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The Analysis of Carotenoids and Retinoids: A Review

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ABSTRACT

In recent years, there has been particular emphasis on obtaining more accurate data on the types and concentrations of various carotenoids and retinoids in foods for various health and nutrition activities. A great deal of attention has thus been given to improved methodologies for the study of these two groups of compounds. This review is aimed at defining the state-of-the-art of analytical methods for the study of carotenoids and retinoids, particularly in foods and blood samples. The first part of the review discusses various preliminary sample treatment procedures, including precautions to be taken during handling of samples. The greater part of the discussion is devoted to reviewing the various methods reported for the separation and quantitation of these two groups of compounds.

A pre-requisite in the analysis of carotenoids and retinoids is preliminary sample treatment in order to release, isolate and extract the compounds of interest from the food matrix. Numerous different preliminary treatment procedures have been reported in the literature. In general, however, these procedures follow a few basic steps, upon which various modifications have been made. For convenience, the treatment procedures for plant materials, animal foods and blood samples are separately treated in this review. Because of the unstable nature of the carotenoids and retinoids, especially towards

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light, heat, oxygen and acids, various precautions have to be taken during saponification, extraction and chromatography steps of the analytical procedure. Precautions against oxidation, such as the use of antioxidants, protection against exposure to direct light, and proper storage of solutions are discussed.

After sample extraction, most investigators endeavoured to separate the carotenoids and retinoids from interfering substances in order to obtain more accurate results. A wide variety of separation and detection and quantitation procedures have been used in studies of these compounds. There has been a general trend in the change of these methodologies over the last two decades. An early technique used for the separation of carotenoids in plant materials, mainly in the 1960s, was countercurrent distribution. A few early studies using paper chromatography were also reported. In the 1970s, a few studies of carotenoids and retinoids using gas–liquid chromatography and gel-permeation chromatography were encountered. A number of studies employing thin-layer chromatography (TLC) as a single technique for the separation of carotenoids in plant materials were encountered throughout the 1970s and the early 1980s. TLC was also used by several investigators in combination with other separation techniques. The procedure was less widely used in studies of retinoids. Adsorption (open) column chromatography, utilizing primarily descending, gravity-flow columns, was widely used for the study of carotenoids and retinoids in foods, even in the 1960s. The procedure remained very much in use in the 1970s and 1980s. Commencing from the late 1970s, a new chromatography procedure became more prominent in the literature. High-pressure liquid chromatography has become a widely used procedure for the separation of carotenoids and retinoids in various materials. Owing to the complicated nature of the required analysis, the associated methodologies are still being developed and improved. Used together with adsorption column chromatography and UV–vis spectroscopy, especially during method development stage, HPLC would be the method of choice for arriving at more accurate data on the content and composition of carotenoids and retinoids in foods.

INTRODUCTION

Since the discovery of vitamin A as a fat-soluble growth factor in the early part of this century, research into carotenoids and retinoids has attracted the attention of many scientists. These two related groups of compounds are still being actively studied all over the world, since many gaps in knowledge exist, and new frontiers are being pursued. Recent developments in studies into the possible roles of carotenoids and retinoids beyond their classical functions in vision have created a great deal of excitement in the biomedical community.

Vitamin A deficiency remains one of the major public health nutritional

problems in many developing countries, and is an important cause of preventable blindness. It is estimated that at least 8–9 million people may be suffering from non-corneal xerophthalmia (WHO, 1982). An estimated 20 000 to 100 000 young children go blind each year as a result of a lack of vitamin A in their diet (De Luca *et al.*, 1979). Vitamin A deficiency has a wide geographical distribution, with most cases concentrated in South-East Asia. It is a major nutritional problem particularly among preschool children, and is often associated with protein-energy malnutrition, parasitic infestation, and diarrhoeal disease (Srikantia, 1982).

Since pre-formed vitamin A in meat, liver, and eggs is out of reach of the economically deprived, the main source of vitamin A in the diet of the rural communities is carotenoids, the precursors of vitamin A, from vegetables and fruits. Although there is abundant vegetation in South-East Asia, the consumption of these foods among children is known to be low. Small amounts of carotenoids are ingested. To compound the problem, the diet which is poor in fat and protein makes for the poor absorption of whatever carotenoids are ingested. At the same time, the major carotenoids in fruits and vegetables contributing to the vitamin A activity are also not well defined. These are obviously important areas for studies and intervention in order to overcome this debilitating disorder among young children.

There have been major advances in the understanding of the role of retinoids on cell differentiation and proliferation, one of the most basic problems of biology (Roberts & Sporn, 1984). Fundamental to these new studies is the realization that retinoids are highly potent agents for control of these processes. In this regard, the retinoids have had a particularly striking role in helping to illuminate the problem of malignant cell differentiation. Similarly, in addition to their role as vitamin A precursors, the carotenoids are now thought to play specific roles in mammalian tissues, for example as antioxidants, similar to their roles in plant tissues (Ritenbaugh, 1987). Carotenoids, with their highly reactive conjugated double bonds, act as free radical traps or antioxidants. Based on the ability of carotenoids to protect plants and bacteria against photosensitivity, trials have been carried out in the use of these pigments for the treatment of erythropoietic porphyria, a condition in which patients suffer from an extreme degree of photosensitivity (Mathews-Roth, 1981). These led to studies in the use of β-carotene and other carotenoids in the treatment of various skin tumours. Thus, a new frontier in vitamin A research has been the examination of a possible association between carotenoids and retinoids and the development and prevention of cancer.

A basic tool in carotenoid and retinoid research and development activities is the content of these two groups of compounds in foods. In recent years, there has been particular emphasis on understanding the types and

concentrations of various carotenoids in foods. It is thought that previously reported values of vitamin A activity in food composition tables may have been unreliable since methodologies used were not sufficiently discriminative and thus had included carotenoids that do not possess vitamin A activity. Advances in studies into the structure and properties of various carotenoids have shown that only a handful of the hundreds of carotenoids occurring in nature possess vitamin A activity. Some of these may occur in higher concentrations than β-carotene, the most potent precursor of vitamin A.

Even epidemiological studies in the area of cancer prevention would require more accurate data on retinol and carotenoid content of foods. Currently, association is on a broad basis, linking vegetable consumption or 'carotene' content of foods to risk of developing cancer. Better methods of determination would help narrow down the compound(s) of interest, not only in foods but in blood serum as well. Furthermore, there is also the possibility that carotenoids, not possessing vitamin A activity, may be associated with lower cancer risk.

The authors have embarked on a systematic study to develop improved methodologies for the identification of foods rich in retinol and several carotenoids, and the levels therein in various commonly consumed Malaysian foods. This review was undertaken as an initial step in the study, and is aimed at defining the state-of-the-art of analytical methods for the study of carotenoids and retinoids, particularly in foods and blood samples. A clear picture of developments in the subject will assist in understanding past achievements and current status, in order to arrive at appropriate analytical methodologies suitable for easy application in laboratories.

PRELIMINARY SAMPLE TREATMENT

A pre-requisite in the analysis of carotenoids and retinoids is preliminary sample treatment in order to release, isolate and extract compounds of interest from the food matrix. A search through the literature indicates numerous procedures for the preliminary treatment of these compounds. In general, however, the procedures follow a few basic steps, upon which various modifications have been made. The procedures of the Association of Official Analytical Chemists methods (Williams, 1984) are first described to illustrate the basic steps involved. Major modifications made by various investigators are next discussed. For convenience, treatment procedures for plant materials, animal foods, and blood samples are separately discussed. A short discussion on the precautions to be taken during handling of samples will also be given in view of the unstable nature of these compounds.

For plant materials

For the analysis of carotenes in plant materials, in the AOAC procedure (Williams, 1984), the well-blended material is extracted with a mixture of acetone–hexane in the presence of magnesium carbonate. The extraction mixture is filtered, and the residue further extracted with aliquots of acetone and hexane. Acetone is then removed from the pooled extract using water. The carotenoids are made up in a solution of acetone–hexane mixture for chromatography in a column of magnesia and Hyflo Super Cel mixture.

For the determination of carotenes and xanthophylls, especially in dried plant materials, the AOAC method includes a saponification step (Williams, 1984). Two procedures have been suggested; namely, hot and cold (overnight) saponification. After extracting with an extractant consisting of hexane–acetone–absolute alcohol–toluene, the mixture is saponified using methanolic potassium hydroxide at 56°C for 20 min for hot saponification or left to stand in the dark for 16 h if cold saponification is preferred. The former is for rapid extraction and for samples containing xanthophyll esters. For samples containing xanthophylls rather than xanthophyll esters, cold saponification is suggested since xanthophylls are particularly sensitive to heat. Hexane is then added to the saponification mixture which is then made up to volume with sodium sulphate solution. The upper phase of this mixture is used for column chromatography.

Most investigators employing open column chromatography for the analysis of carotenoids in plant materials have used extraction procedures similar to those outlined above. Slight modifications to the extracting solvent have been used, for example using petroleum ether or diethyl ether in place of hexane. Quackenbush *et al.* (1970) employed a mixture of hexane–acetone–ethanol–toluene for overnight extraction of carotenoids.

Similar extraction procedures were also used by investigators employing HPLC as the separation process, for example early reports on HPLC by Sweeney and Marsh (1970), Van de Weerdhof *et al.* (1973), and Reeder and Park (1975). The hexane or petroleum ether extracts were injected directly into magnesia or alumina columns for normal-phase HPLC.

Non-polar solvents were also used by some investigators for extraction although reversed-phase HPLC employing a polar mobile phase was used for separation of the carotenoids. In the studies by Zakaria *et al.* (1979) and Braumann & Grimme (1981), petroleum ether was used for extraction. A small volume of the extract was injected directly into various reversed-phase columns and the carotenoids were eluted using mobile phases containing polar solvents such as acetonitrile, methanol. water and chloroform. Hsieh and Karel (1983) diluted the petroleum ether extract with the mobile phase (chloroform and acetonitrile mixture) before injection for reversed-phase

HPLC in order to ensure greater compatibility. Bushway and Wilson (1982) employed two approaches to the analysis of fruits and vegetables. For samples with high carotenoids, extraction was carried out using tetrahydrofuran (THF) and the extract used directly for reversed-phase HPLC. For the other samples, petroleum ether was used for extraction, evaporated dry and the residue re-dissolved in THF for chromatography. Mejia *et al.* (1988) and Khachik & co-workers had also employed THF as the extractant of carotenoids from various root and leafy vegetables (Khachik & Beecher, 1985, 1987, 1988; Khachik *et al.*, 1986, 1988). This solvent is usually stabilized with butylated hydroxytoluene to retard oxidation. Speek *et al.* (1986*a*, 1988), and Pepping *et al.* (1988) used a similar approach. Diisopropylether used for extraction was evaporated and the residue re-dissolved in the mobile phase for HPLC. In the study by Simon and Wolff (1987), hexane used as the extractant was evaporated and the carotenoids were dissolved in acetonitrile for HPLC. These are some examples of approaches used by various investigators in the extraction of carotenoids and preparation of the extract for HPLC.

