HarvestPlus
TECHNICAL MONOGRAPH SERIES 2

HarvestPlus Handbook for Carotenoid Analysis

Delia B. Rodriguez-Amaya and Mieko Kimura

HarvestPlus

HarvestPlus Technical Monographs are designed to provide a forum for HarvestPlus research outputs.

Technical Monographs can be of two kinds:
- state-of-the-art reviews that help to establish and define HarvestPlus research questions or
- "gold standard" procedures to be followed in HarvestPlus research.

HarvestPlus technical monographs are written by recognized experts in the field and are externally peer reviewed prior to publication.

Delia B. Rodriguez-Amaya and **Mieko Kimura**, 2004.
Harvestplus Handbook for Carotenoid Analysis.
HarvestPlus Technical Monograph 2.
Washington, DC and Cali: International Food Policy Research Institute (IFPRI) and
International Center for Tropical Agriculture (CIAT).

HarvestPlus Handbook for Carotenoid Analysis

*Delia B. Rodriguez-Amaya and **Mieko Kimura

* Departamento de Ciência de Alimentos, Faculdade de Engenharia de Alimentos Universidade Estadual de Campinas, C.P. 6121, 13083-862 Campinas, SP, Brasil

** Departamento de Engenharia e Tecnologia de Alimentos, Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista, 15054-000 São José do Rio Preto, SP, Brasil

Breeding Crops for Better Nutrition

Table of contents

Preface

Carotenoid analysis is inherently difficult and error prone. Despite substantial improvements and refinements in the methods for analyzing these fascinating but complicated compounds, discrepancies in analytical data can still be perceived in the literature. Analysts must be well informed about the nature and properties of carotenoids and the problems associated with their identification and quantification.

Carotenoid analysis consists of several steps that can be carried out efficiently in various ways. Thus, in this handbook several procedures are given for some steps to enable the analyst to choose the procedure that best suits his/her laboratory conditions and experience. These procedures have been put together and evaluated for HarvestPlus crops in our laboratory, but we recognize that other procedures may also produce reliable results. It is important that analysts test the methods in their own laboratory prior to performing the actual analyses. Knowledge of the purpose and possible sources of error in each step will assist the analyst in appraising the performances of the methods. Indeed, the key element in the accurate determination of carotenoids is undoubtedly the analyst. Even a well-validated method may perform badly in the hands of an analyst who does not seek the appropriate information and does not pay attention to the many important details that are necessary to ensure the accuracy of the analytical results.

The authors wish to thank HarvestPlus for the opportunity to publish this handbook. A special thanks is extended to Penelope Nestel, Nutrition Coordinator of HarvestPlus, without whom this handbook would not exist. We also gratefully acknowledge the financial support of the Brazilian Ministry of Science and Technology (MCT) through the National Council of Scientific and Technological Development (CNPq), under the Program for Research Groups of Excellence (PRONEX CNPq 662307/1996-8), which has enabled us to gain the knowledge and experience we are sharing through this handbook. Thanks are also due to Barbara Underwood, Jing Tan, and Julia Humpries for reviewing the manuscript.

Delia B. Rodriguez-Amaya
Mieko Kimura

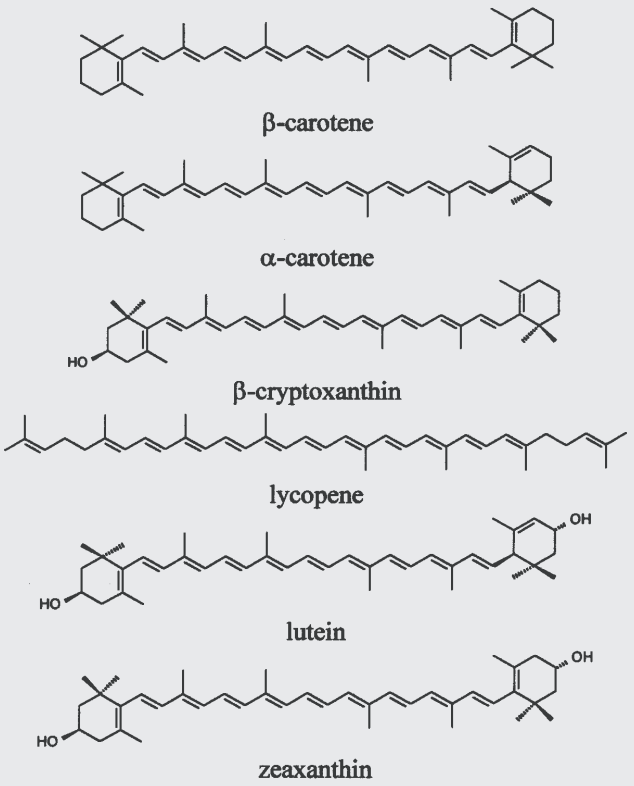
I | Carotenoids In Foods

Carotenoids are notable for their wide distribution, structural diversity, and various functions. More than 600 carotenoids, not including *cis* and *trans* isomers, have been isolated and characterized from natural sources (Pfander 1987). This impressive figure includes the enormous array of carotenoids in algae, bacteria, yeast, and fungi. Only a fraction of the carotenoids recorded to date are found in foods; nonetheless the carotenoid composition of foods can still be complex.

Carotenoids in foods are generally C_{40} tetraterpenoids formed from eight C_5 isoprenoid units joined head-to-tail, except at the center where a tail-to-tail linkage reverses the order, resulting in a symmetrical molecule (Figure 1). An important feature is a centrally located, extended conjugated double-bond system, which constitutes the light-absorbing chromophore that gives carotenoids their attractive color and provides the visible absorption spectrum that serves as a basis for their identification and quantification. The basic skeleton may be modified in many ways, including cyclization, hydrogenation, dehydrogenation, introduction of oxygen functions, rearrangement, chain shortening, or combinations thereof, resulting in a multitude of structures.

FIGURE 1

STRUCTURES OF THE PRINCIPAL CAROTENOIDS IN FOODS AND ZEAXANTHIN



Hydrocarbon carotenoids (e.g., β–carotene, lycopene) are known as carotenes, and oxygenated derivatives are called xanthophylls. Common oxygen substituents are the hydroxy (as in β–cryptoxanthin), keto (as in canthaxanthin), epoxy (as in violaxanthin), and aldehyde (as in β–citraurin) groups. Carotenoids can be acyclic (e.g., lycopene), monocyclic (e.g., γ–carotene), or dicyclic (e.g., α– and β–carotene). In nature carotenoids exist primarily in the more stable all-*trans* (or all-*E*) form, but small amounts of *cis* (or *Z*) isomers do occur.

Because plants are able to synthesize carotenoids *de novo*, the carotenoid composition of plant-derived foods is enriched by low levels of biosynthetic precursors and derivatives of the main components. Carotenoids are not as widely distributed in animal-derived foods and are present at much lower levels. Animals are incapable of carotenoid biosynthesis, and hence depend on dietary carotenoids, which are selectively or unselectively absorbed, converted to vitamin A, deposited as such or slightly altered to form carotenoids typical of animal species.

Importance to human health

Figure 1 shows the principal carotenoids found in foods, together with zeaxanthin, which is not as ubiquitous. β–Carotene, α–carotene, β–cryptoxanthin, lutein, and lycopene are also the carotenoids most commonly found in human plasma. These carotenoids, together with zeaxanthin, have been shown to have health-promoting effects.

β–carotene, α–carotene, and β–cryptoxanthin are provitamins A. Structurally, vitamin A (retinol) is

essentially one-half of the β–carotene molecule. Consequently, β–carotene is the most potent pro-vitamin A; it is also the most widespread (Rodriguez-Amaya 1993). The minimum requirement for a carotenoid to have vitamin A activity is an unsub-stituted β–ring with an 11-carbon polyene chain. Thus, α–carotene and β–cryptoxanthin exhibit about 50% of the vitamin A activity of β–carotene.

Carotenoids, whether provitamins A or not, have been credited with other beneficial effects on human health: enhancement of the immune response and reduction of the risk of degenerative diseases such as cancer, cardiovascular diseases, cataract, and macular degeneration (Astrog 1997, Bendich 1994, Burri 1997, Gaziano and Hennekens 1993, Krinsky 1993, Mayne 1996, Olson 1999a, Olson and Krinsky 1995). The action of carotenoids against diseases has been attributed to an antioxidant property, specifically, their ability to quench singlet oxygen and interact with free radicals (Palozza and Krinsky 1992). However, other mechanisms have been reported: modulation of carcinogen metabolism, inhibition of cell proliferation, enhancement of cell differentiation, stimulation of cell-to-cell communication, and filtering of blue light (Olson 1999a,b).

The ability of carotenoids to quench singlet oxygen has been linked to the conjugated double bond system, the maximum efficiency being shown by carotenoids with nine or more conjugated double bonds (Foote et al. 1970). The acyclic carotenoid lycopene was found to be more efficient than the dicyclic β–carotene (Di Mascio et al. 1989), despite both compounds possessing 11 conjugated double bonds. The effects of lycopene on human health have drawn considerable interest in recent years (Clinton 1998, Gerster 1997, Giovannucci 1999, Sies and Stahl 1998, Stahl and Sies 1996), the current evidence being stronger for lycopene protection against lung, stomach, and prostrate cancer.

Lutein and zeaxanthin make up the yellow pigment in the macula of the human retina (Bone et al. 1988, Handelman et al. 1988), and dietary intake or plasma levels of these carotenoids have been found to have a statistically significant inverse association with risk of macular degeneration [EDCC (Eye Disease Case-Control) STUDY GROUP 1993, Seddon et al. 1994], the principal cause of irreversible blindness in the elderly. These carotenoids have also been consistently associated with reduced risk for cataract (Moeller et al. 2000).

Factors affecting carotenoid composition

Foods vary qualitatively and quantitatively in their carotenoid composition. Green vegetables, leafy and non-leafy, have a defined qualitative pattern with lutein, β–carotene, violaxanthin, and neoxanthin as the principal carotenoids. The relative proportions of these carotenoids are fairly constant, but the absolute concentrations vary considerably. Green vegetables additionally contain minor carotenoids such as α–carotene, α– or β–cryptoxanthin, zeaxanthin, antheraxanthin, and lutein-5,6-epoxide. The xanthophylls are unesterified.

The composition of carotenoids in fruits and fruit vegetables is much more complex and variable, with variations even in the principal carotenoids. Typically, fruits contain a few major carotenoids along with a series of minor carotenoids at trace or very low levels. Eight major patterns can be discerned (Goodwin 1980, Goodwin and Britton 1988): (a) insignificant levels of carotenoids (e.g., pear); (b) small amounts generally of chloroplast carotenoids, (e.g., grape); (c) considerable amounts of lycopene (e.g., tomato, watermelon, red-fleshed guava, papaya); (d) predominance of β–carotene and/or β–cryptoxanthin (e.g., apricot, peach, loquat); (e) large amounts of epoxides (e.g., mango, carambola); (f) preponderance of unusual or species-specific carotenoids (e.g., red pepper); (g) substantial amounts of poly-*cis*-carotenoids (e.g., tangerine tomato); and (h) significant levels of apocarotenoids (carotenoids with a shortened carbon skeleton) (e.g., citrus species). Some merging of these patterns is seen in some fruits. In ripe fruits carotenoids are located in the chromoplasts and hydroxycarotenoids are mostly esterified with fatty acids.

Carotenes predominate in the few carotenogenic root crops (e.g., carrot, sweetpotato), and xanthophylls predominate in maize (seed).

In a given food qualitative and, especially, quantitative differences also exist due to factors such as cultivar/variety, stage of maturity, climate/geographic site of production, part of the plant utilized, conditions during agricultural production, post-harvest handling, processing, and storage conditions.

Differences among cultivars of the same food are well documented and can be either both qualitative and quantitative or only quantitative (Gross 1987, 1991, Rodriguez-Amaya 1993). The mean β–carotene content of sweetpotato cultivars, for example, varies from 10 to 26,600 μg/100 g (Almeida-Muradian and Penteado 1992, Hagenimana et al. 1999, Huang et al. 1999, K'osambo et al. 1998, Takahata et al. 1993). In squashes and pumpkins, some cultivars have α– and β–carotene as principal carotenoids, whereas lutein predominates in others (Arima and Rodriguez-Amaya 1988, 1990, Azevedo-Meleiro and Rodriguez-Amaya 2002, Markovic et al. 2002).

Stage of maturity is the one factor that decisively affects carotenoid composition. Maturation in vegetables and ripening in fruits are generally accompanied by enhanced carotenogenesis (Arima and Rodriguez-Amaya 1988, Gross 1987, 1991, Mercadante and Rodriguez-Amaya 1998, Porcu and Rodriguez-Amaya 2003, Rodriguez-Amaya 1993). Young and mature leaves generally have similar qualitative carotenoid patterns but different carotenoid concentrations; for example, the carotenoid levels in lettuce and endive leaves increase three- to four-fold during maturation (Azevedo-Meleiro and Rodriguez-Amaya 2004c, Ramos and Rodriguez-Amaya 1987). In fruits, the carotenoids increase markedly both in number and quantity during ripening.

Elevated temperature and greater exposure to sunlight increase carotenogenesis in fruits. Thus, tropical climates favor carotenoid biosynthesis, with fruits produced in such climates containing distinctly higher carotenoid concentrations (Cavalcante and Rodriguez-Amaya 1992, Kimura et al. 1991). Similarly, leafy vegetables produced in greenhouses or in plots covered with plastic roofing show higher carotenoid concentrations in the summer (Azevedo-Meleiro and Rodriguez-Amaya 2004b,c, Kimura et al. 2003). In contrast, carotenoid levels in leafy vegetables cultivated in open fields are significantly higher in the winter than in the summer (Heinonen et al. 1989, Ramos and Rodriguez-Amaya 1987), suggesting that photodegradation prevails over heightened carotenogenesis.

Most fruits and fruit vegetables have higher carotenoid levels in the peel than in the pulp (Gross 1987, 1991, Rodriguez-Amaya 1993, Rodriguez-Amaya and Kimura 1989), with the exception of fruits like pink-fleshed guava, in which lycopene is concentrated in the pulp (Padula and Rodriguez-Amaya 1986).

Farming practices may also influence the carotenoid composition. For example, comparison of kale of the same cultivar at the same stage of maturity produced on neighboring farms, one a natural farm and the other a conventional farm using agrochemicals, revealed significantly higher concentrations of all constituent carotenoids in samples collected from the natural farm (Mercadante and Rodriguez-Amaya 1991). In contrast, a study comparing conventionally produced and hydroponic leafy lettuce found no significant difference in the constituent carotenoids (Kimura and Rodriguez-Amaya 2003).

Effects of processing

Many carotenogenic foods are seasonal and processing at peak harvest is necessary to minimize losses, make the products available all year round, and permit transportation to places other than the site of production. Processing and storage of foods should, however, be optimized to prevent or reduce degradation while accentuating bioavailability.

Alteration or loss of carotenoids during processing and storage of foods occurs through physical removal (e.g., peeling), geometric isomerization, and enzymatic or non-enzymatic oxidation (Rodriguez-Amaya 1999b, 2002).

Measures should be taken to ensure maximum retention of carotenoids. Although attention is often focused on industrial processing, home preparation can, at times, cause even greater losses of carotenoids.

Percent retention or loss of carotenoids during processing and storage of food has been reported in numerous papers. However, the reported data are often inconsistent and difficult to interpret for the following reasons (Rodriguez-Amaya 1997): (a) processing and storage conditions are not or are only partially described; (b) different foods are processed differently, making comparisons of processing methods difficult; (c) different conditions (e.g., time and temperature) are used for the same method of processing; and (d) the procedure followed for calculating losses is not specified or the calculation is faulty. Additionally, care must be taken to ensure that any isomerization or oxidation of carotenoids during analysis and/or during sample storage prior to analysis is not erroneously attributed to the processing or storage of foods. However, despite some experimental inadequacies and discrepancies in the data, some conclusions can be drawn (Rodriguez-Amaya 1997):

- Carotenoid biosynthesis may continue, raising the carotenoid content, in fruits, fruit vegetables, and root crops even after harvest, provided the plant materials are kept intact, preserving the enzymes responsible for carotenogenesis. In leaves and other vegetables, post-harvest degradation of carotenoids may prevail, especially at high storage temperatures and under conditions that favor wilting.

- Carotenoids are naturally protected in plant tissues; cutting, shredding, chopping, and pulping of fruits and vegetables increase exposure to oxygen and bring together carotenoids and enzymes that catalyze carotenoid oxidation.

- The stability of carotenoids differs in different foods, even when the same processing and storage conditions are used. Thus, optimum conditions for carotenoid retention during preparation/processing vary from one food to another. Carotenoids *per se* have different susceptibilities to degradation.

- The major cause of carotenoid destruction during food processing and storage is enzymatic or non-enzymatic oxidation. Isomerization of *trans*-carotenoids to the *cis*-isomers, particularly during heat treatment, alters their biological activity and discolors the food, but not to the same extent as oxidation. In many foods, enzymatic degradation of carotenoids may be a more serious problem than thermal decomposition.

- Reported increases in carotenoid content during cooking or thermal processing are not likely to be true increases but are artifacts of the analytical/calculation procedure associated with loss of carotenoids in fresh samples due to enzymatic activity during sample preparation for analysis, greater extractability of carotenoids from processed samples, and unaccounted loss of water and leaching of soluble solids during processing.

- In home preparation, losses of carotenoids generally increase in the following order: microwaving < steaming < boiling < sautéing. Deep-frying, prolonged cooking, combination of several preparation and cooking methods, baking, and pickling all result in substantial losses of carotenoids.

- Whatever the processing method, carotenoid retention decreases with longer processing time, higher processing temperature, and cutting or puréeing of the food. Retention is significantly improved by reducing the processing time, lowering the temperature, and shortening the time lag between peeling, cutting, or puréeing and processing. Rapid processing at high temperature is a good alternative.

- Blanching may provoke some losses of carotenoids, but the inactivation of oxidative enzymes that occurs in this type of heat treatment prevents further and greater losses during holding before thermal processing, slow processing, and storage.

- Freezing (especially quick-freezing) and frozen storage generally preserve carotenoids, but slow thawing can be detrimental, particularly when the product has not been properly blanched.

- Peeling and juicing result in substantial losses of carotenoids, often surpassing those of heat treatment.

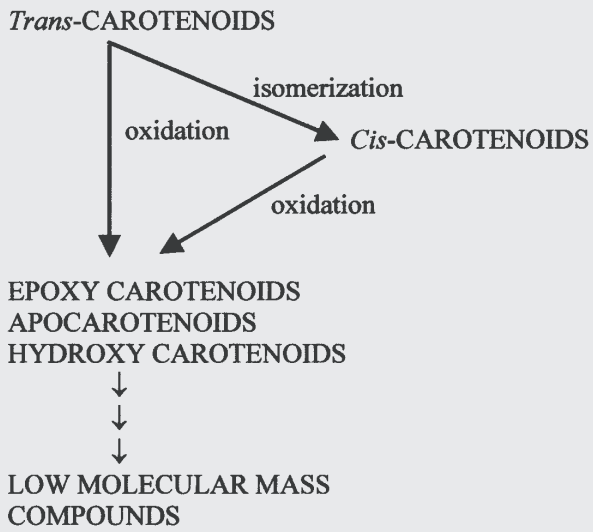
- Traditional sun-drying, although the cheapest and most accessible means of food preservation in poor regions, causes considerable carotenoid destruction. Drying in a solar dryer, even of simple and inexpensive design, can appreciably reduce losses. Protecting the food from direct sunlight also has a positive effect.

- Natural or added antioxidant and sulfiting agents may reduce carotenoid degradation.

- Exclusion of oxygen (e.g., through vacuum or hot filling, oxygen-impermeable packaging, inert atmosphere), protection from light, and low temperature diminish carotenoid decomposition during storage.

FIGURE 2

Possible scheme for carotenoid degradation (Rodriguez-Amaya 1999a)



Being highly unsaturated, carotenoids are prone to isomerization and oxidation (Figure 2). Isomerization of *trans*-carotenoids, the usual configuration in nature, to the *cis*-isomers is promoted by contact with acids, heat treatment, and exposure to light.

The principal *cis*-isomers of β–carotene are shown in Figure 3. The release of organic acids during slicing or juicing of fruits is sufficient to provoke *trans–cis* isomerization. However, this isomerization occurs to a greater extent during thermal processing.

Cis-provitamin A carotenoids have long been attributed lower vitamin A activity than the *trans*-isomers. In recent years, *trans*-β–carotene was found to be preferentially absorbed over 9-*cis*-β–carotene in humans (Ben-Amotz and Levy 1996, Gaziano et al. 1995, Stahl et al. 1995) and ferrets (Erdman et al. 1998). However, for the vitamin A inactive lycopene, the *cis*-isomer was observed to be more bioavailable than *trans*-lycopene in ferrets (Boileau et al. 1999).

The highly reactive, electron-rich carotenoid molecule suffers oxidation under food processing and storage conditions, the magnitude of which depends on the carotenoids present, available oxygen, exposure to light, temperature, and the presence of enzymes, metals, prooxidants, and antioxidants.

Enzyme-catalyzed oxidation can occur prior to heat treatment, during peeling, slicing, pulping, or juicing. Thus, it is recommended that foods be consumed or thermally processed immediately after these operations. Enzymatic oxidation can also take place in minimally processed (Azevedo-Meleiro and Rodriguez-Amaya 2004b,c) and in unblanched frozen foods.

Marketing minimally processed fruits and vegetables is an increasing trend, stimulated by consumer demand for high-quality, nutritive, fresh-like, and convenient to use products. Because drastic processing conditions are not employed, minimally processed products are

expected to retain fresh or fresh-like properties and have good nutritive quality. However, tissue disruption by cutting or shredding allows substrate/enzyme interactions and makes these products more susceptible to physiological/biochemical changes than intact raw commodities. Greater exposure of plant components to oxygen also enhances oxidative degradation.

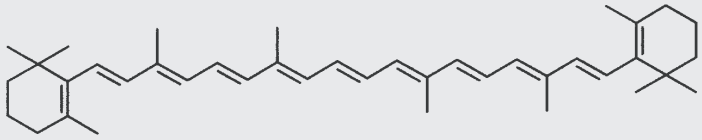
In contrast to the wealth of information available on lipid oxidation, knowledge of carotenoid oxidation remains fragmentary. Carotenoid oxidation is often accompanied by isomerization, and both the *cis*- and *trans*-isomers are subject to oxidation (Figure 2). It is generally accepted that the initial stages of oxidation involve epoxidation and cleavage to apocarotenals (Figure 2). Subsequent fragmentations result in compounds with low molecular masses, similar to those produced in fatty acid oxidation. Now devoid of color and biological activity, these compounds can give rise to desirable flavors (e.g., wine, tea) or off-flavor (e.g., dehydrated carrot).

