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Pink shrimp (*P. brasiliensis* and *P. paulensis*) residue: Influence of extraction method on carotenoid concentration

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ABSTRACT

The main residue from the shrimp processing is formed by head and carapace and represents from 40 to 50% (w/w) of the integral shrimp. The recovery of the carotenoid fraction from this residue stands for an alternative to increase its aggregated value. Therefore, the objective of this study was to use the pink shrimp waste as raw material to obtain carotenoid enriched extracts, evaluating different pre-treatments and extraction methods. The shrimp waste was supplied by a local public market (Florianópolis, SC, Brazil). The investigation of the different pre-treatments applied to the raw material shows that cooking associated with milling and drying produced the extract richest in carotenoid fraction. The extraction methods considered in this work were Soxhlet, maceration and ultrasound by means of different organic solvents and also a vegetable oil as solvent. The extracts were evaluated in terms of yield, carotenoid profile, total carotenoid content (TCC), UV–Visible scanning spectrophotometry and mid-Fourier transform infrared spectroscopy (FTIR). The results indicate that shrimp waste can provide carotenoid enriched extracts, particularly astaxanthin, in concentrations up to 252 $\mu g_{astaxanthin}\, g^{-1}_{extract}$. The most adequate solvents were acetone and hexane: isopropanol (50:50, v/v) used in the maceration procedure. The UV–Vis results revealed the presence of carotenoids and flavonoids in the extracts while the FTIR spectroscopy indicated the existence of fatty acids, proteins, and phenolics.

1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), the worldwide capture production of marine crustaceans was, in 2007, the total of 5840 thousands ton [1]. The Brazilian participation in this global production is continuously increasing, particularly by means of the State of Santa Catarina, which is the largest Brazilian producer of marine fishery. In the same year (2007), the Santa Catarina State (Brazil) reached 39% increase in the crustacean production, compared to 2006 [2].

The shrimp, one of the major crustacean's subclasses, can be commercialized fresh (*in natura*) or processed. The processed shrimp generates an industrial waste composed mainly by head and carapace, or the cephalothorax [3]. The head consists of 35–40% (w/w) of raw material, whereas the carapace is around 7% (w/w). Therefore, these residues represent from 40 to 50% (w/w) of raw material [4] and are potentially considered as environmental contaminants when improperly disposed [5,6].

The shrimp residue is a potential source of food ingredients or additives due to the presence of important components in the cephalothorax. Therefore, the study and the development of new products by means of the shrimp residue, such as extracts, are relevant because of the high aggregated value of the final product (residue extract) and because of the reduction in the environmental impact due to the processing of shrimp residue. Consequently, the shrimp waste can be used to obtain extracts that contain carotenoid components, like astaxanthin, which are bioactive substances highly demanded by the food and pharmaceutical industries.

The strong association between the carotenoids and other macromolecules from the residue, such as proteins and fatty acids, demands an adequate raw material pre-treatment. According to the literature, the most successful pre-treatments used to recover carotenoids from microorganisms, crustaceans and algae are: cooking and drying [7], ultrasound, chemical treatment with liquid nitrogen and dimethyl sulfoxide [8,9], milling and acid or enzymatic hydrolysis by means of lysozyme, lipase, lyticase or catalase [8,10,11].

Organic solvents have been used for the extraction and recovery of carotenoids from natural sources [12–14] and their efficiency is related to the high temperatures used and to the solvent–solute interactions [15]. After the extraction, the recuperation of the

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solvent is essential due to environmental and economical problems. The limitations of the organic solvent extraction procedures, the so called conventional methods, are: high energy costs; elevated amount of solvent; time consuming process; use of high temperatures, affecting thermolabile substances; and presence of solvent vestiges in the solute (extract) [16,17].

Vegetable oils have also been successfully used as solvents for the extraction of carotenoid components from vegetal sources and crustaceans [6,12,18]. The advantage of using vegetable oils is that they are considered as a good barrier against oxygen, which reduces the oxidation processes. The vegetable oils can also contribute as energy source if the product (carotenoid in vegetable oil) is subsequently applied in food formulations [6].

Therefore, considering the environmental, economical, quality and process aspects discussed above, the main objective of this work is to evaluate the application of pink shrimp residue as a source of carotenoid compounds. Different pre-treatments of the raw material were evaluated in order to make the carotenoid fraction available for the extraction procedures, performed by different techniques and solvents. The methods were then evaluated by the quality of the extracts by means of the analysis of the chemical composition and the recovery efficiency of the carotenoid fraction from the shrimp residue.

2. Materials and methods

The procedure proposed in this work consisted of: (a) selection of the pre-treatment for the raw material, including cooking, drying and milling, according to the carotenoid content of the treated material, measured by a classical extraction procedure; (b) study of different extraction methods: after defined the adequate pre-treatment (selected in step a), the treated raw-material was submitted to different extraction procedures (Soxhlet, maceration and ultrasound, with different organic solvents and with edible oil as solvent) and the resulting extracts were evaluated in terms of carotenoid profile. The experimental procedures are detailed below.

2.1. Raw material

The raw material consists of pink shrimp (*Penaeus brasiliensis* and *Penaeus paulensis*) waste, composed essentially by head, carapace, and tail. The residue was provided by *Peixaria Nelson Santos* (Florianópolis, Santa Catarina, southern Brazil), sited at the local public market. The residue from the shrimp processing was supplied as one sample, representative from the high season production in May 2010.

2.2. Pre-treatment methods

The shrimp waste was submitted to the following pre-treatments, before the extraction procedures: (a) *in natura* (without pre-treatment); (b) *in natura* milled; (c) dried and milled; (d) cooked; (e) cooked and milled; (f) cooked, dried and milled, according to the procedure presented below. The efficiency of different pre-treatments was evaluated according to the availability, for the extraction, of the carotenoid compounds present in the pre-treated raw material. The effectiveness of these procedures was determined by the classical method of carotenoid extraction and the quantification as presented in Sections 2.3 and 2.4, respectively.

2.2.1. Cooking

The raw material was submitted to a quick cooking in a heating bath with water at 100 °C for 10 min, according to the procedure presented by Perdigão et al. [7].

2.2.2. Drying

The dried/milled and cooked/dried/milled samples were kept at 60 °C for 5 h in an oven with air circulation (DeLeo, DL-SE, Porto Alegre/RS/Brazil), according to the method described by Perdigão et al. [7].

