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CAROTENÓIDES EM ALIMENTOS PREPARADOS PARA O CONSUMO: COMPARAÇÃO DE ANÁLISE DIRETA E CÁLCULO PELOS DADOS DE RETENÇÃO

PARECER

Este exemplar corresponde à redação final da tese defendida por **Marcela Colognesi de Sá** aprovada pela Comissão Julgadora em 11 de julho de 2001.

Campinas, 11 de julho de 2001

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Não se contentem com as migalhas,

vocês só têm uma vida, mirem alto.

Não se contentem com as

pequenas alegrias, procurem as grandes.

Procurem a plenitude da alegria.

Não se contentem com as pequenas coisas,

pois Deus as quer grandes.

(*Chiara Lubich*)

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Aos meus pais, *Rubens* e *Maria Luzia*

que são em grande parte responsáveis

por todas as minhas conquistas

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MUITO OBRIGADA

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INTRODUÇÃO GERAL

A importância dos carotenóides para a saúde humana é reconhecida tanto pela atividade pró-vitamínica A que alguns destes compostos apresentam, como pelas suas ações antioxidante e imunomoduladora, que levam à diminuição do risco de doenças degenerativas como o câncer, a degeneração macular e as doenças cardiovasculares. Portanto, a obtenção de data-bases confiáveis e mais completas sobre carotenóides é prioritária no mundo inteiro.

Dados sobre a composição de carotenóides em alimentos preparados para o consumo ainda são poucos e a grande maioria se restringe ao β-caroteno. Há, portanto, necessidade de geração de dados nos alimentos como são consumidos, por meio de análise conduzida de acordo com plano de amostragem representativo e métodos validados, ampliando a análise para os outros carotenóides principais dos alimentos.

A análise de carotenóides nos alimentos cozidos pode apresentar algumas dificuldades diferentes daquelas de amostras cruas, como a formação de produtos de degradação e presença de excesso de óleo acrescido durante o cozimento, que podem servir como interferentes.

A análise direta é difícil, trabalhosa e cara e por isso dados estão sendo obtidos através de cálculos por meio das taxas de retenção, uma prática que merece ser avaliada.

Este trabalho teve como objetivos: (a) adequar a metodologia analítica para determinação de carotenóides em amostras cozidas (fervidas ou refogadas); (b) obter concentrações dos carotenóides principais em alimentos cozidos por meio de análise direta; (c) obter as taxas de retenção e de mudança na concentração dos carotenóides durante o cozimento e (d) comparar as concentrações de carotenóides obtidas por análise direta com aquelas obtidas por meio de cálculos.

RESUMO GERAL

Tendo em vista os vários efeitos benéficos à saúde, os carotenóides têm sido bastante investigados, porém, dados em alimentos na forma como são consumidos ainda são escassos. Obter dados por análise direta é uma atividade difícil, trabalhosa e cara, e por isso, valores vêm sendo obtidos através de cálculos utilizando taxas de retenção. Neste trabalho, as concentrações dos carotenóides principais de vegetais verdes cozidos (brócolos fervido e refogado, chicória refogada, couve refogada e vagem fervida e refogada) foram obtidas por análise direta de amostras compradas em restaurantes, perfazendo um total de 75 análises. O procedimento analítico por cromatografia líquida de alta eficiência, estabelecido no nosso laboratório para folhas *in natura*, sofreu várias modificações para adaptá-lo às amostras analisadas. Para a maioria das amostras não houve diferença significativa na composição de carotenóides entre as amostras colhidas de diferentes restaurantes. Violaxantina foi o carotenóide que mais sofreu degradação durante o cozimento. Foram também determinadas as porcentagens de retenção e de mudança de concentração em amostras cozidas no laboratório, simulando o cozimento doméstico. Os resultados de análise direta foram comparados com valores obtidos por cálculo utilizando porcentagens de retenção ou de mudança de concentração. Os resultados mostraram claramente que o uso da porcentagem de mudança de concentração proporcionou os mesmos valores obtidos por análise direta das amostras cozidas. As porcentagens de retenção mostram corretamente as perdas dos carotenóides durante o cozimento, uma vez que as mudanças no peso são compensadas, mas não fornecem a concentração dos carotenóides (quantidade de carotenóide por unidade de peso da amostra) nas amostras cozidas. Esta constatação, porém, não foi tão clara quando as porcentagens foram aplicadas

aos dados publicados de amostras cruas, pois há interação de vários fatores, como variedade, clima e estado de maturação da amostra crua e condições de cozimento. Os valores calculados com a porcentagem de retenção algumas vezes coincidiram melhor com os fornecidos por análise direta do que os calculados com a porcentagem de mudança de concentração. De qualquer forma, a análise direta é a melhor maneira de se obter valores de carotenóides nos alimentos cozidos, mas uma boa aproximação pode ser conseguida, desde que as taxas de mudança sejam melhor determinadas.

CAPÍTULO 1

DATA-BASE DE CAROTENÓIDES: IMPORTÂNCIA DA OBTENÇÃO DE DADOS DE ALIMENTOS PREPARADOS PARA O CONSUMO

Artigo a ser enviado ao Archivos Latinoamericanos de Nutrición

Data-base de carotenóides: Importância da obtenção de dados de alimentos preparados para o consumo

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RESUMO. Tabelas de composição de alimentos têm sido utilizadas por mais de um século para fins nutricionais, científicos, industriais e políticos. Para cumprirem bem seus objetivos, as tabelas precisam ser confiáveis, atuais, específicas para cada país e, sempre que possível, baseadas em análises originais e conduzidas de acordo com um esquema de amostragem representativo e métodos validados. Além disso, devem apresentar os dados dos alimentos na forma como são habitualmente consumidos. No Brasil foi lançada uma nova tabela com dados compilados, porém, há grande necessidade de geração de dados novos. Além disso, data-bases sobre carotenóides, que normalmente não são incluídos, são necessárias devido às suas atividades biológicas. Muitos alimentos ricos em carotenóides são consumidos cozidos ou refogados. Porém, dados sobre alimentos nesta forma são escassos e a maioria dos dados disponíveis é restrita ao β-caroteno. É uma prática calcular valores para alimentos preparados por meio das taxas de retenção, mas este procedimento deve ser bem avaliado. Por outro lado, discrepâncias podem ser constatadas nos dados existentes sobre carotenóides e deve ser verificado se estas são provenientes de fatores naturais, como estado de maturação, variedade, local de cultivo e modo de preparo para consumo, ou de erros analíticos. Três estudos interlaboratoriais foram realizados a fim de avaliar a precisão e exatidão dos métodos analíticos e de detectar as etapas mais críticas da análise. Os métodos ainda precisam de aprimoramento e deve-se continuar com os esforços colaborativos para determinar com maior confiabilidade a composição de carotenóides nos alimentos.

Palavras-chave: data-base, composição de alimentos, carotenóides, alimentos preparados.

SUMMARY. Food composition tables have been used for more than a century for nutritional, scientific, industrial and political ends. To fulfill the objectives, these tables should be reliable, up-to-date, specific for each country and, whenever possible, based on original analyses, carried out according to a representative sampling scheme and using validated methods. Additionally, the data should be on foods as habitually consumed. In Brazil a new table consisting of compiled data was launched recently, however, there is great need for generation of new data. Moreover, databases on carotenoids, which are usually not included, are necessary because of their biological activities. Many carotenoid-rich foods are consumed cooked, but data on foods in this form are scarce and most of the available data is restricted to β-carotene. Values for prepared foods are sometimes calculated by means of retention percentages, but this procedure should be well evaluated. On the other hand, discrepancies can be observed in the existing data on carotenoids and it should be verified if they are coming from natural factors, such as maturity stage, variety, site of production and mode of preparation for consumption, or from analytical errors. Three interlaboratory studies were conducted in order to evaluate the precision and accuracy of analytical methods and to point out the more critical steps of the analysis. The methods still need to be refined and collaborative efforts should continue in order to determine the carotenoid composition of foods with greater reliability.

Key Words: database, food composition, carotenoids, prepared foods.

INTRODUÇÃO

As tabelas de composição de alimentos vêm sendo utilizadas desde o século 19, sendo que as primeiras compilações de dados datam de aproximadamente 150 anos (1,2).

O avanço nas metodologias analíticas, o melhoramento genético de vegetais e animais, as mudanças de hábitos da população e os constantes lançamentos de novos produtos no mercado, dentre outros fatores, fazem com que as tabelas de composição de alimentos tenham que ser sempre atualizadas, a fim de que sejam as mais completas possíveis e reflitam o que a população está atualmente consumindo.