For foods of animal origin

For foods of animal origin, mixed foods and processed foods, the AOAC method requires an initial alkaline hydrolysis to be carried out (Williams, 1984). After grinding the food, the sample is saponified using ethanolic potassium hydroxide. Retinoids (and carotenoids, if present) in the unsaponifiable fraction are then extracted several times into hexane. The hexane extract, after diluting to a suitable volume, is then chromatographed on a column of alumina.

Similar preliminary treatment procedures were used by investigators employing HPLC for the separation of carotenoids and retinoids. Most investigators carried out initial saponification of the sample using ethanolic potassium hydroxide, carrying out the refluxing at about 80°C. Stancher and Zonta (1982, 1984*a*, *b*), however, preferred cold alkaline digestion overnight in their studies of retinol in cheese, fish and fish oils. In a number of studies of various foods including oil and margarine, breakfast cereals, and infant formula, Landen & co-workers did not saponify the samples studied (Landen & Eitenmiller, 1979; Landen, 1980; Granade, 1982). In a recent study of various fortified foods such as dairy products, breakfast cereal and vitamin supplement, Ashoor and Knox (1987) also avoided saponification in order to limit loss of retinol. Barnett *et al.* (1980), on the other hand, incubated the infant formula and dairy products with lipase for 1 h at 37°C.

As for the analysis of carotenoids, earlier studies tended to rely on normal-phase HPLC. An example of such early study was that by Dennison

and Kirk (1977). Samples (cereal products) were saponified and retinol extracted using hexane. The combined hexane extract was evaporated and the residue redissolved in the mobile phase (mixture of hexane and chloroform). Also studying retinol in cereal products, Widicus and Kirk (1979) used chloroform as the extractant, which was subsequently evaporated and the residue dissolved in the same mobile phase as the previous study. Most of the other investigators relied on reversed-phase HPLC. Hexane was again commonly used as the extractant, while some investigators used petroleum ether or diethyl ether. Usually, the solvent was evaporated and the residue redissolved in the mobile phase or various components of the eluting solvent (e.g. acetonitrile, methanol, water, and ethyl acetate). Some examples of studies on various foods and feeds (infant formula, butter, margarine, milk and eggs) are Soderhjelm and Andersson (1978), Henderson and McLean (1979), Sivell *et al.* (1982), Wickroski and McLean (1984), and Reynolds and Judd (1984). A few investigators used one of the polar solvents for extraction, for example methylene chloride (Landen & Eitenmiller, 1979; Landen, 1980; Granade, 1982).

For serum and plasma

Saponification is usually not required for blood samples. Over 95% of the vitamin A normally found in fasting blood is bound to its carrier, a retinol-binding protein. Only after ingestion of a meal rich in vitamin A, after the administration of a high dose of vitamin A, or in certain disease states (e.g. vitamin A toxicity, severe liver disease, or severe protein-energy malnutrition) are lipoprotein-bound retinyl esters found in circulation (Arroyave *et al.*, 1982).

Samples are usually first treated with ethanol (or methanol) to precipitate the protein. Perchloric acid has also been used to denature the protein (Peng *et al.*, 1983; Nierenberg, 1985). The sample is next extracted with an organic solvent, e.g. petroleum ether or hexane. After separation from the protein, the absorbance of the extract may be read in a spectrophotometer to determine carotene and an aliquot reacted with antimony trichloride or trifluoroacetic acid to quantitate retinol. The organic extract may also be separated by high-pressure liquid chromatography (HPLC). If reversed-phase HPLC is used, the organic extract injected has to be compatible with the generally polar mobile phase used. Hence, if petroleum ether or hexane is used for extracting carotenoids and retinoids, the solvent has to be evaporated and the residue redissolved in a suitable solvent such as isopropanol (Puglisi & de Silva, 1976; Katrangi *et al.*, 1984) prior to separation by HPLC. On the other hand, solvents such as a mixture of tetrahydrofuran and ethyl acetate (Peng *et al.*, 1983; Nierenberg, 1985) and

chloroform (Broich *et al.*, 1983) may be used for extracting carotenoids and retinoids, and the extract used directly for HPLC.

Smidt *et al.* (1988) used a different approach to the preparation of rat liver and plasma for the analysis of retinol and α-tocopherol. Lipid was extracted using dichloromethane with added butylated hydroxytoluene and saponified. After extraction of non-saponifiable matter, the vitamins were isolated on a digitonin-impregnated celite column and subsequently subjected to gas–liquid chromatography.

Precautions in handling of samples

The main problems associated with work on retinoids and carotenoids arise from the inherent instability of these compounds, especially towards light, heat, oxygen and acids (Tee, 1988). To prevent oxidation of the vitamins during saponification, extraction and chromatography, some investigators have reported the use of various antioxidants during these processes. These include the use of ethoxyquin (Nelson & Livingston, 1967), pyrogallol (Thompson & Maxwell, 1977; Henderson & McLean, 1979), ascorbic acid (Gross *et al.*, 1971; Egberg *et al.*, 1977; Soderhjelm & Andersson, 1978; Lawn *et al.*, 1983; Stancher & Zonta, 1982, 1984*a*, 1984*b*; Wickroski & McLean, 1984; Reynolds & Judd, 1984), sodium ascorbate (Speek *et al.*, 1986*a*), and hydroquinone (Baloch *et al.*, 1977; Santoro *et al.*, 1982). Butylated hydroxytoluene (BHT) appears to be one of the most commonly used antioxidants, used by various investigators over the years (Stewart & Wheaton, 1972; Reeder & Park, 1975; Stewart, 1977*a*; Klein & Perry, 1982; Chow & Omaye, 1983; Grace & Bernhard, 1984; Nierenberg, 1985; Bureau & Bushway, 1986; Tan *et al.*, 1986; Speek *et al.*, 1986*b*; Ashoor & Knox, 1987; Lauren *et al.*, 1987; Khachik & Beecher, 1988; Khachik *et al.*, 1988; Ng & Tan, 1988).

The antioxidants have been used in several ways; for example, they are added to the sample during grinding, or saponification, or added to solvents such as THF and diisopropylether, or to the standard carotenoids and retinol. The advantage of using antioxidants has not been clearly shown. In many cases, it has not been shown that adding antioxidants improved results, although they did not seem to have adverse effects, either (Parrish, 1977). The AOAC procedures have not suggested the use of these antioxidants in the analysis of retinoids and carotenoids (Williams, 1984).

Besides the use of antioxidants, other measures have also been taken to prevent oxidation of these compounds. Oxygen-free nitrogen has often been used to create an inert atmosphere. This is frequently used when evaporating solvents, usually at elevated temperatures, from test solutions to reduce the volume or to enable the use of another solvent. The use of a stream of

nitrogen not only excludes oxygen from the warm solution, but also speeds up the evaporation process. Other precautions that need to be taken include protection of samples from acids and metal ions which may be present in the solvents used. Particular attention should therefore be given to the use of suitable solvents, or to carry out purification procedures of the solvents. In using HPLC as the separation procedure, another factor to be remembered is the possibility of interference from impurities, particularly when monitoring at low wavelength.

The need for protection of analytical samples from direct exposure to light, particularly sunlight, has often been emphasized. Carotenoids and retinoids undergo structural photo-transformations, particularly when exposed to light in the ultra-violet region (below 350 nm). Various approaches can be taken to minimize this destruction of the compounds. Sunlight in the laboratory can be greatly reduced by using suitable blinds or tinted windows and glass panes. Amber (low-actinic) glassware may be used, although it is rather expensive and difficult to obtain. Aluminium foil may also be used to wrap containers or chromatography columns.

Proper storage of solutions in carotenoid and retinoid analysis has often been emphasized. Solutions should be stored in the dark (e.g. in amber containers), at about $-20°C$, and, if possible, under nitrogen. Containers should be scrupulously cleaned, with well-sealed containers. Standard solutions are not stable over long periods, and their concentration and purity must be checked before use. Routine UV–vis spectral analysis of solutions would be an easy way to detect gross changes in standard or sample solutions.

SEPARATION AND QUANTITATION PROCEDURES

General trend in procedures

β-Carotene and several carotenoids are coloured and absorb maximally at about 450 nm. These compounds can be easily detected and quantitated in a spectrophotometer in the visible range. Similarly, retinol may be detected and quantitated by measurements at 325 nm. It is thus possible to quantitate these compounds without any prior separation procedure. For the determination of β-carotene and retinol in serum, hexane extracts may be read at the two wavelengths mentioned (Bessey *et al.*, 1946; Underwood & Stekel, 1984). Several reports were also encountered in the literature where food extracts were read directly in a spectrophotometer at wavelengths around 450 nm (Thomas, 1975; Mudambi & Rajagopal, 1977; Maeda & Salunkhe, 1981; and Picha, 1985). The most important limitation of such

procedures is that, because no prior separation process is carried out, absorbance readings taken at selected wavelengths are not specific for the compounds of interest. Thus, for carotene estimation at 450 nm, the results so obtained would, at best, be referred to as total carotenoid concentrations. Similarly, absorbance at 325 nm should be related to retinoids rather than retinol.

Another approach that was adopted for the quantitation of retinol was to react the vitamin with various reagents to produce a coloured compound which was then read in a spectrophotometer. The search for a colorimetric method to replace the more tedious biological test based on growth promotion of experimental animals for quantitating vitamin A was initiated even in the early part of the century. One of the earliest reagents studied was sulphuric acid which gave a transient purple colour with liver oils rich in vitamin A (Drummond & Watson, 1922). These investigators demonstrated that there was a positive correlation between the colours produced by oils of various concentration and growth-promoting ability when fed to experimental animals. Since the colour produced disappeared too rapidly for quantitative colorimetric comparison, various other reagents were investigated. Rosenheim & Drummond (1925) described their experiences with the use of arsenic chloride and trichloroacetic acid, both of which produced a brilliant blue colour, which persisted long enough to allow colorimetric comparison with a suitable standard.

