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Sources of errors in the quantitative analysis of food carotenoids by HPLC

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SUMMARY. Several factors render carotenoid determination inherently difficult. Thus, in spite of advances in analytical instrumentation, discrepancies in quantitative results on carotenoids can be encountered in the international literature. A good part of the errors comes from the pre-chromatographic steps such as: sampling scheme that does not yield samples representative of the food lots under investigation; sample preparation which does not maintain representativity and guarantee homogeneity of the analytical sample; incomplete extraction; physical losses of carotenoids during the various steps, especially during partition or washing and by adsorption to glass walls of containers; isomerization and oxidation of carotenoids during analysis. On the other hand, although currently considered the method of choice for carotenoids, high performance liquid chromatography (HPLC) is subject to various sources of errors, such as: incompatibility of the injection solvent and the mobile phase, resulting in distorted or split peaks; erroneous identification; unavailability, impurity and instability of carotenoid standards; quantification of highly overlapping peaks; low recovery from the HPLC column; errors in the preparation of standard solutions and in the calibration procedure; calculation errors. Illustrations of the possible errors in the quantification of carotenoids by HPLC are presented.
Key Words: Carotenoids, quantitative analysis, HPLC, analytical errors.

RESUMEN. Fuentes de errores en el análisis cuantitativo de carotenoides en alimentos por HPLC. Varios factores hacen inherentemente difícil la determinación de carotenoides. A pesar de los avances en la instrumentación analítica, se pueden encontrar en la literatura internacional, discrepancias en los resultados cuantitativos relacionados a los carotenoides. Una gran parte de los errores provienen de las etapas precromatográficas tales como: esquema de muestreo que no produce muestra representativa del lote en estudio; preparación de muestra que no mantenga la representatividad y garantice la homogeneidad de la muestra analítica; extracción incompleta; pérdidas físicas de los carotenoides durante las varias etapas, especialmente durante la partición o lavado y por adsorción en las paredes de vidrios de los recipientes; isomerización y oxidación de los carotenoides durante el análisis. Sin embargo, a pesar de que actualmente el método escogido para análisis de carotenoides es HPLC, éste está sujeto a varias fuentes de errores, tales como: incompatibilidad del solvente de inyección y la fase móvil, resultando en picos distorsionados o divididos; identificación errónea; indisponibilidad, impureza e inestabilidad de los patrones de carotenoides; cuantificación de picos altamente superpuestos; baja recuperación de las columnas de HPLC; errores en la preparación de las soluciones de patrones y en el procedimiento de calibración; errores de cálculo. Se presentan ilustraciones de los posibles errores en la cuantificación de carotenoides por HPLC.
Palabras clave: Carotenoides, análisis cuantitativo, HPLC, errores analíticos.

INTRODUCTION

The day when we can say that most of the data on carotenoid composition of foods are finally reliable is still eluding us. Although there have been tangible strides, and an appreciable part of available analytical information is now reliable, incoherence in published results persists, in spite of the introduction of high performance liquid chromatography (HPLC), currently regarded as the method of choice. A quick look at recent literature illustrates this point, even in terms of only the principal carotenoids. Errors are understandably magnified when minor or trace carotenoids are considered. Tables 1-3 show some recent results on three foodstuffs, obtained in several countries. Lycopene and ß-carotene levels in tomato, from four countries using two analytical techniques, agree well (Table 1), except for the lycopene content of Malaysian tomato. The α-carotene and ß-carotene contents of carrot, obtained in six countries, are more variable, but seems to be mostly a reflection of natural sample variation (Table 2). On the other hand, the carotenoid data for the leafy vegetable *Ipomoea aquatica*, called water spinach by Wills and Rangga (13) of Australia and Hulshof et al. (12) of Indonesia, swamp cabbage by Tee and Lim (4) of Malaysia and water convolvulus by Chen and Chen (11) of Taiwan, are so different that analytical factors must have been involved. Results such as these justify continued strong effort on analytical refinement, so that analytical variability is not mistaken for natural variation of samples.

TABLE 1
Data on principal carotenoids (μg/g) of tomato

Reference, chromatographic technique	Cultivar	ß-Carotene	Lycopene
Hart & Scott (1), UK, HPLC	9 cultivars	4.3-17	12-50
Khachik et al. (2), USA, HPLC	not specified	2.8±0.2	39±1
Tavares & Rodriguez-Amaya (3), Brasil, OCC	Santa Cruz	5.1±1.1	31±20
Tee & Lim (4), Malaysia, HPLC	not specified	3.6	7

