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Electrospray tandem mass spectrometric analysis of zeaxanthin and its oxidation products

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Carotenoids have been implicated in protection of the eye from light-mediated photo-toxicity caused by free radicals. Under conditions of normal oxidative stress the carotenoids serve as protective antioxidants; however, when the oxidative stress exceeds the antioxidant capacity, carotenoids can be oxidized into numerous cleavage products. The determination and identification of oxidized carotenoids in biological samples remains a major challenge due to the small sample size and low stability of these compounds. We investigated the reaction of various zeaxanthin cleavage products with *O*-ethyl hydroxylamine to evaluate their levels in a biological sample. For this, a sensitive and specific electrospray tandem mass spectrometry (ESI-MS/MS) was developed, avoiding the classical lower sensitive and specific HPLC-UV and fluorescence absorption methods. Protonated molecules $[M + H]^+$ of carotenoids upon collision-induced dissociation produced a number of structurally characteristic product ions. A series of complicated clusters of product ions differing in 14 (CH_2)and 26 (C_2H_2)Da was characteristic of the polyene chain of intact carotenoids. All carotenoid ethyl oximes of zeaxanthin cleavage products were characterized by the losses of 60 and 61 Da in their MS/MS spectra. Through the application of the LC/MS/MS method, we identified two oxime derivatives of 3-hydroxy-β-ionone and 3-hydroxy-14′-apocarotenal with protonated molecules at *m/z* 252 and *m/z* 370 respectively, in a human eye sample. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: electrospray ionization; tandem mass spectrometry; oxime; oxidized carotenoids; analysis

INTRODUCTION

Age-related macular degeneration (AMD) and cataract are the leading causes of visual loss in aged populations[1,2]. Studies on AMD and cataract show that physiological changes are due to the formation of reactive oxygen species[3,4]. Several epidemiological studies have indicated that the inverse relationship between micronutrients in plasma and the risk of vision disorders are related to free radical generation[4,5]. There is evidence to indicate that consumption of fruits and vegetables rich in carotenoids lutein and zeaxanthin can be beneficial in delaying and possibly protecting against AMD through their antioxidative effects[6]. Many carotenoids quench singlet oxygen and this action is a function of the number of conjugated double bonds in the molecules. Carotenoids containing increasing number of oxo and hydroxy functional groups show higher antioxidative ability[7,8]. Several products, such as β-ionone and apocarotenals have been identified that are derived from oxidative cleavage of β-carotene[9,10]. The major carotenoids present in human plasma are β-carotene, lycopene, β-cryptoxanthin, lutein, α-carotene, and zeaxanthin (Fig. 1). However, only lutein and zeaxanthin are in the human lens[11,12] and they are also the only carotenoids present in high concentrations in the macula lutea and retina[13,14]. Elevated long-term intake of lutein and zeaxanthin is believed to be associated with a reduced risk to develop lens opacities[15,16].

Previous studies have demonstrated that the carotenoids can be oxidized to form cleavage products by various ways, including free radical reactions[7,9,10,17,18]. Radical treatment of β-carotene can also result in the formation of carbonyls or β-apocarotenals. These cleavage products, being very reactive towards biomolecules[19,20], might be harmful, especially to smokers, when β-carotene is given in high doses[21,22]. It is critical to assess the occurrence of these cleavage products before making conclusions about their beneficial or harmful effects.

Therefore, a sensitive and specific analytical method is needed to analyze the oxidized forms of the carotenoids.

