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One-Solvent Extraction of Astaxanthin from Lactic Acid Fermented Shrimp Wastes

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Free astaxanthin one-solvent extractions with ethanol, acetone, and liquid 1,1,1,2-tetrafluoroethane from raw and lactic acid fermented (ensilaged) shrimp residues were investigated. The total carotenoid recovery from ensilaged shrimp wastes was higher than that from non-ensilaged ones as assessed by HPLC analyses. Acetone gave the highest extraction yields of free astaxanthin with up to 115 μg/g of material. Moreover, liquid tetrafluoroethane is reported for the first time in a successful one-solvent extraction of carotenoids from shrimp.

KEYWORDS: Astaxanthin; tetrafluoroethane; lactic acid fermentation; shrimp wastes

INTRODUCTION

There has been growing interest in recent decades to develop new processes directed toward the reuse of waste materials from fisheries due to environmental issues as well as the potential commercialization of valuable byproducts. The discarded residues from shrimp are one of the main pollutants in coastal areas, which accounts for ca. 40 wt % of the total caught or harvested (*1*). Shrimp wastes are an important source of chitin and proteins as well as astaxanthin, which is the main pigment present in this crustacean (*1–3*). Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a red carotenoid produced by marine plankton, and it gives a characteristic pigmentation to some crustaceans, fishes, and birds (*4*). The shrimps cannot synthesize de novo this pigment, and it is incorporated in the diet by the consumption of algae or animals (*5*).

Astaxanthin is reported to be an excellent antioxidant and anticarcinogenic, as well as a precursor of retinal (*6, 7*). It can be used for animal feed in the aquaculture industry or for cosmetics, among other applications; therefore, its demand is expected to grow in the future (*5, 8, 9*). In this sense, the extraction from crustacean residues offers an ecological alternative to those synthetic ones to obtain this valuable compound. An important issue is the way the astaxanthin is stored in the shrimp residues to avoid earlier degradation, its final color, and its overall extraction quality. Astaxanthin extraction from yeasts and algae, which are so far the most studied sources of natural astaxanthin, usually include an alkaline pretreatment to hydrolyze the astaxanthin-formed esters with fatty acids (*10*). However, the astaxanthin from crustaceans is mainly found as non-covalent complexes with proteins (*11*). The additions of organic or inorganic acids in chemical silage are effective methodologies, and as well they are useful processes to preserve shrimp wastes from decomposition; however, the low pH (1.7) can degrade the carotenoids and also can limit biological applications (*12*). An alternative procedure to extend the shelf life of the shrimp wastes is the biological silage, which involves the production of organic acids by fermentation with lactic acid bacteria, as reported by Shirai et al. (*13*). The ensilage process stabilizes the shrimp wastes during long-term storage, and it also allows the recovery of other valuable products (*1, 13, 14*). It has also been reported that high extents of proteolysis promoted during ensilation by native proteases as well as bacteria resulted in the removal of proteins from chitin. Moreover, the lactic acid produced solubilized calcium and other minerals from the cuticle (*1*).

The present paper deals with the evaluation of the biological shrimp ensilage in the effective extraction of carotenoids as compared to its extraction from raw material. Herein, free astaxanthin was quantified from the total carotenoid extraction. A one-solvent extraction with generally recognized as safe (GRAS) solvents has been investigated to increase the potential uses of the extracted material. Acetone and ethanol as well as liquid 1,1,1,2-tetrafluoroethane were chosen for this study. Liquid 1,1,1,2-tetrafluoroethane is an alternative compressed fluid (CF) to the earlier reported supercritical carbon dioxide ($scCO_2$) for carotenoid extraction (*15–18*), as it requires lower operating pressures (1–2 MPa); moreover, it presents some polarity that should increase the affinity for the extraction of xanthophylls (*19–22*).

