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"Gheorghe Asachi" Technical University of Iasi, Romania

"GREEN" EXTRACTION OF BIOACTIVE MOLECULES FROM VEGETABLES AND FISH INDUSTRY BY-PRODUCTS

Giovanna Ficano*, Gerardina Galdi, Francesco De Sio, Mariateresa Rapacciuolo, Luca Sandei, Domenico Cacace

SSICA (Stazione Sperimentale per l'Industria delle Conserve Alimentari) Research Foundation
Viale F. Tanara 31/A, 43100 Parma (PR) - Italy

Abstract

Food companies are increasingly interested in the effective application of sustainable and innovative techniques able of making by-products management less expensive if not even a source of additional incomes. Since several by-products coming from the food processing industry are rich in high-power bioactive molecules, the recovery of such substances is currently the most promising means, either for reusing them as ingredients in functional foods or for applications in many sectors such as the packaging one. Indeed, combining bioactive molecules extracted from vegetables and fishery by-products can contribute to make biofilm with antimicrobial and antioxidant effects. Moreover, from the point of view of sustainability, it is necessary to replace chemical extraction techniques, characterized by heavy environmental side effects. In this paper, the chitin extraction from deep-water shrimp (*Aristeus antennatus*) by-products was performed by using enzymes and organic acids in alternative of harsh chemicals. As for the vegetable products, the ultrasound-assisted extraction (UAE) was applied for a more sustainable extraction of the bioactive molecules from tomato pomace by-products (seeds and peels). Preliminary results showed that the enzymes reduced significantly the protein content in the shrimp by-products, while formic acid was able to remove 100% of the minerals, being as effective as hydrochloric acid. The UAE, performed using water as the "green" solvent, for 20 min at temperature below 40°C, was effective in the complete extraction of polyphenols from tomato pomace by-products. Moreover, an amount of β-carotene of 8.64 mg/100g DW, equal to 50% of what reported in the literature was extracted.

Key words: bioactive molecules, green extraction technologies, shrimp by-products, tomato by-products

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1. Introduction

The seafood production sector has been increasing over the years in order to meet the growing demand for seafood (FAO, 2022). As described in the report FAO (2022), the global production of crustaceans exceeded 10 million tons in 2020, most of which produced from the aquaculture sector. This overwhelming amount of crustaceans has generated a considerable amount of by-products coming from the processing sector, mainly represented by heads, tails, shells, which can account up to 70% of the raw material total weight (Yan and Chen, 2015; Zhang et al., 2023). In developing countries, crustacean by-products are often just dumped in landfill or the sea, while in developed countries, disposal can be very expensive, representing up to US$150 per ton in Australia, for example (Yan and Chen, 2015). Therefore, companies are increasingly interested in research and development of technological solutions capable of making waste management less costly if not even a source of additional income. Indeed, a lot of valuable compounds can be recovered from these by-products such as chitin, astaxantin, hydrolysates, that could be reused in many sectors, e.g. pharmaceutical, cosmetic, food, feed, wastewater

* Author to whom all correspondence should be addressed: e-mail: giovanna.ficano@ssica.it, Phone: +39 0815133724

treatment sectors and so on, increasing the prospective of sustainability, profitability and competitiveness of food companies (FAO, 2022; Zhang et al., 2023).

Chitin, the world's second most abundant polymer in nature after cellulose, is present in large quantity in crustacean shells (ranging from 15 to 40%), depending on the species, organism, nutrition status, processing methods and other factors (Yan and Chen, 2015). Considering the high amount of chitin, as well as other valuable compounds, as calcium carbonate, proteins and pigments, in crustacean shell, an important challenge in the view of sustainability, is for sure the extraction of these compounds in such a way that does not adversely affect the environment (Yan and Chen, 2015). In most cases, the chitin extraction is carried out by using chemical solvents as sodium hydroxide and hydrochloric acid to remove proteins and minerals respectively (Pakizeh et al., 2021), resulting unfriendly for the environment and making the obtained minerals and proteins unsuitable as human and animal nutrients (Pakizeh et al., 2021; Yan and Chen, 2015). To avoid the use of hazardous chemicals, it is very intriguing and advantageous to explore the feasibility of using enzymes to degrade seafood waste, taking into account their power of producing bioactive peptides and good quality chitin (Mathew et al., 2021). Proteolytic organisms or commercial enzymes, like alcalase, trypsin, delvolase, papain, pancreatin, etc. have been used to achieve enzymatic deproteinization (Islam et al., 2023). Moreover, organic acids, like lactic and formic acid, were used to remove minerals from crustaceans shells, in order to substitute harsh chemicals like HCl (Islam et al., 2023).

