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Liquid chromatography/mass spectrometry of carotenoids

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Abstract: The sensitivity, specificity and selectivity of liquid chromatography/mass spectrometry (LC/MS) and LC-tandem mass spectrometry (LC/MS/MS) make these essential tools for the characterization and identification of carotenoids. LC/MS analysis is particularly important for studies of carotenoids obtained from natural sources, since these compounds are usually present in trace quantities and are often contaminated with biological matrices. To date, five LC/MS techniques have been used for carotenoid analysis including 1) moving belt; 2) particle beam; 3) continuous-flow fast atom bombardment; 4) electrospray; and 5) atmospheric pressure chemical ionization (APCI). Among these LC/MS interfaces, electrospray and APCI are probably the easiest to use and are rapidly becoming the most widely available. These techniques provide comparable sensitivity (at the low pmol level) and produce abundant molecular ions. Whereas electrospray forms primarily molecular ions showing little fragmentation, APCI produces molecular ions and/or protonated or deprotonated molecules depending upon the mobile phase conditions. In addition, APCI produces abundant fragment ions, especially for xanthophylls. Although any of these LC/MS techniques may be combined with tandem mass spectrometry, the majority of LC/MS/MS effort to date has involved the use of continuous-flow fast atom bombardment.

ROLE OF LC/MS IN CAROTENOID ANALYSIS

The vast number of carotenoids found in plants and animals (in excess of 600) and the instability of these compounds to heat, light and air make isolation, identification and quantitation of these natural pigments unusually challenging. Many carotenoids exhibit provitamin A activity (ref. 1), others might function as significant dietary antioxidants, and other beneficial effects of carotenoids that are currently under investigation include inhibition of carcinogenesis (ref. 2), enhancement of the immune response (ref. 3) and prevention of cardiovascular disease (ref. 4). A great need exists for detection and identification of these compounds in complex biological samples such as human tissue and food.

Because carotenoids are thermally labile, separation of mixtures and removal of impurities is usually carried out by reversed phase HPLC (particularly C_{30} HPLC) instead of gas chromatography (refs. 5,6). The small quantities of carotenoids typically isolated from biological matrices such as human serum or tissue preclude structural analysis by nuclear magnetic resonance. Therefore, only the most sensitive analytical methods are adequate such as liquid chromatography/mass spectrometry and HPLC with photodiode-array UV/visible absorbance detection. At the trace level, carotenoid identification may be confirmed by combining data such as HPLC retention times, photodiode-array absorbance spectroscopy, mass spectrometry and tandem mass spectrometry (refs. 7,8).

LC/MS TECHNIQUES USED FOR CAROTENOID ANALYSIS

Five LC/MS techniques have been successfully applied to carotenoid analysis including moving belt, particle beam, continuous-flow fast atom bombardment, electrospray and atmospheric pressure chemical ionization. How each LC/MS technique functions is briefly presented, followed by a discussion of results for carotenoid applications. The order of presentation follows the chronological order in which each LC/MS technique was applied to carotenoid analyses.

Moving Belt Interface

Among the earliest LC/MS techniques and the first to be applied to carotenoid analysis was the moving belt interface, which was combined with electron impact (EI) or chemical ionization (CI) (ref. 5). This is a two-step LC/MS approach in which the solvent is removed from the HPLC mobile phase and then, independently, the sample is ionized in a second step. First, the HPLC eluate is deposited on a belt that passes through a series of heated and evacuated chambers to evaporate the mobile phase. Next, the belt transports the solid analyte into the ion source of the mass spectrometer where it is flash evaporated and then ionized by EI or CI.

The moving belt interface suffers from poor sensitivity for carotenoids because of incomplete analyte volatilization, pyrolysis, and excessive fragmentation. Molecular ions are either low in abundance or not observed. Using a moving belt system, Taylor et al. (ref. 5) observed the molecular ion of β-carotene in low abundance, but lutein formed dehydrated ions, $[M-H_2O]^+$, and many other fragment ions instead of molecular ions. This study was also the first application of LC/MS/MS to carotenoid analysis (ref. 5). Mechanically the most complex LC/MS system, the moving belt interface is not widely used and is no longer manufactured.

Particle Beam Interface

Like the moving belt, the particle beam interface consists of a solvent removal interface followed by a conventional ionization step using EI or CI in the mass spectrometer. The LC eluate, flowing at typical analytical HPLC column rates, is sprayed into a near-atmospheric pressure chamber, which is heated to facilitate solvent evaporation from the droplets. When the solvent has evaporated, an aerosol of analyte particles is separated from the lower molecular weight solvent molecules in a momentum separator. Aggregates of analyte then enter the mass spectrometer ion source where they disintegrate as they strike a heated metal surface. The resulting gas phase sample molecules are subsequently ionized by conventional EI or CI (ref. 9). Molecular ion species are more abundant in particle beam mass spectra than in moving belt spectra, because sample molecules do not require volatilization from the belt (a step that can result in considerable pyrolysis).