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Table 1. Initial Moisture, Ash, Calcium, and Protein Contents in the Shrimp Waste Samples

treatment	ash % w/w (dry wt basis)	calcium % w/w (dry wt basis)	protein content % w/w (dry wt basis)	water content % w/w
non-ensilaged, 40 °C-dried (HRSW)	14.60 ± 0.12	1.35 ± 0.083	30.85 ± 1.19	15.1
non-ensilaged, lyophilized (RSW)	15.98 ± 0.12	1.40 ± 0.086	33.80 ± 1.3	7
ensilaged, 40 °C-dried (HSS)	3.72 ± 0.064	0.102 ± 0.005	8.45 ± 0.56	6
ensilaged, lyophilized (LSS)	3.64 ± 0.063	0.099 ± 0.004	8.27 ± 0.55	8

MATERIALS AND METHODS

Reagents. Acetone and ethanol (technical grade) were purchased from Quimica Barsa (Mexico).

HPLC-grade acetone was purchased from J. T. Baker (Mexico). 1,1,1,2-Tetrafluorethane (R134a, DuPont) was purchased from Refacciones Star (Mexico). Potassium hydroxide was supplied from J. T. Baker (Mexico). Commercial astaxanthin standard (trans isomer, 98% purity) was purchased from Sigma-Aldrich (Mexico) and stored at −78 °C. All other reactants and materials were used as received.

Silage Procedure. The shrimp wastes were obtained from Mexico City's central seafood market and consisted of head and thorax. The shrimp wastes were composed of undetermined mixtures of the species *Litopenaeus vannamei*, *Litopenaeus stylirostris*, and *Litopenaeus setiferus*. The waste was minced through a 0.3 cm sieve using a meat mincer (Sanitary, Chicago, IL) and kept in a freezer (−78 °C) before use. Ensilaged shrimp waste was prepared as follows: *Lactobacillus plantarum* (APG-Eurozym) was cultivated in Man Rogosa Sharpe (MRS) broth during 24 h at 30 °C and added to the nondried mince shrimp wastes at a level of 5% (v/w) and mixed with sucrose 10% (w/w) and inoculated with 5% (v/w) in a static packed bed column bioreactor at 30 °C. The bioreactor was equipped with a filter device that allowed the separation of the liquid protein-rich fraction from the solid fraction, which was mainly composed of chitin, residual protein, and astaxanthin (*1*). The solid fraction of the silage presented a final pH of 4.4 and a total titratable acidity (expressed as lactic acid) of 0.307 mmol/g. The material was kept in a freezer (−78 °C) until use. Ensilaged and raw shrimp wastes were lyophilized using an HETO-CT60e device, minced and sieved through a no. 40 mesh (0.5 mm). For heat-drying tests, a sample of ensilaged material was dried at 40 °C in the oven for 24 h, minced, and sieved to 0.5 mm.

Analyses of Samples. The total nitrogen contents of the ensilaged and raw shrimp wastes were analyzed using an Elemental Analyzer (Perkin-Elmer 2400, Norwalk, CT) (*1*). The percentage of chitin nitrogen was determined after demineralization and deproteination with HCl and NaOH, decolorization, and drying using the Black and Schwartz method (*23*). Corrected protein concentrations were obtained by subtracting chitin nitrogen from total nitrogen. Moisture and ash contents were determined as stated by the AOAC (*24*). Calcium was determined by using an atomic absorption spectrophotometer (Perkin-Elmer) (*24*).

Extractions with Acetone and Ethanol. A series of extractions at 25 °C monitored by UV–visible spectrophotometry were performed to determine the optimum solvent-to-waste ratio. The experimental procedure was carried out as follows: known weights (dry basis) of material were placed in centrifuge tubes with known volumes of each solvent (technical grade). The tubes were centrifuged for 3 min at 3000 rpm. The supernatants were recovered, and the absorbances for each sample were measured at 470 nm in a Perkin-Elmer Lambda-S1 UV–visible spectrophotometer. The pellets were successively resuspended in known volumes of solvent until no absorbance was detected. The series were carried out in three replicates. The best solvent-to-sample weight ratio measured on a dry basis was 25 mL/g of waste material for both solvents. For the extractions, known weights (dry basis) of material were placed in centrifuge tubes with the optimum volumes of the extraction solvent (technical grade, 25 mg/mL). Aliquots were taken for HPLC analyses. The standard deviation of each sample was obtained from four replicates.