2.2.3. Milling

The raw material samples submitted to milling (*in natura* milled, dried/milled, cooked/milled, and cooked/dried/milled) were grounded in a domestic blender (LiqFaz, Wallita, São Paulo/SP, Brazil), using a fixed mass of 100 g for a period of 15 s.

2.3. Classical method of carotenoid extraction

The extraction of pigments from the carotenoid family followed the classical method presented by Chen and Meyers [19] and was performed in triplicate. Briefly, 5 g of shrimp waste samples (*in natura* and pre-treated) were added to 200 mL of a solvent mixture (petroleum ether: acetone: water, 15:75:10, v/v/v), left to rest for 24 h at 5 °C (protected from light), filtered, and the lipid fraction was evaporated in a rotary evaporator with reduced pressure (Fisatom, 550, São Paulo/SP/Brazil). The results obtained were expressed as extraction yield in % (w/w) in dry basis.

2.4. Total carotenoid content (TCC)

The total carotenoid content (TCC) was based on the carotenoid profile results (Section 2.6.1) by considering the integration of the all carotenoid components present in the extract samples. The TCC was evaluated for the pink shrimp extracts obtained by the conventional method of carotenoid extraction (Section 2.3), applied for the samples submitted to the different pre-treatments (Section 2.2). The TCC analysis was performed in triplicate and the results were expressed in $\mu g\, g^{-1}$ of extract.

The most adequate pre-treatment, selected among the procedures described in Section 2.2 for use in the second part of this work (study of the different extraction methods), was defined based on the higher TCC value, allied with the higher extraction yield by the classical method (Section 2.3).

2.5. Extraction techniques

The different extraction techniques, applied to the pre-treated shrimp residue (pre-treatment selected according to Section 3.1), were compared in terms of yield and extract quality. The pre-treated raw material was also characterized in terms of the following physical chemistry aspects: total nitrogen content, moisture content, and total ash content, according to the A.O.A.C. [20] methods 991.20, 925.09 and 923.03, respectively. The results are expressed in % (w/w), wet basis (w.b.).

The extraction methods studied in this work (Soxhlet, maceration, ultrasound and vegetable oil extraction) are described below.

2.5.1. Soxhlet extraction (SOX)

Soxhlet extraction (SOX) method was performed in triplicate and based on the procedure described by Campos et al. [16]. Briefly, the raw material (5 g) was packed inside a cartridge and transferred to a 250 mL extractor device and submitted to 8-h recycling extraction with 150 mL solvent at boiling temperature. The extraction was performed with the following solvents (P.A., Nuclear, CAQ Ind. e Com. LTDA., Brazil): *n*-hexane (Hx); ethanol (EtOH); acetone (Ac); isopropanol and isopropanol:hexane (50:50, v/v). All of the used solvents are authorized as General Recognized as Safe (GRAS) solvents and are usually used in food industry [21]. The extracts were concentrated in a rotatory evaporator and the extraction yield was calculated in dry basis (d.b.) and expressed in % (w/w), d.b.

2.5.2. Maceration (MAC)

The maceration (MAC) method consists in a cold extraction of the pre-treated shrimp residue in organic solvent to avoid thermal degradation. The procedure was performed according to Sachindra et al. [22]. Briefly, it consists of transferring 25 g of the sample into 100 mL of selected organic solvent for five days at room temperature, light protection and one daily manual agitation. Each extraction was performed in triplicate using the solvents Hx, EtOH, Ac, IPA, and the binary Hx:IPA (50:50, v/v). The mixture of raw material, extract and solvent was separated by means of cellulose filtration, followed by concentration under vacuum. The extraction yield was calculated in dry basis (d.b.) and expressed in % (w/w), d.b.

2.5.3. Ultrasound extraction (UE)

The ultrasound extraction (UE) was performed in triplicate and adapted from Gu et al. [11]. Briefly, it consists of adding 5 g of raw material in 150 mL of EtOH, maintaining the solution into an ultrasound apparatus (UltraCleaner 700, 55 kHz, 40 VA, Unique, Indaiatuba/SP/Brazil) for 10 min. The extract was separated from the raw material by centrifugation (Q222T, Quimis, Diadema, SP, Brazil) at 2000 × *g* for 20 min and concentrated under vacuum. The extraction yield was then calculated in dry basis (d.b.) and expressed in % (w/w), d.b.

2.5.4. Hot and cold oil extraction (OilH and OilC)

The extraction methods using vegetable oil as solvent were classified according to the solvent temperature. The cold extraction (OilC) and the hot extraction (OilH) with vegetable oil, performed according to the procedure presented by Sachindra and Mahendrakar [23] and realized in triplicate. The extraction yield was evaluated using sunflower oil and soybean oil as solvents (Liza, Cargill Inc., Brazil). The method consists of mixing 10 g of raw material with 40 mL of vegetable oil in a 250 mL flask (light protected), submitted to hot plates with 2-h agitation period at room temperature (OilC) or at 70 °C (OilH). Further, the oil extracts were recovered by cellulose filtration and the extraction yield of the pigments (carotenoids) was quantified by the Total Carotenoid Content, as expressed by Section 2.6.1.

2.6. Extract quality

The quality of the different extracts from shrimp residue obtained by the various extraction methods (Section 2.5) was determined in terms of carotenoid profile, UV–Visible scanning and Infrared spectroscopy, as presented below.

2.6.1. Carotenoid profile

Shrimp residue extracts obtained through the above described protocols were properly homogenized in *n*-hexane (2 mL) and the resulting solution was centrifuged at 3000 × *g* for 5 min. The extracts were analyzed by high performance liquid chromatography (HPLC) and followed the procedure described by Kuhnen et al. [24] by using a 5 μL extract sample, in a triplicate analysis. The HPLC equipment (Shimadzu LC-10A) was assembled with a thermostatized (40 °C) C_{18} reverse-phase column (Vydac – BioRad, 218TP54, 250 mm × 4.6 mm, 5 μm) coupled with a column-guard (Vydac – BioRad, 218TP54, 30 mm × 4.6 mm, 5 μm), and an UV–Visible detector ($\lambda = 460$ ηm). The mobile phase was acetonitrile: methanol mixture (90:10, v/v – Tedia, USA – HPLC grade) at a flow rate of 0.8 mL/min. The identification of the carotenoids was performed by retention time and co-chromatography of standard compounds (Sigma Aldrich, St. Louis, MO, USA). The standard components were submitted to the same HPLC conditions as of the extracts analysis. The relative quantification of the identified components was based on the area integration of the chromatogram peaks for each compound, coupled with the use of standard curves ($y = 44\,231.57x$, $r^2 = 0.994$, for free astaxanthin, esterified astaxanthin, and β-cryptoxanthin; and $y = 1\,000\,000x$, $r^2 = 0.991$, for α-carotene, *trans*-β-carotene, and *cis*-β-carotene). The quality of the extract samples analyzed by HPLC was expressed in terms of (1) carotenoids concentration, obtained by the relative quantification of each components; (2) total carotenoid content (TCC), obtained by the sum of the amount from all carotenoids identified; (3) astaxanthin yield calculated considering extraction yield (Section 2.5) and the astaxanthin content.

