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Determination of carotenoids and all-*trans*-retinol in fish eggs by liquid chromatography–electrospray ionization–tandem mass spectrometry

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Abstract

A novel method was developed for the combined determination of carotenoids and retinoids in fish eggs, which incorporates prior analyte isolation using liquid-liquid partitioning to minimize analyte degradation, and fraction analysis using high-performance liquid chromatography–electrospray (positive)–quadrupole mass spectrometry (LC–ESI(+)–MS; SIM or MRM modes). Eggs from Chinook salmon (*Oncorhynchus tshawytscha*) were used as the model fish egg matrix. The methodology was assessed and validated for β-carotene, lutein, zeaxanthin, and β-cryptoxanthin (molecular ion radicals $[M]^{\bullet +}$), canthaxanthin and astaxanthin ($[M+\mathrm{Na}]^+$ adducts) and all-*trans*-retinol ($[(M+\mathrm{H})\text{–}\mathrm{H_2O}]^+$). Using replicate egg samples ($n=5$) spiked with β-cryptoxanthin and β-carotene before and after extraction, matrix-sourced ESI(+) enhancement was observed as evidenced by comparable %matrix effect and %process efficiency values for β-cryptoxanthin and β-carotene of 114–119%. In aquaculture-raised eggs from adult Chinook salmon astaxanthin, all-*trans*-retinol, lutein and canthaxanthin were identified and determined at concentrations of 4.12, 1.06, 0.12 and 0.45 μg/g (egg wet weight), respectively. To our knowledge, this is the first report on a method for LC–MS determination of carotenoids and retinoids in a fish egg matrix, and the first carotenoid-specific determination in any fish egg sample.
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Keywords: LC–electrospray–tandem (quadrupole) MS; Carotenoids; Retinoids; Astaxanthin; Fish eggs; Chinook salmon

1. Introduction

Carotenoids are lipid-soluble pigments and important antioxidants that are synthesized by plant and photosynthetic microorganisms and acquired by animals via the diet [1,2]. More than 600 specific carotenoids have been identified, although a far smaller number are found in blood and other tissues of most animals [3]. Retinol is a metabolite of β-carotene in humans [4] and other mammals, while xanthophylls such as astaxanthin, canthaxanthin and zeaxanthin have been reported to be the primary specific precursors of retinol in fish [5].

High-performance liquid chromatography (HPLC) using reversed-phase C8, C18 and C30 bonded phase columns is the preferred approach for the separation of carotenoids in sample extracts isolated from biological matrices [6–10]. UV–vis detection has been the most common detection [6,7,11–14], however it is not capable of providing molecular structure information for identification, especially for unknown carotenoids in complex sample matrices.

Since 1995, HPLC–mass spectrometric (LC–MS)-based methods incorporating atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) coupled with quadrupole and ion trap mass analyzers [4,9,10,15–21], have been used increasingly for carotenoids and other analyte determinations in samples. LC–MS analysis of mostly carotenoids, and to a lesser extent retinoids, has been reported for matrices such as food items, plant and vegetables, whole blood, plasma, serum, liver and prostrate glands [9,10,15–17,21]. Sample preparation and LC–MS analytical

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and spectroscopic parameters require unique developmental optimization given the varying complexity of the matrices. Although the mechanism is not fully understood, unknown matrix components in the mobile phase and sample (i.e., salts, amines and fatty acids, etc.) may enhance or reduce the MS ionization efficiency of analytes [22,23]. With the exception of the most recent reports, matrix effects on ionization suppression in LC–MS-based bioanalytical methods have generally not been assessed, which can have serious ramifications on quantitative precision, accuracy and reproducibility [9,15,18].

To enhance aquaculture marketability, the flesh pigmentation of salmonid fish is enhanced through synthetic canthaxanthin or naturally sourced astaxanthin feed additives [24]. In most species of fish including salmonids, astaxanthin is the primary natural dietary carotenoid and appears to be preferentially mobilized and transferred in ovo for egg production, which is generally associated with improved rates of fertilization, hatching and survival [25]. Astaxanthin content in the eggs of fish can be an indicator protection against, e.g., oxidative stress [26].

Despite the increasing use of LC–MS approaches for carotenoid identification and determination in samples, to our knowledge there are no reports for methods in the analysis of either fish and poultry eggs [27,28]. There are extremely limited reports of carotenoids in eggs of aquaculture-raised or wild salmonids, however total carotenoid levels have been determined based on single wavelength (maximum 480 nm) analysis with no prior chromatographic separation [29–34]. We presently report on the development and matrix effect assessment of a LC–ESI(+)–tandem (quadrupole) MS-based method for the sample isolation, identification and quantitative determination of carotenoids and retinoids from fish eggs using samples from Chinook salmon (*Oncorhynchus tshawytscha*) raised under aquaculture conditions, as well for commercial chicken eggs. Quantitative LC–ESI(+)–MS and LC–UV–vis analysis of the salmon egg extracts are also compared.