For a long time the major carotenoid-related concern in food processing was minimizing the loss of these compounds. In recent years, however, attention has shifted to the effect of processing on the bioavailability of carotenoids.

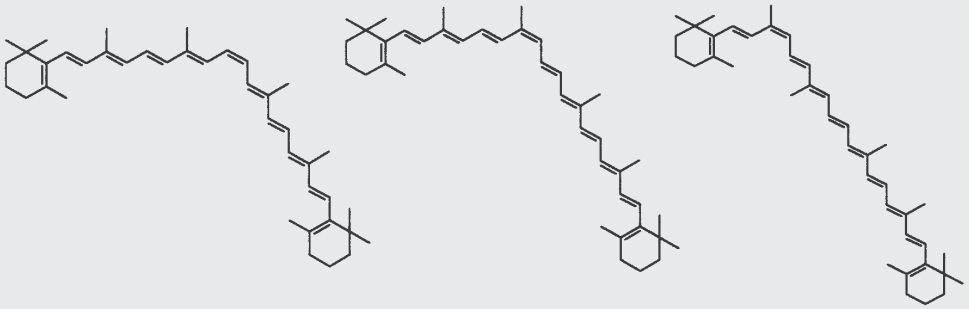
Carotenoids in nature are protected by the cellular structure, the destruction of which renders carotenoids vulnerable to degradation. However, this natural protection also limits carotenoid bioavailability. Processing denatures proteins and breaks down cell walls, facilitating the release of carotenoids from the food matrix during digestion. For example, the bioavailability of β–carotene in human subjects has been shown to be enhanced by mincing or processing of spinach, and processing of carrots (Castenmiller et al. 1999, Rock et al. 1998). In addition, lycopene bioavailability was found to increase in heat-processed tomatoes compared with unprocessed tomatoes (Gärtner et al. 1997, Stahl and Sies 1992, van het Hof et al. 2000). Current knowledge therefore suggests that processing conditions should be optimized to minimize losses of carotenoids while enhancing their bioavailability.

FIGURE 3

COMMON GEOMETRIC ISOMERS OF β-CAROTENE



All-*trans*-β-carotene



15-*cis*-β-carotene 13-*cis*-β-carotene 9-*cis*-β-carotene

II | General Procedure for Carotenoid Analysis

Carotenoid analysis usually consists of (a) sampling and sample preparation, (b) extraction, (c) partition to a solvent compatible with the subsequent chromatographic step, (d) saponification and washing, (e) concentration or evaporation of solvent, (f) chromatographic separation, (g) identification, and (h) quantification (Rodriguez-Amaya 1999a).

Sampling

To obtain meaningful and reliable analytical data, the sample must be representative of the entire lot under investigation and adequately prepared for analysis. According to Kratochvil and Taylor (1981), the major steps in sampling are:

- identification of the population from which the sample is to be obtained;
- selection and withdrawal of valid gross samples of this population;
- reduction of each gross sample to a laboratory-size sample suitable for analysis.

Horwitz (1988) defined anything sent to the laboratory as a laboratory sample and considered reduction of the laboratory sample to a test sample for analysis as part of the sampling process. Pomeranz and Meloan (1994) differentiated sampling and sample preparation as follows: The aim of sampling is to secure a portion of the material that satisfactorily represents the whole, while the purpose of sample preparation is to homogenize the large sample in the laboratory and subsequently reduce it in size and amount for analysis. Following this rationale, in this handbook sample preparation includes all operations between the receipt of the laboratory sample and the weighing of the sample to be analyzed.

In designing a sampling plan, the following factors should be considered (Kramer and Twigg 1970, Kratochvil and Taylor 1981):

- the purpose of the analysis (information sought);
- the nature of the population to be studied;
- the nature of the analyte (substance to be measured);
- the distribution of the analyte within the population;
- desired accuracy and precision of the analytical results;
- the analysis to be performed.

The more heterogenous the material, the greater the difficulties and effort required to obtain a representative sample. Also, the more sensitive modern methods become, the smaller the portions of the original lots that are submitted to analyses, making it more challenging to minimize sampling errors.

Because food samples are typically heterogeneous, a large number of samples should ideally be analyzed. In practice, however, the sampling procedure adopted is usually a compromise between heterogeneity considerations and the cost of the operation. An acceptable sampling program should include at least the following (Keith et al. 1983):

- a sampling plan that takes into account the goals of the studies and the expected uncertainties associated with the number of samples collected and the population variability;
- instructions for sample collection, labeling, preservation, and transport to the analytical facility;
- training of personnel in the sampling techniques and procedures specified.

The program should contemplate the reasons for choosing sampling sites, the number of samples, the timing of sample acquisition, and the expected levels of fluctuation due to heterogeneity. Once the sampling site and time of collection are decided, the following questions should be addressed (Kratochvil and Taylor 1981):

- How many samples should be taken?
- How large should each be?
- From where in the bulk material (population) and how should they be taken?
- Should individual samples be analyzed, or should a composite be prepared?

If an average compositional value is desired, a large number of randomly selected samples can be obtained, combined, and blended to obtain a

reasonably homogenous composite, of which sub-samples may be analyzed (ACS Committee on Environmental Improvement 1980, Keith et al. 1983).

Random sampling involves drawing increments from different parts of the entire lot, with each part of the lot having the same probability of being collected. However, this process is not as simple as it seems. On the one hand, increments taken so randomly may not constitute a representative sample. On the other hand, incremental collection cannot be so defined that the protocol may reflect bias (Kratochvil and Taylor 1981).

To evaluate changes in composition as a function of variables such as time, temperature, and location, systematic sampling should be used and the results should be statistically analyzed.

Sample preparation

The sample that is brought to the laboratory is usually too large, both in bulk and in particle size, for direct analysis. It must therefore be transformed into a homogenous, small sample for analysis, while maintaining its representativity. Homogenization and sub-sampling may be done simultaneously, or consecutively in either order. Physical operations, such as chopping, cutting into pieces, mixing, milling, blending, and sieving, are carried out, along with bulk reduction, such as quartering or riffling. The process can be performed manually or using commercially available mills, blenders, grinders, and riffle cutters. Because the food product is usually analyzed in the form in which it is commonly used, inedible portions (e.g., peel, seed, shell) are removed prior to sample preparation.

The problems encountered by analysts in the preparation of samples for analysis include (Pomeranz and Meloan 1994):

- difficulty in obtaining representative small samples from large samples;
- loss of plant material;
- difficulty in removal of extraneous material from plants without removal of plant constituents, including the analyte;
- enzymatic changes before and during analysis;
- compositional changes during grinding;
- changes in unstable components.

The sample preparation procedure should be adapted to the nature of the food, the analyte, and the analytical method, as well as to the distribution of the analyte in the food.

In our laboratory, the sampling and sample preparation schemes depend on the food under investigation. Sampling for average carotenoid composition is done in distribution centers, supermarkets, groceries, and other retail outlets so as to represent the composition of foods as offered to the consumer. Perennial crops are sampled at different times during the year, and seasonal produce at different times during the season. Each laboratory sample is obtained by taking several increments at random from different parts of the big lot at the sampling site. Depending on the food under investigation, 200 g to 1 kg are usually taken to the laboratory. At the laboratory, inedible portions are removed. For small fruits or fruit vegetables, several fruits are randomly selected from the laboratory sample and homogenized in a food processor; duplicate portions are then weighed and extracted immediately to avoid enzymatic oxidation. Larger fruits or fruit vegetables are taken at random from the laboratory sample, quartered longitudinally, and then opposite sections from each fruit are combined and homogenized in a food processor Vegetables like leafy vegetables and green beans are cut into small pieces and mixed. For headed vegetables such as cabbage, and bunches such as unheaded lettuce, the head or bunch is opened at the center and a proportional number of young and mature leaves are taken from each side prior to cutting. For commercial processed products, which would normally undergo homogenization during processing, at least two units are taken at random from the same production lot and mixed before weighing the sample for analysis. To investigate the effects of influencing factors, sampling has to be designed such that variables are controlled.

Extraction

A good extraction procedure should release all the carotenoids from the food matrix and bring them into solution, without altering them. Because carotenoids are found in a variety of foods, the extraction procedure should be adapted to suit the food being analyzed. The solvent chosen should efficiently extract all carotenoids present in the sample.

Extraction, partition, and open column chromatography (OCC) should be carried out under a fume hood to protect the analyst from inhaling solvent vapor. Breathing hexane, for example, should be avoided due to neurotoxicity of some of its oxidative metabolites (Schiedt and Liaaen-Jensen 1995).

Because the solvents used in extraction or partition will ultimately be removed or at least reduced by evaporation, solvents with low boiling points should be chosen to avoid prolonged heating. Thus, the lower boiling fractions of petroleum ether (b.p. 35–60^{o}C) should be used instead of the higher boiling fractions.

When extracting carotenoids from biological samples, such as foods, which contain large amounts of water, a water-miscible organic solvent (e.g., acetone, methanol, ethanol, or mixtures thereof) should be used to allow better solvent penetration. Dried materials can be extracted with water-immiscible solvents, but extraction is usually more efficient if the samples are rehydrated first and then extracted with water-miscible solvents. Acetone has been widely used in carotenoid extraction; however, the advent of high performance liquid chromatography (HPLC) has seen tetrahydrofuran (THF) become a popular extraction solvent.

The sample is generally homogenized with the solvent in a suitable mechanical blender for 1 to 2 min or with a mortar and pestle. A Waring blender is fast and efficient for mechanical disruption and homogenization of soft fruits and juices. For samples like fresh leaves, however, a mortar and pestle is better because small pieces of leaves, which can escape the blender blades, can be well ground. A combination of the two can also be used, starting with the blender and finishing with the mortar and pestle. Rapid, uniform homogenization can also be achieved with a Polytron homogenizer. Another alternative is vortexing, although this method should be used only for samples that are finely ground and easy to extract.

Leaves and other difficult-to-extract matrices may need previous soaking in the extraction solvent (about 15 minutes for leaves) to soften the cell wall. Prolonged soaking should, however, be avoided to prevent isomerization and degradation of the carotenoids. Celite or Hyflosupercel is often added during extraction to facilitate both tissue disintegration and the subsequent filtration.

$MgCO_3$ or another neutralizing agent is often added to neutralize the acids liberated during tissue disintegration in order to prevent isomerization and degradation. In our laboratory, the practice of keeping the time lag between sample maceration and extraction as short as possible not only prevents enzymatic oxidation, but also makes the addition of neutralizing agents unnecessary.

Filtration can be done with a sintered glass funnel (porosity 3; pore size 20–30 µm) or with a Buchner funnel. Using the latter is less expensive and pore clogging is not a problem. The filter paper should, however, be properly fitted so that the celite and the fine sample particles cannot pass through.

After filtration, the solid residue is returned to the blender and re-extracted with fresh solvent. Extraction and filtration are repeated until the residue is colorless (three extractions is usually sufficient).

Partition

The extract usually contains a substantial amount of water, which can be removed by partitioning to hexane, petroleum ether, diethyl ether, or dichloromethane, or mixtures of these solvents. Diethyl ether or a mixture of this solvent with hexane or petroleum ether is preferred for extracts with large amounts of xanthophylls, part of which is lost during partitioning with pure hexane or

petroleum ether. Partitioning is an integral part of open column methods, so that chromatography can be started at a low mobile-phase polarity, which is then increased during the separation process. In HPLC methods, the extract is sometimes directly evaporated to dryness and then dissolved in the mobile phase or a solvent compatible with the mobile phase.

Partitioning is best done by adding small portions of the acetone, methanol, or THF extract to petroleum ether or another appropriate solvent in a separatory funnel. After the addition of each portion, water is added gently to avoid formation of an emulsion, preferably by allowing it to flow along the walls of the funnel. The two layers are allowed to separate, without agitation, and the lower aqueous phase is discarded. When the entire extract has been added, the petroleum ether phase is washed four or five times with water to remove residual acetone, methanol, or THF.

Saponification

Saponification is an effective means of removing chlorophylls and unwanted lipids, which may interfere with the chromatographic separation and shorten the column's life. In samples like fruits, saponification hydrolyzes the carotenol esters. This simplifies the chromatographic separation, identification, and quantification because the free carotenols are analyzed instead of the carotenol esters, which are usually present in a difficult-to-separate mixture of esters with a variety of fatty acids. Saponification and the subsequent washing, however, can result in losses of carotenoids, especially xanthophylls. Hence it should be omitted from the analytical procedure whenever possible.

When indispensable, saponification is best carried out after transferring the carotenoids to petroleum ether or hexane, and then adding an equal volume of 10% methanolic KOH. The resulting mixture is left overnight at room temperature in the dark, and then the carotenoid solution is washed five times with water to remove the alkali. To avoid losing carotenoids, especially the more polar ones, into the washing water, this step should be done in the same manner as in the partitioning procedure described above.

For high-lipid samples, such as red palm oil, a better procedure for eliminating lipids should be pursued. In our laboratory, palm oil samples are dissolved in acetone and left in a freezer (–15°C) for 4–5 hours to solidify the lipids (Trujillo et al. 1998). The lipids are then separated by filtration with a sintered glass funnel; this operation, which removes about 90% of the lipids, is carried out in the freezer compartment to maintain the low temperature. After partition to petroleum ether, the carotenoid solution is saponified with an equal volume of 20% KOH in methanol overnight at room temperature, with the addition of butylated hydroxytoluene (BHT).

To follow the chromatography rule that the sample be introduced into the chromatographic system in the smallest volume possible, the carotenoid solution, after partitioning for unsaponified samples and after washing for saponified samples, is dried with anhydrous sodium sulfate and then concentrated for open column chromatography (OCC) or evaporated to dryness to be taken up in the mobile phase or another appropriate solvent for HPLC.

Chromatographic separation

Food samples typically contain both the apolar carotenes and the more polar xanthophylls. Whatever the method used, the chromatographic process should be able to cope with this polarity range.

Chromatography in descending, gravity-flow (often with slight pressure provided by a water aspirator) columns, which has come to be known as OCC, is the classical method for separating carotenoids for quantitative analysis. It is also useful in isolating and purifying carotenoids to be utilized as standards for HPLC. Separation of the carotenoid pigments is followed visually. Low pressure may also be applied at the top of the column (e.g., with N_2 gas); this technique is called flash column chromatography.

Thin-layer chromatography (TLC), although efficient in monitoring the progress of chemical tests for identification purposes, is not adequate for quantitative analysis because of the danger of degradation and isomerization

on a highly exposed plate (Liaaen-Jensen 1971, Taylor 1983). Carotenoids are particularly prone to oxidation by air when adsorbed on TLC plates. Additionally, it is not easy to quantitatively apply the sample on the plate and quantitatively recover the separated carotenoids from the plate for measurement. Gas chromatography (GC) is also inappropriate because of the thermal lability and low volatility of carotenoids.

In OCC, the column has to be packed for each analysis. A definite advantage of HPLC over OCC is that reproducible separations can be performed by using a reusable column under controlled conditions without undue exposure to air or light.

Reversed-phase HPLC on C_{18} columns has been the preferred mode for separating carotenoids for quantitative analysis. The popularity of the C_{18} column derives from its weak hydrophobic interactions with the analytes (which should make it less destructive than the polar forces in normal-phase OCC), compatibility with most carotenoid solvents and with the polarity range of carotenoids, and wide commercial availability. Many different C_{18} reversed-phase materials are available from different manufacturers, varying in the degree of carbon loading, end capping, and the nature of the bonded phase (i.e., monomeric or polymeric).

The majority of carotenoid separation has been carried out with 5 µm C_{18} spherical particles packed in a 250 x 4.6 mm column. However, shorter and narrower (narrow bore) columns, smaller particles, and C_{30} stationary phases are increasingly used.

Monomeric phases are simpler to use and more reproducible. Polymeric C_{18} phases have been found to have excellent selectivity for structurally similar carotenoids such as the geometric isomers of β–carotene (Lesellier et al. 1989, Craft 1992), and lutein and zeaxanthin (Epler et al. 1992). However, the total carbon load is lower in the wide-pore polymeric phases, resulting in weak retention of carotenoids (Craft 1992). Additionally, compared with monomeric columns, the peaks tend to be broader and columns from different production lots are more variable.

Guard columns, which should be changed frequently, are used for food samples to prevent particulate material and impurities from entering the analytical column, thus prolonging the column's life. Guard columns can, however, cause band broadening and could potentially retain carotenoids.

The most important properties to be considered in selecting the mobile phase are polarity, viscosity, volatility, and toxicity. In addition, it must be inert with respect to the carotenoids. Many solvent systems have been suggested as mobile phases for carotenoids, but the primary solvents are acetonitrile and methanol, with most systems being slight modifications of some basic combinations (Craft 1992). Acetonitrile has been widely used because of its lower viscosity and slightly better selectivity for xanthophylls when a monomeric C_{18} column is used (Khachik et al. 1986). Epler et al. (1992) reported, however, that methanol-based solvents gave higher recoveries of carotenoids than acetonitrile-based solvents in almost all of 65 columns tested. Methanol is also more available, less expensive, and less toxic than acetonitrile. Addition of triethylamine to acetonitrile-based solvents was found to enhance carotenoid recovery (Hart and Scott 1995).

Small amounts of other solvents are added to obtain the desired retention, increase solubility, and improve resolution. Chlorinated solvents (e.g., chloroform, dichloromethane) are frequently used for this purpose on account of their good solvent properties and effects on selectivity, although these solvents can be contaminated with traces of HCl. Other solvents used as modifiers are THF, ethyl acetate, hexane, acetone, and water. In some cases methanol has been added to an acetonitrile-based mobile phase. Craft (1992) investigated nine solvent modifiers and found THF to be the most beneficial modifier of methanol. Analysts tend to use mixtures of three or more solvents; however, Craft (1992) cautioned against this practice because it can complicate the method, enhance demixing, and result in different evaporation rates, causing variation in the retention times during the course of the day.

Gradient elution should only be employed when the analysis cannot be done isocratically. Isocratic separation is rapid, can be performed with simple equipment (a single high-pressure pump and premixed solvent), and results in a stable baseline and more reproducible retention times. It is usually sufficient for the determination of provitamin A carotenoids or the principal carotenoids of food samples.

Gradient elution has the advantages of greater resolving power, improved sensitivity, and elution of strongly retained compounds. It is more likely to resolve the whole range of carotenoids found in a given food. However, it has several disadvantages: (a) increased complexity, (b) requirement for more sophisticated and expensive equipment, (c) need for column reequilibration between runs, (d) greater differential detector response (i.e., different detector signals for the same concentration of different compounds), and (e) often poor reproducibility. The column must be brought back to the starting solvent and equilibrated for 10 to 30 minutes in this solvent before a new run is commenced. Good solvent miscibility is required to prevent baseline disturbance due to outgassing and refractive index effects (Craft 1992).

Because of the qualitative and quantitative variations in the carotenoid compositions of foods, it is doubtful whether a single chromatographic system can be established that is applicable to different foods. At least some modification of the mobile phase is needed when changing from one food to another.

Because *cis*-isomers have different biological potencies than their *trans* counterpart, it is necessary to separate and quantify *cis*-isomers when they are present in appreciable amounts. This level of detail, however, makes the analysis even more complicated. The polymeric C_{30} column was developed specifically for this purpose (Sander et al. 1994, Emenhiser et al. 1996). This column, with an isocratic solvent system consisting of methanol:methyl-*tert*-butyl ether (89:11), was used for the quantification of *cis-trans* isomers of provitamin A carotenoids in fresh and processed fruits and vegetables (Lessin et al. 1997).

The injection solvent must be compatible with the HPLC mobile phase. If the carotenoids are much more soluble in the injection solvent than in the mobile phase, the carotenoids will precipitate on injection, leading to peak tailing, or they will remain in the injection solvent while passing though the column, resulting in broad bands and doubled peaks (Craft 1992). These problems are particularly pronounced if the carotenoid solution is nearly saturated. The carotenoids will not dissolve completely if the solvent is too weak. Samples can be injected in the mobile phase to avoid this incompatibility problem. However, because of the solubility range of carotenoids in food samples, another solvent may be preferred for solubilization and injection.

Temperature regulation is recommended to maintain within-day and day-to-day reproducibility. Variations in column temperature result in substantial fluctuation of the carotenoids' retention times. Temperature may also influence selectivity.

The color enables analysts to monitor the different steps of carotenoid analysis. Loss or change of color at any time during the analysis gives an immediate indication of degradation or structural modification. Color permits visual monitoring of the separation of carotenoids in OCC, and mainly for this reason, this classical technique is still a viable option for carotenoid analysis.

Identification

The chromatographic behavior and the UV/visible absorption spectrum provide the first clues for the identification of carotenoids. Both the wavelengths of maximum absorption (λ_{max}) and the shape of the spectrum (spectral fine structure) are characteristic of the chromophore. The λ_{max} values of the carotenoids commonly found in foods are listed in Table 1. In the discussion below, the λ_{max} values cited are those of the carotenoids in petroleum ether.

Most carotenoids absorb maximally at three wavelengths, resulting in a three-peak spectrum. As the number of conjugated double bonds increases, the

λ_{max} shifts to longer wavelengths. Thus, the most unsaturated acyclic carotenoid, lycopene, with 11 conjugated double bonds, is red and absorbs at the longest wavelengths (λ_{max} at 444, 470, 502 nm) (Figure 4). ζ-Carotene (light yellow), which is also acyclic, also has three well-defined peaks, but at much lower wavelengths (λ_{max} at 378, 400, 425 nm) (Figure 5), commensurate with seven conjugated double bonds. The two colorless carotenoids that precede ζ–carotene in the desaturation stage of the biosynthetic pathway, phytoene (three conjugated double bonds) and phytofluene (five conjugated double bonds), have

TABLE 1

UV/VISIBLE ABSORPTION DATA FOR COMMON FOOD CAROTENOIDS

Carotenoid	Solvent	λ_{max} (nm)			% III/II
α-Carotene	acetone	424	448	476	55
	chloroform	433	457	484	
	ethanol	423	444	473	
	hexane, petroleum ether	422	445	473	55
β-Carotene	acetone	(429)	452	478	15
	chloroform	(435)	461	485	
	ethanol	(425)	450	478	25
	hexane, petroleum ether	(425)	450	477	25
α-Cryptoxanthin/Zeinoxanthin	chloroform	435	459	487	
	ethanol	423	446	473	60
	hexane	421	445	475	60
β-Cryptoxanthin	chloroform	(435)	459	485	
	ethanol	(428)	450	478	27
	petroleum ether	(425)	449	476	25
Lutein	chloroform	435	458	485	
	ethanol	422	445	474	60
	petroleum ether	421	445	474	60
Lycopene	acetone	448	474	505	
	chloroform	458	484	518	
	ethanol	446	472	503	65
	petroleum ether	444	470	502	65
Zeaxanthin	acetone	(430)	452	479	
	chloroform	(433)	462	493	
	ethanol	(428)	450	478	26
	petroleum ether	(424)	449	476	25

References: Britton 1995, Davies 1976.
Parentheses indicate a shoulder.
%III/II is the ratio of the height of the longest-wavelength absorption peak, designated III, to that of the middle absorption peak, designated II, taking the minimum between the two peaks as the baseline, multiplied by 100.

maximum absorptions in the UV region (at 276, 286, 297 nm and 331, 348, 367 nm, respectively) (Figure 5).

