Fatty acids and astaxanthin composition of two edible native Mexican crayfish *Cambarellus (C.) montezumae* and *Procambarus (M.) bouvieri*

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SUMMARY: The content and composition of the fatty acids (FAs) and astaxanthin (AST) in the edible forms of crayfish: the whole animal of *Cambarellus (C.) montezumae*, and the tail meat (TM) of *Procambarus (M.) bouvieri* were determined by GC and HPLC. The exoskeleton (EXK) of *P. (M.) bouvieri* was also studied. Unsaturated FAs, and mostly oleic acid (C18:1 n-9), were predominant in both edible forms. The contents of the polyunsaturated eicosapentaenoic (C20:5 n-3, EPA), arachidonic (C20:4 n-6, ARA) and docosahexaenoic acid (C22:6 n-3, DHA), were higher in the TM of *P. (M.) bouvieri* than in the complete *C. (C.) montezumae* (p<0.05). Total carotenoids ranged between 2.31 ± 0.33 μg·g^{-1} and 66.3 ± 3.91 μg·g^{-1}, and were composed mainly of AST (>79.50%). AST esters were enriched with saturated FAs in *C. (C.) montezumae* and with PUFAs in EXK of *P. (M.) bouvieri*. We conclude that both *C. (C.) montezumae* and the TM of *P. (M.) bouvieri* are traditional foods rich in n-3 PUFAs and *C. (C.) montezumae* in AST. The EXK of *P. (M.) bouvieri* is a rich potential source of AST, n-3 PUFAs, and the combination AST-DHA.

KEYWORDS: *Acocil; Astaxanthin esters; Mexican crayfish; Omega 3 fatty acids*

RESUMEN: ***Composición de ácidos grasos y astaxantina de dos especies comestibles de acociles nativos de México, Cambarellus (C.) montezumae y Procambarus (M.) bouvieri***. Se determinó por GC y HPLC el contenido y composición de ácidos grasos (AGs) y astaxantina (AST), en dos formas comestibles de acocil: el animal completo de *Cambarellus (C.) montezumae*, y el músculo de la cola (MC) de *Procambarus (M.) bouvieri*. Adicionalmente, se estudió el exosqueleto (EXK) de *P. (M.) bouvieri*. En ambas formas comestibles predominaron los AGs insaturados. Los contenidos de ácido eicosapentaenoico (C20:5 n-3, EPA), araquidónico (C20:4 n-6, ARA) y docosahexaenoico (C22: 6 n-3, DHA), fueron mayores en el MC que en *C. (C) montezumae* (p <0,05). Los carotenoides totales oscilaron de 2.3 ± 0.3 μg·g^{-1} a 66.3 ± 3.9 μg·g^{-1}, con predominancia de AST (>79.50%). Los ésteres de AST en *C. (C.) montezumae* fueron enriquecidos con AGs saturados mientras que los del EXK de *P. (M.) bouvieri* con AGs poliinsaturados. Se concluyó que tanto *C. (C.) montezumae* como el MC de *P. (M.) bouvieri*, son alimentos tradicionales ricos en PUFAs n-3, y *C. (C.) montezumae* en AST. El EXK de *P. (M.) bouvieri* abunda en AST, PUFAs n-3, y en la combinación AST-DHA.

PALABRAS CLAVE: *Acocil; Acociles mexicanos; Ácidos grasos; Ésteres de astaxantina; Omega 3*

1. INTRODUCTION

Wild crayfish have been caught and consumed by native Mexicans since pre-Hispanic times. *Cambarellus (Cambarellus) montezumae* (de Saussure, 1857), (*C. (C.) montezumae*), and *Procambarus (Mexicambarus) bouvieri* (Ortman, 1909), (*P. (M.) bouvieri*), are two autochthonous species among the 56 crayfish of the Cambaridae family that inhabit the fresh waters of Mexico (Alvarez *et al.*, 2012). Commonly known as "acocil", derived from the Nahuatl language "acotzilli", they are ingredients of traditional food. *C. (C.) montezumae,* is the most widely distributed species along the neo-volcanic axis of Mexico (Alvarez *et al.*, 2012). This organism has a small average adult size (32±4.3 mm total length), with a soft and thin EXK so that the consumption of the entire animal is common, either boiled, fried, or roasted. The *P. (M.) bouvieri* is found in Uruapan, Michoacan, Mexico. It is larger than *C. (C.) montezumae* (with an average adult size of 70.5±8.1 mm total length). As in other edible crayfish only the TM is consumed. When whole crayfish are boiled in a soup, the TM is eaten and the EXK is discarded. Both species are caught by the natives and are not marketed. At present, there is no crayfish aquaculture in Mexico, although some prospective studies consider its feasibility (Cerón-Ortiz *et al.*, 2015).

