

Identification and Quantification of Astaxanthin Esters in Shrimp (*Pandalus borealis*) and in a Microalga (*Haematococcus pluvialis*) by Liquid Chromatography–Mass Spectrometry Using Negative Ion Atmospheric Pressure Chemical Ionization

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Negative ion liquid chromatography–atmospheric pressure chemical ionization mass spectrometry [negative ion LC-(APCI)MS] was used for the identification of astaxanthin esters in extracts of commercial shrimp (*Pandalus borealis*) and dried microalga (*Haematococcus pluvialis*) samples. A cleanup step using a normal phase solid phase extraction (SPE) cartridge was applied prior to analysis. Recovery experiments with astaxanthin oleate as model compound proved the applicability of this step ($98.5 \pm 7.6\%$; $n = 4$). The assignment of astaxanthin esters in negative ion LC-(APCI)MS was based on the detection of the molecular ion ($M^{\bullet-}$) and the formation of characteristic fragment ions, resulting from the loss of one or two fatty acids. Quantification of individual astaxanthin esters was performed using an astaxanthin calibration curve, which was found to be linear over the required range ($1\text{--}51 \mu\text{mol/L}$; $r^2 = 0.9996$). Detection limits, based on the intensity of $M^{\bullet-}$, a signal-to-noise ratio of 3:1, and an injection volume of $20 \mu\text{L}$, were estimated to be $0.05 \mu\text{g/mL}$ (free astaxanthin), $0.28 \mu\text{g/mL}$ (astaxanthin–C16:0), and $0.78 \mu\text{g/mL}$ (astaxanthin–C16:0/C16:0), respectively. This LC-(APCI)MS method allows for the first time the characterization of native astaxanthin esters in *P. borealis* and *H. pluvialis* without using time-consuming isolation steps with subsequent gas chromatographic analyses of fatty acid methyl esters. The results suggest that the pattern of astaxanthin-bound polyunsaturated fatty acids of *P. borealis* does not reflect the respective fatty acid pattern found in triacylglycerides. Application of the presented LC-(APCI)MS technique in common astaxanthin ester analysis will forestall erroneous xanthophyll ester assignment in natural sources.

KEYWORDS: LC-(APCI)MS; astaxanthin esters; shrimp; *Pandalus borealis*; *Haematococcus pluvialis*; carotenoid

INTRODUCTION

Astaxanthin, a symmetric ketocarotenoid (3,3'-dihydroxy- β,β -carotene-4,4'-dione), naturally occurs in a wide variety of marine and aquatic organisms. The bright red to pink color of Crustaceae (shrimp, krill) and Salmonidae (salmon, rainbow trout) results from accumulation of astaxanthin (1). Because animals are not capable of de novo synthesis of astaxanthin, microalgae serve as their natural dietary source. Apart from coloration, astaxanthin is considered to be essential for growth processes and as a vitamin A precursor in fish (2, 3). Thus, astaxanthin is commonly used as a feed supplement in worldwide fish farming to grow healthy and well-colored individuals. The astaxanthin molecule bears two hydroxy functions located at C3/C3' of the β -ionone moieties. Thus, astaxanthin may be present in its free as well as in its mono- or diesterified form

(Figure 1), depending on the respective organism and the storage site (e.g., flesh, skin, ovaries) (4).

A natural source of astaxanthin is the green microalga *Haematococcus* sp., which accumulates high concentrations of monoesterified astaxanthin (5). Nonesterified astaxanthin is found in the yeast *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*), another high-accumulating organism (6). Analysis of the highly complex ester pattern found in *Haematococcus* sp. has attracted interest in the past. Yuan et al. (7, 8) provided a high-performance liquid chromatography (HPLC) method for the separation and analysis of astaxanthin esters present in *H. lacustris*, but they did not focus on individual ester identification. However, the authors noted that astaxanthin monoesters were the main components, accompanied by only trace amounts of astaxanthin diesters. Renstrom and Liaaen-Jensen (9) identified xanthophyll esters by means of thin-layer chromatography followed by gas chromatographic (GC) detection of fatty acid methyl esters. Predominant fatty acids in astaxanthin esters of

