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FACULDADE DE ENGENHARIA DE ALIMENTOS

APRIMORAMENTO DA METODOLOGIA PARA DETERMINAÇÃO DE CAROTENÓIDES EM ALIMENTOS

PARECER

Este exemplar corresponde à redação final da tese defendida por Mieko Kimura, aprovada pela Comissão Julgadora em 22 de março de 2000.

Campinas, 22 de março de 2000.

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À minha mãe Fujiko (in memorian)

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orientação firme na adolescência, apoio constante na
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ÍNDICE

INTRODUÇÃO GERAL

Pelo seu papel como corante natural e suas funções/ações biológicas como atividade pró-vitamínica A, fortalecimento do sistema imunológico, prevenção de doenças degenerativas como câncer, doenças cardiovasculares e degeneração macular, os carotenóides tem sido considerados entre os constituintes mais importantes dos alimentos. A necessidade de se obter dados confiáveis sobre sua composição é reconhecida em várias áreas da ciência e tecnologia. No entanto, a determinação de carotenóides é uma análise inerentemente complexa devido a existência de um grande número de carotenóides conhecidos de ocorrência natural, as composições de carotenóides nos alimentos diferirem quali e quantitativamente, as concentrações dos carotenóides variarem sobre uma faixa muito grande em um alimento e as moléculas altamente insaturadas dos carotenóides serem susceptíveis à isomerização e oxidação.

A introdução da CLAE (cromatografia líquida de alta eficiência) propiciou grandes avanços na análise de carotenóides, porém, dados conflitantes de um mesmo alimento podem ser ainda encontrados na literatura.

A quantificação por CLAE é um procedimento complexo devido ao fato dos carotenóides absorverem em diferentes comprimentos de onda e apresentarem diferentes coeficientes de absorção. Há necessidade constante de padrões, que devem ser obtidos por isolamento e purificação de fontes naturais, já que apenas dois são disponíveis comercialmente.

Portanto, o aprimoramento da metodologia para determinação da composição de carotenóides continua prioritário e é o grande objetivo de muitos pesquisadores no mundo

inteiro. A exatidão dos métodos analíticos deve ser tal, que variações analíticas não sejam confundidas com variações naturais entre amostras, como pode ser observado em vários trabalhos publicados.

O presente trabalho teve como objetivos: (a) apontar as fontes de erros na análise de carotenóides; (b) estabelecer uma estratégia para a análise quantitativa dos carotenóides por CLAE, utilizando verduras folhosas como amostra; (c) verificar a ocorrência de γ-caroteno em tomate e extrato concentrado de tomate; e (d) comparar a composição de carotenóides em verduras folhosas hidropônicas e nas produzidas convencionalmente.

RESUMO GERAL

Devido à grande importância que os carotenóides representam em várias áreas do conhecimento, é consenso mundial que uma maior disponibilidade de dados confiáveis sobre a sua composição em alimentos é necessária. No entanto, a determinação de carotenóides não é uma tarefa simples e apesar dos avanços na instrumentação analítica, discrepâncias nos resultados quantitativos podem ser encontrados na literatura internacional. Assim, o aprimoramento da metodologia para determinação de carotenóides continua prioritário e é o grande objetivo de muitos pesquisadores no mundo inteiro.

Atualmente, a cromatografia líquida de alta eficiência (CLAE) é a técnica preferida para a determinação de carotenóides devido ao menor tempo envolvido na análise, reutilização da coluna, menor exposição à luz e oxigênio e melhor capacidade de separação, principalmente dos componentes minoritários. Entretanto, esta técnica está sujeita a varias fontes de erros como: incompatibilidade entre solvente de injeção e fase móvel, identificação equivocada, indisponibilidade, impureza e instabilidade dos padrões de carotenóides, quantificação de picos altamente sobrepostos, baixa recuperação da coluna cromatográfica, erros na preparação das soluções padrões e procedimento de calibração e erros de cálculo. Ilustrações dos possíveis erros na quantificação de carotenóides por CLAE são apresentados e discutidos no capítulo 1.

Para se obter uma identificação e quantificação correta é imprescindível que uma boa resolução dos componentes durante a separação cromatográfica seja alcançada. A cromatografia clássica foi considerada por muito tempo, mais arte que ciência, uma vez que o seu êxito dependia largamente da habilidade e experiência do analista. A introdução da

CLAE e os avanços na fabricação de colunas tornaram a cromatografia moderna mais ciência, mas que ainda depende da habilidade do analista para escolher adequadamente a combinação coluna e fase móvel. Os vários parâmetros que devem ser considerados para se chegar às combinações apropriadas são discutidos no capítulo 2. As melhores condições dependem do objetivo da análise, isto é, determinação da composição total ou apenas dos carotenóides principais, e da composição de cada tipo de amostra. Ilustrações de várias combinações para quatro tipos de amostras são apresentadas.

Tendo em vista que a principal limitação para aplicação da CLAE para determinação da composição quantitativa de carotenóides é a indisponibilidade comercial, impureza e manutenção de padrões, um esquema para isolamento de padrões por cromatografia em coluna aberta (CCA) e quantificação por CLAE foi desenvolvido (capítulo 3). A pureza obtida foi de 91-97% para neoxantina, 95-98% para violaxantina, 97-100% para lactucaxantina, 92-96% para luteína e 90-97% para β-caroteno. A quantificação de cada cada componente pode ser feita por recalibração por 1 ponto, pela equação da reta da curva padrão e fator de resposta relativo a um dos carotenóides. A comparação entre os procedimentos, utilizando vegetais folhosos como amostras, mostrou um coeficiente de variação de 1,6 a 4,0%, bem menor que a variação natural entre as amostras (6,1-42,5%). Embora os fatores de resposta relativo ao β-caroteno possam ser utilizados para quantificar as xantofilas, não é recomendável utilizar luteína como padrão de referência para β-caroteno. A quantificação pela equação da reta mostrou ser adequada, ao menos pelo período de um mês após a construção das curvas padrão.

Apesar do tomate e seus produtos terem sido bem estudados em termos de carotenóides, existem ainda discrepâncias entre dados reportados. Por exemplo, altos teores

de γ-caroteno foram encontrados em produtos de tomate dos Estados Unidos, ao passo que este pigmento não foi detectado em produtos brasileiros. Portanto, no capítulo 4, a composição qualitativa de carotenóides de tomate "in natura" e processado foi estudada verificando a ocorrência de γ-caroteno em extratos de tomate concentrados brasileiros e americanos. No tomate "in natura" foram identificados 11 carotenóides: *trans*-licopeno, fitoeno, fitoflueno, β-caroteno, luteína, dois *cis*-licopenos, γ-caroteno, *trans*-ζ-caroteno, *cis*-ζ-caroteno e neurosporeno. No extrato concentrado de tomate foram detectados além desses carotenóides, *cis*-β-caroteno e quatro outros carotenóides não identificados. γ-Caroteno foi encontrado em concentrações comparáveis nos extratos brasileiros e americanos e aparentemente abaixo do nível de detecção da CCA. Diferenças causadas pela remoção da casca e estágio de maturação do tomate "in natura" não foram suficientes para explicar a perda de γ-caroteno nos produtos processados, indicando que ocorreu degradação durante o processamento. De qualquer forma, os resultados não apoiam os níveis apreciávias de γ-caroteno encontrados nos Estados Unidos.

Como exemplo de variações naturais, a composição de carotenóides de vegetais folhosos hidropônicos mais comercializados foi determinada por HPLC. A lactucaxantina foi quantificada pela primeira vez em quatro tipos de alface. A luteína foi o carotenóide predominante do agrião, rúcula e almeirão (75,4±10,2 μg/g; 57,0±10,3 μg/g; 52,2±12,6 μg/g, respectivamente). Nas alfaces, β-caroteno e luteína foram os carotenóides principais (9,9±1,5 – 24,6±3,1 μg/g e 10,2±1,0 – 22,9±2,6 μg/g, respectivamente). Alfaces produzidas por hidroponia apresentaram teores menores de todos os carotenóides, com exceção da lactucaxantina, em comparação aos produzidos convencionalmente.

CAPÍTULO 1

SOURCES OF ERRORS IN THE QUANTITATIVE ANALYSIS OF FOOD CAROTENOIDS BY HPLC

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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 solution and in the calibration procedure; calculation errors. Illustrations of the possible errors in the quantification of carotenoids by HPLC are presented.

Keywords: carotenoids, quantitative analysis, HPLC, analytical errors.

RESUMEN. Las fuentes de errores en el análisis cuantitativo de carotenoides en alimentos por HPLC. Varios factores tornam inherentemente difícil la determinación de carotenoides. Entonces, 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 garanta 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: inconpatibilidad del solvente de inyección y la fase móvil, resultando em picos distorcidos 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.

TABLE 1

Data on principal carotenoids (μg/g) of tomato

Reference, chromatographic technique	Cultivar	β-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	β-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±0.4
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

TABLE 3

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

Carotenoid	Chen & Chen(11) Taiwan, water convolvulus	Hulshof *et al.*(12) Indonesia, water spinach	Tee & Lim(4) Malaysia, swamp cabbage	Wills & Rangga(13) Australia, water spinach
β-Carotene	100±8	27±10	19	4
Cis-β-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.

Lycopene and β-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 β-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.

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.

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.

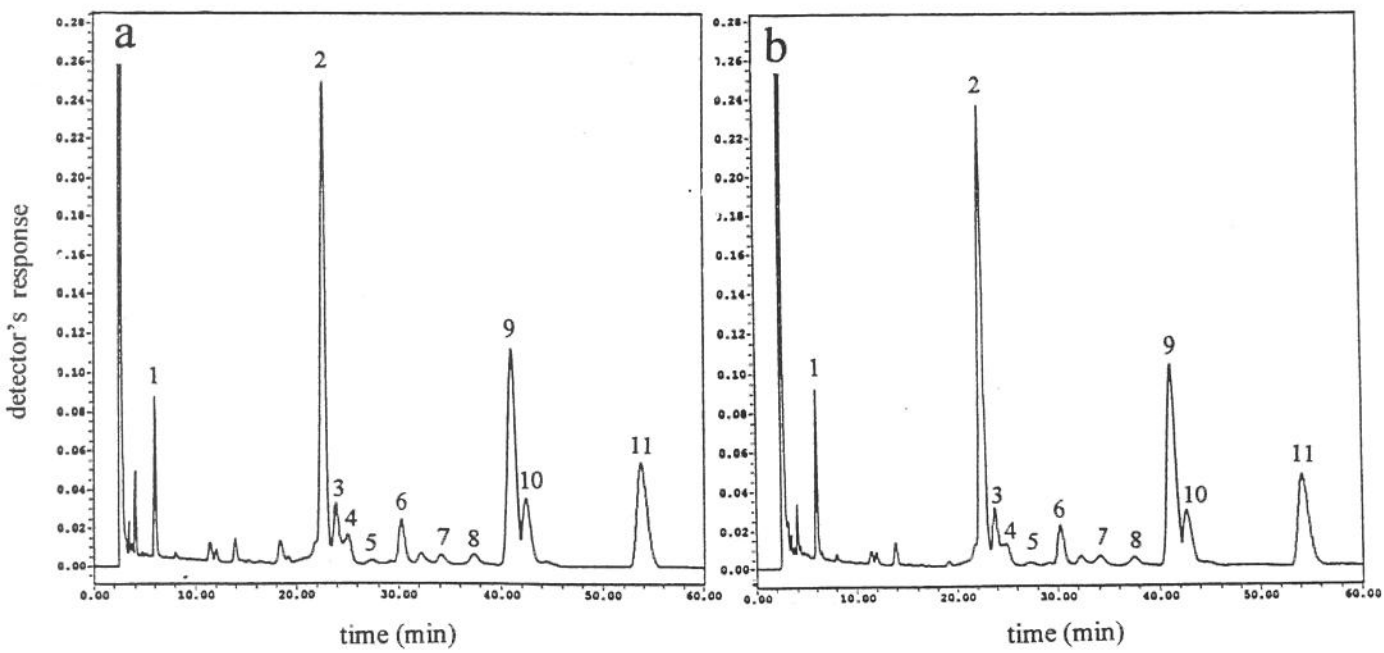
The addition of $MgCO_3$ and other neutralizing agent is often done to neutralize the acids liberated from the sample during tissue disintegration to prevent isomerization and degradation. In our laboratory, keeping the time lag between sample maceration and extraction as short as possible, not only 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 β-carotene being detected in unneutralized tomato with the Vydac column which is capable of separating these geometric isomers.

FIGURE 1

HPLC chromatograms of tomato extracts obtained with (a) and without (b) the use of $MgCO_3$. Conditions: Waters Spherisorb S5 ODS2 column, 5μm, 2.0 x 250 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-β-carotene, 10-phytofluene, 11-phytoene.



Possible losses during saponification have received more attention. This step 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, β-carotene, γ-carotene and β-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 (butylated hydroxytoluene) 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).

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:dichloromethane, 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: 1-5,6 monoepoxy-β-cryptoxanthin, 2-β-cryptoxanthin, 3- lycopene, 4-β-carotene, 5-esters.

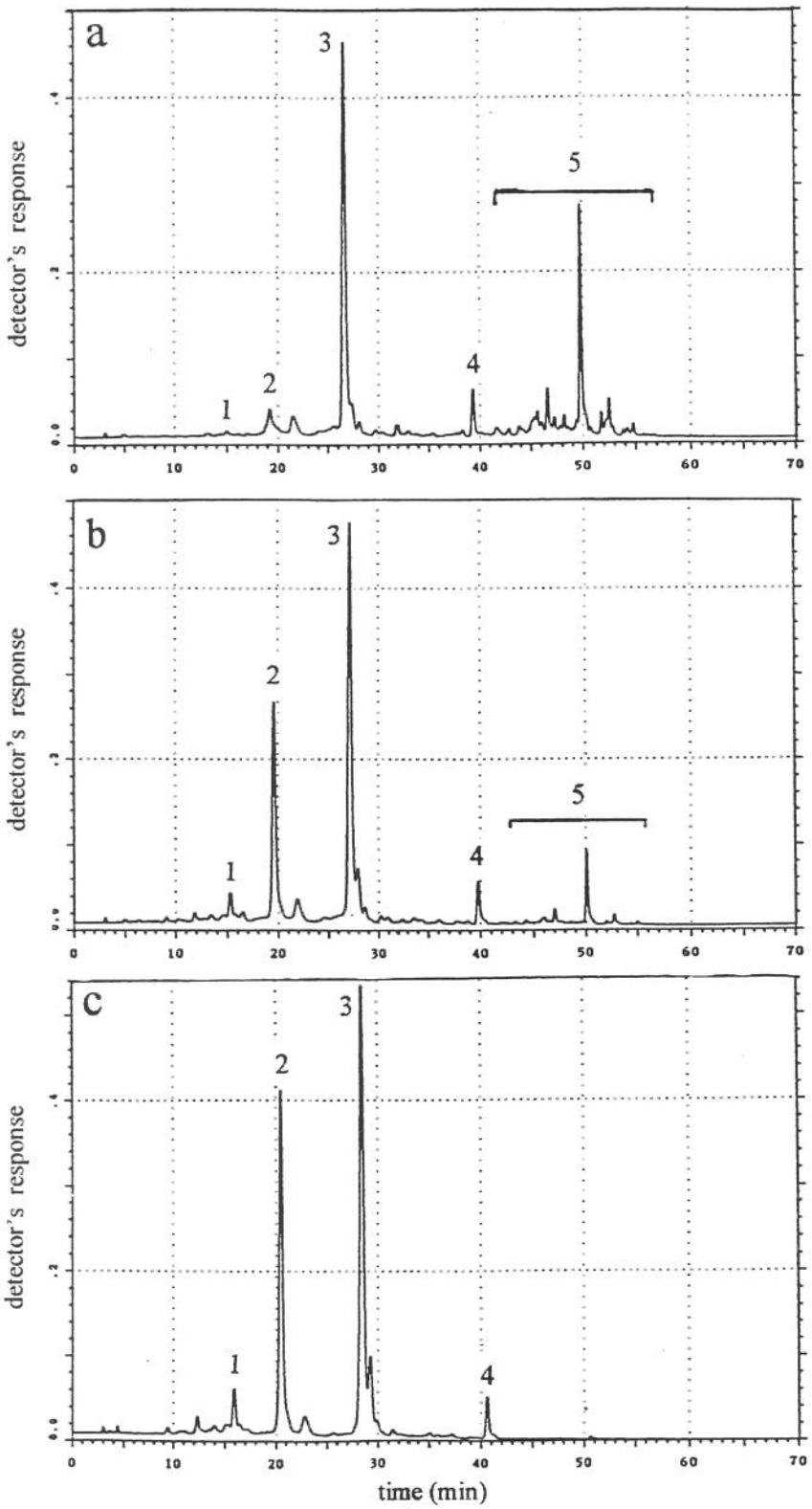
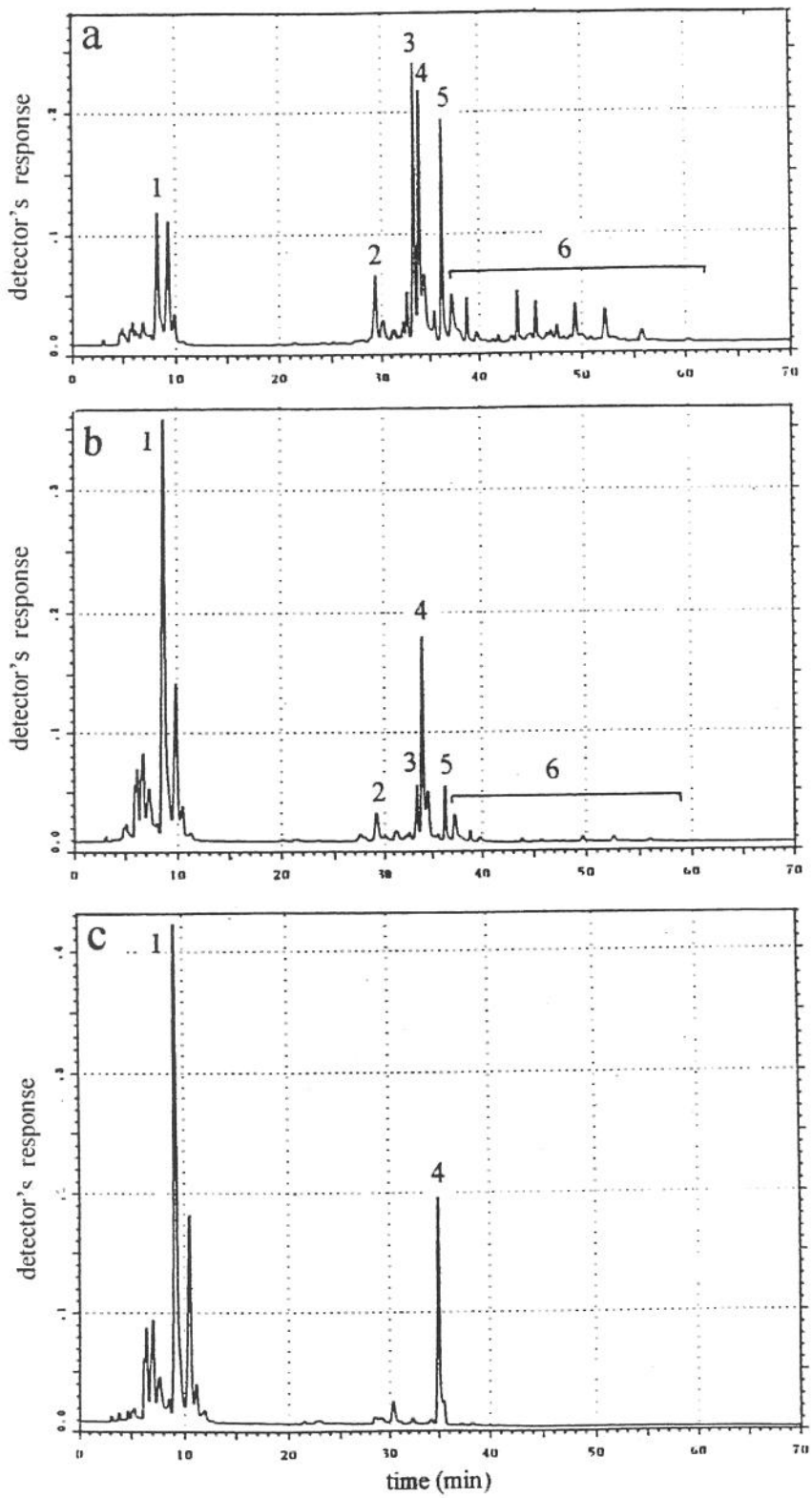


FIGURE 3

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:dichloromethane, 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-β-carotene, 2,3,5 and 6-esters.