2.6.2. UV–Visible scanning

The extracts (20 mg) were transferred to 10 mL amber flasks, homogenized with 10 mL of *n*-hexane and, then, manually agitated. The UV–Visible scanning profile of samples was obtained through an UV–Visible spectrophotometer (Hitachi, model U-1800), for a spectral window of 200–750 ηm, and performed in triplicate.

2.6.3. Infrared spectroscopy

The mid-Fourier transform infrared (FTIR) vibrational spectroscopy analysis was carried out using a Bomem spectrometer (FTLA2000, Bomem, Inc., Quebec, Canada) equipped with DTGS (deuterated triglycine sulfate) detector, according to the method of Silverstein [25]. Briefly, the extract sample (ca. 5 mg) was mixed to KBr previously pulverized and the powder was compressed in stainless steel tablets. The spectra (5 replicate spectra, 128 co-added scans before Fourier transform) were recorded at the transmittance mode from 3000 to 600 $\mathrm{cm^{-1}}$. The extract sample spectra were normalized and the baseline-corrected in the region of interest. Therefore, the resolution enhancement (*k* factor of 1.5) was applied by means of the Fourier self deconvolution, through the Bomem Easy Software Program. The extract samples analyzed by the FTIR procedure were obtained by maceration with ethanol and by acetone, selected for this analysis because of their discrepant profiles (different classes of components detected, as presented in Section 3.2.4) determined by UV–Vis scanning spectrophotometry in comparison to other samples.

2.7. Statistical analysis

The extraction yield, the TCC results and the carotenoid contents from HPLC were statistically evaluated by a one-way analysis of variance (ANOVA), using the statistical package Statistica for Windows 6.0 (Statsoft Inc., USA). The significant differences at level of 5% were analyzed by Tukey test.

3. Results and discussion

3.1. Raw material pre-treatment

The yield and the TCC values, by means of the classical carotenoid extraction, for all pre-treatments applied to the shrimp residue are presented in Table 1. According to these results, the lowest yield was achieved by the *in natura* sample (a), when no pre-treatment was used (12 ± 3%). This yield result was statistically equal to all values from other samples, except for the cooked/dried/milled sample which, otherwise, showed the highest yield value (23.5 ± 0.5%).

Comparing the yield data from Table 1 for the pre-treatments (a), (b), (d) and (e) we observe that, the milling procedure did not statistically improve the extraction yield. The effect of cooking and drying pre-treatments are observed by comparing the results for

Table 1
Extraction yield and total carotenoid content (TCC) for extracts of pink shrimp (*P. brasiliensis* and *P. paulensis*) waste submitted to different pre-treatments and obtained by the conventional extraction for 24 h, in a solvent/raw material ratio of 40 mL g^{-1}.

Raw material pre-treatment	Solvent polarity index[a]	Extraction yield (%, w/w, d.b.)[b,c]	TCC ($\mu g\ g^{-1}_{extract}$)[b]
In natura	4.74	$12 \pm 3^{(1)}$	$14.2 \pm 0.2^{(1)}$
In natura milled	4.74	$16 \pm 3^{(1)}$	$18.1 \pm 0.2^{(2)}$
Dried and milled	4.74	$18.6 \pm 0.2^{(1,2)}$	$15 \pm 1^{(1)}$
Cooked	4.74	$15 \pm 2^{(1)}$	$34.2 \pm 0.5^{(5)}$
Cooked and milled	4.74	$13.5 \pm 0.6^{(1)}$	$27 \pm 1^{(4)}$
Cooked, dried and milled	4.74	$23.5 \pm 0.5^{(2)}$	$29.2 \pm 0.3^{(4)}$

[a] Ref. [46].
[b] Same superscript number on same column indicates no significant difference ($p < 0.05$).
[c] Obtained by the conventional method for carotenoid extraction.

assays (a)–(d); (b)–(e) and (b)–(c), where no statistical significance was detected on yield results. Besides that, the cooking procedure affected significantly the extraction yield, especially when combining drying and milling processes, as observed comparing assays (a) and (b) with (f). These results suggest that milling and drying processes do not significantly affect the yield results for non cooked samples, probably because the cooking procedure breaks the carotenoid–protein complex from the raw material, increasing the extraction yield.

The conventional extraction method provided the maximum TCC value of 34.2 ± 0.5 $\mu g\ g^{-1}_{extract}$ for the (d) cooked sample, followed by 29.2 ± 0.3 $\mu g\ g^{-1}_{extract}$ for the (f) cooked/dried/milled sample, while the minimum TCC value was 14.2 ± 0.2 $\mu g\ g^{-1}_{extract}$ for the (a) *in natura* sample (Table 1). The milling procedure affected significantly the TCC values for *in natura* samples (milled and not milled), probably due to the increase in the amount of exposed solute (outside the solid particles), caused by the enlargement in surface area with the particle size reduction and, then, enhancing the mass transfer by the convection mechanism. Then, the particle reduction is necessary to diminish the solvent pathway to reach the solute inside the solid matrix, e.g., improving also the diffusion mechanism [26].

Perdigão et al. [7] evaluated the carotenoids extraction from *in natura* and cooked lobster carapaces and reported low extraction yield from *in natura* samples (without cooking) compared to the cooked ones. The authors suggested that the cooking process can break the carotenoid–protein complex, releasing the carotenoid compounds and facilitating its extraction. Additionally, the authors concluded that a higher extraction yield was obtained for lower moisture content, emphasizing the importance of the drying procedure. Hornero-Méndez and Mínguez-Mosquera [27], studying the cooking effect on carotene bioavailability in carrots reported that, although thermal treatment during cooking showed a negative impact on the carotenoid content, it also promotes a positive effect on carotene micellarisation and, therefore, on their bioavailability.