HPLC Analyses. All extracts were analyzed on a HP1100 series HPLC (Hewlett-Packard) equipped with an autosampler/injector and a photodiode array detector (DAD). A column X-terra RP18 (C18), 150 mm (Waters, Inc.), was used with a mixture of methanol/acetonitrile/ethyl acetate/water (80:10:5:5), with a flow of 1 mL/min. Astaxanthin and other chromophores were measured at a wavelength of 470 nm. The peak identities of *trans*-astaxanthin were confirmed by their retention times and characteristic spectra of standard chromatograms. They were quantified from their peak areas in relation to the *trans*-astaxanthin reference standard.

Alkaline Hydrolysis. Lyophilized shrimp residues were weighed and treated in centrifuge tubes with aqueous KOH 1% w/v at 25 °C as reported elsewhere (*13*). The tubes were sealed and stored in the dark under a nitrogen atmosphere for 12, 24, and 36 h. Then they were centrifuged for 5 min at 3000 rpm. The supernatants were discarded, and the pellets were set for extractions.

Batch Extraction with Liquid 1,1,1,2-Tetrafluoroethane. Lyophilized shrimp wastes (0.5 g, raw and ensilaged) were contained in cellulose bags, which were placed into a house-made 100 mL st.316 reactor equipped with a magnetic stirrer. The reactor was pressurized with tetrafluoroethane at 2 MPa in all of the experiments by means of a syringe pump ISCO 100XD (ISCO Inc.). The working temperature was monitored by a double-thermocouple system. One hour extraction experiments were carried unless otherwise stated. The reactors were cooled at −5 °C to atmospheric pressure prior to release the compressed fluid. The cellulose bags were removed, and the astaxanthin extracted inside the reactor was recovered with acetone (technical grade) and analyzed by HPLC.

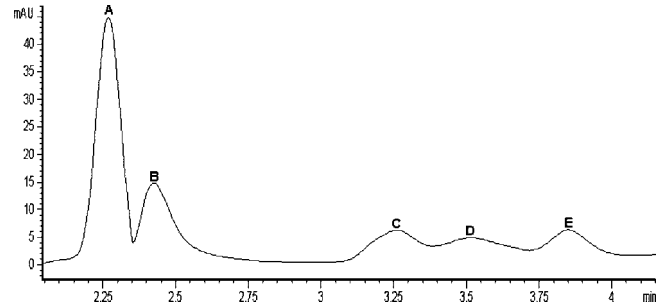

Figure 1. Representative HPLC chromatogram of the extract obtained with acetone from the nonhydrolyzed LSS sample (**Table 1**). Peak A in the chromatogram is identified as astaxanthin. Peaks B−E are nonidentified compounds present in the extract.

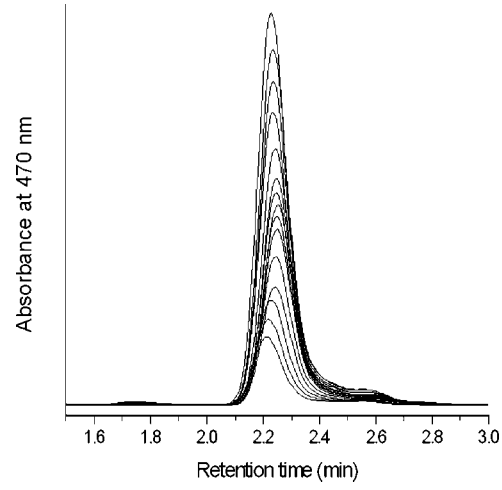

Figure 2. HPLC chromatograms with the retention time of the reference standard of astaxanthin (trans isomer) at different concentrations between 1 and 21 μg/mL.

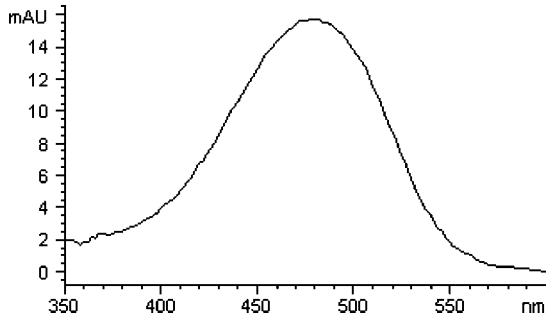

Figure 3. Representative absorption spectrum of peak A in the chromatograms. The other peaks showed identical absorption spectra.