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 solution 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

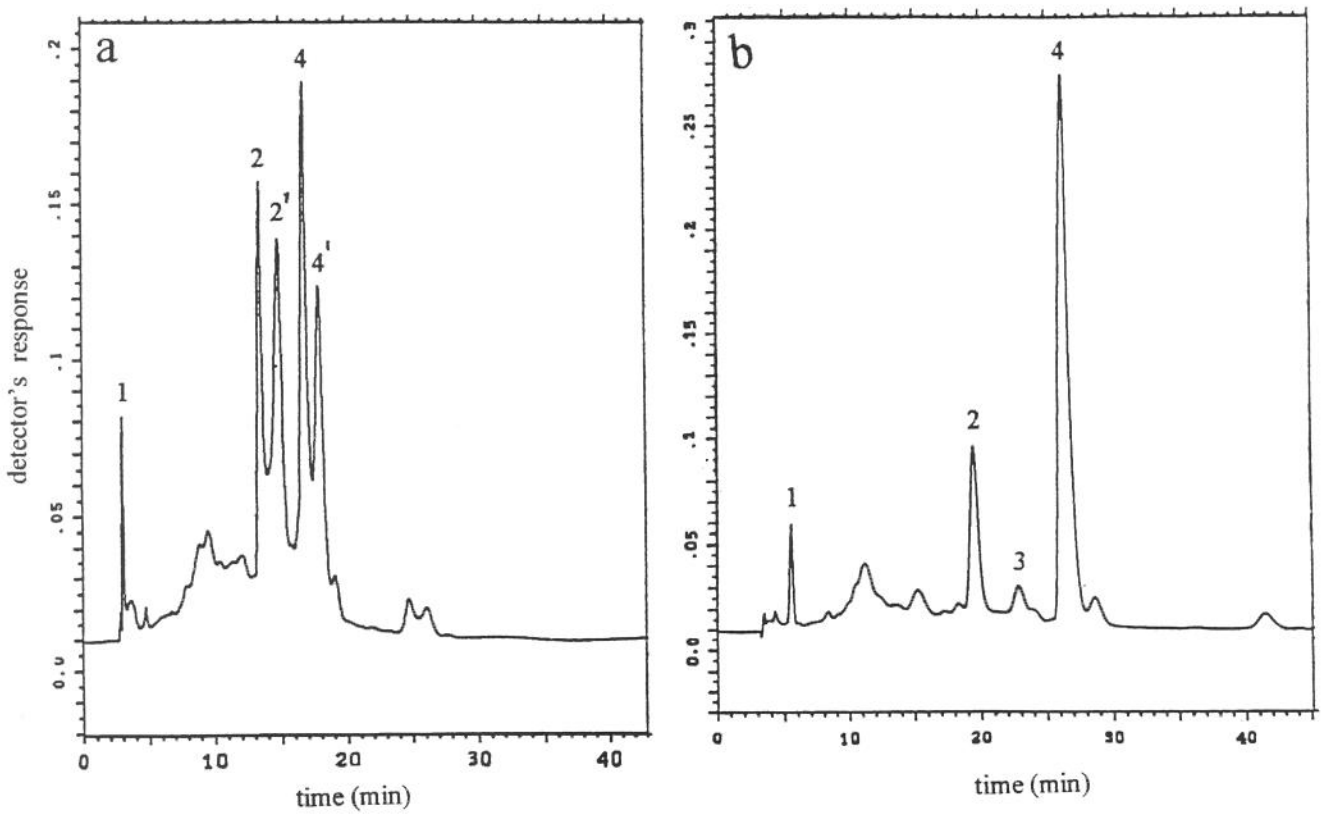
broadening and double or tailing peaks, especially when the extract is concentrated (24). On the other hand, a weak injection solvent will not dissolve the carotenoids completely.

Khachik *et al.* (25) reported peak splitting when carotenoids were injected in dichloromethane, chloroform, tetrahydrofuran, benzene or toluene with a monomeric C18 column and a mobile phase consisting of a mixture of acetonitrile, methanol, dichloromethane 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'-β-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, chloroform, 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 β- 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 β-carotene, both having two β-rings, were greater than those of lutein and α-carotene, both containing one β- and one ε-ring. Within the group of β,β-carotenoids, recovery increased as polarity decreased. Recovery increased in the following order: zeaxanthin (dihydroxy) < β-cryptoxanthin (monohydroxy) < echinenone (monoketo) < β-carotene. For the two β,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 β-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) extracts. 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-β-carotene, 3-γ-carotene in raw tomato and mixture in tomato paste, 4-lycopene.

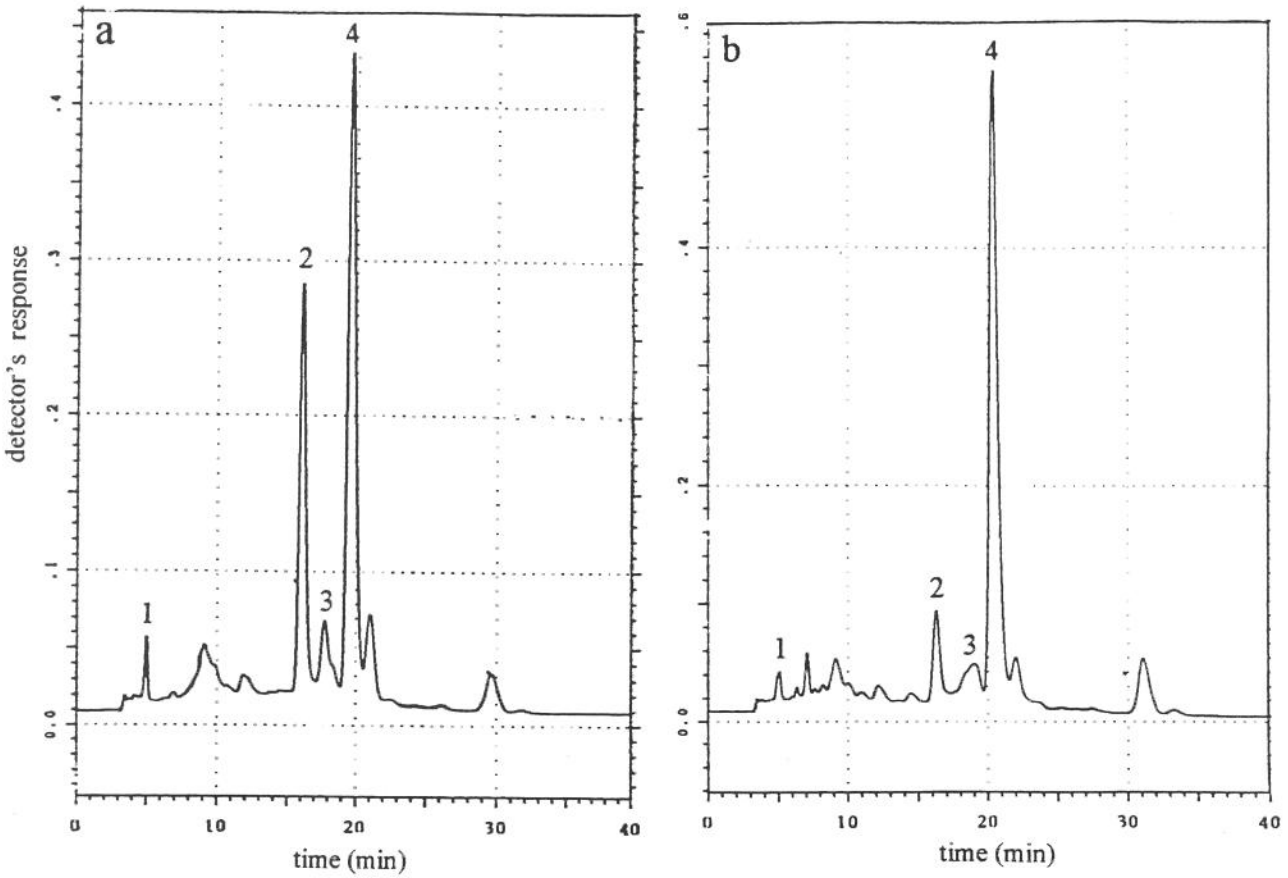
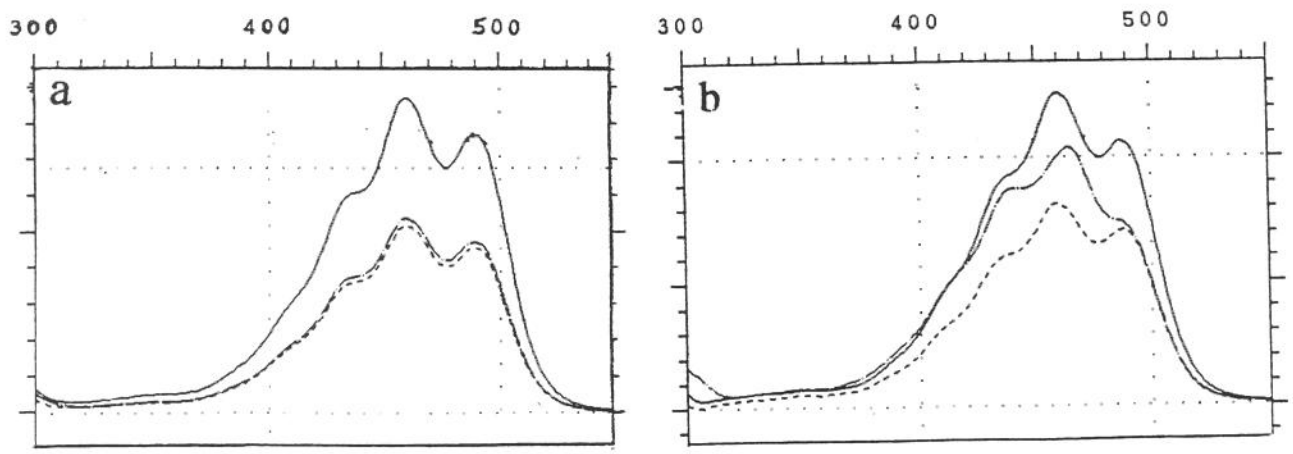


FIGURE 6

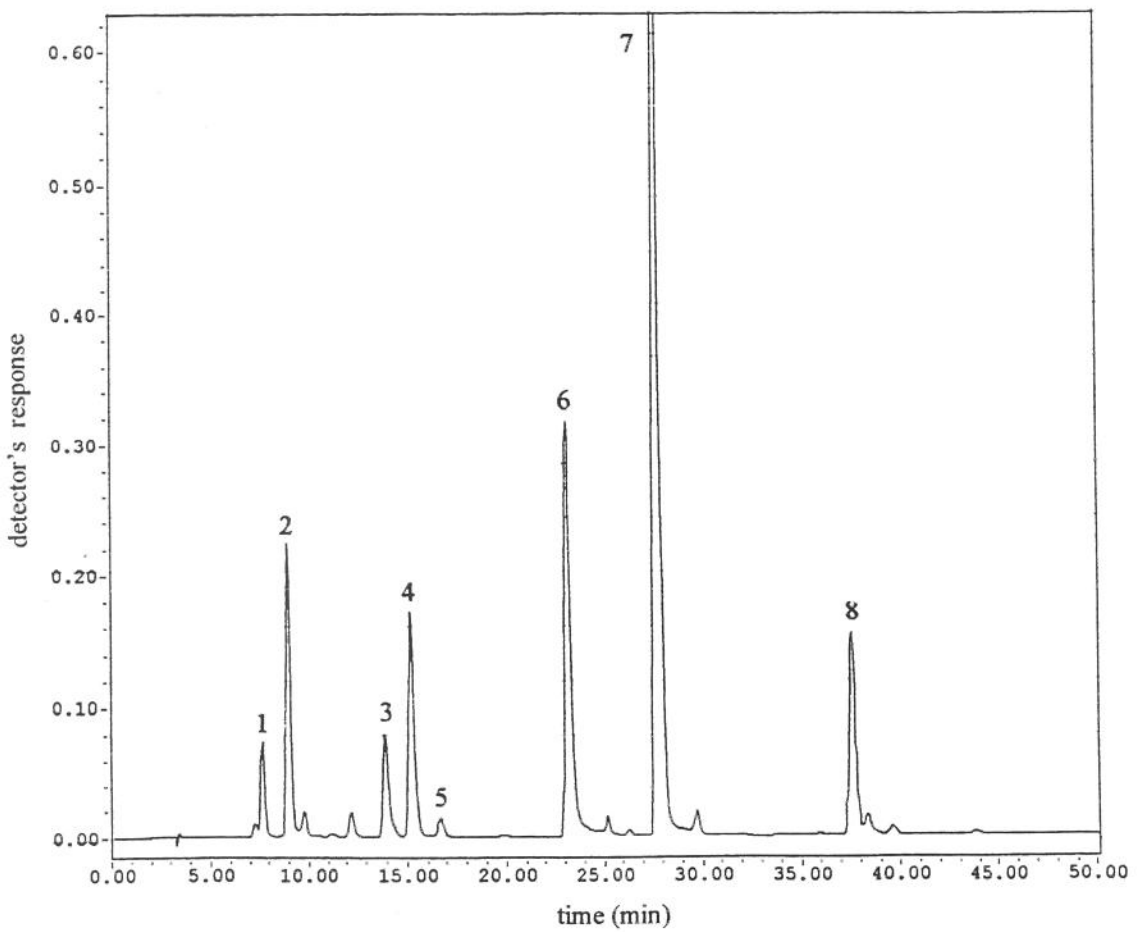
Absorption spectra corresponding to peak 3 of Figure 5 obtained with the photodiode array detector of raw tomato (a) and tomato paste (b) at maximum (——), upslope (-----) and downslope (-.-.-.-.).



Unlike fruits and roots, leaves have been known to contain the same principal carotenoids: lutein, β-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.

FIGURE 7

HPLC chromatogram of lettuce extract. Conditions: column Spherisorb S3 ODS2, 3 μm, 4.6x150 mm. Mobile phase: acetonitrile:methanol:ethyl acetate, convex gradient of 95:5:0 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, 6,7- chlorophylls, 8-β-carotene.



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.

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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CAPÍTULO 2

ESCOLHA DE COLUNA E FASE MÓVEL PARA A ANÁLISE DE CAROTENÓIDES EM ALIMENTOS POR CLAE: UMA CIÊNCIA OU UMA ARTE?

Artigo a ser enviado ao Boletim da Sociedade Brasileira de Ciência e Tecnologia de Alimentos

ESCOLHA DE COLUNA E FASE MÓVEL PARA A ANÁLISE DE CAROTENÓIDES EM ALIMENTOS POR CLAE: UMA CIÊNCIA OU UMA ARTE?

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RESUMO

Ao longo de muitos anos, a cromatografia clássica foi considerada uma arte, uma vez que o seu êxito dependia largamente da habilidade e experiência do analista, especialmente na separação de misturas complexas como os extratos de carotenóides provenientes de alimentos e outras amostras biológicas. A introdução da cromatografia líquida de alta eficiência (CLAE) renovou a esperança de tornar a resolução de carotenóides uma ciência, com o desempenho cromatográfico determinado por parâmetros bem definidos e passíveis de serem estabelecidos sistematicamente. De fato, com os avanços na tecnologia de fabricação de colunas, proporcionando colunas eficientes, e o aprofundamento dos conhecimentos quanto ao comportamento das fases estacionárias e móveis, separações nunca antes possíveis são agora conseguidas, com várias opções de condições cromatográficas. O uso de detector por conjunto de diodos também ajuda marcantemente na obtenção de dados seguros com maior facilidade. No entanto, pela natureza complexa e variável da composição de carotenóides, exigindo máxima eficiência da instrumentação e competência científica do analista, a cromatografia moderna se tornou mais ciência, mas que necessita, ainda, de um toque de arte.

Palavras-chave: carotenóides, CLAE, colunas cromatográficas, fase móvel

SUMMARY

For a long time, classical chromatography was considered an art because its outcome depended heavily on the analyst's skill and experience, especially for the separation of complex mixtures such as carotenoid extracts from foods and other biological sources. The introduction of high performance liquid chromatography (HPLC) renewed the hope of turning the resolution of carotenoids into a science, with the chromatographic performance determined by well defined parameters, which could be established systematically. In fact, with the advances in column technology, providing highly efficient columns, together with the greater understanding of the properties and behavior of the stationary and the mobile phases, hitherto impossible separations are now achieved, with various options of chromatographic conditions. The use of a photodiode array detector has also markedly enhanced the acquisition of reliable results with greater ease. However, given the complex and variable nature of carotenoid composition, demanding the maximum efficiency of instrumentation and scientific competence of the analyst, modern chromatography has become more of a science, but still needing a touch of art.

Key words: carotenoids, HPLC, chromatographic columns, mobile phase.

1 – INTRODUÇÃO

Pelo seu papel como corante natural e suas funções/ações biológicas (atividade pró-vitamínica A, fortalecimento do sistema imunológico, prevenção de doenças degenerativas como câncer, doenças cardiovasculares e degeneração macular), os carotenóides têm sido considerados entre os constituintes mais importantes dos alimentos. A necessidade de se obter dados confiáveis sobre sua composição é reconhecida em várias áreas da ciência e tecnologia. Porém, a determinação de carotenóides não é uma tarefa simples, devido a vários fatores que tornam esta análise inerentemente difícil (1-3):

a – Existem mais de 600 carotenóides conhecidos de ocorrência natural, e apesar do número de carotenóides encontrados em alimentos ser muito menor, a composição dos mesmos pode ser ainda muito complexa.

b – As composições de carotenóides nos alimentos variam quali e quantitativamente. Assim, o procedimento analítico, principalmente a etapa cromatográfica precisa ser adaptada à composição de carotenóides de cada tipo de amostra. A possibilidade de uma identificação equivocada é muito grande, e de fato, identificações inconclusivas e incorretas ainda são encontradas na literatura.

c – As concentrações dos carotenóides em um alimento variam sobre uma faixa muito grande. Geralmente, um a quatro carotenóides principais estão presentes com uma série de carotenóides em níveis baixos e até em traços. A separação, identificação e quantificação desses carotenóides minoritários representam um grande desafio para os analistas. As curvas de calibração são construídas em faixas muito diferentes.

d – A molécula altamente insaturada é susceptível à isomerização e oxidação, reações que podem facilmente ocorrer durante a análise.

Assim, o aprimoramento da metodologia para determinação da composição de carotenóides continua prioritário e é o grande objetivo de muitos pesquisadores no mundo inteiro. Sendo uma análise difícil, exigindo o melhor desempenho de cromatografia, as lições aprendidas nesta análise podem ser estendidas com ótimo aproveitamento à análise de outros analitos.

As fontes de erros mais comuns na determinação dos carotenóides são: amostra analisada não representativa, preparo inadequado da amostra para análise, extração incompleta, separação cromatográfica incompleta, identificação equivocada, quantificação incorreta e isomerização e degradação dos carotenóides durante a análise.

A análise de carotenóides geralmente consiste de extração, partição ou transferência para um solvente compatível com a etapa cromatográfica subsequente, saponificação (para amostras oleosas ou aquelas que contem ésteres de carotenóides), separação cromatográfica, identificação e quantificação. É evidente que uma boa resolução dos componentes durante a separação cromatográfica é imprescindível para se obter uma identificação e quantificação correta. Assim, o presente artigo focalizará somente esta etapa.

A extensão com que a separação cromatográfica deve ser realizada depende das informações desejadas. Os objetivos dos trabalhos citados na literatura podem ser classificados em determinação apenas dos carotenóides pró-vitamínicos, determinação dos carotenóides principais, tanto pró como não vitamínicos, e determinação da composição completa.

Considerando que a determinação da composição completa é mais complexa, demorada e cara, e a importância tanto dos carotenóides pró como dos não vitamínicos, métodos que determinam corretamente os carotenóides principais parecem ser suficientes

para obtenção de dados para tabelas de composição de alimentos e para pesquisas na área de saúde humana.

Como as amostras alimentícias geralmente contém carotenóides apolares e polares, o processo cromatográfico utilizado deve ser capaz de cobrir toda essa faixa de polaridade.

A cromatografia em coluna clássica, também chamada de cromatografia em coluna aberta (CCA), é uma boa técnica para determinação quantitativa e também muito útil para separação e purificação de carotenóides para serem utilizados como padrões nos métodos por CLAE. Os métodos por CCA permitem determinar a composição dos carotenóides principais de forma correta e comparável à obtida por CLAE (4-6).

A maior vantagem da CCA é a simplicidade e baixo custo, mas a reprodutibilidade e eficiência de separação depende da habilidade, paciência e experiência do analista, principalmente no empacotamento da coluna e ajuste dos volumes e proporções dos solventes de eluição, assim como na visualização das bandas. Esta alta dependência da competência do analista fez com que a cromatografia fosse considerada mais arte que ciência.

A CLAE é uma técnica com maior poder de resolução, mais reprodutível com coluna reutilizável e realizada sob condições controladas, sem exposição indevida ao ar e à luz, além de possibilitar a análise de um grande número de amostras em menor tempo. Atualmente, é considerada a técnica ideal para a análise de carotenóides.

2 - COLUNAS PARA CLAE

A CLAE com coluna de fase reversa C_{18} tem sido a modalidade preferida dos pesquisadores. As razões para a popularidade das colunas C_{18} são: sua fraca interação com

os analitos, minimizando a degradação de analitos sensíveis, compatibilidade com a maioria dos solventes apropriados aos carotenóides e a larga faixa de polaridade dos carotenóides, e grande disponibilidade comercial. As propriedades e qualidade de um mesmo tipo de coluna, porém, variam consideravelmente entre marcas, entre lotes e mesmo dentro do mesmo lote (7). Assim, alguns ajustes são muitas vezes necessários quando métodos citados na literatura são utilizados. Características como tamanho e forma das partículas, diâmetro dos poros, porcentagem de carbono (grau de cobertura), grau de desativação por reações de capeamento dos grupos silanóis ("end-capping") e natureza da camada de C_{18} (monomérica ou polimérica) influenciam na separação cromatográfica.

