

# INNOVATIONS IN CAROTENOID ANALYSIS USING LC/MS



**C**arotenoids are primarily C<sub>40</sub> polyisoprenoid compounds that have an extensive conjugated double-bond system and strongly absorb UV and/or visible light. Many carotenoids exhibit brilliant colors and are responsible for the red of tomatoes (lycopene), the yellow of sweet corn (lutein and zeaxanthin), and the orange of carrots ( $\alpha$ -carotene and  $\beta$ -carotene) and sweet potatoes ( $\beta$ -carotene). Carotenoids are also assimilated in the plumage of birds and the exoskeletons of crustaceans; the red carotenoid, astaxanthin, is responsible for the pink color of flamingos and salmon, as well as for the red color of boiled crab and lobster.

Carotenoids are synthesized in photosynthetic microorganisms and plants but not in animals, which must consume other organisms to obtain these substances.

*LC/MS can be used to determine thermally labile carotenoids such as lutein and  $\beta$ -carotene*

More than 600 carotenoids have been identified, and about 50 of these are metabolically converted to vitamin A, which is essential for vision, cellular differentiation, and embryological development. In the absence of adequate preformed vitamin A in the diet, carotenoids serve as a significant source of the vitamin.

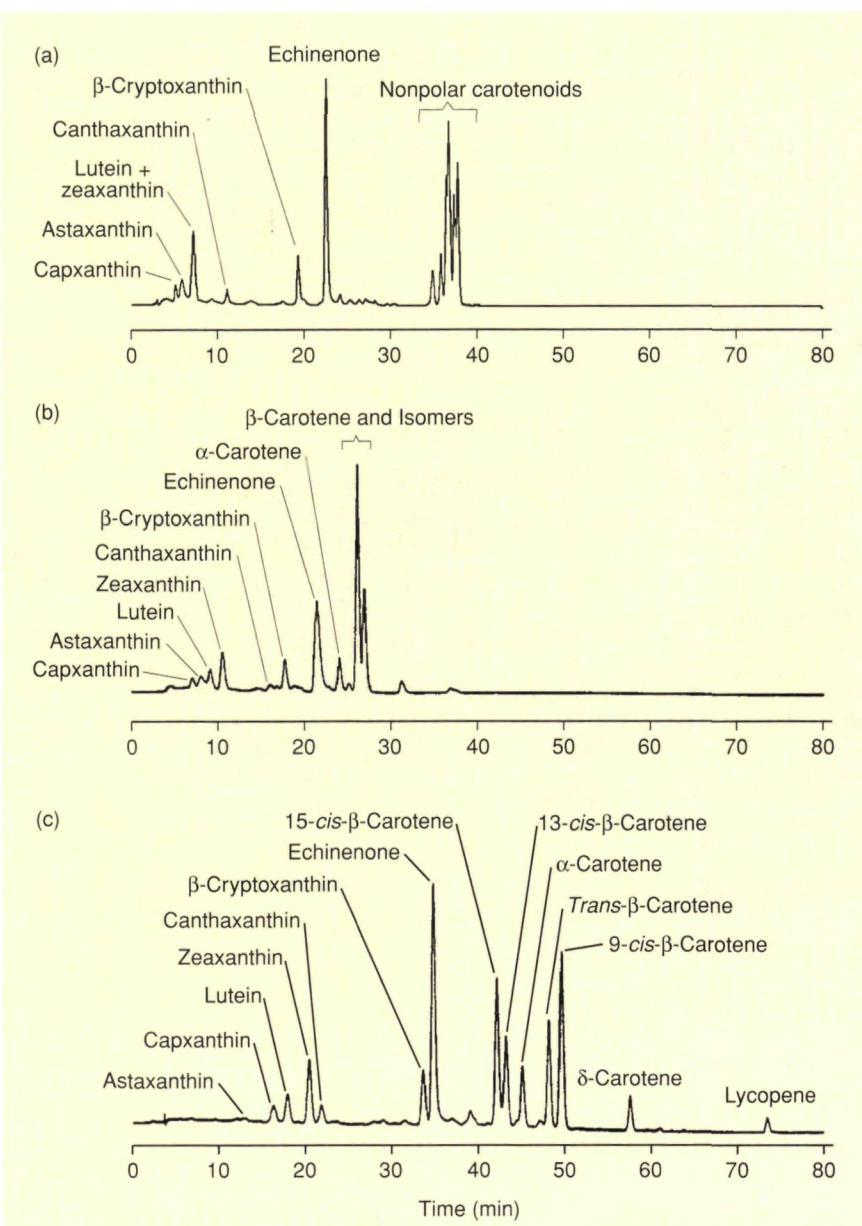
In addition to conferring strong absorbance in the visible and UV regions, the carotenoid system of conjugated double bonds (see box on p. 301 A) enables these com-