As well as seafood production sector, another fundamental sector to be better investigated is the tomato processing chain, because of the huge amount of the global production, the waste generated from the industrial processing, and the valuable molecules that could be recovered from their by-products.

Italy is the third producer of tomatoes for industrial use in the world, behind USA and China. The Italian tomato processing industry is the leader in Europe, followed by Spain, Turkey and Portugal. Indeed, in the Mediterranean area, Italy plays a major role for the tomato derivatives production, with 13% of the total world production and almost 50% of the European one, with a turnover of more than 4 billion €. The tomato processing by-products are unsuitable tomatoes for industrial processing, MOT (Material Other than Tomatoes), cleaning and transport water used in the line of production and, finally, by-products produced in the processing lines (tomato peels, seeds and pomace). In a "circular economy concept", and considering that the waste produced during the industrial processing operations represents generally the 2.3-2.5% of the total fresh matter, the recovery of these by-products and their reuse to valorise them have to be considered in the future (Sandei et al., 2015). A valid alternative is the possibility of using Tomato Processing By-products (TPB) as a potential source of bioactive substances, including carotenoids, polyphenols, which have antioxidant, anti-inflammatory and chemo preventive properties, and prevent cardiovascular diseases (Coelho et al., 2023). With this regard, it is important to use "green" extraction techniques in order to replace the conventional extraction methods with high environmental impact. One of the emerging "Green Technologies" considered in this work is the Ultrasound-Assisted Extraction (UAE) that can increase the extraction yield of the bioactive molecules naturally present in the TPB produced during the common industrial operations (tomato pomace – peels and seeds) by using eco-friendly solvents (water and or ethanol). UAE is an high-potential technology that can accelerate heat and mass transfer and has been successfully used in the extraction of several classes of food components such as aromas, pigments, antioxidants, and other organic and mineral compounds (Chemat et al., 2011). This technology, with affinity to hydrophilic molecules, fails to recover all the lipophilic molecules that are even present in the waste and therefore has to be combined with other techniques such as supercritical CO_2 to recover all bioactive compounds (Riera et al., 2004) .

The need of the hour, it is not only to valorise agri-food wastes and by-products, by extracting from them bioactive molecules in a green and sustainable way, but even to find solution that prolong seafood shelf life, and of all food items, characterized by a very high perishability, preferably employing natural compounds, more and more demanded by consumers. In that point of view, a very sustainable attempt could be represented by biofilms that are made of molecules extracted from both seafood and vegetables, which with their combined power can act as natural preservatives. Towards this direction, the first step and the main objective of the present work was to verify the goodness and effectiveness of "green" techniques (not using chemicals) for the extraction of some bioactive molecules from vegetables and fish industry by-products, in order to contribute to valorise such by-products in a sustainable, efficient and economically profitable way.

In particular, the potentiality of the UAE of extracting bioactive molecules from TPB was investigated, as well as the use of enzymes and organic acids for the deproteinization and demineralization of chitin from deep-water shrimp (*Aristeus antennatus*), to replace harsh chemicals normally used in the extraction of chitin.

2. Materials and methods

2.1. Sample supplying and preparation

Deep-water shrimp (*Aristeus antennatus*) by-products (heads, shells and tails) were supplied by local restaurants and fish markets in Campania (Italy), immediately transported to the laboratory under refrigerated conditions and frozen at -18 °C until use. For the extraction of chitin from shrimp by-products,

the samples, after thawing, were washed several times with distilled water to remove impurities, dried in an oven at 55 ºC for 12h, and then crushed into powder by using a mixer for solid samples (Bosch VitaPower Serie 4).

The peel and seed (35 kg) used for the "green" extraction of bioactive molecules were obtained during the tomato processing operations carried out at the SSICA technology laboratory during summer 2023 (Fig. 1).