Crayfish lipids, as in other crustaceans, are important components characterized mainly by their high phospholipid (PL) content and essential fatty acids (EFA), the polyunsaturated (PUFAs) linoleic acid (C18:2n-6, LA), α-linolenic acid (C18:3n-3, ALA), and chiefly the readily available essential eicosapentaenoic (C20:5n-3, EPA), and docosahexaenoic acid (C22:6n-3, DHA) (Stanek *et al.*, 2011; Harlioğlu *et al.*, 2012; Harlioğlu *et al.*, 2015). There is a current criterion about the beneficial effect of the intake of n-3 PUFAs to prevent many degenerative diseases. To this end, scientific international committee have recommended a dietary intake of 250 mg per day of EPA+DHA (FAO, 2010). Along with FAs, the carotenoid astaxanthin (AST) (3,3′-dihydroxy-β,β′-carotene-4,4′-dione), is found in different crustacean tissues in free and esterified forms (Sachindra *et al.*, 2005). The physiological functions of this fat-soluble antioxidant have been widely documented *in vitro*, *in vivo*, and in pre-clinical studies and covered extensively for its health benefits in a recent review (Dose *et al.*, 2016). AST associated to EPA and DHA has been demonstrated to have a great effectiveness for improving human brain function (Konagai *et al.*, 2013), and neuro-protection (Barros *et al.*, 2014). In this respect, Saw *et al.* (2013), confirmed that low doses of AST combined with DHA or EPA have a synergistic antioxidant action mediated by the Nrf2 signaling pathway.

The fatty acids and carotenoids in crayfish have been sparsely investigated compared to marine crustaceans. The North American species *Astacus leptodactylus* (Harlioğlu *et al.*, 2012; Harlioğlu *et al.*, 2015), and *Procambarus clarkii* (Meyers and Bligh, 1981); the European *Astacus astacus* (Ackefors *et al.*, 1997), and the Australian *Cherax quadricarinatus* (Sagi *et al.*, 1995; Harlioğlu *et al.*, 2012), have been the most investigated in the world so far. Particularly, *C. (C.) montezumae* post-larvae has recently been studied regarding the FA composition (Cerón-Ortiz *et al.*, 2015), but not about carotenoid content while *P. (M.) bouvieri* has not yet been studied with regards to these topics. Thus, the aim of the present study was to determine the content and composition of FAs and AST in the two edible forms of these crayfish species: the whole animal of *C. (C.) montezumae*, and the TM of *P. (M.) bouvieri*. In addition, the study was extended to the EXK of *P. (M.) bouvieri* as a potential source of these important molecules.

2. MATERIAL AND METHODS

2.1. Reagents and materials

Organic solvents were of analytical grade and, when necessary, of HPLC grade, obtained from the Sigma-Aldrich Corporation (St Louis, MO, USA). Synthetic AST was obtained from Hoffmann-La Roche (Basel, Switzerland). β-carotene (purity: 97%), and fatty acid methyl esters (FAME) standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.2. Equipment

Capillary gas chromatography (GC) was carried out in a CP-3380 gas chromatograph equipped with a flame ionization detector and a CP-8400 autosampler (Varian Inc., Palo Alto, CA, USA). HPLC analysis was performed on a HPLC Waters system with a 717 autosampler, 1525 binary pump, and a dual 2487 UV/VIS absorbance detector (Milford, MA, USA). Sample absorbances were measured in a DU-60 Beckman spectrophotometer (Beckman, Fullerton, CA, USA). Vacuum evaporation was carried out on a rotary evaporator (Rotavapor R-134, Büchi, Brinkmann Instruments, Inc., N. Y., USA).

2.3. Animals and sample preparation

Adult *C. (C.) montezumae* of both sexes (with a total length size >30 mm), were caught in June in the nearby canals of Xochimilco, Mexico City. They were transported to the laboratory alive in three polyethylene bags with water, and placed over crushed ice (4 °C). Then, the bags were drained, wrapped with

Aluminium foil and frozen at –80 °C. As these animals are consumed entire without sex selection, the experimental sample consisted of a mixed capture. After defrosting, three kilograms of dead animals were washed with tap water and left to dry in a pilot plant cabinet dryer (Apex, model SSE 17M) at 50 ± 2 °C for 24 h, with natural air circulation. The dried sample was ground in a Wiley model 3379-K41 knife mill (Thomas Scientific, Philadelphia, PA, USA) using a 2 mm mesh. The meal was kept in a polyethylene bag at –20 °C until analysis. Adult *P. (M.) bouvieri* (total length size >70 mm), were caught in June in an Uruapan stream, Michoacan, transported to the laboratory alive in a cooler with crushed ice (4°C) and stored in plastic containers at –80 °C until analysis. After thawing, eighty dead animals with a mean individual body weight of 17.4 ± 0.6 g, were separated into the TM portion (14.7±2.5%, mean yield of the body weight), and EXK. The TM was analyzed fresh. EXK, composed of cephalothorax and abdomen shell, was manually and carefully freed from soft tissue, washed under running water, dried and processed to a meal as described above. The final moisture content of *C. (C.) montezumae* meal was 18.5 ± 1.3% and that of EXK was 12.2 ± 0.9%. The resulting meals were split into three parts and each analyzed in triplicate. In both species the animals were caught and processed in bulk, without sex separation.