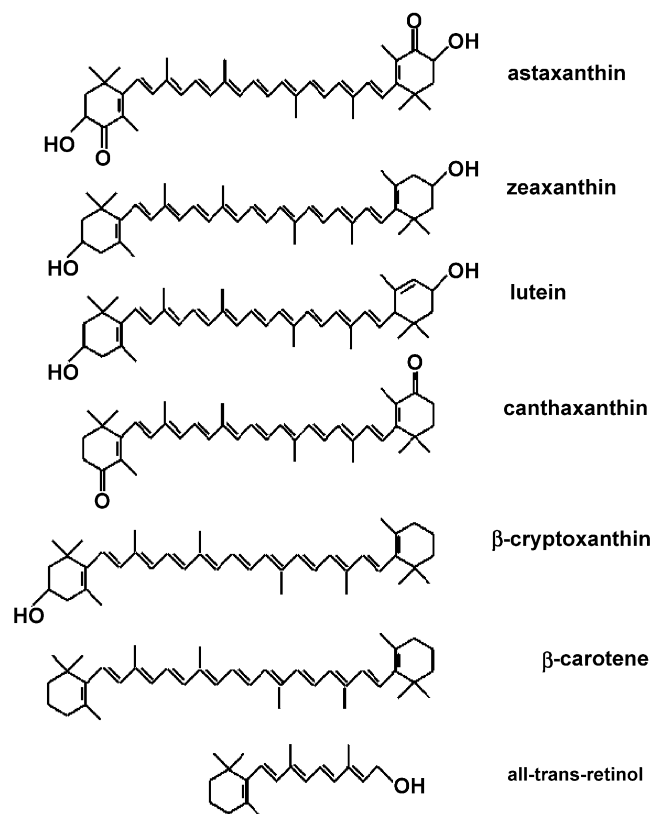

Fig. 1. Chemical structures of all-*trans*-retinol and carotenoids investigated in this study. Hydrogen atoms have been omitted for clarity.

2. Experimental

2.1. Materials, reagents and egg samples

Chemically pure analytical standards of all-*trans*-retinol and astaxanthin were obtained from Alexis Corp. (through Fisher Scientific), and β-carotene, lutein, zeaxanthin, canthaxanthin and β-cryptoxanthin were generously donated by Roche Vitamins Canada Inc. (Fig. 1). HPLC grade methanol, methyl-*tert*-butyl ether (MtBE) and acetone were obtained from Merck (Darmstadt, Germany). All other reagents and solvents were of high analytical grade supplied by VWR Scientific Products (Suwanee, GA, USA). Water was obtained from a Milli-Q (Millipore, San Jose, CA, USA) filtration system equipped with a 0.22 μm filter.

Eggs of Chinook salmon (*Oncorhynchus tshawytscha*) originated from hatchery stock of Yellow Island Aquaculture Ltd. (YIAL) (Quadra Island, BC, Canada) from the 2002 fall spawning season. The grow-out feed for the Chinook adults contained 50 ppm of astaxanthin from the algae *Haematococcus pluvialis*. Collected eggs were frozen at −20 °C and stored at −80 °C until analysis. Commercial chicken eggs were obtained from a local supermarket (Windsor, Ont., Canada).

2.2. Standard solutions

Carotenoids were generally soluble in acetone, acetone/methanol mixtures and MtBE. Stock solutions of 1.0 mg/mL in acetone were prepared for each individual carotenoid and all-*trans*-retinol. A carotenoid standard mixture was prepared by mixing volumes of each of the individual standard stock solutions of astaxanthin, canthaxanthin, β-cryptoxanthin, β-carotene, all-*trans*-retinol, lutein and zeaxanthin, and diluted with acetone so that each compound was 10 ng/μL. The standard stock and mixture solutions were aliquoted into cryo-vials, flushed with nitrogen, sealed and stored at −80 °C and protected from light until further use. Just prior to sample fraction analysis, an aliquot of standard mixture solution was thawed and serially diluted with methanol to provide six calibration standard solutions with analyte concentrations ranging from 52 to 1667 pg/μL. Since

carotenoids are photo-labile and oxidize readily with exposure to air, the stock standard mixture and calibration standard solutions were prepared fresh on the day of analysis, and measures were taken to protect from light exposure, e.g., diffuse daylight and darkened room conditions.