Cyclization results in steric hindrance between the ring methyl group at C-5 and the hydrogen atom at C-8 of the polyene chain. This hindrance takes the π electrons of the ring double bond out of plane with respect to those of the chain, causing a hypsochromic shift (displacement of λ_{max} to shorter wavelength), a hypochromic effect (decrease in absorbance), and loss of fine structure (spectrum with less defined peaks). Thus, the dicyclic molecule β–carotene, despite possessing the same number of conjugated double bonds as lycopene, is yellow-orange and exhibits absorption peaks at 450 and 477 nm and a mere shoulder at 425 nm (Figure 4). Monocyclic γ-carotene is red-orange and exhibits a spectrum intermediate to those of lycopene and β–carotene, in both l_{max} and shape, reflecting a structure that is intermediate with respect to the other two carotenoids. The double bond in the ε-ring of α–carotene is out of conjugation, leaving 10 conjugated double bonds (9 in the polyene chain and 1 in the β–ring); thus, this carotenoid is light yellow and its absorption spectrum is slightly better defined with λ_{maxs} at slightly shorter wavelengths (422, 445, 473 nm) compared with the spectrum of β–carotene.

The introduction of hydroxy substituents into the carotenoid molecule does not affect the chromophore and therefore has virtually no effect on the absorption spectrum. Thus, the spectra of lutein, zeinoxanthin, and α–cryptoxanthin resemble that of α–carotene, and those of β–cryptoxanthin and zeaxanthin are indistinguishable from that of β–carotene.

Cis-isomerization of a chromophore's double bond causes a slight loss in color, a small hypsochromic shift (usually 2 to 6 nm for mono-*cis*), and a hypochromic effect, accompanied by the appearance of a *"cis"* peak about 142 nm below the longest-wavelength absorption maximum of the *trans*-carotenoid when measured in hexane (Figure 6) (Davies 1976, Britton 1995). The intensity of the *cis*-band is greater when the *cis* double bond is nearer the

FIGURE 4

Visible absoption spectra of lycopene (____), γ-carotene (- - -), β-carotene (-.-.-.) and α-carotene (....) in petroleum ether

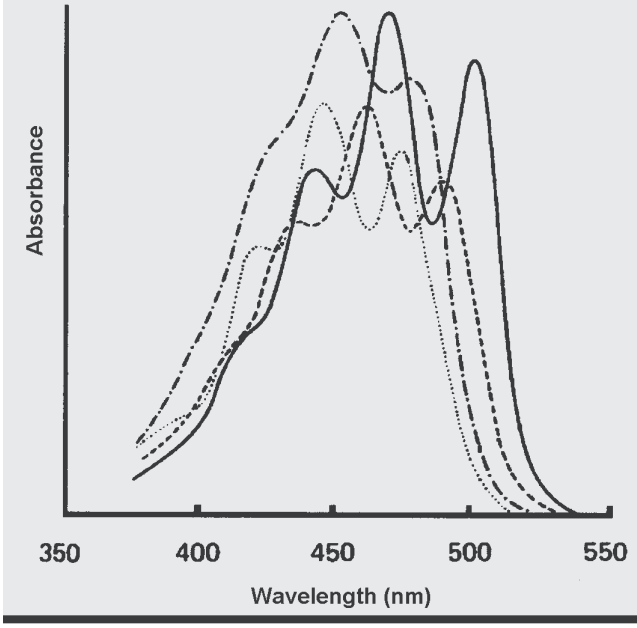
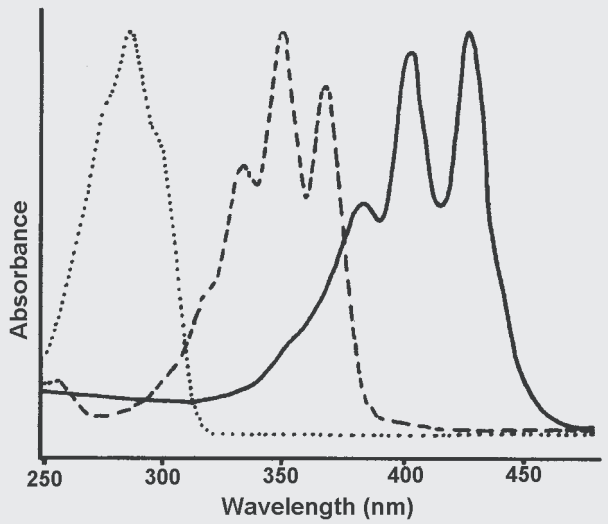


FIGURE 5

Photodiode array spectra of ζ-carotene (_____), phytofluene (----) and phytoene (....). Mobile phase: acetonitrile/ethyl acetate/methanol (85:10:5)



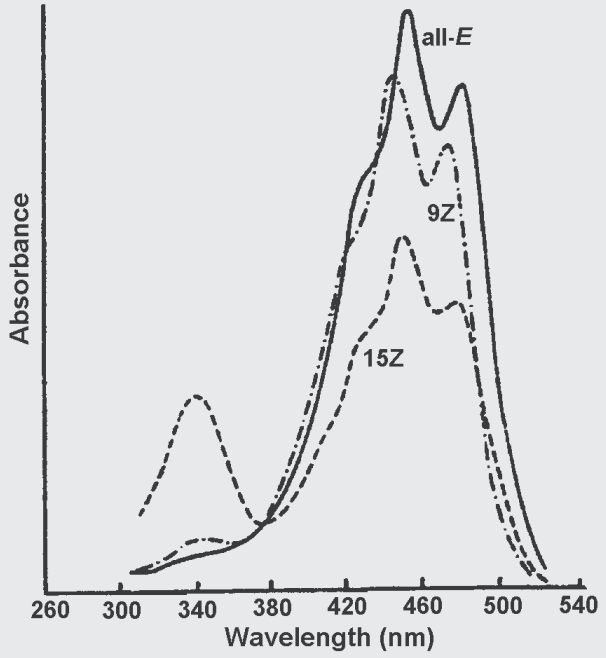
center of the molecule. Thus, the 15-*cis* isomer, in which the *cis* double bond is at the center of the molecule, has a very prominent *cis*-peak.

The λ_{max} values reported in the literature for the same carotenoid vary slightly, which is understandable considering that the error in spectrophotometer readings in the 400–500 nm region is about $\pm$1–2 nm. Instruments should be calibrated to minimize errors, for example by using a holmium oxide filter and recording the spectra of authentic carotenoid standards.

The absorption spectra of carotenoids are markedly solvent dependent (Table 1). This characteristic of carotenoids must be remembered when analyzing spectra recorded by the photodiode array detector in HPLC because such spectra are taken in mixed solvents in isocratic elution and in varying mixed solvents in gradient elution.

FIGURE 6

Absorption spectra of geometric isomers of β-carotene in hexane



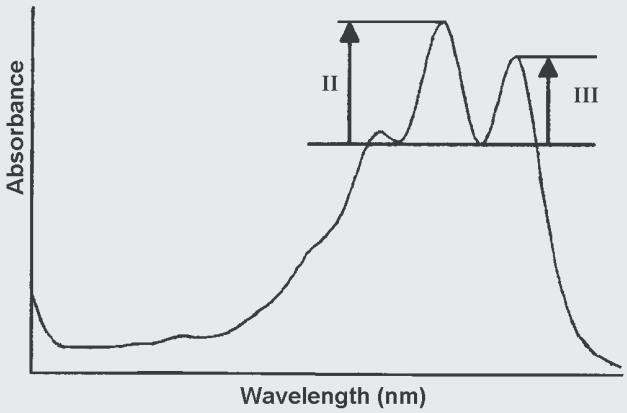
The λ_{max} values of carotenoids in hexane or petroleum ether are practically the same as in diethyl ether, methanol, ethanol, and acetonitrile, and higher by 2–6 nm in acetone, 10–20 nm in chloroform, 10–20 nm in dichloromethane and 18–24 nm in toluene (Britton 1995).

Absorption spectra are now rarely presented in publications on carotenoids. To give an idea of the spectral fine structure, the %III/II (Figure 7) can be presented, along with the λ_{max} values. The %III/II is the ratio of the height of the longest-wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the two peaks as the baseline, multiplied by 100 (Britton 1995). The relative intensity of the *cis*-peak is expressed as $\%A_B/A_{II}$ (Figure 8), which is the ratio of the height of the *cis*-peak, designated A_B, and that of the middle main absorption peak, designated A_{II}, multiplied by 100 (Figure 8).

The chromatographic behavior of carotenoids bears a definite relationship with their structures; however, carotenoids cannot be identified based on chromatographic data alone. Nevertheless, these data serve as useful complementary information.

FIGURE 7

Calculation of %III/II (III/II x 100) as an indicator of spectral fine structure



In normal-phase OCC, the adsorption affinity depends on (a) the number of conjugated double bonds, (b) cyclization, (c) presence of oxygen substituents.

The influence of the number of conjugated double bonds is best illustrated by the adsorption affinities of the acyclic carotenoids, which elute in order of increasing conjugation length: phytoene, phytofluene, ζ–carotene, neurosporene, and lycopene. Comparing monocyclic and bicyclic carotenes, δ-carotene elutes before γ-carotene, and α–carotene before β–carotene.

Cyclization decreases the adsorption affinity. Thus, β–carotene is more weakly adsorbed than g-carotene, which in turn, elutes before lycopene.

The presence of oxygen substituents increases adsorption affinity, the extent of this increase depending on the type, number, and location of the functions. This is demonstrated in a silica thin layer developed with 3% methanol in benzene or 5% methanol in toluene, in which all carotenes elute with the solvent front whereas the xanthophylls are distributed along the plate according to the number and type of substituents present (Figure 9). The hydroxyl group greatly influences absorption behavior. For example, in the TLC chromatogram in Figure 9, the dihydroxycarotenoid appears near the origin whereas the monohydroxycarotenoid appears near the middle of the chromatogram. The effects of more than one oxygen substituent are not always additive; a second substituent in the same end group tends to have less influence than the first.

In reversed-phase HPLC, the order is more or less the reverse of that seen in normal-phase adsorption OCC. The more polar xanthophylls (the trihydroxy-5,6-epoxy neoxanthin, the dihydroxy-5,6,5',6'-epoxy violaxanthin and the dihydroxy lutein and zeaxanthin) elute well before the carotenes (Figure 10), while the monohydroxy carotenoids elute between these two groups. Elution of carotenes does not always follow the expected pattern and varies depending on the type of column (monomeric or polymeric) and the mobile phase, with β–carotene eluting after or before lycopene. α–Carotene usually elutes before β–carotene, as in normal phase chromatography.

FIGURE 8

Calculation of $\%A_B/A_{II}$ (A_B/A_{II} x 100) as an indicator of *cis*-peak intensity

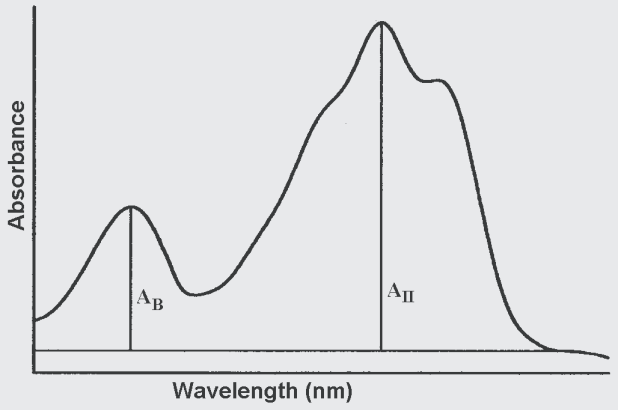
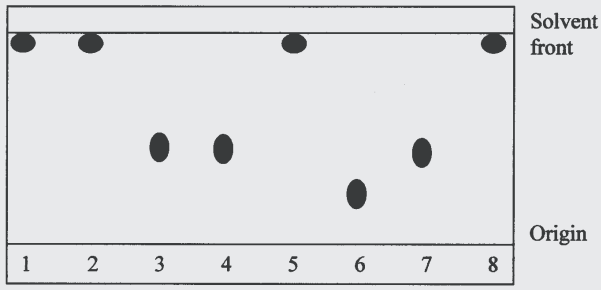


FIGURE 9

TLC silica gel chromatogram of carotenoids and reaction products, developed with 5% methanol in toluene. (1) β-Carotene, (2) lycopene, (3) β-cryptoxanthin, (4) β-cryptoxanthin methylated with acidic methanol - negative response, (5) β-cryptoxanthin acetylated with acetic anhydride, (6) lutein, (7) lutein methylated with acidic methanol, (8) lutein acetylated with acetic anhydride



In HPLC, the availability of a photodiode array detector allows the on-line acquisition of spectra, facilitating the use of spectral characteristics in the identification of carotenoids (Figure 10). Spectra can be recorded, stored, and subsequently compared with those of standards. Spectra taken at points across the peak provide a means of verifying the peak purity (i.e. absence of interfering substances).

Identification of carotenoids based solely on retention times and absorption spectra may lead to erroneous conclusions. Retention times are difficult to reproduce even in the same laboratory, and may vary during the course of a day. Even when carotenoid standards are available and co-chromatography (i.e. spiking) can be done, identification is still not conclusive because different carotenoids can have the same retention time in a given chromatographic system. By the same token, different carotenoids may have the same chromophore and thus exhibit the same absorption spectrum. The widespread use of these two parameters as the only criteria for carotenoid identification has led to various cases of misidentification in the literature. Thus, it has been recommended that the following minimum criteria be fulfilled for identification (Pfander et al. 1994, Schiedt and Liaaen-Jensen 1995):

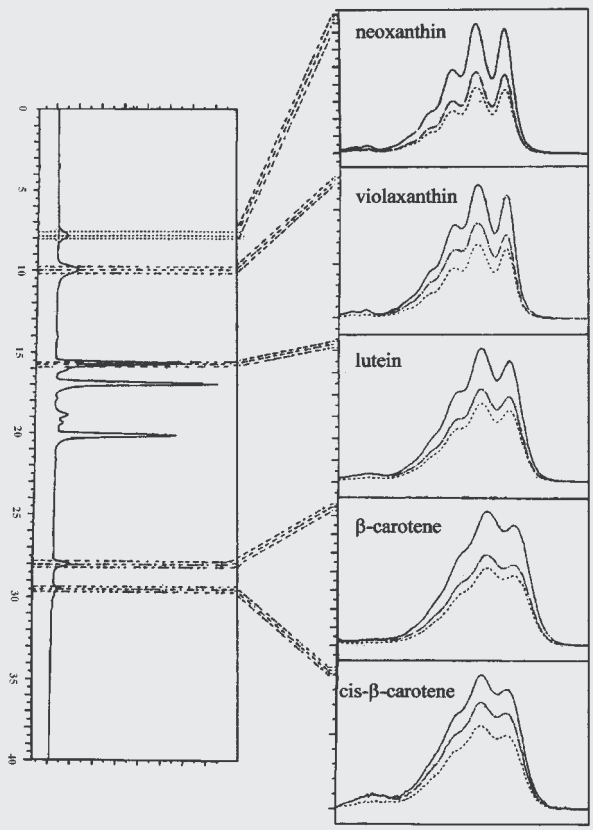
- the visible (or UV for shorter chromophores) absorption spectrum (λ_{max} and fine structure) in at least two different solvents must be in agreement with the chromophore suggested;
- chromatographic properties must be identical in two systems, preferably TLC (R_F) and HPLC (t_R) and co-chromatography with an authentic sample should be demonstrated;
- a mass spectrum should be obtained, which allows at least confirmation of the molecular mass.

The requirement of a mass spectrum would, however, limit carotenoid analysis to a very few laboratories around the world, precluding its execution in areas where it is very much needed. Moreover, common carotenoids can be conclusively identified by the judicious and combined use of chromatographic data, absorption spectra, and specific chemical reactions, with the latter being used to confirm the type, location, and number of functional groups (Davies 1976, Eugster 1995, Rodriguez-Amaya 1999a, Azevedo-Meleiro and Rodriguez-Amaya 2004a).

Xanthophylls undergo functional-group reactions that can serve as simple chemical tests for their identification. These reactions can be performed quickly, with only a small amount of the test carotenoid, and are amenable to rapid monitoring by UV/visible spectrometry, TLC, or HPLC (Davies 1976, Eugster 1995, Rodriguez-Amaya 1999a).

FIGURE 10

HPLC chromatogram and photodiode array spectra of the carotenoids of water cress. Column - polymeric C_{18} Vydac 218TP54, 4.6 x 250 mm, 5 μm; mobile phase - gradient of 10% H_2O to 10% THF in methanol. The other major peaks are of chlorophylls



For example, primary and secondary hydroxy groups are acetylated by acetic anhydride in pyridine. Allylic hydroxyls, isolated or allylic to the chromophore, are methylated with acidic methanol. In both reactions, a positive response is shown by an increase in the silica TLC R_F value or the retention time in reversed phase HPLC, the extent of the increase depending on the number of hydroxy substituents.

Quantification

Carotenoids in solution obey the Beer–Lambert law, that is, their absorbance is directly proportional to the concentration. Thus, carotenoids are quantified spectrophotometrically, provided accurate absorption coefficients in the desired solvent are available. Some published values may contain significant levels of error or uncertainty (Britton 1995), and some discrepancies can be noted in the values presented in Table 2. Given that different authors choose different coefficients for some carotenoids (in the same solvents), this alone can account for a good part of the variations in analytical results.

In OCC methods, the quantification step is fairly straightforward. The separated carotenoid fractions are simply collected and quantified spectrophotometrically using their tabulated absorption coefficients.

The absorption coefficient $A^{1\%}_{1cm}$ of a carotenoid (absorbance at a given wavelength of a 1% solution in 1 cm light-path spectrophotometer cuvette), used in the calculation of the concentration, also shows a marked solvent dependence (Table 2).

TABLE 2

Absorption coefficients ($A^{1\%}_{1cm}$) of common food carotenoids

Carotenoid	Solvent	λ_{max} (nm)	$A^{1\%}_{1cm}$
α–Carotene	petroleum ether	444	2800
	hexane	445	2710
β–Carotene	petroleum ether	450	2592
	ethanol	450	2620
	chloroform	465	2396
α–Cryptoxanthin/Zeinoxanthin	hexane	445	2636
β–Cryptoxanthin	petroleum ether	449	2386
	hexane	450	2460
Lutein	ethanol	445	2550
	diethyl ether	445	2480
	diethyl ether	445	2600
Lycopene	petroleum ether	470	3450
Zeaxanthin	petroleum ether	449	2348
	ethanol	450	2480
	ethanol	450	2540
	acetone	452	2340

Taken from Britton 1995.

In quantitative analysis by HPLC the following facts should be considered:

- Carotenoids absorb maximally at different wavelengths and have different absorption coefficients.
- Solvent effects on absorption are substantial. Tabulated absorption coefficients and λ_{max} values refer to single solvents. The mobile phase in HPLC isocratic elution is usually a mixture and in gradient elution, the mixture's composition varies during the chromatographic process.
- Obtaining and maintaining carotenoid standards, which are required for calibration, is difficult.

Modern liquid chromatographs allow measurement of carotenoids at the wavelengths of maximum absorption. In older chromatographs, more than one injection for the same sample may be necessary for samples containing phytoene, phytofluene, or ζ-carotene, along with other carotenoids.

HPLC quantification is carried out by means of internal or external calibration, for which the concentrations of the standards are also determined spectrophotometrically as in OCC. A constant supply of carotenoid standards is needed, and the accuracy of the analytical results depends on how accurately the concentrations of the standard solutions are known. Unfortunately, only a few carotenoid standards (e.g., β-carotene, lycopene) are available commercially. Moreover, commercial β-carotene standards have been shown to have widely varying purity (Quackenbush and Smallidge 1986, Craft et al. 1990).

Other carotenoids have to be isolated and purified from natural sources by the analyst. This can be done by OCC or by accumulating separated fractions from several HPLC runs. Both procedures are time-consuming and tedious, and require experience and patience.

An ideal commercially available internal standard has yet to be encountered due to the difficulty of finding a readily available and stable compound that has chemical and spectral properties similar to those of carotenoids. In the calibration process, the analyst has to prepare standard solutions of varying concentrations, inject each of these solutions, and construct the standard curve. This curve should be linear and pass through the origin, and must bracket the concentrations of the food samples. Khachik et al. (1992) suggested the following guidelines for the validity of the standards and the instrumentation: (a) the correlation coefficient should be greater than 0.9, (b) the intercept should be very close to zero, and (c) the relative standard deviation of the regression (standard error of the estimate divided by average concentration of standards multiplied by 100) should be less than 5%. If any of these parameters is out of range, the standards as well as the HPLC instrumentation should be carefully examined and the standard curve rerun. Mantoura et al. (1997) recommended that the correlation coefficient should be greater than 0.95.

The wide concentration range of carotenoids in any given food is more of a problem in HPLC than in OCC, because in OCC each fraction can simply be diluted or concentrated to obtain an adequate concentration for spectrometric measurement.

Aside from the internal standards for calibration, standards also termed internal standards have been added at the beginning of the analysis to appraise losses of carotenoids during extraction and the entire work-up process. Given the differing stabilities of carotenoids and the standards themselves, it is questionable whether recovery percentages of these standards do in fact reflect the true losses of the carotenoids. Additionally, use of these standards does not evaluate the extraction step because they are not intimately linked with the food matrices and are, therefore, more easily extracted compared with endogenous carotenoids.

III | Sources of Errors in Carotenoid Analysis

Carotenoid analysis is inherently difficult due to several factors (Rodriguez-Amaya 1989, 1990, 1999a, Rodriguez-Amaya and Amaya-Farfan 1992):

- There are many naturally occurring carotenoids. Thus, conclusive identification, a pre-requisite to accurate quantification, is not easily accomplished. The limited commercial availability of carotenoid standards is also a serious deterrent to carotenoid analysis.