2.4. Preparation of oil extracts

Meal samples of *C. (C.) montezumae*, the TM and the EXK of *P. (M.) bouvieri*, were subjected to analytical procedures under reduced white light. A 20 g meal sample from each species, or 50 g TM (wet weight 75.4 ± 3.5 g·100 g^{-1}) were ground in a mortar and extracted repeatedly with cold acetone (4 °C) until no further pigment was extractable. The colored acetone extracts were pooled and filtered through a Whatman No.1 filter paper under vacuum, phase-separated in 50 mL of petroleum ether (PE) in a separating funnel of 250 mL and washing repeatedly with water to remove traces of acetone. The organic phase was then filtered through a coarse-sintered glass funnel containing anhydrous sodium sulfate, rinsing the pigments with additional PE. Finally, the solvent was evaporated to dryness in a rotary evaporator at 45 °C under vacuum to obtain the oil extract which was stored in the dark under nitrogen at –20 °C until analysis.

2.5. Determination of fatty acids

Fatty acids in the oil extracts of all the samples were converted into methyl esters with 14% methanolic boron trifluoride, according to the AOAC method 969.33 (AOAC, 2000). Chromatographic separation was performed with a DB-23 capillary column

(length 30 m, i. d. 0.25 mm, film thickness 25 μm) (J and W Scientific, Folsom, CA, USA). Nitrogen was used as carrier gas at a flow rate of 2 mL·min^{-1}. Oven temperature was programmed for a linear increase of 10 °C·min^{-1} from 120 °C to 200 °C and 5 °C·min^{-1} from 200 °C to 230 °C. Myristoleic acid (C14:1) (Sigma-Aldrich), was used as internal standard. The chromatographic peaks were identified by comparison of their retention times with those of standards and quantified with the Star Workstation program (Varian Inc.), using a mixture of pure standards to detect retention times. Each identified FA was expressed as a percentage of the total FAs recorded in the sample. Additionally, the quality of the oil extracts was calculated by the formulae of the atherogenicity index (AI) and thrombogenicity index (TI), proposed by Ulbricht and Southgate, (1991):

$$AI = [(C12:0 + (4 \times C14:0) + C16:0)] / [MUFA + (PUFA\ n\text{-}6 + n\text{-}3)] \quad (Eq.\ 1)$$

$$TI = [C14:0 + C16:0 + C18:0] / [(0.5 \times MUFA) + (0.5 \times PUFA\ n\text{-}6) + (3 \times PUFA\ n\text{-}3) + PUFA\ n\text{-}3/PUFA\ n\text{-}6] \quad (Eq.\ 2)$$

2.6. Total carotenoid quantification

Oil extracts from each replicated extraction of samples were taken in a known volume of *n*-hexane and the carotenoids were quantified by determining the optical density at 470 nm (absorption maximum of AST in *n*-hexane). Since AST is the main carotenoid in crustaceans, the total concentration of carotenoids was referred to as this carotenoid in the oil extracts, according to Sachindra *et al.* (2005), by means of the formula:

$$Carotenoid\ content\ (μg\ AST \cdot g^{-1}\ sample) = A \times V \times D/0.2 \times W \quad (Eq.\ 3)$$

where, A = absorbance at 470 nm; V = volume of the extract in *n*-hexane; D = dilution factor; 0.2 is the absorbance value (λ = 470 nm) of 1 μg·mL^{-1} AST standard in *n*-hexane, and W is the weight of meal or TM sample (g).

The absorption maximum and chromatographic features of the AST standard were obtained. To this end, 1.5 mg of synthetic compound were dissolved in 5 mL chloroform and brought up to 50 mL with *n*-hexane (stock solution). A standard solution of AST was made from the stock solution in *n*-hexane:chloroform (95.5:4.5, v/v), and absorption spectra were determined between 360 and 600 nm.

2.7. Chromatographic separation of carotenoids

Individual carotenoids in total oil extracts were separated, identified and their relative composition was established by chromatography, except those of

TM, whose separation was not possible due to insufficient quantity.

2.7.1. Open column chromatography (OCC)

Two g of oil extract diluted in 25 mL *n*-hexane were eluted from a silica gel column 32 × 420 mm (Si 60, particle size 0.40–0.63 mm), with stepwise 5% decreasing proportions of *n*-hexane in acetone, following the procedure described by Coral-Hinostroza and Bjerkeng (2002). The yellow non-polar lipids (fraction F1), were first eluted with pure *n*-hexane; the next two orange-red colored F2 and F3 were separated with a mixture of *n*-hexane:acetone elution solvent in a proportion of 95:5 (v/v), and 90:10 (v/v), respectively; and the last red fraction (F4) with 85:15 (v/v). To increase the purity of the F2, F3, and F4 fractions, they were re-chromatographed with the same mixture of solvents starting with pure *n*-hexane. The obtained fractions were evaporated to dryness, re-dissolved in HPLC grade *n*-hexane for TLC and HPLC tests, and subjected to spectroscopy for obtaining their absorption spectra (360 to 600 nm) and maximum wavelengths (λ_{max}).