2.3. Sample extraction

The extraction of Chinook salmon eggs and chicken egg yolk homogenate was based existing methodology, which describes the isolation of only carotenoids [29], with major modifications to permit the further matrix separation and co-isolation of carotenoids and retinoids. All extractions were carried out at temperatures at or below ambient, and all extraction solvents were cooled on ice prior to use, to prevent carotenoids/retinoid degradation. Three to four grams of sample were thawed and homogenized with glass mortar and pestle, and extracted with 10 mL of acetone. The mixture was vortexed for 30 s and then centrifuged at 3500 rpm for 5 min at 4 °C. The acetone extraction was repeated two more times. The combined acetone extracts were pooled, and mixed vigorously with an equivolume of MtBE, and 5 mL distilled water to facilitate aqueous/organic phase separation. The aqueous phase was extracted an additional three times with the same MtBE volume. The combined MtBE phases were concentrated by roto-evaporation under reduced pressure at 20 °C until less than a 5 mL volume remained. The extract was transferred into a 15 mL calibrated centrifuge tube (rinsing glassware three times with 1 mL MtBE) and evaporated to dryness under a gentle stream of nitrogen. The residue was resuspended in 0.5 mL MtBE, vortexed and passed through Teflon membrane filter (0.45 μm) to remove particulates, sealed under nitrogen in brown glass vials and stored in the dark at −20 °C.

2.4. HPLC–MS

HPLC–ESI (positive ion)–tandem (quadrupole) MS (LC–ESI(+)–MS) analyses were carried out on a Waters 2695 HPLC equipped with a Finesse Genesis C18 column (150 mm length, 2.1 mm i.d., 4 μm particle size; Jones Chromatography, Hengoed, Mid-Glamorgan, UK), and a Phenomenex C18 guard column (40 mm length, 2.0 mm i.d., 4 μm particle size; Phenomenex, Torrance, CA, USA). The HPLC was coupled to a Micromass Quattromicro^{TM} tandem quadrupole mass spectrometer equipped with a Z-spray ESI interface (Manchester, UK). System control and data acquisition was performed using Masslynx v3.5 software. Shortly after extraction, the extracts were thawed and 10 μL was injected into the HPLC. An isocratic mixture of methanol (90%), MtBE (5%) and water (5%) was maintained from time 0 to 5 min, followed by a linear gradient until 8 min at which time mobile phase composition was methanol (67%), MtBE (30%), and water (3%). From 8 to 9 min, the gradient was changed resulting in a final composition of methanol (57%), MtBE (40%), and water (3%), which was maintained until 20 min. All constituents of the mobile phase contained the pH additive formic acid (0.1%, v/v). The HPLC flow rate was 200 μL/min and the column temperature was maintained at room temperature. The entire effluent volume was directed to the ESI(+)–MS. After each sample run, the HPLC system was flushed with MtBE/methanol/water (40:57:3, v/v/v) for 5 min to remove only strongly retained residues, followed by a 10 min equilibration time with the initial mobile phase before the next injection.

For ESI(+) high purity nitrogen was used as the nebulization and desolvation gas at flow rates of 50 and 300 L/h, respectively. Source and desolvation temperatures were 100 and 300 °C, respectively. The capillary voltage and cone voltage were 4 kV and 30 V, respectively. Optimal ESI(+)–MS in the SIM and full scan (*m/z* 100–650) modes, as well as ESI(+)–MS–MS in the multiple reaction monitoring (MRM) mode, were assessed and compared for quantification of the carotenoids and all-*trans*-retinol. Optimum ESI(+)–MS–MS (MRM) conditions were determined using direct injection of the 1.0 ng/μL solution of the working standard mixture at a flow rate of 10 μL/min. ESI(+)–MS–MS (MRM) was performed using argon as the collision-induced dissociation (CID) gas at a pressure of 3.3×10^{-3} mbar, and the CID energy was optimized for each carotenoid and all-*trans*-retinol at 20 eV.

2.5. HPLC/UV–vis

A Waters 487 Dual-Channel UV–vis detector was coupled in-line to the Waters 2695 HPLC, and the same analytical and guard C18 columns were used for LC–UV–vis as described for LC–ESI(+)–MS analysis. UV–vis absorption spectra were recorded in the wavelengths 480 nm (carotenoid absorption maximum) and 325 nm (retinoid absorption maximum). LC–UV–vis analysis were run separately, and the mobile phase did not contain the pH additive formic acid. Therefore, the LC–UV–vis mobile phase composition was different, i.e., it consisted of two components: (A) methanol and (B) MtBE. The solvent program was as follows: 90% A from 0 to 12 min, followed by linear gradients of 90–60% A from 12 to 13 min, maintained until 22 min, followed by linear gradient back to 90% A initial conditions until end of run at 30 min. The flow rate was 0.8 mL/min.

2.6. Quantification and data analysis

All carotenoids and all-*trans*-retinol in sample extracts were identified and determined by LC–ESI(+)–MS by comparison of the chromatographic retention times and full-scan (*m/z* 100–650) mass spectra with those of the reference standards. For LC–ESI(+)–MS and LC–UV–vis quantification, β-cryptoxanthin was employed as an internal standard as it was not detected in either salmon egg or chicken yolk samples. Calibration curves were generated based on the relative response (RR) of the mass chromatographic peak area/amount ratios of each analyte versus β-cryptoxanthin.