A maioria das separações de carotenóides tem sido realizada em colunas de 4,6 x 250 mm, empacotadas com partículas esféricas de C_{18} de 5μm, mas hoje já existem no mercado colunas com outras características como comprimento maior (390 mm), diâmetro menor (2mm), partículas menores (3μm) e fase estacionária C_{30}, as quais podem proporcionar maior eficiência de separação.

A coluna monomérica, na qual uma monocamada de C_{18} é ligada à superfície de sílica usando monoclorosilanos, é mais barata, reprodutível e simples de utilizar. A coluna polimérica, na qual uma camada polimérica de C_{18} é ligada à superfície de sílica usando triclorosilanos na presença de quantidades limitadas de água, oferece maior seletividade a carotenóides estruturalmente similares, como os isômeros geométricos de carotenóides (8). No entanto, apresenta maior variação entre os lotes e pode provocar alargamento de bandas, dificultando a fiel reprodução dos resultados encontrados na literatura (9).

Numa avaliação de 60 colunas comerciais e cinco colunas experimentais de CLAE, Epler *et al.* (10) mostraram que colunas monoméricas geralmente proporcionam maiores

recuperações dos carotenóides, (usando metanol ou fase móvel baseado em metanol), mas não foram capazes de separar luteína e zeaxantina, uma separação conseguida em coluna polimérica.

Uma boa separação, tanto dos carotenóides polares (xantofilas) e apolares (carotenos), bem como de seus isômeros, foi conseguida em coluna polimérica C_{30}, 5 μm, não capeada, desenvolvida por Sander *et al.* (11) . O fator limitante para o seu uso na elaboração de tabelas de composição, que envolvem um grande número de análises, é o alto custo do modificador (metil-tert-butil éter) da fase móvel.

3 - FASE MÓVEL

Na escolha da fase móvel, as propriedades mais importantes a serem consideradas são a polaridade, viscosidade, volatilidade e toxicidade.

As fases móveis mais utilizadas geralmente contêm metanol ou acetonitrila como solvente básico com modificadores como acetato de etila, diclorometano ou tetrahidrofurano em diferentes proporções para eluição isocrática ou por gradiente.

O metanol é mais barato, menos tóxico e apresenta alta taxa de recuperação dos carotenóides na maioria das colunas. Já a acetonitrila proporciona melhor separação dos carotenóides polares (xantofilas) e por ser menos viscoso, faz com que a pressão do sistema seja mais baixa, aumentando a vida útil da bomba. No entanto, é mais tóxico, mais caro e apresenta menor taxa de recuperação dos carotenóides. A recuperação pode ser melhorada adicionando-se trietilamina (TEA) e acetato de amônia (12,13).

A adição de modificadores como diclorometano, acetato de etila e tetrahidrofurano à fase móvel tem por objetivo encontrar a retenção desejada, aumentar a solubilidade e alterar

a seletividade da fase móvel (8). A eficiência de cada um depende dos carotenóides a serem separados e da fase estacionária utilizada. Diclorometano é mais tóxico e deve ser usado com cautela, pois, pode conter traços de HCl. Tetraidrofurano deve ser testado antes de ser utilizado, pois pode acumular peróxidos e a adição de antioxidantes como o BHT (butilato hidroxitolueno) é aconselhável.

Dentre nove modificadores (acetonitrila, tetrahidrofurano, diclorometano, acetato de etila, clorofórmio, acetona, éter etílico, tolueno e hexano), Craft, Wise e Soares Jr. (9) observaram que o tetraidrofurano foi o mais eficiente quanto à seletividade e recuperação de sete padrões de carotenóides (luteína, zeaxantina, β-criptoxantina, equinenona, α-caroteno, β-caroteno e licopeno), utilizando coluna C_{18} polimérica e metanol como base da fase móvel.

A eluição isocrática pode ser realizada usando uma única bomba de alta pressão e solventes pré-misturados, que resultam numa linha de base mais estável, tempos de retenção mais reprodutíveis e menor quantidade de resíduo de solvente, uma vez que o processo cromatográfico pode ser realizado a fluxos baixos. A eluição por gradiente permite a separação de uma faixa mais ampla de analitos, melhora a seletividade, e permite a eluição de compostos altamente retidos. No entanto, necessita de equipamento mais sofisticado e tempo de reequilíbrio da coluna após cada corrida. Pode produzir maior variabilidade nos tempos de retenção e consequentemente erros na identificação e quantificação, e dispende maior quantidade de solvente. Assim, a eluição por gradiente só é aconselhável quando a separação dos carotenóides de interesse não é alcançada por eluição isocrática.

4 - COMBINAÇÕES ADEQUADAS DE COLUNA-FASE MÓVEL

A escolha da combinação coluna-fase móvel é, sem dúvida, o principal fator limitante do sucesso do método cromatográfico. Desde a introdução da CLAE no campo dos carotenóides, numerosos trabalhos foram publicados sobre a separação cromatográfica decorrente desta técnica. Na maioria dos casos, o sucesso foi limitado e existem trabalhos publicados com análises quantitativas baseadas em cromatogramas com picos sobrepostos, que constituem uma das principais causas de resultados discrepantes encontrados na literatura. Ultimamente, com os avanços na tecnologia de fabricação de colunas, aliados à maior informação sobre os parâmetros que levam a uma boa resolução, cromatogramas com picos bem resolvidos podem ser conseguidos, desde que o analista sempre atualize os seus conhecimentos e tenha habilidade de chegar às combinações apropriadas. Várias opções são agora disponíveis para conseguir a mesma separação. Quatro exemplos próprios serão discutidos para ilustrar esta afirmação.

Uma das amostras alimentícias problemáticas são os vegetais folhosos. Embora a composição qualitativa seja mais ou menos constante, com β-caroteno, luteína, violaxantina e neoxantina como carotenóides principais, a separação destes entre si, com os carotenóides minoritários, e com as clorofilas nem sempre é fácil de se conseguir. A coluna Vydac polimérica, 5 μm, 4,6 x 250 mm, tem sido considerada a melhor coluna para este tipo de amostra. De fato, a Figura 1a mostra boa separação entre os carotenóides principais e as clorofilas. No entanto, o cromatograma apresenta uma sobreposição dos picos da luteína e zeaxantina, apesar de Epler *et al.* (10) afirmarem que a separação destes dois carotenóides só é possível em coluna C_{18} polimérica.

Contrariando os resultados citados para colunas C_{18} monoméricas de 5 μm, a Figura 1b revela que uma coluna C_{18} monomérica de 3 μm permite uma ótima separação entre luteína e zeaxantina, além de separar *cis*-violaxantina, que aparece como ombro do pico da *trans*-violaxantina no cromatograma obtido com coluna Vydac.

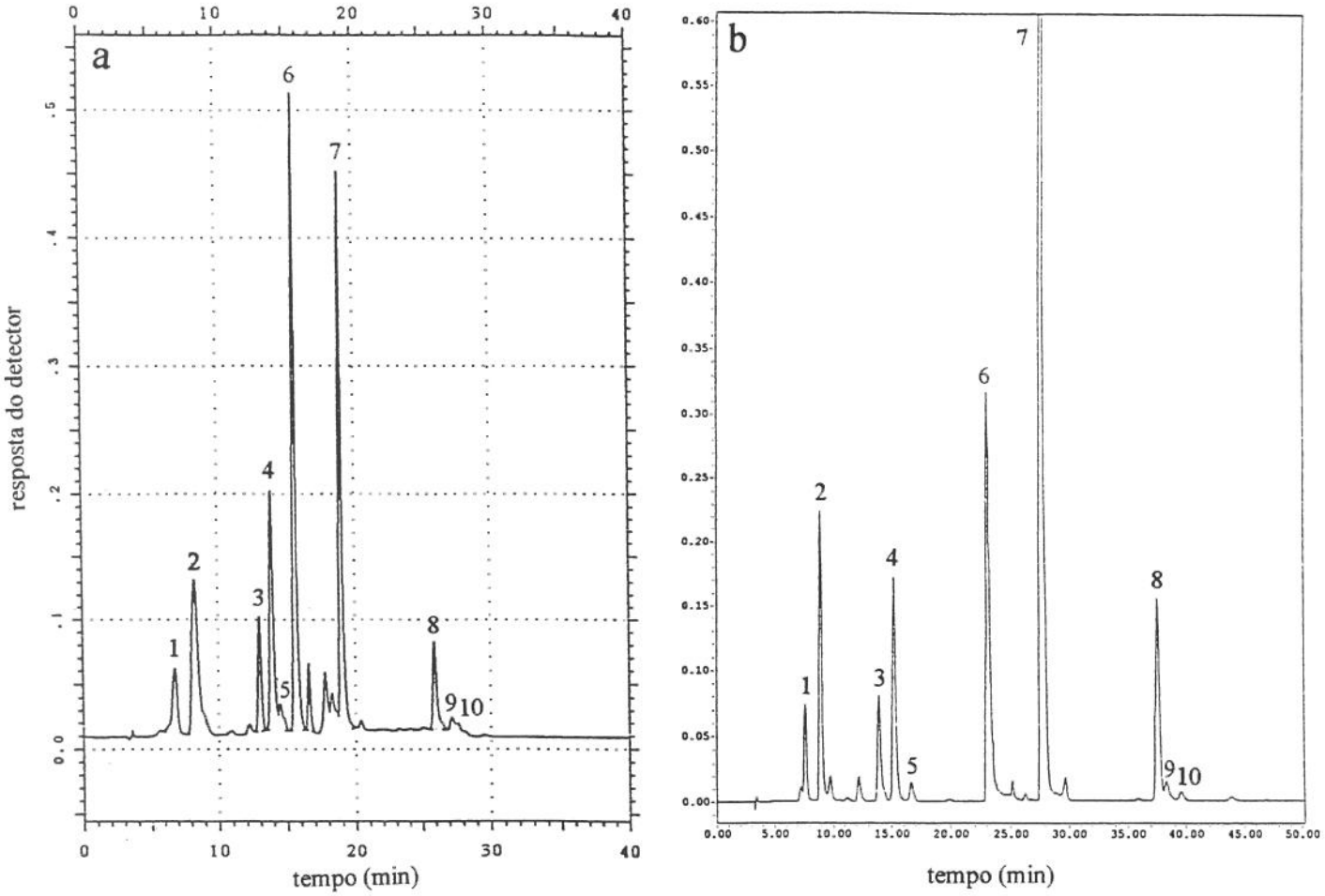

Figura 1. Cromatogramas de extrato de alface crespa obtido por HPLC. 1-neoxantina, 2-violaxantina, 3-lactucaxantina, 4-luteína, 5-zeaxantina, 6,7-clorofilas, 8-β-caroteno, 9,10-*cis*-β-caroteno. (a) Coluna Vydac 218 TP54, 5 μm, 4,6 x 250 mm. Fase móvel: metanol:água:tetrahidrofurano. Gradiente linear de 90:10:0 a 90:0:10 em 20 minutos. Fluxo: 0,8 mL/min. (b) Coluna Waters Spherisorb S3 ODS2, 3 μm, 4,6 x 150 mm. Fase móvel: acetonitrila:metanol:acetato de etila (0,05% trietilamina). Gradiente convexo (curva 10) de 95:5:0 a 60:20:20 em 20 min. Fluxo: 0,5 mL/min.

Tomate é um dos alimentos mais estudados. Geralmente, pela predominância do licopeno, somente este e o β-caroteno são quantificados por CLAE. Raras vezes, a luteína também é quantificada. Para estes três carotenóides, podem ser usadas as colunas Vydac 5µm (4,6 x 250 mm) ou Spherisorb S5 ODS2 narrow bore, 5µm, 2,0 x 250 mm como mostra a Figura 2. No entanto, para quantificar os carotenóides minoritários, a Spherisorb S5 "narrowbore" é melhor, e para separação de licopeno *cis* e *trans*, a Vydac é mais eficiente.

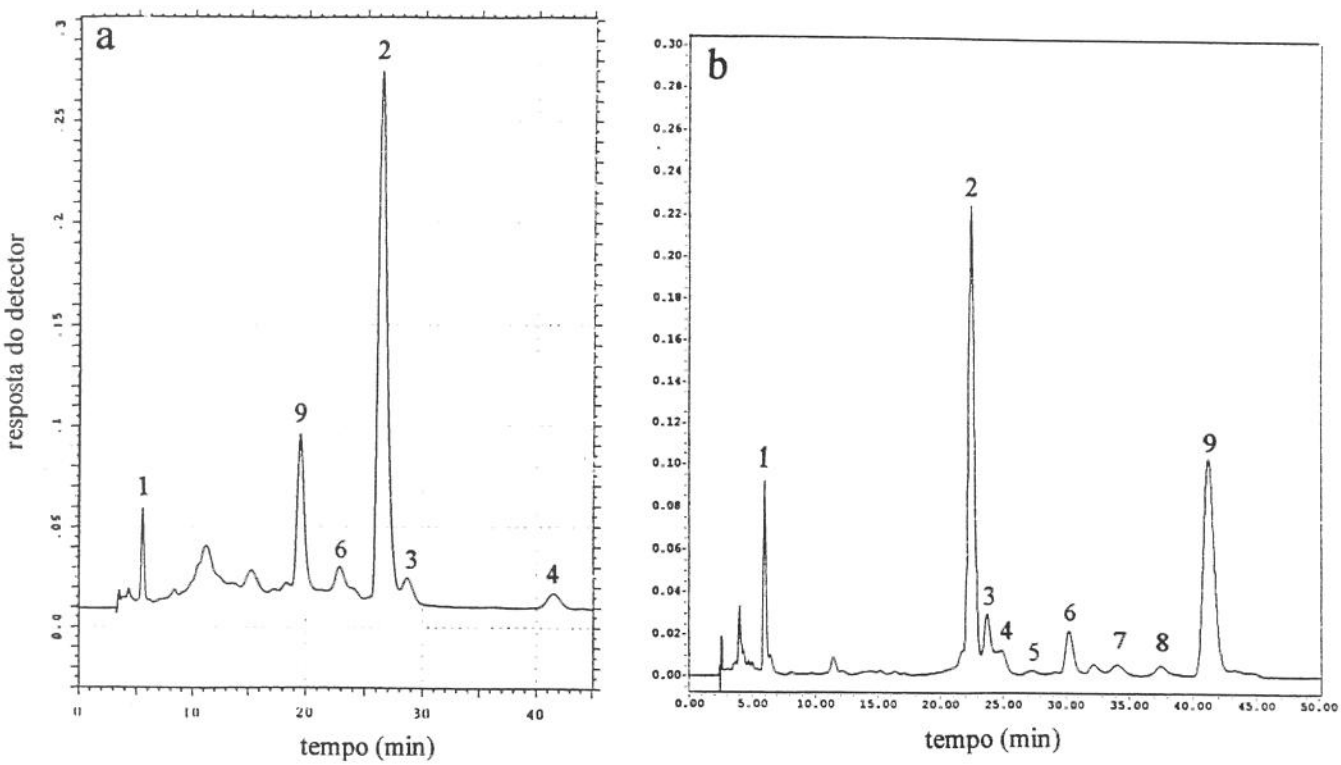

Figura 2. Cromatogramas de extrato de tomate "in natura". 1-luteína, 2-trans-licopeno, 3,4-cis-licopeno, 5-neurosporeno, 6-γ-caroteno, 7-cis-ζ-caroteno, 8-trans-ζ-caroteno, 9-β-caroteno. (a) Coluna Vydac 218 TP54, 5 µm, 4,6 x 250 mm. Fase móvel: metanol:tetraidrofurano 95:5. Fluxo: 0,8 mL/min. (b) Coluna Waters Spherisorb S5 ODS2, 5 µm, 2,0 x 250 mm. Fase móvel: acetonitrila:metanol:acetato de etila 73:20:7. Fluxo: 0,25 mL/min.

A Figura 3 apresenta os cromatogramas de extrato de mamão em coluna Novapak 4μm (3,9 x 350 mm) e Spherisorb S5 ODS2 5μm (2,0 x 250 mm). A separação entre os carotenóides majoritários β-criptoxantina, β-criptoxantina-5,6-monoepóxido, licopeno e β-caroteno são semelhantes nas duas colunas, com resolução ligeiramente melhor dos isômeros *cis* e *trans* do licopeno na coluna "narrowbore". A utilização da coluna "narrowbore" envolve menor gasto de solvente e menor volume de resíduo, já que pode ser operada a fluxos baixos.

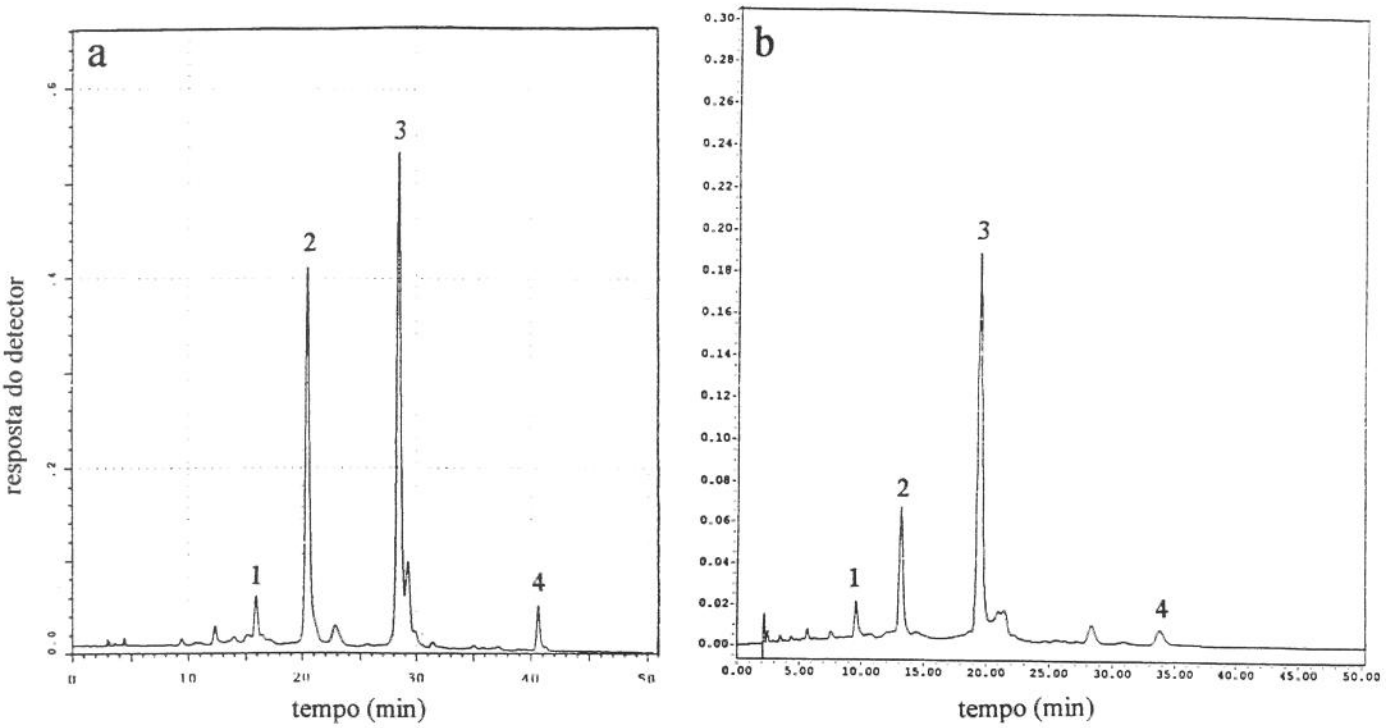

Figura 3. Cromatogramas de extrato de mamão. 1-5,6,monoepóxido-β-criptoxantina, 2-β-criptoxantina , 3-licopeno, 4-β-caroteno. (a) Coluna Novapak 4 μm, 3,9 x 350 mm. Fase móvel: acetonitrila:metanol:diclorometano. Gradiente linear de 80:20:0 a 65:20:15 em 30 min. e a 40:20:40 em 20 min. Fluxo: 0,5 mL/min. (b) Coluna Waters Spherisorb ODS 5 μm, 2,0 x 250 mm. Fase móvel: acetonitrila:metanol:acetato de etila 73:20:7. Fluxo: 0,25 mL/min.