HPLC- high performance liquid chromatography; OCC- open column chromatography

TABLE 2
Data on principal carotenoids (μg/g) of carrot

Reference, chromatographic technique	α-Carotene	ß-Carotene
Abdel-Kader (5), Egypt, HPLC	34	63
Chen et al. (6), Taiwan , HPLC	28±3	54±6
Godoy & Rodriguez-Amaya (7), Brasil, OCC	19±1	38±4
Granado et al. (8), Spain, HPLC	29±3	66±0
Hart & Scott (1), UK, HPLC	27 , 36[a]	85 , 108[a]
Heinonen et al. (9), Finland, HPLC	22-49[b]	46-103[b]
Lessin et al. (10), USA, HPLC	39	56
Tee & Lim, Malaysia (4), HPLC	34	68

[a]Two sample lots analyzed in May and September.
[b]19 cultivars.
HPLC- high performance liquid chromatography; OCC- open column chromatography

It is recognized that carotenoid analysis is inherently difficult, the main reasons being: (a) the existence of a large number of naturally occurring carotenoids; (b) the highly variable qualitative and quantitative carotenoid composition of foods; (c) the wide range in concentration of the constituent carotenoids of any given food; and (d) isomerization and degradation of carotenoids during analysis or storage of samples prior to analysis (14-16).

Regardless of the analytical method adopted, a major source of errors is the susceptibility of the highly unsaturated carotenoid molecule to isomerization and oxidation. Thus, special precautions should be taken during analysis, such as: (a) completion of the analysis within the shortest possible time; (b) exclusion of oxygen; (c) protection from light; (d) avoiding high temperature; (e) avoiding contact with acids; (f) use of high purity solvents, free from harmful impurities (e.g. peroxides).

The general procedure in carotenoid analysis consists of: (a) sampling and sample preparation, (b) extraction, (c) partition or transfer 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 quantification. Evidently, errors can be introduced in each of these steps. Thus, aside from errors arising from the isomerization and oxidation of carotenoids during analysis, other common sources of errors are: (a) analytical samples not representing the food lots under investigation, (b) incomplete extraction, (c) physical losses during the different steps, (d) inefficient chromatographic separation, (e) misidentification, (f) faulty quantification or calculation. Another serious source of error is enzymatic oxidation, which occurs between cutting or disintegration of sample and extraction.

TABLE 3
HPLC data on carotenoids (μg/g) of *Ipomoea aquatica*

Carotenoid	Chen and Chen (11) Taiwan, water convolvulus	Hulshof et al. (12) Indonesia, water spinach	Tee and Lim (4) Malaysia, swamp cabbage	Wills and Rangga (13) Australia, water spinach
ß-Carotene	100±8	27±10	19	4
Cis-ß-carotene	6.8±0.8	4.3±2.2	nd	nd
Lutein	78±7	nd	3.4	6
Violaxanthin	60±5	nd	nd	25
Neoxanthin	50±5	nd	nd	16
Lutein epoxide	29±3	nd	nd	nd
Cis-lutein	11±1	nd	nd	nd
Zeaxanthin	nd	nd	nd	5

HPLC- high performance liquid chromatography; nd- not determined.

Errors in the pre-chromatographic steps

Errors incurred in the steps preceeding chromatography may surpass chromatographic errors and will not be compensated for, no matter how modern and sophisticated the analytical instrumentation may be. In a series of European interlaboratory studies (17), the preliminary conclusion was that preparation of the carotenoid extract for HPLC might account for about 13% of the overall variance of around 23%.

In the interlaboratory studies mentioned above, the same homogenous and stable vegetable mix was analyzed by the different laboratories, thus sampling and sample preparation were not part of the investigation. However, these two initial steps in the analytical process could be major sources of errors.

Several factors markedly influence the carotenoid composition of foods: (a) cultivar or variety; (b) part of plant analyzed; (c) stage of maturity; (d) climate or geographic site of production; (e) harvesting and postharvest handling; and (f) processing and storage. Thus, representative sampling and sample preparation are critical and difficult operations, which, unfortunately, are not well focalized in the carotenoid field. Referring to food analysis in general, Rund (18) eloquently writes, "Are we conscious that the magnitude of sampling errors often exceed three-fold those of the analysis? Why should we be so enamored of new, extremely expensive, and highly sensitive laboratory instrumentation with miraculous detectability characteristics when the gross sample from which the laboratory portion has been extracted was possibly obtained with antiquated equipment and procedure often neither based on scientific fact nor trained personnel?"

Because of the influencing factors cited above, aside from insuring representative sampling and sample preparation, pertinent information must accompany analytical results, such as origin, cultivar, part of plant analyzed, stage of maturity, postharvest handling conditions.