The cooking effect on carotenoid recovery was also observed in the present study. The best pigment recovery, represented by the highest TCC values, was achieved by the cooked samples, as presented in Table 1. The use of "cooking" as the only pre-treatment process results in higher TCC value, when compared to cooked/dried/milled procedure, although it provides lower extraction yield. Therefore, based on previous results, we suggest that all pre-treatment methods were important to improve the carotenoid extraction (yield and quality) from shrimp waste. Additionally, the combined effect of cooking/drying/milling (assay f) on extraction yield associated with TCC, presented the best ratio of 0.8 (yield/TCC), among the cooked procedures (assays d, e and f), indicating higher extraction efficiency. As a consequence, this pre-treatment procedure (cooking/drying/milling) was selected for the following studies to evaluate the different extraction techniques.

3.2. Extraction techniques

3.2.1. Extraction yield

According to the results for the pre-treatment evaluation (Table 1), the raw material submitted to cooking, drying, and milling presented the best results (yield and TCC) and was selected for the extraction systems study. Therefore, the pre-treated raw material was evaluated according to its physical–chemical composition and the results revealed a total nitrogen content of $26.09 \pm 0.01\%$ (w/w, w.b.), a moisture content of $46.30 \pm 0.06\%$ (w/w, w.b.), and an ash content of $17 \pm 2\%$ (w/w, w.b.). The predominance of proteins and minerals (nitrogen and ash contents) from the shrimp residue studied in this work was also detected by Heu et al. [28] for the residue from Nothern pink shrimp (*P. borealis*) and spotted shrimp (SS, *Trachypena curvirostris*). The protein content of $26.09 \pm 0.01\%$, observed in this work (residue from *P. brasiliensis* and *P. paulensis*), was superior to the head residue from other shrimp species presented in the literature [29], e.g., *P. mondon* (11.3%), *P. indicus* (12.3%), and *M. monocerus* (11.2%). The mineral content of $17 \pm 2\%$ was also superior to that observed by Babu et al. [29], which varied from 8.2% to 11.8% for the species cited above. The differences between the physical–chemical results of raw material from the present work and from the literature suggest the influence of shrimp species and the part of shrimp waste treated.

The extraction yield and TCC values for the cooked, dried and milled raw material (pre-treatment), obtained by means of the different extraction techniques are presented in Table 2, together with the solvent polarity index, the solvent/solid ratio and the extraction time. Comparing the conventional method of carotenoid extraction (Table 1) with other extraction techniques (Table 2) we observed that only the Soxhlet with ethanol method presented an extraction yield much higher than the conventional method, with values of $68 \pm 6\%$ and $23.5 \pm 0.5\%$, respectively. Alternatively, when we compare the results in terms of TCC values, the data from Table 2 indicate that several procedures were more efficient in obtaining carotenoid compounds, with values up to 253 ± 7 $\mu g\ g^{-1}_{extract}$ for the maceration with acetone, compared to the value of 29.2 ± 0.3 $\mu g\ g^{-1}_{extract}$ for the conventional result (Table 1). The conventional method (Table 1) requires high amount of solvent and 24 h of extraction time to obtain yield and carotenoid values inferior to the ones reached by other non conventional extraction procedures (maceration with hexane, hexane:isopropanol, acetone, ethanol; Soxhlet with: hexane, hexane:isopropanol, isopropanol, acetone; and ultrasound with ethanol – Table 2). The results from Table 2 show the importance of the optimization of the extraction techniques to achieve the maximum TCC value. Furthermore, because of the considerable quality of the extracts (high TCC values) obtained by the extraction methods listed in Table 2, we suggest that pink shrimp residue is a suitable raw material to obtain valuable by-products.

Evaluating the results from Table 2 we detect that Soxhlet method shows higher yield values compared to maceration, for

Table 2
Extraction yield and total carotenoid content (TCC) of extracts from pink shrimp (*P. brasiliensis* and *P. paulensis*) waste obtained according to extraction technique and solvent.

Technique solvent[a]	Solvent/raw material ratio ($mL\,g^{-1}$)	Extraction time (h)	Solvent polarity index[b]	Extraction yield (%, w/w, d.b.)[c]	TCC ($\mu g\,g^{-1}_{extract}$)[d]
MAC-Hx	4	120.00	0.00	$2 \pm 1^{(4)}$	$188 \pm 5^{(2)}$
MAC-HxIPA	4	120.00	1.95	$9.4 \pm 0.2^{(3)}$	$154.3 \pm 0.5^{(3)}$
MAC-IPA	4	120.00	3.90	$18 \pm 1^{(2)}$	$26 \pm 1^{(7)}$
MAC-Ac	4	120.00	5.10	$4 \pm 1^{(3,4)}$	$253 \pm 7^{(1)}$
MAC-EtOH	4	120.00	5.20	$23.3 \pm 0.6^{(2)}$	$87 \pm 3^{(5)}$
SOX-Hx	30	8.00	0.00	$19 \pm 2^{(2)}$	$35.2 \pm 0.1^{(6)}$
SOX-HxIPA	30	8.00	1.95	$11 \pm 1^{(2)}$	$198 \pm 3^{(2)}$
SOX-IPA	30	8.00	3.90	$22.5 \pm 0.4^{(2)}$	$78 \pm 7^{(5)}$
SOX-Ac	30	8.00	5.10	$20 \pm 2^{(2)}$	$97 \pm 2^{(4,5)}$
SOX-EtOH	30	8.00	5.20	$68 \pm 6^{(1)}$	$25.6 \pm 0.5^{(7)}$
UE-EtOH	30	0.17	5.20	$18.0 \pm 0.7^{(2)}$	$38 \pm 2^{(6)}$
OilH-Sunflower	4	2.00	Null	$32 \pm 2\ (\times 10^{-4})^{(\beta,4)}$	$4.5 \pm 0.2^{(\gamma,8)}$
OilH-Soy	4	2.00	Null	$25.15 \pm 0.05\ (\times 10^{-4})^{(\gamma,4)}$	$3.87 \pm 0.04^{(\delta,8)}$
OilC-Sunflower	4	2.00	Null	$36.4 \pm 0.6\ (\times 10^{-4})^{(\beta,4)}$	$5.18 \pm 0.09^{(\beta,8)}$
OilC-Soy	4	2.00	Null	$44 \pm 4\ (\times 10^{-4})^{(\alpha,4)}$	$6.7 \pm 0.6^{(\alpha,8)}$

[a] MAC, maceration; SOX, Soxhlet extraction; UE, ultrasound; OilC, extraction with cold oil; OilH, extraction with hot oil; Hx, hexane; HxIPA, hexane + isopropanol (50:50); IPA, isopropanol; EtOH, ethanol; Ac, acetone.