Subsequently, efforts were made to further improve the colorimetric method for the quantitation of vitamin A. The search was for similar reagents that could give more quantitative and reproducible results. Carr and Price (1926) proposed the use of a solution of antimony trichloride in chloroform as the colour reagent. The blue colour produced was said to be more intense and more permanent than other reagents tried, with more reproducible results being obtainable. The Carr–Price reaction became well received by many analysts and was widely used in various analytical procedures. Adapted for use for serum samples, hexane extracts were evaporated and the residue reacted with antimony trichloride reagent for the quantitation of retinol. The procedure has been used widely for the assessment of the vitamin A status of communities (Interdepartmental Committee on Nutrition for National Defence, 1957). Some investigators subjected the serum extract to an initial alumina column chromatography procedure, but a separation step was often not included, especially when a large number of samples needed to be analyzed.

The Carr–Price reaction has also been widely used for the quantitation of retinol in food samples. In the AOAC method, the reaction is used in conjunction with the alumina column chromatography method, after initial separation of retinol from the carotenoids (Williams, 1984). This will be

further elaborated in the section dealing with adsorption column chromatography.

Several problems have, however, been encountered in the use of the Carr–Price reaction. It has been established that even small amounts of moisture will result in turbidity of the antimony trichloride reagent. The evanescent nature of the blue colour obtained presents considerable practical difficulty in taking readings in a colorimeter. The search for an even more suitable colour reagent thus continued. Trifluoroacetic acid was subsequently offered as an alternative. The reagent has been said to retain the sensitivity and specificity of the antimony trichloride reaction, but does not exhibit the turbidity property of the antimony trichloride reagent (Neeld & Pearson, 1963). It is also less toxic and does not form a tenacious film on the cuvette. The blue colour formed with trifluoroacetic acid, however, is also transitory.

Retinol and its esters fluoresce in ultra-violet light, and this property has been used for quantitating the vitamin. Fluorescence may be determined using an excitation wavelength of 330 nm and emission of 480 nm. Fluorometric measurements of serum extracts have been described by Thompson *et al.* (1971) and Wu *et al.* (1981). It has been suggested that corrections be made for fluorescence by interfering compounds. The carotenoid phytofluene was felt to be the only significant fluorometrically active contaminant in human serum (Thomas *et al.*, 1971). Fluorescence detection has also been used for the analysis of vitamin A in foods. The technique has been used after initial fractionation of food samples using high-pressure liquid chromatography (HPLC). Several studies employing this approach will be cited in the section dealing with HPLC.

Most of the studies encountered in this review generally involved a separation prior to the quantitation of carotenoids and retinoids. As can be expected, this results in better quantitation of the compounds of interest. This review is thus mainly concerned with procedures involving various separation procedures.

A wide variety of separation and detection and quantitation procedures have been used in studies of carotenoids and retinoids. A general trend in the change of these methodologies is evident over the the last two decades. An early technique used for the separation of carotenoids in plant materials, mainly in the 1960s, was countercurrent distribution. A few early studies using paper chromatography were also reported. In the 1970s, a few studies of carotenoids and retinoids using gas–liquid chromatography and gel-permeation chromatography were encountered. A number of studies employing thin-layer chromatography as a single technique for the separation of carotenoids in plant materials were encountered throughout the 1970s and the early 1980s. TLC was also used by several investigators in combination with other separation techniques. The procedure was less

widely used in studies of retinoids. Adsorption (open) column chromatography, utilizing primarily descending, gravity-flow columns, was widely used for the study of carotenoids and retinoids in foods, even in the 1960s. The procedure remained very much in use in the 1970s and 1980s. Commencing from the late 1970s, a new column chromatography procedure became more prominent in the literature. High-pressure liquid chromatography has become a widely used procedure for the separation of carotenoids and retinoids in various materials.

This review discusses the development in methodologies for the separation and quantitation of carotenoids and retinoids, particularly in food and blood samples. Each procedure will be separately treated, and examples of reports using the technique will be given to illustrate its use. Wherever possible, the limitations and advantages of each method will be discussed. For each procedure, its use for the analysis of carotenoids will first be dealt with, followed by its application to retinoid analysis.

Counter-current distribution

Liquid–liquid partitioning between immiscible solvents has been used in the purification and characterization of carotenoids. The technique may be used as a useful preliminary to the chromatographic stages of purification and isolation of these compounds (Davies, 1976). Counter-current distribution, a refinement of liquid–liquid partitioning, was one of the earliest methods for the separation of carotenoids. It has been used by a few investigators for the separation of carotenoids into different polarity subgroups, which could then be fractionated further by adsorption column chromatography. Examples of studies employing this technique are those by Curl (1962, 1964, 1967), Brossard & Mackinney (1963), Morgan (1967), Yokoyama & White (1967), and De La Mar & Francis (1969). All these studies were carried out on vegetables and fruits, such as bell pepper, persimmon, orange peel and grapefruit. Counter-current distribution usually resulted in the separation of carotenoids into hydrocarbons, monols, and diols. The various fractions were usually re-chromatographed on columns of magnesia, or mixtures of magnesia and Celite, kieselguhr, or Hyflo Super Cel. Identification of pigments was carried out by a combination of techniques, including co-chromatography with known pigments, observation of order of elution, absorption spectra of the various bands, and chemical methods.

Counter-current distribution has not been widely used; no reports using the technique were encountered from 1970 onwards. The main limitation of the procedure is thought to be the high cost of the equipment, and the fact that superior separations may be achieved by using adsorption chromatography (Davies, 1976). It has also been pointed out that prolonged

exposure of carotenoids to extraneous influences during counter-current distribution may be undesirable as it may lead to the formation of artefacts.

Paper chromatography

Adsorption chromatography on paper was another early method used for the separation of carotenoids. Only a few studies using the procedure were reported in the literature, all before the 1970s. The procedure was based on chromatography on paper impregnated with other adsorbents, e.g. zinc carbonate (Bayfield *et al.*, 1967), magnesium oxide (Katayama *et al.*, 1971), and silica gel or aluminium hydroxide (Sherma, 1971). The papers were chromatographed in the typical ascending manner, employing both one- and two-dimensional chromatography. Plant materials such as leaves and fruits were studied in these reports. Bayfield *et al.* (1968) also reported the study of carotenoids in serum or plasma using the technique.

Only one report using paper chromatography for the fractionation of retinoids was encountered in the literature, i.e. the study by Jungalwala and Cama (1962) on fish liver oils. Circular chromatography paper was impregnated with vaseline for the separation of vitamins A1, A2 and allied substances, and β-carotene.

Davies (1976) compared the techniques of chromatography on thin layers and adsorbent-loaded (impregnated) papers. Both methods were said to be roughly equivalent in terms of sample size and speed of development, although some thin-layer chromatographic systems run more slowly. The latter technique was said to be capable of giving higher sample recoveries and more reproducible R_f values. Thin-layers, however, were said to be more versatile in that more adsorbents are available and may give better resolution. Like thin-layer chromatography, carotenoids and retinoids may be subjected to degradation and isomerization during paper chromatographic analysis, and thus cannot be used in isolation for the positive identification of these compounds (Taylor, 1983).

Gas–liquid chromatography (GLC)

As for paper chromatography, very few studies of retinoids and carotenoids have been reported using GLC. These were mostly in the early 1970s and very few of them dealt with studies on food samples or biological specimens. For example, Fenton *et al.* (1973) studied the separation of retinol and retinol acetate using GLC fitted with a flame ionization detector. Taylor and Davies (1975) reported separating standard preparations of various carotenoids using GLC. Vecchi *et al.* (1973) studied the separation of vitamin A isomers and related compounds using GLC and high-pressure

liquid chromatography (HPLC). The latter technique was found to give better separation of the compounds studied. One of the few studies employing GLC for the analysis of retinol and α-tocopherol in rat serum and liver was reported by Smidt *et al.* (1988). A fused-silica capillary column coated with a film of methylsilicone was used with a flame ionization detector. A mass spectrometer was coupled to the gas chromatograph to confirm identity of peaks.

GLC has been found to be not particularly suited to the analysis of carotenoids and retinoids. These two groups of compounds cannot be analyzed directly by GLC because of the inherent thermal instability of their polyene chain and because of their low volatility (Liaaen-Jensen, 1971; Taylor & Davies, 1975). In spite of this limitation, Taylor (1983) felt that prior hydrogenation of the compounds and subsequent GLC may be useful for rapid, tentative identification, as well as providing information on structural features and molecular size. Only a very limited number of carotenoid derivatives have been analysed successfully by the method. Examples of these are separation of perhydrolycopene, perhydro-γ-carotene, and perhydro-β-carotene (Liaaen-Jensen, 1971). Similarly, combination of gas chromatography and mass spectrometry (GC/MS) has had limited applications in carotenoid and retinoid analysis.

Gel-permeation chromatography (GPC)

Even fewer studies have been reported on the use of gel-permeation chromatography. Holasova and Blattna (1976) reported the study of retinol, retinyl esters and carotenes in margarine and liver using Sephadex in a glass column.

More recently, Landen and his co-workers reported several studies using a combination of high-pressure liquid chromatography (HPLC) and high-pressure gel-permeation chromatography (HP–GPC) for the determination of retinol and carotene in several food products. Reports include Landen and Eitenmiller (1979) (oil and margarine), Landen (1980) (breakfast cereals), Landen (1982), Granade (1982) and Landen *et al.* (1985) (infant formulae). These will be discussed in the section on high-pressure liquid chromatography. This is a relatively new technique, and few other reports were encountered in this review of the literature.

Thin-layer chromatography (TLC)

There have been a number of studies employing thin-layer chromatography as a single technique for the separation of carotenoids in plant materials. These were encountered throughout the 1970s as well as in the early

1980s. Two early reports were those by Nelson and Livingston (1967) and Sanderson *et al.* (1971), studying alfalfa meal and tea leaves, respectively. Separation was carried out on layers of silica gel G. Spots on chromatograms were scraped off, eluted and their absorbance spectra were obtained. Silica gel G layers were also used by Martin *et al.* (1974) in the study of carotenoids in yam, Csorba *et al.* (1979) in their study on spinach, and Barro and Gonzalez (1981) studying changes of carotenoids of four *Vicia* species at five different stages of growth. Csorba *et al.* (1979) reported better separation when ammonium sulphate was added at a concentration of 10% to the silica gel. Other adsorbents used included magnesium oxide and Celite mixture (Knowles & Livingston, 1971), cellulose impregnated with coconut oil in petroleum ether (Chan *et al.*, 1975), a mixture of Hyflo Super Cel and magnesium oxide (Baloch *et al.*, 1977), and magnesium oxide (Sadowski & Wojcik, 1983). A variety of solvent mixtures have been used to develop the chromatograms, including benzene–butanol, petroleum ether–benzene–ethanol, cyclohexane–ethyl ether, acetone in petroleum ether, isooctane–acetone–diethyl ether, hexane–acetone, and methanol–acetone–water.