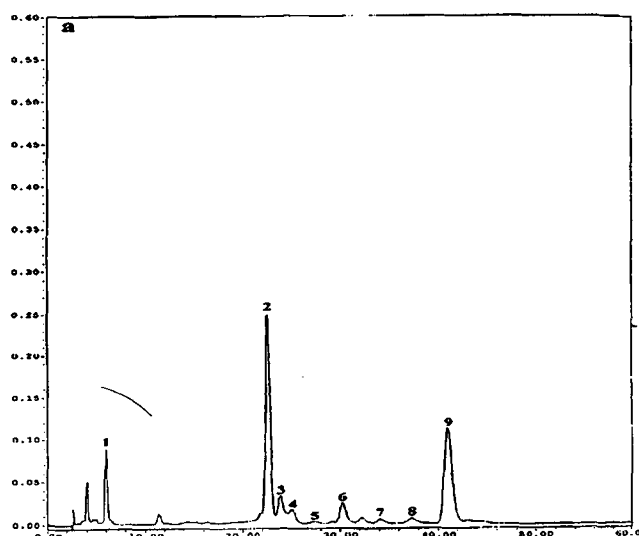
Because of the varying nature of food matrices, including the degree of natural protection conferred on carotenoids, incomplete extraction may be a more common source of error than presently acknowledged. Physical losses, including that resulting from tight adherence of carotenoids in concentrated solutions on the glass walls of containers, are also often overlooked.

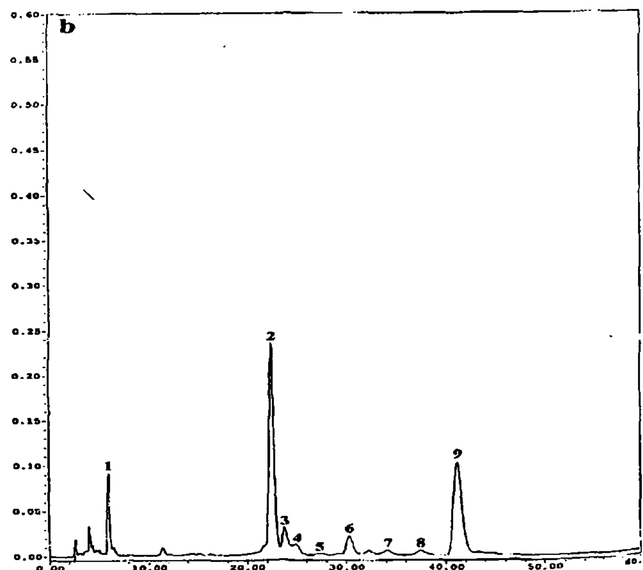
The addition of $MgCO_3$ and other neutralizing agent is often done to neutralize the acids liberated from the sample during tissue disintegration to prevent isomerization and degradation. In our laboratory, keeping the time lag between sample maceration and extraction as short as possible, not only prevents enzymatic oxidation, but also makes the addition of $MgCO_3$ unnecessary. No significant difference in the carotenoid concentrations of tomato, an acidic sample, and kale, were observed with or without the addition of $MgCO_3$ (19).

It could be argued that the effect of $MgCO_3$, under the conditions described above, might not be perceptible in terms of the concentration of the constituent carotenoid, but could be seen in terms of isomerization. The chromatogram of the carotenoids of tomato, obtained with or without the use of $MgCO_3$ (Figure 1) are identical and does not support this contention, no *cis*-isomers of ß-carotene being detected in unneutralized tomato with the Vydac column which is capable of separating these geometrical isomers.

FIGURE 1

HPLC chromatograms of tomato extracts obtained with (a) and without (b) the use of $MgCO_3$. Conditions - Column: Spherisorb ODS 5 µm, 2.0x250 mm. Mobile phase: acetonitrile:methanol:ethyl acetate 73:20:7. Flow rate 0.25 mL/min.





Peak identification: 1- lutein, 2- *trans*-lycopene, 3,4- *cis*-lycopene, 5- neurosporene, 6- γ-carotene, 7- *cis*-ζ-carotene, 8-*trans*-ζ-carotene, 9- ß-carotene.

Possible losses during saponification have received more attention. This steps is carried out to remove chlorophylls and unwanted lipids and to hydrolyze carotenol esters, thus simplifying the chromatographic separation, identification and quantification of the carotenoids. However, artefact formation and degradation of carotenoids can occur, the extent of which depends on the carotenoid present and on the saponification conditions (20). The provitamin A carotenoids α-carotene, ß-carotene, γ-carotene and ß-cryptoxanthin can resist saponification (19, 20), but xanthophylls such as lutein, violaxanthin and other dihydroxy and trihydroxy carotenoids can suffer considerable losses (20-22). Thus, saponification should be omitted whenever possible (e. g. analyses of leafy vegetables, tomatoes and carrots) and when indispensable, mild conditions should be used. Saponification of carotenoids dissolved in petroleum ether with an equal volume of 10% KOH overnight at room temperature in the dark, preferably with the addition of BHT and under an atmosphere of N_2, has been generally found to be adequate in our laboratory. Care should also be taken during the subsequent washing as xanthophylls can be easily lost with the water.