A cenoura possui composição de carotenóides mais simples e os seus três componentes principais, β-caroteno, α-caroteno e luteína podem ser facilmente separados isocraticamente, tanto em coluna polimérica Vydac, 5μm, quanto em coluna monomérica Novapak 4μm (3,9 x 350 mm) ou Spherisorb ODS2, 5μm, "narrowbore" (Figura 4). Apesar

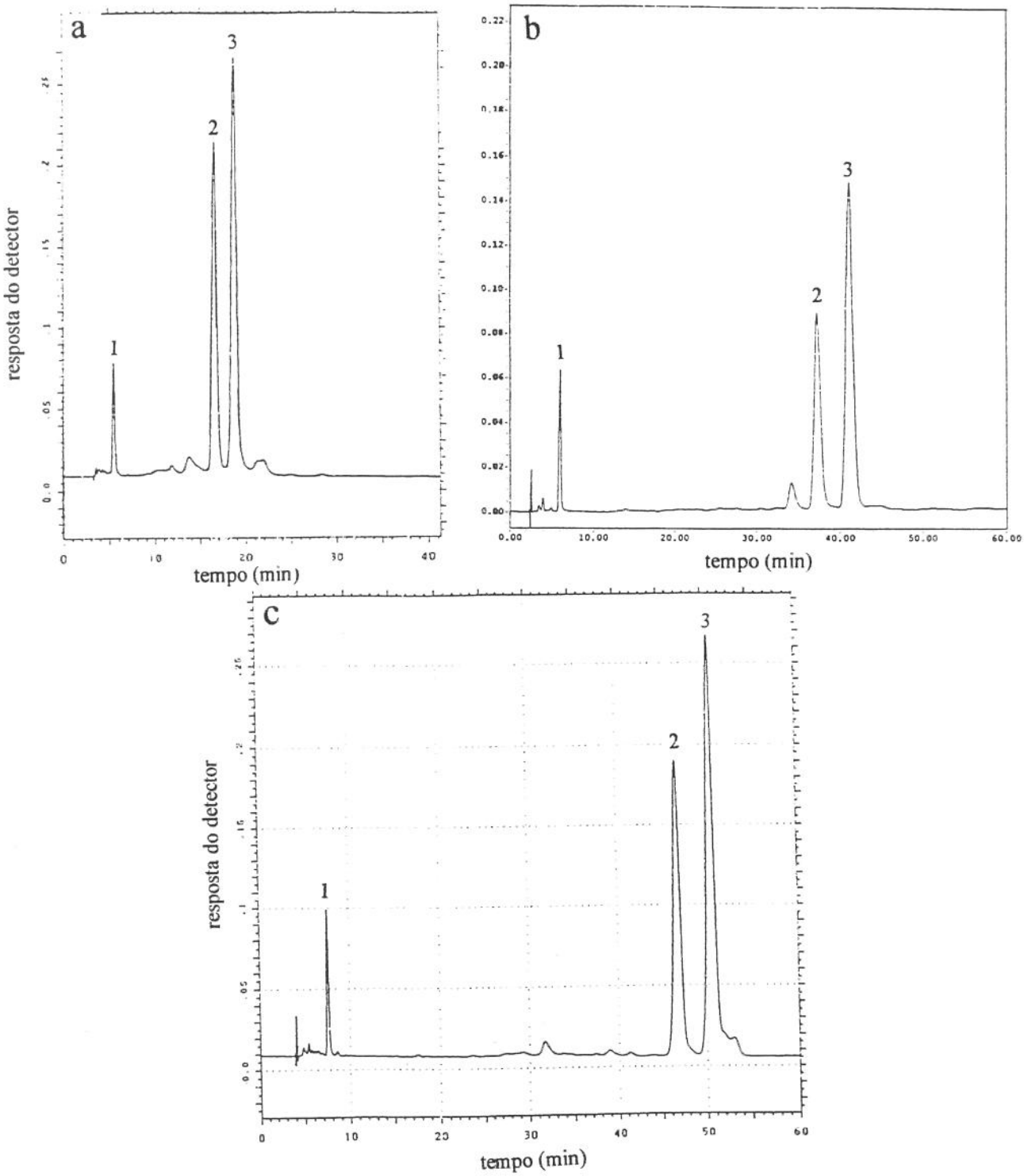

Figura 4. Cromatogramas de extrato de cenoura. 1-luteína, 2-α-caroteno, 3-β-caroteno. (a) Coluna Vydac 218 TP54, 5 μm, 4,6 x 250 mm. Fase móvel: metanol:tetraidrofurano 98:2. Fluxo: 0,8 mL/min. (b) Coluna Waters Spherisorb S5 ODS2 5 μm, 2,0 x 250 mm. Fase móvel: acetonitrila:metanol:acetato de etila 73:20:7. Fluxo: 0,25 mL/min. (c) Coluna Novapak 4 μm, 3,9 x 350 mm. Fase móvel: acetonitrila:metanol:diclorometano 65:20:15. Fluxo: 0,5 mL/min.

da resolução entre o α-caroteno e β-caroteno ser melhor na coluna "narrowbore", a coluna Vydac proporciona boa separação num tempo menor.

5 - USO DO DETECTOR POR CONJUNTO DE DIODOS

Uma das inovações mais úteis em CLAE é o uso do detector por conjunto de diodos, que permite a obtenção de espectros de absorção "on line". Além de auxiliar na identificação, este detector permite também a verificação da pureza dos picos.

Com este detector foi fácil verificar a separação dos isômeros *cis* e *trans* do β-caroteno, licopeno e ζ-caroteno (Figuras 5, 6 e 7), sobreposição de luteina e zeaxantina (Figura 8), além da separação dos carotenóides das clorofilas (Figura 9).

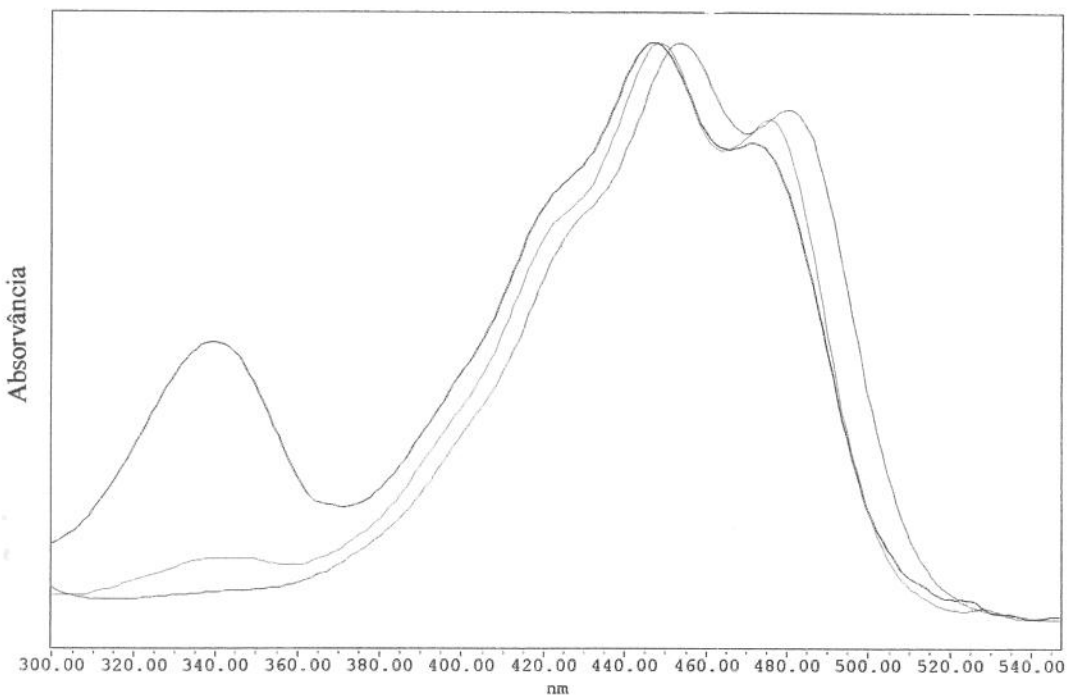

Figura 5. Espectros de absorção dos picos 8-*trans*-β-caroteno (————), 9-*cis*-β-caroteno (————) e 10-*cis*-β-caroteno (————) da figura 1b obtidos pelo detector por conjunto de diodos.

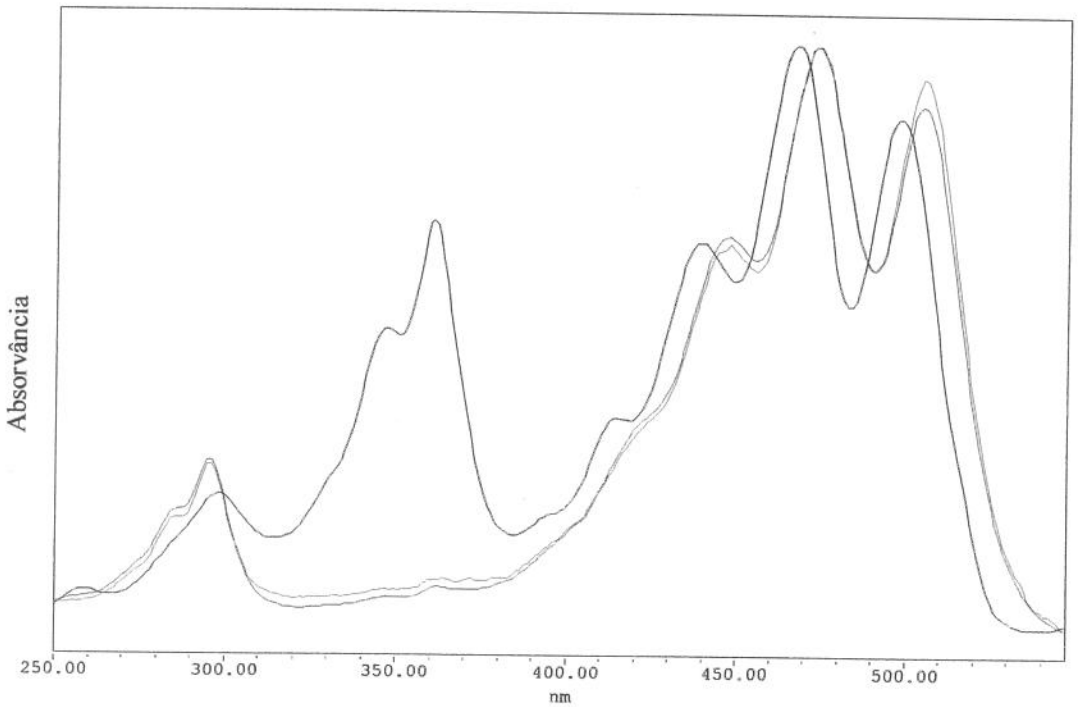

Figura 6. Espectros e absorção dos picos 2-*trans*-licopeno (————), 3-*cis*-licopeno (————) e 4-*cis*-licopeno(————) da figura 2b obtidos pelo detector por arranjo de diodos.

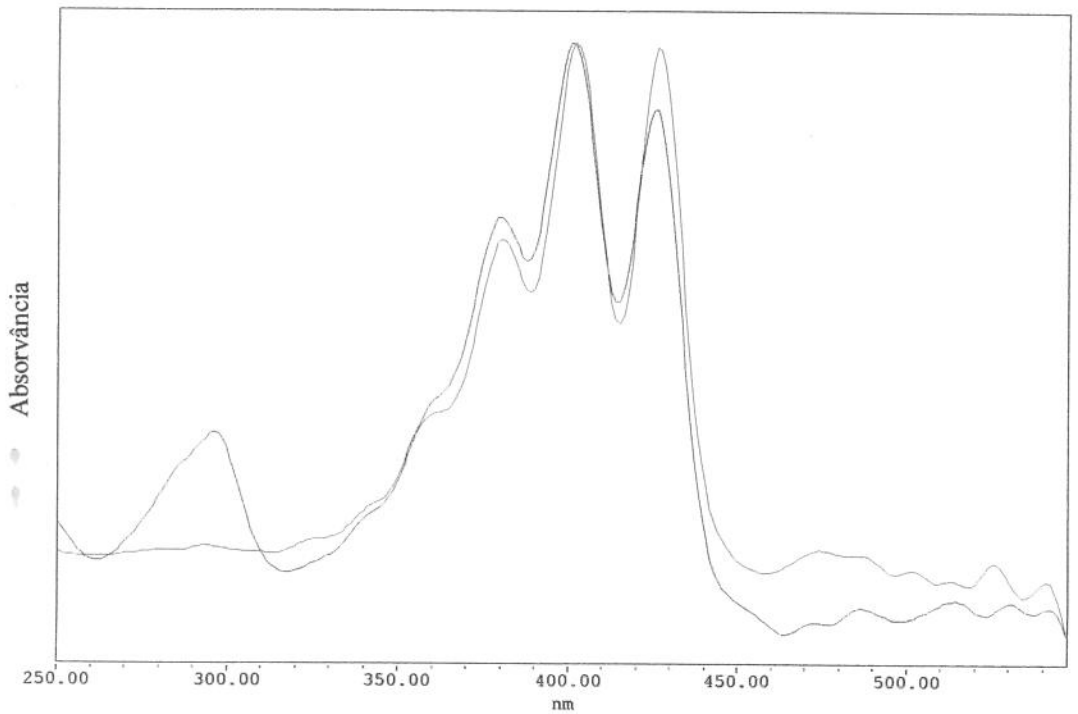

Figura 7. Espectros de absorção dos picos 7-cis-ζ-caroteno (————) e 8-trans-ζ-caroteno (————) da figura figura 2b obtidos pelo detector por conjunto de diodos.

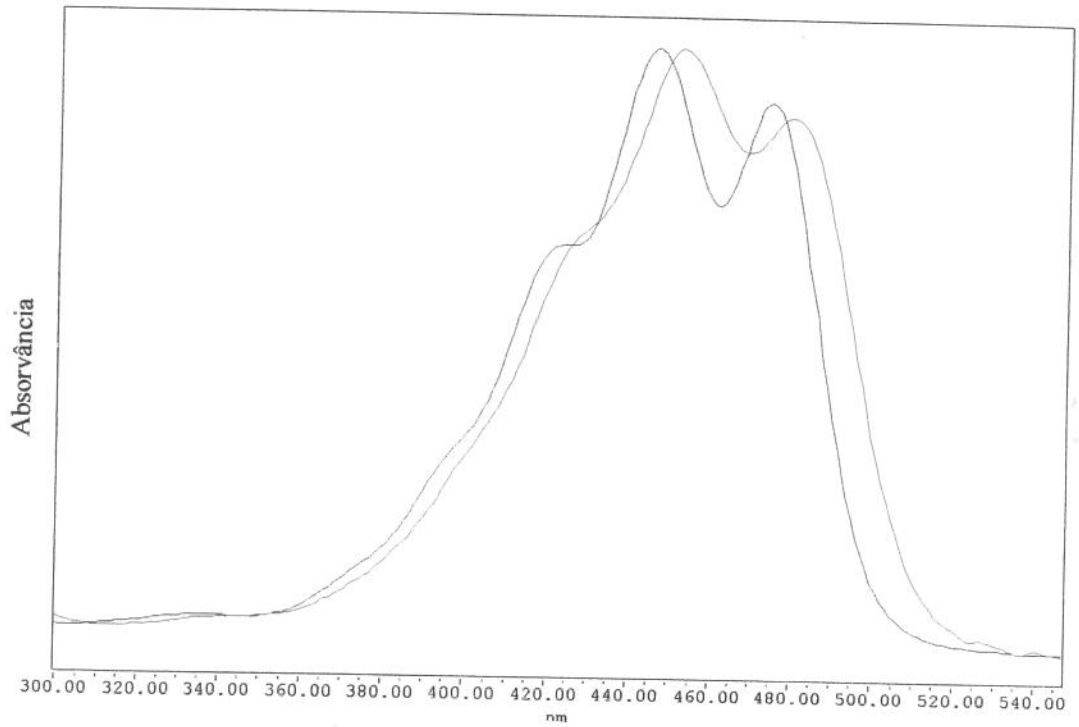

Figura 8. Espectros de absorção dos picos 4-luteína (————) e 5-zeaxantina (————) da figura 2b obtidos pelo detector por conjunto de diodos.

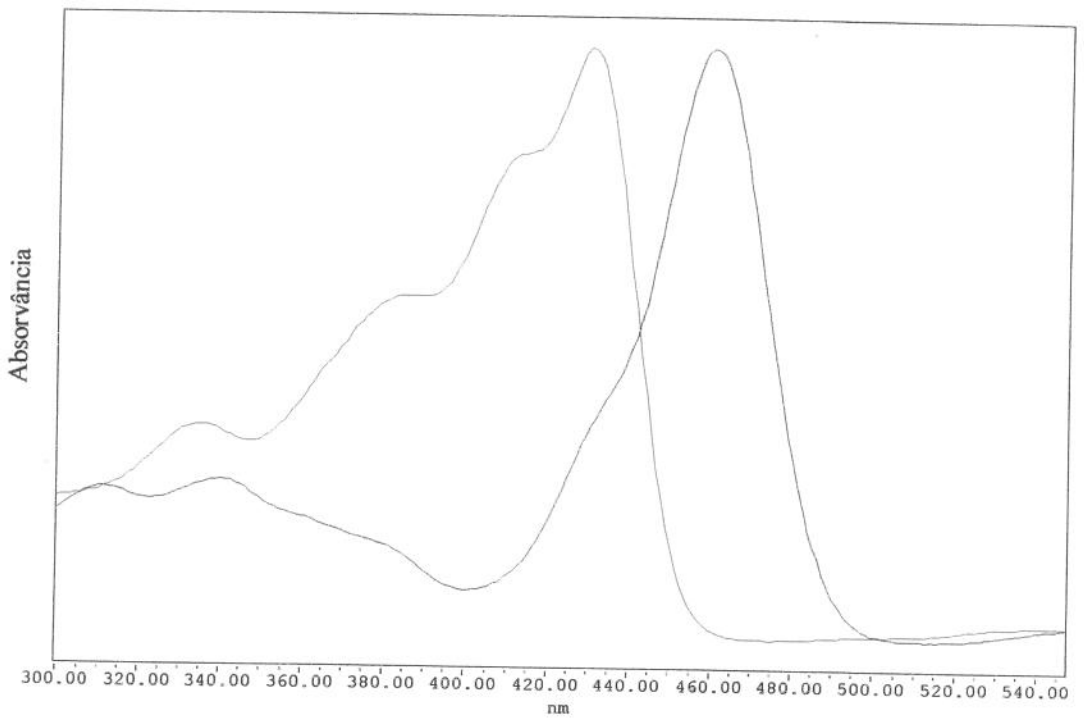

Figura 9. Espectros de absorção dos picos 6 e 7 da figura 1b referentes às clorofilas obtidos pelo detector por conjunto de diodos.

6 – CONSIDERAÇÕES FINAIS

Houve, recentemente, avanços marcantes na fabricação de colunas eficientes de CLAE e na compreensão dos parâmetros que contribuem para o êxito de uma análise de carotenóides. No entanto, para se chegar à combinação ideal de coluna-fase móvel que garanta boa resolução e recuperação dos carotenóides, a ciência ainda precisa de um toque de arte.

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CAPÍTULO 3

A SCHEME FOR OBTAINING STANDARDS AND HPLC QUANTIFICATION OF LEAFY VEGETABLE CAROTENOIDS

Artigo a ser enviado ao Food Chemistry

A SCHEME FOR OBTAINING STANDARDS AND HPLC QUANTIFICATION OF LEAFY VEGETABLE CAROTENOIDS

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ABSTRACT

The reliability of the results obtained by high performance liquid chromatography (HPLC) directly depends on the accuracy of the standardization. Thus, the major difficulty in HPLC analysis is obtaining and maintaining pure standards. Most carotenoids need to be isolated from natural sources. This work presents a strategy for the isolation of standards by open column chromatography and quantification by HPLC, using leafy vegetables as examples. The purity of standards evaluated by HPLC, was 91-97% for neoxanthin, 95-98% for violaxanthin, 97-100% for lactucaxanthin, 92-96% for lutein and 90-97% for β-carotene. Procedures for the calculation of the samples' carotenoid composition were also evaluated, demonstrating that results obtained with one-point recalibration, straight line equation (until 30 days after the construction of the full calibration curves) and response factors relative to β-carotene are similar (CVs of 1.6 to 4.0 %), being well below between- sample lot natural variation (CVs of 6.1 to 42.5 %). The scheme proposed is low-cost, provides a constant supply of carotenoid standards, including those unavailable commercially, and permits high sample throughput.

Keywords: carotenoid, HPLC, analysis, standards

1. Introduction

There is a worldwide consensus that greater and more reliable data on food carotenoids are urgently needed.

Food carotenoid analysis has been carried out to different extent, determining: (1) only the provitamin A carotenoids, (2) the principal provitamin A and nonprovitamin A carotenoids and (3) the complete carotenoid composition. Since it is now recognized that carotenoids enhances the immune system and have a preventive role against some degenerative diseases, this action not being linked to the provitamin A activity, the quantification of only the provitamin A carotenoids is no longer regarded as sufficient. On the other hand, determination of the complete carotenoid composition is complicated, costly and time-consuming. Considering that foods typically contain one to four or five principal carotenoids with minute or trace amounts of many other carotenoids, the added cost, time and complexity involved in identifying and quantifying minor carotenoids do not seem to be justified. Thus, the second approach appears to be the most appropriate for generating data for food data bases.

The major food carotenoids can be reliably determined either by open column chromatography (OCC) or by high performance liquid chromatography (HPLC) (Carvalho *et al.*, 1992; Wilberg & Rodriguez-Amaya, 1995; Adewusi & Bradbury, 1993). OCC has the advantage of using common laboratory equipment (recording UV-Visible spectrophotometer) and does not require a constant supply of carotenoid standards since separated fractions are directly quantified spectrophotometrically, using published

coefficients of absorption. However, the sample throughput is low and reliability of results depends heavily on the expertise of the analyst. HPLC is expensive, especially in developing countries, and reliability of results directly depends on the accuracy of the standardization. Thus, the major difficulty in HPLC analysis of carotenoids is obtaining and maintaining pure standards. The highly unsaturated carotenoids are prone to isomerization and oxidation and only two or three carotenoid standards are commercially available and are of varying purity (Quackenbush & Smallidge, 1986; Craft, 1992). Most carotenoids need to be isolated from natural sources.

Beyond having good analytical methods, the organization of the execution of the analyses will determine the analytical capacity of the laboratory and will also have a direct bearing on the reliability of the results. We present in this paper a strategy, which takes advantage of the best features of OCC and HPLC, for the HPLC determination of the carotenoid composition of foods, using leafy vegetables as examples.