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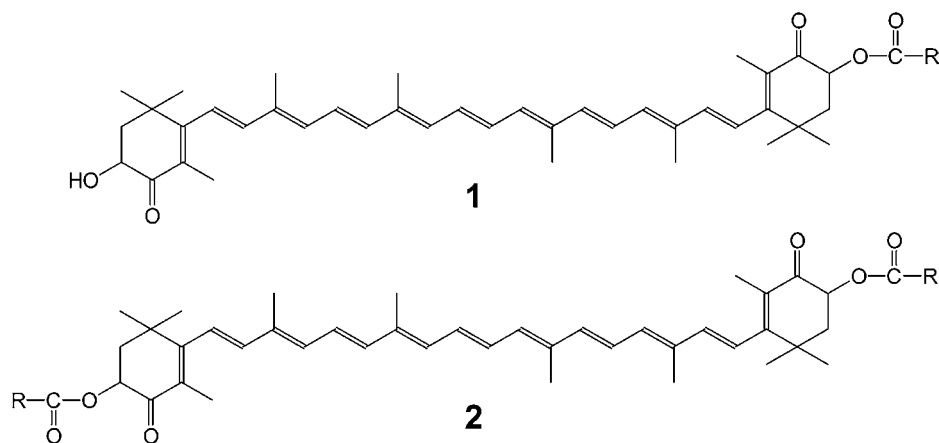


Figure 1. Structures of astaxanthin mono- (1) and diesters (2) (R = saturated or unsaturated alkyl chains).

H. pluvialis were C16:0 (7%), C18:0 (7%), C19:0 (6%), C20:0 (25%), and C18:1 (56%). The presence of oleic acid as the major fatty acid found in the astaxanthin ester fraction of *H. pluvialis* was affirmed by other studies (10, 11).

Renstrom and Liaaen-Jensen (9) provided further information on the astaxanthin ester pattern found in shrimp (*Pandalus borealis*). The main fatty acids esterified with astaxanthin were C12:0 (4–5%), C14:0 (2–3%), C16:0 (3–8%), C16:1 (9–10%), C18:1 (17–44%), C22:1 (21–39%), C20:5 (2–5%), and C22:6 (5–19%). Accordingly, oleic acid and C22:1 formed the principal part. Polyunsaturated fatty acids (PUFAs) such as C20:5 (eicosapentaenoic acid) and C22:6 (docosahexaenoic acid), typical native constituents of various fish oils, also occurred in significant amounts. In an older study, fatty acids bound to astaxanthin derived from brown shrimp (*Crangon vulgaris* Fabr.) were investigated by GC-FID (12). Again, C14:0, C16:0, C16:1, C18:0, and C18:1 were identified, but quantitative data were not given. A recent investigation on the red crab langostilla (*Pleuroncodes planipes*) provided detailed information on the composition of fatty acids acylating astaxanthin in three diester fractions and in one monoester fraction (13). The main fatty acids found in the diester fractions were C14:0 (5–9%), C16:0 (17–30%), C20:0 (0–23%), C16:1 ω -7 (12–15%), and C18:1 (15–24%), whereas C20:5 (47%) and C22:6 (11%) were predominant in the monoester fraction. Recently, the fatty acid pattern of esterified astaxanthin of Antarctic krill (*Euphausia superba*) was published (14). Molecular masses of the carotenoid esters were measured by field desorption mass spectrometry. The fatty acid moieties were additionally transmethylated and analyzed by GC. The authors reported only five fatty acids in the mono- and diester fractions: C12:0, C14:0, C16:0, C16:1 and C18:1. Surprisingly, PUFAs were not found in the respective fractions.

The present study uses HPLC coupled to mass spectrometric detection to obtain a deeper insight into the astaxanthin ester pattern of *H. pluvialis* and *P. borealis* without time-consuming isolation steps of individual esters and without the use of transmethylation of fatty acids. For removal of accompanying lipids, normal phase solid phase extraction (SPE) was applied as a single cleanup step. Because reference substances were not available commercially, astaxanthin mono- and diesters were synthesized independently to facilitate peak assignment in complex mixtures. This study did not focus on the stereoisomeric profile of astaxanthin esters or on the presence of minor xanthophylls (e.g., echinenone, canthaxanthin).

MATERIALS AND METHODS

Chemicals. Light petroleum (boiling fraction 40–60 °C), methanol, ethyl acetate, and acetone were purchased from Merck (Darmstadt, Germany); *tert*-butyl methyl ether (TBME), *n*-hexane, pyridine (99.8%, over molecular sieve), astaxanthin (98%), and acyl chlorides (98–99% each; lauroyl, myristoyl, palmitoyl, stearoyl, oleoyl, and linoleoyl chloride) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Filter paper (no. 610) was from Schleicher & Schuell (Dassel, Germany). All solvents were distilled before use. High-purity water was prepared with a Milli-Q 185 Plus water purification system (Millipore, Eschborn, Germany). SPE cartridges for normal phase chromatography (Sep-Pak; 910 mg florisil plus/cartridge) were purchased from Waters GmbH, Eschborn, Germany.