pounds to quench singlet oxygen and free radicals. The antioxidant properties of carotenoids protect plants and microorganisms from sunlight damage and are thought to be responsible, in part, for human health-protecting characteristics such as an enhanced immune response, inhibition of carcinogenesis, and lowered incidence of cardiovascular disease. The accumulating evidence of an association between disease prevention and carotenoids in the diet has stimulated a great deal of scientific interest in these substances.

Many traditional methods of separating and identifying olefins have proven inadequate for the analysis of carotenoids contained in biological tissues because of their structural similarity and thermolability and the presence of interfering compounds. The wide range of carotenoid concentrations in various samples requires a highly sensitive and specific detection system. GC/MS, the technique most often used for the determination of nonpolar an-

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**Figure 1. Chromatographic resolution of carotenoids using reversed-phase LC columns.**

(a) Monomeric C<sub>18</sub>, (b) polymeric C<sub>18</sub>, and (c) C<sub>30</sub> stationary phases. (Adapted from Reference 1.)

alytes, is unsuitable for the determination of carotenoids because many of these compounds undergo extensive isomerization and degradation at the elevated temperatures ( $> 200^{\circ}\text{C}$ ) required to volatize them. In this Report the various LC-based methods used to determine carotenoids will be presented.

#### LC with UV-vis detection

Standard carotenoid separations by LC have been performed using hydrophobic interaction or reversed-phase LC. Resolution of the analyte mixture is based on differential

interaction between the hydrophobic carotenoid molecule and the C<sub>18</sub> alkyl chain of the stationary phase.

Although isocratic mobile phases can be used, gradients shorten the analysis time for hydrophobic compounds that interact strongly with the stationary phase and are preferred when a sample contains a diverse collection of carotenoids. Under reversed-phase conditions, polar carotenoids such as xanthophylls elute first, followed by increasingly nonpolar carotenes. The recent introduction of a C<sub>30</sub> stationary phase (1) that provides sig-

nificantly more interaction between analyte and solid phases than the C<sub>18</sub> stationary phase has enhanced chromatographic resolution of carotenoids containing even the most subtle structural differences (Figure 1).

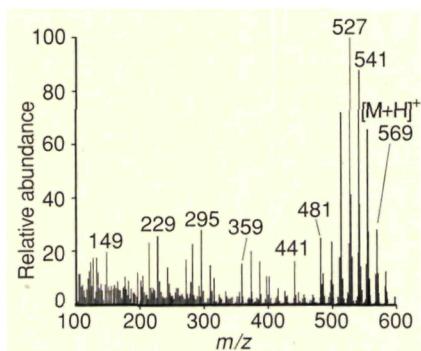
Coupling the photodiode array UV-vis spectrometer with LC allows continuous collection of spectrophotometric data during analysis. However, the complexity of the mixtures in which these carotenoids are found and their structural similarity limit the ability to make unequivocal identifications based solely on chromatographic retention and UV spectrometric data. For this reason, much effort has been devoted to developing an LC/MS interface that could add the specificity of mass spectrometric detection to the LC/UV-vis determination of carotenoids.

#### Evolution of the LC/MS interface

An LC/MS method suitable for carotenoids must have the ability to selectively remove large quantities of mobile phase while simultaneously allowing carotenoid molecules (or ions) into the mass spectrometer (sample enrichment). It must also avoid high temperatures or energetic ionization mechanisms that rearrange or degrade carotenoids. The ionization process must be capable of generating molecular ions from both polar xanthophylls and hydrocarbon carotenes that lack polarizing functional groups.