A more recent development is the use of reversed-phase TLC (RP–TLC) in the separation of carotenoids. Isaksen and Francis (1986) studied the separation of various carotenoid standards on commercially coated 0·25 mm RP-18 plates. A wide variety of solvent compositions consisting of petroleum ether–acetonitrile–methanol were found to be suitable for the separation of the normal range of carotenoids on RP-18 thin layers. The method was said to be markedly superior to normal-phase TLC, which required serial chromatography to achieve similar separation.

TLC has also been used by several investigators in combination with other separation techniques. It may be used for further purification of carotenoids already separated by column chromatography. For instance, in the study of Shamouti orange juice by Gross *et al.* (1971), the extract was first separated on a column of magnesium oxide and Hyflo Super Cel mixture. The hydrocarbon, monol, diol and polyol fractions obtained were then further separated by TLC. The first two fractions were separated using calcium hydroxide–silica gel G mixture as adsorbent, while silica gel G was used for the polyol fraction. Various solvent mixtures were used to develop the chromatograms. Identification of the pigments was on the basis of chromatographic and spectrophotometric properties, as well as chemical tests. Quantitation for some of the carotenoids was carried out. The procedure was also used by these investigators in the study of carotenoids in avocado pear (Gross *et al.*, 1972). El-Difrawi and Hudson (1979) used an alumina column for the initial separation of pigments in Lupin seeds. Separation was achieved by stepwise increases in polarity of the eluting solvent by successive increases in the proportion of acetone:hexane in

mixtures of the two solvents. Eluates from the column were further examined by TLC in two separate adsorbents, cellulose–silica gel and silica gel. UV spectrophotometric studies were carried out on the separated fractions. In the study of tomatoes by Jen (1974) and Thomas and Jen (1975), TLC was similarly used to assist in the identification of carotenoids after an initial adsorbent column chromatography.

In a slightly different approach, Buckle and Rahman (1979) first employed TLC of *Capsicum* fruit extracts on layers of cellulose using four solvent systems. The separated bands were further chromatographed on a column of magnesium oxide and Hyflo Super Cel. Elution of pigments was carried out using hexane containing increasing proportions of benzene. Pigments that were still unresolved were further purified on an alumina column, and the chromatogram was developed with increasing amounts of diethyl ether in petroleum ether.

More recently, TLC has also been used in conjunction with high-pressure liquid chromatography (HPLC); for example, in assisting in the identification of carotenoids. In the study of α- and β-carotene in several fruits and vegetables by Bushway and Wilson (1982) using reversed-phase HPLC, TLC was used to confirm the peaks obtained from the former technique. Two TLC systems were used, one using silica gel G with an eluant of methanol–petroleum ether and the other a reversed-phase system with a solvent mixture of acetonitrile–tetrahydrofuran–water. In a recent study, Schwartz and Patroni-Killam (1985) used a two-dimensional TLC procedure to assist in the study of *cis*- and *trans*-carotene isomers in standard preparations as well as a few vegetables using HPLC. The adsorbent used for the TLC procedure was calcium oxide, and the chromatogram was developed using *p*-methylanisole in petroleum ether for 1–1·5 h in each direction. In the recent study by Philip *et al.* (1988) of orange juice concentrates, TLC on silica gel G layers was first carried out for initial separation of the pigments. Solvent systems used were 5% acetone in petroleum ether for carotenes, diesters, and cryptoxanthin esters, and 20% acetone in petroleum ether for monoesters of dihydroxy carotenoids and citraurin esters. The TLC bands were scraped off, extracted with acetone, and further separated by reversed-phase HPLC.

In a more complicated study, Simon and Wolff (1987) used a combination of HPLC, column chromatography and TLC for the study of carotenoids in carrot. Extracts were chromatographed separately by reversed-phase HPLC, chromatography on a column of silica gel followed by magnesia-Hyflo Super Cel column, and thin-layer chromatography with magnesium oxide-kieselguhr. The four bands from column chromatography were further chromatographed individually with TLC and HPLC. Likewise, the

six bands from TLC were chromatographed individually with column chromatography and HPLC. Total carotenoids, α- and β-carotenes were thus separated and quantitated.

TLC has been shown to be a widely used technique for the separation of carotenoids, mainly because of its low cost and simplicity. It appears to be a particularly useful technique when used in combination with other techniques for further separation and purification of fractions, or in assisting in the identification of carotenoid fractions. Davies (1976) has described some useful practical aspects of TLC of carotenoids including choice of adsorbents, preparation and running of chromatograms, detection of carotenoids, and elution of carotenoids from thin layers.

The procedure was less widely used in studies of retinoids. Most of the studies encountered were carried out on standard preparations of various retinoids; for example, retinol, retinol esters, retinal, and retinoic acid (Fung *et al.*, 1978), or their degradation products (Parizkova & Blattna, 1980). Studies have also been carried out on mixtures of retinoids with other fat-soluble vitamins (Davidek & Blattna, 1962; Lovelady, 1973), or with carotenoids (Keefer & Johnson, 1972). The usual adsorbents were used, including alumina, magnesium hydroxide, and silica gel.

John *et al.* (1965) reported the separation of vitamins A1 and A2 and allied compounds (including β-carotene) in fish liver oils and rat liver extracts using thin layers of Kieselgel mixed with plaster of Paris. Developed plates were examined under UV light and reaction with antimony trichloride reagent. Spots were scraped off the plates and eluted with a diethyl ether–petroleum ether mixture, and their absorption spectra were obtained. The use of TLC for analysis of retinoids in biological specimens was also reported by Kahan (1967). Extracts of human sera and post-mortem liver samples were separated on layers of silica gel G using various solvent systems. Reversed-phase TLC was also carried out, where plates were immersed in paraffin oil in petroleum ether and developed using methanol saturated with paraffin oil. Various reagents and UV light were used in the characterization of the compounds separated.

Adsorption column chromatography

Unlike other previously discussed separation techniques, adsorption column chromatography has been relatively widely used in the study of carotenoids and retinoids in foods. The technique has also been referred to as open column chromatography. Basically, it utilizes descending, gravity-flow columns, as distinguished from columns running under pressure, such as those in high-pressure liquid chromatography (HPLC). In the following

discussion, its use in the analysis of carotenoids in plant materials will first be discussed, followed by the use of this technique for the separation of retinoids.

Adsorption column chromatography has been widely used for the fractionation of carotenoids in plant materials, even in the 1960s. Most of these studies have employed procedures very similar to those described in the AOAC manual, which described methods for two groups of plant materials; namely, carotenes in fresh plant materials and silages, and carotenes and xanthophylls in dried plant materials and mixed feeds. The procedures described in the latest edition of the manual (Williams, 1984) have not changed very much from those prescribed in the earlier edition (Horwitz, 1970). The only major change was the inclusion of the alternative of hot saponification in the later edition. The technique has developed over at least two decades. Davies (1976) and Taylor (1983) have discussed in some detail several practical considerations in the use of the technique, including choice of adsorbent and solvent, and preparation of column for chromatography.

In the AOAC method for determination of carotenes in fresh plant materials, chromatography is carried out in a 22 mm (o.d.) × 175 mm glass column, packed with a mixture of activated magnesia (Sea Sorb 43) and diatomaceous earth (Hyflo Super Cel). The carotene band, visible on the column, is eluted using a solvent mixture of acetone–hexane. The carotenes pass rapidly through the column, leaving behind bands of xanthophylls, carotene oxidation products and chlorophylls on the column.

After the eluate is made up to a suitable volume in a mixture of acetone–hexane, the absorbance of the solution is read in a spectrophotometer at 436 nm. Quantitation of the sample is carried out by comparing absorbance reading with a standard curve prepared from β-carotene.

Various workers have used procedures very similar to those described above for the study of carotenoids in plant materials. One early study was that by Lewis and Merrow (1962), who reported the separation of carotenes from butternut squashes using magnesia and Hyflo Super Cel mixture as adsorbent. The method was compared to a separation technique using a silica gel column. Another study in the 1960s was that by Yokoyama and Vandercook (1967), who reported the separation of a wide range of carotenoids from lemon using the same adsorption columns, except that there was a preliminary chromatography on a column of silica gel. Other studies using similar techniques were reported by Rymal and Nakayama (1974), Jen (1974), Thomas and Jen (1975), Watada *et al.* (1976), Raymundo *et al.* (1976), and Liu and Luh (1977), all of which were studies on carotenoids in tomatoes. The separation of carotenoids in Shamouti orange juice was reported by Gross *et al.* (1971, 1972), in cashew apple by Cecchi and

Rodriguez-Amaya (1981), a number of vegetables by Klein and Perry (1982), in Brazilian fruit by Rodriguez-Amaya *et al.* (1983), in lettuce by Rouchaud *et al.* (1984), and in Brazilian guavas by Padula and Rodriguez-Amaya (1986). Begum and Pereira (1977) reported the use of the procedure for the determination of the carotene content of thirty-two types of edible plants. Most of the studies cited above used increasing proportions of acetone in hexane or petroleum ether to effect the elution of the various carotenoids. In some of these studies, fractions with more than one carotenoid were further chromatographed on a column of alumina. TLC using silica gel layer was also used by a few investigators to aid in the identification of carotenoids.

For the determination of carotenes and xanthophylls in dried plant materials and mixed feeds, AOAC has designated a different chromatographic procedure (Williams, 1984). The adsorbent used in a 12·5 mm (i.d.) × 30 cm glass column is a mixture of silica gel G and diatomaceous earth (Hyflo Super Cel). Carotenes are eluted from the column using a mixture of hexane–acetone (96 + 4), leaving behind the xanthophylls. Monohydroxy pigments (zeinoxanthin and cryptoxanthin) are eluted from the column using a mixture of hexane–acetone (90 + 10), whilst the dihydroxy pigments (lutein, zeaxanthin and their isomers) are eluted using hexane–acetone (80 + 20). The polyhydroxy pigments are said to remain on the column. Absorbance of solutions of the eluted carotenes, and mono- and dihydroxy pigments are obtained at 436 nm for the former and 474 nm for the latter two. Quantitation of these pigments is by comparing absorbance readings with that obtained using C.I. Solvent Yellow 14 dye.

If the concentration of total xanthophylls is required for these dried plant materials and mixed feeds, chromatography has to be performed on a mixture of activated magnesia and diatomaceous earth as adsorbent. Carotenes are eluted from the column with hexane–acetone (90 + 10), whilst total xanthophylls are eluted using hexane–acetone–methanol (80 + 10 + 10). Quantitation of these two fractions is then carried out as described in the previous paragraph.