Dados sobre a composição dos alimentos são utilizados para fins nutricionais, científicos, industriais e até mesmo políticos (3-5). Por meio deles, autoridades da saúde pública podem avaliar a adequação das dietas da população, identificar a necessidade de fortificação de alimentos para corrigir deficiências nutricionais, apoiar a educação alimentar, adequar refeições institucionais e estabelecer metas nutricionais e guias alimentares que levem a uma dieta mais saudável. Atualmente, cientistas estão realizando intensamente estudos epidemiológicos que relacionam a dieta com o risco de doenças, estudos estes que dependem de inquéritos alimentares que utilizam como base a composição tabelada dos alimentos. Também está sendo promovido o melhoramento genético de animais e vegetais, visando modificar a qualidade nutricional dos alimentos e promover boa saúde populacional por meio da dieta. Por outro lado, indústrias podem modificar seus produtos com a finalidade de alterar os níveis dos nutrientes e de outras substâncias desejáveis ou indesejáveis à saúde, adotando, voluntariamente ou por legislação, rotulagem nutricional a fim de auxiliar os consumidores na escolha dos alimentos. Instituições governamentais podem incentivar a importação ou a exportação de

alimentos nutricionalmente importantes e tomar decisões quanto à política de alimentação baseadas nas tabelas de composição de alimentos. Além disso, as tabelas são educativas por si mesmas e qualquer pessoa pode constituir sua dieta levando em consideração a composição dos alimentos.

Portanto, para evitar decisões ou conclusões equivocadas, as tabelas de composição de alimentos precisam ser confiáveis, atualizadas, completas tanto em relação à descrição dos alimentos quanto aos valores apresentados, incluindo alimentos crus e preparados para o consumo. Também devem ser específicas para cada país, baseadas o máximo possível em análises originais e conduzidas de acordo com plano de amostragem representativo e métodos validados (6-9).

Os dados para uma tabela de composição de alimentos podem ser obtidos por meio de análise direta, retirados de outras fontes, estimados de alimentos similares ou calculados para alimentos cozidos e os que contêm vários ingredientes. Dentre estas, a melhor forma evidentemente é a análise direta, porém requer recursos materiais consideráveis e recursos humanos treinados. As outras formas devem ser utilizadas com bastante cautela para fornecer dados representativos (5).

Com a crescente demanda de dados de composição de alimentos nos últimos anos, esforços internacionais estão se voltando para esta área. Deste modo, uma das atividades das Nações Unidas é o estabelecimento de uma rede internacional de sistemas de dados sobre alimentos (INFOODS), que tem como incentivadores a Organização das Nações Unidas para a Agricultura e a Alimentação (FAO) e a Universidade das Nações Unidas (UNU). Foram estabelecidos centros regionais para obtenção e divulgação de dados, tendo como objetivos gerar, compilar e divulgar dados de boa qualidade que satisfaçam as diversas necessidades dos usuários. A colaboração regional é bastante incentivada, visto

que pode levar à redução de custos para a obtenção dos dados, diante da necessidade cada vez maior de informações composicionais (10).

Com a orientação do INFOODS, a compilação dos dados avançou consideravelmente, com muitos países lançando novas tabelas de composição de alimentos. Grande parte destas tabelas, no entanto, contém dados não locais, mas sim importados de outros países, e dados compilados, que muitas vezes não são provenientes de uma amostragem representativa e de métodos validados. Portanto, os esforços atualmente estão voltados para a geração de novos dados de cada país.

Mesmo em países desenvolvidos, dados de alimentos como são consumidos são escassos. Dos valores apresentados nesta forma, uma grande parte ainda foi calculada por meio das taxas de retenção.

No Brasil, as tabelas que vinham sendo utilizadas eram antigas e as mais completas eram de origem estrangeira. As tabelas chamadas nacionais eram publicadas no país, mas continham tanto dados nacionais como obtidos em outros países (11). Portanto, o surgimento de ações de colaboração entre países latino-americanos para obtenção de dados de alimentos regionais vem ao encontro das necessidades do país. Recentemente, a Universidade de São Paulo disponibilizou na Internet uma tabela nova, elaborada por meio da compilação de dados nacionais existentes. Urge, portanto, a geração de dados baseados em análises realizadas com amostragem representativa e métodos analíticos validados, por laboratórios de capacidade laboratorial comprovada.

Tabela de composição de carotenóides

Na área de carotenóides, as tabelas são necessárias devido à atividade pró-vitamínica A que alguns destes compostos apresentam e, de acordo com investigações mais recentes, ao seu potencial de ação na diminuição de risco de doenças degenerativas como o câncer, a catarata, as doenças cardiovasculares e a degeneração macular, além do aumento da resposta imunológica.

Enquanto nos países em desenvolvimento ainda persiste a deficiência de vitamina A, nos países desenvolvidos a maior preocupação com a saúde é com relação às doenças degenerativas. Deste modo, uma tabela completa e com dados confiáveis sobre carotenóides é de importância mundial.

Na geração de novos dados sobre carotenóides, Rodriguez-Amaya (12) sugere que as seguintes observações devam ser levadas em consideração:

- erros analíticos persistem na literatura, portanto, o aprimoramento dos métodos analíticos deve continuar;
- a análise de carotenóides é inerentemente difícil, assim os analistas devem ser bem informados da natureza e propriedades dos carotenóides e dos problemas associados com sua identificação e quantificação;
- vários fatores influem na composição de carotenóides;
- as separações cromatográficas e, conseqüentemente, as quantificações individuais dos carotenóides são realizadas em diferentes extensões (i. e. somente as pró-vitaminas A, os carotenóides principais, carotenóides *cis-trans*, todos os carotenóides);
- existe uma grande diversidade de fontes de carotenóides nos países em desenvolvimento e a maior parte ainda não foi analisada.

Os principais fatores que afetam a composição de carotenóides de um alimento são: cultivar ou variedade do vegetal, parte da planta analisada, estado de maturação, condições pós-colheita, localização geográfica do cultivo (efeito climático) e modo de preparo habitual para o consumo (13). Destes, o estado de maturação é geralmente o fator mais influente.

Esta variação natural explica a necessidade de gerar dados locais, uma amostragem bem planejada para garantir a representatividade das amostras e uma descrição mais adequada dos alimentos nas tabelas (por exemplo, citando variedade/cultivar, procedência e estado de maturação).

Composição de carotenóides em alimentos preparados para o consumo

As tabelas de composição de alimentos devem, idealmente, apresentar os dados dos alimentos como são consumidos. Vários alimentos ricos em carotenóides são consumidos habitualmente cozidos ou refogados. Dos artigos publicados sobre a composição de carotenóides em alimentos, poucos apresentaram valores para alimentos preparados e destes, a grande maioria só apresenta dados para β-caroteno.

Na Índia, Padmavati et al. (14) determinaram os teores de β-caroteno em 12 vegetais preparados para o consumo por seis diferentes procedimentos, cinco destes envolvendo alguma forma de cozimento. Ainda na Índia, as concentrações de oito micronutrientes, incluindo β-caroteno, foram obtidas por Agte et al. (15), em 24 vegetais verdes cozidos tradicionalmente.

No Brasil, Almeida-Muradian e Penteado (16) quantificaram β-caroteno em quatro cultivares de batatas-doces cozidas. Godoy e Rodriguez-Amaya (17), por sua vez,

determinaram as concentrações dos carotenóides pró-vitamínicos A (α-caroteno, β-caroteno, α-criptoxantina e β-criptoxantina), inclusive os isômeros *cis*, em sete vegetais brasileiros cozidos e em dois refogados. No mesmo trabalho, doze vegetais crus foram analisados.

Lessin et al. (18) também quantificaram carotenóides pró-vitamínicos A e seus isômeros *cis* em dez alimentos processados, apenas brócolos era cozido, enquanto os demais eram enlatados ou pasteurizados.

Os teores de β-caroteno e de luteína em vagens cozidas por diferentes métodos (fervura em água, cozimento a vapor, cozimento sob pressão e microondas) foram investigados na Espanha por Cruz-García et al. (19).

Granado et al. (20) e Hart e Scott (21) determinaram a composição dos principais carotenóides em alimentos cozidos consumidos habitualmente na Espanha e Inglaterra, respectivamente. Hart e Scott (21) determinaram os teores de luteína, zeaxantina, β-criptoxantina, licopeno, α-caroteno, β-caroteno e *cis*-β-caroteno em 20 alimentos cozidos e em um frito, enquanto que no mesmo trabalho foram analisados 30 alimentos crus. Granado et al. (20), por sua vez, analisaram 15 alimentos cozidos e 21 crus.