- The carotenoid composition of foods differs qualitatively and quantitatively. Thus, the analytical procedure, principally the chromatographic steps, has to be adapted to the carotenoid composition of each food sample.

- The carotenoid levels vary between samples of the same food and the distribution of the carotenoids in a fruit, vegetable, grain or root is not uniform. Thus, statistically sound sampling and sample preparation procedures should be established.

- The carotenoid concentrations in any given food vary over a wide range. Typically, one to four principal carotenoids are present, with a series of other carotenoids at low or trace levels. The separation, identification, and quantification of these minor carotenoids is a formidable challenge for food analysts. In most cases, the information sought is provided by quantification of only the principal carotenoids.

- The nature of the matrix and the keeping quality of food samples differ. Thus sample preparation, extraction, and storage conditions should be established for each food.

- The highly unsaturated carotenoid molecule is susceptible to isomerization and oxidation, which can easily occur during analysis and/or during storage of samples prior to analysis. Thus, measures to prevent these reactions in the sample and in the carotenoid standards should be standard practices in carotenoid laboratories.

Special precautions in carotenoid analysis

The main problem in the analysis of carotenoids stems from their instability. Thus, whatever the analytical method chosen, precautionary measures to avoid artifacts and quantitative losses should be taken. These include (Davies 1976, Britton 1991, Schiedt and Liaaen-Jensen 1995):

- completion of the analysis within the shortest possible time;
- exclusion of oxygen;
- protection from light;
- avoiding high temperatures;
- avoiding contact with acid;
- use of high purity solvents, free from harmful impurities.

Oxygen, especially in combination with light and heat, is highly destructive. The presence of even traces of oxygen in stored samples (even at deep-freeze temperatures) and of peroxides in solvents (e.g., diethyl ether and THF) or of any oxidizing agent, even in crude extracts of carotenoids, can rapidly lead to bleaching and the formation of artifacts such as epoxy-carotenoids and apocarotenals (Britton 1991). Oxygen can be excluded at several steps during analysis and during storage through the use of vacuum and a N_2 or argon atmosphere. Antioxidants (e.g., BHT, pyrogallol, ascorbyl palmitate) may also be used, especially when the analysis is prolonged. They can be added during sample disintegration or saponification, or added to solvents (e.g., THF), standard solutions, and isolates.

Exposure to light, especially direct sunlight or UV light, induces *trans-cis* photoisomerization and photodestruction of carotenoids. Thus, carotenoid work must be done under subdued light. Open columns and vessels containing carotenoids should be wrapped with aluminum foil, and TLC development tanks should be kept in the dark or covered with dark cloth or aluminum foil. Fluorescent lights, notorious

emitters of high energy, short-wavelength radiation, can be covered with polycarbonate shields. These shields absorb radiation of 375–390 nm and shorter wavelengths, allowing the use of full, "normal" light in laboratories. However, these shields should still be used in conjunction with measures such as covering flasks and columns.

Speed of manipulation and shielding from light are especially important in extracts containing chlorophylls (e.g., extracts of green, leafy or nonleafy, vegetables) or other potential sensitizers. In the presence of these sensitizers, photodegradation and isomerization occur very rapidly, even with brief exposure to light (Britton 1991).

Because of their thermolability, carotenoids should be heated only when absolutely necessary. Carotenoid extracts or solutions should be concentrated in a rotary evaporator at reduced pressure, at a temperature below 40°C, and solvent evaporation should be finished with N_2 or argon. Care should be taken not to allow the extract to go to complete dryness in the rotary evaporator as this may result in degradation of carotenoids, especially lycopene (Tonucci et al. 1995). Additionally, part of the carotenoids, especially the more polar ones, may adhere strongly to the glass walls, precluding quantitative removal from the flask.

Carotenoids may decompose, dehydrate, or isomerize in the presence of acids. The 5,6-epoxides of cyclic carotenoids such as violaxanthin and neoxanthin readily undergo rearrangement to the corresponding 5,8-epoxides. Most carotenoids are stable under alkali conditions. A neutralizing agent (e.g., $CaCO_3$, $MgCO_3$, or $NaHCO_3$) may be added during extraction to neutralize acids liberated from the food sample itself. Strong acids and acidic reagents should not be used in rooms where carotenoids are handled.

Reagent-grade, UV/Vis-grade or HPLC-grade solvents should be employed. If only technical-grade solvents are available, these should be purified, dried, and freshly distilled before being used for extraction or chromatography. Diethyl ether and THF should be tested for peroxides, which can be removed by distillation over reduced iron powder or calcium hydride. Because it easily accumulates peroxides, THF is usually supplied stabilized with the antioxidant BHT, but there is a time limit to its use.

Chloroform is best avoided due to the difficultly of removing all traces of HCl from it. In addition, it is generally stabilized with 1% ethanol, which can affect its properties as a solvent for chromatography. Benzene, although an excellent solvent, should also be avoided because of its toxicity. Chloroform can be replaced by dichloromethane and benzene by toluene.

Fractions or isolates should be kept dry under N_2 or argon or dissolved in a hydrocarbon solvent, e.g., petroleum ether or hexane, and kept at –20°C or lower, when not in use. Leaving carotenoids in solvents such as cyclohexane, dichloromethane, diethyl ether (Craft and Soares 1992), and acetone can lead to substantial degradation. In our laboratory, carotenoids extracted with acetone are immediately transferred to petroleum ether.

It must also be remembered that storing carotenoids in flammable volatile solvents, such as ethyl ether, in a refrigerator is a safety hazard and should be avoided. An explosion-proof refrigerator is also recommended.

Common errors in carotenoid analysis

Errors can be introduced in each step of the analytical procedure. The error of the final result is the sum of the errors arising from sampling, sample preparation, the analysis itself, and interpretation (Figure 11). A good understanding of the purpose of each step, and the possible sources of error, is therefore needed.

Common sources of error in carotenoid analysis are:

- samples not representing the food lots under investigation;
- incomplete extraction;
- physical losses during the different steps, such as incomplete transfer of carotenoids from one solvent to the other during partitioning or loss of carotenoids in the washing water, partial recovery of carotenoids

adhering to container walls when carotenoid solutions are evaporated to dryness;
- incomplete chromatographic separation;
- erroneous identification;
- faulty quantification and calculation;
- isomerization and oxidation of carotenoids during analysis and/or during storage of food samples before analysis.

Given the various factors that affect the carotenoid composition of foods, as discussed earlier, proper sampling and sample preparation to obtain representative and homogeneous samples for analysis are of paramount importance. In addition, results should be accompanied by pertinent information, such as the variety, stage of maturity, season, geographical origin, and part of the plant analyzed. Errors incurred in sampling and sample preparation can easily surpass those incurred in analysis *per se*.

Laboratory work should be planned so that the samples are analyzed soon after collection because it is difficult to avoid changes in carotenoid composition during sample storage, even at very low temperature. Because carotenoid concentration is expressed per unit weight of sample, changes in the food's weight during storage also affect the final result.

When storage is unavoidable, samples should be stored at –20°C (or even lower temperatures for longer periods) and tissue disintegration should be postponed until after storage and then carried out immediately before or simultaneously with extraction. Degradative enzymatic reactions during thawing can be minimized by allowing the sample to thaw in a refrigerator (4 to 6°C) (Schiedt and Liaaen-Jensen 1995).

Lyophilization is widely considered the appropriate way to preserve biological samples that have to be stored before carotenoid analysis. However, degradation of carotenoids does take place during lyophilization (Park 1987, Craft et al. 1993, Ramos and Rodriguez-Amaya 1993), and this processing technique additionally increases sample porosity, increasing exposure of carotenoids to oxygen during storage.

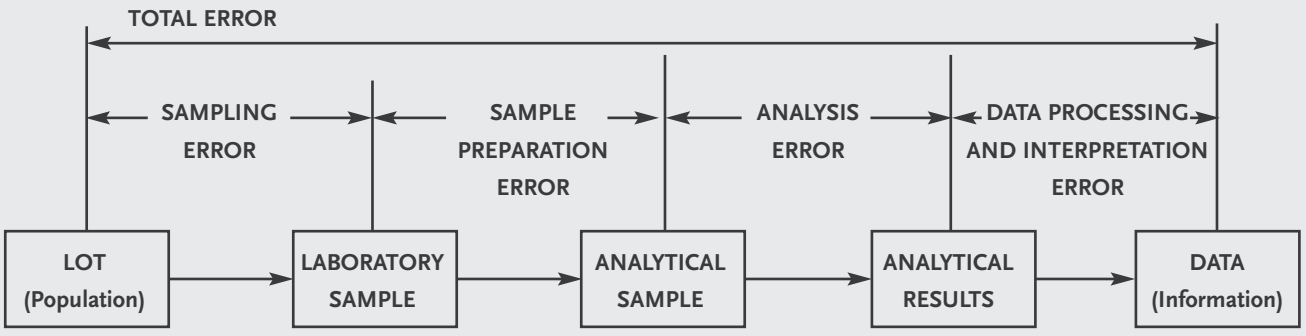
Errors in the pre-chromatographic steps

Sampling, sample preparation, and the steps preceding chromatography, which are often given only cursory attention, can introduce considerable errors that cannot be compensated for in the measurement steps, no matter how modern or sophisticated the analytical instrumentation may be.

To prepare a homogenous, representative sample for analysis and to facilitate the extraction, samples are cut into small pieces or minced. Once this is done, extraction should immediately follow because tissue disruption liberates enzymes (e.g., lipoxygenase), which catalyze substantial carotenoid oxidation, and

FIGURE 11

ILLUSTRATION OF THE TOTAL ERROR IN CAROTENOID ANALYSIS



release acids that promote *trans–cis* isomerization. In fact, sample maceration/homogenization and extraction with an organic solvent are usually carried out simultaneously.

Oxidation can be reduced by directing nitrogen into the blending vessel or by adding dry ice prior to homogenization. These measures, however, increase the cost of analysis. In our experience, using cold acetone (left in the refrigerator for about 2 hours before use) and performing the extraction rapidly are sufficient to prevent errors in this step.

Because of the variable nature of food matrices, incomplete extraction may be a more common source of error than has been acknowledged. Physical losses, including those due to the adherence of carotenoids to the walls of glass containers, are also often overlooked.

Saponification extends the analysis time, and may provoke artifact formation and degradation of carotenoids. The extent of carotenoid degradation during saponification depends on the conditions used, being greater when a higher concentration of alkali or hot saponification is used (Kimura et al. 1990).

Although provitamin A carotenoids (α–carotene, β–carotene, γ-carotene, β–cryptoxanthin) may resist saponification (Rodriguez-Amaya et al. 1988, Kimura et al. 1990), considerable losses of lutein, violaxanthin, and other dihydroxy, trihydroxy, and epoxycarotenoids can occur during saponification and the subsequent washing step (Khachik et al. 1986, Rodriguez-Amaya et al. 1988, Riso and Porrini 1997).

Saponification should therefore be included in the analytical procedure only when indispensable. It is unnecessary, for example, in the analysis of leafy vegetables, tomato, and carrot, all of which are low-lipid materials and essentially free of carotenol esters. The chlorophylls co-extracted with carotenoids from leaves can be separated during chromatography.

Concern about the possible negative effects of saponification has recently led researchers to shorten the duration of ambient temperature saponification (e.g., to 1 or 2 hours). In our experience, however, longer saponification times are required for complete hydrolysis of carotenol esters; for example, the carotenol esters of papaya were completely hydrolyzed only after overnight saponification (Kimura and Rodriguez-Amaya 1999).

Errors in the chromatographic step

Although HPLC is currently the preferred method for carotenoid analysis, it is subject to several sources of errors (Kimura and Rodriguez-Amaya 1999):
(a) incompatibility of the injection solvent and the mobile phase, (b) erroneous identification, (c) quantification of highly overlapping peaks, (d) low and variable recovery of the carotenoids from the HPLC column, (e) errors in the preparation of standard solutions and in the calibration procedure, and (f) erroneous calculations.

Khachik et al. (1988) observed peak splitting when *trans*-carotenoids were injected in dichloromethane, chloroform, THF, benzene, or toluene, and the mobile phase was a mixture of methanol, acetonitrile, dichloromethane, and hexane. No such splitting occurred when the injection solvent was acetone, acetonitrile, methanol, or hexane. In our experience (Kimura and Rodriguez-Amaya 1999) and that of other authors (Lietz and Henry 1997), acetone is a good injection solvent because it efficiently dissolves the range of carotenoids in foods and has polarity and solubility properties similar to those of the mobile phases often used.

Khachik et al. (1988) also showed the importance of injection volume, demonstrating that HPLC peak distortions resulting from the injection solvents mentioned above can be eliminated if the injection volume is reduced to 5 or 10 µL.

Metal surfaces, particularly stainless steel frits in the guard and analytical columns, have been reported to damage carotenoids (Scott 1992). Thus, the use of metal-free columns, for example those with "biocompatible" teflon frits (Craft et al. 1992) and PEEK

(polyether ether ketone) tubing for column connections (Hart and Scott 1995) has been recommended. However, Epler et al. (1992) reported no significant recovery difference in using stainless steel, titanium or "biocompatible" (hastalloy) frits, although slightly lower recoveries were observed for stainless steel frits.

In both OCC and HPLC, accurate quantification requires conclusive identification and optimal separation of the carotenoids. The accuracy of HPLC quantification of carotenoids depends on how well the chromatographic areas are measured. Especially in earlier studies, data on food carotenoids have been obtained by quantifying highly overlapping and tailing peaks. However, improvements in column efficiency have made it possible to obtain chromatograms with well-resolved, symmetrical peaks.

Recovery from the HPLC column have been shown to differ with different carotenoids (Epler et al. 1992). Special attention should be given to lycopene because distinctly higher intralaboratory (Hart and Scott 1995) and interlaboratory (Scott et al. 1996) coefficients of variation and a lower range of linearity (Riso and Porrini 1997) have been found for this carotenoid. Konings and Roomans (1997) observed considerable loss (~40%) of lycopene even when the "biocompatible" hastalloy frit was used.

In HPLC, the concentrations of analytes are calculated by comparing the detector response for the analyte with those of standard solutions of known concentrations. Inaccuracies in the preparation of the standard solutions, in the determination of the concentrations, and in the construction of the calibration curves will obviously be reflected in the results. The purity of the standards should be verified, the standards repurified if necessary, and the concentrations of the standard solutions corrected according to the purity percentage.

The instability of carotenoid standards is a serious problem. Standard carotenoid crystals should be sealed in ampoules under N_2 or argon and stored at –20°C, or better at –70°C, until use. Stock and working solutions, even when kept at low temperature, have limited validity; the analyst should know when degradation commences under the conditions of his/her laboratory.

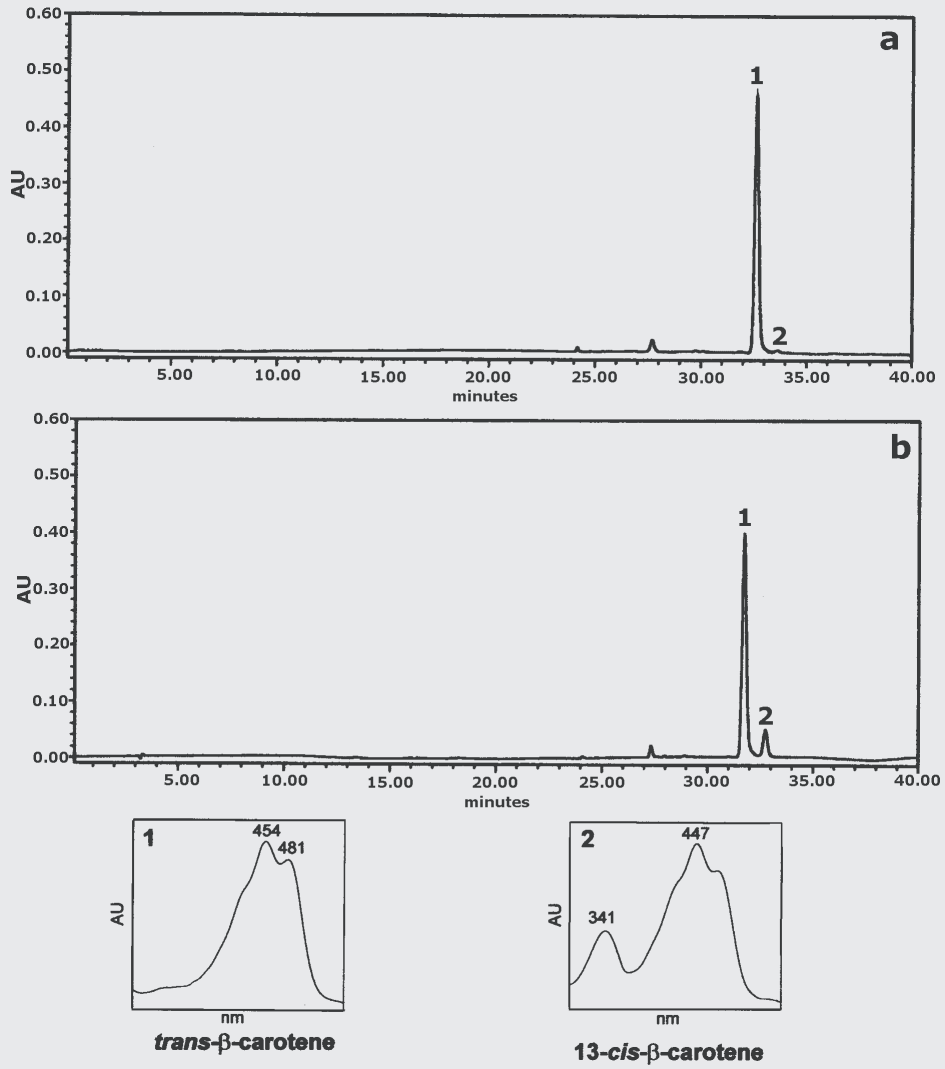
Notwithstanding the inherent difficulties and the many possible errors, careful and well-informed analysts can obtain reliable analytical data on food carotenoids.

IV | Principal Carotenoids Of Harvestplus Crops

There is substantial qualitative and quantitative variation in the carotenoid composition of foods. Even with a particular food, compositional variation occurs due to such factors as variety/cultivar, geographic or climate effects, season, maturity, and part of the plant utilized. Thus, conclusive identification of the carotenoids in a food sample should be accomplished before quantification is carried out. In general, it is sufficient to quantify only the principal carotenoids. Quantifying the minor carotenoids increases analytical complexity, requiring chromatographic resolution, identification, and standards of the different carotenoids, and can introduce more errors besides making the analysis longer, laborious, and costly. The additional results obtained are often of no practical use.

FIGURE 12a AND 12b

HPLC chromatograms and photodiode array spectra of the carotenoids of (a) raw and (b) boiled, mashed orange-fleshed sweetpotato, variety Resisto. Column - monomeric C_{18} Spherisorb ODS2, 3 μm, 4.6 x 150 mm; mobile phase – acetonitrile:methanol:ethyl acetate (80:10:10); flow rate – 0.7 mL/min



When numerous samples have to be analyzed, such as in selecting varieties or breeding lines that meet the desired provitamin A level, it is costly and unnecessary to go directly to HPLC quantification. A high degree of accuracy is not needed at this point. Simple, inexpensive, and rapid screening methods that verify if a sample is above or below the target level can be used to select those that are likely to meet the desired levels. The accurate but expensive HPLC method can then be used only for the chosen samples.

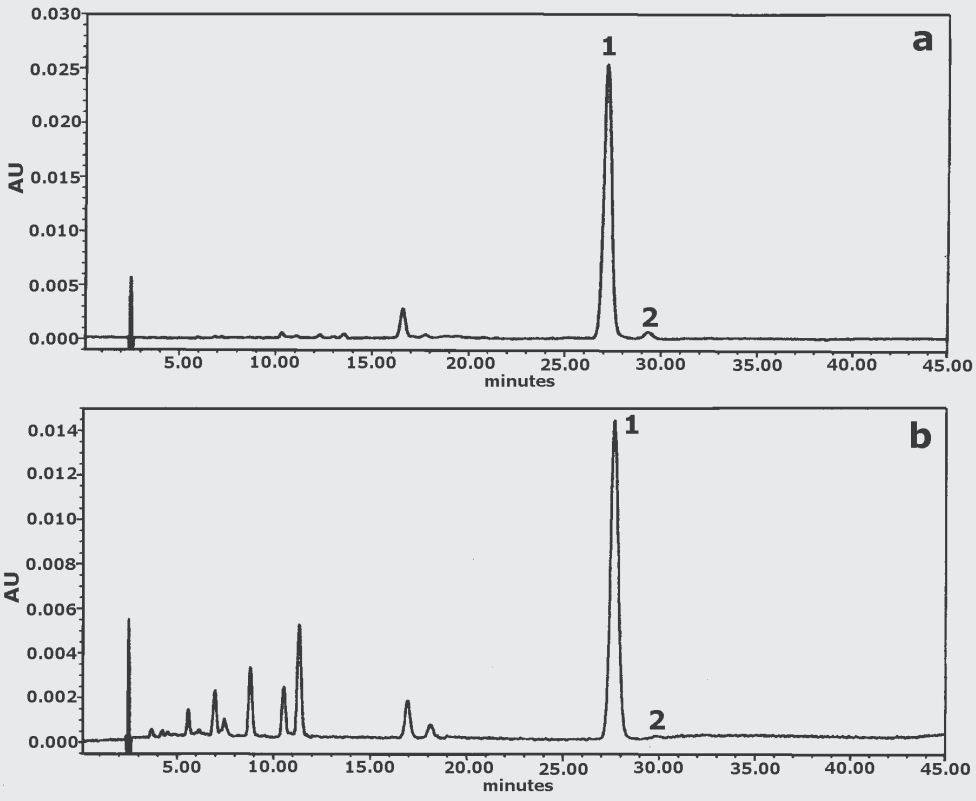
Carotenoids of sweetpotato

Figures 12 and 13a show the predominance of *trans*-β–carotene in the orange-fleshed sweetpotato variety Resisto and a salmon-fleshed EMBRAPA (Empresa Brasileira de Pesquisa Agropecuaria) breeding line CNPH 477-2, respectively. 13-*Cis*-β–carotene and a few other unidentified carotenoids can also be noted, but in much smaller amounts. *Cis*-β–carotene increases in boiled and mashed sweetpotato (Figure 12b), but the level remains very low compared with *trans*-β–carotene. Thus, the β–carotene content of these sweetpotato can be quantified by extracting and determining the concentration spectrophotometrically, without resorting to HPLC.