Samples. Four shrimp samples (*P. borealis*), produced from different manufacturers (sample I, without brine; samples II–IV, with brine), were obtained from local supermarkets. The brine was removed and the shrimp were dried with a dust-free cloth prior to homogenization. Two dried *H. pluvialis* samples were kindly provided by the Microalgal Biotechnology Laboratory (Ben-Gurion University of the Negev, Israel; Prof. S. Boussiba) and by Cyanotech Corp. (Hawaii Ocean Science Technology Park).

Preparation of Samples. Extraction. Shrimp (150 g) were minced with an Ultra Turrax T 25 (Janke & Kunkel, Staufen, Germany) for 1 min. An aliquot of the homogenate (80 g) was suspended in methanol/ethyl acetate/light petroleum (1:1:1 v/v/v; 100 mL) by mixing thoroughly by hand. The extract was separated from the tissue by employing a Büchner funnel covered with a filter paper under vacuum (500 mbar). Direct homogenization of shrimp samples with the ternary solvent mixture resulted in a slurry, which easily blocked the Büchner funnel. Thus, this method was not applicable. To allow for complete extraction, additional portions of solvent were poured onto the filter cake and vacuumed until the filter cake was completely colorless (6 × 100 mL). The resulting extract was transferred into a separating funnel. To assist phase separation, 4 mL of a saturated sodium chloride solution was added. The water layer was discarded, and the organic layer was transferred into an Erlenmeyer flask, dried with anhydrous sodium sulfate, filtered, and evaporated to dryness. For cleanup, the residue was dissolved in *n*-hexane (3 mL). *H. pluvialis* samples (150 mg) were suspended in water (10 mL), mixed with an aliquot of a saturated sodium chloride solution (4 mL), and extracted with methanol/ethyl acetate/light petroleum (1:1:1 v/v/v; 20 mL each) in a separating funnel until the extracts were colorless (3 × 20 mL). To ensure complete extraction, sonication (30 s) was applied prior the last extraction step. The upper organic phases were combined and treated as described above.

Cleanup Procedure. Five SPE columns were directly coupled in-line by sticking them together and conditioned with *n*-hexane. The use of five columns was necessary to provide sufficient sorbent for good separation. Each entire extract was poured completely onto the first column using a syringe. For removal of interfering compounds, the whole column was washed with *n*-hexane (30 mL) and mixtures of

acetone in *n*-hexane [10, 20, 30, 40, and 50% (v/v); 30 mL each]. Elution of astaxanthin esters adsorbed between columns two and four was performed with pure acetone (30 mL). Colored fractions were combined, the solvent was evaporated (50 mbar, 30 °C), and the residue was dissolved in TBME/methanol (1:1 v/v; 3 mL). After membrane filtration (0.45 μ m), samples were subjected to HPLC with diode array detector (DAD) or liquid chromatography–mass spectrometry using an atmospheric pressure chemical ionization interface [LC-(APCI)MS] analyses. All procedures were performed under dim light. Samples were analyzed immediately after cleanup.

Evaluation of the Cleanup Step. To determine possible losses of astaxanthin esters during the cleanup procedure, recovery experiments using synthesized astaxanthin oleate as reference compound were performed. Aliquots (2 mL) of an astaxanthin oleate solution ($c = 75 \mu\text{g/mL}$ in *n*-hexane) were placed with a syringe directly on the SPE column and eluted as described (final volume = 2 mL). The concentration of this sample is referred to as c_2 . For preparation of a reference solution, another aliquot (2 mL) was evaporated under vacuum and redissolved in TBME/methanol (1:1 v/v; 2 mL). The concentration of this reference solution is referred to as c_1 . Recoveries of astaxanthin oleate were calculated as follows: % recovery = $c_2 \times 100/c_1$. The following recovery was obtained ($n = 4$): $98.5 \pm 7.6\%$.

Quantification of Astaxanthin Esters by HPLC-DAD. The concentration of an astaxanthin standard solution was determined spectrophotometrically. Because the solubility of astaxanthin is rather poor in most solvents, astaxanthin (~20 mg) was dissolved in toluene (50 mL), although no ϵ_{mol} value was available. The concentration calculated using the data set given for canthaxanthin ($\epsilon_{\text{mol}} = 118200$; 480 nm) (15) was $51.2 \mu\text{mol/L}$ (30.6 mg/L). Because the influence of the two hydroxy groups of astaxanthin on the spectroscopic behavior is negligible, quantitative results are considered not to be influenced. For calibration, an aliquot (40 mL) was evaporated to dryness, redissolved in TBME/methanol (1:1 v/v; 40 mL), and further diluted to the required concentrations (1.0–51.2 $\mu\text{mol/L}$). Aliquots were subjected immediately to HPLC-DAD analysis. For quantification, the calibration curve was created by plotting the peak area ($\text{mAU} \times \text{s}$) versus the concentration ($\mu\text{mol/L}$). The calibration graph was found to be linear over the required range (1–51 $\mu\text{mol/L}$; $r^2 = 0.9996$). Concentrations of astaxanthin esters were calculated according to the respective molecular masses.

