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FONTES DE CAROTENÓIDES IMPORTANTES PARA A SAÚDE HUMANA

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RESUMO GERAL

Bancos de dados de fitoquímicos como carotenóides são muito importantes para servir como base de estudos epidemiológicos, inquéritos alimentares, programas e pesquisas para combater deficiências (como a hipovitaminose A) e prevenir doenças degenerativas.

A determinação de novas fontes de luteína e zeaxantina, carotenóides relacionados à proteção contra degeneração macular e catarata pode significar um grande avanço na prevenção destas doenças. A análise dos carotenóides da *Tropaeolum majus* L. apresentada no capítulo 2, revelou que esta flor representa uma rica fonte de luteína (450 μg/g e 350 μg/g de luteína nas flores amarelas e alaranjadas, respectivamente). As folhas de *Tropaeolum majus* L. também apresentam valores altos de carotenóides quando comparadas às folhas em geral (136 μg/g de luteína, 69 μg/g de β-caroteno, 74 μg/g de violaxantina e 48 μg/g de neoxantina). Foram ainda identificados violaxantina, anteraxantina, zeaxantina, zeinoxantina, β-criptoxantina, α-caroteno e β-caroteno na flor em quantidades muito pequenas.

Os novos dados de composição de vegetais consumidos em saladas determinados no capítulo 3 concordam com a maioria dos resultados apresentados na literatura, mostrando que as folhas verdes são boas fontes de luteína (de 7,7 a 56,1 μg/g) e β-caroteno (de 2,7 a 35,3 μg/g), as cenouras de α-caroteno (35,0 μg/g) e β-caroteno (61,5 μg/g), enquanto o tomate de licopeno (35,4. μg/g). Outra fonte equivalente deste último carotenóide é a melancia (de 34,6 a 36 μg/g), conforme determinado no Capítulo 4, uma fruta consumida largamente no mundo inteiro, entretanto, pouco enfatizada como fonte de licopeno. Uma dieta rica e variada em frutas e vegetais fornece a gama de carotenóides relacionados com a proteção às doenças degenerativas e fortalecimento da resposta imunológica.

GENERAL ABSTRACT

Databases on phytochemicals, such as carotenoids, are very important to serve as bases for epidemiological studies, dietary intake surveys, programs and research to combat deficiencies (such as vitamin A deficiency) and to prevent degenerative diseases.

The search for new sources of lutein and zeaxanthin, carotenoids implicated in the protection against macular degeneration and cataract, may contribute to the prevention of these diseases. The determination of carotenoids in *Tropaelum majus* L., presented in Chapter 2, revealed the flower as rich source of lutein (450 μg/g and 350 μg/g of lutein in the yellow and orange flowers, respectively). The leaves of *Tropaelum majus* L also presented high levels of carotenoids (136μg/g lutein, 69 μg/g β-carotene, 74 μg/g violaxanthin and 48 μg/g neoxanthin) compared to leaves in general. Violaxanthin, anteraxanthin, zeaxanthin, zeinoxanthin, β-cryptoxanthin, α-carotene and β-carotene were also detected in the flower at very low amounts.

New data on the composition of vegetables consumed in salads are presented in Chapter 3, which are in agreement with majority of the results in the literature, showing that green leaves are good sources of lutein (7.7 to 56.1 μg/g) and β-carotene (2.7 to 35.3 μg/g), carrot of α-carotene (35.0 μg/g) and β-carotene (61.5 μg/g), and tomato of lycopene (35.4 μg/g). As presented in Chapter 4, another equivalent source of the latter carotenoid is watermelon (34.6 to 36.0 μg/g), a fruit widely consumed in the entire world but rarely cited as source of lycopene. A diet rich and varied in fruits and vegetables provides the range of carotenoids associated with the protection against degenerative diseases and enhancement of the immunological system.

INTRODUÇÃO GERAL

Além da reconhecida importância como pró-vitamínicos A, os carotenóides possuem propriedades que resultam em possíveis funções biológicas benéficas à saúde humana como o fortalecimento do sistema imunológico e a diminuição do risco de doenças degenerativas (certos tipos de câncer, doenças cardiovasculares, degeneração macular e catarata). A quantificação de carotenóides em fontes existentes e a descoberta de novas fontes de carotenóides com atuação demonstrada na saúde são de fundamental importância para os estudos que correlacionam a ingestão dos carotenóides e a incidência de doenças, assim como para os programas que promovem a saúde e o bem-estar da população.

O presente trabalho teve como objetivos: (a) identificar e quantificar os carotenóides majoritários das flores e folhas de *Tropaeolum majus L.*; (b) determinar os principais carotenóides dos vegetais mais consumidos pela população brasileira na forma crua em saladas; (c) quantificar os principais carotenóides da melancia de maior produção brasileira proveniente de dois estados.

CAPÍTULO 1

A importância dos carotenóides na saúde humana:

Revisão

Artigo a ser enviado ao Instituto Adolfo Lutz

A importância dos carotenóides na saúde humana: Revisão

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RESUMO. Além da reconhecida importância como pró-vitamínicos A, os carotenóides possuem propriedades que resultam em ações biológicas benéficas à saúde humana, como o fortalecimento do sistema imunológico e a diminuição do risco de doenças degenerativas (p.e. certos tipos de câncer, doenças cardiovasculares, degeneração macular e catarata). Desde a década de 80 o interesse e o número de estudos relacionados a essas ações vem crescendo muito. O presente artigo tem como objetivo apresentar uma revisão bibliográfica dos trabalhos relevantes à relação dos carotenóides com a saúde humana.

PALAVRAS-CHAVE. Carotenóides; saúde humana; câncer; doenças cardiovasculares; degeneração macular; catarata.

ABSTRACT. Besides their well-known importance as provitamins A, carotenoids have properties that result in biological actions beneficial to human health, such as enhancement of the immunological system, reduction of the risk of degenerative diseases (e.g. certain types of cancer, cardiovascular diseases, macular degeneration and cataract). Since the 1980s, the number of studies on these health-promoting effects has increased considerably. The objective of the present article is to review relevant studies on the relation of carotenoids with human health.

KEY WORDS. Carotenoids; human health; cancer; cardiovascular disease; macular degeneration; cataract.

INTRODUÇÃO

A introdução da cromatografia líquida de alta eficiência (CLAE) para a análise de carotenóides na década de 70 ocasionou um aumento significativo nos estudos de carotenóides, uma vez que possibilitou a separação e a quantificação mais exata destes compostos, que permitiram a determinação da composição dos carotenóides, tanto nos alimentos quanto no plasma e em tecidos animais.

Os carotenóides mais estudados em relação à saúde humana são o β-caroteno, α-caroteno, licopeno, β-criptoxantina e luteína, por serem os carotenóides mais encontrados no plasma humano[13,65] e a zeaxantina, por apresentar uma concentração muito alta na retina[12,37].

O β-caroteno, α-caroteno e β-criptoxantina são pró-vitamínicos A, sendo que o primeiro apresenta aproximadamente o dobro de atividade do que os demais. A luteína e zeaxantina são os carotenóides relacionados com a proteção à degeneração macular e catarata[75,79,52]. O licopeno, devido ao seu alto potencial como antioxidante natural[24], vem sendo relacionado com a proteção contra câncer e doenças cardiovasculares[23,30, 81].

ATIVIDADE PRÓ-VITAMÍNICA A

Uma função já conhecida e comprovada dos carotenóides é a atividade pró-vitamínica A. Em países em desenvolvimento, onde os produtos de origem animal (fontes de vitamina A pré-formada) não são economicamente acessíveis para toda a população, a vitamina A da dieta é proveniente principalmente das pró-vitaminas A[78]. A ingestão de pró-vitamina A tem a vantagem desta só ser bioconvertida pelo organismo quando há carência, evitando-se assim a hipervitaminose. Os carotenóides que podem ser convertidos em vitamina A são aqueles que

possuem pelo menos um anel β-ionona não substituído, ligado a uma cadeia poliênica conjugada de no mínimo 11 carbonos.

A transformação dos carotenóides pró-vitamínicos em vitamina A ocorre por clivagem central (mecanismo principal), onde o carotenóide é dividido ao meio, formando duas moléculas de retinal no caso do β-caroteno ou uma molécula no caso dos demais carotenóides pró-vitamínicos-A, que são posteriormente transformadas em retinol. Alternativamente, ocorre através da clivagem excêntrica em que segmentos são retirados de uma das extremidades da molécula do carotenóide, formando apocarotenóides e eventualmente retinal[59].

A bioconversão em vitamina A é influenciada por muitos fatores: os que influenciam na atividade da enzima responsável pela clivagem central (β-caroteno-15,15'-dioxigenase) e os que interferem na biodisponibilidade da pró-vitamina A. A biodisponibilidade depende de fatores relacionados ao alimento como a quantidade e estrutura do carotenóide ingerido; biocompetição entre os carotenóides; presença de fibras, gorduras, compostos oxidantes e antioxidantes na dieta; modo de preparação e tamanho das partículas dos alimentos. É influenciada também por fatores intrínsecos ao indívuduo como estado nutricional e incidência de doenças que possam interferir na absorção dos carotenóides[59].

AÇÃO CONTRA DOENÇAS DEGENERATIVAS

Estudos epidemiológicos demonstraram uma associação entre a ingestão ou nível plasmático dos carotenóides e a diminuição do risco ou proteção contra diversas doenças degenerativas tais como: diversos tipos de câncer, doenças cardiovasculares, degeneração macular, catarata.

1. Carotenóides e câncer

Existe uma clara variação na incidência e mortalidade por causa do câncer em diferentes regiões e em populações ao redor do mundo. Em geral os países em desenvolvimento da África, América Latina e Ásia, têm em comum uma taxa relativamente alta de câncer de boca, faringe, laringe, esôfago, fígado (primário) e cérvix. Em contraste, países economicamente desenvolvidos da Europa, América do Norte e Austrália, tendem a uma taxa relativamente alta de câncer de cólon, reto e dos hormonalmente relacionados como o de mama, endométrio e próstata. No mundo inteiro a incidência de câncer de estômago vem diminuindo nas gerações mais recentes, enquanto o de esôfago e pulmão vêm aumentando. Portanto, alguns tipos de câncer variam em taxas de incidência e mortalidade no decorrer dos tempos, de acordo com o envelhecimento das populações, as imigrações, urbanização, entre outros, o que fornece uma clara evidência de que as principais causas destes tipos de câncer são fatores ambientais (incluindo a dieta e estilo de vida), e que podem ser então, preveníveis[29].

Estudos associaram uma baixa ingestão de frutas e vegetais com o aumento do risco de câncer[14,80,95]. A combinação dos resultados de estudos epidemiológicos, com ensaios em animais e estudos *in vitro*, demonstraram uma proteção dos carotenóides em relação ao câncer[6,9,69,70].