In the commercial sweetpotato variety IAC 60-M-3-Brasilia that is yellow-fleshed with some salmon portions other minor carotenoids are present, which sum up to an appreciable amount (~36% of total carotenoid)

FIGURE 13a AND 13b

HPLC chromatograms of the carotenoids of (a) salmon-fleshed EMBRAPA breeding line CNPH 477-2 and (b) yellow-fleshed variety IAC 60-M-3-Brasilia sweetpotato. Column - monomeric C_{18} Spherisorb ODS2, 3 μm, 4.6 x 150 mm; mobile phase – acetonitrile:methanol:ethyl acetate (80:10:10); flow rate – 0.7 mL/min. Peak identification: 1. *trans*-β-carotene; 2. 13-*cis*-β-carotene

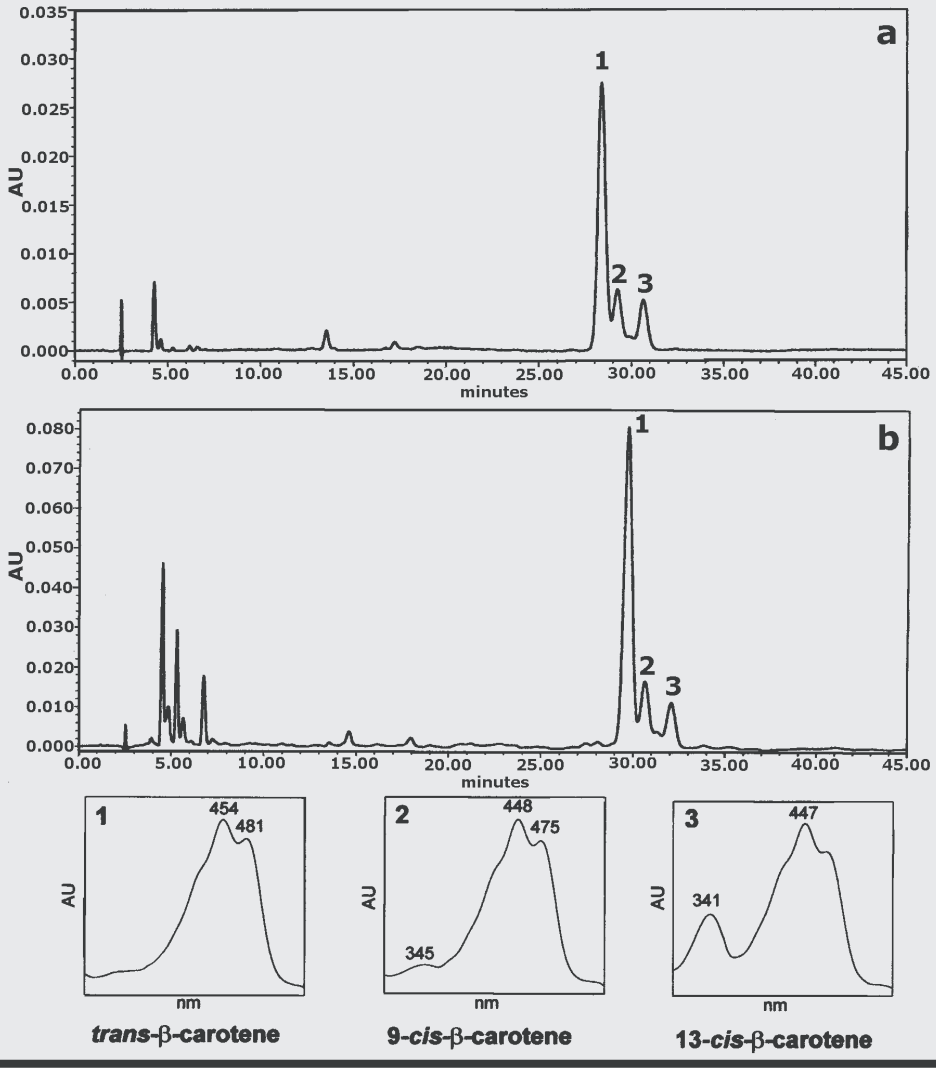


(Figure 13b). Spectrophotometric screening can still be done, but the quantification of β–carotene has to be carried out spectrophotometrically after separation by OCC or by HPLC.

Of the three crops, sweetpotato is the easiest to analyze. Aside from the advantage that only one carotenoid needs be determined, the matrix is easy to extract, the lipid content is low, and there are no esterified carotenoids, making saponification unnecessary. The β–carotene content is very high, way above the limit of quantification of any analytical method. In a work carried out at the Medical Research Council, Cape Town, South Africa, medium-sized sweetpotato taken from the same harvest batch and analyzed individually had 13,200 to 19,400 µg/100 g β–carotene (van Jaarsveld et al. 2004).

FIGURE 14

HPLC chromatograms and photodiode array spectra of the carotenoids of (a) cream-fleshed variety IAC 576-70 and (b) yellow-fleshed variety BRA 005771 cassava. Column - monomeric C_{18} Spherisorb ODS2, 3 µm, 4.6 x 150 mm; mobile phase – acetonitrile:methanol:ethyl acetate (80:10:10); flow rate – 0.7 mL/min

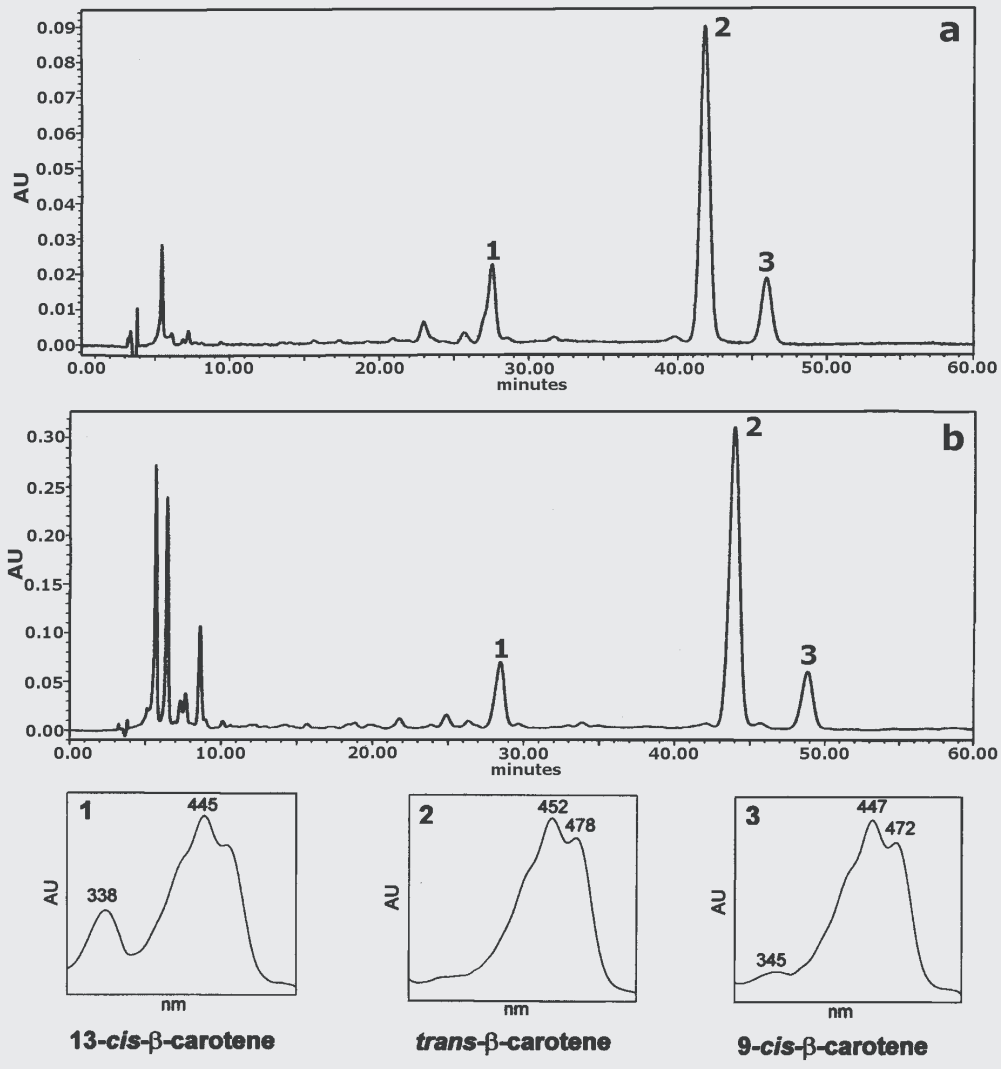


Carotenoids of cassava

β–Carotene is the predominant carotenoid in cassava, but as a mixture of the *trans*- and *cis*-forms (Figures 14 and 15). Because the *cis*-isomers are known to have lower vitamin A activity and are present in significant levels compared with the *trans*-form, the quantitative method should determine the *trans*- and *cis*-isomers individually. However, this makes the analysis more expensive and complicated. Separation of the geometric isomers is better in the C_{30} column (Figure 15), but this column and the methyl-*tert*-butyl ether used as the mobile phase are expensive and not readily available in developing countries. Although there is some peak overlap in the chromatogram obtained using the C_{18} column, the quantitative results obtained in our laboratory using the two columns are equivalent.

FIGURE 15

HPLC chromatograms and photodiode array spectra of the carotenoids of (a) cream-fleshed variety IAC 576-70 and (b) yellow-fleshed variety BRA 005771 cassava. Column – YMC polymeric C_{30}, 3 μm, 4.6 x 250 mm; mobile phase – methanol:methyl-*tert*-butyl ether (80:20); flow rate – 0.8 mL/min



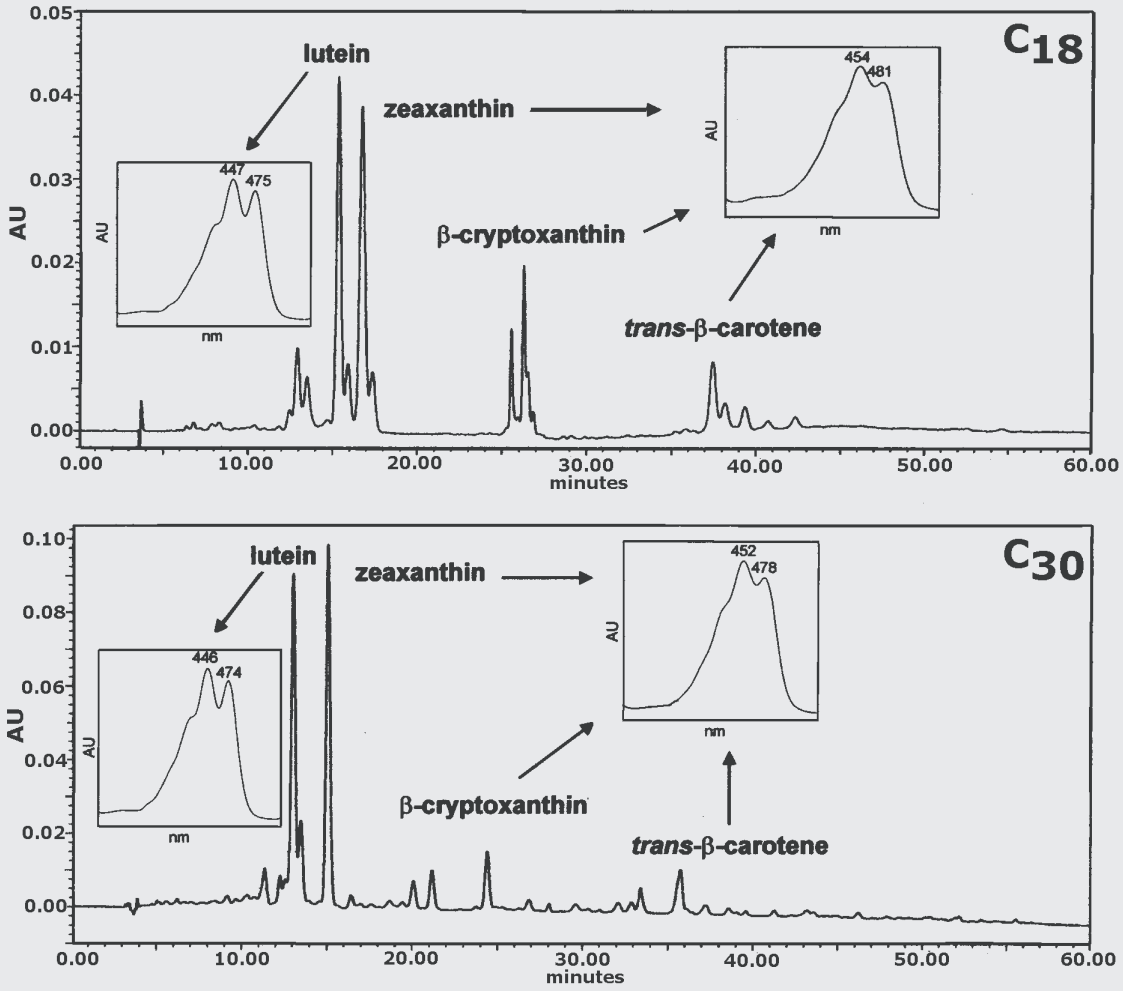
Because the *cis*-isomers of β–carotene are difficult to obtain, their quantification is done using the *trans*-β–carotene curve, and the values can only be considered as estimates.

Cassava does not contain esterified carotenoids and has a low lipid content; hence saponification is unnecessary.

Despite of the presence of both isomers, the presence of β–carotene as the predominant carotenoid means that screening can still be done by extraction and spectrophotometric measurement. The value obtained in this manner will be a good estimate of the total β–carotene content when the carotenoids at the beginning of the chromatogram are in small amounts (Figure 14a), but will overestimate the β–carotene

FIGURE 16

HPLC chromatograms and photodiode array spectra of the carotenoids of dry corn, variety Assum Preto. C_{18} column: monomeric Spherisorb ODS2, 3 μm, 4.6 x 150 mm; mobile phase – acetonitrile:methanol:ethyl acetate, 95:5:0 increasing to 60:20:20 in 20 min (concave gradient); flow rate – 0.5 mL/min, re-equilibration – 15 min. C_{30} column: YMC polymeric, 3 μm, 4.6 x 250 mm; mobile phase – methanol:methyl-*tert*-butyl ether , 90:10 increasing to 40:60 in 60 min (linear gradient); flow rate – 0.8 mL/min, re-equilibration – 15 min. Zeaxanthin, β-cryptoxanthin, and β-carotene have the same chromophore and therefore the same absorption spectrum



content in varieties or breeding lines in which these other carotenoids are present in appreciable amounts (Figure 14b). Screening is appropriate at this time, that is, when β–carotene levels of existing cassava varieties and breeding lines are still low. According to our analyses, the total carotenoid content of breeding lines from EMBRAPA, Brazil, varied from 160 to 770 µg/100 g. When the levels approach the desired point, quantitative analyses should be carried out.

Carotenoids of corn

Figure 16 confirms that zeaxanthin and lutein are the major carotenoids in corn, with β–carotene and β–cryptoxanthin being present in much smaller amounts. The same pattern was found by Moros et al. (2002). Because both lutein and zeaxanthin are vitamin α–inactive (but have important roles in human health in terms of their action against macular degeneration and cataract) and HarvestPlus aims to breed high β–carotene corn, the quantitative method should be able to determine lutein, zeaxanthin, and β–carotene. β–cryptoxanthin has about one-half of the provitamin A activity of β–carotene, and it is questionable whether it is worth quantifying unless its concentration is higher than that of β–carotene. It must be remembered that quantifying a greater number of carotenoids entails obtaining and maintaining pure standards for all the relevant carotenoids, which increases the difficulties involved in the carotenoid analysis.

The amount of esterified carotenoids in corn is negligible, and these esters can be separated by HPLC, thus saponification is not necessary for hydrolysis. Saponification may be necessary to remove the lipids. However, because saponification can give rise to errors, some other means of removing the lipids should be investigated or the chromatographic conditions should be chosen so that the lipids do not accumulate in the column.

The objective of HarvestPlus is to increase the β–carotene levels in corn, which means that screening cannot be done by extraction followed by spectrophotometric measurement because the absorptions of lutein and zeaxanthin will completely mask that of β–carotene. Thus, β–carotene should be separated from lutein, zeaxanthin, and β–cryptoxanthin before spectrophotometric measurement.

Conclusive identification of the principal carotenoids of HarvestPlus crops

Conclusive identification is obviously a prerequisite for the accurate determination of the carotenoid composition of foods. The identifying parameters (Davies 1976, Britton 1995, Eugster 1995, Rodriguez-Amaya 1999a) for the principal carotenoids of HarvestPlus crops are discussed below. Unless otherwise stated, the discussion refers to the *trans*-carotenoids.

α–Carotene. With nine conjugated double bonds in the polyene chain and one conjugated double bond in the β–ring, α–carotene has a spectrum with λ_{max} at 422, 445, 473 nm in petroleum ether (PE). Because one of the conjugated double bonds lies in a ring, the spectrum loses fine structure (%III/II = 55). The absence of substituents can be demonstrated by silica gel TLC developed with 5% methanol in toluene, in which, as a carotene, it runs with the solvent front and by the HPLC retention time (t_R). Co-chromatography can be done by TLC and HPLC, using a commercial α–carotene standard or α–carotene isolated from carrot by OCC.

β–Carotene. With 11 conjugated double bonds, two of which lie in β–rings, β–carotene has λ_{max} at 425 (shoulder), 450, and 477 nm in PE and little fine structure (%III/II = 25). The HPLC t_R and running with the solvent front in the TLC plate reflect the absence of functional groups. For TLC or HPLC co-chromatography, a commercial β–carotene standard or β–carotene isolated from carrot or sweetpotato can be used.

Cis-isomers of β–carotene. The absorption spectra of the *cis*-isomers of β–carotene resemble that of the *trans*-isomer, except for a small hypsochromic shift in λ_{max} (usually 2 to 6 nm for mono-*cis*) and the appearance of a peak, designated *cis*-peak, about 142 nm below the

longest-wavelength absorption maximum of the *trans*-form. The intensity of the *cis*-peak is greater as the *cis* double bond is closer to the center of the molecule. Thus, $\%A_B/A_{II}$, which is an indicator of the *cis*-peak intensity; equals 10, 45, and 56 for 9-*cis*-β–carotene, 13-*cis*-β–carotene and 15-*cis*-β–carotene, respectively (Mercadante et al. 1999).

Zeinoxanthin. This monohydroxy derivative of α–carotene has the same chromophore and, therefore, the same absorption spectrum as α–carotene. The presence of the single hydroxy group, indicated by the R_F on TLC (around 0.56) and the t_R in HPLC, is confirmed by acetylation with acetic anhydride, resulting in a product that behaves almost like a carotene on TLC. Since the hydroxyl is not in the allylic position, response to methylation with acidified methanol is negative.

α–Cryptoxanthin. This carotenoid has the same chromophore, and thus the same absorption spectrum, as zeinoxanthin and α–carotene. It has the same chromatographic behavior as zeinoxanthin. α–Cryptoxanthin differs structurally from zeinoxanthin only in the location of the hydroxy group in the ε–rather than the β–ring, placing this group in an allylic position. Thus, α–cryptoxanthin responds positively not only to acetylation but also to methylation.

β–Cryptoxanthin. This xanthophyll has the same chromophore as β–carotene, and thus exhibits the same visible spectrum. The presence of the hydroxy group, which is manifested by the chromatographic behavior in HPLC and TLC (R_F around 0.44), is confirmed by its positive response to acetylation with acetic anhydride. That the hydroxyl is not in the allylic position is demonstrated by the negative response to methylation with acidified methanol. β–Cryptoxanthin for co-chromatography can be isolated from papaya by OCC.

Zeaxanthin. The visible spectrum of this derivative of β–carotene resembles that of β–carotene. That it is a dihydroxy carotenoid is reflected in its behavior in HPLC and TLC (R_F is around 0.19). The presence and non-allylic position of the hydroxy groups are shown by its positive response to acetylation with acetic anhydride and negative response to methylation with acidified methanol, respectively. Partial acetylation of this carotenoid yields two acetylated products, one near the solvent front and the other at the middle of the silica TL developed with 5% methanol in toluene, the latter corresponding to the acetylation of only one of the hydroxyl groups. Complete acetylation yields one product with both hydroxyls acetylated, running near the solvent front on TLC.

Lutein. This carotenoid has the same chromophore and, consequently, the same spectrum as its parent carotenoid, α–carotene. Lutein differs from zeaxanthin only in the location of one of the terminal conjugated double bonds; however, chromatographic separation of these compounds, while difficult, is possible. It exhibits multi-zoning on TLC, appearing as two spots, with the principal spot having an R_F of around 0.21. The presence of two hydroxy groups can be confirmed by acetylation, as for zeaxanthin. The allylic position of one of the hydroxyls is verified by its positive response to methylation with acidic methanol, producing a compound that behaves like a monohydroxy carotenoid on TLC. For co-chromatography, lutein can be isolated from green leaves such as parsley and water cress.

V | Calibration of the Spectrophotometer

To obtain reliable results, analytical determination should be done with adequate grade solvents, calibrated pipettes and volumetric flasks, and regularly calibrated instruments (see manufacturer's recommendations).

Wavelength accuracy

Verify the spectrophotometer's wavelengths with holmium perchlorate (15% w/v) in 10% perchloric acid. Peak maxima between 400 and 500 nm are at 416.0, 450.5 and 484.5 nm (Scott et al. 1996).

Absorbance accuracy

With the same holmium perchlorate solution, check absorbance accuracy. The absorbance at 451 nm should be 0.832, using 10% perchloric acid solution as blank (Scott et al. 1996). Additionally, according to a procedure of the Association of Official Analytical Chemists (AOAC 1997), prepare a solution of 0.0400 g K_2CrO_4 per liter of 0.05 N KOH and measure the absorbance in a 1 cm cell, using 0.05 N KOH solution as blank. The expected absorbances at specified wavelengths are shown below.

Wavelength (nm)	Absorbance
230	0.171
275	0.757
313.2	0.043
375	0.991
400	0.396

VI | Sampling and Sample Preparation for Harvestplus Crops

The sample subjected to analysis must be representative of the lot under investigation. The sample taken to the laboratory should be made up of increments taken from different parts of the field of production or from the big lot under investigation. This laboratory sample must be reduced in amount and particle size, and homogenized to arrive at the analytical sample. The analytical sample (the sample weighed and subjected to extraction) should not be too small. The smaller the analytical sample, the more difficult it is to guarantee representativity. In our laboratory, the smallest weight of analytical sample is 2 g, even for HPLC analysis.