Synthesis and Isolation of Astaxanthin Esters. Astaxanthin (1 mg) was dissolved in dry pyridine (5 mL) and reacted with the respective acyl chloride as described earlier for zeaxanthin esters (16). Mixed esters were prepared by adding both acyl chlorides dropwise one after another. The crude products were subjected to semipreparative HPLC on C30 material as described by Weller and Breithaupt (16). For elution, isocratic solvent mixtures consisting of TBME and methanol (v/v) were used (for peak numbering see Table 1): 50:50 (6, 9, 13), 40:60 (3, 4, 5, 15, 16), 30:70 (12, 18, 20, 21), 20:80 (10, 11, 19). Retention times were between 5 min (15) and 29 min (11). After isolation, combined fractions of multiple separations were evaporated to dryness, immediately redissolved in TBME/methanol (1:1 v/v; 3 mL), and stored at $-20 \text{ }^\circ\text{C}$. All standard solutions were analyzed by LC-(APCI)MS.

HPLC and LC-(APCI)MS. The HPLC consisted of a modular system HP1100 (Hewlett-Packard GmbH, Waldbronn, Germany) with diode array detector (480 nm). A $250 \times 4.6 \text{ mm}$ i.d. YMC analytical column (YMC Europe, Schermbach, Germany) equipped with $5 \mu\text{m}$ C30 reversed phase material including a $10 \times 4.0 \text{ mm}$ i.d. precolumn was used (35 °C). The mobile phase consisted of mixtures of methanol/TBME/water [81:15:4 v/v/v (A) and 6:90:4 v/v/v (B)]. The following gradient was used (min/% A): 0/99; 39/44; 45/0; 50/99; 55/99. The flow rate was 1 mL/min and the injection volume, 20 μL . LC-(APCI)MS was performed on an HP1100 modular HPLC system, coupled to a Micromass (Manchester, U.K.) VG platform II quadrupole mass spectrometer. The following MS parameters were used: APCI source, 150 °C; APCI probe, 400 °C; corona voltage, 3.7 kV; HV lens, 0.5 kV. Nitrogen was used as sheath (75 L/h) and drying gas (300 L/h). Various cone voltages (CV; 30, 45, 60 V) were tested in the positive and in the negative ionization mode. Further details were given by Breithaupt et al. (17).

Table 1. Negative Ion LC-(APCI)-MS Data (CV 45 V) Used for Identification of Astaxanthin Esters in *H. pluvialis* and Shrimp (*P. borealis*) Extracts^a

compound	<i>m/z</i>		
	$\text{M}^{\bullet-}$	$[\text{M}^{\bullet-} - \text{FA}]^-$	$[\text{M}^{\bullet-} - \text{FA}_2]^-$
A-C18:4 (1) ^b	854.6 (100%)	578.4 (85%)	
A-C18:3 (2) [*]	856.6 (100%)	578.4 (90%)	
A-C18:2 (3)	858.6 (100%)	578.4 (130%)	
A-C18:1 (4)	860.6 (100%)	578.4 (75%)	
A-C16:0 (5)	834.6 (100%)	578.4 (127%)	
A-C18:0 (6)	862.6 (100%)	578.4 (114%)	
A-C20:0 (7) [*]	890.7 (100%)	578.4 (33%)	
A-C18:1/C18:3 (8) [*]	1120.8 (100%)	838.6 (32%)	842.6 (26%)
A-C18:1/C18:2 (9)	1122.9 (100%)	840.6 (12%)	842.6 (14%)
A-C16:0/C18:2 (10)	1096.9 (100%)	840.6 (10%)	816.6 (25%)
A-C18:1/C18:1 (11)	1124.9 (100%)	842.6 (22%)	
A-C16:0/C16:0 (12)	1072.8 (100%)	816.6 (18%)	
A-C18:0/C18:1 (13)	1126.9 (100%)	842.6 (13%)	844.6 (16%)
A (14)	596.4 (100%)		
A-C12:0 (15)	778.6 (24%)	578.4 (100%)	
A-C14:0 (16)	806.6 (30%)	578.4 (100%)	
A-C20:1 (17) [*]	888.7 (31%)	578.4 (100%)	
A-C12:0/C12:0 (18)	960.7 (100%)	760.5 (173%)	
A-C12:0/C14:0 (19)	988.8 (100%)	788.6 (74%)	760.5 (63%)
A-C12:0/C18:1 (20)	1042.8 (100%)	842.6 (71%)	760.5 (109%)
A-C12:0/C16:0 (21)	1016.8 (100%)	816.6 (71%)	760.5 (49%)
A-C12:0/C20:1 (22) [*]	1070.8 (100%)	870.6 (46%)	760.5 (68%)
A-C12:0/C22:1 (23) [*]	1098.9 (100%)	898.7 (43%)	760.5 (52%)
A-C18:1/C20:1 (24) [*]	1152.9 (100%)	870.6 (76%)	842.6 (77%)