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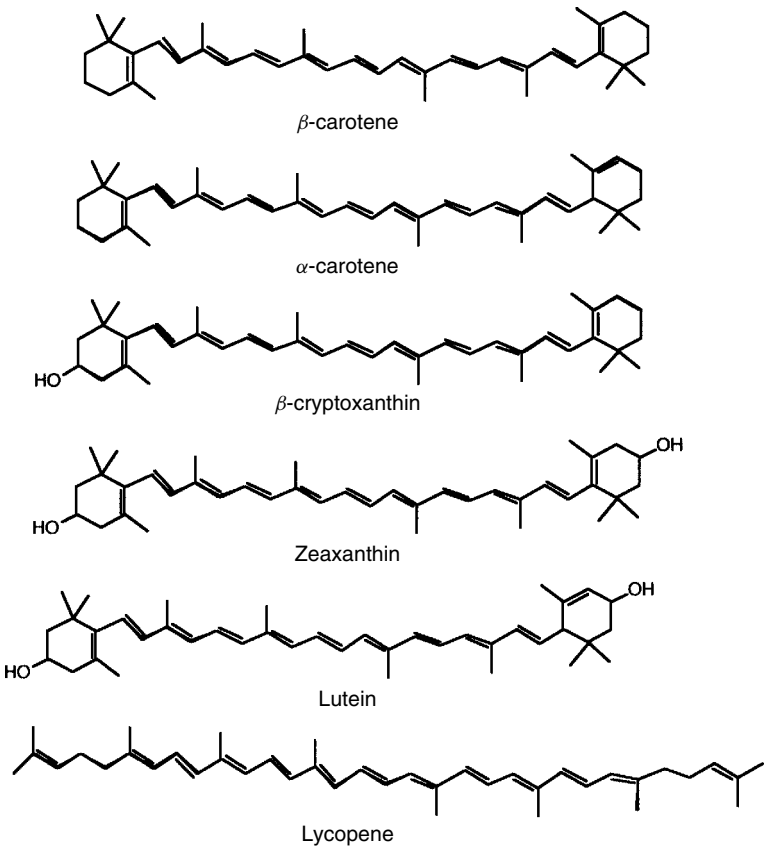

Figure 1. Chemical structures of common carotenoids.

There are reports on separation and identification of carotenoids and their oxidation products in biological samples based on HPLC and HPLC-MS[23,24]. However, these systems are less specific and their analysis is based on comparison of chromatograms of analytes with those of standards. LC-NMR has been utilized to obtain unambiguous identification of carotenoids[24]. However, this method is not applicable to the small sample sizes from a complex biological matrix.

There are several reports on LC-MS and MS/MS using atmospheric chemical ionization (APCI) and electrospray ionization source (ESI) methods for carotenoids analysis[25–30]. Recently, ESI-tandem mass spectrometry has been used to determine carotenoids and retinoids in fish eggs[31]. Despite the increasing use of the LC-MS methods for carotenoid analysis in biological samples, to our knowledge, systematic studies using tandem mass spectrometry of zeaxanthin and its oxidative products in biological samples have not been performed. Tandem mass spectrometric analysis offers added selectivity and specificity and it requires minimal sample clean-up procedure, leading to a high sample throughput. Furthermore, tandem mass spectrometric analysis of carotenoids provides the opportunity for quantitative analysis. In view of this, we studied the fragmentation patterns of the standard carotenoids and their synthesized ethyl oxime derivatives using tandem mass spectrometric method. Using the insight gained from the product ion analysis, we developed a method for detection of oxidized carotenoids in a biological sample. This approach can be extended for quantification of carotenoids and their oxidative metabolites both *in vitro* and *in vivo* studies.

EXPERIMENTAL

Materials

Zeaxanthin was a generous gift of Hoffmann-La Roche, Nutley, NJ. β-ionone and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co, (St Louis, MO). Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$), lutein, b-carotene and *O*-ethyl hydroxylamine were from Fluka, Ronkonkoma, NY. Sodium sulfate (Na_2SO_4), acetonitrile, methanol, hexane, ammonium acetate, and formic acid were of HPLC grade and obtained from Fisher Chemical (Norcross, GA, USA). Ethanol was from Midwest Grain Products Co., Weston, MI. All other chemicals were the best grades available. The concentration of the carotenoid standards was determined from their absorbance at 450 nm using a UV-2101 scanning spectrophotometer (Shimadzu, Columbia, MD) with a 1-cm path length.

Synthesis of zeaxanthin oxidation products and derivatization to oximes

Zeaxanthin (1 mg) dissolved in dichloromethane (1 ml) and methanol (1 ml) was oxidized by the addition of 80 mM NaOCl (in 0.25 ml water) at room temperature for 15 min, as previously described[10]. To the reaction mixture, 5 ml of dichloromethane was added, followed by vortex mixing for 1 min; then 2.5 ml water was added, followed by additional vortex mixing. The samples were centrifuged for 2 min at 1000 g, and the lower organic phase was collected. The extraction was repeated and the organic phases were pooled. The solvents were evaporated under argon, and the extracts were dissolved in methanol.