Kohler *et al.* (1967) reported the separation and quantitation of carotenes and total xanthophylls from fresh and dehydrated alfalfa and grass meal using the adsorbent and eluant described earlier. Quackenbush (1970) and Quackenbush *et al.* (1970) reported the separation of carotenes, mono-, di- and polyhydroxy pigments in alfalfa meals, corn products and feed materials. Other studies on alfalfa and other feed materials using similar chromatographic procedures were those by Knuckles *et al.* (1971, 1972), Livingston *et al.* (1971, 1973), Livingston (1986), Knowles *et al.* (1972), and Quackenbush (1973, 1974). Quackenbush and Miller (1972) reported the use of the procedure in the study of marigold petal, Lee *et al.* (1981) in sweet corn, and Edwards and Lee (1986) in carrots and green peas. To effect the

separation of several carotenoids in pumpkin of various colours, Hidaka *et al.* (1987) first subjected the extracts to phase separation using hexane and methanol. The fractions were then chromatographed in a column of magnesia and Hyflo Super Cel. Pigments were eluted with a stepwise increase of acetone in hexane.

Other adsorbents have also been used for the separation of carotenoids in open-column chromatography. However, studies involving these alternative adsorbents are relatively few. In one of the earliest studies in this field, the fractionation of carotenoids in corn grain was reported by Quackenbush *et al.* (1961) using a magnesia column. Hydrocarbons were eluted from the column with 5% acetone in hexane, zeinoxanthin and cryptoxanthin with 10% acetone in hexane, and lutein with acetone–hexane–ethanol mixture. Curl (1967) used a column of magnesia and Celite for the separation of carotenoids in orange peel. In the same year, Morgan (1967) fractionated several carotenoids in watermelon using a mixture of magnesium carbonate and kieselguhr, developed with increasing proportions of acetone in petroleum ether. Both these studies in 1967 employed an initial counter-current distribution procedure for a preliminary separation of the pigments. Yokoyama and White (1967) also employed an initial counter-current distribution procedure, followed by three separate column chromatographic procedures using magnesia-Hyflo Super Cel, alumina, and Microcel C to effect separation of carotenoids in grapefruit.

Schaller and von Elbe (1971) used a column of silicic acid saturated with methanol for the separation of carotenoids in cherries into hydrocarbons, monols, and diols and polyols. Each of the three fractions was then further fractionated on a column of magnesia–Celite mixture. Ogunles and Lee (1979) also used the same adsorbent for initial separation of pigments in carrot, but the fractions were further chromatographed on a column of magnesia-Hyflo Super Cel. Magnesia–Celite mixture was also used by Buescher and Doherty (1978) for the study of carotenoids in tomatoes.

Alumina was used by several investigators for column chromatography of carotenoid pigments. For the separation of carotenoids in hibiscus flowers, leaves and buds, Hanny *et al.* (1972) carried out initial chromatography using an alumina column to obtain fractions of hydrocarbons and xanthophylls. The former fraction was further chromatographed using a column of magnesia–Celite. The xanthophyll fraction was separated on a thin-layer of vegetable oil-coated kieselguhr. An alumina column was also used by Head (1973) in the separation of carotenoids in *Pyrethrum* flowers, followed by TLC separation of the various fractions. In the study by Begum and Pereira (1977), thirty-two edible plants were analysed for β-carotene content. Buckle and Rahman (1979) used an alumina column as well as a column of magnesia-Hyflo Super Cel for further purification of pigments

obtained from chromatography of *Capsicum* fruit extracts on a thin-layer of cellulose powder. Yet another example of the use of alumina columns was provided by El-Difrawi and Hudson (1979), studying lupin seeds. More recently, Tan *et al.* (1986) described the use of aqueous deactivated alumina for the separation of carotenoids in palm oil. In the last two reports cited, TLC was used for further fractionation of eluates from the column. Thus, several of the reports cited above also illustrate the use of a combination of TLC and column chromatography techniques in the fractionation of carotenoids. In a recent study of various seed oils, Essien *et al.* (1989) had employed an alumina column for initial separation of the pigments, followed by rechromatography on a magnesium oxide column.

Adsorption column chromatography has thus been widely used by various investigators for the separation of carotenoids. Various plant materials have been studied, including some fruits, vegetables, cereals, and feed materials. Besides the studies cited in this review, the method has been used by many laboratories for the generation of data for food composition tables. Most of the studies cited in this review had reported fractionation of a wide range of carotenoids. Using a combination of column chromatography and TLC, Gross *et al.* (1971) reported the fractionation of fifty carotenoids in Shamouti orange juice. To achieve the required fractionation, most of the studies had used somewhat complicated procedures, including the use of stepwise elution of pigments from the column and combination of column and thin-layer chromatography. Such procedures would, of course, not be practical for routine use in the determination of carotenes in a large number of samples.

The AOAC procedures, and other adsorption column chromatographic techniques summarized above have been noted to have various disadvantages. First, these procedures are long and laborious, and only a few samples may be handled at a time. Other potential sources of errors in these procedures include incomplete solvent extraction, destruction and stereochemical changes during extraction and chromatography (Beecher & Khachik, 1984).

The major problem in adsorption column chromatography procedures is related to the quantitation of vitamin A activity of the sample, based on the separation obtained. For fresh plant materials, carotenes are separated from xanthophylls, but the method does not separate individual carotenes, their *cis* isomers, or carotenoid esters. The carotenes eluted are assumed to be total β-carotene and quantitated as such. This assumption gives rise to considerable error in the calculated vitamin A activities, since the carotenes possess widely differing biological activity. In the method for dried plant materials, individual carotenes are again not separated, whilst xanthophylls may be further separated as mono- di- and polyhydroxy pigments using

other adsorbents and eluting solvent mixtures. This non-separation of the various carotenes and xanthophylls is a major drawback of these procedures and constitutes a major source of analytical errors in the vitamin A values obtained.

Throughout the 1970s and early 1980s, several studies on the use of adsorption column chromatography for the study of retinoids were reported. These were, however, much fewer in number compared with those dealing with separation of carotenoids in plant materials. One of the earliest studies using this procedure was reported by Erdman *et al.* (1973), where the investigators studied a variety of foods including cereals, meat, margarine and butter. The methods reported in the literature are essentially those in the AOAC manual (Horwitz, 1970). After saponification of the food to remove fat-soluble interfering substances, vitamin A and carotenoids remaining in the unsaponifiable fraction were extracted into hexane. The extract prepared was next chromatographed on a column of deactivated alumina to further purify the extract. Carotenes in the extract were separated in the process and eluted using 4% acetone in hexane. Retinol remaining on the column, visible by brief inspection with UV light, was eluted using 15% acetone in hexane. The volume of the eluates was suitably adjusted and read in a spectrophotometer. The carotene eluate was read at 450 nm, while retinol was reacted with antimony trichloride (Carr–Price reaction) and read at 620 nm.

Other reported works using the procedure include those by Parrish (1974*a*, *b*) for the analysis of mixed feeds and foods, and Parrish *et al.* (1980) for several cereal products. Parrish (1980) also reported some modifications to the procedure for the analysis of margarine and butter, and included the use of trifluoroacetic acid to replace antimony trichloride. Cohen and Lapointe (1978) reported the analysis of vitamin A in animal feed using this procedure, and compared results with a HPLC method. Egberg *et al.* (1977) also compared the results obtained for the analysis of several food products using HPLC and column chromatography methods. The adsorbent used in the latter was silica gel. Both groups of investigators reported that results obtained by the HPLC and AOAC methods were comparable.

The method has not changed very much since it was reported in the 11th edition of the AOAC manual (Horwitz, 1970). The 14th edition of the publication (Williams, 1984) retains all the main features of the method. Whilst previously it was specified for the analysis of mixed feeds, the latest edition of the manual has extended it to the analysis of pre-mixes and foods as well. It has become a widely used procedure, mainly because it does not require sophisticated instrumentation. It is, however, a rather tedious method, requiring several hours for the separation. Because of this long separation time, retinoids and carotenoids in the open column chroma-

tography procedure are exposed to the destructive influences of light and oxygen (Taylor, 1983). In addition, resolution of the compounds to be separated is not entirely satisfactory. In the AOAC procedure, only separation of retinols from carotenes is achieved, while separation of individual retinols and carotenes is not effected. Cryptoxanthin and similar pigments are eluted in the retinol fraction. If these are present in significant amounts, a correction has to be made to the readings obtained, since they also react with antimony trichloride.

Another major problem with the method is related to quantitation of the separated retinol. Difficulties encountered in using antimony trichloride colour reagent have been previously discussed. Attempts to overcome these difficulties included finding alternatives to antimony trichloride, e.g. trichloroacetic acid (Kamangar & Fawzi, 1978), and trifluoroacetic acid (Wang *et al.*, 1978).

An alternative to the procedure was to read the eluate directly in the ultraviolet region. In the IMR procedure, vitamin A is measured by its absorption at 325 nm (Tee *et al.*, 1987). This also has several limitations, including the fact that the absorbance is only about one-third of that as with the antimony trichloride reagent. More importantly, direct ultraviolet absorption of vitamin A is non-specific, since other compounds are likely to contribute to the absorption at 325 nm.

High-pressure liquid chromatography

From the late 1970s, numerous studies of carotenoids and retinoids using high-pressure liquid chromatography (HPLC) were found in the literature. It is quite obvious that the technique is replacing other methods, including the once widely used column chromatography procedure. The discussion below serves to provide some examples of the use of the technique in the study of various food samples and biological specimens, to illustrate the technique involved and the capabilities of the method.

Carotenoids in plant materials

One of the earlier studies on plant materials using HPLC made use of a mixture of calcium and magnesium hydroxides for the separation of carotene stereoisomers in several vegetables (Sweeney & Marsh, 1970). Rather large diameter (15 mm i.d.) columns were used, specially designed to withstand pressures of up to 20 psi. Seven isomers were eluted using *p*-methylanisole in petroleum ether and acetone in petroleum ether. Fractions were concentrated and studied in a spectrophotometer. In the following year, Stewart and Wheaton (1971) reported the use of a method that bears closer resemblance to the present-day HPLC procedure. A pump capable of

operation up to 2000 psi was used, and several gradient systems were studied. Both glass and stainless steel columns were used, and a variety of adsorbents were tried. Magnesium oxide was selected for the separation of carotenes and zinc carbonate for xanthophylls. Pigments were eluted using hexane and tertiary pentyl alcohol mixtures, and effluent monitored at 440 nm in a spectrophotometer. TLC (on layers of zinc carbonate) was used to aid in the identification of carotenoids.