2.7.2. TLC Chromatography

Fractions F2, F3, and F4 were applied individually to Aluminium backed plates pre-coated with silica (0.2 mm, Merck DC-alufolien, Kieselgel 60 F254, Darmstadt, Germany), and their retention factor (R_f) was calculated in parallel with the synthetic AST, AST-monoester and AST-diester standards. The last two were prepared by separation and purification from the red crab *Pleuroncodes planipes* (Coral-Hinostroza and Bjerkeng, 2002). Spots were eluted in a mobile phase of *n*-hexane:acetone (70:30, v/v).

2.7.3. HPLC chromatography

Carotenoids were analyzed by HPLC on a H_3PO_4-modified Spherisorb SW column (250 mm × 4.6 mm i.d., 5 µm, Waters) at a controlled temperature (30 °C) with a mobile phase of *n*-hexane: acetone (86:14, v/v). The flow rate and injection volume were 1.0 mL min^{-1} and 20 µL, respectively, and the detection wavelength was set at 470 nm. Fractions F2, F3, and F4, were filtered with a pore size 0.45 µm PVLP membrane Durapore filter (Millipore, Bedford, MA, USA), and injected. The HPLC spectra of the synthetic AST and the prepared AST-ester standards were obtained under the same chromatographic conditions as the samples. Carotenoid separation of the complete oil extracts was also obtained by dissolving them (1:100, v/v) in HPLC grade *n*-hexane, through a 0.45 µm pore size PVLP Durapore membrane (Millipore, Bedford, MA, USA), and eluting for 15 min under the same conditions as the

standards. The identification of carotenoids was based on the comparison of the retention times of sample peaks with those of standards. The relative content of total AST was obtained by summing up the areas under the curves of monoester, diester, and free AST, considering the total carotenoid content as 100%. The content of free AST was calculated by summing up the areas under the curves of its geometrical isomers.

2.8. Qualitative analysis of fatty acid composition of the AST esters

Qualitative analysis of FA composition in AST-monoester, and AST-diester fractions obtained from the OCC separation were undertaken using gas chromatography after the methylation step following the procedure explained above.

2.9. Statistical analyses

Results are expressed as mean values of three determinations ± standard deviation (SD). An analysis of variance (ANOVA) was carried out to determine the difference among the examined group of samples. A comparison of mean values was carried out by Duncan's multiple range test and statistically significant differences were reported at $p < 0.05$. Data analyses were performed using the Statistical Package for Social Science (SPSS 11.0 for Windows; SPSS, Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1. Fatty acids of oil extracts

The FA profiles of the oil extracts from *C. (C.) montezumae*, and the TM of *P. (M.) bouvieri* are summarized in Table 1. Palmitic acid (C16:0) and oleic acid (C18:1n-9) were the prevailing saturated (SFA) and monounsaturated fatty acids (MUFA), in both analyzed samples, whereas EPA and LA were the dominant PUFAs. SFA pentadecanoic (C15:0) was found only in the TM of *P. (M.) bouvieri*. The general composition pattern of FAs in both edible forms are similar to those found in complete *C. (C.) montezumae* (Cerón *et al*., 2015), and to other crayfish species (Stanek *et al*., 2011; Harlioğlu *et al*., 2012; Harlioğlu *et al*., 2015). Mean percentage of SFAs (26.52 ± 0.41%) and MUFAs (42.80 ± 0.57%), were significantly higher in *C. (C.) montezumae* than in the TM of *P. (M.) bouvieri* (SFAs, 24.04 ± 0.15% and MUFAs, 34.22 ± 0.82%) ($p<0.05$). The high levels of SFAs and MUFAs in the entire animals of *C. (C.) montezumae* were probably derived from organs, such as the hepatopancreas and gonads of possible female animals in the sample, of high lipid content, mainly C18:1n-9, which is stored as an energy source (Gonzalez-Baro and Pollero, 1988).

TABLE 1. Fatty acid profile and lipid quality indices of the oil extracts of the two crayfish species, *Cambarellus (C.) montezumae* and *Procambarus (M.) bouvieri*, expressed as percentage (%) of the total fatty acids