Table 1
Linear regression analysis of the linear dynamic response range for carotenoids and all-*trans*-retinol using the LC–electrospray (+)–MS in three mass spectrometric modes[a]

Compound	SIM	MRM	Full-scan (*m/z* 100–650)
Astaxanthin	$y = 133497x - 78842$, $r^2 = 0.9929$	$y = 16429x - 14759$, $r^2 = 0.9806$	$y = 208352x - 144491$, $r^2 = 0.9881$
All-*trans*-retinol	$y = 394292x - 256314$, $r^2 = 0.9938$	$y = 4222x - 3320$, $r^2 = 0.9971$	$y = 425643x - 449071$, $r^2 = 0.9779$
Lutein	$y = 329671x - 213287$, $r^2 = 0.9933$	$y = 483x - 514$, $r^2 = 0.9725$	$y = 253965x - 214538$, $r^2 = 0.9808$
Canthaxanthin	$y = 190378x - 103487$, $r^2 = 0.9925$	$y = 16436x - 13250$, $r^2 = 0.9711$	$y = 334256x - 382717$, $r^2 = 0.9865$
β-Carotene	$y = 78727x - 21729$, $r^2 = 0.9962$	$y = 409x - 80$, $r^2 = 0.9813$	$y = 278762x - 244839$, $r^2 = 0.9709$

[a] Linear regression analysis of each analyte in serial dilutions [6] from a single standard mixture (520–16,670 pg). Calibration curves were based on the ratio of the mass chromatographic peak area response of the analyte to the internal standard (β-cryptoxanthin) versus amount of each analyte injected.

From the original standard mixture (10 ng/μL), six serial dilutions of 1667 pg/μL down to 52 pg/μL were prepared. The equations and correlation coefficients (r^2) of the linear regression analysis of all carotenoids and all-*trans*-retinol for LC–ESI(+)–MS in the SIM and MRM (MS/MS) and full-scan modes (*m/z* 100–650) are listed in Table 1. For LC–UV–vis analysis, the linear equations and r^2 values for calibration standards of astaxanthin ($y = 26x - 11$, $r^2 = 0.9987$), lutein ($y = 27x - 66$, $r^2 = 0.9989$), canthaxanthin ($y = 14.688x + 27.543$, $r^2 = 0.9993$), β-cryptoxanthin ($y = 13x - 73$, $r^2 = 0.9945$) were also determined.

Using the lowest concentration (52 pg/μL) of the serial dilution of the standard mixture, instrumental limits of quantification (ILOQs, pg/μL (injected) or part-per-billion (w/v)) were calculated. ILOQs for LC–UV–vis and LC–ESI(+) in the MS–MS (MRM), MS (SIM) and MS (full-scan) modes for each analyte were defined as the mass in picograms giving a minimum signal-to-noise (S/N) ratio of 10.

To assess carotenoid recovery efficiency and method limits of quantification (MLOQs, ng/g (egg wet weight) or parts-per-billion (w/w)), using HPLC–ESI(+)–MS (SIM), ground salmon eggs (1 g) were spiked with 0.5 mL of the individual standards, i.e., 1.0 ng/μL of β-cryptoxanthin and β-carotene (Fig. 1), and $n = 5$ replicates were analyzed. Similar to the approach described by Matuszewski et al. [23], to assess matrix effects on ESI(+), the MLOQs were also determined for β-cryptoxanthin and β-carotene standards spiked to samples ($n = 5$) after extraction. Percent matrix effect (%ME) equals the MLOQ (after extraction)/ILOQ, and %process efficiency equals the MLOQ (before extraction)/ILOQ [23]. For and LC–UV–vis the spiking level of β-cryptoxanthin was 0.25 mL of 50 μg/L to $n = 4$ replicates samples of 1 g salmon egg. MLOQs for β-cryptoxanthin and β-carotene were based on the significance of variation using Student's *t*-values according to $SV = SD_{MLOD} \times t_{(n-1,95\%)}$, where $t_{(n-1,95\%)}$ is the *t*-distribution constant for $n - 1$ degrees of freedom. The commonly accepted MLOQ is 3 SV [30]. β-Cryptoxanthin and β-carotene were not detectable in the eggs, and these quantification parameters were considered representative of all carotenoids and all-*trans*-retinol under study. The method based on only LC–ESI(+)–MS (SIM) analysis was further tested by determining the carotenoids in a pseudo-standard reference material, i.e., the yolks of commercial chicken eggs.