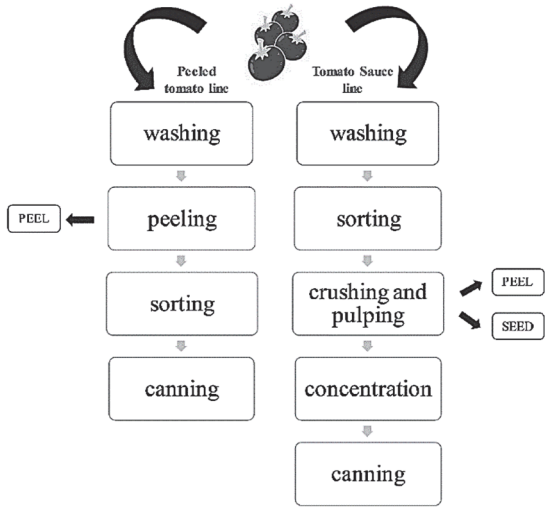

Fig. 1. Processing plant with peel and seed sampling points

2.2. Deproteinization of shrimp by-products

The enzymatic hydrolysis was performed in triplicate in an enzymatic digestion unit (GDE, VELP Scientifica) on 10 g of dried by-products at which 100 mL of distilled water was added (1:10 g/mL). The mixture was heated at 90ºC for 15 minutes to inactivate the endogenous enzymes, then temperature was set at 55ºC, pH adjusted at 8, and two proteases (Protamex® from *Bacillus* sp. or Alcalase® from *Bacillus licheniformis,* Sigma-Aldrich) were added to the mixture (5% w/w of by-products powder). The reaction was performed for 180 minutes under constant agitation (80 rpm) and continuous pH monitoring and addition of NaOH 2M to maintain the pH of 8. At the end of the reaction, temperature was set at 90ºC for 15 minutes to inactivate the enzymes.

For chemical deproteinization, the dried powder was treated with 0.75 M NaOH at a ratio of 1:6 (g/mL) for 24h at room temperature under constant agitation (80 rpm). After reactions, the samples were drained, washed several times with distilled water until neutral pH and oven-dried at 55 ºC. The upper layer, containing the hydrolysates, was stored at -18ºC.

2.3. Demineralization of shrimp by-products

Demineralization was performed on the deproteinized powder using 5% solution of three organic acids (citric, or lactic or formic) (Carlo Erba) at a ratio of 1:10 g/mL for 1h at room temperature and under constant agitation (200 rpm).

As for the chemical demineralization, the dried samples were treated with 1.25 M HCl at a ratio of 1:10 (g/mL) for 1h at room temperature under constant stirring (200 rpm). After reactions, the samples were drained, washed several times with distilled water until neutral pH and oven-dried at 55ºC.

2.4. Ultrasound-assisted extraction (UAE) of bioactive molecules from tomato by-products

The by-products from tomato processing lines were collected from SSICA pilot plants and stored in under vacuum bags, then immediately blast chilled and stored at -18°C, to reduce oxidative phenomena affecting the bioactive molecules content. In a second moment, the tomato by-products were thawed and treated in a crushing process directly with the UAE equipment. The UAE used for this extraction process was "Ecotecne ES15" equipped with a crusher with ECO 10 generator settled directly by the company (Fig. 2).

Fig. 2. Ultrasound extractor ES15

Extraction tests were performed mixing the tomato by-products with water at a ratio of 1:10. The extraction process was carried out as follows: maximum power and frequency of 25.3 KHz (I_{out} = 2.3 A, V_{out} = 310 V, P_{out} = 710 VA), temperature variation between the start and end of the process of about 10 °C, without exceeding 40 °C (Sengkhamparn and Phonkerd, 2020). At the same time, in order to have a reference sample, a similar extraction was performed using the same ES15 UAE system, but using only the stirring mode, without the ultrasound generator.

At the end of the process, each extract was separated from the coarse material through a 0.4-mm fine-mesh sieve and subsequently filtered with a TNT EC1223 cloth, then stored in aluminised vacuum bags at -18°C.

At the end of the extraction process, a total volume of 350 litres of aqueous extract was produced and then 15-fold concentrated by means of evaporation and concentration steps using a vacuum

concentrator, a semi-industrial pilot plant, at the Experimental Station for Food Processing Industry.