Subsequent studies made use of improved instrumentation as well as smaller-diameter (3–5 mm) columns constructed of stainless steel. Reeder and Park (1975) reported the separation of carotenoids in orange juice using two columns: basic alumina for carotenes and Spherisorb for cryptoxanthin. The eluant used for the first column was benzene–hexane, while tetrahydrofuran–hexane was used for the second column. Peaks were detected at 440 nm and plotted on a recorder. Also working on orange juice, Stewart (1977*a*) reported an improved procedure employing a single column of magnesia and gradient elution using acetone–hexane to effect the separation. α- and β-Carotenes and cryptoxanthin were thus quantitated at 440 nm. In the same year Stewart (1977*b*) reported the separation of the same carotenoids in several citrus juices using essentially the same procedure, but with improved gradient elution.

The stationary phases used in the studies discussed above were all normal-phase columns, employing non-polar primary solvents such as *n*-hexane or petroleum ether. Another mode of HPLC introduced made use of reversed-phase columns in which a non-polar hydrocarbonaceous layer is chemically bonded onto the surface of the silica gel by silylation of the free hydroxyl groups with an alkyl trichlorosilane. The latter was usually octyl or octadecyltrichlorosilane, referred to as C_8 or C_{18} (ODS) columns, respectively. In addition to the main column, a pre-column (or guard column) was usually included to act as a trap for contaminants and to protect the analytical column. Packing material for the pre-column is usually the same as that in the analytical column, except that the particle size is larger. Polar solvent mixtures were usually employed in reversed-phase HPLC, e.g. water, methanol and acetonitrile. Zakaria *et al.* (1979) reported the use of four of these reversed-phase columns for the separation of α- and β-carotene and lycopene in tomato. The best separation was said to have been obtained using a 5-μm particle size Partisil column and a mobile phase consisting of 8% chloroform in acetonitrile was used to elute the pigments isocratically. The pigments were detected at 470 nm, and identification was based on retention of standards and stopped-flow visible spectra of the peaks. These investigators pointed out that non-polar reversed-phase columns possess several advantages over the normal-phase polar adsorbents. The former are neutral to the sample and are unaffected by the presence

of water or changes in the mobile phase, and are therefore more suitable for routine sample analysis.

Numerous subsequent studies had made use of reversed-phase HPLC for the study of carotenoids in plant materials. Studies cited in the following few paragraphs are of interest in that they have examined a number of samples, and emphasized a few carotenoids, mainly α- and β-carotene. Bushway and Wilson (1982) reported the analysis of α- and β-carotene in six raw and processed vegetables using a Partisil 5 ODS column, and a mobile phase consisting of a mixture of acetonitrile, tetrahydrofuran, and water. The carotenes were detected at 470 nm, and quantitated using authentic standards. TLC and visible spectrophotometry were used to aid in identification of the pigments. Extending from this work, and using the same HPLC conditions, Bureau and Bushway (1986) reported the study of twenty-two types of fruits and vegetables for the concentration of β-cryptoxanthin in addition to these two carotenes. Vitamin A activities obtained for several vegetables using this procedure were compared with values cited in the USDA Handbook. In the same year, Bushway (1986) reported the study of the two carotenes in nine fruits and vegetables using similar conditions, except for column and mobile phase. A Vydac 218TP54 column was used, and a non-aqueous solvent mixture of acetonitrile, methanol and tetrahydrofuran was used as the mobile phase.

Several other recent studies also concentrated on the analysis of α- and β-carotene and a small number of other carotenoids in various fruits and vegetables. Hsieh and Karel (1983) quantitated the concentration of these two carotenes in six vegetables using RP–HPLC. A μ-Bondapak C_{18} column was used together with a guard column packed with C_{18} Corasil. The mobile phase used was chloroform–acetonitrile, and the pigments were detected at 436 nm. The same column and guard column were also used by Schwartz and Patroni-Killam (1985) for the study of four raw and processed vegetables. The study is interesting in that it emphasized the detection of *cis-trans* isomers of α- and β-carotene. The solvent system employed was a mixture of methanol, acetonitrile and chloroform. A dual-wavelength detector, set at 436 and 340 nm, was used, and the peak height ratio, 340 nm/436 nm, was calculated to enable detection of *cis*-isomers. A two-directional TLC using calcium hydroxide layers was also used to separate the individual isomers, which were subsequently studied by absorption spectra. Schwartz reported another study of *cis-trans* α- and β-carotene, in some ten fresh and processed vegetables, two years later using the same detection procedure (Chandler & Schwartz, 1987). In this later study, however, a slurry-packed calcium hydroxide column with acetone–hexane as the mobile phase was used. Satisfactory results were obtained using this normal-phase column. In fact, the authors reported complete separation of

α- and β-carotene. In all previous studies of these two carotenes using reversed-phase HPLC, only partial or near-complete separation was obtained, although sufficiently separated for quantitation. Fisher and Rouseff (1986) also reported the satisfactory separation of α- and β-carotene in orange juice using a Zorbax ODS column and a mobile phase of acetonitrile–methylene chloride–methanol. The two carotenes and β-cryptoxanthin were detected at 450 nm. A photodiode array detection system was also used to obtain UV–visible spectra for chromatographic peak identification and to determine peak purity.

Carrot was a vegetable that was studied by various investigators. The following studies all made use of a C_{18} column for reversed-phase HPLC, and a guard column. Khachik and Beecher (1985) reported the separation of α- and β-carotene using an isocratic system of methanol, acetonitrile, and methylene chloride–hexane with a Microsorb C_{18} column. Peaks were detected at 450 nm and absorption spectra of eluted peaks obtained. Decapreno-β-carotene was used as an internal standard. A much more complicated study of several carrots of different genetic backgrounds was reported by Simon and Wolff (1987) using a combination of column chromatography, TLC and HPLC. In the last method, a Whatman Partisil 5 ODS column was used with a mobile phase consisting of acetonitrile, methylene chloride and methanol. Lycopene, α-, β-, γ-, and zeta-carotene, β-zeacarotene, and apo-8′-carotenal (as internal standard) were detected at 436 and 340 nm. Following on their earlier study two years ago, Khachik and Beecher (1987) reported the separation of several carotenoids in carrot and several other yellow/orange vegetables; namely, sweet potato, pumpkin and red palm oil. A Brownlee RP-18 column and an isocratic system of methanol, acetonitrile and methylene chloride were used for the separation and quantitation of lycopene, *cis*- and *trans*-zeta-carotene, α-carotene, and *cis*- and *trans*-β-carotene. All the carotenes were detected at 475 nm, except for zeta-carotene which was monitored at 402 nm. C-45-β-carotene (one isoprene unit more than α- and β-carotene) was used as an internal standard.

Speek and his co-workers (1986*a*, 1988) provided data on vitamin A activity for a large number of fruits and vegetables using a reversed-phase HPLC. A home-packed ODS-Hypersil column was used, while a mixture of four solvents consisting of methanol, acetonitrile. chloroform, and water was used as the mobile phase. The procedure was also different from studies previously described in that total carotenoids were also determined by absorption spectrophotometry in addition to the determination of β-carotene concentration by HPLC (Speek *et al.*, 1986*a*). Using this procedure, Speek *et al.* (1988) reported the analysis of fifty-five Thai fruits and vegetables. When compared with values tabulated in the Thai Food Composition Table, the analytical values were generally lower. The effect of processing (cooking, frying, fermenting and sun-drying) on carotene content

was also studied. Pepping *et al.* (1988) reported the α- and β-carotene contents of several East African vegetables, tubers, cereals and legumes using a similar reversed-phase HPLC system with a four-solvent mobile phase comprising acetonitrile, methanol, hexane and dichloromethane. The contents of these two carotenes in various South Pacific root crops were reported recently by Singh and Bradbury (1988). Various solvent mixtures were tried, and a mixture of acetonitrile, methanol and chloroform was found to be able to effect the required separation.

Several other recent studies also reported the content of a few major carotenoids in various plant materials using reversed-phase HPLC. Mejia *et al.* (1988) reported the separation of α-, β- and γ-carotenes in Mexican peppers using a mixture of acetonitrile, methanol and tetrahydrofuran. For the separation of cryptoxanthin, a separate mobile phase was used, in which methanol was replaced with water. The studies by Heinonen and co-workers are of interest, since they covered a large number of samples. In a study of fourteen ready-to-eat foods, the contents of α- and β-carotene, lycopene, and lutein were separated on a C_{18} column using a simple ternary mixture of acetonitrile, dichloromethane and methanol (Heinonen *et al.*, 1988). In a subsequent study, sixty types of vegetables and fruits were studied for their contents of α- and β-carotenes, lutein, γ-carotene, cryptoxanthin, lycopene and 15-*cis*-β-carotene (Heinonen *et al.*, 1989). In addition to the chromatography system previously used, a gradient system was also employed to effect the separation.

Other studies encountered in the literature had determined a large number of various carotenoids. These were, however, carried out on one or a few plant materials. One early study employing reversed-phase HPLC was reported by Braumann and Grimme (1981). Chlorophylls and eight carotenoids were separated from extracts of spinach and green alga using methanol–acetonitrile–water in a linear gradient system. A more recent report by Khachik *et al.* (1986) is another example of the complexities of such studies and the need for various analytical techniques for the identification and quantitation of these pigments. Several green vegetables of the genus *Brassica* were studied using two sets of complicated HPLC conditions. An isocratic system of methanol, acetonitrile, methylene chloride and hexane effected the separation of all-*trans*-β-carotene and its 15,15′-*cis* isomers from decapreno-β-carotene (internal standard for hydrocarbon carotenoids). In the second system, a combination of isocratic and gradient chromatography separated the oxygenated carotenoids and β-*apo*-8′-carotenal (internal standard for xanthophylls) from chlorophylls and the hydrocarbon carotenoids. In both systems, a Microsorb C_{18} column, protected with a guard cartridge was packed with spheri-5-C_{18}. Eighteen components, belonging to three classes of compounds, xanthophylls, chlorophylls and their derivatives, and the hydrocarbon carotenoids, were separated from the

vegetable extracts. The major constituents were also separated by semi-preparative TLC and HPLC and were identified by such tools as mass spectroscopy, NMR, and UV–visible spectroscopy. Khachik and co-workers reported further detailed studies of squash using a combination of isocratic and gradient reversed-phase HPLC systems to effect the separation of some twenty-five carotenoids (Khachik & Beecher, 1988; Khachik *et al.*, 1988). More recently, Khachik *et al.* (1989) report the study of several carotenoids and carotenol fatty acids in various dried and canned fruits using two C_{18} reversed-phase HPLC columns, each with a different adsorbent. Various eluants, comprising mixtures of acetonitrile, methanol, methylene chloride and hexane were used for isocratic and gradient elution. As in previous studies, various tools were used in the identification of carotenoids.