A composição completa de carotenóides em couve de bruxelas e couve cozidas foi determinada por Khachik et al. (22).

Embora os dados existentes sejam poucos, alguns deles podem ser comparados. No caso do espinafre cozido (Tabela 1), os dados concordam entre si. As variações encontradas podem ser provenientes dos vários fatores naturais que afetam a concentração dos carotenóides nos alimentos, além das diferentes condições de preparo utilizadas. Já no caso

da vagem cozida (Tabela 2), os dados são coerentes, exceto em uma das determinações, onde a fonte de variação provavelmente não seja um fator natural.

TABELA 1 - Teores de luteína e β-caroteno em espinafre cozido

Referência	Modo de preparo	Técnica analítica utilizada	Concentração (μg/g)	
			Luteína	β-Caroteno
(21)	Fervura em 2 cm de água por 10 minutos em panela tampada	CLAE	74	45
(20)	Fervura em água por 10 minutos em panela tampada	CLAE	64	46
(15)	"tradicional para consumo", condições não especificadas	Espectrofotometria	nd	39
(17)	Fervura em água por 3 minutos	CCA	nd	21

CLAE, Cromatografia líquida de alta eficiência; CCA, cromatografia em coluna aberta; nd, não determinado

TABELA 2 - Teores dos principais carotenóides em vagem cozida

Referência	Modo de preparo	Técnica analítica utilizada	Concentração (μg/g)		
			luteína	α-caroteno	β-caroteno
(21)	Fervura em água por 10 minutos	CLAE	5,5	0,3	3,2
(20)	Fervura em água por 35 minutos em panela tampada	CLAE	4,8	0,8	2,4
(19)	Fervura em água por 30 minutos em panela tampada	CLAE	16,1	nd	48,1
(17)	Fervura em água por 5 minutos	CCA	nd	0,2	1,0

CLAE, Cromatografia líquida de alta eficiência; CCA, cromatografia em coluna aberta; nd, não determinado

Retenção de carotenóides durante o cozimento

Vários estudos já foram realizados com a finalidade de investigar as mudanças que ocorrem nos níveis de carotenóides durante o preparo para o consumo, mas na maioria dos estudos o total de carotenóides foi estimado espectrofotometricamente na base da absorção máxima. Dados com carotenóides individuais ainda são poucos e bastante discrepantes. A avaliação dos resultados já publicados é difícil por várias razões (13):

- as condições do preparo não são descritas ou são apenas parcialmente descritas;
- alimentos são preparados de modos diferentes, dificultando a comparação;
- condições diferentes (p. e. tempo e temperatura) são utilizadas para o mesmo método de preparo;
- o procedimento seguido para o cálculo da retenção ou perda não é especificado;
- não se faz correção ou compensação para as mudanças de peso durante o cozimento ou processamento e para a maior eficiência de extração dos carotenóides em alimentos preparados em relação às amostras cruas durante a análise

Speek et al. (23) avaliaram a retenção de β-caroteno em vegetais tailandeses preparados de forma tradicional. Após cozimento por fervura em água por 2-8 minutos em recipiente tampado, a retenção do β-caroteno nos nove vegetais analisados variou de 50 a 89%, com uma média de 76% de retenção. Já nos seis alimentos refogados, a retenção variou de 19 a 92%, tendo uma média de 59% de retenção.

Em outro estudo realizado na Tailândia, Wasantwisut et al. (24) analisaram a retenção de β-caroteno em quatro vegetais preparados por três modos diferentes. Três deles, cozidos a 98°C por cinco minutos, apresentaram retenção de 89 a 95%. Um dos vegetais,

fervido a 97°C por cinco minutos apresentou retenção de 96% e outro, fervido à mesma temperatura, mas por dois minutos, reteve 80% do β-caroteno. Três dos vegetais foram refogados, para os quais a retenção variou de 58 a 82%.

Vários vegetais indianos foram investigados por Padmavati et al.(14) quanto à estabilidade de β-caroteno frente a diferentes métodos de preparo. Nos alimentos picados e refogados, houve uma retenção média de 63% do conteúdo de β-caroteno, sendo que nos alimentos picados, macerados e cozidos por maior tempo a retenção foi, em média, 40%. Os resultados mostraram que um menor tempo de exposição ao calor e menor número de etapas no preparo levaram a uma maior retenção de β-caroteno.

Também na Índia, Reddy et al. (25) verificaram a retenção de β-caroteno em oito vegetais preparados de quatro maneiras diferentes. No cozimento sem tampa, a retenção variou de 17 a 100%, com média de 47%. No cozimento com tampa, houve retenção de 31 a 100% (54% em média). Os vegetais cozidos a vapor apresentaram retenção de 17 a 100% (57% em média), enquanto as amostras refogadas tiveram as maiores retenções, que foram de 46 a 100% (68% em média). Neste mesmo estudo foram investigadas as retenções de β-caroteno em cenoura e abóbora aquecidas por tempo prolongado. Nesta condição, a retenção foi de 48% para cenoura e apenas 10% para abóbora.

Rahman et al. (26) avaliaram a retenção de β-caroteno em catorze vegetais de Bangladesh, preparados de três modos tradicionais diferentes. A retenção variou de 57 a 69% nos vegetais que foram cozidos e depois refogados, de 86 a 89% nos alimentos cozidos por fervura em recipiente tampado e aberto esporadicamente e de 89 a 98% nos vegetais cozidos em cima do arroz em cozimento.

No Brasil, a retenção de β-caroteno em quatro cultivares de batatas-doces foi investigada por Almeida-Muradian e Penteado (16). Após cozimento por dez minutos em água fervente, a retenção de β-caroteno variou de 88 a 95%.

Quanto menor o tempo de cozimento e a temperatura, maior a preservação do conteúdo de α-caroteno, β-caroteno e carotenóides totais durante o cozimento de cenouras (27). Neste estudo brasileiro, os níveis de retenção de α-caroteno e β-caroteno variaram de 69 a 89% e 56 a 78%, respectivamente. As maiores perdas ocorreram quando as cenouras foram cozidas e em seguida assadas, e o método de preparo que mais conservou estes carotenóides foi o cozimento convencional (fervura em água).

Em estudo sobre o efeito do preparo de tomate e vegetais verdes para consumo, Khachik et al. (28) constataram que maiores perdas ocorreram com violaxantina e epóxido de luteína. No caso da violaxantina, a retenção variou de 33 (brócolos cozido ao vapor) até 66% (espinafre cozido ao vapor) e para epóxido de luteína a retenção variou de 32% (vagem fervida em água) até 66% (brócolos cozido em microondas). Para os outros carotenóides investigados (neoxantina, luteína, α-caroteno e β-caroteno), as diferenças entre alimentos crus e cozidos não foram estatisticamente significativas.

Porcentagens de retenção encontradas por Granado et al. (20) variaram de 100 até 600%. As taxas de retenção para os carotenóides quantificados em vários alimentos cozidos variaram da seguinte forma: luteína, de 103 (aspargo) a 409% (couve-flor); zeaxantina, de 116 (repolho) a 618% (batata); β-criptoxantina, de 107 (pimentão vermelho) a 326% (batata); α-caroteno, de 129 (cenoura) a 313% (vagem); e β-caroteno, de 101 (aspargo) a 344% (cebola). As taxas de retenção foram calculadas com base no peso seco, que na maioria dos casos superestima a retenção (29). Também Lessin et al. (18), utilizando

cálculo com base em peso seco, encontraram taxas de retenção acima de 100% nos vegetais analisados.

Cruz-García et al. (19), em investigação sobre efeitos de vários tratamentos sobre pigmentos naturais de vagem, observaram um aumento nos níveis de luteína e β-caroteno. Enquanto os níveis de luteína aumentaram independentemente do modo de preparo, o aumento de β-caroteno foi maior com cozimento a vapor e em água fervente. Este resultado foi atribuído pelos autores ao maior tempo de aquecimento nestes modos de preparo, levando à maior facilidade e extração dos carotenóides da matriz, do que no cozimento em microondas e sob pressão, os outros procedimentos testados.

Mosha et al. (30) relataram níveis de retenção de β-caroteno em cinco vegetais da Tanzânia, que variaram de 143 a 182% nos vegetais cozidos por quinze minutos, 122 a 200% quando a cocção foi de 30 minutos e 154 a 131% na cocção por 60 minutos.