^a FA, fatty Acid; A, astaxanthin. Masses of the fragment ions and the signal intensities (in parentheses) are given. Assignment of 1–24 corresponds to peak numbering in Figure 3. ^b The asterisk indicates that assignment is based on the fragmentation pattern of LC-(APCI)MS analyses only.

Optimization of LC-(APCI)MS Parameters. To optimize conditions for LC-(APCI)MS analyses, a standard solution containing synthesized homogeneous astaxanthin diesters was used in preliminary experiments. Because xanthophylls are well-known to form protonated quasimolecular ions $[\text{M} + \text{H}]^+$ (16), the solution was first analyzed in the positive ion mode (CV 30 V). As expected, the quasimolecular ions as well as the respective fragment ions formed by loss of one or two fatty acids were generated. However, when cleaned shrimp extracts were analyzed, an extremely high background noise hampered the interpretation of the fragmentation pattern. These interferences may originate from residual triacylglycerides, also forming positive ions. Increasing the energy imparted to the analytes from 30 to 60 V enhanced xanthophyll ester fragmentation. Subsequently, the standard mixture was analyzed using the negative ion mode (CV 30, 45, 60 V). Under these conditions, negatively charged molecular ions ($\text{M}^{\bullet-}$) were formed, whereas $[\text{M} - \text{H}]^-$ ions were present with low intensity. The same pattern was found with free astaxanthin. The mass spectra obtained from standard solutions of free astaxanthin showed the predicted molecular ion at m/z 596.4 (100%) and a $\text{M} - 1$ signal with a relative intensity of 16%.

Detection Limit of Astaxanthin Esters [LC-(APCI)MS]. For estimating the detection limits of astaxanthin esters and astaxanthin, synthesized astaxanthin-C16:0, astaxanthin-C16:0/C16:0, and free astaxanthin were employed as model compounds in equal concentrations (stock solutions of 10 $\mu\text{mol/L}$ each; TBME/methanol, 1:1 v/v). Solutions were further diluted with TBME/methanol (1:1 v/v). On the basis of the intensity of the respective molecular ion ($\text{M}^{\bullet-}$), a signal-to-noise ratio of 3:1, and an injection volume of 20 μL , the detection limits were estimated to be as follows: free astaxanthin, 0.08 $\mu\text{mol/L}$ (0.05 $\mu\text{g/mL}$); astaxanthin-C16:0, 0.33 $\mu\text{mol/L}$ (0.28 $\mu\text{g/mL}$); astaxanthin-C16:0/C16:0, 0.73 $\mu\text{mol/L}$ (0.78 $\mu\text{g/mL}$).

RESULTS AND DISCUSSION

Fragmentation of Astaxanthin Esters. An example of a mass spectrum obtained from a mixed astaxanthin ester (C12:0/C16:0) determined in a shrimp (*P. borealis*) extract is