Leafy vegetables are the most accessible year-round sources of carotenoids worldwide. Leaves have a constant qualitative carotenoid pattern with lutein, β-carotene, violaxanthin and neoxanthin as principal carotenoids. The quantitative composition varies considerably between leaves and HPLC data of the same leaf are highly variable. In the Asian leafy vegetable *Ipomoea aquatica*, for example, Chen & Chen (1992) reported in μg/g: 100 ± 8 β-carotene, 78 ± 7 lutein, 60 ± 5 violaxanthin and 50 ± 5 neoxanthin. Wills & Rangga (1996) obtained much lower values (μg/g): 4 β-carotene, 6 lutein, 25 violaxanthin and 16 neoxanthin. Hulshoff *et al.* (1997) found 27 ± 10 μg/g β-carotene and Tee & Lim (1991), 19 μg/g β-carotene and 34 μg/g lutein. In kale, the reported β-carotene content varied from 87 to 146 μg/g and the lutein concentration from 186 to 396 μg/g (Khachik *et al.*, 1986; Micozzi *et al.*, 1990; Müller, 1997). For spinach, β-carotene and lutein ranged

from 32 to 82 μg/g and 42 to 159 μg/g, respectively (Bureau & Bushway, 1986; Khachik *et al.*, 1986; Quackenbush, 1987; Heinonen *et al.*, 1989; Micozzi *et al.*, 1990 ; Tee & Lim, 1991; Hart & Scott, 1995; Müller, 1997; Hulshof *et al.*, 1997;). Although natural variation, due to such factors as variety or cultivar, climate, stage of maturity, may account for part of the divergence, the differences for same foods are so wide that analytical inaccuracies appear to be involved.

2. Materials and methods

2.1. Materials

Cress was the preferred source of standards because of the high carotenoid content and the ease with which carotenoids could be extracted. When lettuce was among the samples for analysis, this vegetable was used as source because it was the only one that contained lactucaxanthin.

2.2. Isolation of standards and preparation of the standard solution

Figure 1 shows the scheme proposed. To obtain standards, the carotenoids were extracted with cold acetone, partitioned to petroleum ether, concentrated in a rotary evaporator and separated in an open column of MgO:Hyflosupercel (1:1 activated for 2 hours at 110°C) (Rodriguez-Amaya *et al.*, 1988), adjusting the mobile phase, not to separate all the carotenoids present, but to isolate the desired carotenoids as quickly and efficiently as possible. The separation pattern is shown in Figure 2. Since the objective was not quantitative analysis, only the main portion of each band of carotenoid was collected, avoiding contamination from the other bands. This was done especially with lutein and violaxanthin which eluted close to each other. A detailed description of OCC is given in Rodriguez-Amaya (1999). Isolates eluted with petroleum ether containing acetone were

washed three or four times with water in a separatery funnel to remove acetone and then dried with Na_2SO_4.

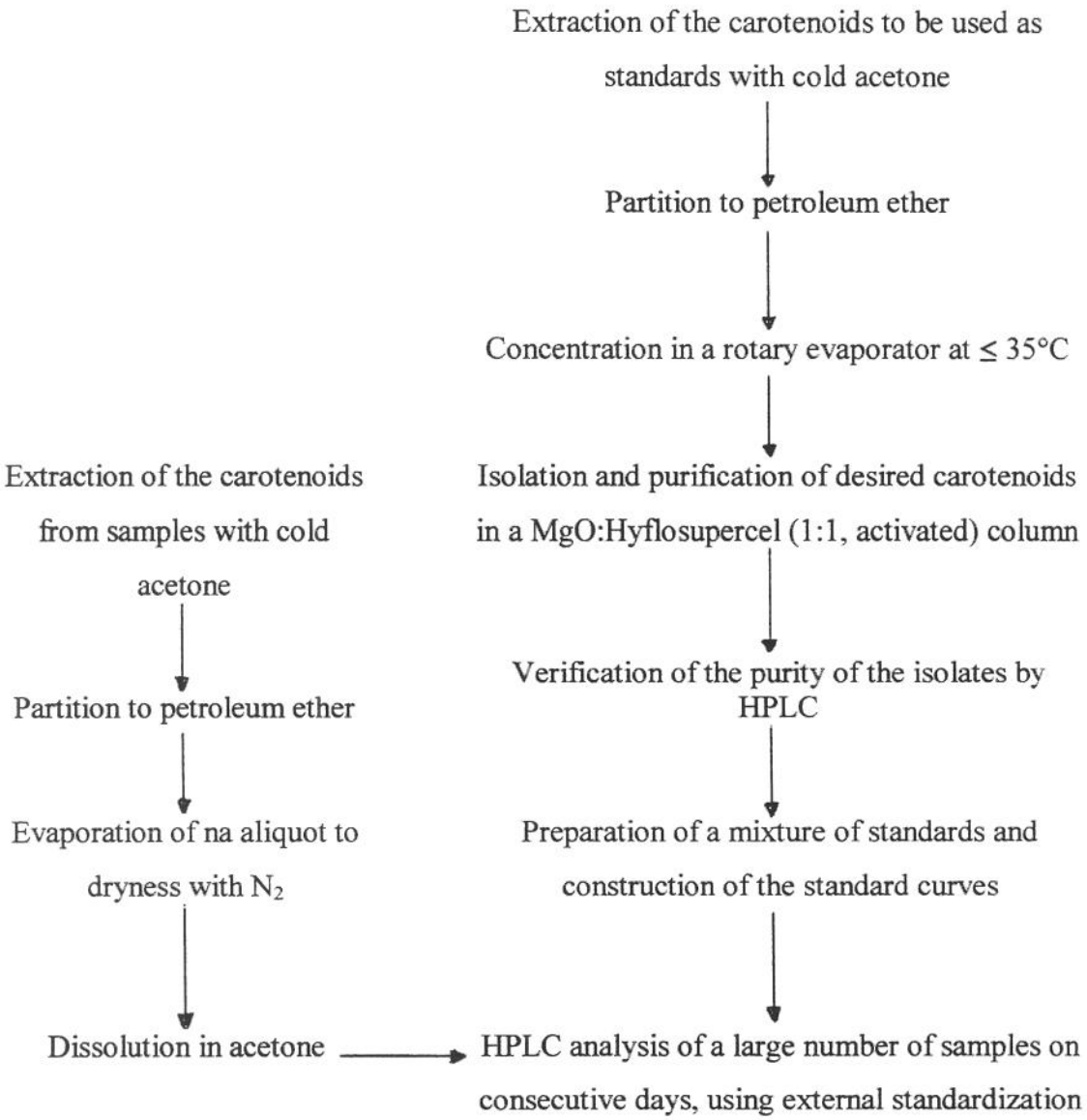

Fig. 1. Proposed scheme for obtaining standards by OCC and quantitaive analysis by HPLC.

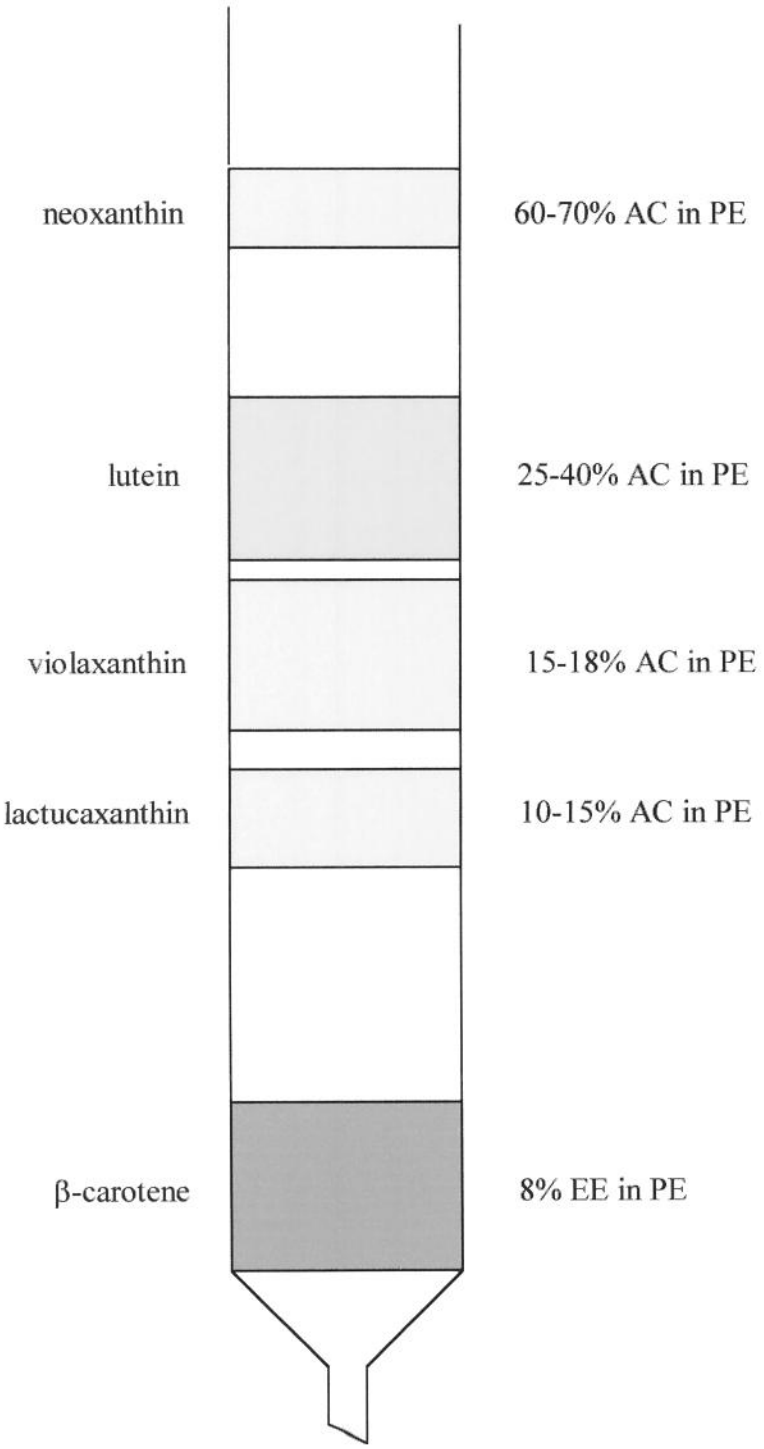

Fig. 2. Isolation of the carotenoid standards from unsaponified lettuce extract by OCC (EE = ethyl ether, AC = acetone, PE = petroleum ether)

The adsorption affinity of MgO can differ between brands and sometimes between batches of the same brand, thus some adjustment of the mobile phase may be needed. In cases when separation of violaxanthin and lutein does not yield pure standards, the fraction corresponding to these carotenoids can be collected together and rechromatographed on a neutral alumina column (activity I), using 25 – 40% of acetone in petroleum ether to elute lutein and acetone to elute violaxanthin.

An aliquot was taken from each isolate to verify the purity by HPLC (i.e. chromatogram showing a single peak corresponding to the carotenoid, giving the same characteristic spectra, obtained with a photodiode array detector, at the ascending and descending slopes and at the maximum). All aliquots were dried under N_2 and dissolved in acetone immediately before injection.

The concentrations of the pure standards were determined spectrophotometrically. A mixed solution consisting of the desired carotenoids was then prepared and quantitatively divided into aliquots, each aliquot being placed in an air-tight screw-topped brown bottle (or transparent bottle wrapped with aluminum foil), dried under N_2 and stored at -18°C or lower. A larger aliquot was set aside for the construction of full calibration curves. These curves, constructed with five different concentrations for each carotenoid, each concentration in triplicate, 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.

2.3. Analysis

Once the standard solution had been prepared and the standard curves demonstrated the required characteristics, a convenient number of samples could be analyzed each day on three to four consecutive days during the week.

Carotenoids were extracted from representative samples with cold acetone and partitioned to petroleum ether as described for the OCC method (Rodriguez-Amaya, 1999). Aliquots were taken, dried under N_2, dissolved in acetone, filtered in 0.22 μm sample filters (Millipore) and injected into the HPLC equipment.

2.4. Quantification

The opportunity was also taken to evaluate the procedures for the calculation of the carotenoid concentrations to verify if this step contributed to the variability of analytical data perceived from the literature. Calculation of the concentrations can be carried out in different ways, all of which attempt to compensate changes in detector's response: (a) using full standard curves constructed at each day of analysis, (b) construction of full standard curves to verify linearity over the samples' concentrations and passage through the origin and one-point recalibration on each day of analysis and (c) use of response factors. Most carotenoid papers do not specify the calculation method used. The first is the ideal procedure but it takes a long time, leaving little time for the samples on each day of analysis, thus limiting sample throughput. It also uses a lot of standards. Following gas chromatographic practices, the second procedure can be used. Injection of a standard of known concentration on each day of analysis in effect verifies any change in the slope of the standard curve (i.e. change in detector's sensitivity). Although much simpler and more rapid, it has to be done

carefully because there is a danger that this single point can be an outlier. The samples' carotenoid concentrations are calculated by the formula:

$$C_X\,(\mu g/g) = \frac{A_X \times C_S\,(\mu g/mL) \times \text{total volume of extract}}{A_S \times \text{sample weight (g)}}$$

where C_X is the concentration of the carotenoid X, A_X is the peak area of the carotenoid X, C_S is the concentration of the standard and A_S is the peak area of the standard.

Use of response factors is also a simplification because a single reference carotenoid standard is injected on each day of analysis. The response factor of each carotenoid relative to the reference carotenoid is calculated by the formula (Asshauer & Ullner, 1986; Hart & Scott, 1995):

$$RF_X = \frac{\text{peak area of carotenoid X (1}\mu g/mL)}{\text{peak area of reference carotenoid (1}\mu g/mL)}$$

The carotenoid concentration in the sample is calculated by the formula:

$$C_X\,(\mu g/g) = \frac{A_X \times \text{total volume of extract (mL)}}{RF_X \times A_{ref} \times \text{sample weight (g)}}$$

where C_X is the concentration of the carotenoid X, A_X is the peak area of the carotenoid X, RF_X is the response factor of the carotenoid X and A_{ref} is the peak area of 1μg/mL of the reference carotenoid.

In the present study different leafy vegetables were quantified by HPLC using external standardization, the calculation being made by one-point recalibration, the straight line equation and response factors.

3. Results and discussion

3.1. Purity of the isolated standards

Figure 3 shows the HPLC chromatograms of the isolated standards. The purity calculated as the percentage of the carotenoid's peak area relative to total area was 91-97% for neoxanthin, 95-98% for violaxanthin, 97–100% for lactucaxanthin, 92-96% for lutein and 90-97% for β-carotene. The concentration of the standards were corrected accordingly. The β-carotene in leaves generally contains *cis*-isomers that cannot be separated in the MgO:Hyflosupercel column, decreasing the purity of the standard. This *cis*-isomers can be separated using a $Ca(OH)_2$ column (Tavares and Rodriguez-Amaya, 1994; Godoy and Rodriguez-Amaya, 1994), but this will prolong the analysis substantially. In any case, the purity percentages obtained are highly satisfactory.

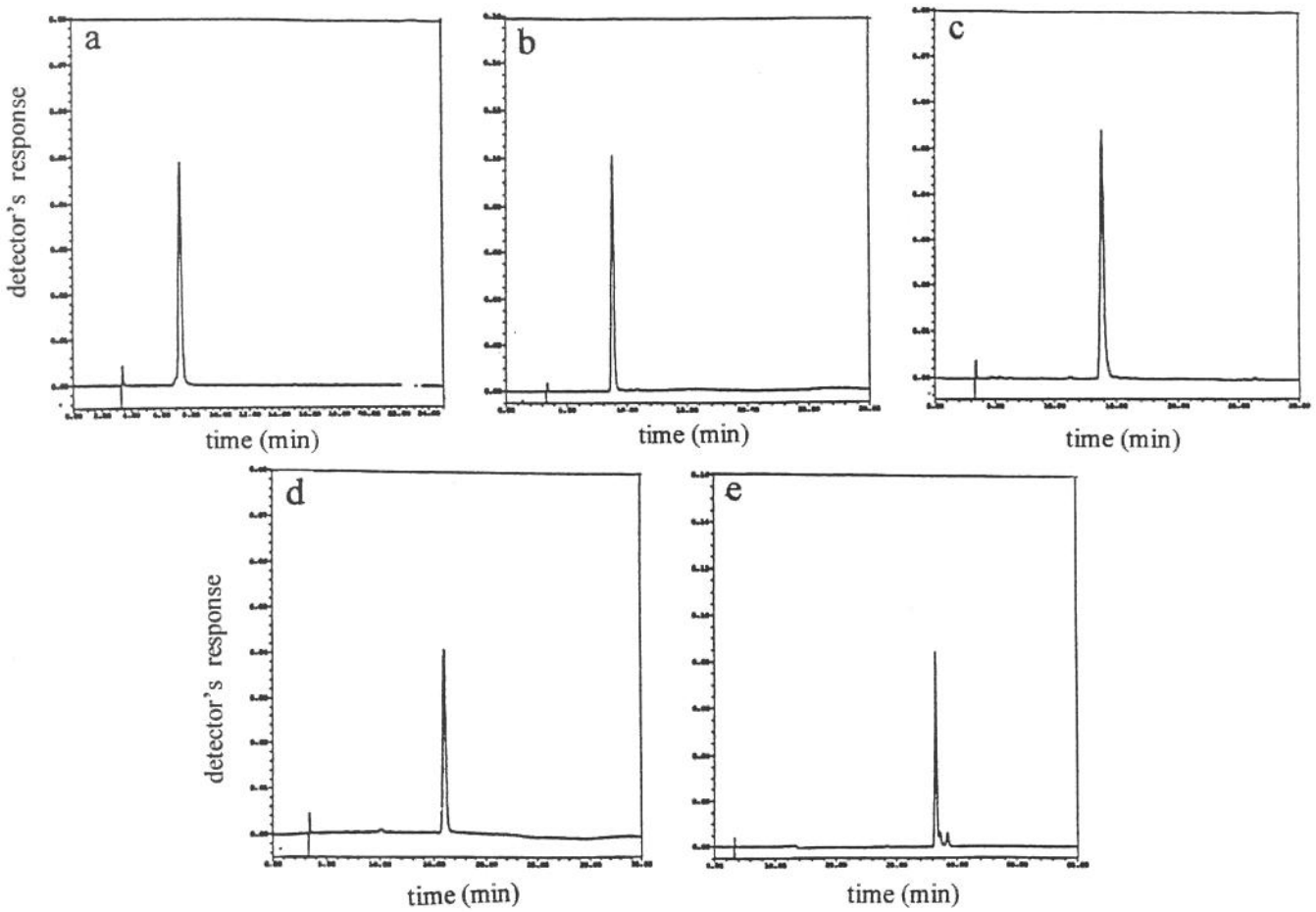

Fig. 3. HPLC chromatograms of carotenoids standards isolates from curly lettuce: neoxanthin (a), violaxanthin (b), lactucaxanthin (c), lutein (d) and β-carotene (e). HPLC conditions are described in text.

Quackenbush and Smallidge (1986) evaluated the purity of commercial β-carotene and the purity by spectral absorbance ranged from 2.4 to 95.6%. Deterioration was attributed principally to autoxidation after packaging. These authors had to recrystallize the commercial β-carotene before use. Craft *et al.* (1990) found that the impurities separated by HPLC accounted for 16-75% of the absorbance of commercial β-carotene preparations at 450 nm. Based on these observations, these authors estimated that all-*trans*-β-carotene measurements could only be 1/50 of reported values.

Hakala & Heinonen (1994) isolated lycopene from tomato puree, using more sophisticated techniques: solid-phase extraction (silica cartridges) and three purifications with semipreparative HPLC. However, the purity obtained with the method developed was only 77% (20% of *cis*-isomers and 3% of xantophylls).

Figure 4a shows a typical chromatogram of the mixture of standards. Calibration with a mixture rather than individual injection of standards saves a lot of time and the calibration chromatogram approximates that of the samples, thereby decreasing relative errors. In fact the chromatogram of the mixture of isolated standards simulates that of the leafy vegetable (Figure 4b), without the peaks corresponding to chlorophylls. Moreover, because the standards were isolated from a leafy vegetables, the concentration ratios also mimic those of the samples, making it easier to bracket the samples' concentrations.

The standard curves of each of the carotenoids passed through the origin and showed linearity, with coefficients of correlation of 0.999 for neoxanthin, violaxanthin and lactucaxanthin and 0.998 for lutein and β-carotene.