2.5. Chemical analyses

The shrimp by-products and chitin extracted were analysed in order to determine total nitrogen, chitin content, total lipids, proteins, ash and moisture contents. The analyses were carried out in triplicate by the following methods: moisture by the AOAC 90.15:1993, lipid content by the Soxhlet method (AOAC 920.39), total nitrogen by AOAC 954.01/988.05 and the ash content by AOAC 938.08. Chitin and protein contents were evaluated by determining total nitrogen (Nt) and non-nitrogen compounds (i. e. ash, moisture and lipids) as described above and applying the Eqs. (1-2) (Díaz-Rojas et al., 2006):

$$Chitin\,\% = (Nt \cdot Cp + K - 100) \cdot Cq \,/\, (Cp - Cq) \quad (1)$$

$$Protein\% = (Nt \cdot Cq + K - 100) \cdot Cp \,/\, (Cq - Cp) \quad (2)$$

where: K is equal to the sum of the non-nitrogen compounds, while Cp (6.25) and Cq (14.5) are conversion coefficients that relate the mass fraction of nitrogen with protein and chitin, respectively (Díaz-Rojas et al., 2006).

The demineralization and deproteinization degrees (DDM% and DDP%, respectively) were calculated by using Eqs. (3-4):

$$DDM\% = \{[(MO \cdot O) - (MR \cdot R)]/\, MO \cdot O\} \cdot 100 \quad (3)$$

$$DDP\% = \{[(PO \cdot O) - (PR \cdot R)]/\, PO \cdot O\} \cdot 100 \quad (4)$$

where: *MO* and *MR* are the ash contents (%) before and after demineralization; while, PO and PR are the protein concentrations (%) before and after deproteinization. O is the mass (g) of original sample and R represents the demineralized residue (Eq. 3) or hydrolysed residue (Eq. 4), in dry weight basis.

On the aqueous extracts obtained from tomato by-products, total polyphenols, were determined by means of Folin-Ciocalteu reagent (Sigma-Aldrich), and using gallic acid as a standard (Sigma-Aldrich) (Hudz et al., 2019). On the concentrated aqueous extract, dry mass, degree brix, organic acid, sugars, total polyphenols and carotenoids were determined.

Dry mass and degree brix were determined following methods described in the Italian Ministerial Decree (1989), while citric acid and malic acid by enzymatic kits (Citric acid and D-malic acid kits, R-Biopharm). The speciation of sugars was performed by HPLC-ELSD (Ma et al., 2014). D-(+)-glucose, D-(-)-fructose and sucrose were obtained from Sigma-Aldrich. Lycopene and beta-carotene were determined by HPLC-UV (De Sio et al., 2001). Standards of lycopene and b-carotene were purchased from Sigma-Aldrich.

2.6. Statistical analysis

Experiments and analyses were carried out in triplicate. One-way analysis of variance (ANOVA) and Tukey HSD multiple comparisons were performed using RStudio software (2023.12.1+402) to analyse significant differences ($p < 0.05$).

3. Results and discussion

3.1. Composition of shrimp by-products

By-products composition of *A. antennatus* is shown in Table 1. These findings were similar to those obtained by Liu et al. (2021), who analysed the meat and by-products composition of five species of shrimps. Composition of *A. antennatus* by-products indicated they can be used as a rich source of bioactive compounds to develop natural nutraceuticals, or to be employed in other sectors, such as food packaging.

Table 1. Proximate composition of *A. antennatus* by-products (g/100g, mean ± SD)

Proximate composition	***g/100 g of by-products***
Protein	6.00 ± 0.32
Fat	1.44 ± 0.03
Ash	15.30 ± 0.27
Chitin	12.56 ± 0.05
Moisture	64.70 ± 0.78

3.2. Deproteinization and demineralization steps

Commercial proteases can be useful for the extraction of chitin, because, unlike crude proteases extracted from microorganisms, they can give a more reliable product and can be easily scaled up for industrial extraction of chitin/chitosan (Mathew et al., 2021). Usually, after enzymatic deproteinization, 5-10% of proteins remains adhered to chitin, and are not completely removed, unlike what done by chemical deproteinization (Mathew et al., 2021). However, the latter method could damage proteins extracted and negatively affect the safety of the hydrolysates preventing their use as animal feeding or human nutraceuticals.