To minimize fragmentation and enhance sensitivity, negative ion electron capture ionization (also called negative ion CI or NCI) has been used in all published particle beam LC/MS analyses of carotenoids. As a result, this method has been limited to the analysis of xanthophylls, since nonpolar carotenes lack the electronegative atoms like oxygen that are necessary for efficient electron capture. For example, Khachik et al. (ref. 10) used particle beam LC/MS with NCI to analyse polar carotenoids extracted from human serum. They observed abundant molecular anions, M^-, and fragmentations such as loss of water from the molecular ion for xanthophylls like lutein. No ions were reported for carotenes like β-carotene or lycopene.

Continuous-Flow Fast Atom Bombardment (CF-FAB)

FAB is a matrix-mediated technique in which an analyte of low volatility is dissolved in a liquid of low volatility (i.e., glycerol, 3-nitrobenzyl alcohol, etc.) and then subjected to energetic bombardment by a beam of atoms or ions (usually xenon atoms at 3,000-10,000 V or cesium ions at 10,000-20,000 V). The matrix facilitates the transfer of energy from the fast atoms (or ions) to the analyte so that analyte molecules are desorbed from the solution phase into the gas phase with a minimum of fragmentation. Ionization occurs either in solution prior to desorption (due to protonation or deprotonation of the analyte

by the matrix) or in the gas phase directly above the matrix. For FAB ionization of carotenoids, the most effective matrix is 3-nitrobenzyl alcohol (ref. 7), which facilitates the formation of molecular ion cations, M^+, for both xanthophylls and carotenes. No other molecular ion species are formed, and essentially no fragmentation is observed.

Since FAB ionization requires that the analyte be in solution, this technique may be readily interfaced to a solvent pump to provide a steady infusion of analyte in solution (i.e., eluate from an HPLC separation). During CF-FAB, the matrix is ideally mixed with the mobile phase and analyte in the ion source by coaxial-flow addition (ref. 10). The more volatile mobile phase quickly evaporates leaving behind a thin film of matrix containing the analyte, which is exposed to the FAB beam.

Advantages of CF-FAB over both previous LC-MS techniques for carotenoids (moving belt and particle beam) include the formation of much more abundant molecular ions, lack of fragmentation, and high sensitivity for both xanthophylls and carotenes (9-28 pmol per analysis). Because all the carotenoid ion current is concentrated into an abundant molecular ion during CF-FAB, this ion is easy to select for collision induced dissociation and then analysis of structurally significant fragment ions during LC/MS/MS analysis (ref. 10). Disadvantages include an upper flow limit of only 10 µL, so that the vacuum system is not overloaded with solvent, and the need to clean the ion source approximately every three hours as the 3-nitrobenzyl alcohol matrix fouls the sample stage and reduces sensitivity. As a result of these practical limitations, CF-FAB cannot be set up to run large numbers of samples in an automatic, unattended mode.

Electrospray

Unlike previous LC/MS techniques, electrospray is both an ionization technique and an efficient solvent removal interface. During electrospray, a fine mist of charged droplets is formed from the HPLC eluate at atmospheric pressure by spraying the solution through a capillary electrode at high potential (usually 2,000-7,000 V). As the charged droplets are electrostatically attracted towards the opening of the mass spectrometer, they encounter a cross-flow of heated nitrogen curtain gas that increases solvent evaporation and prevents most of the solvent molecules from entering the mass spectrometer. When the droplets shrink in size until the electrostatic repulsion between ions in each droplet exceeds the combined energy of solvation and surface tension, ions are ejected from the droplets into the gas phase or else the droplet disintegrates and releases analyte ions. Because of the efficiency of this combined ionization and desolvation process, electrospray is compatible with HPLC flow rates from a few µL/min up to 1 mL/min.

Although ions produced by electrospray are usually preformed in solution by acid/base reactions (i.e., $[M+nH]^{n+}$ or $[M-nH]^{n-}$), carotenoid ions are probably formed by a field desorption mechanism at the surface of the droplet which appears to be enhanced by the presence of halogenated compounds such as heptafluorobutanol (ref. 11). As a result of this unusual ionization process, electrospray of carotenoids produces abundant molecular cations, M^+, with little fragmentation, and no molecular anions. For example, the positive ion electrospray LC/MS analysis of an extract of fresh sweet potatoes is shown in Fig. 1. Fresh sweet potatoes are rich in all-*trans* β-carotene. Note the superior signal-to-noise in the mass chromatogram compared to the chromatogram recorded by the photodiode array absorbance detector.