3. Results and discussion

3.1. HPLC–ESI(+)–MS–MS parameters

Under optimal ESI(+)–MS conditions, mass spectra obtained for all carotenoids except that of astaxanthin and canthaxanthin were characterized by abundant molecular radical cations $[M]^{\bullet+}$ (Table 2). Abundant $[M]^{\bullet+}$ ions were also reported in the ESI(+) mass spectra of both xanthophylls and carotenes [18], and for β-carotene and some xanthophylls using LC–turboionspray–MS [15]. Also observed for β-carotene was a significant, ESI(+) generated $[M - 92]^+$ (*m/z* 444) ion, which is a typical fragment ion formed by free-radical fragmentation from $[M]^{\bullet+}$ resulting in the loss of toluene [17]. In the case of carotenoids not containing keto-groups (Fig. 1), such as lutein, β-cryptoxanthin and β-carotene, the generation of $[M]^{2+}$ likely occurs via electrochemical oxidation [31]. Electrophoretic charging and field ionization at the metal–liquid interface of the electrospray capillary, where the electrospray interface may be viewed as a electrolytic cell, results in the generation of $[M]^{2+}$ for non-keto-group containing carotenoids.

For astaxanthin and canthaxanthin, which were the only carotenoids studied that contain keto-groups on the end ring systems (Fig. 1), $[M + Na]^+$ adducts were dominant with much less abundant $[M]^{\bullet+}$. Carotenoids such as β-carotene lack keto-groups, and thus protonated ion or sodium adducts of the molecular are not likely to form during ESI, and require solution oxidants post-column to facilitate $[M + H]^+$ or $[M + Na]^+$ formation [18]. Like the present system, with no obvious source of sodium, Careri et al. [15] observed abundant $[M + Na]^+$ adduct ions for astaxanthin

Table 2
Parameters for electrospray ionization (positive)–tandem quadrupole mass spectrometry (ESI(+)–MS/MS) by multiple reaction monitoring (MRM) transitions in the analysis of carotenoids and all-*trans*-retinol

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)
Astaxanthin	$[M + Na]^+$ (619)	$[(M + Na)–C_6H_6CH_2]^+$ (527)
All-*trans*-retinol	$[(M + H)–H_2O]^+$ (269)	$[C_6H_6CH_3]^+$ (93)
Lutein/zeaxanthin	$[M]^{\bullet+}$ (568)	$[M - C_6H_6CH_2]^+$ (476)
Canthaxanthin	$[M + Na]^+$ (587)	$[(M + Na)–C_6H_6CH_2]^+$ (495)
β-Cryptoxanthin	$[M]^{\bullet+}$ (552)	$[M - C_6H_6CH_2]^+$ (460)
β-Carotene	$[M]^{\bullet+}$ (536)	$[M - C_6H_6CH_2]^+$ (444)

and canthaxanthin, but not for lutein, zeaxanthin, β-cryptoxanthin and β-carotene, using LC–turboionspray–MS. However, supernatant extracts can contain residual, matrix-associated Na, K, etc. based salts [22], and thus in our case a matrix source of Na would be a possible explanation for $[M+Na]^+$ formation. The mass spectrum of all-*trans*-retinol (not shown) showed a base peak of *m/z* 269 corresponding to $[M+H–H_2O]^+$, and lesser ions (e.g., *m/z* 93), which is consistent with the APCI(+) mass spectrum reported by Wang et al. [32].

Consistent with ESI(+)–MS mass spectra, and under optimal CID conditions for product (daughter) ion formation, the major ESI(+)–MS–MS (MRM) daughter ions of the carotenoids were $[(M+H)–92]^+$ or $[(M+Na)–92]^+$ (resulting from loss of toluene), and for all-*trans*-retinol was protonated toluene (*m/z* 93) (Table 2) [17].

The influence of polarity on the elution time is exemplified by the centrosymmetric xanthophyll zeaxanthin (Fig. 2D), which elutes much more rapidly than its dehydroxylated analog β-carotene (Fig. 2G). Complete chromatographic resolution of all carotenoids using the present RP-C18 HPLC column was observed (Fig. 2A), except for lutein and zeaxanthin, which are structural isomers (Fig. 1), and other studies have reported co-elutions [6]. However, with C30 phases Dachtler et al. [21] recently reported baseline separation. In this study no further attempt was made to resolve lutein and zeaxanthin since they were relatively unimportant carotenoids in the Chinook salmon eggs.