Ethyl oximes of zeaxanthin cleavage products were synthesized as described by Handelman *et al*.[14] Briefly, zeaxanthin oxidized cleavage products (3-hydroxy apocarotenals and 3-hydroxy apocarotenone derivatives) and β-ionone in methanol were incubated with 0.1 M ethyl hydroxylamine (in 0.1 M PIPES buffer, adjusted to pH 6.7) and incubated overnight at room temperature to ensure complete derivatization[14].

Biological sample preparation

Human donor eyes were obtained from the Montana Eye Bank, Missoula, MT. The preparation of the eyes for storage has been previously described[32]. The eye cups were stored at −85 °C under argon, and the interval between death and storage ranged from 3 to 26 h. For carotenoid and cleavage product analysis, a punch around the macula of the frozen eyes was made using a trephine of 6 mm internal diameter, as previously described[33]. The eyes were thawed for 45 min at room temperature, and the tissues were removed and homogenized in the presence of 0.1 M ethyl hydroxylamine as previously described[32–34].

Following a 12-h incubation, 2 ml hexane was added to each sample. This mixture was then vortexed for 2 min and centrifuged at 1800 g for 2 min. The upper layers containing the carotenoids and the ethyl oximes of the cleavage products were collected and passed through Pasteur pipettes containing ~150 μg anhydrous sodium sulfate to remove traces of moisture. The extracted carotenoids and ethyl oximes in hexane are stable for more than 24 h at −20 °C. The hexane phase was evaporated with argon, and the extract was reconstituted in 30 μl mobile phase of which 20 μl were injected onto the HPLC column or onto the LC/MS/MS apparatus.

HPLC separation of zeaxanthin and its cleavage products

The HPLC system comprised a System Gold 125 Solvent Module (Beckman Instruments Inc., Palo Alto, CA), a Beckman 168 Diode Array Detector, and a 7725i Injector valve fitted with a 20-μl loop (Rheodyne Inc., Cotati, CA). Channel A of the detector was set at 450 nm to monitor intact zeaxanthin and channel B was set at 300 nm to detect cleavage products. A 5 μm C_{18} protein and peptide HPLC analytical column, 250 × 4.6 mm (VYDAC, 218TP54, Hesperia, CA), protected by a C_{18} guard column (40 × 4.6 mm) was used as described[35]. The mobile phase consisted of 100% acetonitrile/methanol, 85 : 15, v/v, with 0.01% ammonium acetate, (w/v). The initial flow rate was 0.55 ml/min. At 10 min, the flow rate was increased to 1.2 ml/min over a 5-min ramp. At 23 min, the flow was reverted to the initial rate over 1 min, and the system was re-equilibrated for an additional minute giving a total analysis time of 25 min.

Mass spectrometry

LC/MS/MS analyses were performed using a system consisting of a model SIL-HT refrigerated Shimadzu autosampler (Shimadzu Scientific Instruments, Inc. Columbia, MD) and an API-III triple quadrupole mass spectrometer (PE Sciex, Concord, Canada). Chromatography was carried out on a 100 × 2.1 mm i.d. Waters X-Terra C_{18} column with a 5 × 2.1 mm i.d. guard column. A gradient consisting of solvent A: 85% acetonitrile, 15% methanol and 0.1% formic acid; solvent B: water and 0.1% formic acid, 0–7 min 5% B; 8–15 min 0% B was used. Multiple reaction monitoring (MRM) was used to perform mass spectrometric identification of oxidized carotenoids. The MRM procedure involves the monitoring of a unique product ion from a selected molecular ion that can be monitored and quantified even in the midst of a very complicated biological matrix. The column effluent was introduced into the mass spectrometer using an electrospray ionization interface in the positive mode. The voltage on the ionspray interface was 4900 V and the orifice potentials were set at 60 V (zeaxanthin) and 40 V (β-ionone and oxime derivatives). Selected $[M + H]^+$ or $[M]^{\bullet+}$ were analyzed by collision-induced dissociation with 90% argon–10% nitrogen gas, and product ion spectra were recorded.