Os relatos de retenção acima de 100% não podem ser considerados como aumento verdadeiro uma vez que não há nenhuma possibilidade de acréscimo de carotenóides durante o processamento. O tratamento térmico inativa as enzimas responsáveis pela biossíntese de carotenóides e, de fato, estimula a isomerização e oxidação de carotenóides, resultando em perdas. Várias explicações foram levantadas para as relatadas retenções acima de 100% (13, 31):

- a maior facilidade com que os carotenóides são extraídos de amostras cozidas em relação às cruas durante a análise;
- a oxidação enzimática dos carotenóides nas amostras cruas, diminuindo seu conteúdo de carotenóides;

- lixiviação de sólidos solúveis, que aumenta a concentração de carotenóides por unidade de peso;
- cálculo de retenção que não leva em consideração a mudança do peso da amostra.

Variações da composição de carotenóides devido a erros analíticos

A análise de carotenóides é inerentemente difícil devido a vários fatores como: grande número de carotenóides existentes nos alimentos, variação qualitativa e quantitativa da composição de carotenóides, ampla faixa quantitativa dos carotenóides em um mesmo alimento e susceptibilidade à isomerização e à oxidação durante a análise, devido ao alto grau de insaturação destes compostos (12, 13, 31).

Erros analíticos podem ocorrer em todas as etapas da análise. As fontes de erros nas etapas pré-cromatográficas podem ser (12, 13, 31, 32):

- amostra não representativa do lote analisado;
- preparo da amostra que não assegura a homogeneidade e representatividade da amostra analítica;
- extração incompleta;
- perda dos carotenóides na água de lavagem durante partição e na saponificação e perda de carotenóides por aderência na vidraria;
- perdas devido à isomerização e à oxidação dos carotenóides durante a análise.

Erros podem ser introduzidas nas etapas cromatográficas devido a (12, 31):

- separação cromatográfica incompleta;
- identificação equivocada;
- quantificação ou cálculo errado.

A análise de carotenóides por cromatografia líquida de alta eficiência, embora seja o método de escolha para a análise atualmente, pode ter várias fontes de erros, como (33):

- incompatibilidade entre solvente de injeção e a fase móvel;
- identificação equivocada;
- impureza e instabilidade dos padrões dos carotenóides;
- quantificação de picos sobrepostos;
- baixa recuperação dos carotenóides da coluna cromatográfica;
- inexatidão no preparo da solução padrão e no procedimento de calibração e erro no cálculo

Estudos Interlaboratoriais

Para harmonizar os dados da composição de carotenóides em alimentos, avaliar a precisão e exatidão dos métodos analíticos e verificar as etapas mais críticas da análise, torna-se de grande valia a realização de estudos interlaboratoriais.

Um estudo interlaboratorial bastante extensivo foi realizado com laboratórios europeus por Scott et al. (34). As etapas investigadas que poderiam ser fontes de erros na análise foram os sistemas cromatográficos, as soluções padrão dos carotenóides, a extração e a manipulação dos dados. Em conclusões preliminares, foi afirmado que mais da metade da variação total de aproximadamente 23% foi proveniente do preparo do extrato dos carotenóides.

Schüep e Schierle (35) realizaram um estudo interlaboratorial de determinação de β-caroteno em alimentos comerciais, cobrindo grande variedade de matrizes e faixa ampla de concentração do β-caroteno. Todos os 14 laboratórios envolvidos neste estudo utilizaram o mesmo procedimento para preparo da solução padrão e da amostra. Houve boa precisão entre os resultados, e as diferenças encontradas nos teores de β-caroteno total e *trans*-β-caroteno deveu-se à eficiência variada na separação dos isômeros *cis* nos sistemas cromatográficos utilizados.

Na tentativa de obter um material de referência para carotenóides, Sharpless et al. (36) analisaram o material de referência padrão 2383 do National Institute of Standards and Technology, um composto alimentar infantil. Concordando com Scott et al.(1996), as maiores fontes de variação dos resultados pareciam ser provenientes das etapas pré-cromatográficas. Alguns problemas sugeridos foram: degradação de licopeno durante a extração, isomerização do β-caroteno e desesterificação das xantofilas durante o preparo da amostra. Concentrações certificadas foram obtidas para luteína (incluindo ésteres), zeaxantina (incluindo ésteres), β-criptoxantina (incluindo ésteres), α-caroteno total e β-caroteno total. Para os outros carotenóides analisados, os valores obtidos foram considerados apenas como concentrações de referência (luteína livre, zeaxantina livre, β-criptoxantina livre, *trans*-licopeno e licopeno total, *trans*-α-caroteno, *trans*-β-caroteno, 9-*cis*-β-caroteno, 13-*cis*-β-caroteno, 15-*cis*-β-caroteno, 13-+15-*cis*-β-caroteno). Os autores admitiram que as incertezas relativas constatadas são maiores do que se espera de valores certificados (chegaram a 28% para luteína na porção saponificada), mas representam o estado atual dos métodos para determinação de carotenóides em alimentos. Portanto o aprimoramento dos métodos e esforços colaborativos devem continuar, para que um dia a

composição de carotenóides nos alimentos possa ser determinada com maior confiabilidade nos diversos laboratórios.

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CAPÍTULO 2

OPTIMIZATION OF HPLC QUANTIFICATION OF CAROTENOIDS IN COOKED GREEN VEGETABLES

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Optimization of HPLC Quantification of Carotenoids in Cooked Green Vegetables

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The need for reliable and complete carotenoid data is recognized worldwide. Analysis of cooked food has problems different from those of raw samples. Although cooking softens cell walls and make extraction of carotenoids easier, incorporation of oil and formation of degradation products may pose some analytical difficulties. This work was carried out to optimize the analytical method and adapt it to cooked foods. The HPLC method used in our laboratory for fresh green vegetables was found adequate only for boiled broccoli. For stir-fried broccoli, kale and endive, keeping the acetone extract of carotenoids in the freezer to solidify the oil, followed by filtration, removed the oil, without degrading carotenoids. One-hour saponification (10% KOH in methanol, room temperature, in the dark) was required to remove a chlorophyll degradation product that eluted close to β-carotene in boiled green beans. Overnight saponification of stir-fried beans removed this product and part of the oil.

Key Words: carotenoids, HPLC analysis, cooked vegetables.

INTRODUCTION

Well-appreciated as natural colorants responsible for the yellow to red color of many foods, carotenoids are also credited with important health-promoting functions such as provitamin A activity, enhancement of the immune system and reduction of the risk of degenerative diseases, e.g. cancer, cardiovascular disease and macular degeneration. Thus, the need for reliable and more complete databases on carotenoid composition of foods is felt throughout the world.

Carotenoid analysis, however, is admittedly difficult, even when high performance liquid chromatography (HPLC), currently the method of choice, is used (Rodriguez-Amaya, 1989,1999; Hart and Scott, 1995; Kimura and Rodriguez-Amaya, 1999). With the advent of HPLC, numerous reviews and research articles have been published on its application to food carotenoids. Nevertheless, advances in the HPLC quantification of carotenoids in foods have been slow because an overwhelming majority of published papers dealt with only the chromatographic separation, although the ultimate aim is to obtain accurate quantitative data. Three interlaboratory studies, involving experienced European and American laboratories (Scott et al., 1996; Schüep and Schierle, 1997; Sharpless et al., 1999), demonstrated the difficulties in the HPLC quantification of carotenoids, a major part of the error coming from the pre-chromatographic steps.

On the other hand, most of the quantitative data available are on raw foods, although it is generally recognized that databases should be on foods as consumed. Since many foods are eaten cooked, data on prepared foods are needed. Moreover, with a few exception, data are restricted to β-carotene. More information should be obtained on the

other principal carotenoids, provitamins A or not, considering that the beneficial effects on health attributed to carotenoids are not limited to provitamins A.

Analysis of cooked vegetables has problems somewhat different from those of the raw produce. Although cooking softens the cell walls and makes the extraction of carotenoids easier, incorporation of oil and formation of degradation products during cooking may pose some analytical difficulties. Thus, this work was carried out to optimize the analytical method for carotenoids in cooked samples.

MATERIAL AND METHODS

Reevaluation of the scheme for obtaining carotenoid standards

The procedure for isolating and purifying β-carotene, lutein, violaxanthin and neoxanthin standards was established by Kimura and Rodriguez-Amaya (2001), because of the difficulty in obtaining standards commercially. The scheme, reappraised in the present study, consisted of extraction of the carotenoids of a leafy vegetable with cold acetone, partition to petroleum ether, concentration in a rotary evaporator, chromatographic separation of the carotenoids in a MgO:Hyflosupercel (1:1, activated for 4 hours at 110°C) column developed with petroleum ether containing increasing percentages of ethyl ether (up to 8%) and acetone (up to approximately 60%). The purity of the carotenoids isolated was verified by HPLC. Once the purity of the standards was established, a standard mixture was prepared, mimicking the relative proportion of the carotenoids in the green vegetables to be analyzed. Aliquots of this mixture were placed in test tubes, dried under nitrogen gas

and stored in a vacuum dessicator at -20°C until use. Stability of the standards under this condition was verified.