RESULTS AND DISCUSSION

The aim of this work was to evaluate the efficiency of the extraction of astaxanthin using GRAS solvents from raw and lactic acid fermented Mexican shrimp wastes (head and thorax) for industrial applications. An important issue in a commercial extraction of astaxanthin from shrimp wastes is the initial water content in the materials and its removal as this could play an important role in the extraction efficiency. In our laboratory, lyophilized, nondried, and postensilage heat dried (40 °C) processed samples were evaluated. The water weight percentages of the selected samples under study herein, lyophilized ensilaged (LSS) and raw (RSW), non-ensilaged 40 °C-dried (HRSW), and a postensilaged 40 °C-dried (HSS), are shown in **Table 1**.

The protein, ash, and calcium contents were also evaluated as parameter controls in the efficiency of the ensilaged process. As can be seen in **Table 1**, the protein, ash, and calcium amounts were remarkably reduced during the ensilation. The reductions of protein, ash, and calcium on a dry basis were determined as 73.55 ± 2.52, 75.48 ± 0.32, and 92.72 ± 0.77%, respectively.

Astaxanthin Extraction with Acetone and Ethanol. Acetone and ethanol are GRAS solvents and readily available for industry, and they were chosen to avoid the restrictions involved with products extracted with more toxic solvents. The HPLC chromatograms of all the extracts showed the same pattern. The proportion of astaxanthin in all of the extracts varied between 75 and 80% of the total integrated areas without significant differences among the samples. A representative HPLC chromatogram of the extract obtained with acetone from LSS sample is shown in **Figure 1**.

The quantification of the free astaxanthin trans isomer from the extracts, identified as peak A in the chromatograms, was related to the reference standard (trans isomer). The retention time of the reference standard astaxanthin (trans isomer) at different concentrations is shown in **Figure 2**.

The UV–visible absorption spectra of peak A as interfered from the HPLC photodiode array matched earlier reported spectra of this carotenoid (*25*). Peaks B−E also showed the maximum of absorbance at $\lambda = 470$nm with the same UV–visible absorption spectra as peak A (**Figure 3**). A three-dimensional chromatogram of the separate compounds extracted is presented in **Figure 4**.

Yuan et al. reported the HPLC chromatograms of the separated trans and cis isomers of free astaxanthin using a similar methodology (*26*, *27*). In their paper, the 9-*cis*-astaxanthin isomer peak is eluted after the *trans*-astaxanthin form peak in the chromatograms. On the other hand, the chromatograms corresponding to the standard astaxanthin also displayed peak B after the main peak, which may well correspond to cis form impurities in the commercial samples.

According to the literature and the chromatograms obtained with the commercial astaxanthin, peak B in the chromatogram shown in **Figure 1** could be assigned to the astaxanthin cis isomer. Other papers have shown the detection of astaxanthin in the form of diesters and monoesters at longer elution times in the chromatograms (*28*); however, Mortensen et al. reported a similar UV–visible absorption spectrum for canthaxanthin at $\lambda = 470$ nm, which could also be present in minor proportion in shrimp (*29*).

The combined effects of the solvent, ensilation, and alkaline hydrolysis on astaxanthin extraction yields from samples of LSS and RSW (**Table 1**) are shown in **Figure 5**. As can be observed, the non-ensilaged materials gave remarkably lower extraction yields than the ensilaged ones with both solvents. Acetone gave the highest extraction yield of free astaxanthin, up to 115.5 ± 5.15 μg/g of LSS. There is a general decrease in the free astaxanthin recoveries from the alkali-treated ensilaged samples, which is significant for ethanol. It is reported that saponification