**Moving belt LC/MS.** Among the first LC/MS techniques used to analyze carotenoids from plant extracts was the moving belt interface with electron impact (EI) or chemical ionization (CI) (2). Solute eluting from the LC column is deposited on a belt that then passes through a series of evacuated desolvation chambers containing IR heaters to accelerate solvent evaporation. The belt then moves into the ion source of the mass spectrometer, where the deposited solid analyte is flash evaporated by rapid heating prior to ionization using EI or CI.

Limitations to carotenoid analysis by use of moving belt LC/MS include loss of chromatographic resolution, pyrolysis, and incomplete volatilization from the moving belt during flash evaporation. After volatilization, additional pyrolysis and extensive fragmentation occur during EI



**Figure 2. Positive-ion CI mass spectrum of lutein.**

or CI in a heated (typically 200–300 °C) ion source. As a result, molecular ions are in low abundance or are not detected, and determination of molecular weight (a critical parameter in the identification of carotenoids) may not be possible. Instead, abundant fragment ions and ions of pyrolysis products dominate the mass spectrum (Figure 2).

**Particle beam LC/MS.** To simplify the maintenance and improve the reliability of LC/MS, moving parts such as those used in the moving belt interface have been eliminated from all subsequent types of interfaces. In the particle beam interface, the LC effluent is nebulized and sprayed into a near-atmospheric pressure desolvation chamber. As the solvent evaporates, the aerosol forms a beam of sample particles or aggregates, which are separated from the lower molecular weight solvent molecules in a momentum separator similar to that used for removal of carrier gas during GC/MS. The sample particles enter the ion source of the mass spectrometer, where they strike a heated plate and disintegrate into gas-phase molecules. Freed of solvent, the sample molecules can be ionized by use of conventional EI or CI techniques (3).

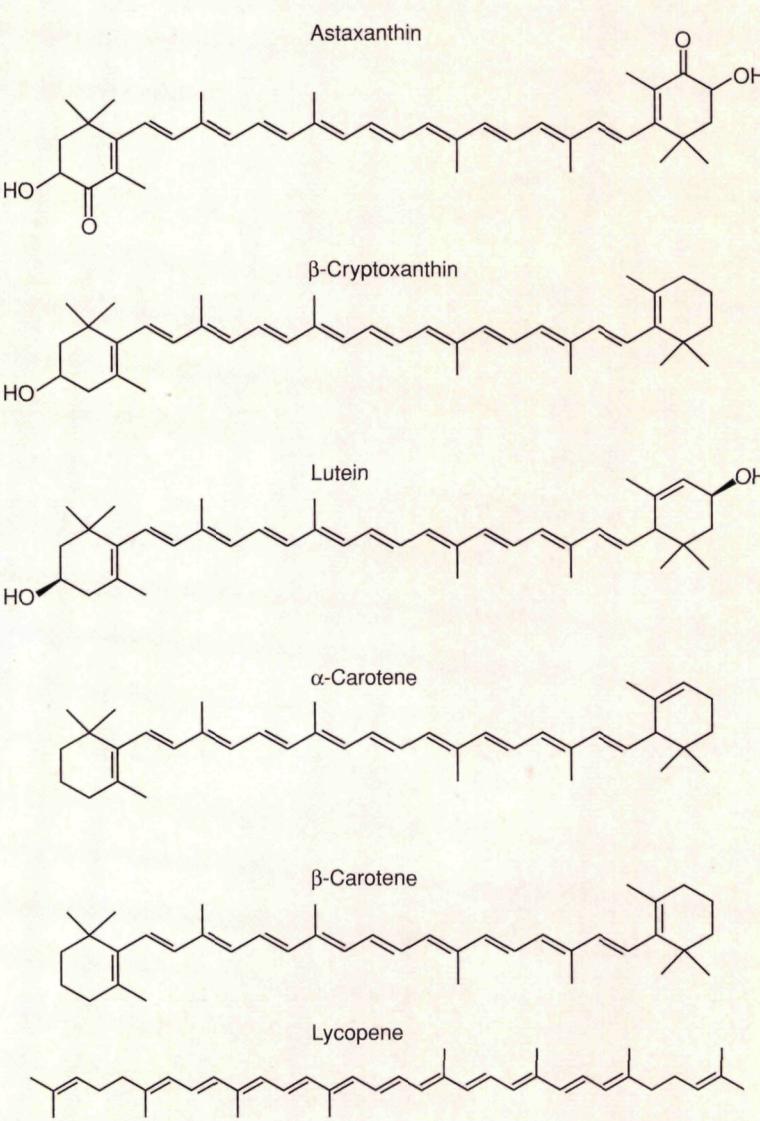
Khachik and colleagues (4) have applied particle beam LC/MS to the analysis of carotenoids extracted from human plasma. To minimize carotenoid fragmentation, enhance the detection of molecular ions, increase selectivity, and improve the level of detection, electron capture negative-ion chemical ionization (NCI) was used instead of EI or positive-ion CI. As a result, abundant molecular ions,  $M^{+}$ , and some fragmentation ions (usually corresponding to elimination of water from