Fatty acid	Edible forms		
	C. (C.) montezumae whole animal	*P. (M.) bouvieri* TM	*P. (M.) bouvieri* EXK
SFA			
C14:0			1.37±0.31
C15:0		0.35±0.05[a]	0.93±0.15[b]
C16:0	20.46±1.02[c]	17.20±0.60[a]	18.31±0.25[b]
C18:0	5.01±0.35[a]	5.78±0.64[a]	5.52±0.33[a]
C20:0	1.05±0.28[b]	0.70±0.06[a]	0.82±0.07[a]
MUFA			
C14:1n-7	10.45±1.69[c]	5.27±0.50[a]	7.67±0.40[b]
C16:1n-7	10.60±0.43[b]	8.36±0.23[a]	10.08±0.43[b]
C17:1n-8			1.61±0.07
C18:1n-9	21.74±1.11[b]	20.60±0.12[b]	18.38±0.23[a]
PUFA			
C18:2n-6	9.31±0.16[a]	9.10±0.40[a]	10.25±0.11[b]
C18:3n-3	4.15±0.88[b]	3.56±0.08[a]	5.19±0.17[b]
C20:4n-6	4.14±0.45[a]	9.83±0.45[c]	5.31±0.06[b]
C20:5n-3	10.35±0.59[a]	13.39±0.15[c]	11.51±0.26[b]
C22:6n-3	2.19±0.30[a]	5.32±0.29[c]	3.02±0.16[b]
ΣSFA	26.52±0.41[b]	24.04±0.15[a]	26.95±0.35[b]
ΣMUFA	42.80±0.57[c]	34.22±0.82[a]	37.74±0.30[b]
ΣPUFA	30.14±1.30[a]	41.19±0.88[c]	35.28±0.18[b]
Total n-3	16.99±1.09[a]	22.26±0.11[c]	19.72±0.19[b]
Total n-6	13.45±0.38[a]	18.93±0.37[c]	15.55±0.11[b]
n-3/n-6	1.26±0.03[b]	1.18±0.04[a]	1.27±0.01[b]
AI	0.28±0.01[b]	0.23±0.01[a]	0.33±0.01[c]
TI	0.32±0.01[c]	0.24±0.00[a]	0.29±0.01[b]

TM, tail meat; EXK, exoskeleton; AI, atherogenicity index; TI, thrombogenicity index.
Means with different superscripts in the same row are significantly different ($p < 0.05$)

Total PUFAs, were significantly greater in TM (41.19 ± 0.88%) than in *C. (C.) montezumae* (30.14 ± 1.30%) ($p<0.05$), with statistically significant predominance of EPA, arachidonic acid (C20:4n-6, ARA), and DHA in the former than in the latter. The discrepancy in the FA content and composition between our samples might be attributed to:1) different animal components in the analyzed sample, and 2) to different habitat conditions of each species, in particular diet composition, in line with Cerón-Ortiz *et al.* (2015). The contents of LA in our studied crayfish were high, contrasting with those of seafood that are characterized by low levels of n-6 and high levels of n-3 (Valfré *et al.*, 2003). There is evidence

that EPA and DHA in the PL form ingested directly possess high bioavailability in humans (Konagai *et al.*, 2013; Cook *et al.*, 2016), and that consumption of krill oil increases these long chain EFA concentrations in plasma and blood cells (Ramprasath *et al.*, 2013). The n-3/n-6 ratio values for *C. (C.) montezumae* (1.26 ± 0.03) was of higher magnitude than that of the TM (1.18±0.04) ($p<0.05$). Lower ratios were found in TM of wild crayfish *Orconectes limosus*, aged 4+ (Stanek *et al.*, 2011), while higher values were determined in the TM of wild *A. leptodactylus* (Harlioğlu *et al.*, 2012). The ratio value for *C. (C.) montezumae* was comparable to those observed in the TM of *A. leptodactylus* (both sexes), after a diet supplementation with 1% n-3 PUFAs (Harlioğlu *et al.*, 2015). Our obtained values were in the range of 1 to 4 as observed for fish meat (Valfré *et al.*, 2003), suggesting the good quality of these crayfish species as a food.

Ulbricht and Southgate (1991) have indicated that fats with high atherogenicity (AI), and thrombogenicity (TI) indices are assumed to be detrimental to human health. As shown in Table 1, the AI of whole *C. (C.) montezumae* oil extracts were 0.28 ± 0.01 and in the TM of *P. (M.) bouvieri* 0.23 ± 0.01. These values are lower compared to those reported for rainbow trout meat (0.57) (Valfré *et al.*, 2003), sardine (0.60), and mackerel (0.48) (Fernandes *et al.*, 2014), and much lower than that of terrestrial animal meat such as lamb (1.00), beef (0.72), pork (0.69), chicken (0.50), and rabbit (0.82) (Bosco *et al.*, 2001). Mean values of TI were 0.24 ± 0.00 and 0.32 ± 0.01 in the TM of *P. (M.) bouvieri* and *C. (C.) montezumae*, respectively, which are in the range of 0.20 to 0.40 just as those found for marine fish meat (Fernandes *et al.*, 2014). From our results, we can suggest that the utilization of both these edible forms of crayfish species for human nutrition offer advantages because they have a good n-3/n-6 balance and lipids of good quality, in the context of the low intake of n-3 PUFAs in the Mexican population as claimed by Ramirez-Silva *et al.* (2011), derived probably from the patterns of food choice and unavailability of fish stocks to supply fish meat.

As shown in Table 1, the two most important FAs by order of magnitude in the EXK of *P. (M.) bouvieri* were C18:1n-9 and C16:0 as was observed in the edible samples. In addition, three different FAs were identified, myristic (C14:0), pentadecanoic (C15:0) and margaroleic (C17:1n-8), although in minimal amounts (<1.6% each). Among the PUFAs, high amounts of EPA and LA where determined in this crayfish component. The n-3/n-6 ratio in it was 1.27 ± 0.01, whereas values of TI (0.29) and AI (0.33) were in the range of those reported for some marine fish (Fernandes *et al.*, 2014). This pattern, associated with a higher n-3 content in the oil extract of EXK of *P. (M.) bouvieri* contributes to a desirable nutritional quality with high potential as a valuable ingredient in the formulation of animal feed.