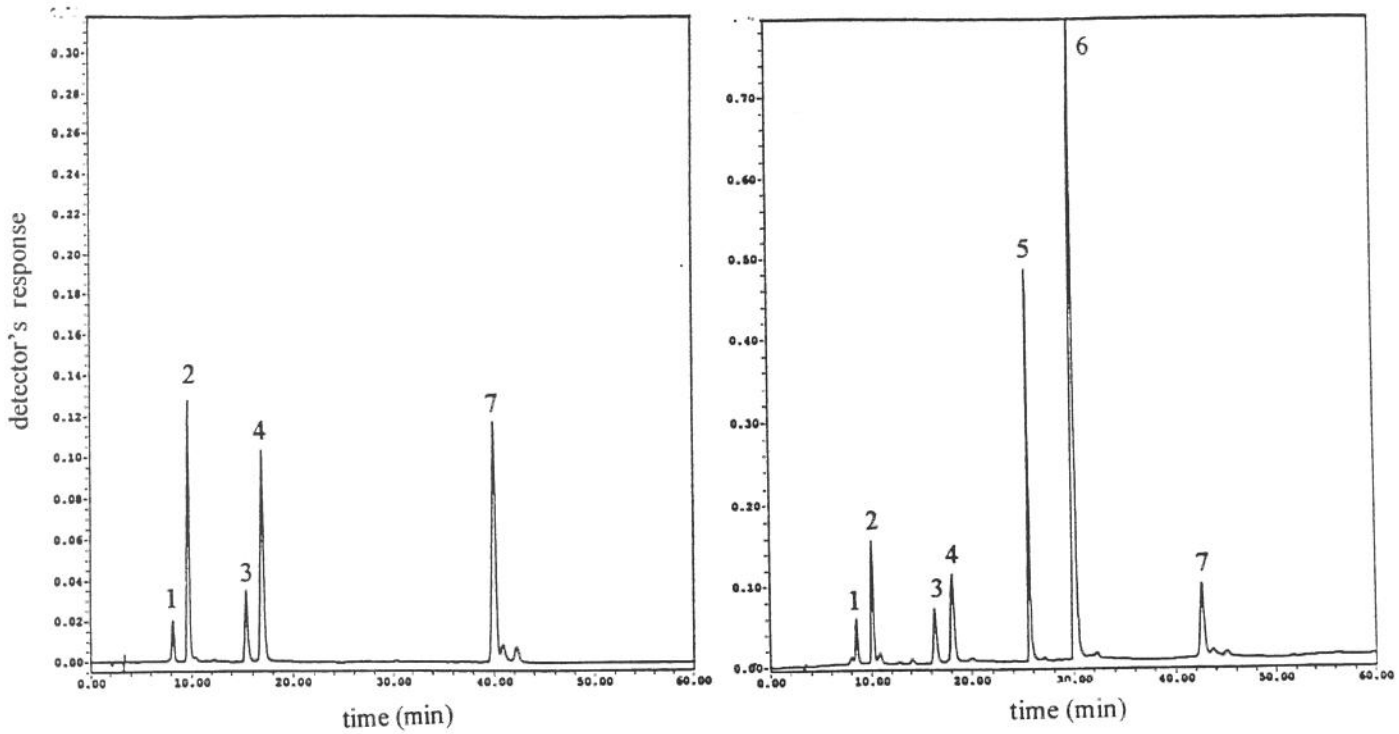

Fig. 4. HPLC chromatograms of a mixture of isolated standards (a) and extract of lettuce (b). Chromatographic conditions are described in text. Peak identification: 1-neoxanthin; 2-violaxanthin; 3-lactucaxanthin; 4-lutein; 5,6-chrolophylls; 7-βcarotene.

3.2. Comparison of calculation procedures

Tables 1-5 presents the carotenoid compositions of the leafy vegetables calculated by one-point recalibration, the straight line equation (of the standard curves constructed at the beginning of the study) and the response factors. The coeficient of variation (CV) was only 1.6-4.0 %, except for β-carotene when response factor relative to lutein was used (5.0-7.5 %). These CVs are much smaller than the lot-to-lot variation (6.1-42.5 %).

Notably, the results obtained with the straight line equation resembled the other results even 30 days after the construction of the full calibration curves. This procedure amounts to using the standard curves obtained on one day to quantify samples analyzed over a one

month period. This means that the detector's response of the chromatograph used did not change during this period. A significant change may occur over a longer period.

Hart & Scott (1995) used β-cryptoxanthin as the reference standard in determining carotenoid concentration, using response factors. Although these authors did not give the reason for this choice, it can be surmised that it was based on the fact the β-cryptoxanthin has an intermediate polarity, between dihydroxy xantophylls and the carotenes, thus serving as a good reference standard for carotenoids of both sides of the polarity range. β-cryptoxanthin, however, is not commercially available. Thus, β-carotene, which is easy to isolate and is commercially available, was used as reference standard in this work, and to verify the effect of polarity, lutein was also utilized. β-Carotene appeared to be an appropriate reference standard for the dihydroxy xantophylls lutein, lactucaxanthin, violaxanthin and neoxanthin. However, response factor relative to lutein appeared to be inadequate for β-carotene.

The scheme proposed can be applied to other food samples. This implies that aside from identifying the carotenoids in the samples conclusively and defining the optimum conditions for HPLC, the procedure for isolating and purifying the carotenoids to be used as standards by OCC should be established before-hand. It is not necessary that standards be isolated from the same types of food as the samples, as was done in the present work for leafy vegetables. For greater ease of isolation, the analyst can use carotenoid-rich foods as sources of standards, such as α-carotene and β-carotene from carrots, β-cryptoxanthin from papaya and lycopene from tomato.

Table 1. Comparison of carotenoids composition (μg/g) of Boston lettuce obtained by one-point recalibration, straight line equation and response factors.

carotenoid	sample number[1]	one-point calibration	straight line equation	RF relative to β-carotene	RF relative to lutein	CV between calibration
neoxanthin	1	7.0	6.7	6.9	7.3	3.0
	2	10.4	10.3	10.3	10.6	1.6
	3	11.5	11.1	10.8	11.1	2.9
CV between samples		24.3	23.9	22.3	21.3	
violaxanthin	1	17.7	16.8	17.1	18.0	3.1
	2	17.7	17.2	17.2	17.8	1.8
	3	21.8	21.4	20.9	21.5	1.7
CV between samples		12.3	14.0	11.7	11.1	
lactucaxanthin	1	11.7	12.0	12.0	12.8	3.9
	2	10.8	11.5	11.4	11.8	3.9
	3	12.6	13.7	13.2	13.7	3.8
CV between samples		7.9	9.4	7.5	7.3	
lutein	1	21.0	19.5	19.7	-	4.0
	2	19.5	18.7	18.7	-	2.4
	3	23.0	22.7	22.1	-	2.1
CV between samples		8.2	10.4	8.4	-	
β-carotene	1	22.9	22.6	-	20.8	5.0
	2	21.7	21.6	-	19.3	6.5
	3	24.5	25.2	-	21.8	7.5
CV between samples		6.2	8.0	-	6.1	

[1] samples analyzed at 0 (1), 15 (2) and 30 (3) days after construction of the full standard curves

RF response factor

CV variation coeficient (%)

Table 2. Comparison of carotenoids composition (μg/g) of curly lettuce obtained by one-point recalibration, straight line equation and response factors.

carotenoid	sample number[1]	one-point calibration	straight line equation	RF relative to β-carotene	RF relative to lutein	CV between calibration
neoxanthin	1	5.6	5.4	5.4	5.6	2.2
	2	6.7	6.6	6.6	6.9	1.6
	3	9.0	8.7	8.4	8.7	2.9
CV between samples		24.7	24.0	22.6	22.3	
violaxanthin	1	15.2	14.2	14.1	14.7	3.5
	2	14.8	14.4	14.5	14.9	1.8
	3	16.7	16.4	16.0	16.5	1.7
CV between samples		6.2	8.1	6.4	6.7	
lactucaxanthin	1	8.7	9.0	8.9	9.3	2.7
	2	7.6	8.1	8.0	8.4	3.9
	3	9.5	10.3	9.9	10.3	3.8
CV between samples		11.0	12.0	10.4	10.4	
lutein	1	15.6	15.0	14.9	-	2.6
	2	15.4	14.8	14.8	-	2.4
	3	17.9	17.7	17.2	-	2.1
CV between samples		8.4	10.2	8.6	-	
β-carotene	1	16.9	17.0	-	15.2	6.4
	2	18.2	18.1	-	16.2	6.5
	3	19.4	20.0	-	17.3	7.5
CV between samples		7.0	8.1	-	6.6	

[1] samples analyzed at 0 (1), 15 (2) and 30 (3) days after construction of the full standard curves

RF response factor

CV (%) variation coeficient

Table 3. Comparison of carotenoids composition (μg/g) of roquette obtained by one-point recalibration, straight line equation and response factors.

carotenoid	sample number[1]	one-point calibration	straight line equation	RF relative to β-carotene	RF relative to lutein	CV between calibration
neoxanthin	1	9.5	9.2	9.1	9.5	2.1
	2	8.1	7.7	7.4	8.0	3.6
	3	13.8	13.3	12.9	13.3	2.8
CV between samples		28.5	28.5	28.4	27.0	
violaxanthin	1	20.9	19.4	19.4	20.1	1.7
	2	12.0	12.2	11.8	12.6	2.8
	3	28.3	27.9	27.2	28.0	1.7
CV between samples		40.1	39.4	39.5	38.1	
lutein	1	49.7	47.7	47.3	-	2.6
	2	33.0	31.9	30.6	-	3.7
	3	67.4	66.6	64.7	-	2.1
CV between samples		34.3	35.6	35.8	-	
β-carotene	1	32.7	32.9	-	29.3	6.3
	2	19.2	19.9	-	17.6	6.1
	3	47.3	48.6	-	42.1	7.5
CV between samples		42.5	42.5	-	41.2	

[1] samples analyzed at 0 (1), 15 (2) and 30 (3) days after construction of the full standard curves

RF response factor

CV (%) variation coeficient

Table 4. Comparison of carotenoids composition (μg/g) of cress obtained by one-point recalibration, straight line equation and response factors.

carotenoid	sample number[1]	one-point calibration	straight line equation	RF relative to β-carotene	RF relative to lutein	CV between calibration
neoxanthin	1	14.4	13.9	13.8	14.4	2.1
	2	13.1	12.9	12.9	13.4	1.6
	3	20.1	19.3	18.8	19.4	2.8
CV between samples		23.6	22.4	20.8	20.6	
violaxanthin	1	20.9	19.4	19.3	20.1	3.5
	2	23.6	22.9	23.0	23.7	1.8
	3	27.7	27.3	26.6	27.4	1.7
CV between samples		14.3	16.9	15.7	15.4	
lutein	1	61.4	59.0	58.5	-	2.6
	2	77.8	74.6	74.6	-	2.4
	3	80.7	79.8	77.4	-	2.1
CV between samples		14.2	15.2	14.6	-	
β-carotene	1	28.4	28.6	-	25.5	6.3
	2	40.5	40.4	-	36.1	6.5
	3	39.4	40.5	-	35.1	7.5
CV between samples		18.5	18.7	-	18.1	

[1] samples analyzed at 0 (1), 15 (2) and 30 (3) days after construction of the full standard curves

RF response factor

CV (%) variation coeficient

Table 5. Comparison of carotenoids composition (μg/g) of chicory obtained by one-point recalibration, straight line equation and response factors.

carotenoid	sample number[1]	one-point calibration	straight line equation	RF relative to β-carotene	RF relative to lutein	CV between calibration
neoxanthin	1	9.1	8.9	8.8	9.2	2.1
	2	18.2	18.0	17.3	18.5	3.6
	3	18.4	17.7	17.2	17.8	2.8
CV between samples		35.3	35.0	35.0	34.3	
violaxanthin	1	15.4	14.4	14.3	14.9	3.5
	2	23.4	23.8	23.0	24.5	2.8
	3	24.9	24.5	23.9	24.6	1.7
CV between samples		23.9	27.1	25.9	26.2	
lutein	1	41.4	39.8	39.5	-	2.6
	2	69.0	66.7	64.0	-	3.7
	3	61.6	60.9	59.1	-	2.1
CV between samples		24.9	25.3	24.0	-	
β-carotene	1	24.9	25.1	-	22.4	6.3
	2	43.1	44.8	-	39.7	6.1
	3	40.9	42.0	-	36.4	7.5
CV between samples		27.3	28.6	-	32.8	

[1] samples analyzed at 0 (1), 15 (2) and 30 (3) days after construction of the full standard curves

RF response factor

CV (%) variation coeficient

For greater efficiency, food samples to be analyzed should be grouped according to the carotenoid composition, and samples of similar composition should be analyzed together so that the same standard solution can be used and the greatest number of samples can be analyzed. The scheme proposed projects a one-week activity. The standards can be isolated and purified, and their purity checked on the first day. The standard solution (mixture) can be prepared and the standard curves made on the second day. Extraction and HPLC analyses of a large number of samples can then be carried out in the next three consecutive days. The limiting factor will be the time for each HPLC run. In the following weeks, one-point recalibration can be employed throughout, increasing sample throughput. Mantoura *et al.* (1997) recommends that full calibration be done every three-four month or when variation of the ratio between concentration and area of standard exceeds 5 %.

In our laboratory, standards are usually used within one week after isolation. It is, however, possible to extract a greater amount of standards and the aliquots stored in sealed glass vials under N_2, at the lowest temperature possible (< -18°C), for use over an extended period.

The strategy herein described is low-cost and provides a constant supply of carotenoid standards, including those which cannot be acquired commercially. Sample throughput is high.

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CAPÍTULO 4

CAROTENOIDS OF TOMATO AND TOMATO PASTE: VERIFICATION OF THE OCCURRENCE OF γ-CAROTENE

Artigo a ser enviado ao Journal of Agricultural and Food Chemistry

CAROTENOIDS OF TOMATO AND TOMATO PASTE: VERIFICATION OF THE OCCURRENCE OF γ-CAROTENE

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ABSTRACT

Tomato and tomato paste are among the most consumed foodstuffs worldwide. Although widely studied in terms of its carotenoid composition, some inconsistencies in the results reported can be discerned. In Brazil, γ-carotene was detected in fresh tomato but not in various tomato products, using open column chromatography (OCC). On the other hand, very high amounts (eight times the β-carotene content) of this carotenoid were obtained in American tomato products, using high performance liquid chromatography (HPLC). Thus, this work was carried out to verify if the difference in data is due to natural variation of the samples or to artifacts of the analytical process. In fresh tomato, 11 carotenoids were identified: *trans*-lycopene, phytoene, phytofluene, β-carotene, lutein, two *cis*-lycopenes, γ-carotene, *trans*-ζ-carotene, *cis*-ζ-carotene, and neurosporene. In tomato paste, aside from the mentioned carotenoids, *cis*-β-carotene and four other unidentified carotenoids were also detected. γ-Carotene was found in comparable concentrations in Brazilian and American tomato pastes, at levels much lower than β-carotene, and apparently below the detection limit of OCC. The removal of the peel and stage maturity of the fresh tomatoes could not explain the loss of γ-carotene in Brazilian tomato pastes, indicating that degradation was involved. The results do not lend support to the reported high levels of γ-carotene in American tomato products.

Keywords: tomato, tomato products, carotenoids, γ-caroteno

INTRODUCTION

In recent years, the marked improvement in the efficiency of high-performance liquid chromatographic (HPLC) columns, together with the on-line acquisition of UV-Visible spectra with the photodiode array detector , have greatly facilitated the analysis of the complex mixtures of carotenoids in foods. With these refinements in analytical instrumentation, conditions are propitious for the clarification of some diverging data in the literature.

Tomato and tomato products are among the most consumed foodstuffs worldwide and are often the major sources of carotenoids for the population. Although widely studied in terms of its carotenoid composition, some inconsistencies in the results reported can be discerned. For example, γ-carotene was found in 10 sample lots of fresh Brazilian tomatoes at 0.7 ± 0.2 μg/g, but not in 39 samples of tomato products (juice, paste, puree and catchup) (Tavares and Rodriguez-Amaya, 1994). On the other hand, substantial amounts of γ-carotene (means of 15 to 100 μg/g), surpassing β-carotene (means of 2.3 to 15 μg/g), were found in tomato products in the U.S. (total of 52 samples of tomato soup, tomato juice, whole tomatoes, catchup, spaghetti sauce, paste, puree, and sauce), contributing significantly to the vitamin A value of these products (Tonucci et al., 1995).

Based on the data of different laboratories, Gross (1987) noted that the total carotenoid content of raw red tomato varies between 90 and 190 μg/g fresh weight. Lycopene, the major pigment, makes up to 90% of the total, with phytoene and phytofluene constituting 15-30%. Minor pigments are β-carotene, ζ-carotene, γ-carotene, and neurosporene. Recent data obtained by HPLC methods vary from 3.6 to 17 μg/g β-carotene and 7 to 114 μg/g

lycopene (Heinonen et al., 1989; Micozzi et al., 1990; Tee and Lim, 1991; Khachick et al., 1992; Hart and Scott, 1995; Müler, 1997). In tomato products, the carotenoid composition will vary depending on the carotenoid composition of the raw material, and the time and severity of the processing treatment, which result in varying degrees of degradation.

The importance of reliable carotenoid data cannot be overemphasized, and there is a worldwide effort to this end. Thus, this work was carried out to restudy the qualitative composition of tomato and tomato paste so as to verify if the difference in data is due to natural variation of the samples or to artifacts of the analytical process.

MATERIALS AND METHODS

Samples. Fresh tomatoes were purchased from supermarkets in Campinas and analyzed on the same day. Tomato pastes of the three commercial brands with the largest sale volume in Brazil were also bought in Campinas. Three national brands of tomato paste from the United States were acquired from grocery stores in Washington, DC.

Extraction. The fresh tomatoes were homogenized in a Waring blender and 10 to 20 g subsamples were taken for immediate analysis. Since tomato pastes undergo homogenization during processing, the tomato paste samples were simply mixed and 10 g samples were taken for analysis.

Carotenoids were extracted with cold acetone, transferred to petroleum ether and concentrated in a rotary evaporator as described by Rodriguez-Amaya (1999). The concentrated extracts were transferred to vials, brought to dryness with N_2, redissolved in 2

mL HPLC grade acetone with ultrasonic agitation, filtered through PTFE filters of 0.22 μm (Millipore) and injected into the HPLC instrument.

HPLC. Two HPLC equipment were used: (a) Varian model 9010 ternary solvent system equipped with Waters model 994 photodiode array detector, a polymeric C_{18} Vydac 218 TP 54 (Separations Group) column, 5 μm, 4.6 x 250 mm, using as mobile phase methanol: tetrahydrofuran (with 0.01% butylated hydroxytoluene) (95:5) at a flow rate of 0.8 mL/min; (b) Waters model 2690 separations module equipped with Waters model 996 photodiode array detector, a Spherisorb S5 ODS2 "narrowbore" column (Waters), 5μm, 2.0 x 250 mm, with acetonitrile:methanol: ethyl acetate (73:20:7) as mobile phase at a flow rate of 0.25 mL/min. Detection of peaks was done at 450 nm with the Varian HPLC and at the wavelengths of maximum absorption (max plot) with the Waters HPLC. Peak purity was verified through the spectra taken at the ascending and descending slopes and at the maximum by the photodiode array detector.

OCC. A glass column, 25 x 300 mm, packed with MgO:Hyflosupercel (1:1) activated at 110°C for 4 hours was used to separate the total extract into three bands, corresponding to β-carotene, γ-carotene, and lycopene fractions, which were eluted with 12% acetone in petroleum ether, 20% acetone in petroleum ether, and acetone and 10% water in acetone, respectively.

Identification. The peaks were identified by the combined use of retention times, co-chromatography, and the visible absorption spectra.

RESULTS AND DISCUSSION

Occurrence of γ-carotene in Brazilian tomato paste. Typical chromatograms obtained with the Vydac column of Brazilian fresh tomatoes and tomato paste are shown in Figure 1, the carotenoid patterns being similar for the three brands of pastes. The Vydac column is widely used in carotenoid analysis because of its efficiency. Chromatograms of the carotenoids of fresh tomato, taken at 450 nm, had six well defined peaks, which were identified as lutein, β-carotene, γ-carotene, *trans*-lycopene, and two *cis*-lycopenes. More peaks appeared in the chromatograms of the tomato paste carotenoids and the peak corresponding to γ-carotene appeared distorted. The visible spectra taken at different points of the peak revealed that it was a mixture.