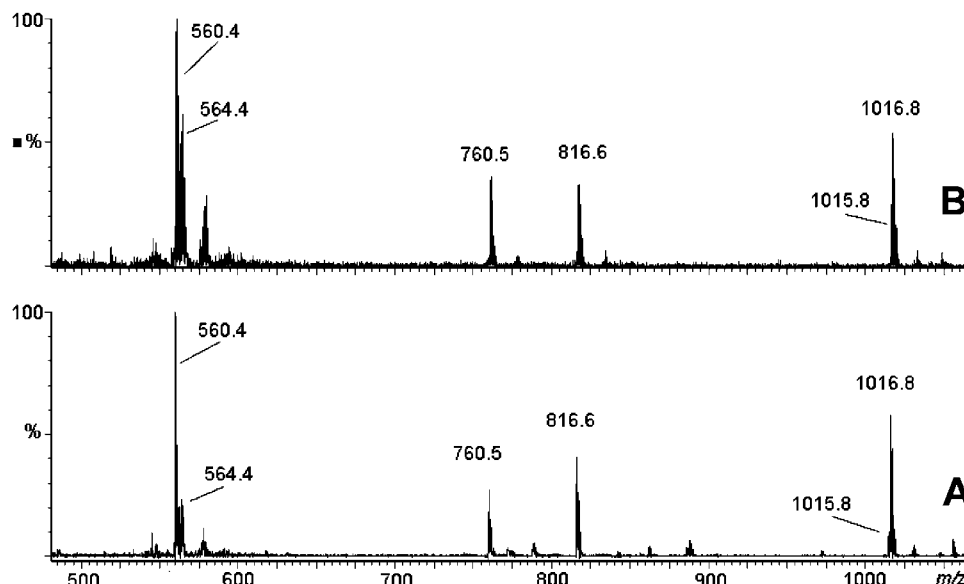


Figure 2. Mass spectrum of astaxanthin-C12:0/C16:0 [negative ion LC-(APCI)MS; CV 45 V], obtained from a shrimp (*P. borealis*) extract (A) and from a standard solution (B). Assignment of the respective ions (m/z) is as follows: 1016.8 ($[M]^-$); 1015.8 ($[M - H]^-$); 816.6 ($[M - C12:0]^-$); 760.5 ($[M - C16:0]^-$); 564.4; 560.4 ($[M - C12:0 - C16:0]^-$).

shown in **Figure 2A**. For direct comparison, the mass spectrum of the chemically synthesized compound is presented in **Figure 2B**. The formation of M^- molecular ions has been described previously by van Breemen et al. (18), who analyzed free xanthophylls in the negative mode. Liquid chromatography coupled to particle beam MS has been used earlier in the negative mode for analyses of β -carotene solutions (19). The fragmentation pattern of astaxanthin esters was dominated by loss of fatty acids as neutral molecules, resulting in the case of mixed diesters in the formation of two $[M - FA]^-$ ions. Enhancing the CV from 30 to 45 and 60 V resulted in a decrease of the molecular ion intensity and in an increase of the fragment ion abundance. The ion representing the backbone of astaxanthin (m/z 560.4) was present in all spectra but varied in intensity and was accompanied by fragment ions with low intensity (**Figure 2**). Additionally, a fragment ion at m/z 564.4 was present, even forming the base peak in some experiments. The origin of this ion is as yet unknown. To obtain the best ratio among intensities of molecular and fragment ions, the CV was set to 45 V in all subsequent experiments. The mass spectrometric data used for the identification of astaxanthin esters in *H. pluvialis* and *P. borealis* extracts are presented in **Table 1**. The reason for the low abundance of monoester molecular ions in the case of **15–24** (*P. borealis*) is not yet clear. However, identification was achievable in most cases. It must be emphasized that relative fragment ion intensities are given for cleaned real samples, not for synthesized reference compounds. The backbone masses m/z 578.4 (monoesters) and m/z 560.4 (diesters) were used to scan the total ion current (TIC) trace for the existence of astaxanthin esters. In a subsequent step, individual mass spectra corresponding to peaks with absorption at 480 nm were produced. Because only mass differences between quasimolecular and fragment ions were used for assignment of acyl chains, LC-MS does not allow for double-bond location. If this information is required, GC represents the method of choice. Alternatively, LC-MSⁿ experiments have to be carried out.

Astaxanthin Esters in *H. pluvialis*. Astaxanthin monoesters, the main derivatives of astaxanthin in *H. pluvialis*, eluted in a

time window between 15 and 25 min, whereas diesters typically showed retention times of 25–35 min (**Figure 3A**). All UV-vis spectra featured one broad maximum at 480–482 nm (DAD, measured in the eluent), which is typical for the astaxanthin skeleton (15). To unequivocally allocate identification based on LC-(APCI)MS, astaxanthin mono- and diesters, presumed to be constituents of the extract, were synthesized independently and added to aliquots of the extract. Cochromatography was consistent with assignment of the respective compounds. Unfortunately, only a few acyl chlorides were available commercially. In particular, fatty acids of the C18 family with more than two double bonds or compounds with chain lengths over 18 carbons were not obtainable. Accordingly, three astaxanthin monoester (**1**, **2**, **7**) and one diester (**8**) were identified only on the basis of their fragmentation pattern. Structural assignment of compounds marked with an asterisk in **Table 1** is consequently not confirmed by an independent method. Interpretation of the mass spectra of the broad peak **9** suggested the presence of a coeluting component, which was presumed to be astaxanthin-C22:0. Synthesis of this ester was not possible, forestalling conclusive identification. However, the main monoesters of *H. pluvialis* unambiguously contained the acyl chains C18:3 (**2**), C18:2 (**3**), C18:1 (**4**), and C16:0 (**5**). Thus, the predominance of oleic acid, stated in the literature (e.g., ref 9), was confirmed by the results of this study.