[b] Ref. [46].

[c] Same superscript number or Greek letter on same column indicates no significant difference ($p < 0.05$).

the same solvent applied. As observed by Mezzomo et al. [30], the high temperature, the solvent recycle and the solvent/solute interactions, detected in Soxhlet method contributed to the highest solubilization of components from the raw material. Also, the use of solvent in its boiling temperature reduces its viscosity and surface tension, allowing the solvent to reach easier the soluble substances inside the solid matrix [15].

Maceration procedure using ethanol and isopropanol (Table 2) shows extraction yield comparable to conventional method using the same pre-treatment (Table 1). Moreover, maceration method does not require the use of heating system during extraction and applies low amount of solvent (4 $mL\,g^{-1}$ of raw material), although high extraction times are needed. According to the literature, xanthophylls such as astaxanthin, which contain oxygenated groups in their β-ionone rings, are adequately extracted by polar solvents like ethanol and isopropanol [11,12,31].

The sample obtained by ultrasound with ethanol showed satisfactory extraction yield, similar to data obtained by Soxhlet with hexane, isopropanol and acetone, and maceration with ethanol and isopropanol. Ultrasound can facilitate swelling and hydration of the shrimp waste, allowing an enlargement in the pores present on matrix cells from the raw material. This effect improves the diffusion process, enhancing the mass transfer of soluble constituents from solid material to solvent phase, especially by diffusion [9,32]. Additionally, ultrasound method can use moderate extraction temperatures and very low process time such as the 10 min applied in the present work, which is equivalent to 1/48 and 1/720 times lower than Soxhlet and maceration processes (Table 2), respectively.

Extractions using vegetal oil as solvent resulted in low yields for all conditions applied (type of vegetable oil and process temperature), where no significant difference ($p < 0.05$) was detected among data, when compared to other techniques (maceration, Soxhlet and ultrasound extraction). Despite that, vegetable oils can be used in a combination with other solvents to improve the extract quality, and the knowledge of the extracting conditions is valuable. Therefore, we applied statistical analysis among the results for oily solvents (OilC and OilH), searching for process optimization. The results, showed in Table 2 (superscript Greek letters), indicate that soy oil was better than sunflower at room temperature, while the opposite was detected at higher temperature. The increase in temperature with soy oil as solvent also reduced significantly the yield extraction (from $44 \pm 4 \times 10^{-4}$ to $25.15 \pm 0.05 \times 10^{-4}\%$). This behavior probably occurred because carotenoids are heat-sensitive compounds that can be degraded at high temperatures.

3.2.2. *Total carotenoid content*

The TCC results for the different extraction systems are shown in Table 2. Comparing these data with the result obtained by the classical extraction method (Table 1) for the sample cooked/dried/milled ($29.2 \pm 0.3\ \mu g\,g^{-1}_{extract}$) we observe that the methods listed in Table 2 presented higher TCC values, except for the sample obtained by Soxhlet with ethanol ($25.6 \pm 0.5\ \mu g\,g^{-1}_{extract}$), by maceration with isopropanol ($26 \pm 1\ \mu g\,g^{-1}_{extract}$) and by ultrasound samples. These results indicate that the optimization of the carotenoid recovery from the shrimp processing waste is feasible by selecting the suitable extraction technique.

The highest TCC observed was $253 \pm 7\ \mu g\,g^{-1}_{extract}$, for the sample obtained by maceration using acetone, followed by Soxhlet with hexane:isopropanol ($198 \pm 3\ \mu g\,g^{-1}_{extract}$). Samples obtained by maceration with hexane:isopropanol and by Soxhlet with acetone, with isopropanol and with ethanol also presented satisfactory TCC values (Table 2) when compared to classical extraction method (Table 1). Actually, astaxanthin can be successfully extracted by alcohols and acetone as solvents due to their high polarity, justifying the results from the present study and suggesting that acetone is the best solvent for carotenoid extraction from *P. brasiliensis* and *P. paulensis* residue.

Comparing the extraction techniques in terms of total carotenoid content, maceration with acetone was statistically superior to other methods, probably because the non-requirement of a heating system during extraction, which avoids the carotenoids thermal degradation, and the high contact time between solvent and raw material. Subsequently, Soxhlet also presented satisfactory TCC, probably due to the high diffusion and solubilization power promoted by this technique, resulting in a good amount of carotenoids extracted from the raw material [30]. This behavior can be observed when comparing the extraction methods maceration (low temperature process) and Soxhlet (high temperature process) by means of the same solvents (hexane, ethanol and acetone). The results from Table 2 show that, in general, maceration samples presented superior TCC values compared to Soxhlet samples, except when isopropanol was used as solvent (pure or in mixture), probably because of the solute/solvent interactions and to the association between carotenoids and macroelements from the shrimp residue.

The ultrasound method is also a promising alternative for processing shrimp residues with no heating application, because ultrasound allows good extraction yield, although the TCC was low. Therefore, the results from Table 2 suggest that the ultrasound method is adequate for the extraction of different groups of compounds (instead of carotenoids), such as lipids and proteins. Alternatively, the carotenoid extraction can be optimized by increasing the extraction time or using other solvent, such as acetone or hexane:isopropanol mixture.

Extraction with vegetal oils as solvent presented the lowest carotenoid content values, contrasted to other techniques (Table 2). Additionally, considering only the samples obtained by vegetable oil extraction we detect: (1) samples obtained by hot vegetal oils have TCC values statistically lower than samples obtained by cold oils, probably due to the low stability of carotenoids in high temperatures; (2) considering the cold oil results, soy oil produced the highest TCC compared to sunflower oil. For instance, the presence of the thermosensible unsaturated fatty acids, such as linolenic acid from the soy oil [33], enhance the interactions among the components presented in the shrimp residue, improving the carotenoid extraction. Besides the low carotenoid content obtained by vegetable oils, in defense of these solvents are the protection against oxidation, the selectivity enhancement and the supply of energy for food products [6,12,19,31].