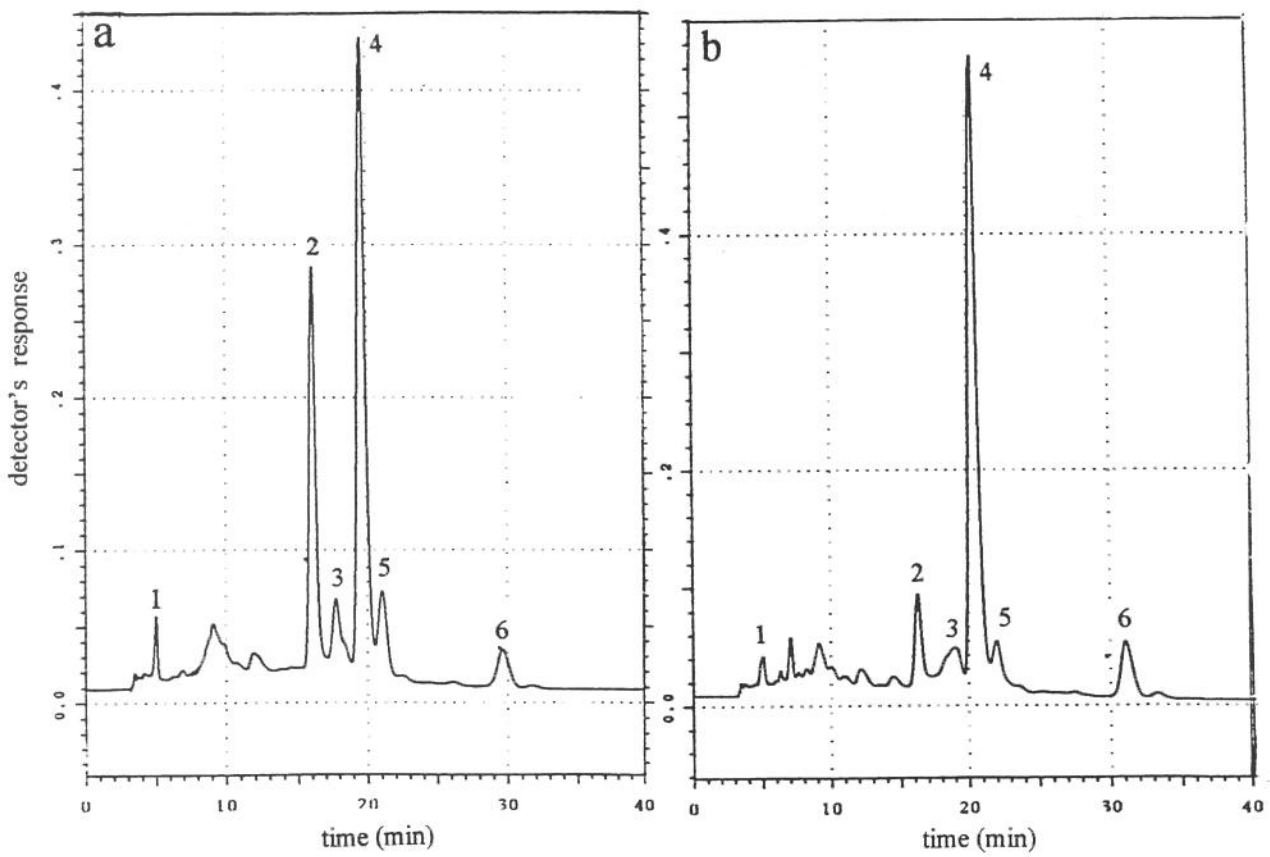

Figure1. HPLC chromatograms of the carotenoids of Brazilian fresh tomato (a) and tomato paste (b) obtained with the Vydac 218 TP 54 column. Chromatographic conditions are described in text. Detection was set at 450 nm. Peak identification: 1-lutein, 2-β-carotene, 3-γ-carotene in fresh tomato and mixture in paste, 4-*trans*-lycopene, 5 and 6-*cis*-lycopene.

Because of the predominance of lycopene, the γ-carotene peak appeared very small. To verify better the occurrence of γ-carotene in tomato paste, preliminary separation on a MgO:Hyflosupercel column was undertaken to separate the β-carotene fraction, the γ-carotene fraction, and the lycopene fraction before HPLC. The chromatograms of the γ-carotene fractions of fresh tomato and tomato paste and the spectra taken at different points of the peak corresponding to γ-carotene are presented in Figure 2. While the purity of the γ-carotene peak of fresh tomato was confirmed, the three spectra resembling each other, that of the tomato paste proved to be γ-carotene mixed with other compounds, probably carotenoid degradation products. If not separated, these compounds would be quantified with γ-carotene, raising its concentration.

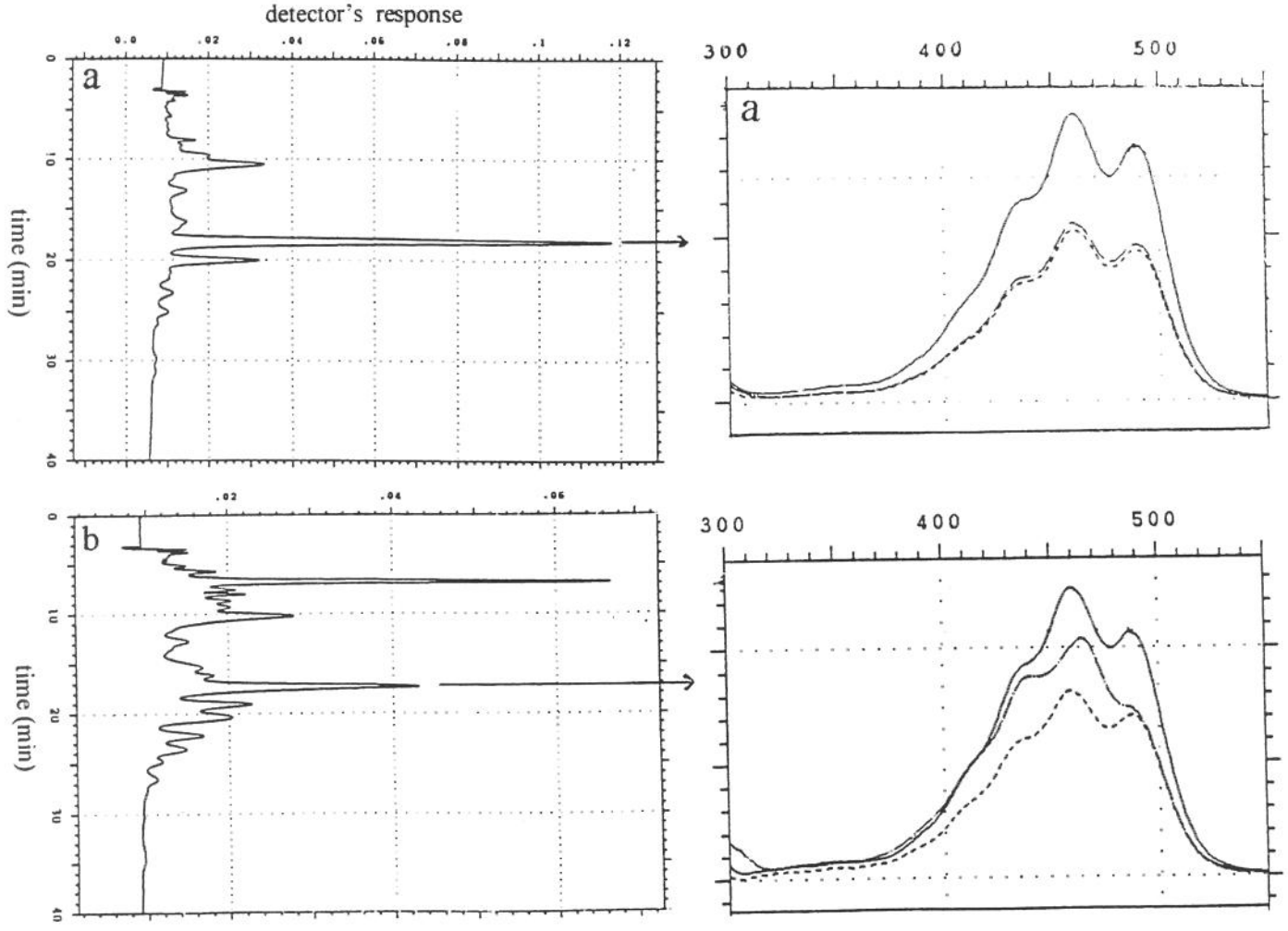

Figure 2. HPLC chromatograms of the γ-carotene fraction obtained with Vydac 218 TP 54 column and absorption spectra of the peak corresponding to γ-carotene of Brazilian fresh tomato (a) and tomato paste (b). Chromatographic conditions are described in text.

The chromatograms obtained with the Vydac column demonstrated that many minor compounds could elute in the region between β-carotene and lycopene. To separate these minor peaks better, other chromatographic conditions were tested.

Better separation was achieved with a monomeric Spherisorb S5 ODS2 narrowbore column, using acetonitrile:methanol:ethyl acetate (73:20:7) as mobile phase (Figure 3). Setting detection at the wavelengths of maximum absorption, 11 carotenoids were identified in fresh tomatoes: lutein, *trans*-lycopene, two *cis*-lycopenes, neurosporene, γ-carotene, *cis*-ζ-carotene, *trans*-ζ-carotene, β-carotene, phytofluene, and phytoene. In the tomato paste, *cis*-β-carotene and four other unidentified carotenoids were also detected.

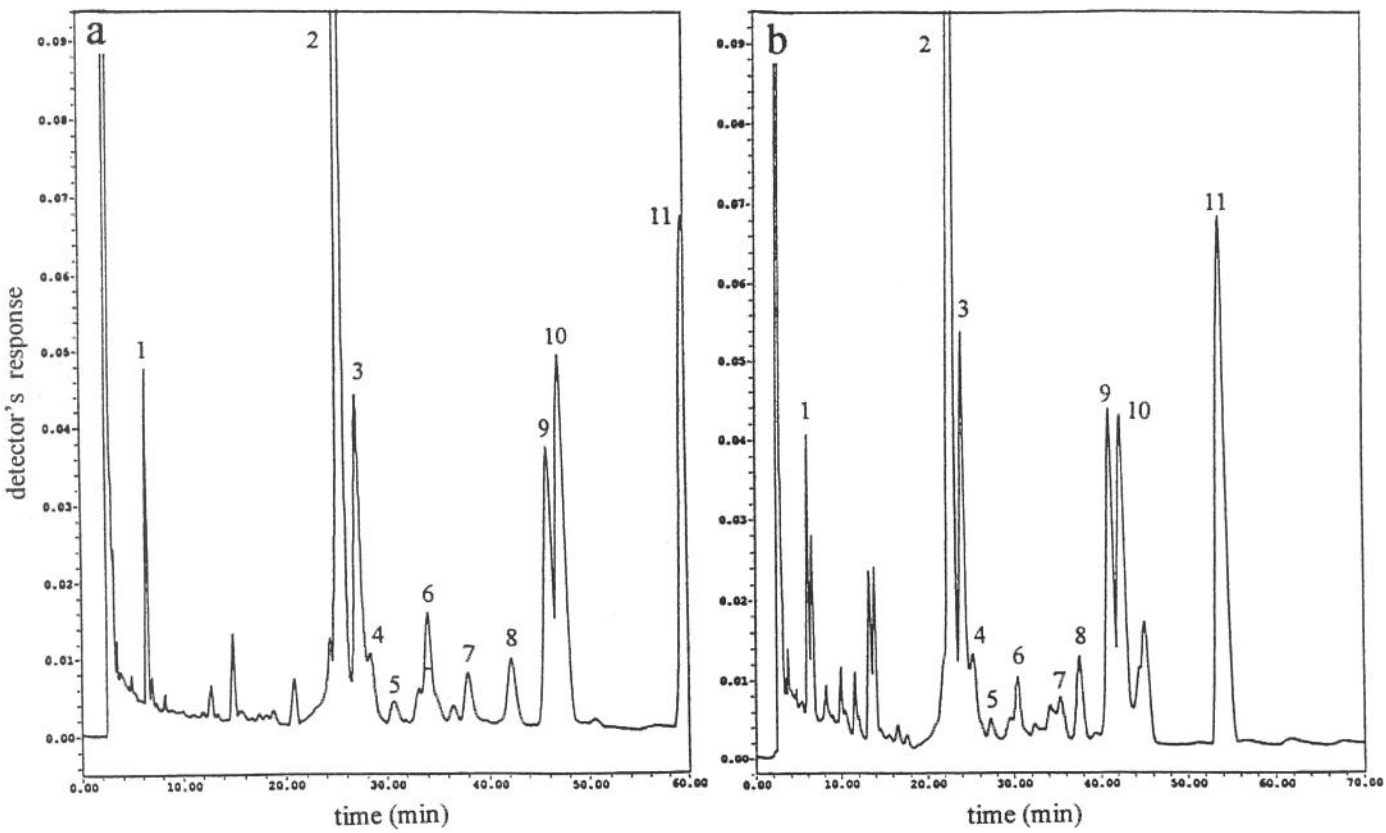

Figure 3. HPLC chromatograms of the carotenoids of Brazilian fresh tomato (a) and tomato paste (b) obtained with Spherisorb S5 ODS2 narrowbore column. Chromatographic conditions are described in the text. Detection was set at the wavelengths of maximum absorption. Peak identification: 1-lutein, 2-*trans*-lycopene, 3,4-*cis*-lycopene, 5-neurosporene, 6-γ-carotene, 7-*cis*-ζ-carotene, 8-*trans*-ζ-carotene, 9-β-carotene, 10-phytofluene, 11-phytoene.

The purity of the γ-carotene peak was confirmed in both the fresh tomato and tomato paste. γ-Carotene was apparently lower in the tomato paste, however. This level was probably below the detection limit of the open column chromatographic method used by Tavares and Rodriguez-Amaya (1994), explaining why γ-carotene was not detected in tomato products in this work.

An attempt was also made to explain the unexpected lowering of the γ-carotene content in Brazilian tomato paste. Considering the °Brix reported by Tavares and Rodriguez-Amaya (1994), the tomato paste samples analyzed would be about five times more concentrated than the fresh tomato. Thus, significant loss of γ-carotene occurred during the processing of Brazilian tomato paste. This loss could be due to any one or a combination of the following factors: (a) removal of the peel, (b) stage of maturity of the tomatoes used as raw materials, and (c) degradation of the carotenoid during processing.

Comparison of the chromatograms of the carotenoids of the same lot of tomatoes (Figure 4) shows that, although β-carotene (peak 9) was much higher in the peel, γ-carotene (peak 6) appeared practically the same in the pulp and the peel.

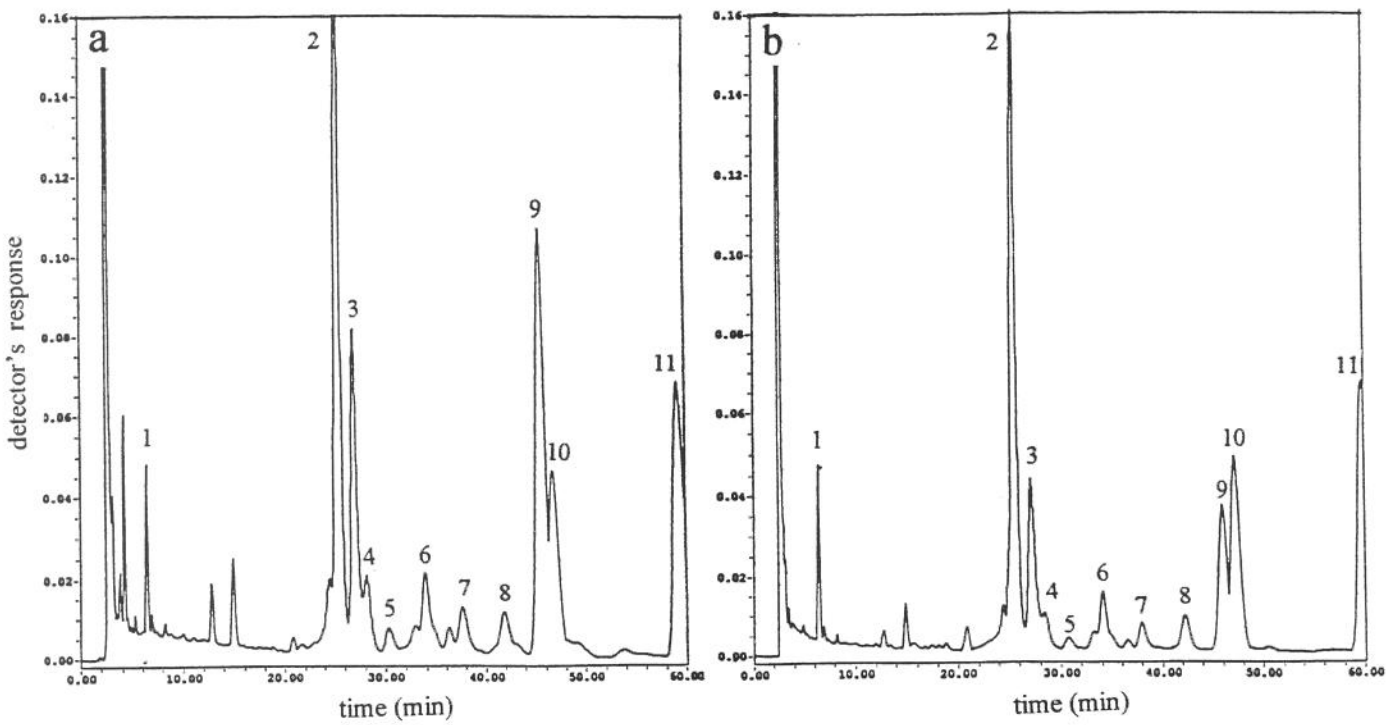

Figure 4. HPLC chromatograms of the carotenoids of the peel (a) and pulp (b) of fresh tomato. Chromatographic conditions and peak identification are the same as Figure 3.

Verification of the effect of the stage of maturity showed that with the exception of β-carotene, which was practically the same at the three stages of maturity, all other carotenoids, including γ-carotene, increased from the almost ripe to the overripe stage (Figure 5). This difference, however, is not sufficient to account for the disappearance of the γ-carotene in processed Brazilian tomatoes.

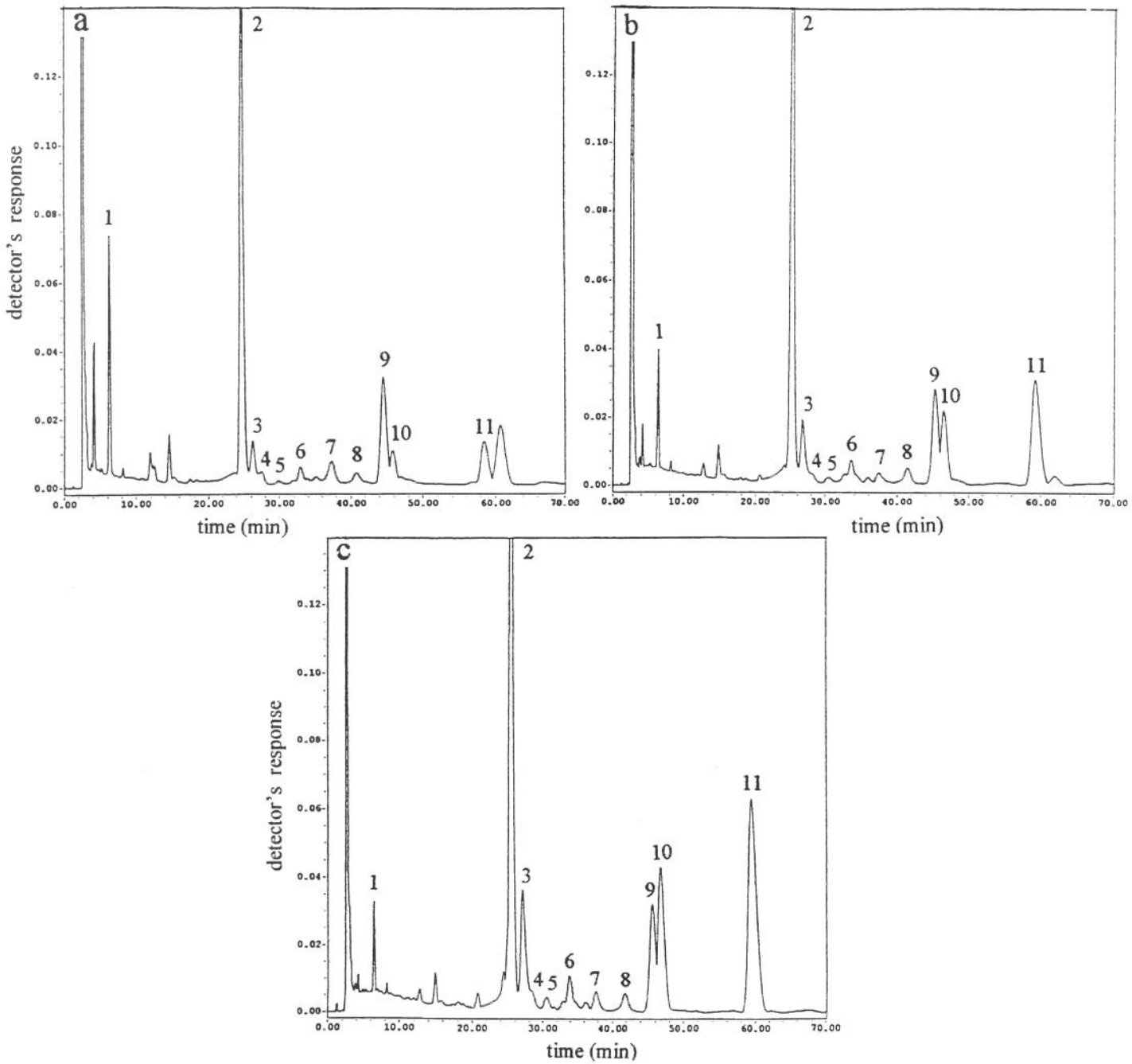

Figure 5. HPLC chromatograms of the carotenoids of fresh tomato at three different stages of maturity: almost ripe (a), ripe (b) and overripe (c). Chromatographic conditions and peak identification are the same as Figure 3.

The major reason for loss of γ-carotene therefore appears to be degradation during processing. The *trans*-lycopene content of the tomato paste products (means of 158 to 183 μg/g) analyzed by Tavares and Rodriguez-Amaya (1994) were in the expected range (about 155 μg/g), calculated from the soluble solids. The β-carotene levels of the pastes (means of 4.3 to 8.7 μg/g) were in the same range as the fresh tomato (5.1 ± 1.1 μg/g), falling short of the expected values (about 25 μg/g). This is partly explained by the removal of the peel, which appeared to have much higher levels of β-carotene than the pulp as mentioned earlier. γ-Carotene content in the paste should be about 3.5 μg/g, this small amount being practically lost during processing.

