Analysis of Retinoids and Carotenoids: Problems Resolved and Unsolved

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ABSTRACT  Progress in nutritional biochemistry has always depended on progress in analysis of nutrients. Animal growth assays were fundamentally important in the discovery and initial isolation of the fat-soluble vitamins. Chromatography, initially introduced by Tswett for separation of plant pigments (including carotenoids), quickly became indispensable for separation of carotenoids and vitamin A compounds; the early open-column methods were eventually superseded by more efficient HPLC techniques, and reversed-phase HPLC has become the current method of choice for analysis of retinoids and carotenoids in biological tissues. Detection and quantitation of retinoids and carotenoids most often has depended on their unparalleled spectral properties; the conjugated polyene structures of these compounds give them unique light absorption spectra and high molar absorbptivities, and hence outstanding lower limits of detection. Other techniques, such as gas chromatography (GC) and mass spectroscopy (coupled with GC and HPLC), immunoassays, supercritical fluid chromatography, and capillary electrophoresis, have proven useful in certain applications. Analysis of retinoid-binding proteins has been mostly by conventional protein methods, although the fluorescence of the retinol ligand has been useful in some instances to provide a highly specific assay. Current challenges in retinoid and carotenoid analysis include the resolution of stereoisomers, and quantitation of these compounds at ultratrace levels in biological tissues. Possible new approaches include accelerator mass spectroscopy, and use of gene expression assays to assess vitamin A status. J. Nutr. 134: 281S–285S, 2004.

KEY WORDS: ● retinoids ● carotenoids ● vitamin A ● analysis

Progress in nutritional sciences, perhaps more so than in most other sciences, depends on advances in analytical techniques. Space does not allow this paper to provide detailed information on appropriate analytical tools and methods, but it reviews the methods traditionally used for vitamin A and carotenoid analysis and provides references for fuller descriptions of these procedures.

Biological assays

The discovery of the vitamins depended on biological assays (1,2), as did their subsequent isolation. The rat growth assay (Fig. 1) provided the means by which McCollum and Davis (3) and Osborn and Mendel demonstrated the nutritional need for a fat-soluble growth factor, soon called “fat soluble A” and later named “vitamin A.” Before the determination of the chemical structure and physical properties of retinol and retinyl esters, animal growth assays [described in detail by Bliss and Roels (4)] necessarily were the only means of determining the vitamin A content of foodstuffs and tissues. The discovery of the visual function of vitamin A is another example of a specialized type of biological assay (5). Later, the finding that retinoic acid is an active metabolite of vitamin A also depended on growth assays (6). In some sense, the recognition of the nuclear receptor proteins (RAR, RXR, PPAR, LXR) also depended on a new type of biological assay.

Chromatography

However, the identification of the carotenoids followed a different path in that their chemical isolation and characterization preceded any conjecture as to their biological functions in animals. Their highly visible color (orange to red) prompted early interest in their isolation and characterization, for example the isolation of bixin from Bixa orellana reported by Boussingault in 1825 (7) and the isolation of β-carotene from carrot root by Wachenroder in 1826 [cited by Palmer (8)]. Early procedures for isolation and characterization of carotenoid studies were reviewed in a delightful monograph by Palmer published in 1922 (8). In fact, the study of carotenoids led to the development of a new and eventually extremely powerful analytical technique. Mikhail Tswett, a botanist...
studying plant pigments, found that plant pigments could be separated into a variety of individual colored zones on columns of powdered calcium carbonate, inulin, or sucrose in glass tubes; he named this new technique “chromatography” (“writing with color”) and described its application in a series of papers (9). Chromatography allowed the discrimination of xanthophylls (oxygen-containing carotenoids, such as lutein and zeaxanthin) from carotenes (hydrocarbon carotenoids, including lycopene, α-carotene and β-carotene) and provided separations within classes. Alumina and silica became the preferred stationary phases for reproducible separations, with mixtures of organic solvents as mobile phases. This separation technique, adsorption chromatography, in time was applied to many other compounds, including retinol and retinyl esters.

Although planar chromatographic techniques (both paper chromatography and thin layer chromatography on silica-coated plates) were applied for analysis of many compounds, these techniques have never been used extensively for analysis of carotenoids and retinoids. Gas chromatography also was little used for analysis of carotenoids and retinoids because of their thermal instability and limited volatility. As a special application, however, gas chromatography coupled with detection by mass spectroscopy (GC-MS) has proven uniquely valuable in determining isotope ratios for isotopic dilution analysis of vitamin A status. Combustion of 13C-carotenoids and retinoids combined with gas chromatography-mass spectrometry of the resulting 13CO2 (isotope ratio-gas chromatography-mass spectrometry, IR-GC-MS) has proven to be extremely sensitive and precise for analysis of isotope ratios in tracer experiments (10).