3.2.3. *Carotenoid profile*

The carotenoid profile determined by HPLC, for the extract samples from the shrimp residue, obtained by the various methods (Soxhlet, maceration, ultrasound, extraction with cold and hot oil) and solvents (hexane, hexane:isopropanol, isopropanol, acetone, ethanol, sunflower and soy oils) are presented in Table 3.

The results from Table 3 show that astaxanthins in free and esterified forms are the most abundant components among the carotenoids (total carotenoids evaluated by HPLC) present in the shrimp residue extracts, independently of the extraction technique applied.

The astaxanthin in its free form is unstable and extremely sensitive to factors such as light, oxygen, acidity, and heat [34]. Under these conditions, the free component is very susceptible to a variety of oxidative conversions, degradation, and isomerization processes. Nevertheless, because of the high possibility to link with free radicals, the astaxanthin in its free form also presents high antioxidant power [35].

The concentration of astaxanthin in the free form decreased progressively for the extract samples obtained by: maceration with acetone (131 ± 2 $\mu g\, g_{\mathrm{extract}}^{-1}$), Soxhlet with ethanol (23.0 ± 0.7 $\mu g\, g_{\mathrm{extract}}^{-1}$), extraction with hot oils (from 0.9 ± 0.4 to 1.9 ± 0.1 $\mu g\, g_{\mathrm{extract}}^{-1}$), and extraction with cold oils (from 0.468 ± 0.003 to 0.67 ± 0.05 $\mu g\, g_{\mathrm{extract}}^{-1}$). These results were found probably because astaxanthin in free form has lower molecular chain compared to the etherified form, especially when linked to fatty acids, resulting in a better extraction performance for more polar solvents. These methods also reached high extraction yield values (Table 2), and consequently low selectivity, i.e., allowed the extraction of chemically distinct compounds (besides carotenoids).

The free astaxanthins can be stabilized by esterification with fatty acids, forming monoesters or diesters of astaxanthin, or with proteins, forming carotenoprotein complex [34]. The concentration of astaxanthin in the esterified form is presented in Table 3 for the extract samples obtained by the different procedures. The values decreased progressively for the extract samples obtained by Soxhlet with hexane:isopropanol (197 ± 3 $\mu g\, g_{\mathrm{extract}}^{-1}$) and by maceration with hexane:isopropanol (154.2 ± 0.5 $\mu g\, g_{\mathrm{extract}}^{-1}$) and with pure hexane ($138 \pm 3$ $\mu g\, g_{\mathrm{extract}}^{-1}$). These treatments used pure solvent or solvent mixture with low polarity index (from 0.00 to 1.95 – Table 2). The polarity of the esterified form of astaxanthin depends on the linked molecule, i.e., proteins enhance the astaxanthin polarity whereas fatty acids reduce it. Therefore, the higher concentration of the esterified form obtained by solvents with low polarity index implies that the pink shrimp extracts are probably mainly esterified by fatty acids (lower polarity), instead of protein (higher polarity). Additionally, the esterified astaxanthin was more efficiently extracted by maceration, probably because of the low temperature and longer contact time between solvent and raw material, compared to other methods.

Besides astaxanthin, secondary metabolite components such as β-cryptoxanthin, α-carotene and β-carotene were also detected in the extracts, mainly for the maceration samples (Table 3). The maceration treatments provide extracts with higher variety and quantity of secondary metabolites, probably due to the longer contact time between solvent and raw material, compared to other methods.

The β-cryptoxanthin is the second most representative carotenoid type detected for the shrimp extracts. The sample obtained by maceration with hexane provided the higher concentration of β-cryptoxanthin (49 ± 1 $\mu g\, g_{\mathrm{extract}}^{-1}$), followed by Soxhlet with hexane (4.8 ± 0.1 $\mu g\, g_{\mathrm{extract}}^{-1}$) and maceration with ethanol (4.2 ± 0.5 $\mu g\, g_{\mathrm{extract}}^{-1}$). Structurally, β-cryptoxanthin is closely related to β-carotene, differing only by the addition of a hydroxyl group to one of the β-ionone ring. Because of its chemical structure, β-cryptoxanthin has antioxidant properties, preventing free radical damage to cells and DNA as well as presenting some pro-vitamin A activity [36]. Recent findings of an inverse

Table 3
Carotenoid profile for pink shrimp (*P. brasiliensis* and *P. paulensis*) waste extracts from different extraction procedures.

Technique-solvent[b]	Carotenoid contents ($\mu g\, g_{\mathrm{extract}}^{-1}$)[a]					
	Etherified astaxanthin	Free astaxanthin	β-criptoxanthin	α-carotene	*trans*-β-carotene	*cis*-β-carotene
MAC-Hx	$138 \pm 3^{(3)}$	–	$49 \pm 1^{(1)}$	$0.61 \pm 0.04^{(2)}$	$0.6 \pm 0.2^{(1)}$	–
MAC-HxIPA	$154.2 \pm 0.5^{(2)}$	–	–	–	–	$0.015 \pm 0.002^{(2)}$
MAC-IPA	$23.8 \pm 0.6^{(7)}$	–	$2.1 \pm 0.7^{(2)}$	$0.009 \pm 0.005^{(1)}$	$0.009 \pm 0.005^{(2)}$	–
MAC-Ac	$121 \pm 4^{(3)}$	$131 \pm 2^{(1)}$	–	–	–	–
MAC-EtOH	$79 \pm 3^{(5)}$	–	$4.2 \pm 0.5^{(2)}$	–	–	–
SOX-Hx	$30.5 \pm 0.2^{(6,7)}$	–	$4.8 \pm 0.1^{(2)}$	–	–	–
SOX-HxIPA	$197 \pm 3^{(1)}$	–	–	–	–	$0.28 \pm 0.05^{(1)}$
SOX-IPA	$78 \pm 7^{(5)}$	–	–	–	–	–
SOX-Ac	$97 \pm 2^{(4)}$	–	–	–	–	–
SOX-EtOH	$2.6 \pm 0.2^{(8)}$	$23.0 \pm 0.7^{(2)}$	–	–	–	–
UE-EtOH	$38 \pm 2^{(6)}$	–	$0.65 \pm 0.08^{(3)}$	–	–	–
OilH-Sunflower	$0.9 \pm 0.4^{(8)}$	$4.3 \pm 0.5^{(3)}$	–	–	–	–
OilH-Soy	$1.9 \pm 0.1^{(8)}$	$4.9 \pm 0.8^{(3)}$	–	–	–	–
OilC-Sunflower	$0.67 \pm 0.05^{(8)}$	$3.9 \pm 0.1^{(3)}$	–	–	–	–
OilC-Soy	$0.468 \pm 0.003^{(8)}$	$3.40 \pm 0.05^{(3)}$	–	–	–	–

[a] Same superscript number on same column indicate no significant difference ($p < 0.05$).