In another example, the electrospray LC/MS analysis of carotenoids extracted from 2 mL of human serum is shown in Fig. 2. Lutein, α-carotene, β-carotene and lycopene were identified based on their HPLC retention times and molecular weights. One monooxygenated carotenoid was detected at *m/z* 552, which corresponds to β-cryptoxanthin or an epoxide of β-carotene. Isomeric with lutein, an unidentified dioxygenated carotenoid was detected at a retention time of 8.0 min (eluting later than zeaxanthin). Note that the carotenoid quantities being analysed in Fig. 2 (levels were not determined) were below the detection limit of the photodiode array absorbance detector. Advantages of electrospray over previous LC/MS techniques for carotenoids include lower limits of detection (see Table 1), abundant molecular ions of both xanthophylls and carotenes, substantially improved ease of operation, reduced maintenance, higher sample throughput (compared to moving belt and CF-FAB), and compatibility with autosamplers for unattended operation. One of the potential limitations of electrospray is the relatively narrow range of linearity of response it shows for carotenoid quantitation (unpublished observation).

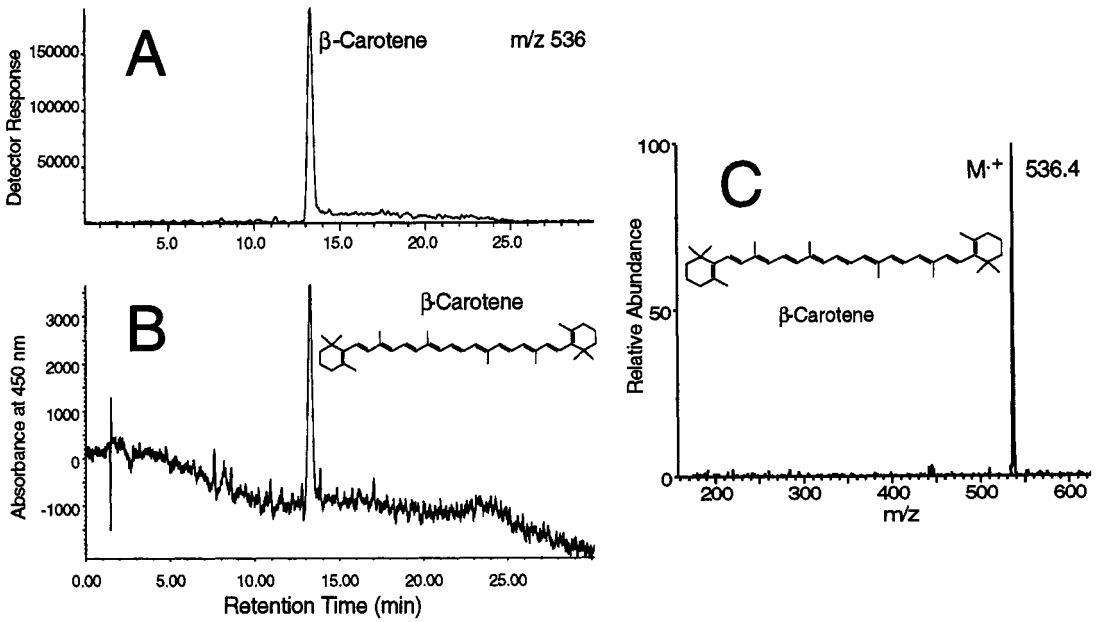

Fig. 1. Positive ion electrospray LC/MS analysis of an extract of fresh sweet potatoes containing approximately 20 ng of β-carotene. A C_{30} HPLC column (4.6 mm x 12.5 cm) was used with a 60 min gradient from 15-100% methyl-*tert*-butyl ether in methanol (containing 1 mM ammonium acetate) at a flow rate of 1.0 mL/min. (A) Computer-reconstructed mass chromatogram showing signal for the molecular ion of β-carotene at *m/z* 536; (B) Absorbance chromatogram at 450 nm recorded online during LC/MS using a photodiode array detector; and (C) electrospray mass spectrum of β-carotene recorded at a retention time of 13.3 min. For similar analyses and more experimental details, see ref. 11.