3.2. Carotenoid content and composition in the oil extracts

The total carotenoid concentration in the whole *C. (C.) montezumae* was 66.30 ± 3.95 (µg AST·g^{-1}), significantly higher than that in the TM of *P. (M.) bouvieri* (2.30 ± 0.29 µg AST·g^{-1}) ($p < 0.05$) (Table 2). The carotenoid amount in the former, measured as AST, is notably higher than that determined in a meal of whole *Procambarus clarkii* (15.74 µg·g^{-1}) (García-Romero *et al.*, 2014). The high carotenoid level in a whole crayfish can be anticipated as it includes organs rich in AST such as the gonads of ovigerous females, eye stalks, antenna, and components such as the EXK where high levels of this pigment compound is deposited as demonstrated in *A. leptodactylus* (Czeczuga, 1971). On the contrary, the carotenoid content in the muscle tissue of these animals is low as affirmed by Czeczuga (1971), in *A. leptodactylus* (1.3 µg g^{-1}) and in *Cherax quadricarinatus* (0.04 to 0.62 µg·g^{-1}) (Sagi *et al.*, 1995). Varying AST concentrations in tissue may be attributed to the available dietary carotenoids of crayfish in their particular environments or to the metabolic needs of particular carotenoid in the organ. The present study has shown the high pigment importance of a *C. (C.) montezumae* meal, with concentrations as much as 2.6 times greater than that reported for the meal of sun-dried shrimp, *Litopenaeus vannamei* (25.5 µg·g^{-1}) (Hernandez *et al.*, 2014), another edible form of a complete crustacean consumed in Mexico.

The EXK of *P. (M.) bouvieri* showed a high content of carotenoids (424.40 ± 9.02 µgAST·g^{-1}) with respect to the edible forms of this study. This content was lower than the one found in the purified carotenoprotein-1 fraction of *Procambarus clarkii* (610 µg·g^{-1}) (Cremades *et al.*, 2003). As noted by Zagalsky *et al.* (1990), AST is incorporated as the prosthetic group into proteins of crustacean shells in which it is mostly deposited and are responsible for coloration. It could then be expected that animals with intense colors provide significant levels of this carotenoid as is the case of *P. (M.) bouvieri* which when alive shows an external dark orange-brown color.

The estimation of the carotenoid composition by OCC resulted in four fractions. F1, β-carotene; F2, diester of AST; F3, monoesters of AST; and F4, free AST with their chromatographic features presented in Table 3. The HPLC profile of the complete oil extracts (Figure 1) of *C. (C.) montezumae* (Chromatogram A) and EXK (Chromatogram B), showed that AST (peak 3) and its esters (peaks 1, 2) were the major carotenoid pigments in both samples (Table 4). In addition to AST, lesser amounts of β-carotene and other not identified compounds were distinguished, more notably in *C. (C.) montezumae* than in EXK, which may correspond to the influence of carotenoids from organs such as the hepatopancreas, whose principal carotenoid is β-carotene (Sagi *et al.*, 1995). The ocurrence of AST as the main carotenoid in species of the current study was congruent with those found in other crayfish species such as *Procambarus clarki* and *Astacus astacus,* as mentioned above.

The relative AST content in the EXK of *P. (M.) bouvieri* was 93.15 ± 5.71%, significantly higher than that of *C. (C.) montezumae* (79.50 ± 1.02%) ($p < 0.05$) (Table 4). Both samples were distinguished by their high content of free AST (61.13 ± 1.44% and 41.88 ± 1.17%, respectively), although the content in EXK surpassed 50% with respect to the esterified forms. A high content of free AST was observed earlier in the waste products of *Procambarus clarki* (40.3% free and 49.4% esterified) (Meyers and Blight, 1981), and in the EXK of the same species where free AST was the main form (Inoue *et al.*, 1988). It seems that in *Procambarus* crayfish species

TABLE 2. Total carotenoid content in the two edible forms of crayfish *Cambarellus (C.) montezumae* and *Procambarus (M.) bouvieri*; and in the exoskeleton of *Procambarus (M.) bouvieri*

Total carotenoid content	Edible forms		P. (M.) bouvieri EXK
	C. (C.) montezumae whole animal	P. (M.) bouvieri TM	
µg AST·g^{-1}	66.30 ± 3.95[b]	2.30 ± 0.29[a]	424.40 ± 9.02[c]

TM, tail meat; EXK, exoskeleton. Data expressed as mean ± standard deviation (SD). Different superscripts in the row are significantly different ($p < 0.05$).