In terms of deproteinization, the proteases used in the present study at the optimal conditions of pH and temperature previously defined, were able to reach a DDP of 95%, very similar to that obtained by using 0.75 M NaOH (DDP = 96%), as shown in Fig. 3, without statistically significant differences observed between treatments ($p>0.05$). In terms of optimization of the deproteinization step, in the future other techniques will be used after enzymatic extraction, like ultrasound, which could be useful to remove the protein residue remained attached to the chitin. The degree of deproteinization obtained in the present work was slightly higher than that obtained in previous studies by using enzymes (Islam et al., 2023).

However, present results confirmed what obtained in terms of high power of alcalase and protease from *Bacillus* sp. to remove proteins from seafood by-products (Dey and Dora, 2014; Dumay et al., 2006; Valdez-Peña et al., 2010). Alcalase® is a bacterial serine endopeptidase prepared from a strain of *Bacillus licheniformis*. Alkaline alcalase was proven to be efficient in obtaining the highest protein recovery and DDP% among the proteases tested from shrimps by-products (De Holanda and Netto, 2006; Dey and Dora, 2014; Valdez-Peña et al., 2010).

Protamex® is a *Bacillus* sp. protease complex, which was used to recover proteins/lipids by *Sardina pilchardus* viscera (Dumay et al., 2006), giving slightly higher protein recovery (61%) than Alcalase (60%), with degrees of hydrolysis quite similar (3.1 and 3.3%, by using Protamex and Alcalase respectively). However, Messina et al. (2021), who performed enzymatic extraction of bioactive peptides from *Parapenaeus longirostris* by-products at a pilot scale, found a higher hydrolysis degree by using Protamex® on *P. longirostris* by-products (10-16%).

Concerning the demineralization step, it was observed that the use of the three organic acids allowed reducing drastically the mineral content from the raw by-products, with statistically significant differences between DDM% obtained from the three organic acids (Fig. 4). In particular, the highest percentage of demineralization was reached by using formic acid, which was able to remove completely the minerals (DDM% = 100%), followed by lactic acid that contribute to reach a DDM% of 60%, and by citric acid with a DDM% = 44%. The controls, i.e. the samples demineralized with HCl, showed a DDM% equal to 100% (Fig. 4). The results of the present study agree with those obtained by Baron et al. (2017), who observed that formic acid was the only acid able to reach a demineralization degree of 99% with a pH of 3.5. Another study (Hu et al., 2020) obtained a slighty lower (90%) demineralization degree of shrimp by-products using a 5% solution of formic acid. As regards the other organic acids, it was found that lactic acid achieved a demineralization degree of 60%, lower than that (90%) obtained by Mahmoud et al. (2007), by using lactic acid (75.6 g L^{-1}) on *Pandalus borealis* deproteinized shells, at a ratio of 1:10 (g shell/mL acid) and room temperature, but considering a reaction time of 2 hours, instead of 1 h considered in the present work. Retention time is one of the most important parameters in the demineralization process, because could affect the quality of the purified chitin. Citric acid was also used as a promising alternative to HCl to demineralize shrimp shells, but as it was shown by Pohling et al. (2022), to have a complete removal of minerals from shrimp shells, it could require much more concentrated citric acid solution and two-steps of demineralization unlike formic acid, for which a single step was enough.

3.3. Total phenolic content

The values of total polyphenols obtained from the analysis of samples taken at regular intervals of 5 min in an extraction test lasting 30 min are shown in Fig. 5. Statistically significant differences ($p<0.05$) in total polyphenol content were observed between the extraction performed with ultrasound and that carried out by agitation, because the ultrasound improved solvent penetration into plant cells and cell wall disruption, facilitating the release of bioactive molecules.

As it can be seen in Fig. 5, an extraction time of 20 minutes allowed a complete extraction of the polyphenols contained in the processed by-products, because any significant variation was observed after that time. Subsequent trials were carried out on the tomato waste for a time of 20 min. The extracts obtained were combined and 15-fold concentrated from 0.41 to 6.20°Bx.