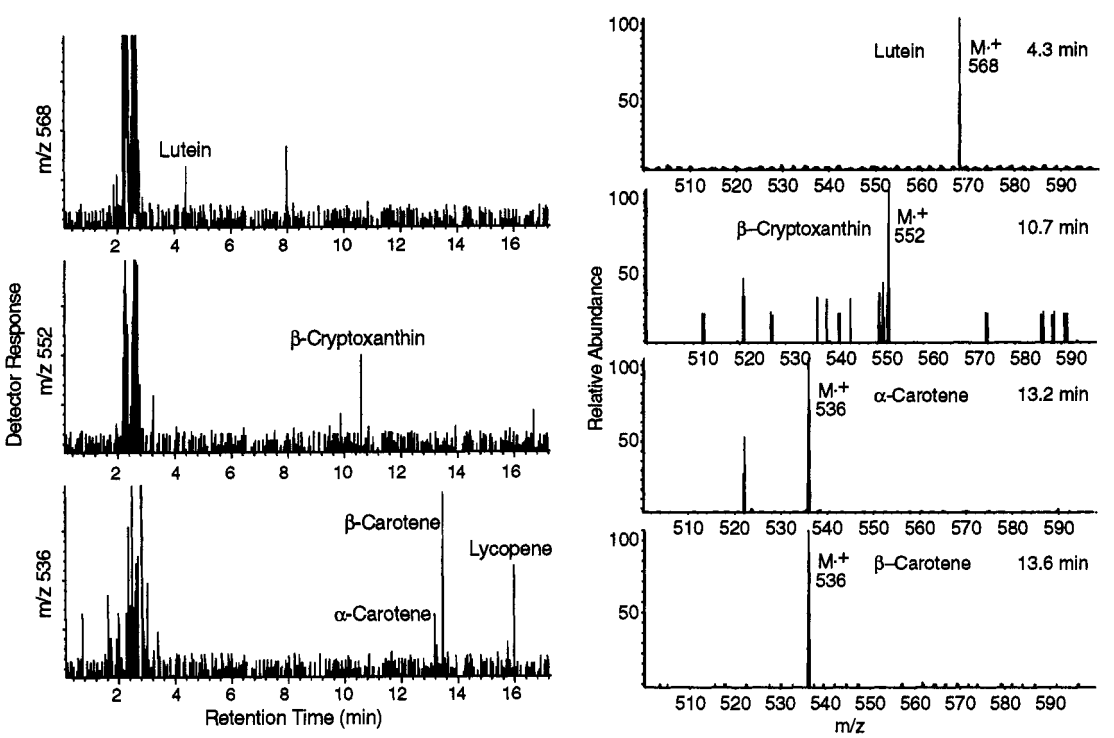

Fig. 2. Electrospray LC/MS analysis of an extract of approximately 2 mL of human serum. HPLC conditions were identical to those described in Fig. 1. (Left) Computer-reconstructed mass chromatograms showing signals for molecular ions corresponding to lutein, β-cryptoxanthin (or a monoepoxide of α- or β-carotene), α-carotene, β-carotene, and lycopene; (Right) Positive ion electrospray mass spectra recorded during the LC/MS analysis shown in the chromatogram on the left.

Atmospheric Pressure Chemical Ionization (APCI)

Although ionization during both electrospray and APCI takes place at atmospheric pressure, APCI uses a heated nebulizer instead of a strong electromagnetic field to facilitate solvent evaporation and obtain a fine spray of the HPLC mobile phase. Ionization takes place by spraying the analyte solution into a corona discharge, which produces numerous reactive species that can ionize the analyte including reagent gas ions formed from the mobile phase. Ions are then drawn into the aperture of the mass spectrometer. As in electrospray, a cross-flow of a curtain or bath gas (usually heated nitrogen) prevents most uncharged solvent molecules from entering the low pressure mass spectrometer.

The first APCI LC/MS analyses of carotenoids were recently published (refs. 12,13) and show some unexpected molecular ion heterogeneity. Molecular ions and protonated molecules were observed in positive ion APCI, and molecular ions and deprotonated molecules were detected during negative ion APCI. The relative abundance of molecular ions and protonated or deprotonated molecules vary with the mobile phase composition. For instance, polar solvents such as alcohols lead to an increased abundance of protonated carotenoids (even protonated β-carotene), and less polar solvents such as methyl-*tert*-butyl ether facilitate the formation of more abundant molecular ions. An example of an LC/MS analysis of a mixture of lutein, α-carotene and β-carotene is shown in Fig. 3. Because both methanol and methyl-*tert*-butyl ether were used during the HPLC separation on a C_{30} column, a mixture of molecular ions and protonated molecules was observed in the mass spectrum of each carotenoid.