Construction of the standard curves

Quantification of the carotenoids was carried out by external standardization. The full standard curve, with 5 points bracketing the samples' concentration range and each point in triplicate, was constructed twice, at the beginning of the study and when the lamp of the detector was changed. The concentration ranges in the first curve were 5.2-26,2μg/g for neoxanthin, 9,5-47.5 μg/g for violaxanthin, 19.4-96.8 μg/g for lutein and 17.5-87.3 μg/g for β-carotene. In the second curve the ranges were 6,4-31.9μg/g for neoxanthin, 9.6-47.9μg/g for violaxanthin, 32.0-160.1μg/g for lutein and 18.4-92.2 μg/g for β-carotene.

Evaluation of the influence of the matrix

The analytical procedure established in our laboratory for fresh leafy vegetables (Kimura and Rodriguez-Amaya, 2001) was initially used for all the samples analyzed. This involved extraction with cold acetone, partition to petroleum ether, concentration in a rotary evaporator and complete removal of solvent with nitrogen gas. The carotenoids were redissolved in acetone and injected immediately into the liquid chromatograph. This procedure as is was found adequate only for boiled broccoli.

For all stir-fried samples, excess oil was a problem. Saponification with 10% KOH at room temperature overnight was not found sufficient to remove the oil of stir-fried kale. Since interference with chlorophyll or its degradation products was not a problem, filtration at freezer temperature (-20°C) was tried. This consisted of leaving the acetone extract of the

carotenoids in the freezer compartment of the refrigerator for two hours, during which the oil solidified, followed by filtration inside the freezer to maintain the low temperature. Partition to petroleum ether was then carried out in the usual manner.

Long periods of exposure to acetone could induce carotenoid degradation. Thus, it was necessary to evaluate if losses happened during the two-hour freezing to solidify the oil. The evaluation was done in triplicate with β-carotene and lutein. A known amount of the isolated carotenoid was left in acetone for two hours and filtered as done with samples. Approximately the same amount of carotenoids found in samples and the same quantity of acetone were used. The carotenoid was then partitioned to petroleum ether and quantified spectrophotometrically.

Saponification was necessary for boiled and stir-fried green beans to remove a chlorophyll degradation product that eluted close to β-carotene and could affect the quantification. Saponification was carried out with 10% KOH in methanol in the dark at room temperature. To avoid degradation of carotenoids during saponification, 0.1% of BHT was added to the extract.

HPLC instrumentation and conditions

HPLC conditions previously established for leafy vegetables (Kimura and Rodriguez-Amaya, 2001) were utilized. The analysis was performed with a Waters separation module (model 2690) equipped with an automatic injector, controlled by a Millenium workstation (version 3.10). A C18 monomeric column (Spherisorb S3 ODS2, 3 μm, 4.6 x 150 mm) was employed and the mobile phase was composed of acetonitrile:methanol:ethyl acetate in a concave gradient, from 95:5:0 to 60:20:20 in

20 minutes, the latter proportion being maintained until the end of the run. Flow rate was set at 0.5mL/min. Acetonitrile contained 0.05% of triethylamine, as recommended by Hart and Scott (1995), to improve carotenoid recovery from the chromatographic column. A UV-Visible photodiode array detector (Waters model 996) was used, detection being at the wavelengths of maximum absorption (max plot) (The spectra of the carotenoids investigated obtained by the photodiode array detector are shown in Anexo I).

RESULTS AND DISCUSSION

Purity and stability of the carotenoid standards

The purity of the standards was 90-97% for neoxanthin (mean 92%), 91-100% for violaxanthin (mean 97%), 90-100% for lutein (mean 93%) and 90-91% for β-carotene (mean 90%) (chromatogram shown in Anexo II). It was evident, however, that in order to achieve these purity percentages, skill, experience and great care are required of the analyst.

Stability of the standards was maintained for 13 days when kept at -20°C in a vacuum dessicator (Figure 1). Thus, standards isolated in the laboratory and stored under the above condition are used within 2 weeks after isolation. When a vacuum dessicator was not used, the standards were stable only during a week.

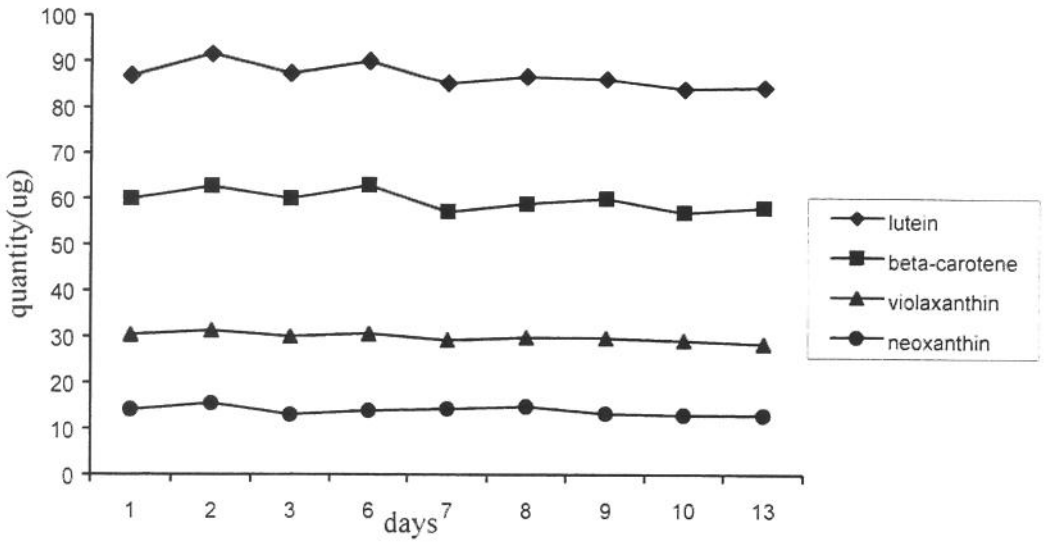

Figure 1: Stability of carotenoid standard solutions during two weeks

Characteristics of the standard curves

The standard curves passed through the origin, showed good agreement among the triplicates and linearity at the concentration ranges studied (Anexos III e IV). The coefficients of variation of triplicates for each point ranged from 1.1 to 5.3% for neoxanthin, 0.3 to 3.0% for violaxanthin, 0.3 to 1.6% for lutein and 0.8 to 2.6% for β-carotene in the first curve and from 0.9 to 4.2% for neoxanthin, 0.7 to 2.4% for violaxanthin, 1.1 to 3.0% for lutein and 0.4 to 2.9% for β-carotene in the second curve. The coefficients of correlation were 0.9861, 0.9954, 0.9992 and 0.9920 for neoxanthin, violaxanthin, lutein and β-carotene, respectively, for the first curve. The corresponding values were 0.9968, 0.9984, 0.9985 and 0.9980 for the second curve. Because of the difficulty in carotenoid analysis, a minimum coefficient of correlation of 0.9 was suggested by Khachik et al.(1992), while Mantoura et al. (1997) recommended a coefficient of correlation greater than 0.95.

Extract preparation and chromatographic separation

For boiled broccoli, a chromatogram (Figure 2) adequate for the quantification of carotenoids was obtained without modification of the procedure used in our laboratory for fresh leafy vegetables.

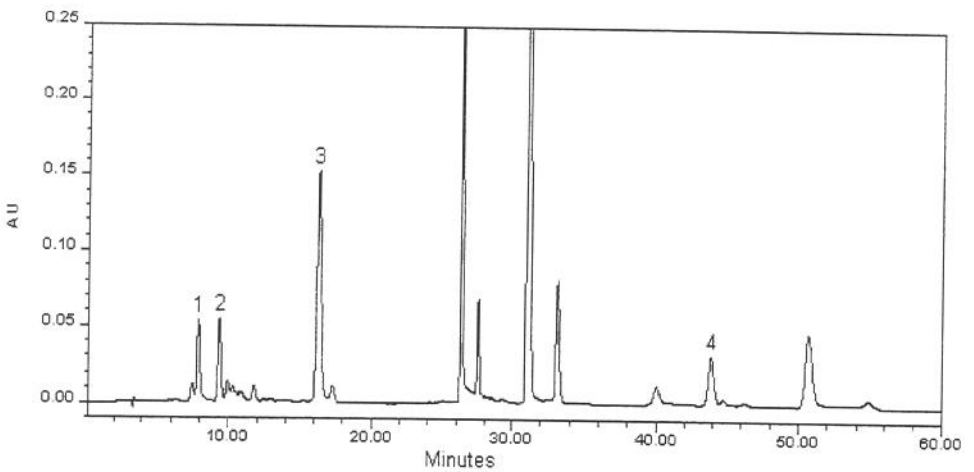

Figure 2. HPLC chromatogram of boiled broccoli extract. Chromatographic conditions are described in the text. Peak identification: 1 - neoxanthin. 2 - violaxanthin, 3 - lutein, 4 - β-carotene.