The sampling and sample preparation procedures should be adapted to the purpose of the analysis, the nature of the food being analyzed, the nature of the analyte and its distribution in the sample, and the desired accuracy of the analytical results. The schemes described below have been established, based on the principles discussed in the chapter on General Procedure for Carotenoid Analysis.

Cassava and sweetpotato

For each genotype, randomly collect at least 15 roots of sweetpotato or cassava from different plants in the field or from different parts of the big batch. In the laboratory, take five roots at random, then wash, peel, wash, dry with absorbent paper, and quarter them longitudinally (from the stem end to the root end). Alternatively, the five roots can be directly taken at random from different plants of the entire field or from different parts of the entire lot. Take two opposite sections from each root, combine, manually cut into small pieces, and mix. Homogenize in a food processor or mixer. Perform this operation rapidly to prevent enzymatic degradation of the carotenoids.

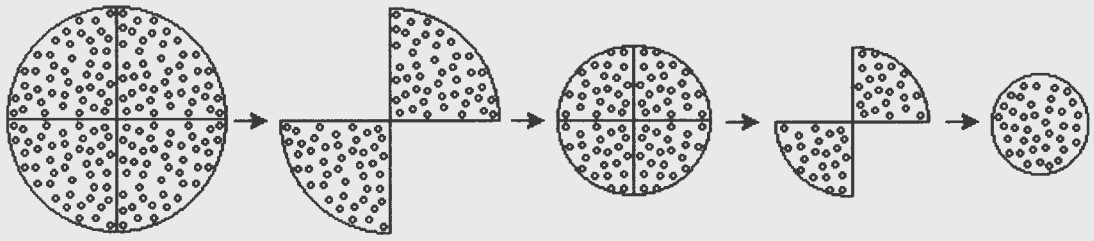
Corn

Randomly take at least 15 ears of corn from different plants in the field or from a big batch. In the laboratory, take at least five ears and dehull. Pile the grains (approximately 1 kg) evenly on a clean surface, flatten the pile and spread into a circle. Make a cross, dividing the circle into four roughly equal parts (Figure 17). Discard two diametrically opposite quarters and remix the remaining two quarters. Repeat the quartering procedure until the amount is reduced to approximately 250 g. Grind the grains and keep the powder in a tightly closed container.

Alternatively, automatic dividers are available that can randomly divide free-flowing particles into 2, 4, or more streams, any one of which can be taken to represent the gross sample.

FIGURE 17

Illustration of quartering of grains and flours



VII | Screening Method for Sweetpotato and Cassava

Extraction

Procedure 1: Extraction with acetone using a mortar and pestle

Weigh a portion (about 2–5 g of sweetpotato, about 5–15 g of cassava) of the homogeneous, representative sample in a beaker. The weight depends on the carotenoid content of the sample. Transfer the sample to a mortar and add a small amount (3 g) of Hyflosupercel (celite). Grind this mixture with 50 mL of cold acetone (acetone refrigerated for about 2 hours). Filter with suction through a sintered glass funnel (or Buchner funnel with filter paper). Wash the mortar, pestle, funnel, and residue with small amounts of acetone, receiving the washings in the suction flask through the funnel. The residue or washings must be devoid of color. If not, repeat the extraction. Return the residue to the mortar, add fresh cold acetone, and macerate again. Filter and wash as before.

Procedure 2: Extraction with acetone using a Polytron homogenizer

It is not necessary to add Hyflosupercel in this case. Weigh the sample (the same weight as in Procedure 1) in the extraction tube. Homogenize the sample with 50 mL of cold acetone for 1 min and filter as described in Procedure 1.

Procedure 3: Extraction with methanol: THF using a Polytron homogenizer

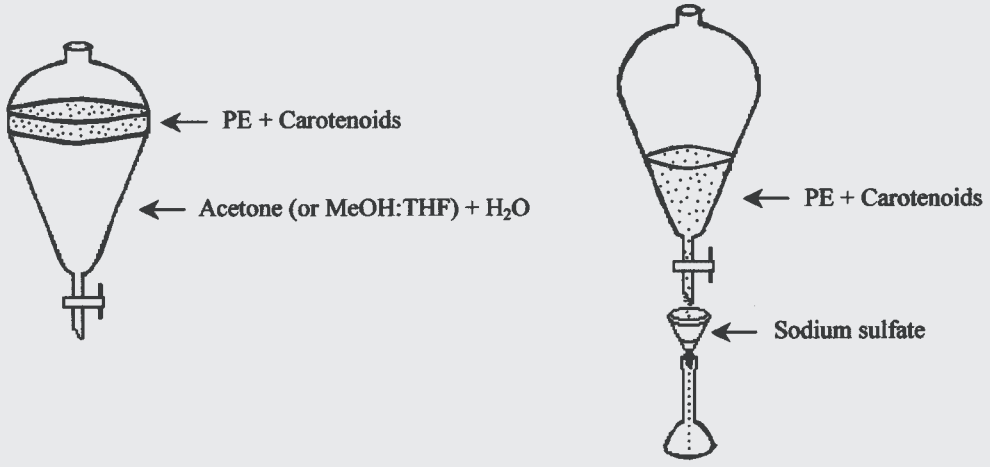
Weigh the sample (the same weight as in Procedure 1) in the extraction tube. Homogenize with 50 mL of methanol:tetrahydrofuran (THF) (1:1) for 1 min and filter as described in Procedure 1.

Partition to petroleum ether (Figure 18)

Put petroleum ether (PE) (~40 mL for sweetpotato and ~20 mL for cassava) in a 500 mL separatory funnel with teflon stop-cock and add the acetone or methanol: THF extract. Slowly add distilled water (~300 mL), letting it flow along the walls of the funnel. To avoid formation of an emulsion, do not shake. (Once formed, an emulsion can be broken by adding saturated sodium chloride solution. When an emulsion is difficult to break, it is better to start the analysis over rather than proceed with an analysis that may give an erroneous result.) Let the two phases separate and discard the lower, aqueous phase. Wash 3–4 times with distilled water (~200 mL each time) to remove residual acetone or methanol:THF. In the last washing, be sure to discard the lower phase as completely as possible, without discarding any of the upper phase.

FIGURE 18

ILLUSTRATION OF PARTITIONING TO PETROLEUM ETHER



Collect the PE phase in a volumetric flask (50 mL for sweetpotato and 25 mL for cassava), making the solution pass through a small funnel containing anhydrous sodium sulfate (~15 g) to remove residual water. (Put a glass wool plug to hold the sodium sulfate.) Wash the separatory funnel with PE, collecting the washings in the volumetric flask by passing through the funnel with sodium sulfate. Alternatively, before transferring to a volumetric flask, the PE phase can be collected in a flask and anhydrous sodium sulfate added until some crystals remain loose.

Spectrophotometric reading and calculation

Make up to volume with PE and take the absorbance at 450 nm. It may be necessary to concentrate or dilute the carotenoid solution (the absorbance should be between 0.2 and 0.8).

Calculate the total carotenoid content using the following formula:

$$\text{Total carotenoid content } (\mu g/g) = \frac{A \times \text{volume}\,(mL) \times 10^4}{A_{1cm}^{1\%} \times \text{sample weight}\,(g)}$$

where A= absorbance; volume = total volume of extract (50 or 25 mL); $A_{1cm}^{1\%}$ = absorption coefficient of β–carotene in PE (2592).

Multiply by 100 to give the carotenoid content in µg/100 g.

Notes:

1. Cassava deteriorates rapidly. Raw cassava should be analyzed within 24 hours of harvest.
2. Because the carotenoid contents of sweetpotato and cassava vary widely, adjusting the weight of the sample and the volume of extraction solvent may be necessary.
3. The funnel with sodium sulfate can be reused during the day, provided it is washed with PE between samples.
4. A screening method should be low-cost, simple (i.e., not requiring sophisticated equipment), and fast. It is designed to analyze a large number of samples to verify if the amount of the analyte in each sample is below or above the target level, thus it is semi-quantitative (i.e., the accuracy required is not as high as that of a quantitative method). However, for orange-fleshed sweetpotato such as the Resisto variety, which contain almost exclusively *trans*-β–carotene, the screening method described here is quantitative.
5. The analyst should be able to organize his/her work so that several samples are analyzed simultaneously, increasing the sample throughput.
6. The screening method described here was developed and evaluated (Kimura et al. 2004), following the guidelines of Rodriguez-Amaya (1999a).

VIII | Screening Method for Dry Corn

Rehydration and extraction

Dry corn is difficult to extract. Rehydration allows efficient penetration of the extraction solvent into the corn tissues. Acetone is used in this method because it is inexpensive and readily available, and it penetrates food tissues well.

Procedure 1: Room temperature rehydration and extraction with mortar and pestle

Weigh 3 g of the ground corn in a beaker. Add sufficient water to cover the ground corn (about 10 mL) and let stand for 30 min. Add about 20 mL of cold acetone and let stand for 15 min.

Filter with suction through a sintered glass funnel or a Buchner funnel. Put the solid in a mortar, grind well with the pestle, add about 50 mL of cold acetone (acetone refrigerated for about 2 hours), and grind again with the pestle to extract the carotenoids.

Filter through the same funnel, collecting the acetone extract in the same suction flask. Wash the mortar and pestle, funnel, and residue with small amounts of acetone, receiving the washings in the suction flask with the extract.

Return the residue to the mortar, add 50 mL of fresh cold acetone, macerate and filter as before. Two extractions/filtrations are usually enough, but if the residue is still colored, repeat the extraction and filtration.

Alternatively, a Polytron homogenizer can be used. A Waring blender is not recommended because part the sample escapes the blades and is not ground with acetone. If a Polytron homogenizer is used, after rehydration transfer the mixture to the extraction tube, add an additional 30 mL of cold acetone and homogenize for 1 min. Filter as described above.

Procedure 2: Hot rehydration and extraction with a Polytron homogenizer

Weigh 3 g of the ground corn in the extraction tube, add 20 mL of water, mix, and let stand for 10 min in a water bath at 85°C, mixing a second time after 5 min. Cool in a water bath.

Homogenize with 50 mL of cold acetone for 1 min. Filter as described in Procedure 1.

Partition to petroleum ether

Place about 20 mL of petroleum ether (PE) in a separatory funnel (we use a 500 mL separatory funnel with a Teflon stop-cock). Add one-third of the extract each time. After each addition, slowly add distilled water (~300 mL), letting it flow along the wall of the funnel. To avoid formation of an emulsion, do not shake. Let the two phases separate and discard the lower, aqueous-acetone phase. Add the second portion and repeat the operation. After the third portion has been transferred to PE, wash 3 times with water (i.e., add about 200 mL distilled water, let the phases separate, and discard the lower phase) to remove residual acetone. In the last washing, be sure to discard the lower phase as completely as possible, without discarding any of the upper phase.

Collect the upper phase in a 25 mL volumetric flask, passing it through a funnel with anhydrous sodium sulfate to remove residual water. Wash the funnel with a small amount of PE, collecting the washings into the volumetric flask. Make up to volume with PE.

Transfer a 20 mL aliquot to a 50 mL round-bottom flask for the separation of the carotene and monohydroxy fractions. Use the remaining extract to measure the absorbance at 450 nm (A_{total}).

Separation of carotene and the monohydroxycarotenoids (Figure 19)

Concentrate the carotenoid solution in the round-bottom flask in a rotary evaporator (temperature must not exceed 35°C) to the smallest possible volume (~1 mL). Do not bring to dryness because this will leave the carotenoids tightly adhered to the glass wall, making complete removal from the flask difficult.

Mount a minicolumn with a Pasteur pipette with the tip cut to 2 cm. Place a small glass wool plug at the bottom of the column. Add about 1 g of neutral alumina of activity III (see Note 3 below). Tap the side of the column 3 or 4 times to better accommodate the adsorbent in the column. Top the column with a 0.5 cm layer of anhydrous sodium sulfate.

With a dropper or pipette, add the carotenoid solution into the column. Rinse the round-bottom flask two times with about 1 mL of PE and add the rinsings to the column. Let the sample layer go down almost to the surface of the sodium sulfate layer before adding the rinsings. (The idea is to keep the carotenoids in as small a volume as possible to diminish band broadening and to prevent the separation from initiating before the entire carotenoid sample has reached the top of the adsorbent.) Continue to add PE and collect the first band (fraction 1) in a 5 mL volumetric flask. Then change to 20% ethyl ether in PE until the second yellow band (fraction 2) is eluted, collecting it in a 10 mL volumetric flask. Leave the bright yellow band (lutein + zexanthin) in the column.

Make up to volume with PE and read the spectrophotometric absorbance of fractions 1 (A_{fr1}) and 2 (A_{fr2}) at 450 nm. Concentrate or dilute the carotenoid solution if necessary (absorbance should be between 0.2 and 0.8).

Calculations

Total carotenoid content

Calculate the total carotenoid content using the formula:

Total carotenoid content (µg/g) =

$$\frac{A_{total} \times \text{volume (mL)} \times 10^4}{A^{1\%}_{1cm} \times \text{sample weight (g)}}$$

where A_{total}= absorbance; volume = total volume of extract (25 mL); $A^{1\%}_{1cm}$ = absorption coefficient of 2500, which is recommended for mixtures.

Carotene content

Calculate the carotene content using the formula:

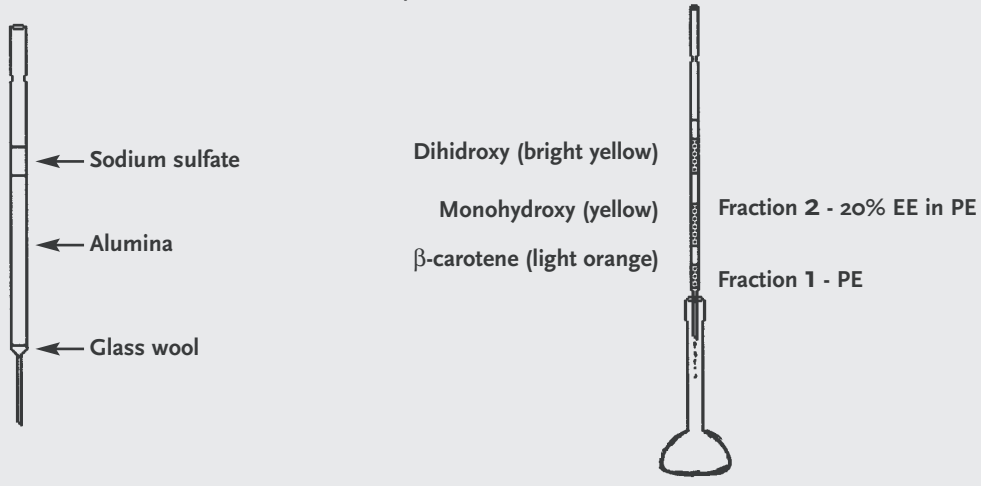
Carotene content (µg/g) = 1.25

$$\frac{A_{fr\,1} \times \text{volume (mL)} \times 10^4}{A^{1\%}_{1cm} \times \text{sample weight (g)}} \times 1.25$$

where $A_{fr\,1}$= absorbance; volume = volume of fraction 1 (5 mL); = absorption coefficient of β–carotene in PE (2592). Multiplying by 1.25 accounts for the use of a 20 mL aliquot taken from a total extract of 25 mL.

FIGURE 19

CHROMATOGRAPHIC SEPARATION IN AN ALUMINA (ACTIVITY III) COLUMN. EE – ETHYL ETHER, PE – PETROLEUM ETHER



Monohydroxycarotenoid content

Calculate the content of monohydroxycarotenoids using the formula:

Monohydroxycarotenoid content (µg/g) =

$$\frac{A_{fr\,2} \times \text{volume (mL)} \times 10^4}{A^{1\%}_{1cm} \times \text{sample weight (g)}} \times 1.25$$

where $A_{fr\,2}$= absorbance; volume = volume of fraction 2 (10 mL); $A^{1\%}_{1cm}$ = absorption coefficient of β–cryptoxanthin in petroleum ether (2386).

β-Cryptoxanthin content

Fraction 2 normally contains zeinoxanthin and β–cryptoxanthin in the same proportion. Estimate β–cryptoxanthin as half of the monohydroxycarotenoid content.

Zeaxanthin + lutein content

To estimate the zeaxanthin + lutein content, subtract the carotene (fraction 1) and monohydroxycarotenoid (fraction 2) contents from the total carotenoid content.

Multiply all values in µg/g by 100 to give µg/100 g.

Notes:

1. The carotenoid contents obtained by the screening method are estimates. They include *cis*-isomers and some other minor carotenoids.
2. Because lutein, zeaxanthin, and cryptoxanthin in corn are mostly unesterified, saponification is not necessary. The presence of lipids in the carotenoid extract does not affect the separation in the alumina column and the spectrophotometric reading (in the visible region).
3. Neutral alumina is usually sold with activity I. To obtain activity III, thoroughly mix alumina activity I with 6% water (w/w). This can be done by vigorously shaking the combined adsorbent and water in a closed container until no lumps are observed. Let stand for about 12 hours to equilibrate. The container should be well closed when not in use.
4. The idea of hot rehydration came from a screening method proposed by Peter Beyer's group (personal communication). Heating for 10 min is sufficient to rehydrate the corn without thermal degradation.
5. Although the method appears tedious or com-plicated at first glance, with practice the analyst can organize his/her work so that several samples can be analyzed simultaneously, increasing the sample throughput.
6. The screening method described here was developed and evaluated (Kimura et al. 2004), following the guidelines of Rodriguez-Amaya (1999a).

IX | Isolation of Carotenoid Standards by Open Column Chromatography

Carotenoid standards are costly, unstable, and often not available commercially. Thus, a carotenoid laboratory should be able to isolate carotenoid standards from natural sources. (e.g., carrot for α–carotene and β–carotene, orange-fleshed sweetpotato for β–carotene, green corn for β–cryptoxanthin, lutein, and zeaxanthin).

Extraction

Homogenize carrot, orange-fleshed sweetpotato, or green corn in a food processor. Weigh about 50 g of carrot, 20 g of sweetpotato, or 120 g of green corn. Transfer to a mortar containing a small amount of Hyflosupercel (about 5 g for carrot and sweetpotato, 20 g for corn). Grind with 50 mL of cold acetone and filter with suction through a sintered glass or Buchner funnel. Rinse the mortar, pestle, and residue with acetone, receiving the rinsings in the funnel. Repeat the extraction and filtration 3–4 times.

Partition to petroleum ether

Place about 100 mL of petroleum ether (PE) in a 500 mL separatory funnel with a teflon stop-cock and add one-fifth of the acetone extract. Slowly add 300 mL of distilled water, letting it flow along the walls of the funnel. To avoid formation of an emulsion, do not shake. (If an emulsion forms, break it by adding a saturated sodium chloride solution.) Let the two phases separate and discard the lower, aqueous-acetone phase. Repeat the process until the carotenoids of the other four portions have been transferred to PE. Then, wash five times with 200 mL water (i.e., add distilled water, let the phases separate, and discard the lower phase). In the final washing, be sure to the discard the lower phase as completely as possible.

For the sweetpotato or carrot extract, collect the upper phase in an Erlenmeyer flask and add anhydrous sodium sulfate until some crystals remain loose. Transfer to a 250 mL round-bottom flask and concentrate in a rotary evaporator ($T \leq 35^{o}C$) until approximately 5 mL remains. Saponify the corn extract to obtain better separation of the carotenoids in the open column.

Saponification

Collect the upper phase in a Teflon-stoppered Erlenmeyer flask and add 0.1% butylated hydroxytoluene (BHT). Add 100 mL of 10% methanolic KOH. Mix and flush with N_2 prior to putting on the stopper. Let the mixture stand in the dark at room temperature overnight (about 16 hours).

Place the mixture in a separatory funnel and collect the lower, methanolic phase. Wash the upper PE phase with water to remove the alkali (about five times). Collect the washed PE phase in an Erlenmeyer flask. Place 50 mL of PE:ethyl ether (1:1) in the separatory funnel. Add the methanolic phase in portions, add distilled water, and discard the lower aqueous-methanol-KOH phase after each addition. When the entire methanolic phase has been added, wash five times with water. Collect the PE-ethyl ether phase in the flask containing the PE phase obtained previously. Dry with anhydrous sodium sulfate. Concentrate in a rotary evaporator to about 5 mL.

Preparation of the column

Mount a chromatographic glass tube (25 x 300 mm) on a suction flask. Place a small glass wool plug at the bottom of the chromatographic tube. Loosely add adsorbent, MgO (Merck, Germany):Hyflosupercel (1:1) activated for 4 hours at 110°C, up to a height of 20 cm. Tap the sides of the column three or four times to better accommodate the adsorbent in the column. Apply a moderate vacuum from a water aspirator for 1 h. Use a flat instrument (such as an inverted cork mounted on a rod or a tamping rod, with a diameter slightly smaller than that of the glass tube so that it fits snugly into the tube) to press down the adsorbent and flatten the surface (the packed column should be about 15 cm high). Top the column with a 1 cm layer of anhydrous sodium sulfate to ensure that no residual water gets into the adsorbent. Pass about one bed volume of PE through the column (the adsorbent surface must be smooth and the solvent flow even) and

adjust the vacuum so that the solvent flow is about two to three drops per second. Once PE has been added to the column, keep the top of the column covered with solvent at all times until chromatography is complete.

Development of the column (Figure 20)

With a dropper or pipette, add the carotenoid PE solution into the column and let the sample layer go down almost to the surface of the sodium sulfate layer before adding the rinsings (PE) from the round-bottom flask. (The objective is to keep the carotenoids in as small a volume as possible to diminish band broadening and to prevent the separation from initiating before the entire carotenoid sample has reached the top of the adsorbent.) Develop the column, adjusting the mobile phase so as to isolate the desired carotenoids as quickly and efficiently as possible. Elute α–carotene with PE, β–carotene with 2% acetone, β–cryptoxanthin with 15–20% acetone, lutein with 25–30% acetone, and zeaxanthin with 40–45% acetone in PE. For α–carotene and β–carotene, leave the other carotenoids in the column after elution of these carotenoids. For β–cryptoxanthin, lutein, and zeaxanthin, discard the carotenoids that elute from the column before these xanthophylls. Because the objective of OCC is not quantitative analysis, only the main portion of each band of the desired carotenoid should be collected, avoiding contamination from the other bands.