RESULTS AND DISCUSSION

We first developed mass spectrometric methods for analyzing zeaxanthin (**1**), β-ionone (**2**) and β-ionone ethyl oxime (**3**). Subsequently, a reaction mixture containing cleavage products derived from oxidation of zeaxanthin was derivatized and the resulting *O*-ethyl oxime derivatives of 3-hydroxy apocarotenals (**5,7** and **8**) and 3-hydroxy apocarotenones **(4** and **6)** were analyzed by tandem mass spectrometric method and their structures are as shown in Fig. 2. The oxidative cleavage products of zeaxanthin bearing reactive carbonyl group(s) and their presence in a biological sample were unequivocally identified by tandem mass spectrometry. This was based on the evidence that the products gave rise to their *O*-ethyl oximes by treatment with ethyl hydroxylamine, characteristic of oximes derivatives of oxidized carotenoids prepared chemically (**3**–**8**).

HPLC detection of oxidized zeaxanthin

When zeaxanthin was completely oxidized with NaOCl, several oxidation products were detected by HPLC with diode array detection (Fig. 3A). The spectra of the peaks eluting between 7 and 10 min were analogous to those known for 3-hydroxy apocarotenone and 3 hydroxy apocarotenals[36]. These oxidized products were further reacted with ethyl hydroxylamine to produce ethyl oximes. Figure 3B shows that the conversion of β-ionone to β-ionone ethyl oxime on reacting with ethyl hydroxylamine was almost 100%.

MS and tandem MS of zeaxanthin (1), β-ionone (2) and β-ionone ethyl oxime (3)

Carotenoids were analyzed with both positive and negative ion modes. Zeaxanthin showed a protonated molecule $[M + H]^+$ at m/z 569 as well as radical cation $[M]^{\bullet+}$ at m/z 568 in the positive ion mode. In the negative ion mode, its deprotonated molecule was very weak. Therefore, the positive ion mode was utilized for carotenoid

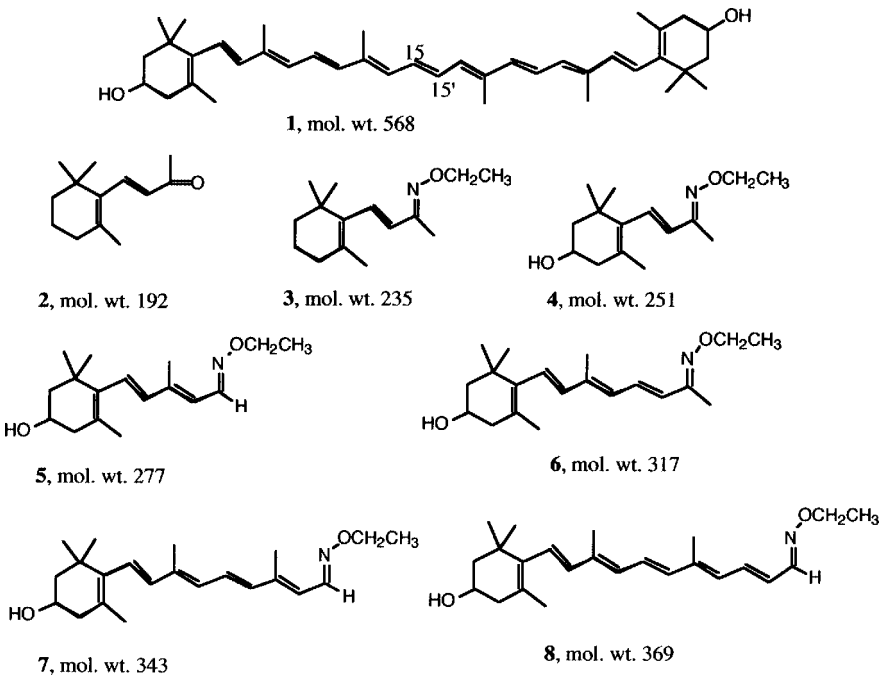

Figure 2. Zeaxanthin (**1**), β-ionone (**2**) and proposed structures of their oxime derivatives (**3**–**8**).