Occurrence of γ-carotene in American tomato paste. An attempt was made to find an explanation for the discrepancy in the data obtained by Tonucci et al. (1995) and Tavares and Rodriguez-Amaya (1994). The difference in the γ-carotene contents of American and Brazilian tomato pastes, can be due to any one or a combination of the following factors: (a) difference in tomato cultivars used as raw materials, (b) difference in the processing condition, and (c) analytical variability.

Typical chromatograms of the carotenoids of tomato pastes produced in the United States, obtained with the Vydac and Spherisorb columns are shown in Figures 6, the three brands showing similar patterns.

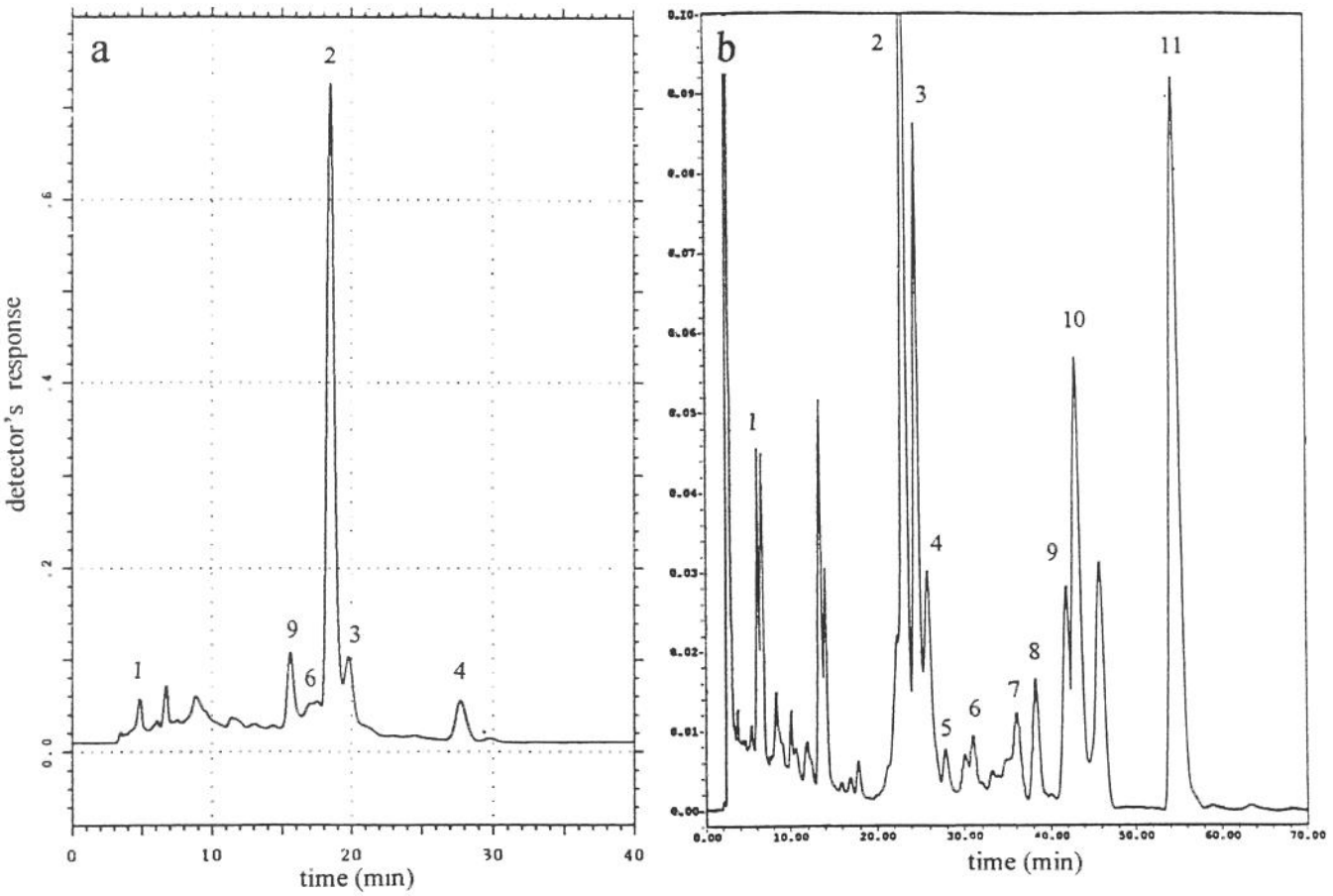

Figure 6. Typical HPLC chromatograms of the carotenoids of American tomato pastes obtained with Vydac 218 TP 54 (a) and Spherisorb S5 ODS2 (narrow bore) columns. Chromatographic conditions are described in the text. Peaks identification are the same as Figure 3.

The major differences in the carotenoid patterns of the tomato paste are: (a) phytoene, *cis*-lycopene, and *cis*-β-carotene are markedly higher in the American tomato paste, and (b) two unidentified peaks, one very close to lutein and the other to γ-carotene are also greater in the American tomato pastes. The γ-carotene peak, however, appears essentially of same magnitude and evidently smaller than the β-carotene peak in both pastes. Thus, this comparison does not shed any light on the huge difference in the γ-carotene content (99.8 ± 11.5 μg/g vs. not detected) of the American and Brazilian tomato pastes analyzed by

Tonucci et al. (1995) and Tavares and Rodriguez-Amaya (1994). According to the chromatograms obtained in Figures 1 and 5, the American and Brazilian tomato pastes should have practically the same amounts of γ-carotene.

The lycopene level (554.5 ± 43.3 vs. means of 158 to 183 μg/g) was about three times in the American tomato paste while the β-carotene content (12.7 ± 2.4 vs. means of 4.3 to 8.7 μg/g) was slightly higher (Tonucci et al., 1995; Tavares and Rodriguez-Amaya, 1994).

A closer look at the chromatogram presented by Tonucci et al. (1995) reveals that the β-carotene peak is substantially greater than the γ-carotene peak. Even considering that the $A_{1cm}^{1\%}$ of γ-carotene (3100 in petroleum ether) is higher than that of β-carotene (2592 in petroleum ether), and the change in the mobile phase composition (a gradient was used), the γ-carotene concentration cannot be much higher (almost eight times) than that of β-carotene. Thus, calculations errors may be involved.

ACKNOWLEDGEMENT

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CAPÍTULO 5

CAROTENOID COMPOSITION OF HYDROPONIC LEAFY VEGETABLES

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CAROTENOID COMPOSITION OF HYDROPONIC LEAFY VEGETABLES

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ABSTRACT

Considering that hydroponic production of vegetables is becoming more common, the carotenoid composition of hydroponic leafy vegetables commercialized in Campinas, Brazil, was determined. Lactucaxanthin was quantified for the first time and was found to have similar concentrations as neoxanthin in the four types of lettuce analyzed. Lutein predominated in cress, chicory, and roquettes (75.4±10.2 μg/g; 57.0±10.3 μg/g; 52.2±12.6 μg/g, respectively). In the lactucaxanthin-containing lettuces, β-carotene and lutein were the principal carotenoids (9.9±1.5 – 24.6±3.1 μg/g and 10.2±1.0 – 22.9±2.6 μg/g, respectively). Comparing hydroponic and field-produced curly lettuce, taken from neighboring farms, the hydroponic lettuce had significantly lower lutein, β-carotene, violaxanthin, and neoxanthin contents than the conventionally produced lettuce. Since the hydroponic farm had a polyethylene covering, less exposure to sunlight and lower temperatures may have decreased carotenogenesis.

Keywords: carotenoids, leafy vegetables, hydroponic

INTRODUCTION

The ever increasing population of the world has put a lot of strain on available resources and has encouraged the search for new techniques of food production. One such technique that is gaining wider application is hydroponic farming. It has several advantages: (a) smaller area required and greater productivity per area; (b) possibility of using areas not suitable for traditional farming; (c) possibility of several harvests during the year because of rapid plant growth; (d) crop rotation not necessary; (e) less consumption of water and fertilizer; (f) greater hygiene and less possibility of contamination with microorganisms, nematodes and insects inherent to the soil, consequently, minimal use of fungicides and insecticides; (g) less manpower needed; and (h) greater control of quality. Some disadvantages are: (a) use of insecticides and fungicides, although in amounts less than those used in conventional systems; (b) high cost of installation; (c) dependence on electricity in automated systems; and (d) need for specialized laborers.

It is always necessary to verify the effect of a new farming practice on the food composition, especially of nutrients and phytochemicals important to human health. At the same time, hydroponic farming offers an excellent opportunity to investigate the influence of enviromental factors on carotenogenesis. Thus, this study was carried out to determine the carotenoid composition of hydroponic leafy vegetables.

MATERIALS AND METHODS

Sampling and sample preparation. To determine the carotenoid composition of marketed vegetables, the samples were purchased from a supermarket at different times during the

winter season, always in the morning, few hours after harvest. Analysis was carried out on arrival at the laboratory.

For each vegetable, 5 bunches collected at different times were analyzed individually. The whole bunch was finely cut, mixed, and 2 to 5 g samples were taken for analysis.

For the comparison of hidroponic and conventionally produced lettuce, three sampling were carried out. At each sampling time, three sample lots were taken from a hydroponic farm and a neighboring conventional farm. Each lot consisted of five heads of lettuce which were cut and mixed, and 5 g samples were taken for analysis.

Analysis. The carotenoid composition was determined according to a procedure described previously (Kimura and Rodriguez-Amaya, 1999). This involved isolation of standards by open column chromatography and quantitative analysis by high performance liquid chromatography. The carotenoids were extracted with cold acetone, partitioned to petroleum ether, concentrated in a rotary evaporator and dried under N_2. The residue was dissolved in 2 mL HPLC grade acetone, filtered through a 0,22 μm PTFE filter (Millipore JBR 610291) and a 10 μL aliquot was injected into the liquid chromatograph.

Lactucaxanthin was first reported by Siefermann-Harms et al. (1981). Found specifically in lettuce, the structure was elucidated by mass spectrometry and nuclear magnetic resonance spectroscopy as ε,ε-carotene-3,3'-diol. In the present work this carotenoid was identified by its chromatographic behavior, reflecting the presence of two hydroxy groups; the absorption spectrum, (466, 436, 412 in petroleum ether and 468, 439, 415 in mobile phase), with defined fine structure (% III/II = 96), consistent with the presence of nine conjugated double bonds in the polyene chain; and the positive response to methylation with

acidic methanol, the R_f value on a silica gel thin-layer plate developed with 5% methanol in toluene increasing from 0.13 before reaction to 0.99 after methylation, demonstrating the allylic position of two hydroxy groups.

The concentrations of standard carotenoids isolated by open column chromatography were determined by visible absorption spectrometry, using the following $A_{1cm}^{1\%}$ values: β-carotene (β,β-carotene), 2592 in petroleum ether; lutein (β,ε-carotene-3,3'-diol), 2550 in ethanol; violaxanthin (5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol), 2550 in ethanol; neoxanthin (5',6'-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro-β,β-carotene-3,5,3'-triol), 2243 in ethanol. For lactucaxanthin a $A_{1cm}^{1\%}$ value of 2944 in petroleum ether was calculated according to the formula which relates the and the molecular masses of two carotenoids of the same chromophore (Davies, 1976), using the $A_{1cm}^{1\%}$ of 3120 of ε,ε-carotene.

HPLC conditions: The HPLC analysis was performed on a Waters separations module (model 2690) equipped with an automatic injector, controlled by Millenium workstation (version 2010), using a monomeric C_{18} column (Spherisorb S3 ODS2), 3 µm, 4.6 x 150 mm. The mobile phase consisted of acetonitrile, methanol and ethyl acetate containing 0.05% of TEA (triethylamine) used at a flow rate of 0.5 mL/min. A concave gradient (curve 10) was applied from 95:5:0 to 60:20:20 in 20 min, maintaining this proportion until the end of the run. Reequilibration took 15 min. A UV-Visible photodiode array detector (Waters model 996) was used. Detection was at the wavelengths of maximum absorption (max plot).

Results of the comparison of hydroponic and field produced curly lettuce were submitted to analysis of variance.

RESULTS AND DISCUSSION

Carotenoid composition of hydroponic leaves. The compositions of four types of lettuce, roquette, cress, and chicory are presented in Table 1. Typical chromatograms of the carotenoids of cress (without lactucaxanthin) and Boston lettuce (with lactucaxanthin) are shown in Figure 1.All the lettuce samples had lactucaxanthin and this is the first report on the quantitative analysis of this carotenoid. The biological significance of lactucaxanthin is not known at the moment.

Table 1. Carotenoid composition (μg/g)[1] of hydroponic leafy vegetables produced in winter.

Sample	Portuguese name	Neoxanthin	Violaxanthin	Lactucaxanthin	Lutein	β-carotene
Curly lettuce	Alface crespa	6.4±1.6	14.3±3.9	8.2±0.9	15.4±1.6	17.1±1.8
French lettuce	Alface crespinha	10.8±2.2	20.1±2.2	11.9±1.3	22.9±2.6	24.6±3.1
Boston lettuce	Alface lisa	9.9±1.7	19.2±1.7	11.8±0.7	21.4±1.4	22.8±1.2
Freelice lettuce	Alface freelice	5.4±1.4	8.1±1.1	6.9±0.4	10.2±1.0	9.9±1.5
Roquette	Rúcula	11.5±2.9	21.0±5.9	nd	52.2±12.6	33.0±9.9
Cress	Agrião	16.8±3.5	25.9±5.3	nd	75.4±10.2	36.9±7.0
Chicory	Almeirão	14.9±4.8	20.7±4.0	nd	57.0±10.3	36.3±7.2

[1] means and standards deviation of five sample lots for each vegetable.

nd. not detected

Of the leafy vegetables analyzed, cress had the highest concentrations of β-carotene, lutein, violaxanthin, and neoxanthin. The lettuce freelice presented the lowest levels of these principal carotenoids. Lutein predominated in roquette, cress, and chicory, whereas β-carotene had slightly higher or equal levels as lutein in the lactucaxanthin-containing lettuce varieties. Having ε-rings and two hydroxy groups, lutein and lactucaxanthin are apparently sharing the same biochemical pathway. As expected, lutein and β-carotene were followed by violaxanthin then neoxanthin, quantitatively.

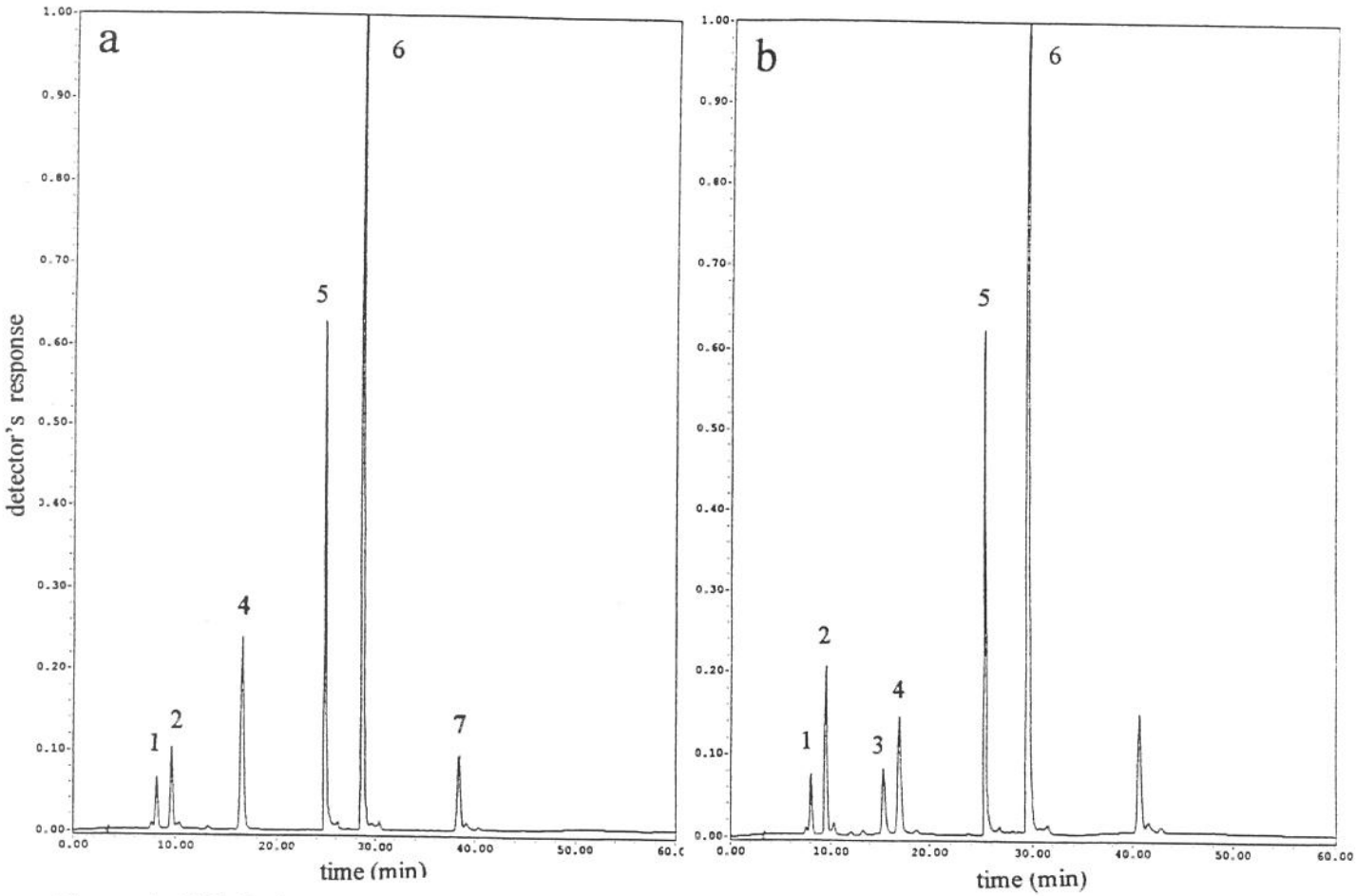

Figure 1. HPLC chromatograms of cress (a) and curly lettuce (b) extracts. Peak identification: 1-neoxanthin; 2-violaxanthin; 3-lactucaxanthin; 4-lutein; 5,6-chlorophylls; 7-β-carotene. HPLC conditions are described in the text.

In a previous paper (Ramos and Rodriguez-Amaya, 1987), the β-carotene contents of conventionally produced curly lettuce, Boston lettuce, roquette, cress, and chicory, analyzed at different times during the year, were 14.5 ± 4.7 μg/g (n=14), 12.6 ± 5.2 μg/g (n=6), 34.6 ± 13.2 μg/g (n=5), 41.5 ± 10.0 μg/g (n=5) and 34.3±9.7 μg/g (n=10), respectively. Except for Boston lettuce, which had lower β-carotene concentration, the results agree well with those of the present study, although the previous data were obtained by open column chromatography and reflected variations during the year as shown by the higher standard deviations.

No comparison can be made in terms of the other carotenoids because no data are available for violaxanthin and neoxanthin whereas the lutein levels were underestimated in the previous study because saponification was carried out. After a thorough investigation of the consequences of the saponification step, under different conditions (Kimura et al., 1990), and considering that chlorophylls can be separated from the carotenoids during chromatography, this step was deleted from the analytical procedure for leafy vegetables.

Comparison of the carotenoid composition of hydroponic and conventionally produced lettuce. A direct comparison of the carotenoid composition of conventionally produced and hydroponic curly lettuce, collected from neighboring farms, was also carried out. The conventionally produced lettuce had significantly higher β-carotene, lutein, violaxanthin, and neoxanthin levels then the hydroponic lettuce (Table 2).

Table 2: Comparison of the carotenoid composition (μg/g)[1] of hydroponic and conventionally produced curly lettuce.

Production	Neoxanthin	Violaxanthin	Lactucaxanthin	Lutein	β-carotene
Conventional	6.2±1.0a	18.7±3.3[a]	7.4±1.3a	16.6±2.6a	19.8±4.3a
Hidroponic	5.1±0.9b	14.7±3.0b	6.4±1.2a	13.3±2.3b	15.3±2.9b

[1] mean and standard deviation of 9 lots; each lot consisted of 5 heads.

values in the same column with different letters are significantly different at $p< 0,05$.

Two processes occur in photosynthetic tissues that have opposite effects on the carotenoid content: enhancement of biosynthesis and photodegradation. Both of these processes are affected by enviromental factors, particularly exposure to sunlight and temperature.

The hydroponic farm from which the samples were taken was covered by a polyethylene roof during the whole year. This controls the amount of sunlight and the temperature to which the vegetables are exposed, which can serve as a protection against photodegradation during the summer. During the winter, the plastic covering may limit exposure to sunlight and lower the temperature to the extent that carotenoid biosynthesis is not stimulated as in vegetables in open fields, explaining the lower carotenoid values of the hydroponic lettuces analyzed in the present study. In a previous investigation, also carried out in the winter, hydroponic curly lettuces harvested on warmer periods presented higher carotenoid concentration than those collected from the same farm on colder days (Miyake and Rodriguez-Amaya, 1998). On the other hand, conventionally produced leaves had been found to contain higher carotenoid content in the winter then in the summer (Heinonen et al,

1989; Mercadante and Rodriguez-Amaya, 1990), during which photodegration of carotenoid could prevail. To provide direct evidence for the above hypotheses, the work herein described should be repeated in the summer.

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