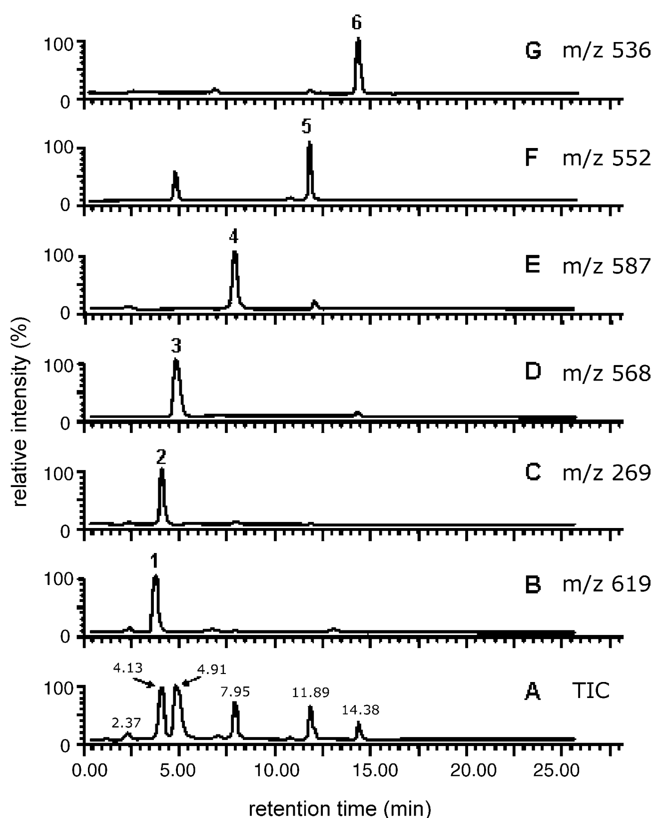

Fig. 2. Total ion (TIC) and extracted ion (EIC) mass chromatograms of carotenoid standards obtained by LC–ESI(+)–MS (SIM): (A) TIC of all precursor ions described in Table 2. EIC of each individual carotenoids in the standard mixture; (B) astaxanthin (peak 1); (C) all-*trans*-retinol (peak 2); (D) lutein + zeaxanthin (peak 3); (E) canthaxanthin (peak 4); (F) β-cryptoxanthin (peak 5); (G) β-carotene (peak 6). For MS and chromatographic conditions see Section 2.

3.2. Quantification parameters

ESI analysis allows for very sensitive (low ppb) analyte quantification, but coeluting interferents in the sample matrix and mobile phase components can decrease sensitivity and precision due to ion suppression [22,23,33]. Ion suppression effects can be minimized in several ways including (1) improved analyte isolation from the sample; (2) improved chromatographic separation from interfering contaminants; (3) reduction of the amount and mobile phase complexity of effluent entering the ESI chamber (leading to increased desolvation, ionization efficiency and ion transfer efficiency); and (4) the use of internal standard correction. In the present study, the LC mobile phase conditions and ESI(+) parameters were optimized for maximum analyte ionization in the LC–ESI(+)–MS (SIM) mode. Wang et al. [32] recently reported that LC–MS using APCI(+) rather than ESI(+) was more sensitive is the determination of retinoic acids and retinol in chemical extracts from small tissues of rat. Regardless, we presently chose to use ESI(+) due to the lower HPLC flow rates required and the small fraction volumes of the egg extracts.

The contribution of co-eluting and ESI suppressing interferents in the present egg extracts was presently minimized by using a comprehensive analyte isolation approach. The present use of formic acid at mobile phase concentrations of ≤0.1% is appropriate, since it has been shown to be a superior pH additive in LC–ESI(+)–MS analysis of a variety on non-carotenoid analytes, relative to optimal concentrations of additives such as trifluoroacetic acid, acetic acid and ammonium hydroxide [22,23,33]. Ion suppression by co-eluting biogenic interferents is suggested to be due to ionization competition, reduction in solvent evaporation in the source or increased surface tension in the droplets [34]. A LC mobile phase fortified with pH additive is often used for analyte separation. In the case of RP–LC, the use of acidic and polar additives facilitates rapid elution and sorbent dislodgement of trapped biogenic molecules, but must be used at appropriate concentration as not to compromise chromatographic efficiency. Further efforts to minimize ion suppression effects were incorporated in the present LC–ESI(+)–MS (SIM) analysis of carotenoids in egg fractions including optimum LC separation, and use of internal standard correction.

For quantitative determinations, the use of MRM transition ions gave only marginally decreased ILOQs for any of the carotenoids or all-*trans*-retinol relative to LC–ESI(+)–MS (SIM) (Table 3). For β-carotene, canthaxanthin, zeaxanthin and lutein, Rentel et al. [19] reported order of magnitude higher LC–ESI(+)–MS–MS (MRM) $([M+Ag]^+$ adducts and $[(M+Ag)–92]^+$ ILOQs (S/N of 4–7). Furthermore, it was

Table 3
Instrumental limits of quantification (ILOQs) for carotenoids and all-*trans*-retinol using optimized LC–ESI(+)–MS (pg/μL (injected)) in three modes[a]

Compound	ESI(+)–MS (SIM)[b]	ESI(+)–MS–MS (MRM)[b]	ESI(+)–MS (full-scan)[c]
Astaxanthin	4.7	5.0	84
All-*trans*-retinol	4.6	7.7	54
Lutein	1.7	7.3	15
Canthaxanthin	4.3	4.7	61
β-Crytoxanthin	4.2	5.6	32
β-Carotene	4.5	6.5	87

[a] See Section 2 for calculation details.
[b] See Table 2 for SIM and MRM ions.
[c] Nominal *m/z* range of 100–650 amu.

also shown that the $[(M+\mathrm{Ag})–92]^+$ ion abundance is only 7% the intensity of $[M]^{\bullet+}$, and thus the use of MRM was not advantageous relative to SIM analysis. In the present study LC–ESI(+)–MS (SIM) (Table 2) was therefore chosen for quantitative determination of the carotenoids and all-*trans*-retinol in sample extracts.