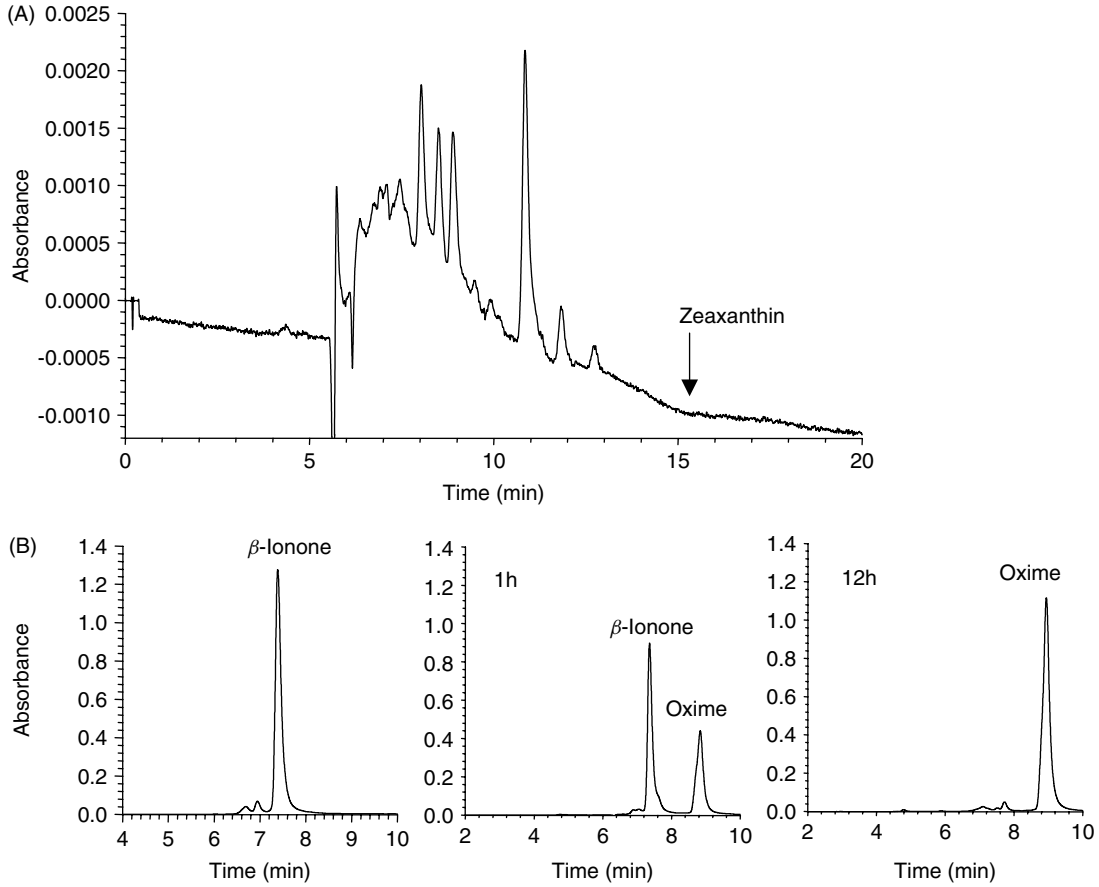

Figure 3. HPLC chromatogram of (A) the oxidized zeaxanthin cleavage products obtained from the reaction of zeaxanthin with NaOCl and (B) β-ionone and its conversion to ethyl oxime derivative. Conditions were as described in the 'Experimental' section.

analysis. We also compared the mass spectrometric sensitivity of carotenoids with ammonium acetate and formic acid. Electrospray ionization source mass spectrometry (ESI-MS) analysis of zeaxanthin in 85% acetonitrile, 15% methanol containing 0.1% formic acid provided better sensitivity.

In a MS/MS experiment, the $[M]^{\bullet+}$ at m/z 568 produced a cluster of ions at m/z 474, 475 and 476 along with a series of

product ions in the mass range *m/z* 100–300. The generation of the ion at *m/z* 476 has also been reported[31]. A prominent product ion at *m/z* 175 probably originated due to the loss of a water molecule (18 Da) and subsequent fragmentation at C_9–C_{10} bond of the protonated zeaxanthin.