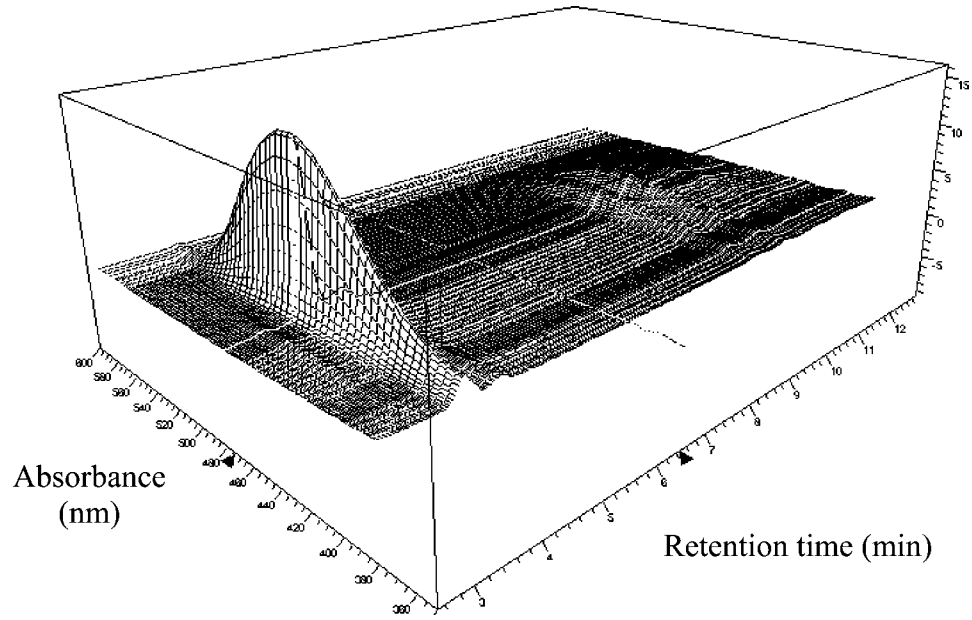

Figure 4. Representative three-dimensional chromatogram of the extracted mixture from LSS sample with acetone.

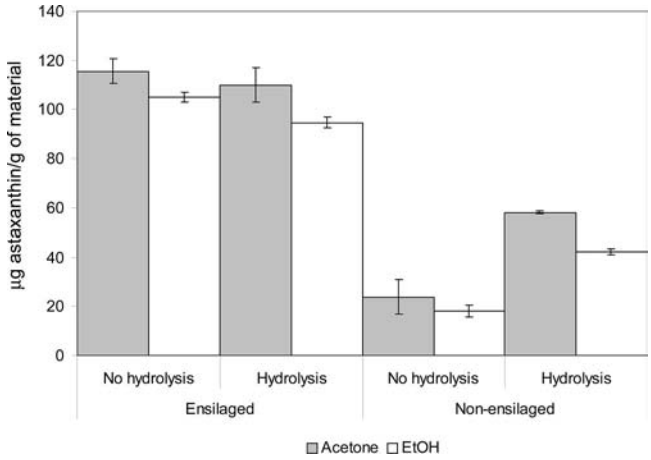

Figure 5. Astaxanthin extraction yields (dry basis) expressed as micrograms per gram with acetone and ethanol from samples of LSS and RSW in **Table 1**. The standard deviations in all assays were obtained from four replicates.

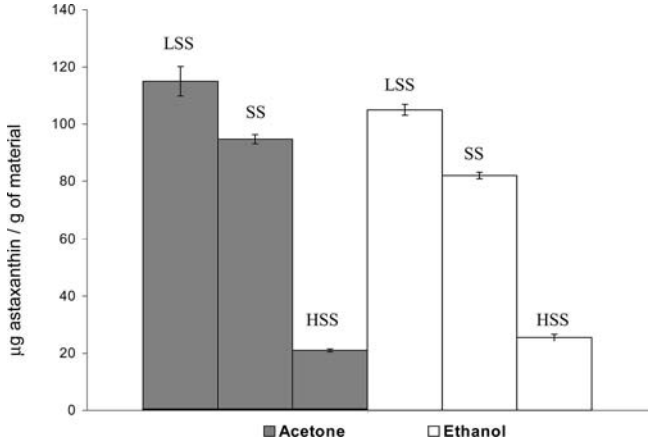

Figure 6. Astaxanthin extraction yields and deviation standards expressed as micrograms per gram on dry basis of ensilaged lyophilized sample (LSS), nondried sample (SS), and 40 °C heat-dried sample (HSS). The standard deviations in all assays were obtained from four replicates.

reduced the extraction yields of free astaxanthin due to the oxidation of the carotenoid (*12*). The yield extractions in non-ensilaged samples subjected to alkaline treatment prior to extraction were relatively higher than those without KOH pretreatment (**Table 1**; **Figure 4**). These results might be explained for disruption of non-covalent bonds of astaxanthin with other compounds, mainly protein, which releases the free astaxanthin form.