The advent of HPLC in the early 1970s, with much enhanced resolution provided by very small stationary phase particle sizes and the reproducibility provided by commercially produced stationary phases, revolutionized retinoid and carotenoid studies. This technique opened a new era in studies of the tissue distribution and metabolism of retinoids and carotenoids. Retinoids and carotenoids in small volume extracts of biological tissues (either plant or animal) could now be analyzed qualitatively and quantitatively in a short time (11,12) (example chromatograms from contemporary practice are shown in Figs. 2 and 3). The sensitivity of this technique opened new opportunities for tissue analysis; limits of detection of retinol and other retinoids by absorbance detection on HPLC are typically 0.35 pmol (0.1 ng). Most retinoid and carotenoid analyses have been performed by partition chromatography (“reversed-phase HPLC”) on octadecylsilane (C18) columns, but within the past several years C30 columns have proven useful for certain demanding separations of carotenoids (13). The most commonly used detectors are UV and visible light absorbance detectors (both single-wavelength and photodiode-array detectors), although fluorescence detectors (retinol and retinyl esters are fluorescent, other retinoids and most carotenoids are not), refractive index detectors, electrochemical detectors, and mass spectrometers have also been used.

FIGURE 1 Lower curve (up to d 18) eight male rats upon pure dietary; upper curve eight similar rats taking 3 c.c. of milk each a day. On d 18, marked by vertical dotted line, the milk was transferred from one set to the other. Average weight in g vertical; time in d horizontal.

FIGURE 2 Reversed-phase high-pressure liquid chromatography of retinoids and carotenoids: isocratic separation of compounds having similar polarity, demonstrating the power of HPLC to resolve similar compounds. A. Absorbance at 290 nm (tocopherols); B. Absorbance at 340 nm (retinoids); C. Absorbance at 445 nm (carotenoids). From Barua and Olson (41).
used. Recent detailed reviews of HPLC of retinoids and carotenoids (and sample preparation techniques) include those of Britton et al. (14), Barua and Furr (15), Eitenmiller and Landen (16), Barua et al. (17) and Song et al. (18).

Supercritical fluid chromatography (using either packed columns or capillary columns) also should be useful for separations of retinoids and carotenoids (as well as a useful technique for extraction of carotenoids and retinoids from biological tissues), but this technique has received relatively little attention (19). Perhaps this is because conventional HPLC equipment has become rugged and widely available and its applications have become relatively standardized.

Spectrometry

Carotenoids and retinoids absorb light particularly well because of their conjugated polyene systems, with light absorption in regions of the visible or ultraviolet spectrum where few other biological compounds absorb (i.e., around 450 nm for carotenoids and around 325–380 nm for retinoids) and with particularly high molar extinction coefficients (absorbance spectra of some retinoids and carotenoids are shown in Fig. 4). Absorbance spectroscopy has become a major tool for quantitation of retinoids and carotenoids, either of pure solutions or of biological extracts. The Bessey-Lowry procedure was used earlier for analysis of retinol in blood plasma and liver extracts (20). Currently, absorbance detectors have become the standard means for quantitative analysis of retinoids and carotenoids in HPLC, and photodiode-array detectors (which can display the complete light absorption spectrum of an eluting peak) have become important tools for qualitative analysis and identification in HPLC. Useful tables of light absorbances (wavelengths of maximum absorption, and molar extinction coefficients) have been compiled for carotenoids (21) and retinoids (17,22,23).

Before the commercial availability of sensitive and rugged spectrophotometers capable of operating in the ultraviolet region, colorimetric assays such as the Carr-Price method proved invaluable in vitamin A and carotenoid analysis: the polyene system of a retinoid or carotenoid is protonated by a strong acid such as antimony trichloride or trifluoroacetic acid in organic solvent to give a characteristic transient blue color of high intensity, providing a selective and very sensitive assay for vitamin A analysis (24,25). Although these methods were used for many years for studies of vitamin A metabolism, the reagents required are corrosive and noxious, and so this method is now little used.

Fluorescence spectroscopy offers greater specificity than ab-

![Figure 3](https://academic.oup.com/jn/article-abstract/134/1/281S/4688308)

**FIGURE 3** Reversed-phase high-pressure liquid chromatography: gradient elution of retinoid compounds having a range of polarities, demonstrating the power of HPLC to analyze a range of compounds. Peak identification: ORA, 4-oxo-retinoic acid; OROL, 4-oxo-retinol; RAG, retinoyl beta-glucuronide; ROL, retinyl beta-glucuronide; RA, retinoic acid; EROL, 5,6-epoxyretinol; ROL, retinol; RAC, retinyl acetate; RL, retinyl linoleate; RP, retinyl palmitate; RS, retinyl stearate. From Barua (42).

![Figure 4](https://academic.oup.com/jn/article-abstract/134/1/281S/4688308)

**FIGURE 4** Absorption spectra of some representative retinoids (in methanol solution) and carotenoids (in hexane solution).
sorbance spectroscopy (because two wavelengths, excitation and emission, can be selected) for analysis of retinol and retinyl esters in biological samples (26,27). Retinol and retinyl esters are intensely fluorescent, but retinal and retinoic acid (and most synthetic retinoids) are not; of the common carotenoids only phytolfluene shows appreciable fluorescence, and fluorescence spectroscopy is not heavily used for routine retinoid and carotenoid analysis.