The protonated *β*-ionone at *m/z* 193 showed a product ion at *m/z* 151 most likely due to the neutral loss of ketene (CH_2=C=O, 42 Da), consistent with the presence of CH_3CO- group in the molecule. A series of product ions (*m/z* 135, 109 base peak, 95, 69 and 43) were observed differing by 26 (CH=CH) or 14 (CH_2) units, representing conjugated hydrocarbon skeleton (Fig. 4A). The ethyl oxime derivative of *β*-ionone at *m/z* 236 produced prominent product ions at *m/z* 176 and 175 with almost equal intensity together with several cluster ions (Fig. 4B). The ions at *m/z* 175 can be rationalized due to the neutral loss of 61 Da ($NH_2OCH_2CH_3$), whereas the *m/z* 176 ion may result from the loss of the methyl group and the ethoxy group (−60 Da). Figure 5 shows the fragmentation mechanism and possible structures of the major product ions observed in the MS/MS experiment of the ion at *m/z* 236.

Identification of ethyl oxime derivatives of oxidation products of zeaxanthin

The reaction of 3-hydroxy apocarotenals and 3-hydroxy apocarotenone with *O*-ethyl hydroxylamine hydrochloride led to the formation of their ethyl oxime derivatives that were separated by reverse-phase HPLC. UV absorption spectra indicated that the oximes were formed. These fractions were collected and further analyzed by ESI-MS and MS/MS. Ions observed at *m/z*, 252, 279, 318, 344 and 370 in a full scan ESI-MS analysis corresponded to various oxime derivatives. They were further characterized in MS/MS experiments.

The ions in the product ion spectrum of the ion at *m/z* 252 showed similarity with those of the ion at *m/z* 236 (Fig. 4C). Because of the similarity in product ions with higher mass units by 18 Da compared to *m/z* 236, the structure of the ion *m/z* 252 is suggested as shown in Fig. 2. The ion at *m/z* 278 (higher homolog of **4**) also shared the common feature of 60 and 61 losses, but it produced a base peak at *m/z* 94 which may be due to $C_6H_8N^+$. Similarly, the ion at *m/z* 318, 344 and 370 (**6**–**8**) generated a series of ions due to similar losses as described in the cases of **3**–**5** (Fig. 6). On the basis of these pieces of evidence, structures of these compounds (**3**–**8**) were proposed as shown in Fig. 2.

The MS/MS conditions were adjusted to maximize the response of each of the precursor-product ion combination. By increasing the orifice voltage, the sensitivity of product ions of protonated zeaxanthin in the lower mass range (*m/z* 100–300) appeared to be enhanced. The optimal collision energy and orifice voltage were 17 eV and 60 V, respectively for zeaxanthin. For *β*-ionone and zeaxanthin oximes, a collision energy (17 eV) and orifice voltage (40 V) provided the best sensitivity.

Detection of oximes products in biological samples

Detection of oxidized zeaxanthin cleavage products in biological samples is a major challenge due to their very low stability. Using insights gained from product ion analyses of carotenoids and their oximes, we developed an LC/MS/MS method to detect oximes of zeaxanthin cleavage products (**4**–**8**) using precursor/product ion transitions *m/z* 252/152, 278/94, 318/258, 344/107, and 370/164 in MRM scans (Table 1). The LC system was adjusted to maximize the response of each of the precursor/product ion combinations. This method was further utilized to detect zeaxanthin oximes in a biological sample. For this, as described in the 'Experimental' section, ethyl hydroxylamine hydrochloride was added to the sample to convert biologically produced oxidized carotenoids to their oxime derivatives. MRM chromatograms of ethyl oxime products in a human