Na década de 80 já existiam evidências científicas que relacionavam o β-caroteno com proteção ao risco de câncer, especialmente de pulmão[66]. Nos anos 90, entretanto, dois estudos de intervenção resultaram em efeito inverso, em que a suplementação com β-caroteno elevou a taxa de desenvolvimento de câncer no pulmão em indivíduos de alto risco (fumantes e trabalhadores expostos ao amianto por tempo prolongado)[61,85,]. Em análise posterior destes estudos, verificou-se que o aumento da incidência de câncer ocorreu em pessoas que fumavam mais do que 30 cigarros por dia ou que reportaram um elevado consumo alcoólico[1,60]. O β-caroteno até então

aclamado benéfico à saúde, passou a ser visto como prejudicial. Um estudo[39] realizado em população de baixo risco, no entanto, não relatou efeito adverso com a suplementação do β-caroteno. Na China, em uma das regiões de maior incidência de câncer gástrico e esofágico do mundo e onde a ingestão de micronutrientes é deficiente, a suplementação com mistura de β-caroteno, vitamina E e selênio[15] foi relacionada com uma diminuição de morte por câncer gástrico. Com estes resultados, foi levantada a possibilidade de que os efeitos negativos encontrados nos primeiros dois estudos foram devidos às elevadas doses de β-caroteno administradas diariamente por tempo prolongado[20]. Outra consideração foi a de que *in vivo* o β-caroteno provavelmente atua em conjunto com outros carotenóides ou outros constituintes alimentares, sendo preferível, portanto, a ingestão de frutas e verduras ricas em carotenóides ao invés de suplemento com um carotenóide isolado.

Desde o final da década de 90, o carotenóide que vem ganhando destaque é o licopeno. Devido ao seu elevado potencial como antioxidante natural[24], vem sendo estudado em relação à proteção contra doenças degenerativas[23,30,69], sendo os resultados mais fortes para o câncer de próstata, estômago e pulmão[30].

O mecanismo mais citado pelo qual o carotenóide confere esta proteção é o da ação antioxidante[26,63]. A capacidade antioxidante dos carotenóides é devido ao sistema de duplas ligações conjugadas[46,51,93]. Outros possíveis mecanismos são a modulação do metabolismo de carcinógenos, a inibição da proliferação celular, aumento da diferenciação de células através dos retinóides, estimulação da comunicação intercelular e aumento da resposta imunológica[58,77,86].

2. Carotenóides e doenças cardiovasculares

Aterosclerose é a condição onde as paredes das artérias danificadas e estreitadas pelo depósito de placas, eventualmente bloqueiam o fluxo sanguíneo. Os depósitos de placa podem resultar em hemorragia ou formação de coágulo. Quando a hemorragia ou o coágulo interrompem o fluxo de sangue pelas artérias, ocorre um ataque do coração ou um derrame. Elevados níveis de colesterol – particularmente o colesterol carregado por lipoproteína de baixa densidade (LDL) - estão associados com o aumento do risco de aterosclerose[3]. A modificação oxidativa do LDL pode ser uma chave nos primeiros passos de aterogênese. Antioxidantes da dieta como α-tocoferol, ascorbato, β-caroteno e flavonóides poderiam diminuir a susceptibilidade à oxidação do LDL *in vitro* e em estudos de suplemementação[28]. De 12 estudos epidemiológicos citados por Kris-Etherton et al.[47], os quais investigaram a associação entre o consumo de frutas e vegetais ricos em carotenóides e o risco de doenças cardiovasculares ou coronárias, 10 encontraram associação inversa, sendo que em um a associação foi somente em relação aos vegetais. Um estudo não encontrou nenhuma correlação e apenas um estudo encontrou associação positiva.

Na avaliação de estudos epidemiológicos de Palace et al.[64], 10 estudos confirmaram a relação positiva entre a ingestão de carotenóides e a proteção contra doenças cardiovasculares, sendo que em seis estudos o carotenóide estudado foi o β-caroteno. Três estudos com o β-caroteno não encontraram nenhuma relação.

Um estudo prospectivo[90] relacionou os níveis de carotenóides do plasma (α- e β-caroteno, luteína, licopeno, zeaxantina, β-criptoxantina), vitaminas A e E com aterosclerose e demonstrou o efeito protetor de altas concentrações de α- e de β-caroteno nos estágios iniciais de aterosclerose. Nenhum efeito foi observado com as vitaminas A e E.

Um estudo de caso-controle[25] foi realizado em Rottendam relacionando os níveis de α-caroteno, β-caroteno, β-criptoxantina, luteína, licopeno e zeaxantina no soro com a incidência de aterosclerose. O estudo sugeriu que o licopeno proporcionou ação protetora contra a doença, sendo os efeitos mais pronunciados em fumantes. Os demais carotenóides não apresentaram nenhuma associação.

Em um grande estudo europeu[80] conduzido na Irlanda, Irlanda do Norte, Espanha, França e Holanda, uma dieta de frutas e vegetais ricos em carotenóides elevou a resistência do LDL contra a oxidação, enquanto a suplementação de carotenóides não teve efeitos. Além disso, altas taxas de carotenóides foram associadas com uma diminuição dos danos no DNA.

3. Carotenóides e degeneração macular e catarata

Alguns estudos têm demonstrado que o elevado consumo de luteína e zeaxantina, particularmente de certos alimentos ricos em xantofilas como espinafre, brócolis e ovos, são relacionados à significante redução da catarata (mais de 20%) e da degeneração macular relacionada à idade (mais de 40%)[52].

Vários carotenóides estão presentes no plasma humano, entretanto apenas a luteína e a zeaxantina são encontradas na retina em consideráveis concentrações[12,16,17,36]. Apesar das funções biológicas dos carotenóides nos olhos ainda não serem plenamente compreendidas, são atribuídos 2 tipos de mecanismos na proteção deste órgão: atividade antioxidante e filtragem de luz azul[18,43], considerada a mais energética, e portanto, a que produz maiores danos aos nervos óticos.

A degeneração macular relacionada à idade (AMD) é a principal causa de cegueira em idosos em países desenvolvidos[88]. A luteína e zeaxantina podem ajudar a melhorar a visão ao

longo da vida através dos efeitos diretos na ótica dos olhos, ou evitando a perda visual com a idade, retardando os efeitos cumulativos dos danos oxidativos na retina[35].

A suplementação com espinafre mostrou elevar a quantidade de luteína e zeaxantina, na região macular[35]. A ingestão de luteína e de zeaxantina ocasionou aumento destes no soro e na densidade ótica do pigmento macular[11,17], diminuindo-se o risco de AMD[49]. Um estudo envolvendo autópsia de pessoas com e sem AMD revelou que naquelas que tinham a doença, os níveis dos dois carotenóides na macula eram bem menores comparado com as que não tinham AMD[48]. Dois grandes estudos epidemiológicos[27,75] concluíram que a elevada ingestão de luteína e zeaxantina ocasionavam um aumento da concentração dos mesmos no soro e eram relacionados com a proteção à AMD.

Snodderly[79] concluiu que a combinação das evidências sugere que carotenóides e vitaminas antioxidantes podem ajudar a retardar alguns dos processos destrutivos do pigmento epitelial da retina que leva à degeneração macular ligada à idade.

As proteínas das lentes oculares podem sofrer oxidação e se aglomerarem, impedindo a transmissão da luz e deixando a lente opaca. Esta opacidade da lente é denominada catarata[42]. Estudos epidemiológicos[19,50,52,82] encontraram correlação entre os carotenóides luteína e zeaxantina dietários e a diminuição do risco de catarata.

Em Hankinson et al.[38] o consumo de espinafre e outros vegetais verdes por pelo menos cinco vezes por semana resultou em um risco 47% menor de incidência de catarata do que o consumo por menos de uma vez ao mês. Chasan-Taber[22] reportou que mulheres que ingeriram uma média de 13,7 μg/dia tiveram um risco 22% menor de incidência de catarata do que as que ingeriram uma média de 1,7 μg/dia. Em Olmedilla et al.[56], a suplementação por 2 anos de luteína

melhorou a função visual em pacientes com catarata, enquanto que na suplementação de α-tocoferol não foi observada melhora significativa.

4. Carotenóides e o sistema imunológico

Como revisado por Bendich[8,9], Olson[57] e Semba[76], a administração de carotenóides em animais e a suplementação de β-caroteno em humanos mostrou elevação de vários índices nas funções imunológicas quando comparados com o controle.

Em um estudo de intervenção clínica[68], β-caroteno e ácido 13 cis-retinóico aumentaram significativamente a população de células imunes dos pacientes envolvidos. Em outro estudo de intervenção[89], no entanto, a suplementação com suco de tomate, apesar de ocasionar o aumento de licopeno no plasma, não afetou significativamente as células relacionadas à resposta imunológica.

O stress oxidativo é o principal responsável pela progressão da infecção com o vírus da imuno deficiência (HIV) para a síndrome da deficiência imunológica adquirida (AIDS), o que é demonstrado pela produção em excesso das espécies reativas de oxigênio (ROS) e perda geral das defesas antioxidantes em pacientes infectados com HIV. Foi observado um grande déficit de carotenóides no plasma de pacientes infectados com HIV, que foi atribuído à sua utilização como antioxidantes pelo organismo[94].

FONTES BRASILEIRAS

Atualmente, a posição perante os carotenóides é de cautela em relação à suplementação, mas de incentivo em relação aos carotenóides dietários. A ingestão de frutas e verduras vem sendo indicada por vários programas de saúde[91,92].

As maiores fontes de carotenóides são as frutas e verduras, mas em quantidades muito menores, eles podem ser obtidos de leite e derivados, gema de ovos, alguns peixes e crustáceos e dos carotenóides adicionados como corantes em alimentos.

Conforme citado anteriormente, os carotenóides mais estudados e considerados de grande importância em relação à saúde humana são: α-caroteno, β-caroteno, β-criptoxantina, licopeno, luteína e zeaxantina. O Brasil, em função do clima e da extensão geográfica, possui uma grande variedade de frutas e verduras que são fontes de carotenóides[73,74].

O β-caroteno pode ser encontrado em grandes quantidades em buriti[32], tucumã[73] e bocaiúva[41] e por estar presente em matriz oleosa, pode ter sua biodisponibilidade aumentada. Entre todas as fontes já analisadas no Brasil, o buriti possui a maior concentração deste carotenóide[32], contendo ainda em concentrações substanciais α-caroteno, γ-caroteno e zeaxantina. O β-caroteno está presente também em acerola[21], e pode ser ainda encontrado em manga[31], algumas variedades de abóbora[4,5,7], cenoura[2,34] e óleo de dendê[87]. Estes três últimos, são também boas fontes de α-caroteno. Algumas variedades de abóbora[7,5] e os vegetais folhosos em geral[40,45,54] são também boas fontes de luteína, além do β-caroteno.

A β-criptoxantina é o carotenóide predominante em muitas frutas de coloração alaranjada como cajá[71] (*Spondias lútea*), mamão alaranjado[44], nectarina[33] e pêssego[34].