Nuclear magnetic resonance (NMR) spectrometry is important for structural determination of organic compounds, and it is particularly useful for assignment of cis and trans isomers. It has found little other use in retinoid and carotenoid analysis, however. Similarly, infrared spectroscopy (IR) has had some use in structural analysis but has not been as important as UV and visible light spectroscopy (28–30). Coupling analytical techniques, for example HPLC with NMR and mass spectrometry, HPLC-NMR-MS (31), provides greatly enhanced sophistication in separation, identification, and quantitation.

Methods useful for analysis of retinoid-binding proteins, and other techniques

Retinol-binding protein (RBP) can be quantitated in small volumes of blood serum or plasma (5 to 20 μL) by radial immunodiffusion, providing an inexpensive means of assessing vitamin A status in the field which requires no sophisticated equipment. The disadvantage, of course, is that the analyte measured is RBP, not retinol itself; however, in many situations the concentrations of retinol and RBP are closely correlated. Concentrations of retinoid nuclear receptor proteins are measured is RBP, not retinol itself; however, in many situations the concentrations of retinol and RBP are closely correlated. Concentrations of retinoid nuclear receptor proteins are routinely assessed by immunoprecipitation techniques (Western blots).

The fluorescence of retinol itself makes it a useful tag for analysis of retinol-binding proteins, including plasma RBP. A portable fluorometer has been adapted for measurement of human plasma retinol-RBP concentrations in field studies (32). Capillary electrophoresis of retinol-RBP requires <10 nL of serum or plasma for quantitative analysis using laser-excited fluorescence detection and can be adapted to analysis of retinol-RBP in dried blood spots (33). Similarly, retinol fluorescence can be used for sensitive quantitation of retinol-RBP separated by size-exclusion HPLC (34,35).

Immunological techniques are of limited use for binding and analysis of retinoids themselves (36,37). Although this approach has not demonstrated high specificity for individual retinoids, it may still have unrealized potential for localization of retinoids in tissues slices by immunohistochemistry (coupled antibody techniques).

Current techniques of retinoid and carotenoid analysis

Analysis of retinoids and carotenoids may now be considered a relatively mature field. Although specific challenges remain, there is a corpus of accepted techniques for extraction of these compounds from biological samples and for their qualitative and quantitative analysis. A number of reviews are available, of which the recent series Carotenoids (edited by Britton, Liaaen-Jensen and Pfander) is especially useful (14,38). Recent reviews that include specific instructions for extraction and analysis procedures were provided by Barua and Furr (15,23), Napoli and Horst (39), Eitenmiller and Landen (16), Barua et al. (17) and Song et al. (18).

Contemporary challenges and new approaches

A continuing problem in retinoids and carotenoids research is the lack of standard compounds for testing biological activity in various systems and for use as analytical standards. In the past a variety of compounds has been available from sources in the vitamins industry, at little or no cost, and the academic research community has been heavily indebted to colleagues in industry. With changes in commercial perspectives and interests, these compounds are less available. Alternative sources of a wide variety of compounds will have to be found in order for research to progress.

The current appreciation of the crucial roles of retinoic acid (all-trans and 9-cis isomers) in control of gene expression, and the possibility that carotenoids also may be involved in control of gene expression, demand more sensitive and selective methods for analysis of these compounds in biological tissues and cell cultures. The likelihood that retinoid metabolism (oxidation of retinol to retinoic acid, isomerization of all-trans retinoids to 9-cis retinoids, and possibly 4-hydroxylazyl(oxidation) plays an indirect role in control of gene expression increases the importance of measuring tissue concentrations of these vitamin A metabolites. Although HPLC has been an invaluable technique for retinoid analysis, perhaps new methods such as capillary electrophoresis will be required to provide adequate levels of sensitivity. Rapid methods for extraction and resolution of stereoisomers that obviate any problems of isomerization during processing are needed not only for studies of retinoids (and carotenoids) in molecular biology, but also in vision research. Improved lower limits of detection continue to be important for helping elucidate metabolism of these compounds.

Looking ahead, I am particularly impressed by the greatly enhanced sensitivity of accelerator mass spectrometry for analysis of ultra-low tracer quantities of 14C-retinoids and carotenoids (40). This technique allows investigation of vitamin A and carotenoid kinetics with truly physiologically low doses of tracer. Noting the sensitivity of expression of a variety of genes to retinoids, I suggest that measurement of the levels of expression of specific target genes could serve as a sensitive indicator of functional vitamin A status.

Assessment of vitamin A status is a continuing problem in human nutrition. Development of analytical techniques has contributed to the variety of methods for assessment of vitamin A status currently available, but there is still need for simple, rugged and inexpensive methods.

Looking back over the past century, it is clear that our understanding of retinoid and carotenoid function and metabolism has depended on the analytical techniques available. Meeting the next set of experimental challenges will depend on resolution of the next set of analytical problems.

LITERATURE CITED

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