3.4. Characterization of the concentrated extract

The results of the characterization of the concentrated extract are shown in Table 2.

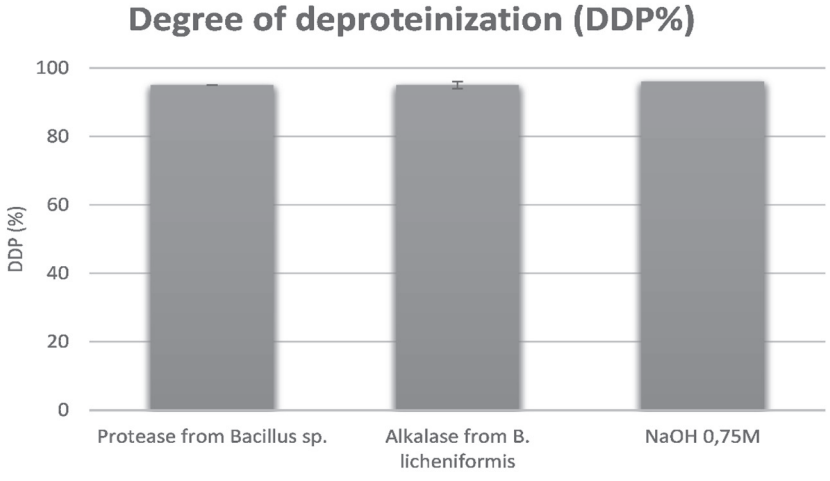

Fig. 3. The DDP% reached by the proteases and sodium hydroxide used for the deproteinization step

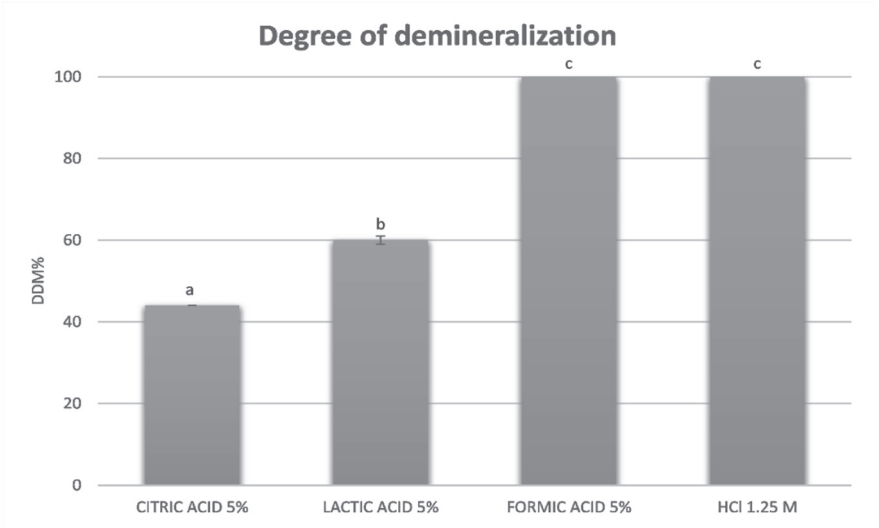

Fig. 4. The DDM% reached by the four acids used for the demineralization step. Different lowercase letters indicate statistically significant differences between treatments ($p<0.05$)

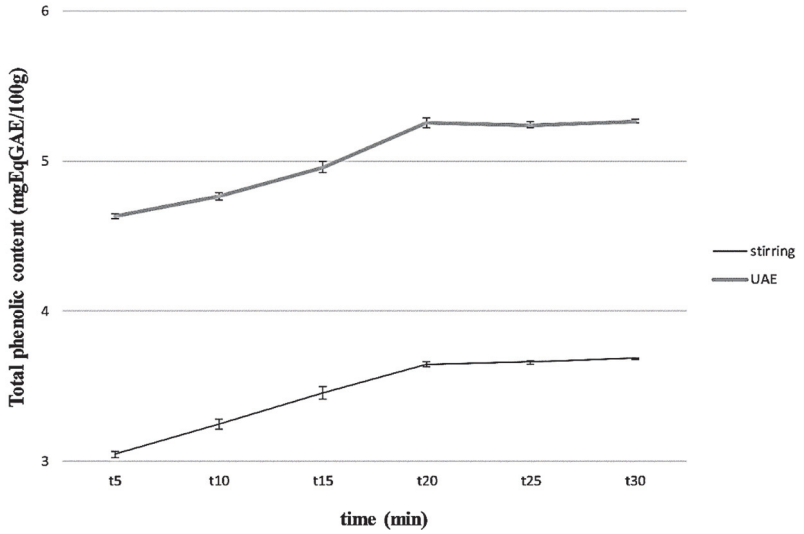

Fig. 5. Total phenolic content at different extraction times with UAE and under agitation.