Astaxanthin Esters in *P. borealis*. **Figure 3B** shows a typical chromatogram of a shrimp extract. In contrast to *H. pluvialis*, astaxanthin diesters clearly formed the main components. Free astaxanthin (**14**) appeared as a minor constituent. Chromatographic resolution in the rear part of the chromatogram was not excellent, but identification of some astaxanthin esters was possible: five astaxanthin monoesters (**4**, **5**, **15–17**) and eight astaxanthin diesters (**11**, **18–24**) were identified. Lack of acyl chlorides impeded cochromatography of three esters comprising C20:1 (**17**, **22**, **24**) or C22:1 (**23**). Coelution with other components was observed for **22** and **23** because signals consistent with the fragmentation pathways of astaxanthin-C14:0/C18:1 and astaxanthin-C14:0/C20:1, respectively, were found. Although it was not possible to calculate the complete astaxanthin ester pattern of *P. borealis*, lauric acid was found

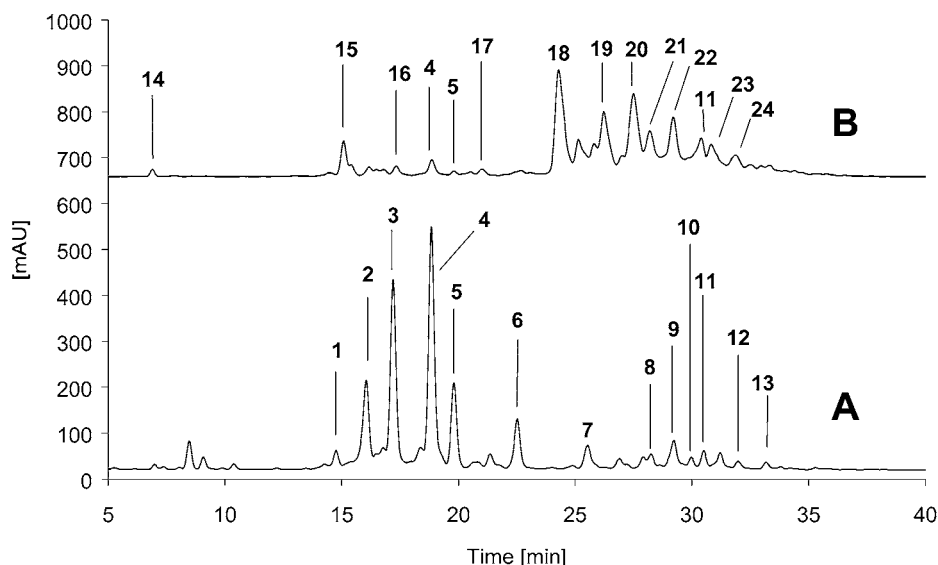


Figure 3. Chromatograms (DAD, 480 nm, extended sections) of an *H. pluvialis* extract (A) and of a shrimp extract (*P. borealis*) (B). For peak assignment see Table 1.

Table 2. Concentrations of Astaxanthin Esters (Micrograms per 100 g of Dry Matter \pm SD; $n = 4$) in *H. pluvialis*^a

compound	sample I	sample II
A-C18:4 (1)	26.8 \pm 2.7	34.2 \pm 0.6
A-C18:3 (2)	179.3 \pm 13.4	76.8 \pm 1.8
A-C18:2 (3)	372.7 \pm 30.6	135.2 \pm 2.8
A-C18:1 (4)	525.2 \pm 37.7	173.0 \pm 5.7
A-C16:0 (5)	182.6 \pm 11.3	139.5 \pm 4.2
A-C18:0 (6)	123.2 \pm 9.5	32.5 \pm 0.8
A-C20:0 (7)	63.9 \pm 5.2	49.7 \pm 1.3
A-C18:1/C18:3 (8)	16.8 \pm 1.0	12.2 \pm 0.3
A-C18:1/C18:2 (9)	87.6 \pm 8.0	47.7 \pm 1.1
A-C16:0/C18:2 (10)	17.8 \pm 2.2	23.0 \pm 0.4
A-C18:1/C18:1 (11)	36.3 \pm 2.6	17.0 \pm 0.3
A-C16:0/C16:0 (12)	17.5 \pm 2.8	16.7 \pm 0.3
A-C18:0/C18:1 (13)	18.6 \pm 1.4	6.5 \pm 0.3

^a A, astaxanthin. Quantitative amounts were calculated on the basis of the respective molecular masses. Numbers of the compounds correspond to Figure 3A.

to be one of the prevalent fatty acids. PUFAs, formerly regarded as typical components in the astaxanthin ester fraction of *P. borealis* (9), were not found as main fatty acids in this study. Thus, the distribution of fatty acids bound to astaxanthin seems not to reflect the native pattern of PUFAs found in the triacylglyceride fraction (20). However, this observation has to be proven in further studies using GC analyses of methylated fatty acids and the method presented here. Discrepancies in the fatty acid pattern of the triacylglyceride fraction on the one hand and the xanthophyll ester fraction on the other hand were already observed by investigating the lutein ester pattern of *Tagetes erecta* (17) and seem to be also valid for *P. borealis*.