[b] MAC, maceration; SOX, Soxhlet extraction; UE, ultrasound; OilC, extraction with cold oil; OilH, extraction with hot oil; Hx, hexane; HxIPA, hexane + isopropanol (50:50); IPA, isopropanol; EtOH, ethanol; Ac, acetone.

association between β-cryptoxanthin and lung cancer risk in several epidemiological studies suggest that β-cryptoxanthin could potentially acts as a chemopreventive agent against lung cancer [37]. Due to the importance of β-cryptoxanthin properties, its high amount in extract obtained by maceration with hexane suggests its potential use as a bioactive product.

The component α-carotene is a primary form of carotene differing from β-carotene by the position of a double bond in the aromatic ring. This carotenoid has shown suppressor growth activity of tumors and cancers, by means of paralyzing the cellular multiplication [38]. In the present work, α-carotene was detected in low concentrations only for the sample obtained by maceration with hexane and with hexane:isopropanol mixture (Table 3).

The β-carotene is a natural pigment with a wide variety of market applications, such as food coloring and antioxidant agent, precursor of vitamin A (retinol) in food and animal feed, and additive to cosmetics and to multivitamin preparations. Moreover, the biological potential depends on the predominance of the *cis*-isomer of β-carotene, which is more efficient in protecting against oxidative damage than the *trans*-isomer [39]. The results from Table 3 shows that *cis*-β-carotene was only detected in samples obtained by maceration and Soxhlet methods using solvent mixture of hexane and isopropanol.

Considering the total astaxanthin content (free and esterified forms), the results from Table 3 inform that maceration was the most adequate method and acetone was the best solvent to extract astaxanthin from pink shrimp residue. The ultrasound method was not efficient for astaxanthin extraction, probably because of the low contact time between solvent and raw material, and also due to the use of ethanol, a solvent with low selectivity to astaxanthin. As for TCC results, the extraction with vegetal oils presented the lowest total value (Table 2). On the other hand, this method provided extracts enriched in astaxanthin among the identified carotenoids, which suggests a highly selective procedure, although it is necessary to optimize the yield of the extraction method.

Furthermore, the higher concentration of the esterified astaxanthin (Table 3), contrasted to the fact that the free form is biologically more active, advice for the necessity of the samples hydrolysis to release the free astaxanthin [40].

Complementarily, there is a lack of studies about carotenoid profile of shrimp residues, particularly *P. brasiliensis* and *P. paulensis,* mostly related to the astaxanthin content in the lipid fraction. Therefore, in order to compare our results with literature data, we considered information related to shrimp and other marine species. The highest astaxanthin concentration (Table 3) was obtained by maceration with acetone (252 ± 6 $\mu g\, g_{extract}^{-1}$), a value superior to the one obtained by Sachindra et al. [41], for head and carapace Indian shrimp (from 35.8 to 153.1 $\mu g\, g^{-1}$), and by Omara-Awala et al. [10], for crawfish puree (123 $\mu g\, g_{lipid}^{-1}$). Also, our results (Table 3) were similar to the ones obtained by Pacheco et al. [42] for shrimp (*Litopenaeus* sp.) residue (280 $\mu g\, g_{silage}^{-1}$). The variation was probably caused by differences in the environmental conditions, species, astaxanthin quantification methods, and other variables like particles size, temperature, moisture content and residue/solvent ratio.

The most effective extraction procedure was defined by the astaxanthin yield (process yield × astaxanthin content). The results for the different methods studied, compared to the standard sample (cooked/dried/milled extract obtained by the conventional method of carotenoid extraction – Table 1) are presented in Fig. 1 in terms of $\mu g_{astaxanthin}\, g^{-1}$ of dried raw material (RM). The data from Fig. 1 show that: Soxhlet with hexane:isopropanol and with acetone reached the highest astaxanthin yields (21 ± 1 and 20 ± 2 $\mu g_{astaxanthin}\, g^{-1}$ RM, respectively), followed by maceration with ethanol and Soxhlet with isopropanol and with ethanol (18.3 ± 0.6, 17 ± 1 and 17 ± 1 $\mu g_{astaxanthin}\, g^{-1}$ RM, respectively). The good performance in terms of astaxhantin yield obtained by Soxhlet with ethanol was probably caused by the higher extraction yield of this method (68%, w/w, d.b.), and not to the relative content of astaxanthin (25.2 $\mu g\, g_{extract}^{-1}$), unlike the extract obtained by Soxhlet with hexane:isopropanol, which the high astaxanthin content (197 ± 3 $\mu g\, g_{extract}^{-1}$) was a result from a more selective extract. Therefore, Soxhlet with hexane:isopropanol could represent a viable and interesting choice to obtain astaxanthin enriched extracts from pink shrimp residue, with application in the pharmaceutical and food formulations. Additionally, a complete economical analysis is necessary to provide an industrial application for each extraction technique, as observed and studied by Mezzomo et al. [43].