A fonte de licopeno mais citada atualmente é o tomate[83]. Entretanto ele pode ser encontrado em concentrações ainda maiores em pitanga[21], goiaba[62,67] e produtos de goiaba[67], em

concentrações similares em melancia[55] e em mamão cultivar Tailândia[44] e em menores quantidades em mamão cultivar Solo e Formosa[44].

A flor comestível *Topaeolum majus* L.[53] é uma rica fonte de luteína, enquanto que a zeaxantina pode ser encontrada em pequi (*Cariocar vilosium*).

De um lado as evidências científicas apontam para a ação protetora dos carotenóides em relação à saúde humana, de outro existe uma variedade de fontes destes compostos disponíveis na imensa gama de frutas e verduras produzidos em toda a extensão brasileira. O que falta, portanto, é a conscientização da população e o desenvolvimento de programas de incentivo ao aumento do consumo de frutas e vegetais.

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CAPÍTULO 2

The flowers and leaves of *Tropaeolum majus* L. as rich source of lutein

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The Flowers and Leaves of *Tropaeolum majus* L. as Rich Sources of Lutein

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ABSTRACT

The lutein content of the edible flowers and leaves of *Tropaeolum majus* L. was determined by HPLC-PDAD, complemented by HPLC-MS for identification. The yellow and brownish orange flowers had 450 ± 60μg/g and 350 ± 50 μg/g lutein, respectively. Violaxanthin, antheraxanthin, zeaxanthin, zeinoxanthin, β-cryptoxanthin, α-carotene, and β-carotene were also detected at very low levels. The leaves had 136 ± 18 μg/g lutein, 69 ± 7 μg/g β-carotene, 74 ± 23 μg/g violaxanthin, and 48 ± 13 μg/g neoxanthin. Lutein was partly esterified in the flowers and unesterified in the leaves.

Keywords: *Tropaeolum majus* L., carotenoids, flowers, leaves, lutein

Introduction

Lutein and zeaxanthin make up the yellow pigment in the macula of the human retina (Bone and others 1988; Handelman and others 1988). Dietary intake and plasma levels of these carotenoids were found to have statistically significant inverse relation with the risk of macular degeneration (EDCC 1993; Seddon and others 1994; Snodderly 1995), the principal cause of irreversible blindness in the elderly. There is also consistent evidence of a protective association between lutein in the diet and cataract (Moeller and others 2000). Thus, a search for sources of lutein and zeaxanthin is going on in several countries, and the need to know the contents of these two carotenoids in foods is widely acknowledged. Lutein is a dihydroxy derivative of α-carotene and zeaxanthin a dihydroxy derivative of β-carotene.

On the other hand, poultry feed must contain lutein or zeaxanthin because chicken selectively accumulate these dihydroxy carotenoids which then color the egg yolk, the skin, and the muscle.

The current commercial source of lutein is the inedible marigold (*Tagetes erecta*) flower. Ingested lutein from marigold extract was shown to increase human macular pigment density, which would reduce the risk of macular degeneration (Landrum and others 1997). Leafy vegetables are good to rich sources of lutein. Increased consumption of spinach and other greens was associated with a significant reduction in the risk for macular degeneration (Seddon and others 1994) and cataract (Hankinson and others 1992; Tavani and others 1996; Chasan-Taber and others 1999; Brown and others 1999).

In this work the principal carotenoids of the flowers and leaves of *Tropaeolum majus* L. were quantified. *T. majus* (Nasturtium) is an ornamental, annual, rapid growing, bushy (about 30 cm tall) or vining (may extend up to 90 cm) plant. It has tender, rounded, blue-green, watercress-

flavored leaves (5-15 cm across), held by long fleshy stalks, and showy trumpet-shaped yellow or orange flowers with reddish patches. The entire plant has a spicy peppery flavor. Leaves, flowers and stems are used fresh in salads, and the green pods can be pickled and used as a substitute for capers. This herbal plant is believed to be medicinal, with antimicrobial, antimycotic, expectorant, and purgative properties, and is used for respiratory, ophthalmologic, and urinary tract infections.

Materials and Methods

Sample collection

The flowers were purchased from three supermarkets in the city of São Paulo (São Paulo, Brazil) in small packages of 7 to 10 g. Since the leaves are not usually sold in markets, leaf samples were purchased in packages of around 20 g from one of the major producing farms in the state of São Paulo. The samples were collected at different times during the year.

For each sample lot, the yellow and the brownish orange flowers of one or two packages were separated, weighed (this amounted to 1 to 5 g), and submitted to analysis. The leaves from three packages were homogenized in a food processor and 2-g subsamples were weighed for analysis. The flowers and the leaves were analyzed without the stem.

Carotenoid analysis

The principal carotenoids of the flowers and leaves of *T. majus* were determined by high performance liquid chromatography (HPLC) using a method developed for leaves (Kimura and

Rodriguez-Amaya 2002) and found to be also appropriate for the flowers, from which carotenoids were easy to extract.

The carotenoids from the flowers were extracted with cold acetone, partitioned to petroleum ether and saponified overnight with 10% KOH in methanol. After washing, the carotenoid solution was concentrated in a rotary evaporator and brought to dryness under nitrogen. Immediately before injection into the liquid chromatograph, the carotenoids were redissolved in acetone. The same procedure was followed for the leaves except that saponification was not carried out, considering that the hydroxy carotenoids were not esterified and the chlorophylls were well separated from the carotenoids in the HPLC chromatogram.

Identification of the carotenoids was done according to Rodriguez-Amaya (1999a). This involved the combined use of retention times, co-chromatography with authentic carotenoids, visible absorption spectra (λ_{max} and spectral fine structure) obtained with a recording spectrophotometer (Beckman DU 640) and with the photodiode array detector, and chemical tests for the xanthophylls. Spectral fine structure was expressed as %III/II, the ratio of the height of the longest-wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the two peaks as baseline, multiplied by 100 (Britton, 1995). Chemical reactions such as acetylation with acetic anhydride of secondary hydroxyl group, methylation with acidified methanol of allylic secondary hydroxyl group, and epoxide-furanoid rearrangement of 5,6-epoxy groups were carried out on carotenoids isolated on a MgO:Hyflosupercel (1:1, activated for 4 h at 110 ^{o}C) column. The progress of the reaction was monitored spectrophotometrically and/or by thin layer chromatography (TLC) on silica gel plates developed with 5% methanol in toluene.

The HPLC chromatographic system consisted of a Waters separation module (model 2690), equipped with an autosampler injector and a UV-visible photodiode array detector (Waters model 996), controlled by a Millenium workstation (version 2010). Detection was at the wavelengths of maximum absorption (max plot). Reversed-phase chromatography was carried out using a monomeric C_{18} column (Spherisorb ODS2, 3 μm, 4.6 x 150 mm). The mobile phase consisted of acetonitrile containing 0.05% triethylamine, methanol and ethyl acetate, 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.

The electron impact mass spectrum of lutein was obtained with a Waters Integrity System equipped with a Thermabeam HPLC-MS interface, the temperatures of the expansion region and nebulizer being 80 ^{o}C and 90 ^{o} C, respectively. The ionizing voltage was 70 eV, and the temperature of the ion source was 210 ^{o}C. The m/z range was 150-650.

Results and Discussion

Identity of the carotenoids

Eight carotenoids were identified in the *T. majus* flowers: violaxanthin (5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol), antheraxanthin (5,6-epoxy-5,6-dihydro-β,β-carotene-3,3'-diol), lutein (β,ε-carotene-3,3'-diol), zeaxanthin (β,β-carotene-3,3'-diol), zeinoxanthin (β,ε-carotene-3-ol), β-cryptoxanthin (β,β-caroten-3-ol), α-carotene (β,ε-carotene), and β-carotene (β,β-carotene). The identifying properties of these carotenoids are presented in Table 1 and discussed below.

The carotenoid identified as violaxanthin had a visible absorption spectrum with well-defined spectral structure, typical of a carotenoid with nine conjugated double bonds in the polyene chain. Positive acetylation and the chromatographic behavior (t_R = 9.4 min, R_F = 0.12) demonstrated the presence of two hydroxyl groups while the epoxide-furanoxide rearrangement (hypsochromic shift of 40 nm) proved the existence of two epoxide groups at the 5,6 and 5',6'-positions.

The visible spectrum of antheraxanthin, with λ_{max} at slightly higher wavelengths than those of violaxanthin and less fine structure, was consistent with a carotenoid having nine of 10 conjugated double bonds in the polyene chain and one in a ring. The presence of two secondary hydroxyls was manifested by the chromatographic behavior (t_R = 12.6 min, R_F = 0.15) and the positive reaction to acetylation, the non-allylic position being shown by the negative response to methylation. That a 5,6-epoxide was also present was demonstrated by the hypsochromic shift of 20 nm on addition of dilute HCl.

Lutein showed the same visible spectrum of antheraxanthin, with λ_{max} and fine structure in accordance with a chromophore of 10 conjugated double bonds, nine in the polyene chain and one in a β-ring. The presence of two secondary hydroxyls was confirmed by the positive reaction to acetylation and the chromatographic behavior (t_R = 15.9 min, R_F = 0.21), the allylic position of one of them being shown by the positive response to methylation, forming a monohydroxylated carotenoid.

The identification of lutein was confirmed by HPLC-MS. The mass spectrum showed the molecular ion prominently at *m/z* 568, consistent with $C_{40}H_{56}O_2$, and characteristic fragments at *m/z* 550 $[M-18]^{\cdot+}$ and at *m/z* 532 $[M-18-18]^{\cdot+}$, corresponding to the loss of one and two molecules of water, respectively. Other peaks were observed at *m/z* 476 $[M-92]^{\cdot+}$, due to the elimination of

toluene from the polyene chain, *m/z* 430 $[M-138]^{\cdot+}$ and *m/z* 338 $[M\text{-toluene-}138]^{\cdot+}$ in which 138 corresponded to either the ε or β end group of lutein.

Zeaxanthin presented a visible spectrum with λ_{max} higher than those of lutein and less definition of the peaks, commensurate with a chromophore of 11 conjugated double bonds, two of which situated in rings. Acetylation and the chromatographic behavior (t_R = 17.1 min, R_F = 0.19) confirmed the presence of the hydroxyl groups, the non-allylic position of which was shown by the negative response to methylation.

Zeinoxanthin showed a visible spectrum similar to those of antheraxanthin and lutein, consistent with a carotenoid of 10 conjugated double bonds, one situated in a β-ring. The presence of a hydroxyl group in a non-allylic position was reflected by the positive response to acetylation and negative methylation, and the chromatographic behavior (t_R = 24.4 min, R_F = 0.56).

β-Cryptoxanthin, has the same chromophore as zeaxanthin, with 11 conjugated double bonds, two of which located in β-rings, and thus the same visible spectrum. The existence of a non-allylic hydroxy substituent was demonstrated by the chromatographic behavior (t_R = 26.3 min, R_F = 0.44) and by the positive reaction to acetylation and negative response to methylation.

Not having functional groups, diagnostic chemical reactions are not done with carotenes and the identification is based mainly on the chromatographic behavior and the λ_{max} and fine structure of the visible spectrum.