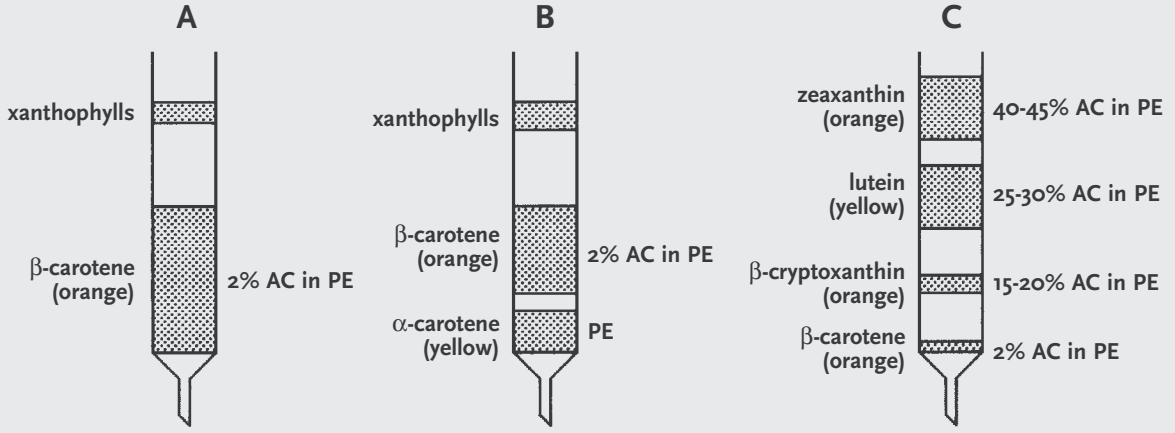
As acetone affects the absorption of carotenoids in PE, remove the acetone from the β–carotene, β–cryptoxanthin, lutein, and zeaxanthin by washing with water in a separatory funnel. Dry the PE solution of the carotenoid with anhydrous sodium sulfate.

Verification of purity and calculation of the concentration of the standards

Take an aliquot from each isolate to verify the purity by HPLC (i.e., chromatogram showing a single peak corresponding to the carotenoid, and the same characteristic spectrum being obtained with the photodiode array detector at the ascending and descending slopes and at the maximum). Dry the aliquot under N_2 and, immediately before injection, dissolve in 1 mL of HPLC-grade acetone, filter through a 0.22 µm PTFE syringe filter (Millipore) directly to a sample vial, and inject into the liquid chromatograph.

FIGURE 20

Separation patterns of the carotenoids of (A) orange-fleshed sweet potato, (B) carrot, and (C) green corn on a MgO:Hyflosupercel column with the eluting solvents, AC - acetone; PE - petroleum ether



Calculate the % purity of the standard solution as follows:

$$\% \text{ purity} = \frac{\text{area of standard peak}}{\text{total area}} \times 100$$

These areas are those obtained at the maximum wavelength of the standard, the total area being the sum of the areas of all peaks at this wavelength.

Once the desired purity is obtained (≥ 90%), determine the concentrations of the pure standards spectrophotometrically, using the following $A^{1\%}_{1cm}$ values: α–carotene, 2800 in PE; β–carotene, 2592 in PE; β–cryptoxanthin, 2386 in PE; zeaxanthin, 2348 in PE; and lutein, 2550 in ethanol. For lutein, put the PE solution of this carotenoid to volume in a 5 mL volumetric flask. Dry under N_2, dissolve the residue with ethanol, making up to volume, and read the absorbance using ethanol as blank.

$$C\ (\mu g/mL) = \frac{\text{absorbance} \times 10^4}{A^{1\%}_{1cm}}$$

Correct the concentration using the respective standard's % purity:

$$\text{Corrected C}\ (\mu g/mL) = \frac{C\ (\mu g/mL) \times \%\ \text{purity}}{100}$$

Commercial standards

Even when commercial standards are used, the purity must be verified and the concentrations of the standard solutions corrected accordingly. If necessary, purify commercial standards by collecting and accumulating the fractions corresponding to the carotenoids of interest in several HPLC runs. Alternatively, purification can be done by OCC as described above.

Notes:

1. The analyst should verify the stability of the carotenoid standards under his/her laboratory storage conditions. In our laboratory the carotenoid standards of β–carotene, lutein, violaxanthin, and neoxanthin, isolated as described above and stored at –20°C in culture tubes (screw cap) in a vacuum desiccator, showed no evidence of decomposition after 13 days (Sá and Rodriguez-Amaya 2004). Thus, we use these standards within 2 weeks of isolation. It is possible to isolate large quantities of standards and the aliquots with BHT stored in sealed vials under N_2, at the lowest possible temperature (< –20°C) , for use over an extended period.
2. The above scheme for isolating standards was established for HarvestPlus crops, based on a previous study (Kimura and Rodriguez-Amaya 2002), and following the guidelines of Rodriguez-Amaya (1999a).

X | Construction Of Standard Curves

Preparation of the standard solutions

Take aliquots of the carotenoid isolates in petroleum ether (PE) in volumes that would give the relative proportion found in the sample, mix and add 0.1% of butylated hydroxytoluene (BHT), concentrate and adjust the volume to 50 mL.

Calculate the concentration of each standard in the mixed standard solution using the formula:

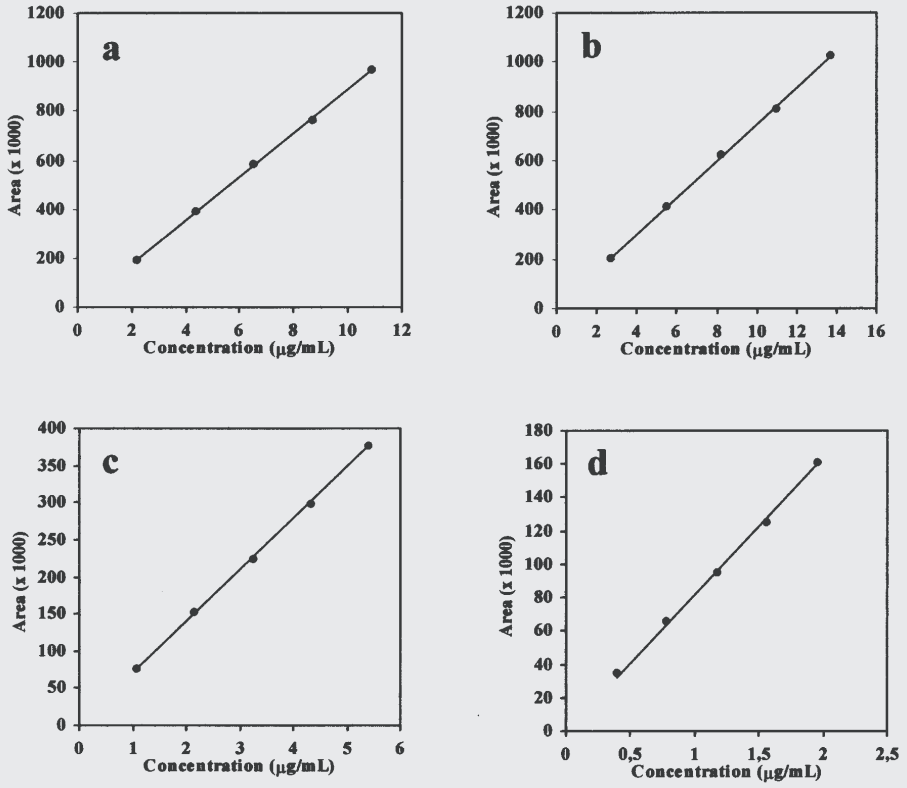
$$\text{Concentration } (\mu g/mL) = \frac{\text{Corrected C} \times \text{Vstd (mL)}}{50}$$

where corrected C = concentration in the isolated standard solution, and V_{std} = volume taken to prepare the mixture.

Transfer aliquots of 1, 2, 3, 4, and 5 mL in triplicate to culture tubes (screw cap), dry under N_2 and, just before injection, redissolve in 1 mL of HPLC grade acetone, filter through a 0.22 mm PTFE syringe filter (Millipore), and inject 10 mL into the HPLC equipment. Use the chromatographic conditions optimized for the food samples being analyzed. Construct the standard curves with five different concentrations for each carotenoid in triplicate, plotting the area against the concentration. The curves should pass through or very near the origin, be linear with a correlation coefficient ≥ 0.95, and should bracket the concentrations expected in the samples. Examples of standard curves for corn carotenoids are shown in Figure 21. For these curves, the purity of the standards was 98% for lutein, 97% for zeaxanthin, 96% for β–cryptoxanthin, and 98% for β–carotene.

FIGURE 21

Standard curves of (a) *trans*-lutein, (b) *trans*-zeaxanthin, (c) *trans*-β-cryptoxanthin and (d) *trans*-β-carotene



The coefficients of correlation were 0.9998, 0.9995, 0.9998, and 0.9983, respectively. The corresponding ranges of the coefficients of variation of the triplicate injections at each of five points were 0–2%, 0–1%, 1–2%, and 1–2%.

Note:

Standard curve construction is necessary to verify the linearity and reproducibility of the detector's response in the concentration range of the samples. However, it is time-consuming. Thus, the recommended approach is one-point calibration for each carotenoid on each day of analysis provided that the point falls on or very close to the curve, and full calibration every 3 to 4 months or when variation of the ratio between concentration and the area of the standard's peak exceeds 5% (Mantoura and Repeta 1997).

XI | HPLC Method for Sweetpotato

Extraction

Procedure 1: Extraction with acetone using a mortar and pestle

Weigh 2 to 5 g of the homogeneous, representative sample of sweetpotato. (The weight depends on the carotenoid content of the sample; a higher amount should be taken for sweetpotato with lower β–carotene content.) With a mortar and pestle, grind the sample with 50 mL of cold acetone (acetone refrigerated for about 2 hours) and about 3 g of Hyflosupercel or celite. Filter with suction through a sintered glass funnel (or Buchner funnel). Wash the mortar, pestle (or homogenizer), and residue with small amounts of acetone, receiving the washings in the funnel. Repeat extraction and filtration if necessary (until the residue is colorless).

Procedure 2: Extraction with acetone using a Polytron homogenizer

Weigh 2 to 5 g of the homogenous, representative sample of sweetpotato in the extraction tube. Homogenize the sample with 50 mL of cold acetone for 1 min and filter as described in Procedure 1.

Procedure 3: Extraction with methanol:THF using a Polytron homogenizer

Weigh 2 to 5 g of the homogenous, representative sample of sweetpotato in the extraction tube. Homogenize with 50 mL of methanol:tetrahydrofuran (THF) (1:1) for 1 min and filter as described in Procedure 1.

Partition to petroleum ether

Place about 40 mL (the volume depends on the color of the extract) of PE in a 500 mL separatory funnel and add the acetone or methanol:THF extract. Slowly add distilled water (about 300 mL), letting it flow along the walls of the funnel. To avoid formation of an emulsion, do not shake. Let the two phases separate and discard the lower, aqueous phase. Wash (i.e., add distilled water, let the phases separate, discard the lower phase) three to four times with water (about 200 mL each time) to remove residual acetone or methanol:THF. In the final washing, be sure to discard the lower phase as completely as possible, without discarding any of the upper phase. Collect the PE phase, passing the solution through a small funnel containing anhydrous sodium sulfate (~15 g). Wash the separatory funnel with PE, combining the washings with the PE solution of carotenoids after passing through the funnel with anhydrous sodium sulfate. Drying can also be carried out by adding anhydrous sodium sulfate to the collected carotenoid solution until some crystals remain loose.

Introduction to the HPLC equipment

Concentrate the extract in a rotary evaporator ($T \leq 35^{o}C$). Dry under N_2, and immediately before injection, redissolve in 1 mL of HPLC grade acetone (for very concentrated samples, it may be necessary to increase the solvent volume), filter through a 0.22 mm PTFE syringe filter (Millipore) directly into sample vials and inject 10 mL into the chromatograph. Sample and standard should be injected in the same volume.

HPLC chromatographic conditions

Monomeric C_{18} column: Waters Spherisorb ODS 2, 3 µm, 4.6 x 150 mm
Mobile phase: acetonitrile:methanol:ethyl acetate (0.05% triethylamine)
Isocratic elution: 80:10:10, flow rate of 0.7 mL/min.
or
Polymeric C_{30} column: YMC C30, 3 µm, 4.6 x 250 mm
Mobile phase: methanol:methyl-*tert*-butyl ether
Isocratic elution: 80:20, flow rate of 0.8 mL/min.

Calculation

Calculate carotenoid concentration using the formula:

$$C_x\ (\mu g/g) = \frac{A_x \times C_s\ (\mu g/mL) \times \text{total volume of extract (mL)}}{A_s \times \text{sample weight (g)}}$$

where C_x = concentration of carotenoid X; A_x = peak area of carotenoid X; C_s = concentration of the standard; A_s = peak area of the standard.

Notes:

1. Because the carotenoid content of sweetpotato varies widely, adjusting the weight of the sample and the volumes of the extraction and injection solvents may be necessary.
2. The funnel with sodium sulfate can be reused during the day, provided it is washed with PE between samples.

XII | HPLC Method for Cassava

Extraction

Procedure 1: Extraction with acetone using a mortar and pestle

Weigh 5 to 15 g of the homogeneous, representative sample of cassava. (The weight depends on the carotenoid content of the sample. It may have to be increased for cassava with very low β–carotene content.) With a mortar and pestle, grind the sample with 50 mL of cold acetone (acetone refrigerated for about 2 hours) and about 3 g of Hyflosupercel or celite. Filter with suction through a sintered glass funnel (or Buchner funnel). Wash the mortar and pestle (or homogenizer) and residue with small amounts of acetone, receiving the washings in the funnel. Repeat extraction and filtration, if necessary (until the residue is colorless).

Procedure 2: Extraction with acetone using a Polytron homogenizer

Weigh 5 to 15 g of the homogenous, representative sample of cassava in the extraction tube. Homogenize the sample with 50 mL of cold acetone for 1 min and filter as described in Procedure 1.

Procedure 3: Extraction with methanol: THF using a Polytron homogenizer

Weigh 5 to 15 g of the homogenous, representative sample of cassava in the extraction tube. Homogenize with 50 mL of methanol:tetrahydrofuran (THF) (1:1) for 1 min and filter as described in Procedure 1.

Partition to petroleum ether

Place about 40 mL (the volume depends on the color of the extract) of PE in a 500 mL separatory funnel and add the acetone or methanol:THF extract. Slowly add distilled water (about 300 mL), letting it flow along the walls of the funnel. To avoid formation of an emulsion, do not shake. Let the two phases separate and discard the lower, aqueous phase. Wash (i.e., add distilled water, let the phases separate, discard the lower phase) three to four times with water (about 200 mL each time) to remove residual acetone or methanol: THF. In the final washing, be sure to discard the lower phase as completely as possible, without discarding any of the upper phase. Collect the PE phase, passing the solution through a small funnel containing anhydrous sodium sulfate (about 15 g). Wash the separatory funnel with PE, combining the washings with the PE solution of carotenoids after passing through the funnel with anhydrous sodium sulfate. Drying can also be carried out by adding anhydrous sodium sulfate to the collected carotenoid solution until some crystals remain loose.

Introduction to the HPLC equipment

Concentrate the extract in a rotary evaporator ($T \leq 35^{o}C$). Dry under N_2 and, immediately before injection, redissolve in 1 mL of HPLC grade acetone, filter through a 0.22 mm PTFE syringe filter (Millipore) directly into sample vials and inject 10 µL into the chromatograph. Sample and standard should be injected in the same volume.

HPLC chromatographic conditions

Monomeric C_{18} column: Waters Spherisorb ODS 2, 3 µm, 4.6 x 150 mm
Mobile phase: acetonitrile:methanol:ethyl acetate (0.05% triethylamine)
Isocratic elution: 80:10:10, flow rate of 0.7 mL/min.
or
Polymeric C_{30} column: YMC C30, 3 µm, 4.6 x 250 mm
Mobile phase: methanol:methyl-*tert*-butyl ether
Isocratic elution: 80:20, flow rate of 0.8 mL/min.

Calculation

Calculate carotenoid concentration using the formula:

C_x (µg/g) =

$$\frac{A_x \times C_s\ (\mu g/mL) \times \text{total volume of extract (mL)}}{A_s \times \text{sample weight (g)}}$$

where C_x = concentration of carotenoid X; A_x = peak area of carotenoid X; C_s = concentration of the standard; A_s = peak area of the standard.

Notes:

1. Cassava deteriorates rapidly. Raw cassava should preferably be analyzed within 24 hours of harvest.
2. Because the carotenoid content of cassava varies widely, adjusting the weight of the sample and the volumes of the extraction and injection solvents may be necessary.
3. The funnel with sodium sulfate can be reused during the day, provided it is washed with PE between samples.

XIII | HPLC Method for Dry Corn

Rehydration and extraction

Procedure 1: Room temperature rehydration and extraction with mortar and pestle

Weigh 3 g of the ground corn in a beaker. Add enough water to cover (about 10 mL) and let stand for 30 min. Add about 20 mL of cold acetone and let stand for 15 min.

Filter with suction through a sintered glass funnel or a Buchner funnel. Place the residue in a mortar, grind well with the pestle, add about 50 mL of cold acetone (acetone refrigerated for about 2 hours), and grind again with the pestle to extract the carotenoids.

Filter through the same funnel, collecting the acetone extract in the same suction flask. Wash the mortar and pestle, funnel, and residue with small amounts of acetone, receiving the washings in the suction flask with the extract.

Return the residue to the mortar, add fresh cold acetone, macerate, and filter as before. Two extractions/filtrations are usually enough, but if the residue is still colored, repeat the extraction and filtration.

A Polytron homogenizer can also be used. After rehydration, transfer the mixture to the extraction tube, add an additional 30 mL of cold acetone, and homogenize for 1 min. Filter as described above.

Procedure 2: Hot rehydration and extraction with a Polytron homogenizer

Weigh 3 g of the ground corn in the extraction tube, add 20 mL of distilled water, mix, and let stand for 10 min in a water bath at 85°C, mixing a second time after 5 min. Cool in a water bath.

Homogenize the sample with 50 mL of cold acetone for 1 min and filter as described in Procedure 1. Extraction and filtration should be repeated if the residue is still colored.

Partition to petroleum ether

Place about 20 mL of PE in a 500 mL separatory funnel. Add one-third of the extract. Slowly add distilled water (~300 mL), letting it flow along the wall of the funnel. To avoid formation of an emulsion, do not shake. Let the two phases separate and discard the lower, aqueous phase. Add the second portion and repeat the operation. After the third portion has been transferred to PE, wash (i.e., add distilled water, let the phases separate, discard the lower phase) three times with water (about 200 mL each time) to remove residual acetone. In the final washing, be sure to discard the lower phase as completely as possible, without discarding any of the upper phase. Collect the upper phase in a 50 mL round-bottom flask, passing the solution through a small funnel containing anhydrous sodium sulfate (~15 g). Wash the separatory funnel with PE, combining the washings with the PE solution of carotenoids after passing through the funnel with anhydrous sodium sulfate. Drying can also be carried out by collecting the carotenoid solution, then adding anhydrous sodium sulfate until some crystals remain loose.

Introduction to the HPLC equipment

Concentrate the extract in a rotary evaporator ($T \leq 35^{o}C$). Dry under N_2 and, immediately before injection, redissolve in 1 mL of HPLC grade acetone, filter through a 0.22 µm PTFE syringe filter (Millipore) directly into sample vials and inject 10 µL into the chromatograph. Sample and standard should be injected in the same volume.

HPLC chromatographic conditions

Monomeric C_{18} column: Waters Spherisorb ODS 2, 3 µm, 4.6 x 150 mm
Mobile phase: acetonitrile:methanol:ethyl acetate (with 0.05% triethylamine)
Gradient elution: 95:5:0, to 60:20:20 in 20 min (concave gradient), staying in this proportion until 40 min, then to 20:40:40 in 60 min (linear gradient) to remove the lipids; flow rate, 0.5 mL/min; reequilibration, 15 min.
or
Polymeric C_{30} column: YMC C30, 3 µm, 4.6 x 250 mm
Mobile phase: methanol:methyl-*tert*-butyl ether
Gradient elution: linear gradient with the initial proportion of 90:10 increasing to 40:60 in 60 min; flow rate, 0.8 mL/min; reequilibration, 15 min.

Calculation

Calculate carotenoid concentration using the formula:

C_x (µg/g) =

$$\frac{A_x \times C_s\ (\mu g/mL) \times \text{total volume of extract (mL)}}{A_s \times \text{sample weight (g)}}$$

where C_x = concentration of carotenoid X; A_x = peak area of carotenoid X; C_s = concentration of the standard; A_s = peak area of the standard.

Note:

Because lutein, zeaxanthin, and cryptoxanthin in corn are mostly unesterified, saponification is not necessary. The very small amount of esters present are separated from β–carotene and do not interfere with its quantification. As a gradient is used, the lipids in the extract are removed from the column.

XIV | Evaluation of Retention of Carotenoids in Cooked/Processed Foods

For human health applications the analytical data should be in terms of the carotenoid concentrations in the food as consumed. Given the instability of carotenoids, it is necessary to determine the carotenoid content of cooked/processed foods and to verify losses during cooking/processing. This data can then be used to make recommendations regarding the conditions that give the greatest retention of these important compounds.

The adequacy of the procedure for assessing carotenoid losses during cooking or processing has been brought into question by the appearance of several reports citing carotenoid retention values of over 100%, calculated on a dry weight basis. These results must be false because carotenoids cannot be biosynthesized during cooking. Specifically, heat treatment inactivates the enzymes responsible for carotenoid biosynthesis and, in fact, promotes isomerization and oxidative degradation of carotenoids.

There are three ways by which the carotenoid levels can be artificially increased in cooked/processed food compared with the corresponding raw food, leading to retentions of over 100%. First, carotenoids can be more easily extracted from cooked/processed samples compared with those of fresh foods, in which the carotenoids are physically protected and/or combined with other food components. Extraction efficiency of fresh samples must be enhanced to make it as equivalent as possible to that of cooked samples (e.g., by soaking the sample in water or extraction solvent prior to extraction), and extraction must be exhaustive. Second, appreciable leaching of soluble solids can occur during processing, as shown in carrots, concentrating the carotenoids per unit weight of cooked food. Third, enzymatic oxidation of carotenoids can substantially lower their concentrations in raw samples, especially when the samples are left standing after being cut or disintegrated.

Losses of carotenoids have been calculated in the literature simply as the difference between the carotenoid concentration before (e.g., µg/g raw weight) and after cooking/processing (e.g., µg/g cooked weight); however, this calculation does not take into account changes in the weight of the food during cooking (e.g., loss of water and/or soluble solids, gain of water or oil) and, therefore, does not represent the true losses of the carotenoids.

