins in extracts was evidenced. PE increases the yield of pellet precipitated at 15 and 30 °C. Ultrasound enhances the liberation of genipin and proteins as well as the formation of a polyelectrolyte complex. The higest yield of genipin was found after ultrasound treatment at 10 °C for 15 min. Ultrasound assisted extraction improves the yield of non-cross-linked genipin by a factor of 8. Ultrasound assisted extraction represents a viable method for genipin recovery at cold temperatures.

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