Table 2. Sugars, organic acids and carotenoids in concentrated aqueous extract (mean ± SD)

Sugars		*Organic acids*		*Carotenoids*	
Glucose (g/100g dw)	***Fructose (g/100g dw)***	***Citric acid (g/100g dw)***	***Malic acid (g/100g dw)***	***Licopene (mg/100g dw)***	***β-carotene (mg/100g dw)***
4.86 ± 0.37	9.96 ± 0.12	0.36 ± 0.01	0.21 ± 0.02	3.24 ± 0.25	8.64 ± 0.63

A direct comparison with data reported in the literature is not possible given the variability of the raw material due to the different percentages of peel, seed and pulp in the by-products, which depends on tomato processing lines (tomato purees and paste, or peeled or diced tomato for example), type of crop, variety and ripeness of the tomato. It is also difficult to find solid data on organic acids and sugars content; only an average of 25% of carbohydrates has been reported (Coelho et al., 2023).

It should be highlighted that although the ultrasonic extraction technique is more suitable to extract hydrophilic substances, the cell-wall rupture also brought into solution a certain amount of carotenoids; the content found was low for lycopene but for β-carotene it was about 50% of the amount reported in the literature (Coelho et al., 2023). In order to obtain a higher amount of carotenoids, the process could be optimized by using a more homogeneous raw material in terms of peel size; this operating condition will be evaluated in the future.

4. Conclusions

As far as the chitin extraction was concerned, the "green" extraction method, carried out by considering enzymes and formic acid, allowed to obtain chitin with less than 1% of protein and mineral

contents, which are recommended both as food ingredient and for chitosan applications in food packaging. Further analyses will be carried out on the extracted chitin in order to characterize it in terms of solubility, degree of acetylation, molecular weight, and finally converted in chitosan.

In terms of scalability, this field has not been much explored so far, but the studies have increased in the last years, with studies that scale up from a laboratory to a pilot scale, providing important results on the feasibility of this kind of shrimp shell processing on an industrial scale. In terms of costs, using enzymes for extracting chitin could be slightly more expensive than using hazardous chemicals but much greener. Indeed, the use of enzymes and organic acids is in accordance to the 12 principles of the green analytical methods (GAC) that focuses on reducing or possibly eliminating hazardous substances used in some processes or analyses to minimize their negative effects on human health and the environment. However, the higher costs can be balanced by the possibility to obtain hydrolysates rich in essential amino acids that, in turn, can be integrated in formulated diets for aquaculture as sources of biologically active peptides.

One of the main goals of this work was to valorise and develop a "green and sustainable process" protocol able to valorise the huge amount of Italian tomato processing by-products, by the application of novel "green" extraction technologies. Preliminary tests of UAE showed that a treatment of tomato by-products for 20 minutes was enough to obtain a complete extraction of the polyphenols as well as a high β-carotene level. Next step of this research will be to apply in a "cascade process model" this technique with others "green extraction systems (i.e. Supercritical CO_2 extraction)" in order to add and exploit the extraction of the lipophilic fraction of the tomato by-products biomolecules.

It is conceivable that the green technique based on the use of ultrasound for extracting bioactive molecules from tomato pomace by-products could be scaled up for industrial application being affordable for the food companies. Indeed, pilot plants of ultrasound are not so costly, how a market research can show, moreover, the use of water as solvent is more sustainable and avoids the necessity of more expensive chemicals.

Moreover, the bioactive molecules successfully extracted from shrimp and tomato pomace by-products in eco-friendly processes, might be thought to be used together in the future to make packaging solutions that can help prolong food shelf life, giving even more support to food companies to make their production more sustainable and profitable.

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