LC–ESI(+)–MS (SIM) MLOQs for spiked salmon eggs, and in comparison to the corresponding ILOQs for β-cryptoxanthin and β-carotene, exemplifies the achievement in minimizing matrix ion suppression effects. The LC–ESI(+)–MS (SIM) MLOQs (S/N = 10) spiked before extraction for β-cryptoxanthin and β-carotene were the low parts-per-billion (pbb) concentrations of 4.2 and 5.3 ng/g (egg wet weight), respectively, which are only marginally higher than the corresponding ILOQs (Table 3). Furthermore, for sample spiked with β-cryptoxanthin and β-carotene after extraction, the MLOQs were 5.0 and 5.2 ng/g (egg wet weight), respectively. Using an ion suppression evaluation approach similar to Matuszewski et al. [23], by comparing the after extraction MLOQs to the ILOQs, the %MEs were 119 and 116% for β-cryptoxanthin and β-carotene, respectively. By comparing the before extraction MLOQs to the ILOQs, the %PEs were 114 and 116% for β-cryptoxanthin and β-carotene, respectively. %ME greater than 100% indicates ionization enhancement due to residual matrix components present after extraction. %PE is a true measure of the recovery value taking into account the observed ESI(+) enhancement effect [23]. The IS-based recovery efficiencies of β-cryptoxanthin and β-carotene were determined to be 95 ± 5%. Accounting for this matrix-sourced ESI(+) enhancement, carotenoid/retinoid %recovery is marginally over estimated, and is more likely around 80%. The LC–UV–vis MLOQ of 8.6 ppb (ng/g (w/w)) for β-cryptoxanthin spiked to salmon eggs was also comparable to the MLOQ (before extraction) for LC–ESI(+)–MS (SIM). This indicates that LC–ESI(+)–MS (SIM) and HPLC–UV–vis are similarly sensitive for carotenoid determination in the fish eggs.

To our knowledge, this is the first extraction methodology to be developed and reported for combined isolation of carotenoids and retinoids from eggs. Craik [29] reported total carotenoid levels based on single maximum wavelength-based determination and direct analysis of the extracted sample from Atlantic salmon eggs, with no prior chromatographic separation. The carotenoids-specific selectivity was therefore non-existent since an absorption maximum of 485 nm was used for quantification, and similar extinction coefficients (2500 L/mol cm) were assumed for all carotenoids. Carotenoid recovery efficiencies were not reported. LC–MS-based quantitative methods for carotenoids and retinoids from various biological matrices have been reported, although no reports exist as yet for the analysis of either fish and poultry eggs [27,28]. For example, Careri et al. [15] selectively quantified astaxanthin, canthaxanthin, lutein, zeaxanthin, β-cryptoxanthin and β-carotene in extracts from microalgae *Spirulina platensis* using LC–turboionspray–MS (SIM). Carotenoids ILOQs were estimated to be in the 0.1–1 ng range as compared to the higher sensitivity of the present method where ILOQs were in the 0.02–0.05 ng range for LC–ESI(+)–MS (SIM) determination (Table 3) using the same SIM ions (Table 2). For retinoid quantification, using similar MS conditions as in the present study, van Breemen et al. [10] reported ILOQs for all-*trans*-retinol using LC–APCI(+)–MS (SIM) (*m/z* 269 amu) of 0.670 pmol (or about 0.2 ng) as compared to an ILOQ of 0.05 ng using the present LC–ESI(+)–MS (SIM) approach (Table 3).

3.3. Carotenoids/retinoids in eggs

As expected from the feeding regime of the parent fish of the present Chinook salmon eggs, astaxanthin was the major, identifiable carotenoid or retinoid, although substantial TIC and EIC responses are also observed for all-*trans*-retinol, lutein and canthaxanthin (Fig. 3). Using LC–ESI(+)–MS (SIM), the concentrations of astaxanthin, all-*trans*-retinol, lutein and canthaxanthin in salmon egg extracts were 4.12, 1.06, 0.12 and 0.45 μg/g (wet weight), respectively. The profile of astaxanthin, all-*trans*-retinol, lutein and canthaxanthin observed in the LC–MS mass chromatogram (Fig. 3) was similar in the LC/UV–vis chromatograms for the same salmon egg extract (Fig. 4). Using the pseudo-SRM of commercial chicken egg yolk, the major carotenoid detected in the extract was lutein, which as measured at a concentration of 2.9 μg/g (fresh yolk weight). Surai and Speake [28] reported lutein concentrations in chicken egg yolk extracts at 13.3 μg/g (fresh yolk weight) using LC–UV–vis determination.