As lutein, α-carotene (β,ε-carotene) had a visible spectrum manifesting a conjugated double bond system with nine double bonds in the polyene chain and one in a β-ring. β-Carotene exhibited a visible spectrum with λ_{max} higher than those of α-carotene and much less spectral fine structure, commensurate with a chromophore of 11 conjugated double bonds, two of which

situated in rings. The absence of functional groups was shown by the chromatographic behavior (t_R = 36.3 min and R_F = 0.99 for α-carotene, t_R = 37.3 min and R_F = 0.99 for β-carotene).

Cis-β-carotene appeared as the last peak in the HPLC chromatogram and was identified by the λ_{max} lower than those of β-carotene and the *cis* peak at 341. This carotenoid was not separated in the open column.

Because inconclusive or incorrect identifications can be noted in the literature, Pfander and others (1994) and Schiedt and Liaaen-Jensen (1995) recommended that the following minimum criteria for identification be fulfilled: 1) the visible (or ultraviolet for shorter chromophores) absorption spectrum (λ_{max} and fine structure) in at least two different solvents must be in agreement with the chromophore suggested; 2) chromatographic properties must be identical in at least two systems, preferably TLC (R_f) and HPLC (t_R) and co-chromatography with an authentic sample should be demonstrated; and 3) a mass spectrum should be obtained, which allows at least the confirmation of the molecular mass. However, the requirement of a mass spectrum would limit carotenoid analysis to a very few laboratories around the world, precluding its execution in areas where it is probably most needed. We have shown in the case of lutein in this work and other carotenoids in previous studies (Mercadante and others 1997, 1998) that identifications based on the chromatographic behavior, visible spectra and chemical tests (for xanthophylls) were all confirmed by the mass spectra. The judicious and combined use of these identifying parameters can conclusively identify carotenoids with known structures. MS is indispensable for the elucidation of the structures of unknown carotenoids. Erroneous identification in the literature can be observed when the retention time/co-chromatography is used as the only basis for identification or in the case of xanthophylls, only retention time and the

visible spectrum. On the other hand, the mass spectrum, especially when some of the characteristic fragments are missing, cannot be used as the sole criterion for identification.

The typical chromatograms of the carotenoids of saponified and unsaponified samples of *T. majus* flowers are shown in Fig. 1 and 2, clearly demonstrating that lutein is partly esterified. The predominance of lutein is also shown, comprising 65-70% of the total carotenoid content. Interestingly, zeaxanthin, the other carotenoid found in the macula, had higher level than the other minor carotenoids.

The yellow flowers had 450 ± 60 μg/g of lutein while the orange flowers had 350 ± 50 μg/g lutein (Table 2). The average weight of the flower was 0.6 g. Thus, one yellow flower would provide 270 μg of lutein and one orange flower 210 μg lutein. These high values make the *T. majus* flower an excellent functional food.

Although marigold flowers had been the subject of several investigations, it is not easy to find in the literature the concentration range of lutein in this flower, making comparison with that of *T. majus* difficult. Gregory and others (1986) reported that the concentration of lutein ester in fresh marigold varied from 4 μg/g in greenish yellow flowers to 790 μg/g in orange brown flowers but only one sample was analyzed for each color. Rivas (1989) and Hadden and others (1999) found that lutein esters represented 95.5 and 88% of the total carotenoids of marigold petals and of an extract, respectively, but only the area percentages were presented. Delgado-Vargas and Paredes-Lopez (1997) reported a total carotenoid content of 11.4 to 17.4 mg/g in untreated dehydrated marigold meal and 18.0 to 24.7 mg/g in dehydrated meal treated with enzymes to enhance carotenoid extraction. In spite of this confusion, the lutein content of *T. majus* flowers appears comparable to that of marigold.

Figure 3 shows a typical chromatogram of an unsaponified sample of the *T. majus* leaves, showing that lutein and the other hydroxy carotenoids are not esterified. Leaves have been consistently shown to have the same carotenoid pattern, the principal carotenoids being lutein, β-carotene, violaxanthin, and neoxanthin. In any case, the first three carotenoids were identified in the present work by the same identifying parameters as described above. Neoxanthin presented a visible spectrum (λ_{max} in PE = 414, 438, 466 nm; λ_{max} in the mobile phase = 415, 439, 467 nm) with defined spectral fine structure (% III/II = 88), consistent with a chomophore of eight conjugated double bonds and an allene group. The presence of three hydroxyl groups, indicated initially by the chromatographic behavior (t_R = 7.7 min, R_F = 0.07), was confirmed by the positive response to acetylation. The 5,6- to 5,8-epoxide rearrangement (hypsochromic shift of 20 nm) reflected the existence of a 5,6- epoxide.

The concentrations of the principal carotenoids of the leaves are presented in Table 3. These levels are much higher than those found in common commercialized leafy vegetables (Ramos and Rodriguez-Amaya 1987; Mercadante and Rodriguez-Amaya 1991; Rodriguez-Amaya, 1999b; Kimura and others 2003). Thus, the *T. majus* leaves are rich sources of lutein and β-carotene, the latter being the most important provitamin A.

Conclusion

The *T. majus* flower is an excellent source of lutein, the level of this carotenoid being equivalent to that found in marigold. The leaf is a rich source of lutein and β-carotene, the concentration of which and of the other principal carotenoids being higher than those found in common commercialized leafy vegetables.

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Table 1. Wavelengths of maximum absorption and response to chemical test of the carotenoids of *Tropaeolum majus* L. flower

Peak	Identification	λ_{max} (nm) [a]	λ_{max} (nm) [b]	%III/II	Response to chemical tests
1	Violaxanthin	417, 441, 470	416, 440, 468	98	positive to 5,6-epoxy test (2 groups) positive to acetylation (2 OH groups)
2	Antheraxanthin	423, 447, 474	421, 443, 471	50	positive to 5,6-epoxy test (1 group) positive to acetylation (2 OH groups)
3	Lutein	423, 447, 475	421, 443, 472	60	positive to acetylation (2 OH groups) positive to methylation (1 allylic OH)
4	Zeaxanthin	(428)a, 454, 480	(425)a, 448, 476	24	positive to acetylation (2 OH groups) negative to methylation
5	Zeinoxanthin	423, 447, 475	421, 443, 472	63	positive to acetylation (1 OH group) negative to methylation
6	β-Cryptoxanthin	(426)a, 454, 481	(425)a, 448, 476		positive to acetylation (1 OH group) negative to methylation
7	α-Carotene	425, 448, 476	422, 444, 472	58	no substituents
8	β-Carotene	(428)a, 454, 480	(424)a, 448, 476	23	no substituents

[a] λ_{max} (nm) in the mobile phase, obtained by DAD.

[b] λ_{max} (nm) in petroleum ether.

[a] Parenthesis indicates a shoulder; %III/II is the ratio of the height of the longest-wavelenght absorption peak, designed II, taking the minimum between the two peaks as baseline, multiplied by 100.

Table 2. Lutein content of *Tropaeolum majus* L. flowers

Flower	Lutein (μg/g)[a]
Yellow	450 ± 60
Orange	350 ± 50

[a]Means and standard deviations of 8 and 7 sample lots of yellow and brownish orange flowers, respectively, collected at different times during the year.

Table 3. Carotenoid content of *Tropaeolum majus* L. leaves.

Carotenoid	Concentration (μg/g)[a]
Neoxanthin	48 ± 13
Violaxanthin	74 ± 23
Lutein	136 ± 18
β-Carotene	69 ± 7

[a] Means and standard deviations of 6 sample lots collected at different times during the year.

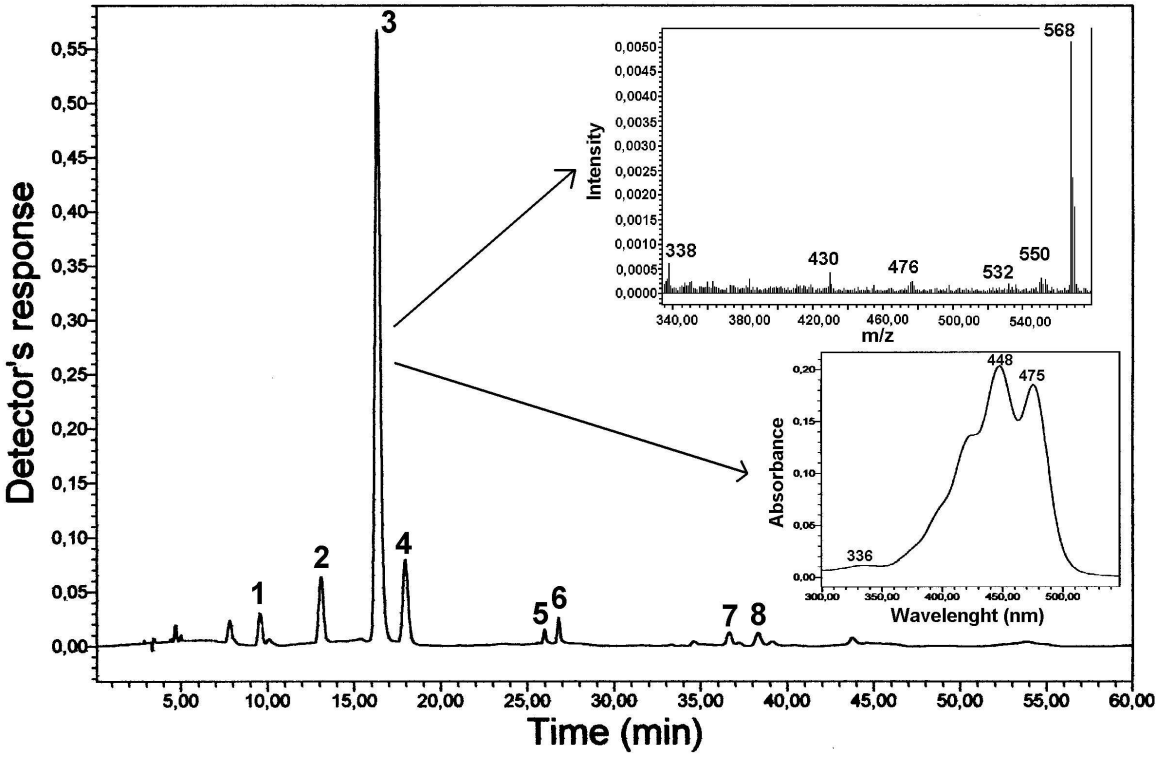

Figure 1. Typical HPLC chromatogram of the carotenoids of a saponified sample of *Tropaeolum majus* L. flowers. For peak identification, see Table 1. Inset: mass and visible absorption spectra.