For stir-fried kale, solidification of the oil and filtration at freezing temperature produced an extract suitable for HPLC quantification of the carotenoids. Interference with chlorophyll or its degradation products was not a problem, so saponification was not necessary (Figure 3). This procedure was extended to stir-fried broccoli (Figure 4) and endive (Figure 5).

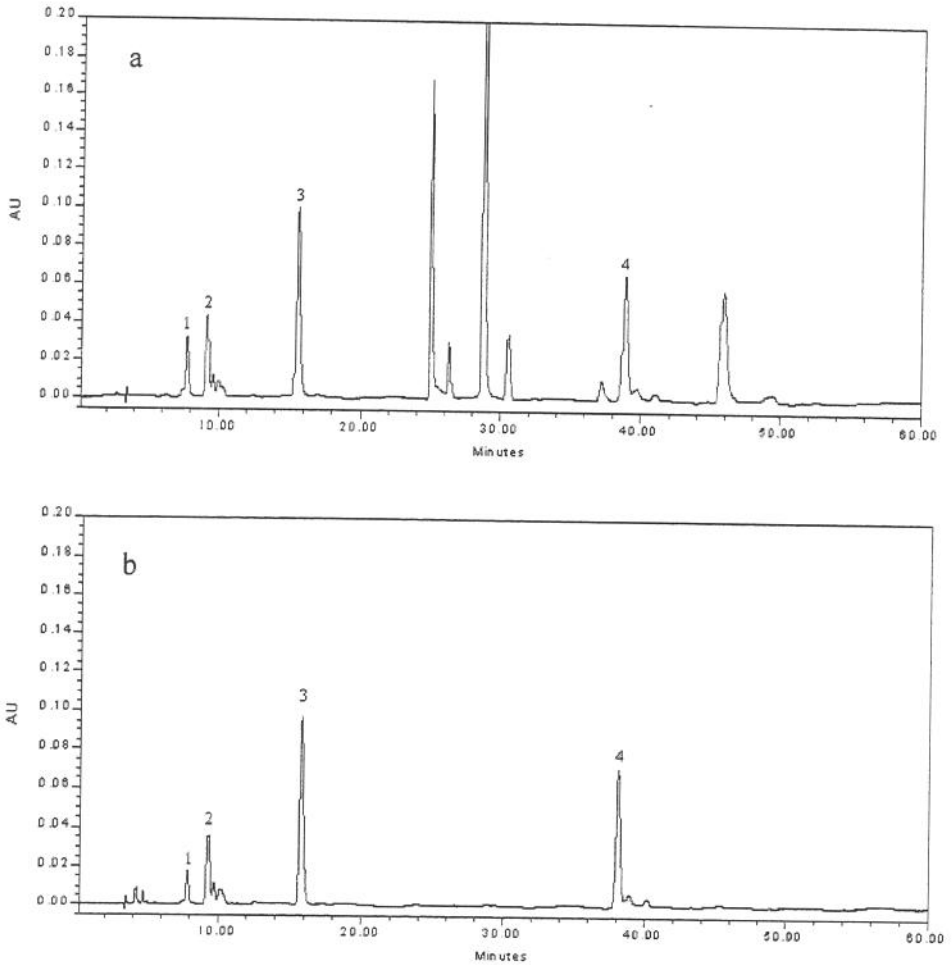

Figura 3. HPLC chromatograms of (a) unsaponified and (b) saponified stir-fried kale extracts. Chromatographic conditions are described in the text. Peak identification: 1 - neoxanthin. 2 - violaxanthin, 3 - lutein, 4 - β-carotene.

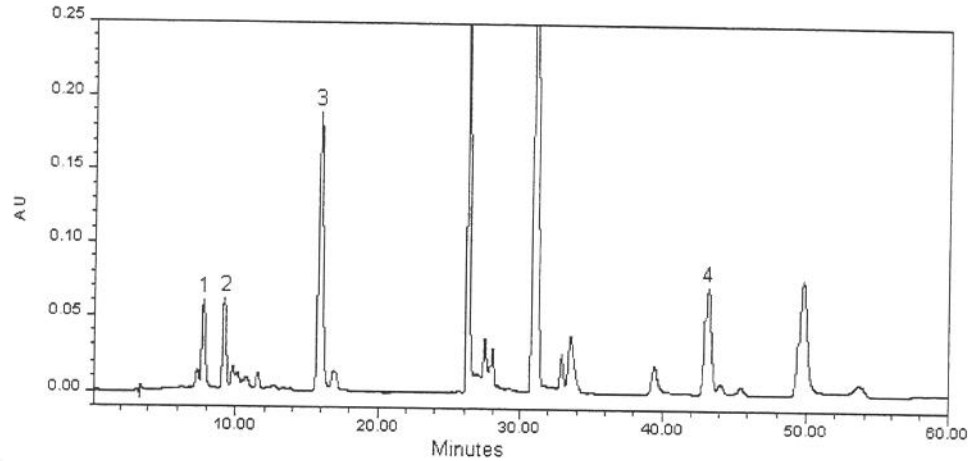

Figure 4. HPLC chromatogram of stir-fried broccoli extract. Chromatographic conditions are described in the text. Peak identification: 1 - neoxanthin. 2 - violaxanthin, 3 - lutein, 4 - β-carotene.

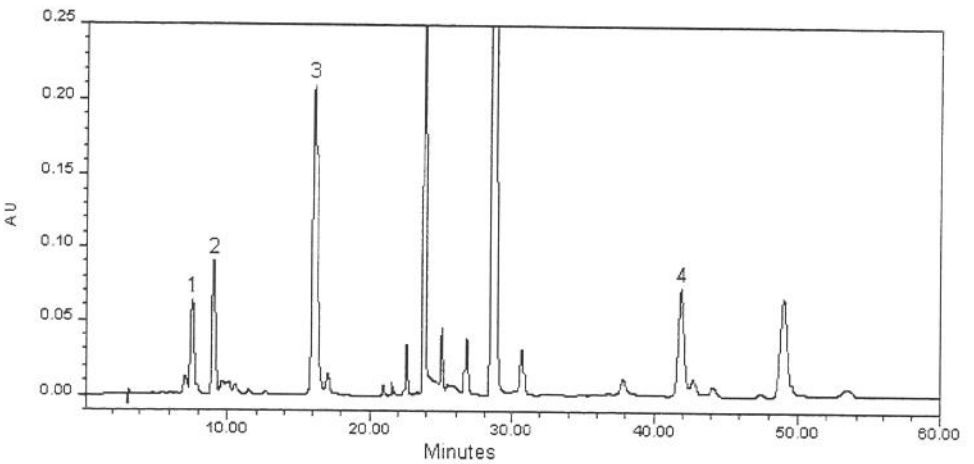

Figure 5. HPLC chromatogram of stir-fried endive extract. Chromatographic conditions are described in the text. Peak identification: 1 - neoxanthin, 2 - violaxanthin, 3 - lutein, 4 - β-carotene.

Table 1 shows that leaving the extract in acetone for two hours in the freezer was not harmful to the carotenoids. The percentage recovery was 99.8% and 100% for β-carotene and lutein, respectively.

Table 1. Recovery of carotenoids during two-hour freezing in acetone[1]

Carotenoid	Amount added (μg)	Carotenoid recovered (μg)
β-Carotene	79.0	78.9
Lutein	74.9	74.9

[1] mean of triplicates

Saponification has long been used in carotenoid analysis to remove chlorophylls and unwanted lipids and to hydrolyze carotenol esters. However, it is a step that should be carried out only when absolutely necessary because it can provoke carotenoid losses and it requires a lot of care and time (Rodriguez-Amaya, 1999). In the case of green beans, saponification was indispensable because there was a clorophyll degradation product formed during cooking that eluted close to β-carotene, as shown in Figure 6.