In the LC–ESI(+)–MS (SIM) analysis of the present Chinook egg extract, an unknown retinoid-like compound eluted at 14.07 min as shown in the TIC and EIC (*m/z* 269) mass

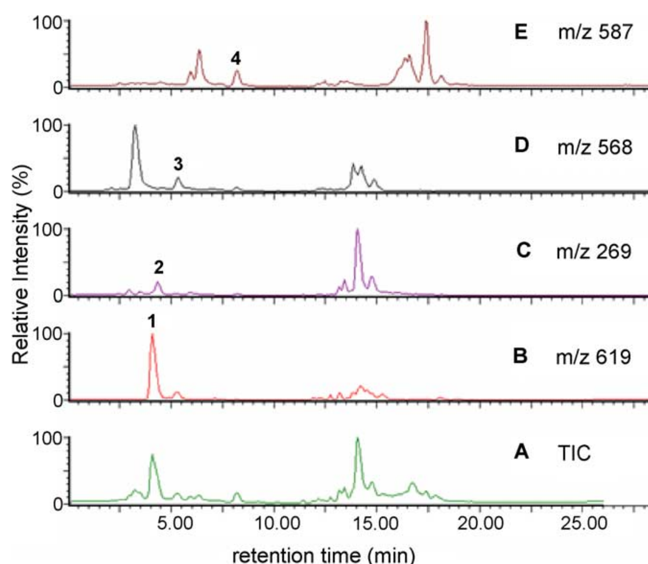

Fig. 3. A total ion chromatogram (TIC; see precursor ions listed in Table 2) and mass chromatograms of individual extracted ions (EICs) for each of the carotenoids and all-*trans*-retinol obtained by LC–ESI(+)–MS (SIM) in egg extracts from Chinook salmon. TIC (A) for astaxanthin, all-*trans*-retinol, lutein + zeaxanthin and canthaxanthin. EIC of each individual carotenoids in the standard mixture; (B) astaxanthin (peak 1); (C) all-*trans*-retinol (peak 2); (D) lutein + zeaxanthin (peak 3); (E) canthaxanthin (peak 4). For MS and chromatographic conditions see Section 2.

chromatograms (Fig. 3A and C). The elution time of this peak was close to that of β-carotene in the standard mixture (Fig. 2G). The retinoid-like peak at 20.07 min in the 325 nm UV–vis chromatogram (Fig. 4D) may be a secondary bioconversion product of all-*trans*-β-carotene, which is known to catalytically transformed to all-*trans*-retinol via 15,15′-carotenoid-dioxygenase [4].

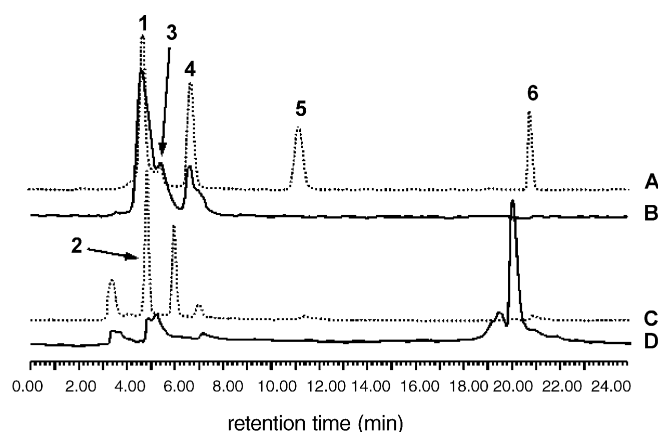

Fig. 4. LC–UV–vis chromatograms of carotenoids and retinoids in egg extracts from Chinook salmon at absorption wavelength maxima of 480 and 325 nm, respectively: (A) standard carotenoid/all-*trans*-retinol mixture at 480 nm; (B) fraction from salmon eggs at 480 nm; (C) standard carotenoid/all-*trans*-retinol mixture measured at 325 nm; (D) fraction from salmon eggs 325 nm and peaks: 1, astaxanthin; 2, all-*trans*-retinol; 3, lutein + xeaxanthin; 4, canthaxanthin; 5, β-cryptoxanthin; 6, β-carotene. Chromatographic conditions are described in Section 2.

4. Conclusions

A method was developed for the chemical extraction and isolation, LC separation and ESI(+)–MS identification and determination of carotenoids and all-*trans*-retinol in the eggs of Chinook salmon. To our knowledge, this is the first carotenoids/retinoid-specific LC–MS-based methodology for the identification and determination in fish or poultry eggs. By incorporation of optimized sample extraction and LC parameters, some matrix-sourced ESI(+) enhancement was observed, rather than ionization suppression, as evidenced by the %ME and %PE for β-cryptoxanthin and β-carotene. LC–ESI(+)–MS (SIM) and LC–UV–vis analysis were found to be comparably sensitive for carotenoid determination. Using LC–ESI(+)–MS (SIM), the major carotenoid identified in aquaculture-raised Chinook salmon eggs was found to be astaxanthin, with much lesser amounts of all-*trans*-retinol, lutein and canthaxanthin.

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