the molecular ion) were reported for polar, oxidized carotenoids, including lutein and zeaxanthin. However, no NCI LC/MS data have been reported for carotenes, perhaps because the absence of heteroatoms in molecules such as lycopene and  $\alpha$ -carotene reduces their electron affinity compared with xanthophylls.

**CF-FAB/LC/MS.** In continuous-flow fast atom bombardment (CF-FAB), a liquid matrix of low volatility is added to the LC effluent by either coaxial-flow or post-column addition. Once exposed to the low pressure of the ion source, the mobile phase rapidly evaporates, leaving behind a thin film of matrix-containing analyte,

which is then bombarded by energetic fast atoms or ions (typically 3000- to 10,000-V xenon or argon atoms or 10,000- to 20,000-V cesium ions). During FAB, the matrix facilitates the transfer of sufficient energy from the fast atoms (or ions) to the analyte to overcome solute–matrix interactions and effect desorption of analyte ions into the gas phase (5, 6).

For carotenoid analysis using CF-FAB, the most effective liquid matrix is 3-nitrobenzyl alcohol (7), which ideally is mixed with the mobile phase in the ion source by using coaxial-flow addition (8). The 3-nitrobenzyl alcohol facilitates the formation of molecular ions  $M^{+}$  for both po-



**Typical carotenoid structures, including xanthophylls (oxygenated carotenoids) and carotenes (hydrocarbons).**

lar xanthophylls and nonpolar carotenes. The appearance of radical cations instead of even-electron protonated molecules is