Concern about losses of carotenoids has recently led researchers to shorten the time of ambient saponification (1,2, 23). However, complete hydrolysis of carotenoid esters from papaya and *Cucurbita maxima* cultivar Exposição was found to be complete only after overnight saponification (Figures 2 and 3).

Errors in the chromatographic step

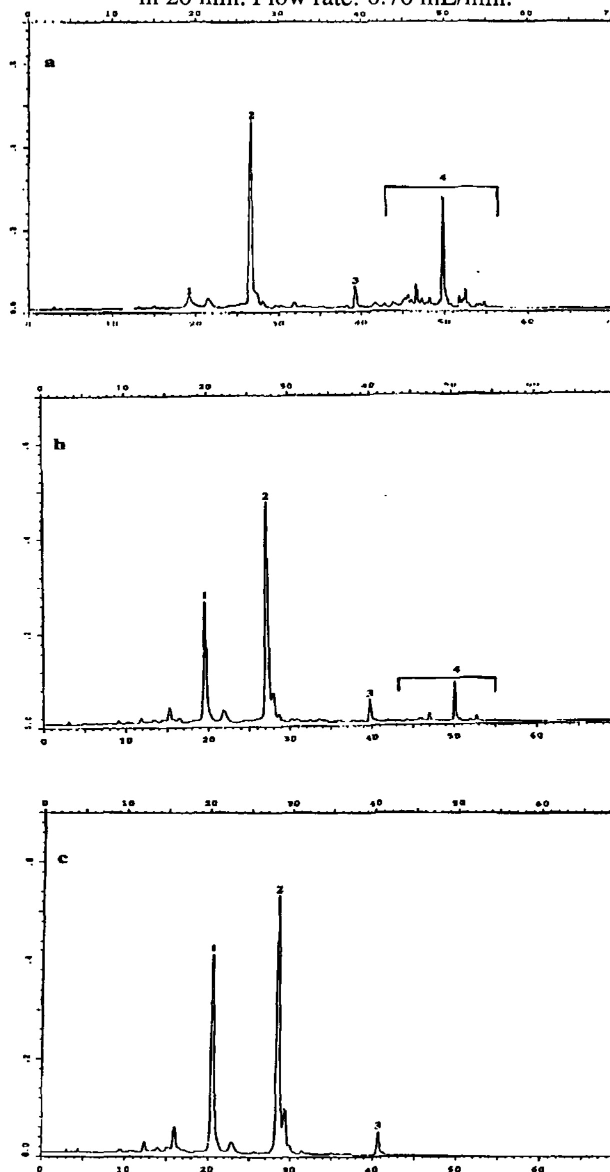
Before carrying out an expensive and complicated analysis, the analyst must clearly define what information is desired. Carotenoid analysis has been carried out at three different levels. For a long time, quantitative analysis of carotenoids involved mainly the determination of the concentrations of only the principal provitamin A carotenoids. With the recognition that vitamin A inactive carotenoids can also be biologically active, determination of major carotenoids, provitamins A or not, have been increasingly carried out. The complete carotenoid composition is the ultimate aim of carotenoid analysis. However, considering that the carotenoid composition of foods typically consist of 1 to 4 principal carotenoids, with a series of carotenoids in minute or trace amounts, it is questionable whether the added information is well worth the greater complexity of the analysis, with greater possibility of errors, higher cost and longer analysis time. In our opinion, the determination of the major carotenoids is adequate for the generation of data for food composition databases.

Although the preferred method for the chromatographic separation of carotenoids, HPLC is subject to several sources of errors: (a) incompatibility of the injection solvent and the mobile phase, (b) erroneous identification, (c) impurity and instability of carotenoid standards, (d) quantification of highly overlapping peaks, (e) low recovery from the HPLC column, (f) errors in the preparation of standard solutions and in the calibration procedure, and (g) erroneous calculation.

The injection solvent must be capable of dissolving all the sample's carotenoids and must also be compatible with the mobile phase. If the injection solvent is much stronger than the mobile phase, the carotenoids can precipitate in the mobile phase, resulting in band broadening and double or tailing peaks, especially when the extract is concentrated (24). On the other hand, a weak injection solvent will not dissolve the carotenoids completely.