Finally, the high extraction yield values allied with low astaxanthin yield, for some extracts from pink shrimp residue, indicates that other components, besides carotenoids, are present in the samples. This behavior advises further chemical investigation of the extract samples. Therefore, a complementary study was performed by UV–Visible scanning spectrophotometry and vibrational

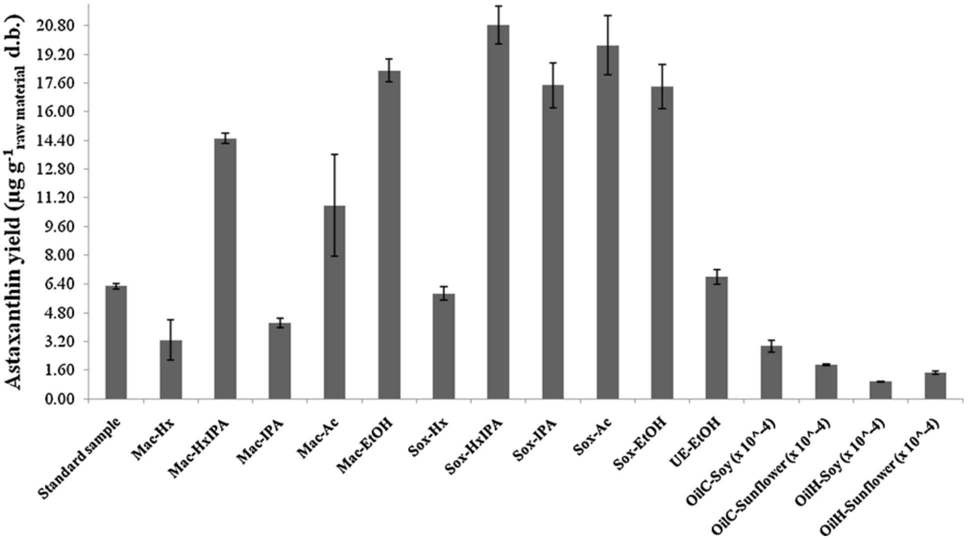

Fig. 1. The astaxanthin yield from the shrimp residue extracts obtained by different methods (MAC, maceration; SOX, Soxhlet extraction; HxIPA, hexane:isopropanol; Ac, acetone; EtOH, ethanol; IPA, isopropanol) and by the standard sample (cooked/dried/milled pre-treatment and conventional technique of carotenoid extraction).

infrared spectroscopy (FTIR), which can provide a better evaluation of the chemical composition of the extracts.

3.2.4. UV–Vis scanning and infrared spectroscopy (FTIR)

The UV–Vis spectra (200–750 ηm) of the shrimp extracts show a different chemical profile according to the extraction procedure used to obtain the samples investigated. The sample obtained by maceration with hexane presented two main bands: one at 280–300 ηm, characterizing phenolic compounds, especially flavonoids; and other at 430–480 ηm, typically illustrating the occurrence of carotenoids [44]. Maceration and Soxhlet with ethanol produced extracts with a large group of components detected between 300 and 750 ηm, which suggest the presence of flavonoids, carotenoids, phenolic compounds, among others. Ultrasound with ethanol showed maximum absorbances in the visible range (from 300 to 500 ηm), inferring the presence of flavonoid-like compounds, besides carotenoids [44]. The extract obtained by cold sunflower oil showed the highest absorbance at 280–300 ηm, characteristic of flavonoids (anthocyanins), differently from the sample obtained by hot soy oil, which presented the highest band intensity at 400–480 ηm, suggesting that the soy oil, in comparison with sunflower oil, promoted highest carotenoid extraction. Finally, maceration with acetone showed maximum absorbances with high intensity at 300–350 nm, also inferring the presence of flavonoid-like compounds. The probable presence of these components in the extracts, especially flavonoids, can be attributed to the content of the shrimp head, mainly formed by the digestive system of the animal, contributing to the presence of compounds from animal feed like marine plants and algae. Therefore, UV–Vis scanning spectrophotometry confirmed the importance of the extracts, suggesting the presence of components with interesting bioactive properties, such as flavonoids. In this context, the spectrophotometric approach herein described is an interesting contribution to detect the occurrence of non-carotenoid compounds in the residual biomass in study, i.e., in quality control process of the extracts.

Furthermore, the infrared spectroscopy was performed in order to provide more information regarding the extract composition, necessary for the industrial application of the extracts. Because the extracts obtained by maceration with acetone and ethanol showed the largest difference in the UV–Vis profile, these samples were selected for the FTIR qualitative analysis. The results indicate a similar qualitative spectral profile for both samples, except for small differences at the range between 1800 and 1300 cm^{-1}, indicating comparable composition.

The sample obtained by maceration with ethanol showed the following stronger bands: at 2954, 2924, and 2854 cm^{-1}, typical for alkyl group from proteins; at 1738 cm^{-1}, relative to esters of fatty acids; at 1583 cm^{-1}, that can be related to aromatic compounds such as primary amines; and at 1467, 1406 and 1238 cm^{-1}, which are characteristic of aromatic compounds or carboxylic acids, such as phenolic compounds [45].

The other extract (maceration with acetone) also contains the same intense bands at 2926 and 2854 cm^{-1}, characteristic from the presence of proteins. Additional strong bands were detected at: 1701 and 1670 cm^{-1}, characteristic of carbonyl and carboxylic acids like proteins and phenolic acids; 1466, 1395, and 1378 cm^{-1}, typical from aromatic compounds, aliphatic amines and carboxylic acids. Minor signals were detected at 2601 and 2118 cm^{-1}, most likely also related to protein components [45].

Similarly to the UV–Vis scanning spectrophotometry, the FTIR results revealed that the shrimp residue extracts were present more than that of carotenoids in their composition, since typical signals for proteins, fatty acids, and phenolic compounds were detected. Such results might be explained by the fact that shrimp waste, composed mainly by head and carapace, contains residues of shrimp meat [29].

4. Conclusions

The carotenoid extraction from *P. brasiliensis* and *P. paulensis* waste by different techniques and pre-treatments showed the feasibility of this residual biomass as a rich source of carotenoids of human health interest. The industrial application of these methods is technically viable depending on the astaxanthin purity of extracts. The best raw material pre-treatment, in terms of yield and total carotenoid content, was obtained by the combination of the procedures cooking, drying, and milling of the shrimp residues. From the studied extraction techniques, the best solvents for carotenoid extraction were acetone and hexane:isopropanol mixture, which promoted high carotenoid content, particularly using the maceration method. Cold oil extraction can be a good alternative to selectively extract astaxanthin and protect the extract, although process optimization is required to enhance the extraction yield. The UV–Vis scanning spectrophotometry indicated the presence of phenolics, carotenoids, and flavonoids, whereas FTIR analysis revealed the occurrence of proteins, fatty acids, and phenolics, mainly due to the wide composition variety of the shrimp diet. The identified composition of the extracts suggests their high potential for applications in food or pharmaceutical industries.

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