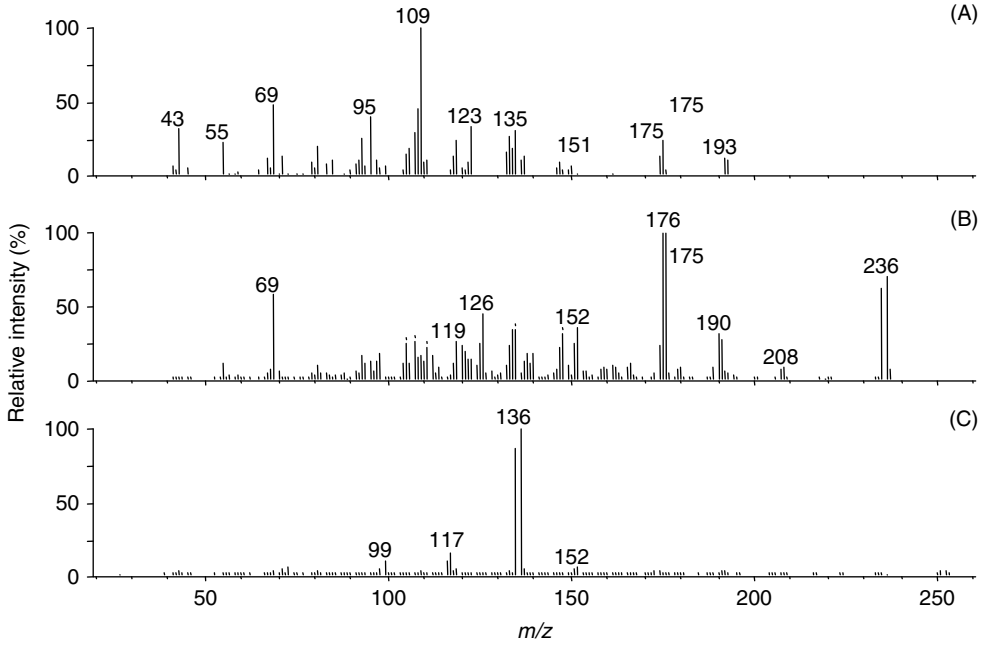

Figure 4. Product ion spectra of the protonated molecule of *β*-ionone at *m/z* 193 (A), *β*-ionone ethyl oxime at *m/z* 236 (B) and 3-hydroxy-*β*-ionone ethyl oxime at *m/z* 252 (C).

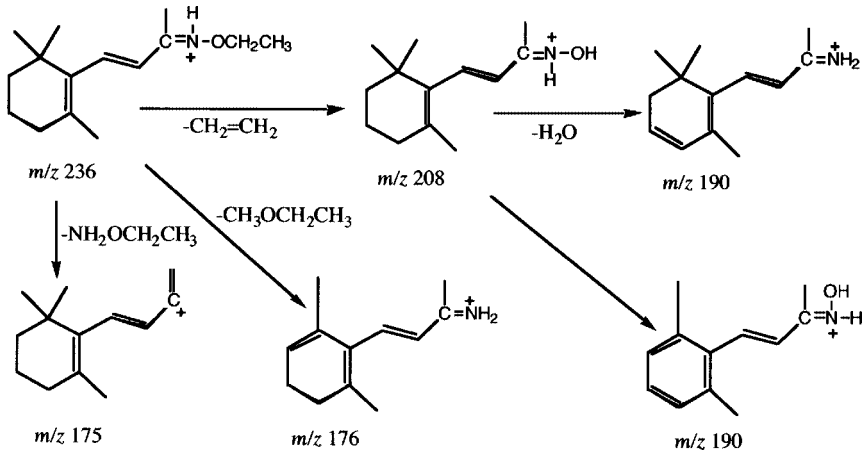

Figure 5. Proposed fragmentation mechanism and possible structures of product ions observed in the MS/MS experiment the ion at *m/z* 236.