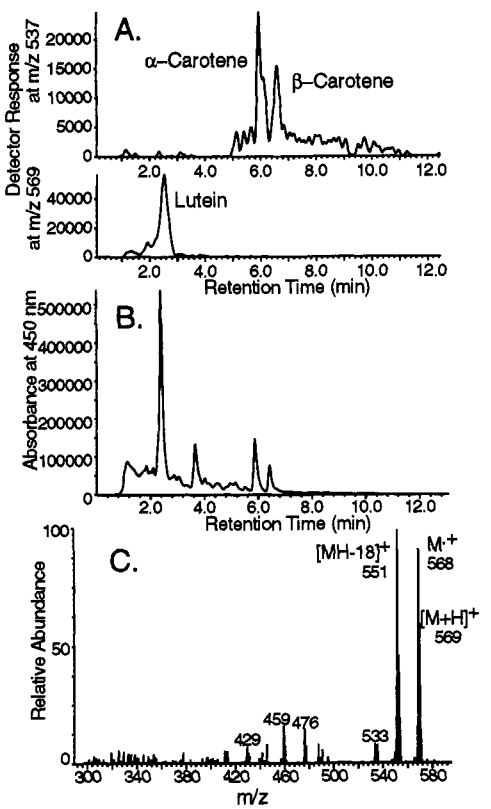

Fig. 3. Positive ion APCI LC/MS analysis of a carotenoid mixture containing lutein, α- and β-carotene. HPLC conditions are similar to those described in Fig. 1, except that a narrow bore C_{30} reversed phased column was used at a flow rate of 300 μL/min. (A) Computer-reconstructed mass chromatograms showing the signal for the protonated molecules of α- and β-carotene and lutein; (B) absorbance chromatogram recorded at 450 nm during the LC/MS analysis; (C) APCI mass spectrum of lutein recorded at a retention time of 2.3 min. Note the presence of both a molecular ion of *m/z* 568 and a protonated molecule of *m/z* 569, and the abundant fragment ion at *m/z* 551 formed by elimination of water from the protonated molecule.

The limit of detection (Table 1), ease of use, compatibility with HPLC solvents and flow rates, and suitability of APCI for automated or unattended LC/MS carotenoid analysis are comparable to those of electrospray. The main advantage of APCI compared to electrospray is its higher linearity of detector response over a carotenoid concentration range of at least three orders of magnitude (ref. 13), which

suggests that APCI LC/MS might become the preferred mass spectrometric technique for carotenoid quantitation. Disadvantages of APCI include the multiplicity of molecular ion species, which might lead to ambiguous molecular weight determinations, and the abundant fragmentation, which tends to reduce the abundance of the molecular ions.

COMPARISON OF LC/MS TECHNIQUES AND FUTURE DIRECTIONS

TABLE 1. Features of Carotenoid Mass Spectra Obtained by Various LC/MS Techniques

LC/MS Technique	Molecular Ion Species	Extent of Fragmentation	Limit of Detection
Moving belt	$M^{+\cdot}$	very high	poor (ref. 5)
Particle beam	$M^{-\cdot}$	medium	not reported
CF-FAB	$M^{+\cdot}$	low-none	9-28 pmol (ref. 10)
Electrospray	$M^{+\cdot}$	low-none	1-2 pmol (ref. 11)
APCI	$M^{+\cdot}$, $[M+H]^+$	low for carotenes	3-13 pmol (ref. 13)
	$M^{-\cdot}$, $[M-H]^-$	medium for xanthophylls	1-3 pmol (ref. 13)

Because the majority of LC/MS systems currently being installed are designed for electrospray and/or APCI, these interfaces will be the most widely available to carotenoid researchers during the next several years. Fortunately, electrospray and APCI provide high sensitivity for carotenoids (low pmol level), are among the easiest LC/MS interfaces to operate and maintain, and may be operated unattended by use of an autosampler. The superior linearity of detector response of APCI for carotenoids (linear over a concentration range of at least four orders of magnitude) suggests that this LC/MS technique may become the standard for carotenoid quantitation. However, the soft ionization of electrospray, which produces molecular ions without fragmentation, greatly facilitates molecular weight confirmation and might be preferable to APCI for identification of carotenoids in mixtures and biological extracts, since APCI can produce abundant fragment ions.

In the immediate future, electrospray and APCI LC/MS sensitivity will probably be enhanced by at least one or two orders of magnitude as quadrupole mass analysers are succeeded by more efficient time-of-flight or ion trap analysers. Finally, what might be the next advance in LC/MS technology? Perhaps we will see the development of a sensitive, low cost, benchtop matrix-assisted laser desorption time-of-flight LC/MS instrument.

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