However, the opposite occurs in the ensilaged samples, where the alkaline hydrolyses decreased the astaxanthin recovery. Earlier studies showed that the lactic acid bacteria promote acidic and reductive environment due to their metabolism and are able to solubilize minerals (*13*). Besides, a protein breakdown occurred, which released the pigment from the carotenoid protein chitin complex; therefore, the free pigment is susceptible to becoming more available for the solvent.

As can be seen in **Table 1**, the protein content is remarkably reduced in the ensilaged sample, LSS, as compared to RSW. According to the experimental results, the hydrolysis of the ensilaged samples is not needed, and the lowering of free astaxanthin extracted during saponification might be due to partial carotenoid degradation by such a process.

Effect of Water Content on Astaxanthin Extraction with Acetone and Ethanol. A nondried ensilaged sample (SS), which contained 64% w/w of moisture, and the LSS and HSS samples (**Table 1**) were analyzed to assess the effect of the initial water content on the extractions as this can be an important issue at industrial scale. The results showed a mean of 94.3 ± 1.74 μg/g (dry basis) of free astaxanthin obtained from SS with acetone, which is close to the mean of 115.5 ± 5.15 μg/g (dry basis) obtained from LSS, despite the former containing the highest moisture content (**Figure 6**). Similar extraction profiles are obtained with ethanol. The silage pretreatment can be the explanation for the measured yield in the SS sample; thus, it proves that the carotenoid−protein complex in this heterogeneous mixture was degraded enough to allow a relevant extraction. On the other hand, the HSS scored only 21 ± 0.54 μg/g of free astaxanthin when the degradation of the carotenoid by the heat treatment was pointed out as a possible explanation.

Sachindra et al. (*2*) reported the comparison of lactic acid fermentation and an acidification process with propionic acid in the total carotenoid extraction from the species *Penaeus indicus* using hexane/isopropanol (60:40 v/v) as solvent mixture. The lactic acid fermentation carried out in that work resulted in a significant increase in the extraction yields. They reported 41.85 ± 1.02 μg/g of total carotenoid extracted determined by a spectrophotometric method. The carotenoid extraction yields determined by HPLC quantification are up to 2.76-fold higher than the maximum amount earlier reported (*2*). These differences

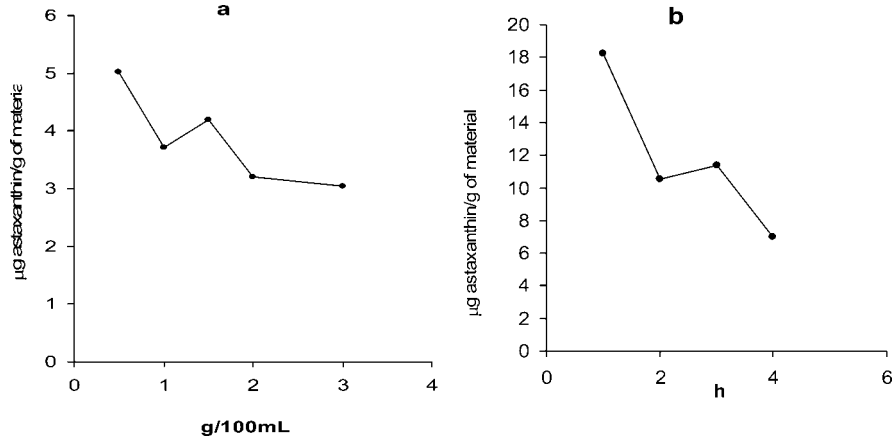

Figure 7. (**a**) Effect of LSS sample weight loaded in the 100 mL reactor with batch extractions with liquid tetrafluoroethane at 20 °C and 2 MPa; (**b**) effect of time in the astaxanthin extraction from LSS samples with liquid tetrafluoroethane at 40 °C and 2 MPa.