Further examples of complex studies of the separation of carotenoids are the studies by Fisher and Kocis (1987) and Gregory *et al.* (1987). Carotenoids in paprika (red bell pepper) were studied using gradient elution from a C_{18} column. In the latter study, thirty-eight carotenoids and esters, including several unidentified, were separated. TLC was used to assist in the separation and identification of the pigments. A combination of TLC and reversed-phase HPLC was also used by Biacs *et al.* (1989) in their study of several carotenoids in paprika. Philip *et al.* (1988) used a similar approach for an elaborate study of carotenoid esters in orange juice concentrates after treatment with hydrochloric acid. Working further on several other fruits, Philip & Chen (1988*a*, *b*) focused on the separation of β-carotene, cryptoxanthin and cryptoxanthin esters in various fruits using a combination of column chromatography using alumina as adsorbent and gradient reversed-phase HPLC. In a subsequent study, these investigators used a combination of TLC, normal-phase HPLC and reversed-phase HPLC for the study of fatty acid esters of several carotenoids in fruits (Philip & Chen, 1988*c*). In the same year, Ng and Tan (1988) reported the separation of at least twelve carotenoids on a Microsorb C_{18} column from extracts of palm oil, with α- and β-carotene as the dominant carotenoids. Isocratic elution was carried out using a ternary system of acetonitrile, methanol and methylene chloride. A UV–visible diode array detector was used to obtain spectra of peaks to aid in the identification of the pigments. Tan (1988) also carried out detailed studies of the carotenoid composition of tomato paste, using a combination of open column chromatography using alumina as adsorbent and reversed-phase HPLC.

Carotenoids in blood

Several studies on the analysis of carotenoids in blood serum or plasma samples were reported. No studies on other biological specimens were encountered. All the studies encountered employed reversed-phase HPLC

on a C_{18} column, and frequently only a few carotenoids were studied. An early report by Puglisi and de Silva (1976) studied phytoene concentration in dog blood after intravenous and oral administration of the carotenoid. A methanol–water mixture was used as the mobile phase and the carotenoid was detected at 280 nm. No other studies on the determination of this straight-chain, 40-carbon precursor in the biosynthesis of β-carotene were encountered. Peng *et al.* (1983) reported the separation and quantitation of α- and β-carotene using an Ultrasphere-ODS column and a mobile phase of 88% acetonitrile–tetrahydrofuran (3:1, v/v) and 12% methanol–ammonium acetate (1%) (3:2, v/v), with the detector set at 436 nm. In the study reported by Nierenberg (1985), lycopene was also determined besides α- and β-carotene. The chromatographic conditions used were similar to those reported by Peng *et al.* (1983), including column, mobile phase, and detector. In both studies, α-carotene appeared to be partially separated and was not given much emphasis. More recently, Sowell *et al.* (1988) reported the study of *cis*-isomers of carotenoids using a column that was maintained at 40°C, and a mixture of ethanol and acetonitrile as the mobile phase. Two detectors, at 450 nm and 340 nm, were used to detect and monitor the carotenoids and the isomers.

Retinoids in food and feed

HPLC was also widely used in the determination of retinol in various foodstuffs. As for determination of carotenoids in plant materials, earlier studies had made use of normal-phase HPLC, whereas later studies tended to favour the use of reversed-phase HPLC. Two early studies using normal-phase HPLC were reported before 1980. Head & Gibbs (1977) reported the determination of retinol in mixed food items or composites of complete meals using a LiChrosorb Si60 column. The pigments were eluted using gradient elution by programmed mixing of hexane from one pump with methylene chloride–isopropanol from another. Retinol in the samples was monitored at 325 nm. In the same year, Dennison and Kirk (1977) reported the analysis of retinol in several cereal products. A μ-Porasil column was used, and retinol eluted isocratically using a mobile phase consisting of a mixture of hexane and chloroform (containing 1% ethanol) and detected at 313 nm. In both studies, the investigators reported that the presence of carotenoids did not interfere with the determination of retinol. The same chromatographic conditions (column, mobile phase and detector wavelength) were also used by Widicus and Kirk (1979) for the determination of retinyl palmitate (and tocopherol) in breakfast cereals. A fluorescence detector was also used for monitoring retinyl palmitate. Recently, Beaulieu *et al.* (1989) reported the analysis of retinyl acetate, retinyl palmitate and 13-*cis* retinyl esters in various multivitamin formulations using an amino bonded

phase column, with hexane as the mobile phase, one of the few studies employing this HPLC configuration.

Numerous other studies were subsequently reported for the analysis of *cis*- and *trans*-retinol, and their esters in foods using reversed-phase HPLC. Egberg *et al.* (1977) reported the study of retinol in several types of foods (including cereal products, cod liver oil, margarine, butter and cat food) using normal-phase and reversed-phase HPLC. The former procedure used a Zorbax silica gel column and a mixture of hexane, methylene chloride and isopropanol as the mobile phase. For the reversed-phase procedure, a Vydac ODS column was used, with acetonitrile–water as the mobile phase. The two isomers of retinol were monitored at 328 nm. The investigators felt that the reversed-phase procedure was preferred because of its ease of operation, while sufficiently resolving the two isomers. In this procedure, the *cis*-isomer appeared as a peak just before the *trans*-isomer. The investigators also compared the results obtained with those obtained with the AOAC Carr-Price procedure. When both isomers were taken into account, results obtained by the reversed-phase HPLC procedure were found to compare favourably with those obtained with the AOAC method.

Cohen and Lapointe (1978) also reported that vitamin A values from the HPLC procedure compared favourably with those from the AOAC method for various animal feed samples. The vitamin was eluted from a μ-Bondapak C_{18} column using a mixture of methanol and water and detected at 280 nm. Vitamins D and E were also determined in this study. The simultaneous determination of vitamins A and E in feeds and various foods (margarine, butter, and infant formula) was also reported by Soderhjelm and Andersson (1978). The same mobile phase and detector wavelength were used. A Spherisorb ODS column was used for the separation. Vitamin A and its derivatives in various fortified foods, including cheese, yogurt, cereals, and vitamin supplements were studied by Ashoor and Knox (1987). Essentially a reversed-phase HPLC procedure was used, except that a linear gradient elution system was adopted, consisting of 0·1% glacial acetic acid in water as solvent A and a mixture of acetonitrile, *n*-butanol and glacial acetic acid as solvent B.

Various studies of vitamin A in milk and milk products were reported in the literature, using very similar chromatographic conditions; namely, a C_{18} column, combinations of acetonitrile, methanol and water as mobile phase, and detection at 280 or 325 nm. Some examples of these studies are Henderson and McLean (1979), Wickroski and McLean (1984), Reynolds and Judd (1984), and Grace and Bernhard (1984). In all four studies, other fat-soluble vitamins were simultaneously determined, especially vitamin D. Another study resorted to more elaborate chromatographic procedures. Barnett *et al.* (1980) made use of two Zorbax ODS columns in series, as well

as flow, solvent, and wavelength programming to effect the separation of vitamins A, D, E and K.

Several other studies of retinol and its esters in various foodstuffs were encountered. These were, however, different from the previously cited studies in several aspects. In the study by Landen (1980) and Landen *et al.* (1985) on the concentration of retinyl palmitate in breakfast cereals and infant formulae, an initial high-pressure gel-permeation chromatographic (HP–GPC) procedure was carried out to obtain the fraction containing the vitamin. This was then subjected to reversed-phase HPLC using a μ-Bondapak C_{18} column, and a methylene chloride–acetonitrile mixture as the mobile phase. Retinyl palmitate was monitored at 313 nm. Except for two other studies reported by Landen and co-workers for the study of infant formulae (Landen, 1982; Landen *et al.* 1985), very few other studies employing HP–GPC for retinol analysis were encountered. In two studies reported by Stancher and Zonta (1984*a*), normal-phase HPLC was employed, as opposed to a reversed-phase procedure used in most other studies. A silica Si-60 column and hexane containing a small amount of isopropanol as the mobile phase were used for the separation of various retinol and dehydroretinol isomers in cod liver oil and the livers and eyes of salt- and fresh-water fishes. In a subsequent study, these investigators modified the mobile phase into a ternary mixture of 1-octanol, isopropanol and hexane (Stancher & Zonta, 1984*b*). Seven retinol and seven dehydroretinol geometric isomers were said to have been fractionated using the system developed.

Another interesting study using normal-phase HPLC was reported by Mills (1985). Milk extracts were analysed for retinol on a LiChrosorb Si-60 column, using an ethyl ether and hexane mixture as the mobile phase and detecting at 313 nm. Samples were also determined by the AOAC Carr–Price colorimetric method. Results given by the two methods were said to be statistically equivalent.

A few investigators had studied other retinoids in food samples. Sivell *et al.* (1982) reported the determination of *cis*- and *trans*-retinol and retinaldehyde in various egg samples. Retinol and retinyl acetate were separated on a reversed-phase HPLC column of C_{22} packing, using a mixture of methanol and water and detecting at 325 nm. Retinaldehyde was reduced to retinol using sodium borohydride followed by saponification; the total retinol so obtained was extracted and determined by HPLC. Total retinol after reduction, minus the total retinol before reduction, was taken to be directly proportional to retinaldehyde in the sample.

Retinoids in blood

As for the analysis of retinoids in foods, early studies of retinoids in blood

were carried out using normal-phase HPLC. De Ruyter and de Leenher (1976) reported the use of a 10-μm silica column with a mixture of petroleum ether, dichloromethane and isopropanol for the determination of serum retinol. Subsequently, these investigators employed reversed-phase HPLC for the simultaneous determination of retinol and retinyl esters in serum (De Ruyter & de Leenher, 1978).

In general, reversed-phase HPLC applied to the determination of vitamin A in blood plasma or serum by various investigators were as described above for various foodstuffs. One early study in this area was reported by De Leenher *et al.* (1979), in which retinol and tocopherol were simultaneously determined. These two fat-soluble vitamins were also determined by Chow and Omaye (1983), Nierenberg and Lester (1985), and Sanz and Santa-Cruz (1986). In the last named study, the detector was first set at 340 nm for the first 3 min and thereafter at 280 nm. Williams (1985) employed fluorescence detection for the determination of retinol and tocopherol in serum. The detector was programmed for excitation and emission at two wavelengths. Huang *et al.* (1986) introduced further sophistication in the detection procedure in their study. Retinol was detected at 313 nm, whereas tocopherols were monitored using electrochemical detection.