TABLE 3. Chromatographic and UV/VIS features of forms of astaxanthin in the oil extract samples of *Cambarellus (C.) montezumae* and *Procambarus (M.) bouvieri* crayfish

Fraction	OCC elution mixture (*n*-hexane:acetone, (v/v)	UV-VIS λ max (*n*-hexane) (nm)	TLC (R$_f$) solvent system (*n*-hexane:acetone) (70:30, v/v)
F1, β-carotene	100	449	0.98 ± 0.02
F2, di-AST	95:5	468	0.94 ± 0.01
F3, mono-AST	90:10	469	0.70 ± 0.02
F4, free AST	85:15	470	0.33 ± 0.01

F2, diester of AST; F3, monoester of AST; OCC, open column chromatography; Rf, retention factor value; UV/VIS, maximum absorption in n-hexane.
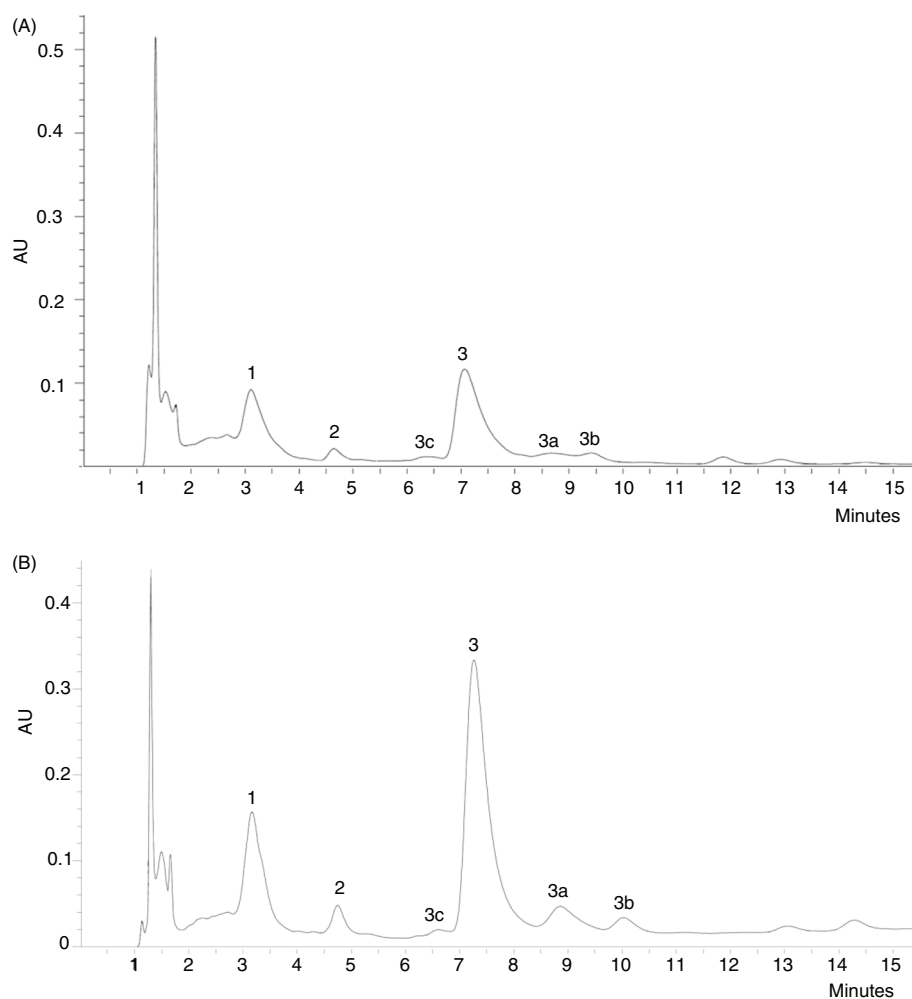
FIGURE 1.   HPLC comparison of astaxanthin (AST) composition in the whole crayfish *Cambarellus (C.) montezumae* (A), and *Procambarus (M.) bouvieri* exosqueleton (B). Peaks were identified by retention time, co-chromatography with synthetic AST and AST-esters. Peak 1: AST-diester; peak 2: AST-monoester; peak 3: trans *all-E* AST showing its geometrical isomers: 3a, 9 *cis*; 3b, 13 *cis*; 3c, probably Di-Z.

the prevailing form of this carotenoid is free and might be attributable to a particular biochemical characteristic.

Taking into account the carotenoid concentration of the whole *C. (C.) montezumae*, and its relative AST content it is worth considering the consumption of this food as a good way to enrich the diet with this bioactive molecule, although its bioavailability in these edible forms remains to be investigated. The geometric isomer composition of free AST in all cases showed a higher proportion of trans *all-E* isomer (around 80%), and minor amounts of 9*cis* and 13*cis* configurations (around 15%), which corresponds to the normal composition of this carotenoid in nature. Di-*Z* configuration could not be confirmed.