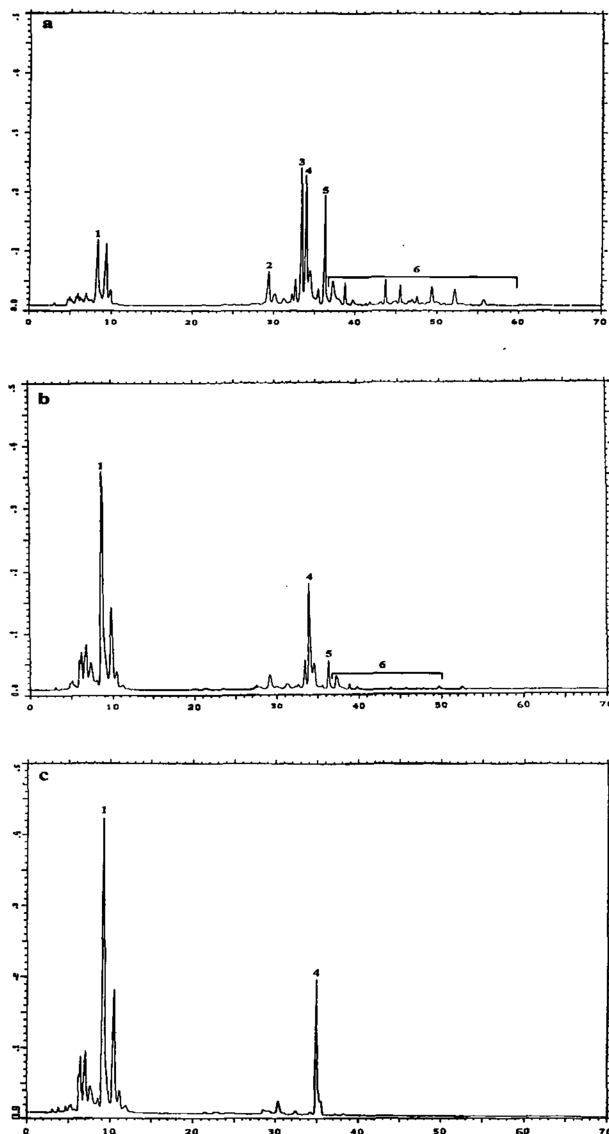
FIGURE 2

HPLC chromatograms of papaya extracts, unsaponified (a), saponified for 4 hours (b) and saponified overnight (c). Conditions - Column: Novapak 4 μm, 3.9x300 mm. Mobile phase: acetonitrile:methanol:dichoromethane, linear gradient of 80:20:0 to 65:20:15 in 30 min and to 40:20:40 in 20 min. Flow rate: 0.70 mL/min.



Peak identification: 1- ß-cryptoxanthin, 2- lycopene, 3- ß-carotene, 4- esters

FIGURE 3
HPLC chromatograms of *Cucurbita maxima* cultivar Exposição extracts, unsaponified (a), saponified for 4 hours (b) and saponified overnight (c). Conditions - Column: Novapak 4 µm, 3.9x300 mm. Mobile phase: acetonitrile: methanol: dichoromethane, linear gradient of 80:20:0 to 65:20:15 in 20 min and to 40:20:40 in 20 min. Flow rate: 0.70 mL/min.

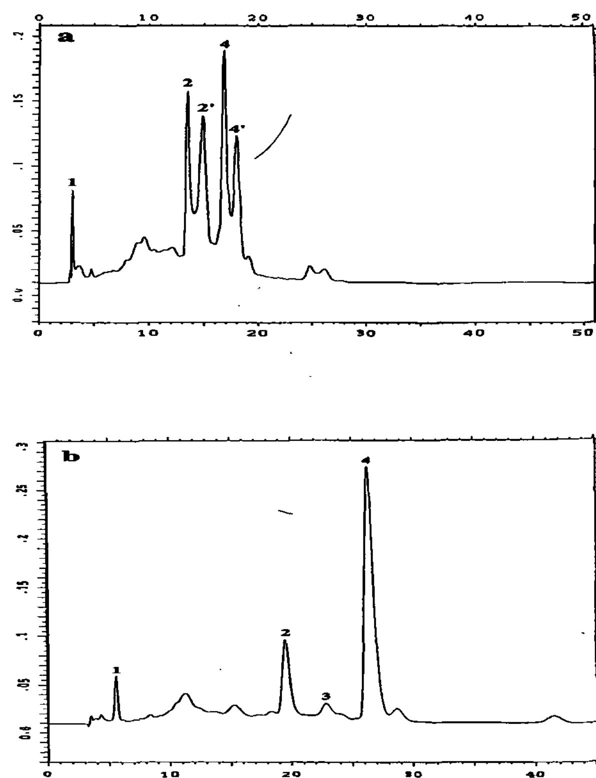


Peak identification: 1- lutein, 4- ß-carotene, 2,3,5 and 6- esters.

Khachik et al. (25) reported peak splitting when carotenoids were injected in dichloromethane, chloroform, tetrahydrofuran, benzene or toluene with a monomeric C_{18} column and a mobile phase consisting of a mixture of acetonitrile, methanol, dicloromethane and hexane. No such splitting occurred when the injection solvent was acetone, acetonitrile, methanol or hexane. On the other hand, Zapata and Garrido (26) observed distorted peaks, especially with the first peaks, when carotenoids were injected in 90% acetone with a gradient of 100% methanol to methanol-acetone (8:2) as mobile phase. No peak distortion was observed when the same extract was injected in 95% methanol or 69% acetone.