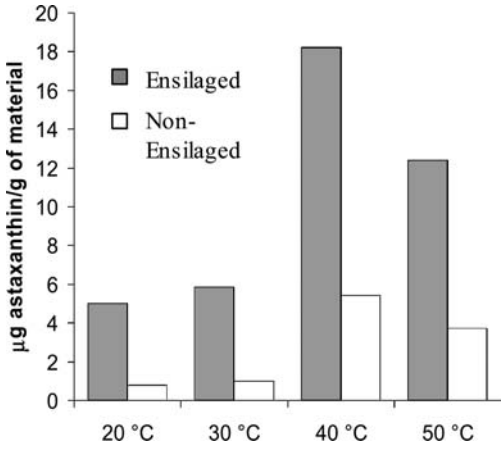

Figure 8. Effect of working temperature on the liquid tetrafluoroethane (2 MPa) batch extraction of astaxanthin from LSS (ensilaged) and RSW (non-ensilaged) samples.

might be explained for the amount of protein and minerals removed during ensilation; however, no further discussion can be done because these data were not reported by the authors.

Batch Extraction with Liquid 1,1,1,2-Tetrafluorethane. The optimum carotenoid extraction from LSS and RSW samples with liquid tetrafluoroethane at 2 MPa pressure was carried out as described under Materials and Methods. Preliminary experiments were carried out at 20 °C by varying the initial weight of LSS samples. The optimum weight of sample for the 100 mL volume reactor was determined (0.5g). In **Figure 7a** is shown the negative effect of increasing the ensilaged sample amount in the extraction efficiency.

The effect of the operation temperature in 1 h experiments was investigated, and the results are shown in **Figure 8**.

Liquid tetrafluoroethane gave higher free astaxanthin recovery from the ensilaged shrimp waste than from non-ensilaged shrimp waste in the whole temperature range. As can be observed, an approximately 3-fold increase was obtained at 40 °C (18 μg/g) compared when to when the temperature was lowered to 30 and 20 °C. Extraction times of >1 h at 40 °C showed a significant decrease in the extraction yield, as shown in **Figure 7b**, which could be also attributed to carotenoid degradation at such temperatures with longer operational times. The extraction carried out at 50 °C showed a significant decrease in the astaxanthin extraction yield, which was assumed to be due to degradation of the carotenoid (**Figure 8**).

The use of this CF as extraction solvent presents several advantages as it is a nonflammable, nontoxic GRAS solvent and does not deplete the ozone layer; therefore, this organic CF in its liquid state, far below its critical state (T_c = 101 °C, P_c = 40.6 bar), has been pointed out as an alternative to $scCO_2$ in synthetic and engineering processes as it requires lower operating pressures and it has higher polarity than the inorganic CF. Indeed, its dipole moment, DM = 2.05, and a dielectric constant, 9.5 kHz, are close to those of dichloromethane or tetrahydrofuran (*19*–*22*), which would favor the extraction of polar compounds, such as xanthophylls. The pressure to become liquid can be as low as 0.65 MPa at 25 °C, at which it possesses adequate physical and chemical properties for materials synthesis and processing (*19*, *22*), such as low viscosity (0.21 cP at 25 °C), which is similar to that of $scCO_2$ (0.1 cP at 200 bar and 33 °C), and low surface tension (8.7 dyn/cm), which is far from the values for common organic solvents (17–73 dyn/cm). The use of a CF in an extraction process has generally the advantage of being completely evaporated at atmospheric pressure; thus, no traces of solvent remained in the extracts, which should extent its applications (*22*). As well, the CFs can be easily recovered and recycled. Earlier reported carotenoid extractions using $scCO_2$ required high pressures ($\geq$50 MPa) as well as polar cosolvents, such ethanol or methanol, to attain a significant extraction yield of total carotenoids (*15*–*18*).

Herein, the use of liquid tetrafluoroethane for one-solvent carotenoid extraction from crustaceans has been demonstrated for the first time. Further optimization of the engineering process in a continuous extraction system is encouraged in order to increase astaxanthin recovery from ensilaged shrimp wastes.

It is concluded that one-solvent extractions of carotenoids with acetone, ethanol, and liquid tetrafluoroethane were significantly increased in ensilaged shrimp wastes as compared to raw material. The selection of an appropriate solvent as well as the extension of water removal should depend on economical factors and also on the final application of the product when at industrial scale.

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