3.3. Fatty acid profile of astaxanthin esters

Fatty acids of purified esters of AST are presented in Table 5. The FA profile of ester fractions of both samples contained the same predominant C18:1n-9 and C16:0 than in the complete oil extracts, although minor FAs appeared in the EXK esters as caprylic C8:0, lauric C12:0, myristoleic C14:1n-7, and araquidic C20:0, at low amounts (<3%). The predominance of C18:1n-9 and C16:0 in the esterified AST was similar to those obtained by Wolfe *et al.* (1965), in *O. rusticus*. A conspicuous difference in the FA composition of AST esters between the two analyzed samples was that concerning the PUFAs components. The AST esters of *C. (C.) montezumae* contained LA and ALA, while AST-esters of EXK

TABLE 4.   Relative composition of major carotenoids in the whole *Cambarellus (C.) montezumae* and in the exoskeleton of *Procambarus (M.) bouvieri* by HPLC

Peak	Carotenoids	*C. (C.) montezumae* whole animal	*P. (M.) bouvieri* EXK
	Total AST	79.50±1.02[a]	93.15±5.71[b]
	β-carotene	15.48±2.60[b]	4.85±5.44[a]
	NI	5.02±3.15[b]	2.00±0.27[a]
	AST fractions*		
1	di-AST	54.39±1.02[b]	35.07±3.00[a]
2	Mono-AST	3.73±0.15[a]	3.81 ±1.56[a]
	Free AST	41.88±1.17[a]	61.13±1.44[b]
	AST isomers**		
3c	Di-Z	1.94±0.16[a]	1.45±0.35[a]
3	all-trans	81.64±0.85[a]	82.71± 0.42[a]
3a	9-cis	8.61± 0.64[b]	6.02± 0.82[a]
3b	13-cis	7.93± 0.44[a]	9.81±0.17[b]

EXK, exoskeleton; Peaks 1, 2, 3c, 3, 3a, and 3b, correspond to Figure 1. (*) Percentages calculated with respect to total AST. (**) Percentage of isomers calculated with respect to free AST. NI: not identified carotenoids. Data were expressed as mean ± standard deviation (SD). Different superscripts in the same row are significantly different ($p < 0.05$).

TABLE 5.   Astaxanthin esters and their fatty acid composition expressed as percentage (%) of the total

Fatty acid	*C. (C.) montezumae* whole animal		*P. (M.) bouvieri* EXK	
	AST-diester	AST-monoester	AST-diester	AST-monoester
SFA				
C8:0			1.50	
C12:0			2.10	0.65
C14:0	3.07	2.23	2.36	2.69
C15:0	2.21	1.82	1.26	0.67
C16:0	18.72	17.08	9.97	10.45
C17:0	1.43	1.36		0.67
C18:0	3.96	4.44	3.84	6.63
C20:0			3.50	2.27
C23:0		2.96		0.47
MUFA				
C14:1n-7				1.68
C16:1n-7	2.47	1.21	1.40	0.81
C17:1n-8	1.45	1.27	1.22	1.17
C18:1n-9	16.62	17.0	14.92	2.97
C22:1n-9	3.77	3.61	4.48	0.83
PUFA				
C18:2n-6	12.87	10.87	8.70	1.29
C18:3n-3	6.02	4.54	4.10	0.53
C20:2n-6			1.77	2.64
C20:4n-6				0.49
C20:5n-3			1.31	1.49
C22:6n-3			7.30	30.46
ΣSFA	40.49	42.99	35.18	35.58
ΣMUFA	33.49	33.21	31.58	10.83
ΣPUFA	26.02	23.79	33.24	53.59

EXK, exoskeleton.

contained noticeable enrichment of DHA in the form of monoester. This feature was not observed in the EXK of *O. rusticus,* which displayed small amounts of LA and ALA and no DHA (Wolfe *et al.*, 1965). Moreover, preferential accumulation of EPA and DHA as monoesters was determined in the marine red crab *Pleuroncodes planipes* (Coral-Hinostroza and Bjerkeng, 2002). To this regard, Vincent and Cecaldi (1988), stated that the unsaturation level of fatty acid esterifying carotenoids increases with the increasing level of molecule oxygenation; thus, AST as one of the most oxygenated carotenoids would be expected to be most esterified with highly unsaturated FAs. We think that during biosynthesis, the steric structure of DHA prevents the esterification of both sites of the carotenoid giving preference to monoesterification. This particular association of DHA and EPA with AST has high pharmacological significance because their antioxidant synergism at low concentrations has been demonstrated (Saw *et al.*, 2013). The recovery of these valuable bioactive components from the EXK of *P. (M.) bouvieri* waste would improve the utilization and economic exploitation criteria of this crayfish.

We conclude that *C. (C.) montezumae* and the TM of *P. (M.) bouvieri* are good sources of n-3 PUFAs of good nutritional and functional quality equivalent to some marine fish. Moreover, *C. (C.) montezumae* is a good source of dietary carotenoids, particularly of AST. The EXK of *P. (M.) bouvieri* represents a valuable potential source of n-3 PUFAs, free AST and the combined n-3 PUFAs-AST for different purposes (food additives, animal feeding, or nutraceuticals). An increased accessibility of crayfish and their by-products through aquaculture might open up new possibilities for obtaining this novel source of bioactive compounds.

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