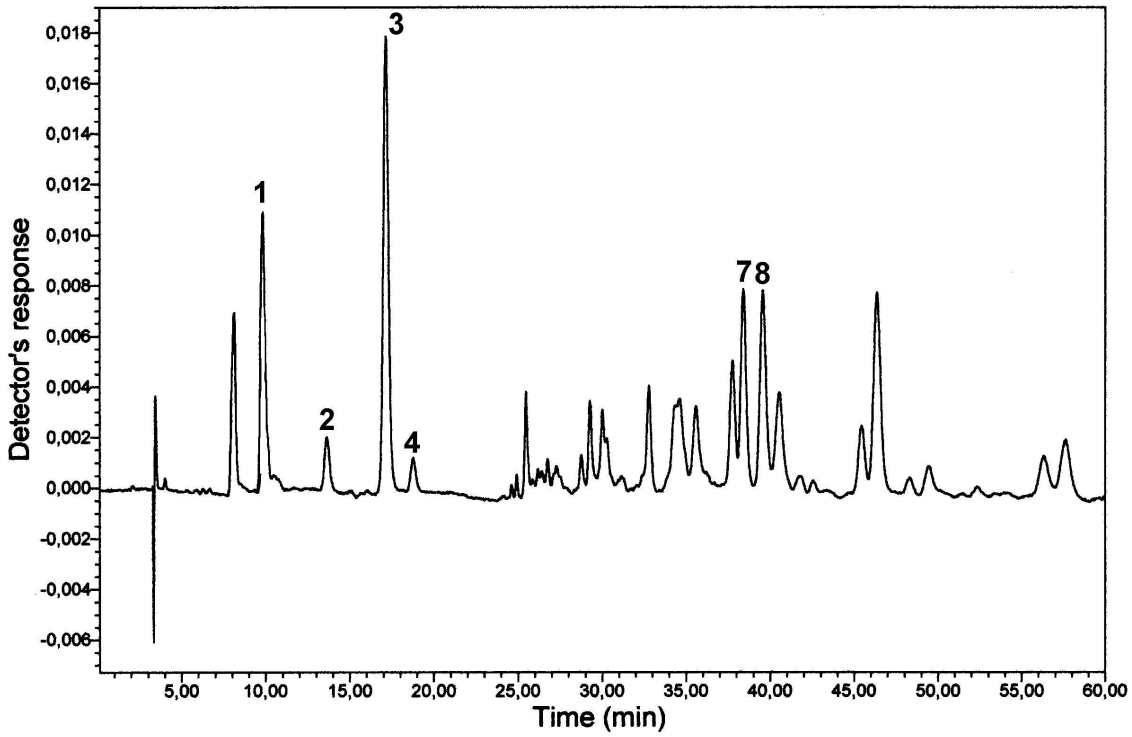

Figure 2. Typical HPLC chromatogram of the carotenoids of an unsaponified sample of *Tropaeolum majus* L. flowers. For peak identification, see Table 1. Peaks in the monohydroxy and carotene region are monoesters and diesters, respectively.

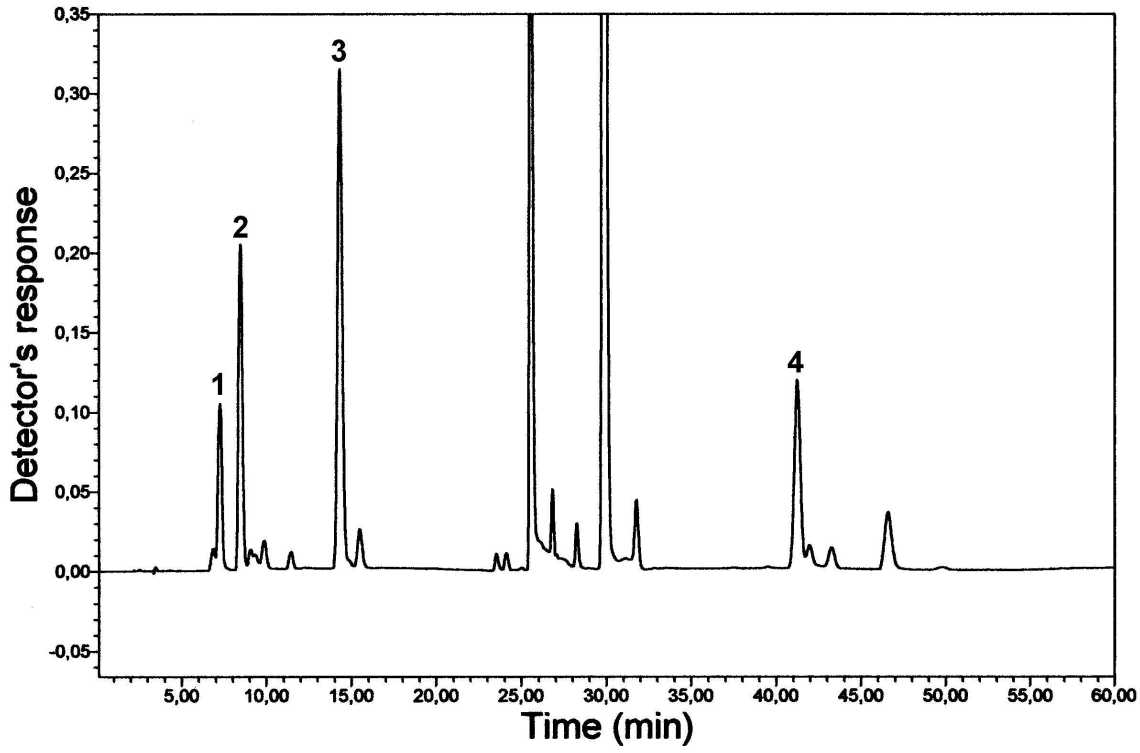

Figure 3. Typical HPLC chromatogram of an unsaponified sample of *Tropaeolum majus* L. leaves. Peak identification: 1. neoxanthin, 2. violaxanthin, 3. lutein, 4. β-carotene. The other principal peaks are those of chlorophylls.

Figure 4. Pictures from yellow and orange flowers and leaves from *Tropaeolum majus* L.

CAPÍTULO 3

New data on the carotenoid composition of raw salad vegetables

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New data on the carotenoid composition of raw salad vegetables

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Abstract

This study was carried out to determine the concentrations of the principal carotenoids of eight vegetables (Nantes carrot, chicory, Boston and curly lettuce, green bell pepper, rucula, Carmen tomato, and cress), which are the most consumed in raw salad by the Brazilian population. The samples were purchased from three major supermarkets in Sao Paulo City. The green vegetables had lutein (7.7 to 56.1μg/g), β-carotene (2.7 to 35.3 μg/g), violaxanthin (4.6 to 31.7 μg/g) and neoxanthin (7.5 to 20.5 μg/g) as principal carotenoids. Boston and curly lettuce also contained lactucaxanthin (7.5 and 6.7 μg/g, respectively). Carrot had α-carotene (35.0 μg/g) and β-carotene (61.5 μg/g) as principal carotenoids and lutein (5.1 μg/g) as minor component. Tomato is a rich source of lycopene (35.4 μg/g) and contains also lutein (1.0 μg/g) and β-carotene (3.2 μg/g) in much smaller amounts.

Keywords: Carotenoids; Leafy vegetables; Bell pepper; Carrot; Tomato.

1. Introduction

Carotenoids are a group of natural pigments responsible for the yellow, orange or red color of many foods. Besides the well-known provitamin A activity of some of these compounds, they have also been associated with lowered risk of developing degenerative diseases such as cancer, cardiovascular diseases, cataract and macular degeneration. Fruits and vegetables are rich sources of these bioactive phytochemicals; many vegetables are accessible year-round sources of carotenoids worldwide.

Reliable data on carotenoids are needed to provide information to consumers and public health workers on food sources of these compounds, to assess dietary carotenoid intake more accurately and to serve as basis for studies on the physiological actions of carotenoids and their relationship to health and diseases. Because of some inherent difficulties, however, carotenoid analysis is not an easy task (Rodriguez-Amaya, 1989, 1997, 1999a). However, tangible improvements in analytical methodology and instrumentation have been achieved in recent years.

Brazil has a wide diversity of carotenoid sources, a good part of which had been analyzed (Rodriguez-Amaya, 1996, 1999b, 2002). Thus, this country has an extensive database on carotenoids. However, more data are needed and recent refinements on the analytical methodology have to be taken advantage of in producing quality data.

In this work, the concentrations of the principal carotenoids of vegetables used in raw salad were determined. The Brazilian population is consuming more salad, and salad bars are becoming more common in restaurants.

2. Materials and methods

2.1 Sampling

The samples were purchased from three big supermarkets in the city of Sao Paulo, Brazil, at different times during the year, totaling six samples analyzed individually for each vegetable. Analysis commenced on arrival of the samples in the laboratory. For the green leaves each sample was composed of two to three bunches of chicory (*Cichorium intybus*) (around 500 g), roquette or rucula (*Eruca sativa*) (around 350 g) or cress (*Nasturtium officinalis*) (around 550 g) and two to three heads of Boston lettuce (*Lactuca sativa*) (around 400 g) or curly lettuce (around 450 g). For the green bell pepper (*Capsicum annum*), each sample consisted of five units (around 800 g), while for carrot (*Daucus carota*) and tomato (*Lycopersicon esculentum*), each sample was composed of 10 units (around 1200 g and 1600 g respectively). The bunches and the heads were cut longitudinally in the middle and one part was combined with those of the other heads or bunches, immediately cut in strips of around 5 cm and homogenized in a food processor, 2 to 6 g being taken for analysis. Green bell pepper, carrot or tomato were quartered, two opposite sections from each unit were combined, immediately cut into pieces of around 5 cm and then homogenized in the food processor. Ten to 17 g of green bell pepper, 2 g of carrot and 3 to 6 g of tomato were taken for analysis.

2.2 Carotenoid analysis

Analyses were carried out in duplicates according to a method developed for leaves (Kimura and Rodriguez-Amaya, 2002). This involved isolation of standards by open column chromatography (OCC) and quantitative analysis by high performance liquid chromatography (HPLC).

Standards of neoxanthin, violaxanthin, lactucaxanthin, lutein, and β-carotene were isolated from lettuce; α-carotene and β-carotene from carrot; lycopene from tomato. Briefly, this involved extraction of the carotenoids with cold acetone, partition to petroleum ether with peroxide-free ethyl ether (10%), concentration in a rotary evaporator, and separation of the carotenoids in a MgO:Hyflosupercel column (for the carotenoids of carrot and tomato, 1:1, activated for 2 h at 110º C; for the carotenoids of leafy vegetables, 2:1, not activated), adjusting the mobile phase, not to separate all the carotenoids present, but to isolate the desired carotenoids as quickly and efficiently as possible. A detailed description of OCC is given in Rodriguez-Amaya (1999a). Purity of each isolate was verified by HPLC. Average purity of the isolated carotenoids was 91%, 97%, 97%, 93%, 99%, 91% and 95% for neoxanthin, violaxanthin, lactucaxanthin, lutein, α-carotene, β-carotene and lycopene, respectively. The concentrations of the standard solution were corrected accordingly.