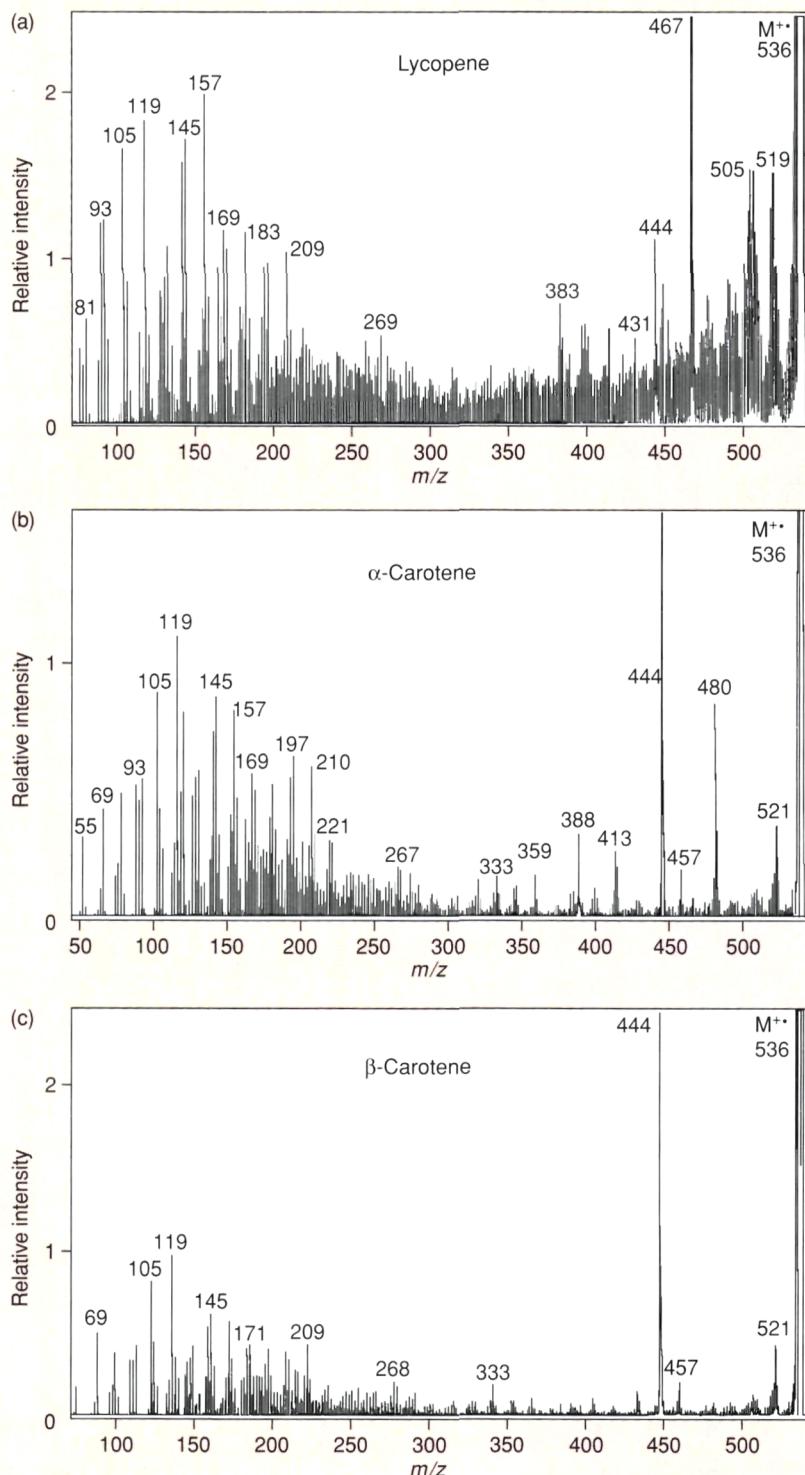
unusual for FAB ionization (especially in the case of oxygenated xanthophylls such as lutein) and probably results from the

low ionization potential of the conjugated bonding system of carotenoids, which facilitates electron transfer to the matrix. Although radical cations are also formed during EI, CF-FAB mass spectra of carotenoids show essentially no fragmentation, and therefore unambiguous molecular weights can be determined.

In the absence of fragmentation, all the carotenoid ion current is concentrated in the molecular ion during CF-FAB LC/MS, which results in a limit of detection that is comparable to UV-vis photodiode array LC detection. For example, the on-column detection limits for lutein and  $\alpha$ -carotene during CF-FAB LC/MS are  $\sim 5$  ng (8.8 pmol) and 15 ng (28 pmol), respectively (8). Despite high sensitivity and unambiguous molecular weight confirmation for both xanthophylls and carotenes, CF-FAB LC/MS is limited by low sample throughput because the 3-nitrobenzyl alcohol matrix fouls the CF-FAB probe, thereby gradually reducing sensitivity. As a result, the FAB probe must be removed and cleaned after  $\sim 3$  h of use. In addition, mobile-phase flow rates are limited to  $< 10 \mu\text{L}/\text{min}$  into the CF-FAB ion source, so that either capillary columns must be used or the column effluent must be split prior to entering the mass spectrometer.