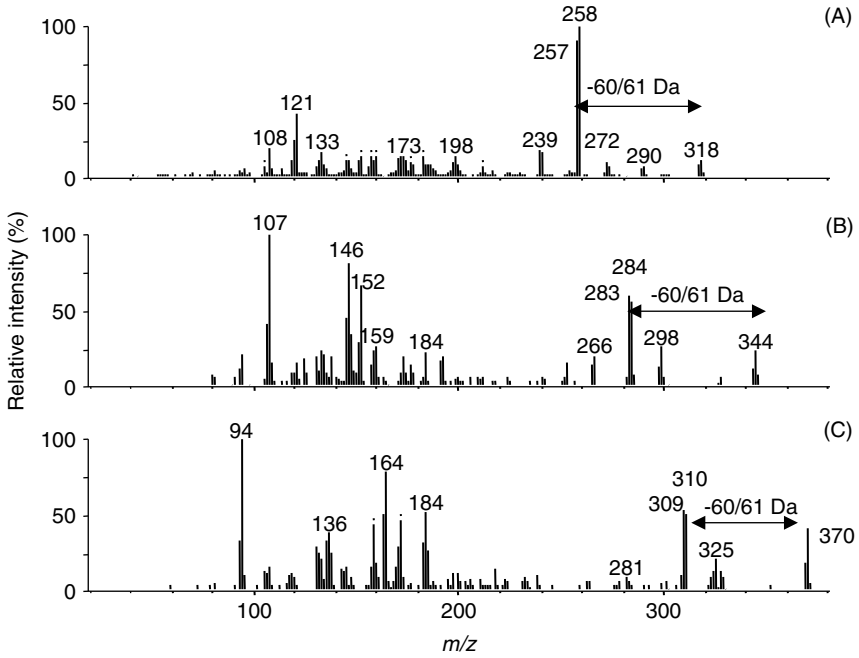

Figure 6. Product ion spectra of the ions at *m/z* 318 (A), 344 (B) and 370 (C).

Table 1. MS/MS transitions used to detect oxime derivatives of oxidized zeaxanthin cleavage products in MRM experiments

Ethyl oximes of oxidized zeaxanthin product	Transitions *m/z*
3-hydroxy-β-ionone	252/152
3-hydroxy-β-ionylidene-acetaldehyde	278/94
3-hydroxy-β-apo-13-carotenone	318/258
3-hydroxy-retinaldehyde	344/107
3-hydroxy-14′-apocarotenal	370/164
3-hydroxy-12′-apocarotenal	410/350

macular retina sample showing the presence of 3-hydroxy-β-ionone (*m/z* 252/152) and 3-hydroxy-14′-apocarotenal (*m/z* 370/164) are shown in Fig. 7. Thus by the application of this method, we were able to identify two oxidized carotenoid cleavage products in the human eye. It is well known that both lutein and zeaxanthin are present in the human retina[13,14], and as shown by the structures in Fig. 1, it is evident that the oxidation products we detected as shown in Fig. 7 can be derived from either zeaxanthin or lutein. Further studies will include detection of these products in healthy and AMD retinas and also in the retinal pigment epithelium.

CONCLUSION

Oximes of zeaxanthin and β-ionone were prepared and analyzed by ESI-MS/MS methods to obtain information on characteristic product ions. This enabled development of an LC/MS/MS method using MRM for detection of carotenoid oximes in a human eye sample. These data will be invaluable in the characterization and quantification of carotenoids and their oxidized metabolites in *in vitro* and *in vivo* studies.

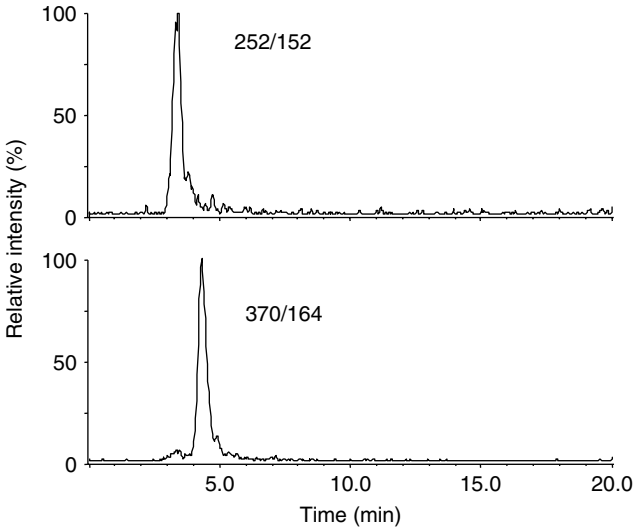

Figure 7. MRM chromatograms of the reaction product of oxidized zeaxanthin and lutein cleavage products and ethyl hydroxylamine hydrochloride in a human eye sample.

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