The carotenoids of the samples were extracted with cold acetone and partitioned to petroleum ether. Saponification was not carried out to avoid losses, especially of the more polar carotenoids (lutein, violaxanthin and neoxanthin) (Kimura et al., 1990). The extract was concentrated in a rotary evaporator (T < 36ºC) and dried under N_2. Immediately before injection, the carotenoids were redissolved in 2 ml HPLC grade acetone and 1 ml was filtered with a 0.22 μm PTFE syringe filter, 10 μL was automatically injected into the HPLC equipment. Quantification was done by external standardization.

The chromatographic system consisted of a Waters separation module (model 2690), equipped with an automatic injector and a UV-visible photodiode array detector (Waters model 996), controlled by a Millennium workstation (version 2010). Detection was at the wavelengths of maximum absorption (max plot). Reversed-phase chromatography was performed using a monomeric C_{18} column (Spherisorb ODS2, 3 μm, 4.6 x 150 mm). For chicory, Boston and curly lettuce, green bell pepper, rucula, and cress, the mobile phase consisted of acetonitrile containing 0.05% triethylamine, methanol and ethyl acetate, 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. For carrot and tomato, the mobile phase consisted of acetonitrile containing 0.05% triethylamine, methanol and ethyl acetate (60:20:20), used at a flow rate of 0.8 ml/min.

Identification of the carotenoids was carried out as described by Rodriguez-Amaya (1999a), involving the combined use of the retention times, co-chromatography with authentic samples, the visible absorption spectra (λ_{max} and spectral fine structure) obtained with a recording spectrophotometer (Beckman DU 640) and with the photodiode array detector, chemical tests such as acetylation with acetic anhydride of secondary hydroxyl groups, methylation with acidic methanol of allylic hydroxyl groups, and epoxide-furanoid rearrangement of 5,6-epoxy groups. Spectral fine structure was expressed as %III/II, the ratio of the height of the longest-wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the two peaks as baseline, multiplied by 100 (Britton, 1995).

3. Results and discussion

The chromatograms of the leafy vegetables and the green bell pepper showed that the principal carotenoids were lutein (β,ε-carotene-3,3'-diol), β-carotene (β,β-carotene), violaxanthin (5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol) and neoxanthin (5',6'-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro-β,β-carotene-3,5,3'-triol) (Figure 1). Lettuce also had lactucaxanthin (ε,ε-carotene-3,3'-diol) as major carotenoid (Figure 1 a). Carrots had α-carotene (β,ε-carotene), and β-carotene as the main carotenoids and lutein as minor component (Figure 1 b). Lycopene (ψ,ψ-carotene) predominated in tomato, which also contained lutein and β-carotene in much smaller amounts (Figure 1 c).

The carotenoid identified as neoxanthin presented a visible absorption spectrum (λ_{max} at 415, 439, 467 nm in the mobile phase and 414, 438, 466 nm in petroleum ether) with defined spectral fine structure (% III/II = 88%), consistent with its chromophore of eight conjugated double bonds and an allenic group located in the polyene chain. The positive response to acetylation confirmed the presence of three hydroxyl groups, initially indicated by the retention time, while a hypsochromic shift of 20 nm on addition of dilute HCl reflected the rearrangement of a 5,6- to a 5,8-epoxide group.

Violaxanthin had a visible absorption spectrum (λ_{max} at 417, 441, 470 nm in the mobile phase and 416, 440, 468 nm in petroleum ether) with well defined peaks (% III/II = 98%), consistent with a chromophore of nine conjugated double bonds, all in the polyene chain. The presence of two hydroxyl groups was demonstrated by the retention time and the positive acetylation. The hypsochromic shift of 40 nm, resulting from the epoxide-furanoxide rearrangement, manifested the presence of 5,6- and 5',6' epoxides.

Lutein displayed a visible absorption spectrum (λ_{max} at 423, 447, 475 nm in the mobile phase and 421, 443, 472 nm in petroleum ether) with less defined spectral structure (% III/II = 60%),

typical of a carotenoid of 10 conjugated double bonds, one located in a β-ring. The retention time and the positive response to acetylation revealed the presence of two hydroxyl groups, the allylic position of one of which was shown by the positive response to methylation, resulting in a monohydroxy product.

Lycopene presented the visible absorption spectrum (λ_{max} at 446, 473, 504 nm in the mobile phase and 442, 468, 500 nm in petroleum ether and % III/II = 65%) of a chromophore of 11 conjugated double bonds in the polyene chain. α-carotene had a visible absorption spectrum similar to that of lutein, consistent with its chromophore of 10 conjugated double bonds, one of which situated in ring. The visible absorption spectrum of β-Carotene (λ_{max} at 454, 480 nm and a shoulder at 428, nm in the mobile phase and at 448, 472 nm and a shoulder at 424 nm in petroleum ether) with little fine structure (% III/II = 25%) agreed with a chromophore of 11 conjugated double bonds, two of which located in β-rings. The absence of substituents in these hydrocarbon carotenoids was manifested by the chromatographic behavior. They also co-chromatographed with authentic carotenoids.

In general, all the vegetables appeared to be good sources of carotenoids, with the exception of green bell pepper which had lower carotenoid content (Tables 1 and 2). The leafy vegetables analyzed had 14 to 56 μg/g of lutein, with cress having the highest level and Boston lettuce the lowest (Table 1). The β-carotene content varied from 15 to 35 μg/g, chicory having the highest and Boston lettuce the lowest concentration. As it is well known, tomato is a rich source of lycopene and carrot of α-carotene and β-carotene (Table 2).

Heinonen (1990), Granado et al. (1992), Khachik et al. (1992), Hart and Scott (1995), Lessin et al. (1997), and Burns et al. (2003) also quantified carotenoids in vegetables. In Brazil Ramos and Rodriguez-Amaya (1986), Almeida and Penteado (1987), Tavares and Rodriguez-Amaya

(1994), Godoy and Rodriguez-Amaya (1998), Kimura and Rodriguez-Amaya (2003) determined the carotenoids in Brazilian vegetables.

In the paper of Ramos and Rodriguez-Amaya (1986), which was basically on provitamin A, the vitamin A inactive lutein was also quantified. However, this work was done before the detailed assessment of the saponification step (Kimura et al., 1990) and lutein was underestimated due to losses during saponification and the subsequent washing. Thus, lutein is not considered in the comparative discussion below.

For chicory, Ramos and Rodriguez-Amaya (1986) reported 35 μg/g of β-carotene. These values are similar to those of the present work, although the former paper employed open column chromatography and the present study HPLC. In Boston and curly lettuce, Ramos and Rodriguez-Amaya (1986) found 16μg/g and 15 μg/g of β-carotene, respectively. Kimura and Rodriguez-Amaya (2003) reported for neoxanthin, violaxanthin, lactucaxanthin, lutein and β-carotene in hydroponic Boston lettuce 10 μg/g, 19 μg/g, 17 μg/g, 21 μg/g, and 23 μg/g, respectively, and in curly lettuce 6 μg/g, 16 μg/g, 7 μg/g, 15 μg/g, and 18 μg/g. These results are generally in agreement with those in Table 1. In an unspecified cultivar of lettuce, Burns et al. (2003) found 52 μg/g neoxanthin, 167 μg/g violaxanthin, 124 μg/g lutein and 230 μg/g β-carotene. These results are about 10 times higher than our results and those of others in the literature and could be calculation errors.

In rucula, Ramos and Rodriguez-Amaya (1986) obtained 35 μg/g for β-carotene and Kimura and Rodriguez-Amaya (2003) for hydroponic leaves, 12 μg/g neoxanthin, 21 μg/g violaxanthin, 52 μg/g lutein and 33 μg/g β-carotene. In cress Ramos and Rodriguez-Amaya reported 42 μg/g for β-carotene and Kimura and Rodriguez-Amaya (2003) found 17 μg/g neoxanthin, 26 μg/g violaxanthin, 76 μg/g lutein and 37 μg/g β-carotene. Hart and Scott (1995) got 107 μg/g for

lutein and 48 μg/g for β-carotene. The lower values found for lutein and for β-carotene in the present work are probably due to the amount of stem incorporated in the sample.

Hart and Scott (1995) also demonstrated that green bell pepper is a poor source of carotenoid. The data for lutein (7 μg/g) and for β-carotene (3 μg/g) are similar to those obtained in the present work. Again Burns et al. (2003) found values 10 times higher (34 μg/g for lutein and 20 μg/g for β-carotene).

The data obtained for carrot in the present work are higher than those reported previously for Nantes carrot produced in Brazil. Godoy and Rodriguez-Amaya (1998) found 17 μg/g α-carotene and 33 μg/g β-carotene; Almeida-Muradian and Penteado (1987) reported 22 μg/g of α-carotene and 34 μg/g of β-carotene, almost half of the values in the present work. Carvalho et al. (1992) obtained for α-carotene the range of 23 to 42 μg/g, and for β-carotene 37 to 54 μg/g, results that are more similar to the present work. The first two papers used open column chromatography and the latter HPLC. The levels reported by Bushway (1986), Heinonen (1990), Hart and Scott (1995) and Lessin et al. (1997) ranged from 20 to 52 μg/g for α-carotene and from 56 to 115 μg/g for β-carotene, The results obtained in the present work fall within this range. Burns et al. (2003) reported even higher results (45 μg/g for α-carotene and 321 μg/g for β-carotene).

Although the cultivar was different, the present data agree with those obtained for the Brazilian Santa Cruz tomato by Tavares and Rodriguez-Amaya (1994), 31 μg/g of lycopene and 5 μg/g of β-carotene. Khachik et al. (1992), Hart and Scott (1995), Konings and Roomans (1997) and O'Neill et al. (2001) got similar results, ranging from 27 to 39 μg/g for lycopene, from 3 to 6 μg/g for β-carotene, and 1 μg/g for lutein. Buns et al. (2003) again reported results 10 times higher, 523 μg/g of lycopene and 56 μg/g of β-carotene.

α-Carotene and β-carotene are provitamin A carotenoids. β-carotene is the most potent provitamin A, having about twice as much activity as α-carotene. Lycopene had been shown to be a more efficient antioxidant than β-carotene (Di Mascio et al., 1989) and had been associated with the lowered risk of developing cancer, the evidence being stronger for lung, prostate and stomach cancer (Giovanucci, 1999), and cardiovascular diseases (Kolmeier and Hastings, 1995). Lutein is the carotenoid implicated in the reduced risk of cataract and macular degeneration (Seddon, 1994; Snodderly, 1995; and Moeller, 2000), together with zeaxanthin. Although possible health benefits of neoxanthin, violaxanthin, lactucaxanthin and other carotenoids have not been shown, their determination is important for future studies, as they have similar structures to those of carotenoids considered important to human health. These carotenoids are not provitamins A, but the antioxidant property is linked with the conjugated double bond system, the maximum protection being given by carotenoids having more than nine double bonds (Foote et al., 1970).

Eating salads of different kinds of vegetables appears to be an inexpensive, healthy and secure way of consuming carotenoids, aside from other phytochemicals that may also be health promoting.