**LC/MS/MS and MS/MS.** Ionization techniques that produce primarily molecular ion species (such as FAB) are ideally suited for coupling with tandem MS. After molecular ions are identified during initial LC/MS analysis, LC/MS/MS may be used to obtain structural information about the analytes. A specific precursor ion (i.e., a carotenoid molecular ion) is selected by one mass spectrometer or sector and fragmented using collision-induced dissociation (CID). The CID product ions are then separated by a second mass spectrometer or sector and recorded as a tandem mass spectrum.

We recently investigated FAB MS/MS analysis of 17 different carotenoids and determined the fragmentation patterns that can be used for structural determination (9). Following CID, MS/MS of carotenoids showed structural features indicative of the presence of hydroxyl groups, ring systems, ester groups, and aldehyde groups, as well as the extent of aliphatic polyene conjugation.



**Figure 3. Positive-ion CF-FAB MS/MS of carotenoids.**

Analyses of the molecular ions at  $m/z$  536 following CID of (a) lycopene, (b)  $\alpha$ -carotene, and (c)  $\beta$ -carotene. Retention times were 32, 46, and 49 min, respectively, during a single separation using a C<sub>18</sub> reversed-phase LC column at 4  $\mu\text{L}/\text{min}$ . (Adapted with permission from Reference 8.)

To illustrate the utility of tandem MS during LC/MS, we used CF-FAB LC/MS/MS to distinguish between isomeric carotenoids in a mixture containing  $\alpha$ -carotene,  $\beta$ -carotene, and lycopene based on the detection of unique fragment ions (Figure 3) (7). Isomers were separated using reversed-phase LC, the molecular ions at  $m/z$  536 were fragmented using CID, and the product ions were analyzed by MS/MS. Elimination of a terminal isoprene group from the acyclic carotenoid lycopene formed a unique fragment ion at  $m/z$  467 that distinguished this molecule from its isomers.  $\alpha$ -Carotene was distinguished from  $\beta$ -carotene by the formation of two unique ions at  $m/z$  480 and 388, which corresponded to retro-Diels–Alder fragmentation of the ring containing an unconjugated carbon–carbon double bond,  $[M-56]^{+}$ , and a combination of retro-Diels–Alder fragmentation and elimination of toluene,  $[M-56-92]^{+}$ . Although structural isomers can be identified unambiguously by MS/MS, geometrical isomers such as cis/trans double-bond configurations have not been successfully distinguished by this technique.

**Electrospray LC/MS.** The electrospray ionization (ESI) process creates a mist of charged droplets at atmospheric pressure by forcing the eluting analyte solution through a capillary electrode at high potential (typically 2000–7000 V) relative to a surrounding counter electrode. As the droplets move toward the MS aperture, they encounter a cross-flow of heated nitrogen gas that increases solvent evaporation from the droplet surface. As evaporation continues, the droplets shrink until electrostatic repulsion between ions in each droplet exceeds the combined energy of solvation and surface tension. Ions are then ejected from the droplets or the droplets explode, releasing analyte into the mass spectrometer. The process continues until all of the solvent has evaporated.

Solvent removal takes place at atmospheric pressure and is highly efficient; therefore, ESI LC/MS is compatible with column flow rates up to 1.0 mL/min without the need for solvent splitting. Because ESI requires no additional gas-phase ionization after removal of solvent from the analyte, pyrolysis and ion fragmentation are minimized or avoided (10).

Compared with CF-FAB or moving belt LC/MS, ESI LC/MS requires far less maintenance and is capable of significantly greater throughput (limited only by the speed of the chromatographic separation). However, analysis of nonpolar carotenoids by ESIMS is not straightforward because these compounds do not easily generate preformed ions in solution that can be evaporated and detected during ESI. Although oxygenated xanthophylls can be protonated by acids in solution and hydroxylated xanthophylls can be deprotonated in solution and detected using positive- or negative-ion ESI, hydrocarbon carotenes will not ionize under these conditions (11).