Initially, lutein was being lost during the washing that followed the saponification. To avoid this loss, the following measures were taken: (a) the petroleum ether layer was separated from the methanolic phase and ethyl ether was added to petroleum ether before washing; (b) acetone was mixed with the methanolic phase before transferring the carotenoids of this phase to petroleum ether-ethyl ether; (c) the transfer was done very slowly, a small volume of the methanolic phase being transferred each time.

One-hour saponification was found sufficient to remove chlorophylls and their degradation products of boiled green beans (Figure 7). For stir-fried green beans, overnight saponification was found necessary to remove part of the oil, along with chlorophylls and their degradation products. To certify the complete removal of the oil, the extract redissolved in acetone before injection was left 30 minutes in the freezer to solidify the oil, which was separated by filtration, producing an extract adequate for HPLC quantification (Figure 8).

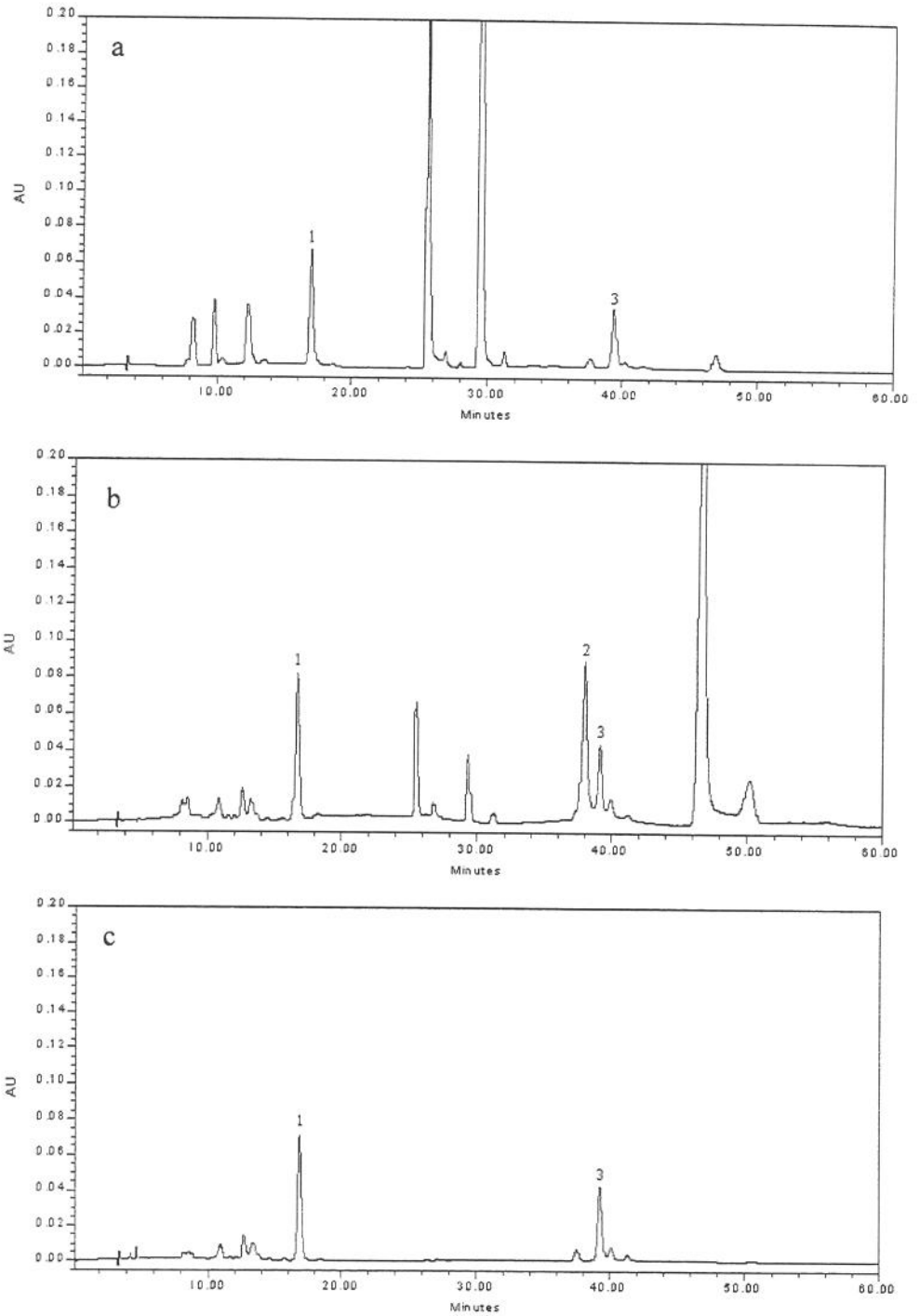

Figure 6. HPLC chromatograms of (a) raw, (b) boiled, unsaponified and (c) boiled, saponified green beans extracts. Chromatographic conditions are described in the text. Peak identification:1 - lutein, 2 - chlorophyll degradation product, 3 - β-carotene.

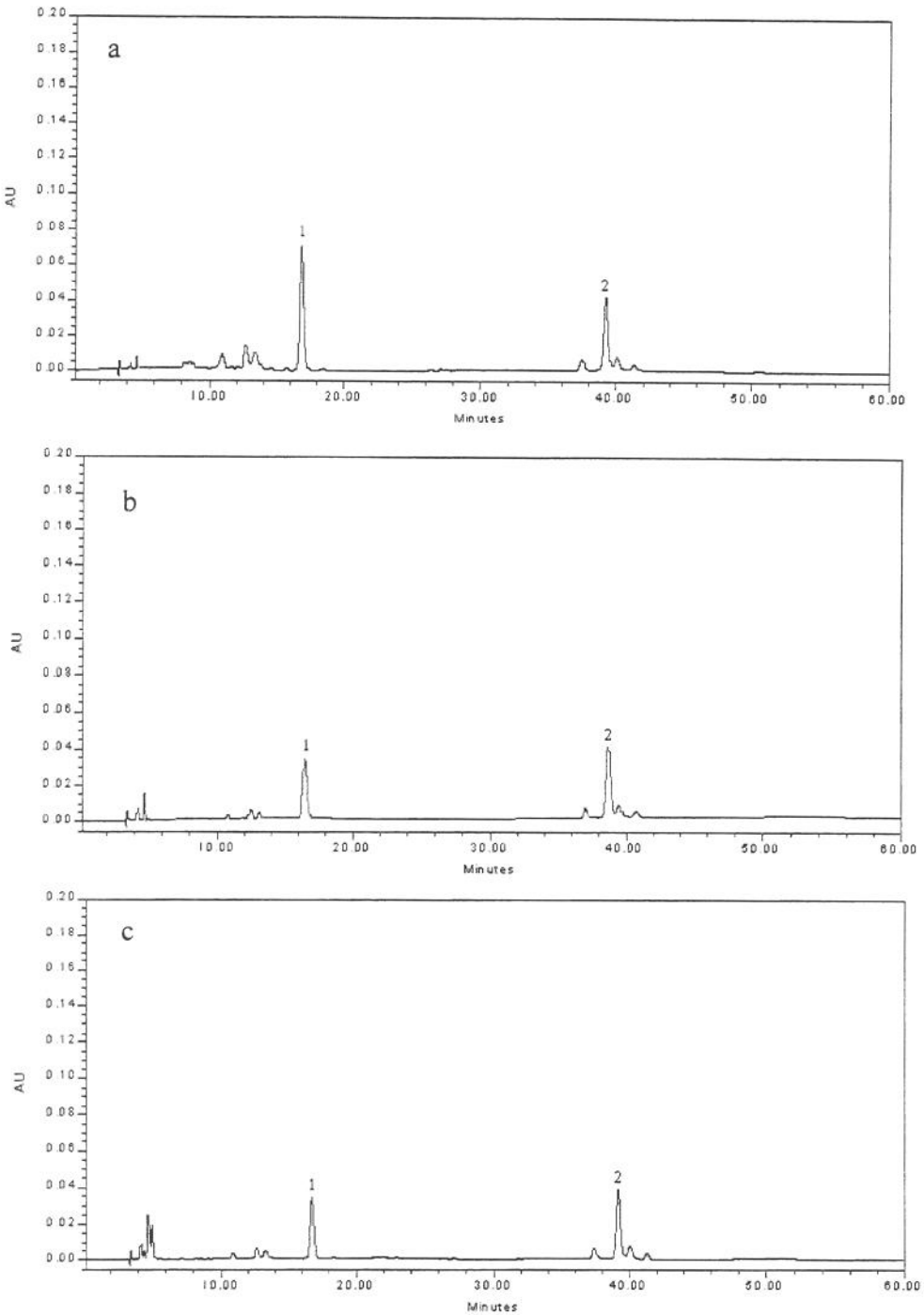

Figure 7. HPLC Chromatograms of boiled green beans extracts saponified for (a) one hour, (b) two hours and (c) overnight. Chromatographic conditions are decribed in the text. Peak identification: 1 - lutein, 2 - β-carotene.