Other studies concentrated on the determination of retinol and its esters in blood samples. Examples were studies by Nierenberg (1984) and Collins and Chow (1984). The latter was different in that a fluorescence detector was used for the detection and quantitation of retinol. Another method of detection was used by MacCrehan and Schonberger (1987). These investigators employed electrochemical detection for the determination of retinol and *cis*-isomers and reported results to correlate well with UV–visible absorbance detection. The study by Speek *et al.* (1986*b*) was different from the other studies on retinol in blood in that normal-phase HPLC was employed. A laboratory-packed Polygosil column was used, with a mixture of hexane, methylene chloride, and 2-propanol as the mobile phase. A fluorescence detector was used to monitor the eluted retinol. The method was said to be suitable for use with the small volume of blood (5 μl) collected from a finger-prick.

Besides retinol, other retinoids in serum or blood samples were also investigated. Besner and Leclaire (1980) reported the determination of retinol and retinoic acid. In the study by Annesley *et al.* (1984), programmed gradient elution was used to effect the separation of various retinoids; namely, retinol, retinyl acetate, *trans*- and *cis*-retinoic acid and etretinate. For the separation of isotretinoin, tretinoin and their 4-oxo metabolites, Wyss and Bucheli (1988) used two reverse-phase columns coupled in series. A fully automated gradient system and the column-switching technique employed.

Carotenoids and retinoids in food samples

Several studies were encountered that determined both carotenes and retinol in various food samples. However, in several of these studies, carotene and vitamin A were eluted and detected using two separate sets of chromatographic conditions. One such study reported in the early 1970s was that by Van de Weerdhof *et al.* (1973), using a liquid chromatography apparatus assembled from separate parts. Alumina in a glass column was used, and vitamin A was eluted using 3% ethanol in benzene, while β-carotene was eluted using 1% ethyl acetate in hexane. Fluorometric detection was used for the former, while carotene was detected using a spectrophotometer set at 460 nm. Thus, in this normal-phase HPLC procedure, vitamin A and carotene were not eluted and detected using a single sample injection.

The same approach was also used by Thompson and Maxwell (1977) in the determination of carotene and retinol in margarine, infant formula and fortified milk. In this study, however, a reversed-phase HPLC procedure was adopted. β-Carotene was eluted from a LiChrosorb reversed-phase column using methanol containing 1% water, and detected at 453 nm. Retinol was eluted using methanol containing 10% water, and the detector was set at 325 nm. Results obtained using the HPLC method were compared with those obtained with the AOAC method, but no statistical analysis was made on the two sets of results. There appeared to be no clear trend in differences in results. In the study by Scott *et al.* (1984), β-carotene and retinol in milk samples were also eluted using two separate chromatographic conditions. A few years after their earlier study in 1977, Thompson and co-workers followed up with another report on similar determinations (Thompson *et al.*, 1980). In this later study, however, normal-phase HPLC procedure was followed, using a silica column and a mobile phase consisting of a mixture of hexane and ethyl ether. Retinyl palmitate was determined rather than the free retinol to avoid destruction and loss of the vitamin. Results obtained for milk samples were said to be generally similar to those given by a fluorometric method, although the latter method tended to give slightly higher results. In a study of various ready-to-eat foods, Heinonen *et al.* (1988) employed a reversed-phase HPLC procedure for the determination of carotenoids, but resorted to normal-phase HPLC for the estimation of retinol and 13-*cis*-retinol. In a recent study, van Dokkum *et al.* (1990) reported the retinol concentration of total diets using the normal-phase HPLC method proposed by de Ruyter and de Leenheer (1976). Total carotenoid and β-carotene concentrations were determined separately using the procedure reported by Speek *et al.* (1986*a*). A direct spectrophotometric method was used for quantitating total carotenoid concentration, whereas β-carotene was determined by a reversed-phase HPLC method employing a mixture of methanol, acetonitrile, chloroform and water as the mobile phase.

Since β-carotene has an absorption maximum at 450 nm but does not

absorb at 325 nm, and the converse is true for retinol, both compounds could not be simultaneously detected at one wavelength. Thus, in the studies cited above, samples had to be injected twice in order to determine both compounds. However, simultaneous determinations could be achieved if two detectors set at 450 and 325 nm could be connected in series for continuous monitoring of the eluate. Alternatively, if a single detector was used, the wavelength could be changed in the middle of a run. The switch could be done manually, which would require close monitoring of the run, and could be rather tedious. More recently, programmable detectors have become available, in which wavelength change can be programmed at a predetermined time. Several studies are cited below to illustrate the approaches taken to achieve simultaneous detection of carotene and retinol.

Landen and Eitenmiller (1979) reported the continuous monitoring of β-carotene at 436 nm and retinol and retinyl esters at 313 nm eluted from a μ-Bondapak C_{18} column using a mixture of methylene chloride and acetonitrile. In this study on oils and margarine, extracts were first fractionated or cleaned-up by using high-pressure gel-permeation chromatography (HP–GPC). This combination of HP–GPC and reversed-phase HPLC procedure was said to be advantageous in that it enabled the quantitation of the more stable vitamin A ester forms. The procedure was described as non-destructive in that no saponification (and, therefore, heating) step was necessary. It was used in a few other studies described earlier in this review (Landen, 1980, 1982; Landen *et al.*, 1985), and also in the report by Granade (1982). In the latter report, besides the detection of vitamin A palmitate at 313 nm and carotene at 436 nm, vitamin E acetate was monitored at 280 nm.

Stancher and Zonta (1982) described their experiences in the simultaneous determination of retinol and its isomer, and α- and β-carotene in cheese. Normal-phase HPLC was carried out using a LiChrosorb Si60 column, and an isocratic mobile phase of methylethylketone-hexane. To enable the detection of retinol and carotene in a single injection, the detector was first set at 450 nm for detection of the latter. After the elution of carotene, the detector was quickly switched to 340 nm for retinol and its isomers. The investigators reported that the system was not able to separate α- and β-carotene.

Carotenoids and retinoids in blood

Several studies on the determination of both carotenes and retinol in blood or serum samples have also been made. As described for food samples above, earlier studies made use of two separate sample injections to enable detection of carotene and retinol in the same sample. Broich *et al.* (1983) reported the determination of lycopene, α- and β-carotene and retinyl esters

using a Supelco C_{18} column and a mobile phase of methanol, acetonitrile and chloroform. The carotenoids were monitored at 446 nm, and retinyl esters at 325 nm using two separate sample injections. In another study, Katrangi *et al.* (1984) reported the separation of lycopene, α-, β-, δ- and γ-carotene using a μ-Bondapak C_{18} column and acetonitrile–chloroform as the mobile phase. The carotenes were monitored at 462 nm. Delta- and γ-carotenes were not separated and usually comprised a small proportion of total carotenoids. α-Carotene was also not separated from β-carotene, appearing only as a shoulder on the latter peak. Total carotenoids in the serum samples were also determined by obtaining absorbance reading of extracts at 450 nm. Retinol was also determined in the study, but using a separate chromatographic system. A 10-μm Radial Pak C_8 reversed-phase column and a mobile phase consisting of methanol–water were used for this determination.

Ohmacht *et al.* (1987) used a totally different approach to the determination of several carotenoids (lycopene, β-carotene, β-cryptoxanthin, lutein, and zeaxanthin) and retinol in human serum. These investigators preferred the use of normal-phase HPLC, and studied two columns, containing amino and cyano groups. To effect the separation of various carotenoids with differing polarities, a gradient elution system was used. Carotenoids were monitored at 452 nm and retinol at 328 nm. It was reported that the stationary phases studied, particularly the amino-phase, were suitable for the qualitative and quantitative determination of serum carotenoids.

CONCLUSION

The analysis of carotenoids and retinoids is complicated and beset with various problems. The analysis is complicated because of the large number of naturally occurring carotenoids (as many as 500 have been reported). There are far fewer retinoids, but the occurrence of *cis*- and *trans*-isomers of carotenoids and retinoids further complicates the analysis. The composition and content of carotenoids in various plant materials and retinoids in foods of animal origin vary widely. Not all of the naturally occurring carotenoids are precursors of vitamin A, and for those with provitamin A activity, the biological activity varies widely. There is also considerable variation in the biological activity of various retinoids.

The main problems associated with work on carotenoids and retinoids arise from the inherent instability of these compounds, especially towards light, heat, oxygen and acids. Various precautionary measures have to be taken during sample preparation and analysis. Thus careful and dedicated workers are required for the analysis. Another obstacle in the analysis is the

difficulty in obtaining authentic reference carotenoids and retinoids as most of them are not available commercially.

It is therefore rather difficult to obtain accurate data on carotenoid and retinoid content and composition. The literature abounds with attempts at improving the analytical techniques. The procedure should be able to effectively remove interfering compounds, separate the carotenoids and retinoids and quantitate them accurately. This review has shown that there has been much work on method development and improvement in this field in the last three decades. From the use of counter-current distribution in the 1960s, thin-layer chromatography and adsorption (open) column chromatography became widely used in the 1970s and 1980s. From the late 1970s, high-pressure liquid chromatography became more prominently used for the analysis of carotenoids and retinoids, mainly because of the ability of the technique to effect rapid separation, its non-destructiveness and, more importantly, the better resolution that is achieved. The ability of HPLC to rapidly separate and quantitate various carotenoids and retinoids, at least in standard preparations, has been demonstrated. Its application to the analysis of foods, however, is still being developed and improved. As with other vitamins, the application of HPLC in the determination of these compounds will see further development and wider application in the future.

Many investigators have shown that the use of other techniques in combination with HPLC will greatly enhance the usefulness of the latter, especially so during method development. The combined use of open-column chromatography and UV–visible absorption spectra, for example, would assist in the identification and confirmation of carotenoids and retinoids.

It has often been said that many laboratories in developing countries will not be able to possess a high-pressure liquid chromatograph. Contributory factors are the high cost in the purchase as well as the maintenance of HPLC. It is also costly to run the instrument, e.g. in the purchase of solvents and columns. In addition, it requires considerable skill to operate. However, it has been shown that for the satisfactory separation of the carotenoids and retinoids, HPLC is the method of choice. It is not necessary to invest in complicated HPLC systems or search for the full range of carotenoids, many of which occur in minute amounts in foods. More accurate results and the considerably shorter time required would make the HPLC a worthwhile investment.

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