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TABLE 1. Concentration of the major carotenoids of raw green vegetable

	Carotenoid (μg/g)[a]				
Vegetable	**Neoxanthin**	**Violaxanthin**	**Lactucaxanthin**	**Lutein**	**β-carotene**
Chicory	**20.5 ± 4.8**	**31.7 ± 8.1**	**-**	**53.7 ± 8.3**	**35.3 ± 5.0**
Boston lettuce	**7.5 ± 2.0**	**18.0 ± 4.9**	**7.5 ± 3.4**	**13.5 ± 4.3**	**14.9 ± 4.6**
Curly lettuce	**7.6 ± 1.6**	**18.7 ± 2.4**	**6.7 ± 1.8**	**14.3 ± 2.4**	**15.5 ± 4.2**
Green bell pepper	**3.1 ± 0.5**	**4.6 ± 1.4**	**-**	**7.7 ± 1.6**	**2.7 ± 0.4**
Rucula	**18.1 ± 5.5**	**29.7 ± 7.3**	**-**	**50.0 ± 4.4**	**28.4 ± 1.5**
Cress	**17.7 ± 1.7**	**26.1 ± 6.3**	**-**	**56.1 ± 7.3**	**27.2 ± 4.5**

[a]Means and standard deviations of 6 sample lots collected at different times during the year.

TABLE 2 Concentration of the major carotenoids of raw carrot and tomato

Vegetable	Carotenoid (μg/g)[a]			
	lutein	lycopene	α-carotene	β-carotene
carrot	5.1 ± 1.0	-	35.0 ± 5.0	61.5 ± 9.0
tomato	1.0 ± 0.2	35.4 ± 9.5	-	3.2 ± 0.6

[a]Means and standard deviations of 6 sample lots collected at different times during the year.

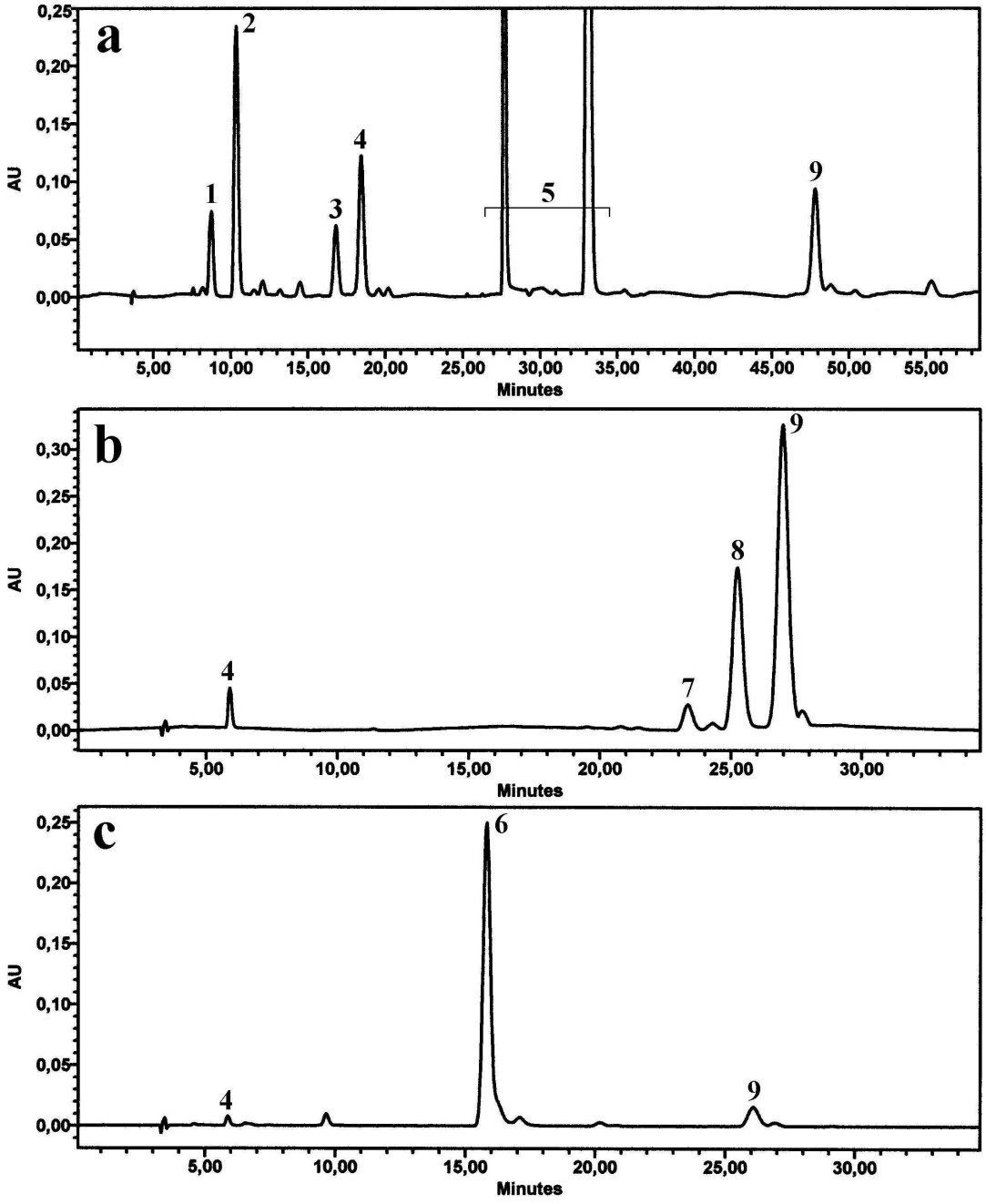

FIGURE 1. HPLC chromatograms of (a) unsaponified green vegetable (lettuce), (b) carrot and (c) tomato. Chromatographic conditions are described in the text. Peak identification: (1) neoxanthin, (2) violaxanthin, (3) lactucaxanthin, (4) lutein, (5) chlorophylls, (6) lycopene, (7) ζ-carotene, (8) α-carotene, (9) β-carotene.

CAPÍTULO 4

A MELANCIA COMO FONTE DE LICOPENO

Artigo a ser enviado ao Instituto Adolfo Lutz

A MELANCIA COMO FONTE DE LICOPENO

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RESUMO.

Este trabalho teve como objetivo quantificar os principais carotenóides da melancia, variedade Crimson Sweet, produzida nos estados de São Paulo e Goiás. As amostras foram colhidas durante o ano da Central de Abastecimento (CEASA) de Campinas, em um total de cinco frutas analisadas individualmente para cada região. As análises foram realizadas em duplicata, consistindo-se na extração com acetona, partição para éter de petróleo e quantificação por cromatografia líquida de alta eficiência (CLAE), coluna Spherisorb C_{18} ODS2, 3 μm, 4,6 mm x 150 mm, eluição isocrática, fase móvel de acetonitrila:metanol:acetato de etila 60:20:20, com um fluxo de 0,8 mL/min, utilizando padronização externa. Os cromatogramas demonstraram que a melancia contém quase exclusivamente licopeno, com uma pequena quantidade de β-caroteno. Os teores (μg/g) de licopeno e β-caroteno foram respectivamente de 36,0 $\pm$ 5,4 e 4,7 $\pm$ 2,4 para as frutas de São Paulo e de 34,6 $\pm$ 2,1 e 2,6 $\pm$ 1,7 para as de Goiás. As concentrações destes dois carotenóides são semelhantes às encontradas em tomate cultivar Carmen (35,4 $\pm$ 9,5 μg/g para licopeno e 3,2 $\pm$ 0,6 μg/g para β-caroteno), evidenciando a melancia como uma importante fonte de licopeno. As diferenças em termos do local de produção não foram significativas.

PALAVRAS-CHAVE. Carotenóides; licopeno; melancia; análise; CLAE.

WATERMELON AS SOURCE OF LYCOPENE.

ABSTRACT.

This work had the objective of quantifying the principal carotenoids of the watermelon cultivar Crimson Sweet obtained from two producing states: São Paulo and Goiás. The samples were purchased during the year from the Central Distribution Center (CEASA) of Campinas, totalling five fruits analysed individually for each region. The analyses carried out in duplicate, consisted of extraction with acetone, partition to petroleum ether and quantification by high performance liquid chromatography (HPLC) (Spherisorb column C18 ODS2, 3 μm, 4,6 mm x 150 mm; isocratic elution, mobile phase of acetonitrile:methanol:ethyl acetate at 60:20:20; flow rate of 0,8 of mL/min), using external standardization. The HPLC chromatogram revealed that the watermelon analyzed contained almost exclusively lycopene, with a small amount of β-carotene. The lycopene and β-carotene contents (μg/g) were, respectively, 36,0 $\pm$ 5,4 and 4,7 $\pm$ 2.4 for the fruits from São Paulo, and 34,6 $\pm$ 2,1 and 2,6 $\pm$ 1,7 for those from Goiás. The concentrations of these two carotenoids resembled those found in tomato cultivar Carmen (35,4 $\pm$ 9,5 μg/g for lycopene and 3,2 $\pm$ 0,6 μg/g for β-carotene), showing watermelon to be an important source of lycopene. The difference in terms of place of production was not significant.

KEY WORDS. Carotenoids; lycopene; watermelon; analysis; HPLC.

INTRODUÇÃO

O consumo de frutas e vegetais tem sido fortemente relacionado com a diminuição do risco de doenças degenerativas e vem sendo recomendado por programas governamentais e não governamentais.

Entre os possíveis fitoquímicos presentes associados a essa proteção estão os carotenóides. Estudos vêm demonstrando a relação dos carotenóides com o fortalecimento do sistema imunológico e com a diminuição do risco de doenças como certos tipos de câncer, doenças cardiovasculares, degeneração macular e catarata[1,7,10,13]. Dentre os carotenóides, o licopeno vem ganhando destaque devido à sua alta eficiência como antioxidante natural[4] e sua possível ação contra doenças degenerativas[3,5,16,21,26], as evidências sendo mais fortes para câncer de próstata, estômago e pulmão[5]. A fonte de licopeno mais investigada atualmente é o tomate[5,11,16,24], mas a busca de outras fontes ocorre em vários países. O licopeno é encontrado também em goiaba vermelha (*Psidium guajava)*[15,18], mamão vermelho (*Carica papaya*)[9] e pitanga (*Eugenia uniflora*)[2]. A melancia (*Citrullus lanatus*) também deve a sua cor ao licopeno e apesar de consumida largamente no mundo todo, tem sido pouco estudada. Segundo o Programa de desenvolvimento da Fruticultura[19] (Profruta) do Ministério da Agricultura, Pecuária e Abastecimento, em 2002, a produção mundial anual de melancia foi de 51.657.568 ton. e a brasileira de 833.666 ton.

O presente trabalho teve como objetivo quantificar os carotenóides majoritários (licopeno e β-caroteno) presentes na melancia, comparando-se frutas de dois estados de grande produção brasileira, São Paulo e Goiás, segundo a Companhia de Entrepostos e Armazéns Gerais de São

Paulo (CEAGESP). Como objeto de estudo foi escolhido o cultivar Crimson Sweet, por ser o mais produzido no Brasil[20].