Carotenoid retention can be calculated, taking into account or compensating for changes in food weight during cooking, using one of the following formulas:

$$\% \text{ retention} = \frac{\text{carotenoid content per g of cooked food} \times \text{g of food after cooking}}{\text{carotenoid content per g of raw food} \times \text{g of food before cooking}} \times 100$$

$$\% \text{ retention} = \frac{\text{carotenoid content per g of cooked food (dry basis)}}{\text{carotenoid content per g of raw food (dry basis)}} \times 100$$

The first formula, recommended by Murphy et al. (1975) for calculating retentions of nutrients in cooked foods, was found by the proponents to give more accurate retention data for a range of nutrients under various weight change situations.

Calculation on a dry weight basis overestimated retentions in nearly all instances. It is not always feasible, however, to obtain data on the weights of foods before and after processing, especially under industrial production conditions; thus calculation on a dry weight basis is used in these cases.

In studies of retention, it is very important to specify the processing and storage conditions (time, temperature, etc.). Paired samples (i.e., equivalent raw and cooked samples) must be used, and the results should be analyzed statistically so that their real meaning can be appreciated (Rodriguez-Amaya 1997, 1999a, van Jaarsveld et al. 2004).

Sampling and sample preparation

To obtain paired samples, both raw and cooked samples must come from the same harvest batch, which should be as homogenous as possible (i.e., all medium-sized sweetpotato or cassava). Preferably, quartering should be performed, as described above for sweetpotato, cassava, and corn. Opposite quarters should be combined and analyzed raw and the other two opposite sections should be combined and cooked or processed. In some cases the processing regime being studied does not allow quartering. In such cases, the sample subjected to processing and the raw counterpart should come from the same batch and several units should be taken for each analysis to compensate for between-unit variations.

References

ACS Committee on Environmental Improvement. Guidelines for data acquisition and data quality evaluation in environmental chemistry. Anal Chem 1980;52:2242-2249.

Almeida-Muradian LB, Penteado MVC. Carotenoids and provitamin A value of some Brazilian sweet potato cultivars (*Ipomoea batatas* Lam.) Rev Farm Bioquím Univ São Paulo 1992;28:145-154.

AOAC. Official methods of analysis of the Association of Official Analytical Chemists. AOAC Inc., Arlington, 1997.

Arima HK, Rodriguez-Amaya DB. Carotenoid composition and vitamin A value of commercial Brazilian squashes and pumpkins. J Micronutr Anal 1988;4:177-191.

Arima HK, Rodriguez-Amaya, DB. Carotenoid composition and vitamin A value of a squash and a pumpkin from Northeastern Brazil. Arch Latinoamer Nutr 1990;40:284-292.

Astrog P. Food carotenoids and cancer prevention: An overview of current research. Trends Food Sci Technol 1997;81:406-413.

Azevedo-Meleiro CH, Rodriguez-Amaya DB. Determination of the carotenoids of *Cucurbitaceae* fruit vegetables by HPLC-DAD and HPLC-MS. Paper presented at the IV Brazilian Meeting on Chemistry of Food and Beverages, Campinas, Brazil. 2002

Azevedo-Meleiro CH, Rodriguez-Amaya DB. Confirmation of the identity of the carotenoids of tropical fruits by HPLC-DAD and HPLC-MS. J Food Comp Anal 2004a;17:385-396.

Azevedo-Meleiro CH, Rodriguez-Amaya DB. Carotenoid composition of kale as influenced by maturity, season and minimal processing. J Sci Food Agric 2004b (in press).

Azevedo-Meleiro CH, Rodriguez-Amaya DB. Carotenoids of endive and New Zealand spinach as affected by maturity, season and minimal processing. J Food Comp Anal 2004c (submitted).

Ben-Amotz A, Levy Y. Bioavailabitlity of a natural isomer mixture compared to synthetic all-*trans* β–carotene in human serum. Am J Clin Nutr 1996;63:729-734.

Bendich A. Recent advances in clinical research involving carotenoids. Pure Appl. Chem 1994;66:1017-1024.

Boileau AC, Merchen NR, Wasson K, Atkinson CA, Erdman JW Jr. *Cis*-lycopene is more bioavailable than *trans*-lycopene in vitro and in vivo in lymp-cannulated ferrets. J Nutr 1999;129:1176-1181.

Bone RA, Landrum JT, Fernandez L, Tarsist SL. Analysis of the macular pigment by HPLC: Retinal distribution and age study. Invest Ophthalmol Vis Sci 1988;29:843-849.

Britton G. . Carotenoids. Meth Plant Biochem 1991;7:473-518.

Britton G. UV/visible spectroscopy. In Britton G, Liaaen-Jensen S, Pfander H (eds), Carotenoids: Spectroscopy, vol 1B. Birkhäuser Verlag, Basel, 1995, pp 13-63.

Burri BJ. Beta-carotene and human health: A review of current research. Nutr Res 1997;17:547-580.

Castenmiller JJM, West, CE, Linssen, JPH, van het Hof KH, Voragen AGJ. The food matrix of spinach is a limiting factor in determining the bioavailability of β–carotene and to a lesser extent of lutein in humans. J Nutr 1999;129:349-355.

Cavalcante ML, Rodriguez-Amaya DB. Carotenoid composition of the tropical fruits *Eugenia uniflora* and *Malpighia glabra*. In Charalambous G (ed), Food science and human nutrition. Elsevier Science Publishers, Amsterdam, 1992, pp 643-650.

Clinton, SK. Lycopene: Chemistry, biology, and implications for human health and disease. Nutr Rev 1998;56:35-51.

Craft NE, Sander LC, Pierson HF. Separation and relative distribution of all-*trans*-β–carotene and its *cis* isomers in β–carotene preparations. J Micronutr Anal 1990;8:209-221.

Craft NE, Soares JH Jr. Relative solubility, stability, and absorptivity of lutein and β–carotene in organic solvents. J Agric Food Chem 1992;40:431-434

Craft NE, Wise AS, Soares JH Jr. Individual carotenoid content of SRM 1548 total diet and influence of storage temperature, lyophilization, and irradiation on dietary carotenoids. J Agric Food Chem 1993;41:208-213.

Craft NE, Wise SA, Soares JH Jr. Optimization of an isocratic high-performance liquid chromatographic separation of carotenoids. J Chromatogr 1992;589:171-176.

Craft NE. Carotenoid reversed-phase high performance liquid chromatography methods: Reference compendium. Meth Enzymol 1992;213:185-205.

Davies BH. Carotenoids. In Goodwin TW (ed), Chemistry and biochemistry of plant pigments. 2nd edition, vol 2. Academic Press,

London, 1976, pp 38-165.

Di Mascio P, Kaiser S, Sies H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch Biochem Biophys 1989;274:532-538.

EDCC (Eye Disease Case-Control) STUDY GROUP. Antioxidant status and neovascular age-related macular degeneration. Arch Opthalmol 1993;111:104-109.

Emenhiser C, Simunovic N, Sander LC, Schwartz SJ. Separation of geometrical isomers in biological extracts using a polymeric C_{30} column in reversed-phase liquid chromatography. J Agric Food Chem 1996;44:3887-3893.

Epler KS, Sander LC, Ziegler RG, Wise SA, Craft NE. Evaluation of reversed-phase liquid chromatographic columns for recovery and selectivity of selected carotenoids. J Chromatogr 1992;595:89-101.

Erdman JW, Thatcher AJ, Hofmann NE et al. All-*trans* β–carotene is absorbed preferentially to 9-*cis*-β–carotene, but the latter accumulates in the tissues of domestic ferrets (*Mustela putorius puro*). J Nutr 1998;128: 2009-2013.

Eugster CH. Chemical derivatization: Microscale tests for the presence of common functional groups in carotenoids. In Britton G, Liaaen-Jensen S, Pfander H (eds), Carotenoids: Isolation and analysis, vol 1A. Birkhäuser Verlag, Basel, 1995, pp 71-80.

Foote CS, Chang YC, Denny RW. Chemistry of singlet oxygen X. Carotenoid quenching parallels biological protection. J Am Chem Soc 1970;92:5216-5218.

Gärtner C, Stahl W, Sies H. Lycopene is more bioavailable from tomato paste than from fresh tomatoes. Am J Clin Nutr 1997;66:116-122.

Gaziano JM, Hennekens CH. The role of beta-carotene in the prevention of cardiovascular disease. Ann New York Acad Sci 1993;691:148-155.

Gaziano JM, Johnson EJ, Russell RM et al. Discrimination in absorption or transport of β–carotene isomers after oral supplementation with either all-*trans*- or 9-*cis*-β–carotene. Am J Clin Nutr 1995;61:1248-1252.

Gerster HY. The potential role of lycopene for human health. J Am Coll Nutr 1997;16:109-126.

Giovannucci E. Tomatoes, tomato-based products, lycopene, and cancer: Review of the epidemiologic literature. J Natl Cancer Inst 1999;91:317-331.

Goodwin TW, Britton G. Distribution and analysis of carotenoids. In Goodwin TW (ed), Plant pigments. Academic Press, London, 1988, pp 61-132.

Goodwin TW. Nature and distribution of carotenoids. Food Chem 1980;5:3-13.

Gross J. Pigments in fruits. Academic Press, London, 1987.

Gross J. Pigments in vegetables. Chlorophylls and carotenoids. Avi: Van Nostrand Reinhold Company Inc, New York, 1991.

Hagenimana V, Carey EE, Gichuki ST, Oyunga MA, Imungi JK. Carotenoid contents in fresh, dried and processed sweetpotato products. Ecol Food Nutr 1999;37:455-473.

Handelman GJ, Dratz EA, Reay CC, van Kujik FJGM. Carotenoids in the human macula and whole retina. Invest Ophthalmol Vis Sci 1988;29:850-855.

Hart DJ, Scott KJ. Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. Food Chem 1995;54:101-111.

Heinonen MI, Ollilainen V, Linkola EK, Varo PT, Koivistoinen PE. Carotenoids in Finnish foods: Vegetables, fruits and berries. J Agric Food Chem 1989;37:655-659.

Horwitz W. Focus on sampling: Sampling and preparation of sample for chemical examination. J Assoc Off Anal Chem 1988;71:241-245.

Huang AS, Tanudjaja L, Lum D. Content of alpha-, beta-carotene, and dietary fiber in 18 sweetpotato varieties grown in Hawaii. J Food Comp Anal 1999;12:147-151.

K'osambo LM, Carey EE, Misra AK, Wilkes J, Hagenimana V. Influence of age, farming site and boiling on pro-vitamin A content in sweet potato (*Ipomoea batatas* (L.) Lam.) storage roots. J Food Comp Anal 1998;11:305-321.

Keith H, Crummet W, Deegan JJr, Libby RA, Taylor JK, Wentler G. Principles of environmental analysis. Anal Chem 1983;55:2210-2218.

Khachik F, Beecher GR, Goli MB, Lusby WR. Separation and quantification of carotenoids in foods. Meth Enzymol 1992;213:347-359.

Khachik F, Beecher GR, Vanderslice JT, Furrow G. Liquid chromatographic artifacts and peak distortion: Sample-solvent interactions in the separation of carotenoids. Anal Chem 1988;60:807-811.

Khachik F, Beecher GR, Whitaker NF. Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. J Agric Food Chem 1986;34:603-616.

Kimura M, Kobori CN, Rodriguez-Amaya DB, Nestel P. Screening and HPLC methods for sweetpotato, cassava and maize. Food Chem 2004 (submitted).

Kimura M, Rodriguez-Amaya DB. Sources of errors in the quantitative analysis of food carotenoids by HPLC. Arch Latinoamer Nutr 1999;49:58S-66S.

Kimura M, Rodriguez-Amaya DB. A scheme for obtaining standards and HPLC quantification of leafy vegetable carotenoids. Food Chem 2002;78:389-398.

Kimura M, Rodriguez-Amaya DB. Carotenoid composition of hydroponic leafy vegetables. J Agric Food Chem 2003;51:2603-2607.

Kimura M, Rodriguez-Amaya DB, Godoy HT. Assessment of the saponification step in the quantitative determination of carotenoids and provitamins A. Food Chem 1990;35:187-195

Kimura M, Rodriguez-Amaya DB, Yokoyama SM. Cultivar differences and geographic effects on the carotenoid composition and vitamin A value of papaya. Lebens Wissen Technol 1991;24:415-418.

Konings EJM, Roomans HHS. Evaluation and validation of an LC method for the analysis of carotenoids in vegetables and fruit. Food Chem 1997;59:599-603.

Kramer A, Twigg BA. Fundamentals of quality control for the food industry, 3rd edition, vol 1. Avi Publishing Co, Westport, CT, 1970.

Kratochvil B, Taylor JK. Sampling for chemical analysis. Anal Chem 1981;53:924A-926A, 928A, 930A, 932A, 934A, 936A, 938A.

Krinsky NI. Actions of carotenoids in biological systems. Annu Rev Nutr 1993;13:561-587.

Lesellier E, Marty C, Berset C, Tchapla A. Optimization of the isocratic non-aqueous reverse phase (NARP) HPLC separation of *trans*/*cis*-a and β–carotenes. J High Resolut Chromatogr 1989;12:447-454.

Lessin WJ, Catigani GL, Schwartz SJ. Quantification of *cis-trans* isomers of provitamin A carotenoids in fresh and processed fruits and vegetables. J Agric Food Chem 1997;45:3728-3732.

Liaaen-Jensen S. Isolation, reactions. In Isler O, (ed), Carotenoids. Birkhäuser Verlag, Basel, 1971, pp 61-188.

Lietz G, Henry CJK. A modified method to minimise losses of carotenoids and tocopherols during HPLC analysis of red palm oil. Food Chem 1997;60:109-117

Mantoura RFC, Repeta DJ. Calibration methods for HPLC. In Jeffrey SW, Mantoura RFC, Wright SW (eds), Phytoplankton pigments in oceanography: Guidelines to modern methods. UNESCO Publishing, Paris, 1997, pp 407-428.

Markovic M, Mülleder U, Neunteufl H. Carotenoid content in different varieties of pumpkins. J Food Comp Anal 2002;15:633-638.

Mayne ST. Beta-carotene, carotenoids, and disease prevention in humans. FASEB J 1996;10:690-701.

Mercadante AZ, Rodriguez-Amaya DB. Carotenoid composition of a leafy vegetable in relation to some agricultural variables. J Agric Food Chem 1991;39:1094-1097.

Mercadante AZ, Rodriguez-Amaya DB. Effects of ripening, cultivar differences, and processing on the carotenoid composition of mango. J Agric Food Chem 1998;46:128-130.

Mercadante AZ, Steck A, Pfander H. Carotenoids from guava (*Psidium guajava* L.): Isolation and structure elucidation. J Agric Food Chem 1999;47:145-151.

Moeller SM, Jacques PF, Blumberg, JB. The potential role of dietary xanthophylls in cataract and age-related macular degeneration. J Am Coll Nutr 2000;19:522S-527S.

Moros EE, Darnoko D, Cheryan M, Perkins EG, Jerrell J. Analysis of xanthophylls in corn by HPLC. J Agric Food Chem 2002;50:5787-5790.

Murphy EW, Criner PE, Gray BC. Comparison of methods for calculating retention of nutrients in cooked foods. J Agric Food Chem 1975;23:1153-1157.

Olson JA. Carotenoids. In Shils ME, Olson JA, Shike M, Ross AC (eds), Modern nutrition in health and disease, 9th edition. Williams & Wilkins, Baltimore, 1999a, pp 525-541.

Olson JA. Carotenoids and human health. Arch Latinoamer Nutr 1999b;49:7S-11S.

Olson JA, Krinsky NI. The colorful, fascinating world of the carotenoids: Important physiologic modulators. FASEB J 1995;9:1547-1550.

Padula M, Rodriguez-Amaya, DB. Characterisation of the carotenoids and assessment of the vitamin A value of Brasilian guava. Food Chem 1986;20:11-19.

Palozza P, Krinsky NI. Antioxidant effects of carotenoids *in vivo* and *in vitro*: An overview. Meth Enzymol 1992;213:403-420.

Park YW. Effect of freezing, thawing, drying, and cooking on carotene retention in carrots, broccoli, and spinach. J Food Sci 1987;52:1022-1025.

Pfander H. Key to carotenoids, 2nd edition. Birkhäuser Verlag, Basel, 1987.

Pfander H, Riesen R, Niggli U. HPLC and SFC of carotenoids – scope and limitations. Pure Appl Chem 1994;66:947-954.

Pomeranz Y, Meloan CE. Food analysis: Theory and practice, 3rd edition. Chapman & Hall, New York, 1994.

Porcu OM, Rodriguez-Amaya DB. Carotenóides de acerola: Efeito de estádio de maturação e remoção de película. Paper presented at the V Simpósio Latino Americano de Ciência de Alimentos, Campinas, Brazil, 2003.

Quackenbush FW, Smallidge RL. Nonaqueous reverse phase liquid chromatographic system for separation and quantitation of provitamins A. J Assoc Off Anal Chem 1986;69:767-772.

Ramos DMR, Rodriguez-Amaya DB. Determination of the vitamin A value of common Brazilian leafy vegetables. J Micronutr Anal 1987;3:147-155.

Ramos DMR, Rodriguez-Amaya DB. Avaliação das perdas de carotenóides e valor de vitamin A durante desidratação e liofilização industrial de espinafre. Arq Biol Tecnol 1993;36:83-94.

Riso P, Porrini M. Determination of carotenoids in vegetable foods and plasma. Int J Vit Nutr Res 1997;67:47-54

Rock CL, Lovalvo, JL, Emenhiser C, Ruffin MT, Flatt SW, Schwartz SJ. Bioavailability of β–carotene is lower in raw rather than in processed carrots and spinach in women. J Nutr 1998;128:913-916.

Rodriguez-Amaya DB. Critical review of provitamin A determination in plant foods. J Micronutr Anal 1989;5:191-225.

Rodriguez-Amaya DB. Provitamin A determination - problems and possible solutions. Food Nutr Bull 1990;12:246-250

Rodriguez-Amaya DB. Carotenoids and food preparation: The retention of provitamin A carotenoids in prepared, processed, and stored foods. Opportunities for Micronutrient Intervention (OMNI), Arlington, 1997.

Rodriguez-Amaya DB. A guide to carotenoid analysis in foods. ILSI Press, Washington DC, 1999a.

Rodriguez-Amaya DB. Changes in carotenoids during processing and storage of foods. Arch Latinoamer Nutr 1999b;49:38S-47S.

Rodriguez-Amaya DB. Effects of processing and storage on food carotenoids. Sight Life Newsletter (Special issue) 2002;3:25-35.

Rodriguez-Amaya DB. Nature and distribution of carotenoids in foods. In Charalambous G (ed), Shelf-life studies of foods and beverages. Chemical, biological, physical and nutritional aspects. Elsevier Science Publishers, Amsterdam, 1993, pp 547-589.

Rodriguez-Amaya DB, Amaya-Farfan J. Estado actual de los metodos analiticos para determinar provitamina A. Arch Latinoamer Nutr 1992;42:180-191.

Rodriguez-Amaya DB, Kimura M, Godoy HT, Arima HK. Assessment of provitamin A determination by open column chromatography/visible absorption spectrophotometry. J Chromatogr Sci 1988;26:624-629.

Rodriguez-Amaya DB, Kimura M. Carotenóides e valor de vitamina A em cajá (*Spondias lutea*). Cienc Tecnol Aliment 1989;9:148-162.

Sá MC, Rodriguez-Amaya DB. Optimization of HPLC quantification of carotenoids in cooked green vegetables – Comparison of analytical and calculated data. J Food Comp Anal 2004;17:37-51.

Sander LC, Sharpless KE, Craft NE, Wise SA. Development of engineered stationary phases for the separation of carotenoid isomers. Anal Chem 1994;66:1667-1674.

Schiedt K, Liaaen-Jensen S. Isolation and analysis. In Britton G, Liaaen-Jensen S, Pfander H (eds), Carotenoids, vol 1A: Isolation and analysis. Birkhäuser Verlag, Basel, 1995, pp. 81-108.

Scott KJ. Observations on some of the problems associated with the analysis of carotenoids in foods by HPLC. Food Chem 1992;45:357-364.

Scott KJ, Finglass PM, Seale R, Hart DJ, Froidmont-Gortz I. Interlaboratory studies of HPLC procedures for the analysis of carotenoids in foods. Food Chem 1996;57:85-90.

Seddon JM, Ajani UA, Sperduto RD, et al. Dietary carotenoids, vitamins A, C and E and advanced age-related macular degeneration. J Am Med Assoc 1994;272:1413-1420.

Sies H, Stahl W. Lycopene: Antioxidant and biological effects and its bioavailability in the human. Proc Soc Exp Biol Med 1998; 218:121-124.

Stahl W, Schwarz W, von Laar J, Sies H. All-*trans* β–carotene preferentially accumulates in human chylomicrons and very low density lipoproteins compared with 9-*cis* geometrical isomer. J Nutr 1995;25:2128-2133.

Stahl W, Sies H. Uptake of lycopene and its geometrical isomers is greater from heat-processed than from unprocessed tomato juice. J Nutr 1992;122:2161-2166.

Stahl W, Sies H. Lycopene: A biologically important carotenoid for humans? Arch Biochem Biophys 1996;336:1-9.

Takahata Y, Noda T, Nagata, T. Varietal differences in chemical composition of the sweet potato storage root. Acta Hort 1993;43:77-80.

Taylor RF. Chromatography of carotenoids and retinoids. Adv Chromatogr 1983;22:157-213.

Tonucci LH, Holden JM, Beecher GR, Khachik F, Davis CS, Mulokozi G. Carotenoid content of thermally processed tomato-based food products. J Agric Food Chem 1995;43:579-586.

Trujillo JA, Muller MC, Kimura, M, Rodriguez-Amaya DB. Um método por HPLC para determinação da composição de carotenóides em óleo de dendê. Paper presented at the Congresso Latino-Americano de Carotenóides em Alimentos, Campinas, Brazil, 1998.

van het Hof KH, de Boer BCJ, Tijburg LBM et al. Carotenoid bioavailability in humans from tomatoes processed in different ways determined from the carotenoid response in the triglyceride-rich lipoprotein fraction of plasma after a single consumption and in plasma after four days of consumption. J Nutr 2000;130:1189-1196.

van Jaarsveld PJ, Marais DW, Harmse E, Nestel P, Rodriguez-Amaya DB. Retention of β–carotene in boiled, mashed orange-fleshed sweet potato. J Food Comp Anal 2004 (in press).

Breeding Crops
for Better Nutrition

c/o International Food Policy Research Institute
2033 K Street, NW, Washington, DC 20006-1002 USA
Phone: 1-202-862-5600 • **Fax**: 1-202-467-4439• **HarvestPlus@cgiar.org**