Quantification of Astaxanthin Esters. To determine the concentrations of individual astaxanthin esters by HPLC-DAD, free astaxanthin was used to create a calibration curve (mAU \times s vs μ mol/L). Assuming similar extinction coefficients of free and acylated forms, this method allows for quantification of individual esters, considering the respective molecular masses. Quantitative results are presented in Tables 2 (*H. pluvialis*) and 3 (*P. borealis*). In both *H. pluvialis* samples, astaxanthin-C18:1 (4) was the main astaxanthin ester; however, concentrations varied considerably between the two samples (525 vs 173 μ g/100 g). This may be due to unknown growth and/or storage conditions. Quantitative data of *P. borealis* proved the homo-

Table 3. Concentrations of Astaxanthin Esters (Micrograms per 100 g of Wet Matter \pm SD; $n = 4$) in Shrimp (*P. borealis*)^a

compound	sample I	sample II	sample III	sample IV
A (14)	10.0 \pm 0.8	14.1 \pm 1.8	18.0 \pm 2.6	5.4 \pm 0.6
A-C12:0 (15)	48.0 \pm 3.2	53.0 \pm 2.5	59.5 \pm 2.1	44.7 \pm 1.8
A-C14:0 (16)	5.7 \pm 0.9	5.7 \pm 0.3	4.9 \pm 0.3	3.4 \pm 0.1
A-C18:1 (4)	25.7 \pm 1.5	28.3 \pm 1.6	26.2 \pm 0.8	15.4 \pm 0.3
A-C16:0 (5)	5.7 \pm 0.3	6.5 \pm 0.2	9.2 \pm 0.3	5.6 \pm 0.1
A-C20:1 (17)	6.6 \pm 0.5	6.4 \pm 0.2	4.1 \pm 0.4	3.6 \pm 0.2
A-C12:0/C:12:0 (18)	129.1 \pm 4.8	146.2 \pm 7.4	116.2 \pm 3.2	151.1 \pm 3.8
A-C12:0/C14:0 (19)	22.7 \pm 1.5	23.6 \pm 1.7	9.0 \pm 0.4	14.4 \pm 0.7
A-C12:0/C18:1 (20)	92.3 \pm 5.3	100.6 \pm 2.9	81.1 \pm 2.4	77.2 \pm 1.8
A-C12:0/C16:0 (21)	24.2 \pm 1.4	28.6 \pm 0.8	33.2 \pm 1.1	29.5 \pm 0.7
A-C12:0/C20:1 (22)	38.8 \pm 2.0	37.9 \pm 2.7	12.7 \pm 1.1	23.8 \pm 0.8
A-C18:1/C18:1 (11)	17.7 \pm 1.5	23.0 \pm 0.7	24.6 \pm 0.8	17.9 \pm 0.4
A-C12:0/C22:1 (23)	16.8 \pm 1.0	19.8 \pm 1.4	15.5 \pm 0.5	12.6 \pm 0.3
A-C18:1/C20:1 (24)	12.0 \pm 0.8	13.2 \pm 0.4	6.7 \pm 0.5	6.9 \pm 0.3

^a A, astaxanthin. Quantitative amounts were calculated based on the respective molecular mass. Numbers of the compounds correspond to Figure 3B.

geneous diester astaxanthin-C12:0/C12:0 (18) to be the predominant compound, followed by a mixed diester astaxanthin-C12:0/C18:1 (20). Notably, the results of samples II-IV, which contained brine, did not differ from those obtained for sample I, containing no brine. Although preliminary saponification experiments using methanolic potassium hydroxide solution led to the disappearance of the respective peaks in *H. pluvialis* and *P. borealis* extracts, total free astaxanthin was not quantified because special care has to be taken to avoid byproduct formation (21).

Taken together, negative ion LC-(APCI)MS provides a possibility to analyze astaxanthin esters in extracts of *H. pluvialis* and *P. borealis* after SPE cleanup without time-consuming isolation or derivatization steps. Quantification can be performed using astaxanthin as reference material. Application of the presented LC-(APCI)MS technique in common astaxanthin ester analysis will forestall erroneous xanthophyll ester assignment in natural sources in future experiments.

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