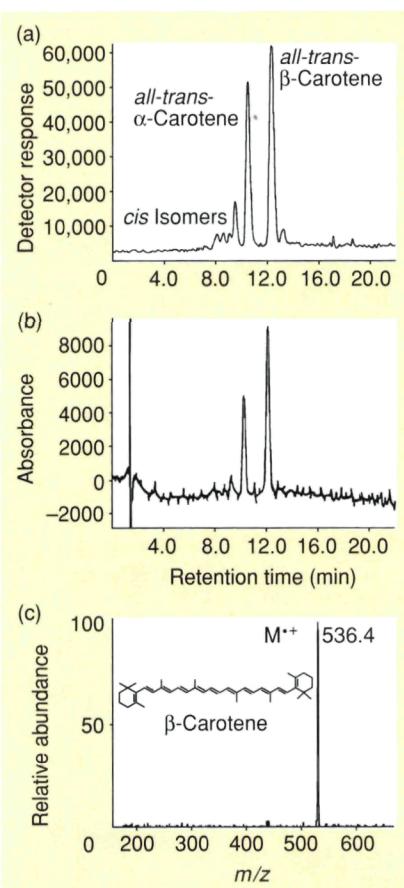
Because of the low ionization potential of the  $\pi$ -electrons in the conjugated polyene chain of carotenoids, molecular ion radicals,  $M^{+}$ , can be generated for xanthophylls and carotenes during ESI by adjusting the electrospray conditions to facilitate electrophoretic charging and field ionization at the metal–liquid interface of the electrospray capillary. This ionization can be enhanced by postcolumn addition of a halogenated oxidant to the mobile phase.

In evaluating a series of fluorinated electron acceptors for this purpose, we found 0.1% heptafluorobutanol to be the additive and concentration that maximized the abundance of radical cations formed from analytes such as  $\beta$ -carotene (11). Other studies have reported generating doubly charged molecular ions ( $M^{2+}$ ) of  $\beta$ -carotene through solution-phase oxidation by trifluoroacetic acid or strong inorganic oxidants in the ESIMS of nonpolar carotenoids (12). Why one method produces singly charged molecular ions and the other doubly charged species is under investigation.

Recently, we used  $C_{30}$  reversed-phase LC columns online with MS for the first time for ESI-LC/MS analysis of carotenoids (11). The positive-ion ESI-LC/MS analysis of a mixture of  $\alpha$ -carotene and  $\beta$ -carotene contained in an extract of raw carrots is shown in Figure 4 along with the corresponding absorbance chromatogram at 450 nm. UV-vis spectra were recorded during LC/MS using a photodiode array detector located between the LC column and the mass spectrometer. We found that the mass spectrometer was

~100-fold more sensitive than the photodiode array absorbance detector and showed a limit of detection of 9.0 and 28 pmol for lutein and  $\alpha$ -carotene, respectively.

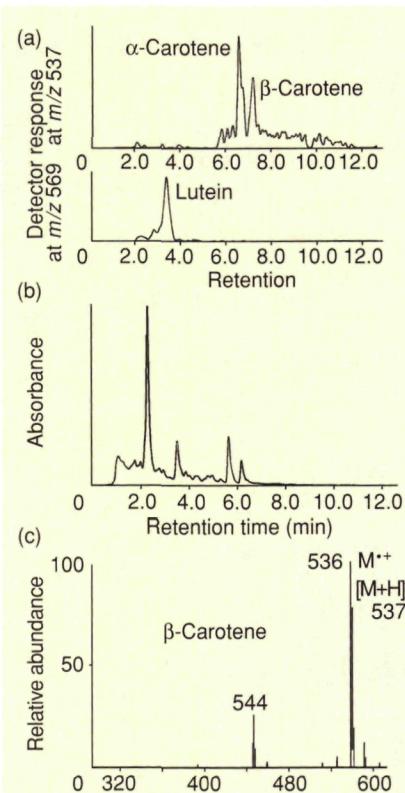
**APCI.** Like electrospray, atmospheric pressure chemical ionization (APCI) combines nebulization and ionization of the LC effluent at atmospheric pressure and is therefore compatible with high LC flow rates. Instead of generating a spray from the mobile phase in a powerful electromagnetic field as in electrospray, the



**Figure 4. Positive-ion ESI LC/MS analysis of an extract of raw carrots.**

A mixture of primarily all *trans*  $\alpha$ -carotene and  $\beta$ -carotene (20 ng each) was analyzed using a  $C_{30}$  reversed-phase LC column at 1 mL/min with postcolumn addition of heptafluorobutanol to enhance ionization. The mobile phase consisted of a 60-min linear gradient from 15% to 100% methyl *t*-butyl ether in methanol (containing 1 mM ammonium acetate).