As with Khachik et al.(25) and Lietz and Henry (27), in our laboratory, acetone has been found to be a suitable injection solvent. With a Vydac C_{18} polymeric column and a mobile phase of methanol-tetrahydrofuran (95:5), peak splitting occurred when tomato extract was injected in hexane (Figure 4a). However, well defined peaks were obtained when the extract was injected in acetone (Figure 4b). Since occurrence of peak distortion and splitting depends on the chromatographic system used, and results of different laboratories diverge somewhat, the analyst should test his own system.

FIGURE 4
HPLC chromatograms of tomato extracts injected in hexane (a) and in acetone (b). Conditions - Column: Vydac 218 TP54, 5 µm, 4.6x250 mm. Mobile phase: methanol:tetrahydrofuran 95:5. Flow rate: 0.80 mL/min.



Peak identification: 1- lutein, 2,2'- ß-carotene, 3- γ-carotene, 4,4'- lycopene.

According to Craft (24), stronger, miscible solvents can be used as injection solvent if the volume is small (≤ 10 µL) and

the concentrations of the carotenoids are not greatly in excess of their solubility in the mobile phase. In fact, Khachik et al. (25) observed that HPLC peak distortion of carotenoids that occurred with injection solvents such as methylene chloride, choloroform, THF, benzene and toluene, could be eliminated if the injection volume of samples in these solvents were reduced to 5-10 µL. Hexane, which resulted in peak splitting of ß-carotene at higher injection volumes, did not do so at an injection volume of 20 µL. In our system, however, peak splitting was seen with hexane even with an injection volume of 10 µL (Figure 4 a).

Porsch (28) observed that anomalous peaks may be formed, even when sample solubility in the mobile phase is sufficient, if the injection solvent and the mobile phase differ substantially in viscosity and/or the injection solvent strength is considerably higher. He suggested that the viscosity ratio should be kept fairly below two and too high elution power of the injection solvent should be decreased by mixing with the mobile phase prior to injection.

After the introduction of HPLC in the carotenoid field, reversed-phase HPLC C_{18} column immediately became the preferred mode. Among the reasons for such popularity is the weak hydrophobic interaction between the carotenoids and the stationary phase, expected to be less destructive than polar forces in normal-phase chromatography. It was later shown, however, that low recovery of carotenoids from the reversed-phase HPLC column can occur.

Epler et al. (29) investigated the effects of mobile phase, type of stationary phase and the column frit material on recovery of seven carotenoids from sixty commercially available and five experimental HPLC columns. All except five columns were C_{18}. On the average, monomeric C_{18} columns yielded higher recoveries than polymeric C_{18} columns, but were unable to resolve lutein and zeaxanthin. On almost all columns tested, using methanol or methanol-based solvents provided higher recoveries of carotenoids than acetonitrile or acetonitrile-based solvent (Table 4). Recovery with acetonitrile-based solvents was improved with the addition of ammonium acetate and triethylamine, an observation later confirmed by Hart and Scott (1).

TABLE 4
Average recovery of carotenoids with different mobile phases

Mobile phase	Number of columns tested	Recovery ± SD (%)[a]
100% methanol	29	84±8
Methanol-tetrahydrofuran	35	86±11
Methanol-ethyl acetate	35	82±12
100% acetonitrile	21	56±19
Acetonitrile-tetrahydrofuran	43	68±17
Acetonitrile-ethyl acetate	43	47±17

[a]Mean and standard deviation
Reference: Epler et al. (29)

Recovery was also found by Epler et al. (29) to be dependent on the carotenoid structure. Losses of zeaxanthin and ß-carotene, both having two ß-rings, were greater than those of lutein and α-carotene, both containing one ß- and one ε-ring. Within the group of ß,ß-carotenoids, recovery increased as polarity decreased. Recovery increased in the following order: zeaxanthin (dihydroxy) < ß-cryptoxanthin (monohydroxy) < echinenone (monoketo) < ß-carotene. For the two ß,ε-carotenoid, the recovery of lutein (dihydroxy) was less than that of α-carotene. Hart and Scott (1) also found differences in the recovery of individual carotenoids, suggesting that on-column losses varied with different carotenoids.

Although recoveries were slightly lower for stainless steel frits, Epler et al. (29) observed no significant difference in recovery in using stainless steel, titanium or "biocompatible" (hastealloy) frits. Degradation of carotenoids provoked by the metal surface of stainless steel frits of the guard and analytical column was, however, reported by several authors in recent years (23,30,31). Thus, the use of the "biocompatible" hastealloy frits was advocated. But even with this frit, Konings and Roomans (23) observed considerable loss (approximately 40%) of lycopene, leading them to suggest that a PAT (peek alloyed with teflon) frit be used.