MATERIAL E MÉTODO

As amostras de melancia (*Citrullus lanatus*) cultivar Crimson Sweet foram coletadas em diferentes épocas durante o ano de 2002, diretamente da Central de Abastecimento (CEASA) de Campinas. Foi analisado para cada procedência um total de cinco frutas maduras escolhidas aleatoriamente de grandes lotes e analisadas individualmente em duplicata. O cultivar Crimson Sweet caracteriza-se por ser uma melancia de coloração verde claro, com listras verde escuro no sentido longitudinal, com formato redondo ovalado de 30-40 cm de comprimento, de 25-35 cm de diâmetro e peso variando de 11 a 14 kg.

O fruto foi quarteado no sentido longitudinal e duas partes opostas foram homogeneizadas em multiprocessador durante 15 segundos, após a remoção da casca e sementes. Amostras de 2,0 a 2,6 g da polpa homogeneizada foram retiradas para análise imediata.

A composição de carotenóides foi determinada por cromatografia líquida de alta eficiência (CLAE), utilizando uma metodologia adaptada de Kimura e Rodriguez-Amaya[8] que envolve o isolamento por coluna aberta e quantificação dos padrões por espectrofotômetro UV/Visível e análise quantitativa das amostras por CLAE, utilizando padronização externa.

A obtenção dos padrões consistiu em extração de polpa de melancia com acetona gelada, partição para éter de petróleo, e evaporação em evaporador rotativo. Os carotenóides licopeno e β-caroteno foram isolados em coluna aberta recheada com MgO:Hiflosupercel (1:2), desenvolvida com éter de petróleo contendo porcentagens crescentes de éter etílico (até 8%) e

acetona (até aproximadamente 20%). A pureza foi verificada por CLAE e variou de 95 a 98% para o licopeno e de 94 a 96% para o β-caroteno. As concentrações da solução padrão foram corrigidas pelas porcentagens de pureza. A quantificação dos padrões foi realizada com espectrofotômetro UV/Vis.

A extração das amostras foi realizada com acetona gelada, partição para éter de petróleo, concentração em evaporador rotativo seguida de evaporação total utilizando nitrogênio. A etapa de saponificação não foi necessária, uma vez que os principais carotenóides não eram hidroxicarotenóides esterificados. Imediatamente antes da injeção no cromatógrafo, as amostras foram diluídas em acetona grau HPLC e filtradas.

A identificação dos carotenóides foi realizada de acordo com Rodriguez-Amaya[22], utilizando em conjunto o tempo de retenção, co-cromatografia com carotenóide autêntico, espectro de absorção UV/Visível ($\lambda_{máx}$ e estrutura espectral fina) obtido com espectrofotômetro (Beckman DU 640) e com detector de arranjo de diodos. A estrutura espectral fina é dada pelo cálculo da razão III/II multiplicada por 100, onde III é a altura do pico correspondente ao maior comprimento de onda e II à do comprimento de onda intermediário, definindo-se o mínimo entre os dois picos como linha de base.

Para a quantificação dos carotenóides por padronização externa, a curva de calibração foi construída com cinco pontos em triplicata e a faixa de concentração utilizada foi de 14,5 a 64,8 μg/g para o licopeno e de 3,7 a 8,1 μg/g para o β-caroteno.

O sistema cromatográfico era composto por um módulo de separação Waters (modelo 2690), controlado por um software Millenium (versão 2010). A detecção foi realizada em comprimento de absorção máxima (max plot). A cromatografia de fase reversa ocorreu em uma coluna monomérica C_{18} (Spherisorb ODS2, 3 μm, 4,6 x 150 mm). A eluição foi isocrática com a

fase móvel composta por acetonitrila contendo 0,05% de trietilamina, metanol e acetato de etila na proporção de 60:20:20, com fluxo de 0.8 mL/min.

RESULTADOS E DISCUSSÃO

A Figura 1 apresenta um cromatograma típico dos carotenóides da melancia cultivar Crimson Sweet, demonstrando a predominância do licopeno (ψ,ψ-caroteno). Este carotenóide foi identificado pelo seu espectro de absorção na região visível (λ_{max} a 442, 468, 500 nm em éter de petróleo e a 446, 473, 504 nm na fase móvel; estrutura espectral % III/II = 65), em acordo com um cromóforo de 11 duplas ligações conjugadas, todas na cadeia poliênica. A ausência de grupos funcionais foi demonstrada pelo comportamento cromatográfico (t_R = 10,9 min.). O β-caroteno (β,β-caroteno) em éter de petróleo apresentou λ_{max} a 448 e 476 nm com um ombro a 424 nm, e na fase móvel a 454 e 480 nm, com ombro a 428 nm, tendo pouca estrutura espectral (% III/II = 25), refletindo um cromóforo de 11 duplas conjugadas também, entretanto, com duas duplas em anéis β. O tempo de retenção (t_R = 17,9 min.) indicou a ausência de substituintes.

A Tabela 1 mostra os resultados obtidos na quantificação de licopeno e β-caroteno. A análise de variância (ANOVA) demonstrou que os resultados obtidos não diferem significativamente ao nível de significância de 5 % entre as duas regiões.

Perkins-Veazie et al.[17], determinaram espectrofotometricamente os teores de licopeno em 11 cultivares de melancia, obtendo valores de 36,5 a 71,2 μg/g. O menor valor encontrado foi no cultivar Crimson sweet (36,5 $\pm$ 1,7 μg/g), nível muito similar ao encontrado no presente trabalho.

Pelos relatos de maiores teores de licopeno em melancia, deve ser considerada no Brasil a produção de cultivares com maiores teores.

Outros trabalhos também relataram o teor de licopeno em melancia, entretanto, sem especificação dos cultivares: Holden et al. [6,28] (48,7 μg/g), O'Neill et al.[14] (37,8 μg/g) e Setiawan et al.[25] (113,89 μg/g). Este último valor, porém, pode ter sido uma superestimação uma vez que estes autores acharam este carotenóide em frutas, como abacaxi, onde jamais foi encontrado.

Uma vez que é consumida no mundo todo e praticamente durante o ano inteiro, a melancia torna-se uma fonte importante de licopeno. Para fins comparativos, outras fontes brasileiras de licopeno encontram-se na Tabela 2. O conteúdo de licopeno encontrado na melancia *cultivar* Crimson Sweet é menor do que na goiaba (*Psidium gujava*) e pitanga (*Eugenia uniflora*), maior do que no mamão (*Carica papaya*), e equivalente ao do tomate (*Licopersicon esculentum*).

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Tabela 1. Concentrações dos carotenóides principais em melancia (Citrullus lanatus) Cv. Crimson Sweet proveniente de duas regiões brasileiras.

Procedência	Licopeno (μg/g de polpa)	β-caroteno (μg/g de polpa)
São Paulo	36a ± 5	4,7a ± 2,4
Goiás	35a ± 2	2,6a ± 1,7

Letras iguais em uma mesma coluna indicam que não houve diferença significativa ao nível de significância de 5%.

Tabela 2. Outras fontes brasileiras de licopeno.

Fonte	Cultivar	Origem da amostra	Licopeno (μg/g)	Referência
GOIABA (*Psidium guajava*)	IAC-4	São Paulo	53 ± 6	15
	Paluma	São Paulo	69 ± 5	18
	Ogawa	São Paulo	58 ± 9	18
MAMÃO (*Carica papaya*)	Solo	Bahia	21 ± 16	9
	Formosa	Bahia	26 ± 3	9
	Tailândia	Bahia	40 ± 6	9
PITANGA (*Eugenia uniflora*)		Pernambuco	73 ± 1	2
TOMATE (*Licopersicon esculentum*)	Santa Cruz	São Paulo	31 ± 20	27
	Carmen	São Paulo	35 ± 10	12

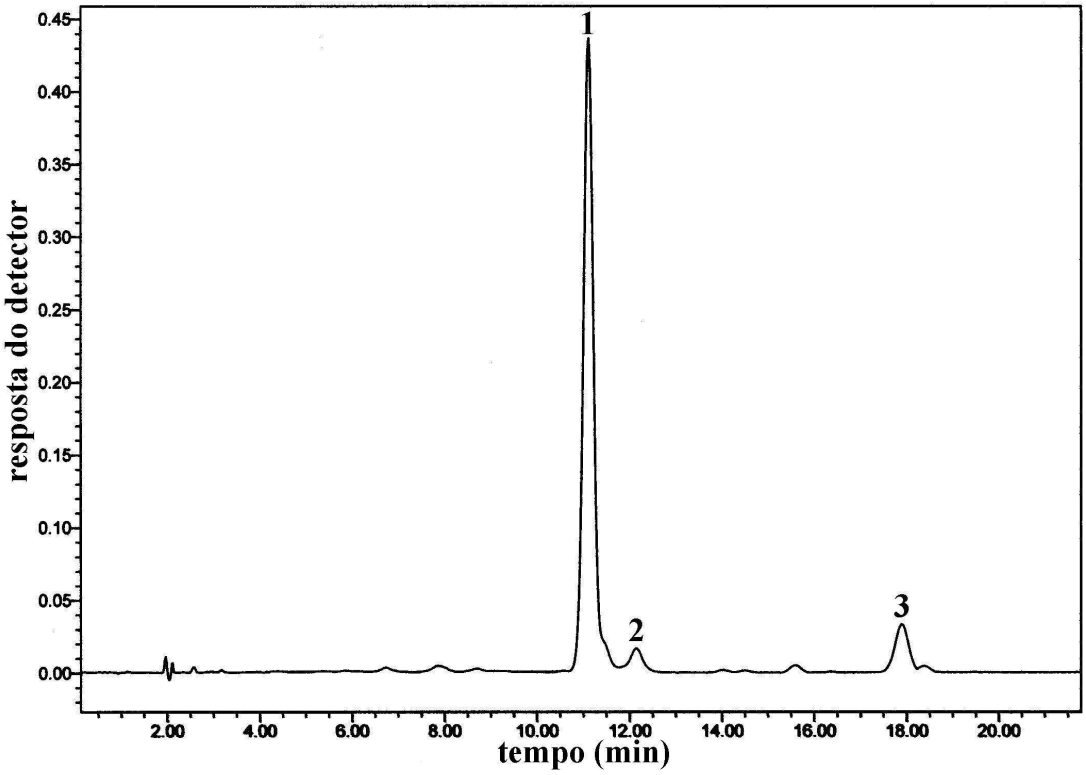

Figura 1. Cromatograma característico dos carotenóides de melancia (*Citrullus lanatus*) Cv. Crimson Sweet**.** Condições cromatográficas: coluna C_{18}, Spherisorb ODS2, 3 μm, 4.6 x 150 mm; fase móvel: acetonitrila contendo 0,05% de trietilamina, metanol e acetato de etila (60:20:20); fluxo: 0.8 mL/min; detector de arranjo de diodos. Identificação dos picos: 1. *trans*-licopeno; 2. *cis*-licopeno; 3. β-caroteno.

Figura 2. Fotografia da melancia (*Citrullus lanatus*) Cv. Crimson Sweet**.**