(a) Computer-reconstructed mass chromatogram of the molecular ions at  $m/z$  536, (b) absorbance chromatogram at 450 nm recorded online during LC/MS using a photodiode array detector, and (c) ESI mass spectrum of  $\beta$ -carotene recorded at a retention time of 12.2 min.



**Figure 5. Positive-ion APCI LC/MS analysis of a mixture of lutein and  $\beta$ -carotene.**

Approximately 20 ng of each carotenoid is present in the mixture, which was analyzed using a narrow-bore C<sub>30</sub> reversed-phase LC column without solvent splitting at a flow rate of 300  $\mu$ L/min. Mobile-phase conditions are identical to those described for Figure 4 except that water at 80  $\mu$ L/min was added postcolumn. (a) Computer-reconstructed mass chromatograms of the protonated molecules of  $\alpha$ -carotene and  $\beta$ -carotene at  $m/z$  537, (b) mass chromatogram of the protonated molecule of lutein at  $m/z$  569, (c) absorbance chromatogram at 450 nm recorded during the LC/MS analysis shown in (a) and (b), and (d) mass spectrum of  $\beta$ -carotene obtained during LC/MS using CID in the ion source to enhance fragmentation.

mobile phase is rapidly heated and sprayed into a corona discharge that facilitates ionization primarily through classical chemical ionization processes such as proton transfer or charge exchange (13). Although developed and produced commercially more than 10 years ago (14), APCI has only recently become a routine technique for the analysis of low molecular weight compounds (< 1000 u).

To date, no APCI LC/MS analyses of carotenoids have been reported in the literature. However, our preliminary results indicate that xanthophylls and carotenes

will form ions during either positive- or negative-ion LC/MS, and that the sensitivity of this technique is similar to that of electrospray (Figure 5). A surprising observation is that all carotenoids can form protonated molecules and molecular ions during positive-ion APCI and deprotonated molecules and molecular ions during negative-ion APCI. The mechanism of formation of unusual protonated hydrocarbons such as  $\beta$ -carotene is under investigation.

Because APCI produces more fragmentation than either CF-FAB or electrospray, structurally significant fragment ions can be generated in the ion source as an alternative to MS/MS. For example,  $\beta$ -carotene fragmented during APCI using CID in the ion source to form an abundant fragment ion at  $m/z$  444, which corresponds to the loss of toluene. During CF-FAB MS/MS with CID, the elimination of toluene from the molecular ion also produces the most abundant fragment ion. The only other effect of CID on the appearance of the APCI mass spectrum is the observation of the molecular ion at  $m/z$  536 as the base peak instead of the protonated molecule. In most quantitative applications, APCI produces a linear detector response/concentration curve over sample concentrations of at least 3 orders of magnitude (13), which gives it the potential to be an excellent technique for quantitation of carotenoids during LC/MS.

### Future work

The combination of C<sub>30</sub> reversed-phase LC and ESI-MS or APCI-MS provides a tool with sufficient sensitivity and selectivity to quantitate carotenoids in complex mixtures and at the low levels typical of many naturally occurring sources. We are currently developing quantitative assays using external standards and concentration/response curves. As stable isotopically labeled carotenoid standards become available, quantitation will be possible by isotope dilution LC/MS, which should improve precision and streamline analysis by eliminating the need for elaborate standard curves.

Investigation of numerous important issues about the physiological effects of dietary carotenoids has been stymied by the lack of an analytical technology with

the necessary sensitivity and selectivity and the capability of routinely performing the large numbers of analyses typical of clinical studies. We believe the current generation of LC/MS technology, such as ESI-LC/MS and APCI-LC/MS, is sufficiently robust to meet these requirements and thus removes a significant impediment to answering some of the fundamental questions about the role of carotenoids in human health.

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