The accuracy of HPLC quantification of carotenoids obviously depends on how well the chromatogram peak areas are measured. Especially in earlier HPLC studies, data on food carotenoids have been obtained by quantifying highly overlapping peaks. Although working with non-carotenoid compounds (naphthalene and anthracene), Meyer (32) gave an idea of the magnitude of the error derived from integration of incompletely resolved chromatographic peaks. Errors increased with increasing size ratio of the fused peaks, increasing tailing and decreasing resolution. Within the range of parameters investigated (size ratio up to 10:1, tailing to 2.0, resolution down to 0.75), the relative error can reach a 40% deviation in peak area.

Highly efficient columns are now available, which with judicious choice of mobile phase, can provide good resolution of even complex mixtures, such as carotenoid extracts from foods.

Errors in the identification step

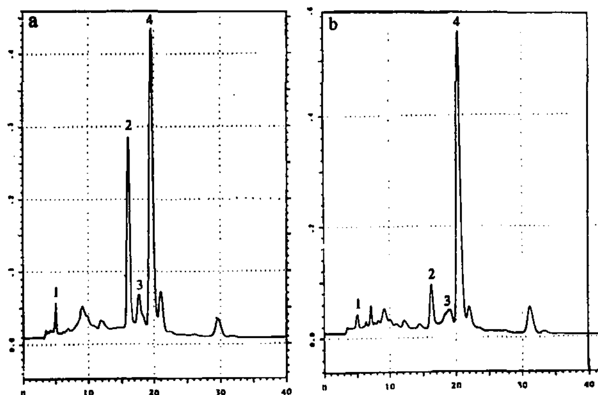
The chromatographic behavior and the UV-visible absorption spectrum are the first tools used to identify carotenoids. The retention time reflects the polarity; and the wavelengths of maximum absorption and the fine structure (shape) of the spectrum reflect the chromophore. However, the use of these two parameters as sole criteria for identification, although a common practice, may not be conclusive and may lead to erroneous identification. Retention times are difficult to reproduce, and even when authentic carotenoids are available for co-chromatography, identification will still be inconclusive since different carotenoids may have the same retention time. Likewise, different carotenoids may have the same

chromophore and thus present the same spectrum. Some examples of misidentifications are given below.

α-Cryptoxanthin and zeinoxanthin both monohydroxy derivatives of α-carotene, differ only in the position of the hydroxy group, thereby presenting identical spectrum and very similar chromatographic behavior. They can be differentiated by simple methylation with acidified methanol, α-cryptoxanthin responding positively because of the allylic position of the hydroxy substituent. Seemingly, these two carotenoids are often confused with each other and even with ß-cryptoxanthin.

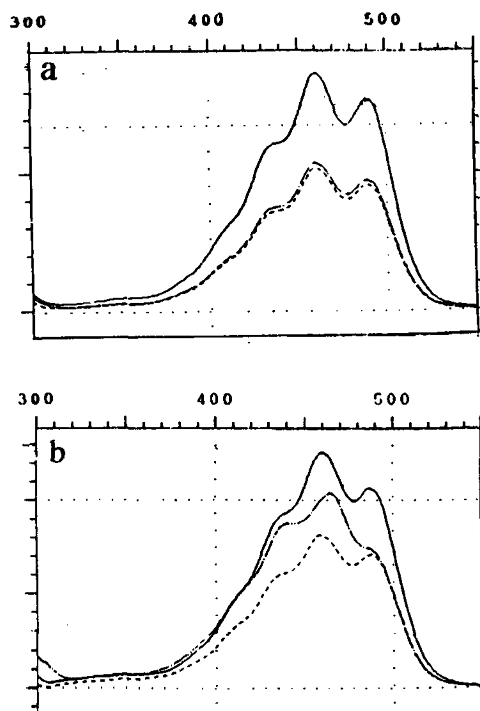
With the photodiode array detector, testing the peak purity is easier, avoiding the identification of a peak of a mixture of carotenoids as that of a sole carotenoid. A quick look at the chromatograms in Figure 5 may give the idea that peak 3 in both the fresh tomato and the tomato paste is γ-carotene. The spectra taken at the ascending and descending slopes and at the maximun show that while peak 3 of the fresh tomato was pure γ-carotene, this peak in the tomato paste was a mixture (Figure 6).

FIGURE 5
HPLC chromatograms of raw tomato (a) and tomato paste (b). Conditions - Column: Vydac 218 TP54, 5 µm, 4.6x250 mm. Mobile phase: methanol:tetrahydrofuran 95:5. Flow rate: 0.80 mL/min.



Peak identification: 1- lutein, 2- ß-carotene, 3- γ-carotene in raw tomato and mixture in tomato paste, 4- lycopene.

FIGURE 6
Absorption spectra corresponding to peak 3 of Figure 5 obtained with the photodiode array detector of raw tomato (a) and tomato paste (b) at maximum (————), upslope (---------) and downslope (-.-.-.-.).



Unlike fruits and roots, leaves have been known to contain the same principal carotenoids: lutein, ß-carotene, violaxanthin and neoxanthin. Siefermann-Harms et al. (23) showed that lettuce also contains lactucaxanthin. Usually overlooked, lactucaxanthin appears to be present in similar or greater amounts than neoxanthin as shown in Figure 7.

In cases where the judicious and combined use of chromatographic data, co-chromatography with authentic samples, UV-visible absorption spectra and chemical reactions do not yield conclusive identifications, mass spectrometry and nuclear magnetic resonance spectroscopy, two techniques required in structure elucidation, will have to be used.

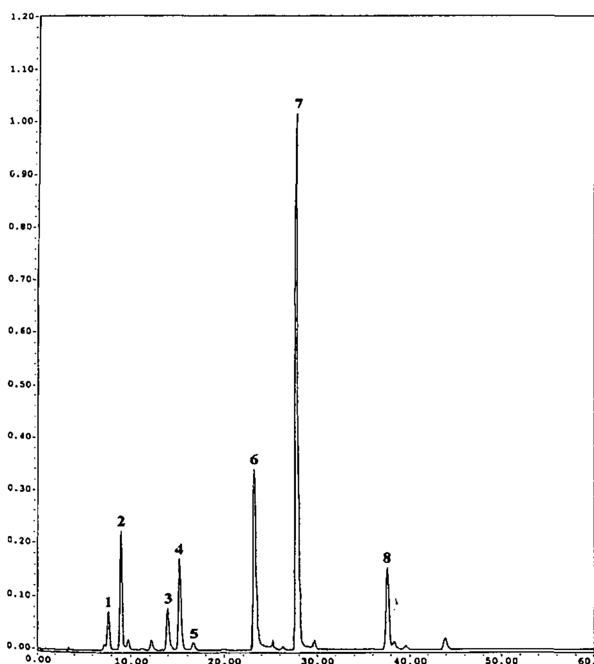
Errors in the quantification step

In HPLC, concentrations of the analytes are determined by comparison with standard solutions of known concentrations. Thus, any error in the preparation and quantification of the standard solutions themselves will be directly reflected in the quantitative data obtained.

Quantification of carotenoids is made difficult by the widely varying purity of commercial standards (34, 35), very limited number of carotenoid standards available commercially

and instability of carotenoids. The purity of carotenoid standards should always be verified and impure standards repurified. Instead of repurifying, Hart and Scott (1) assessed the "purity" of the carotenoid by HPLC, and expressed it as the peak area of the carotenoid as a percentage of the total area of the chromatogram. The concentration of the carotenoid standard calculated from the absorbance reading was corrected accordingly. Carotenoids not available commercially, can be isolated from natural sources, but this is an operation that requires skill, experience and care. Although several authors claim stability of carotenoid stock solutions at -18°C under N_2 for an extended period, it is our experience as well as of others (36) that carotenoid standard solutions can only be used over a very short period.

FIGURE 7
HPLC chromatogram of lettuce. Conditions - Column: Spherisorb ODS 3 µm, 4.6x150 mm. Mobile phase: acetonitrile:methanol:ethyl acetate, convex gradient of 95:5 to 60:20:20 in 20 min. Flow rate: 0.50 mL/min



Peak identification: 1- neoxanthin, 2- violaxanthin, 3- lactucaxanthin, 4- lutein, 5- zeaxanthin, 6 and 7- chlorophylls, 8- ß-carotene.

The standard curve should be linear, pass through or very near the origin and must bracket the concentrations of the food samples. To fulfill the third requirement, the analyst will have to work on vastly different ranges since the carotenoid concentrations of a given food vary over a very wide range.

Khachick et al. (37) cited the following parameters to evaluate 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, (c) the relative standard deviation of the regression should be less than 5%. If any of these parameters is out of range, the standard as well as the HPLC instrumentation should be carefully checked and the standard curve rerun. Mantoura et al. (36) recommended a coefficient of correlation greater than 0.95.

Finally, some calculation errors must be involved since, ocasionally, for a certain foodstuff, a laboratory would come up with a value about 10 times those reported by the other laboratories.

In order to limit analytical variability, in the European interlaboratory studies (17), the following measures were taken by the participating laboratories: (a) the spectrometers were calibrated; (b) the same absorption coefficients and absorption maxima were used; (c) a sample extract was circulated for analysis, using circulated and in-house standards, to verify differences in standards; (d) a common data handling approach was used , including the use of peak area instead of peak height.

In closing, it can be said that HPLC is truly a potentially powerful technique. However, it is very easy to make mistakes with this technique and because the results are precise, lack of accuracy easily passes unnoticed. The analyst should guard against undue confidence that modern instrumentation can inadvertently give.

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