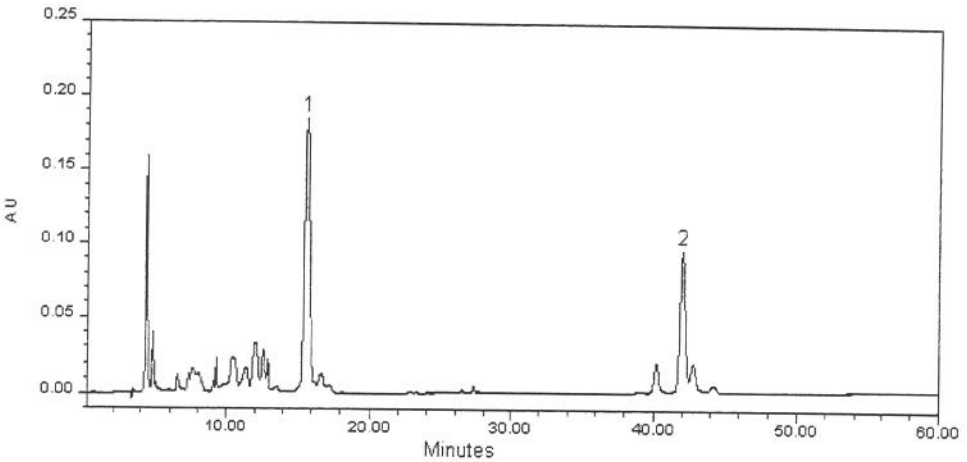

Figure 8. HPLC chromatogram of stir-fried green beans extract. Chromatographic conditions are decribed in the text. Peak identification: 1 - lutein, 2 - β-carotene.

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CAPÍTULO 3

CAROTENOID COMPOSITION OF COOKED GREEN VEGETABLES OBTAINED FROM RESTAURANTS

Artigo a ser enviado ao Food Chemistry

Carotenoid Composition of Cooked Green Vegetables Obtained from Restaurants

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Abstract

It is generally recognized that data in food databases should be given in terms of the food as eaten. However, data obtained by direct analyses of cooked foods are lacking. In this work, the concentrations of β-carotene, lutein, violaxanthin and neoxanthin in cooked green vegetables obtained from restaurants were determined by HPLC. The analytical procedure was the same as that used in our laboratory for fresh leafy vegetables, with some modifications to adapt it to cooked samples. Stir-fried kale and boiled and stir-fried broccoli were found to be rich sources of carotenoids, while cooked green beans had low carotenoid levels. There were no statistical differences in the carotenoid concentrations of vegetables taken from different restaurants, except for β-carotene in stir-fried broccoli and cooked green bean and violaxanthin in stir-fried kale. Violaxanthin, compared to the other carotenoids, was the most labile during cooking. Some discrepancies with published data were noted.

Key words: carotenoid, analysis, cooked vegetable, restaurant food.

1. Introduction

Carotenoids are among the phytochemicals most frequently cited as responsible for the reduction of the risk of developing degenerative diseases such as cancer, cardiovascular disease and macular degeneration. The provitamin A activity of some of these compounds has long been known. Thus, the need for reliable and more complete databases on the carotenoid concentrations of foods is recognized worldwide. Great efforts have been exerted to refine the analytical methods and insure that accurate data are obtained. However, carotenoid analysis is difficult and some errors persist in the literature. In addition, available data are wanting in two aspects: (1) most are restricted to β-carotene; (2) most are on raw food. Information on carotenoids other than β-carotene and in terms of the food as eaten should be generated.

In recent years it has also been reported that cooked/processed foods may have greater bioavailability then the raw commodities (Gärtner, Stahl & Sies, 1997; Rock, Lovalvo, Emenhiser, Ruffin, Flatt & Schwartz, 1998; Stahl & Sies, 1992). Thus, it is recommended that programs designed to alleviate vitamin A deficiency and/or to promote the health benefits of carotenoids should include means of enhancing bioavailability by cooking or processing (Olson, Parker, Reddy, Rodriguez-Amaya, Smitasiri & Tsou, 1999).

In this paper, the concentrations of the principal carotenoids of cooked green vegetables from restaurants at or near the University of Campinas are presented. The reason for deciding on restaurant food, instead of simulating home cooking, was three-fold: (1) a good part of the university's population eat at restaurants daily; (2) data on restaurant foods

are lacking; (3) restaurant foods permit a wider sampling while still representing typical Brazilian home recipes.

Cooked vegetables would have variations in their carotenoid composition brought about by varying cooking conditions (e.g. time and temperature), but also by compositional differences of the raw material, due to such factors as stage of maturity, cultivar, part of the plant utilized, climatic or seasonal effects, agricultural and post-harvest handling.

2. Material and methods

2.1 Samples

The most commonly prepared and consumed cooked vegetables in Brazil were chosen as samples. Five 300 g samples of each vegetable were obtained from each of three restaurants (except for stir-fried samples of broccoli and endive, which were available only in two and one restaurant, respectively) at different times during the year, totaling 75 separate determinations. These cooked samples were prepared by the restaurants from lots of raw vegetables weighing approximately 4-6 kg. Analysis was carried out as soon as the sample was collected. Endive and kale were analyzed only in the stir-fried form because they are not commonly consumed boiled in Brazil.

2.2 Carotenoid determination

The analytical method was optimized and adapted to the sample matrix (de Sá & Rodriguez-Amaya, 2001), based on a procedure established by Kimura and Rodriguez-Amaya (2001).

Standards were isolated from leafy vegetables by open column chromatography (OCC) and samples were analyzed by high performance liquid chromatography (HPLC). Pre-chromatographic steps involved grinding samples in a food processor, weighing

subsamples (36-38 g for green beans and 2.5-4.0 g for the other vegetables), extracting with cold acetone, partitioning to petroleum ether, saponifying when necessary, concentrating in a rotary evaporator, drying under nitrogen, dissolving in 2 ml of HPLC grade acetone, filtering through a 0.22μm PTFE filter and injecting immediately into the liquid chromatograph.

Acetone extracts of stir-fried broccoli, endive and kale, that contained excess oil, were left in a freezer for two hours to solidify the oil and then filtered inside the freezer through a cold glass sintered funnel. Partition to petroleum ether followed. Saponification with 10% KOH in methanol (volume equal to that of the extract) at room temperature in the dark was necessary for green bean samples to remove a chlorophyll degradation product that eluted close to β-carotene. BHT (0,1%) was added to the petroleum ether extract before saponification to prevent degradation of carotenoids. Boiled green bean extracts were saponified for one hour to remove the chlorophyll degradation product, while stir-fried green bean extracts were saponified overnight to remove both the chlorophyll degradation product and part of the oil. Oil was completely removed by keeping the carotenoids after redissolving in acetone for injection, in the freezer for 30 minutes, followed by filtration, before injecting.

2.3 HPLC conditions

HPLC conditions previously determined for fresh leafy vegetables (Kimura & Rodriguez-Amaya, 2001) were used. The analysis was carried out with a Waters separation module (model 2690) equipped with an automatic injector, controlled by Millenium workstation (version3.10). A C18 monomeric column (Spherisorb S3 ODS2), 3μm, 4.6 x 150mm, was used and the mobile phase was composed of acetonitrile:methanol:ethyl acetate in a concave gradient, from 95:5:0 to 60:20:20 in 20 minutes, maintaining this

proportion until the end of the run. The flow rate was 0.5mL/min. Triethylamine (0.05%) was added to acetonitrile, as recommended by Hart and Scott (1995) to improve carotenoid recovery from the column. A UV-Visible photodiode array detector (Waters model 996) was used, detection being set at the wavelengths of maximum absorption (max plot).

2.4 Statistical analysis

The results were submitted to analysis of variance and Tukey's test.

3. Results and discussion

The concentrations of β-carotene, lutein, violaxanthin and neoxanthin in cooked green vegetables obtained from restaurants are presented at Table 1.

Among the samples analyzed, stir-fried kale and both boiled and stir-fried broccoli were rich sources of carotenoids. Cooked green beans had much lower carotenoids levels.

Differences in carotenoid concentrations between restaurants were not found statistically significant in most of vegetable samples analyzed. This result is surprising at first glance because of the many factors that can cause variation in the raw materials, aside from variations brought about by the cooking conditions. However, it is possible that the interplay of so many factors might have masked the individual effects. Moreover, taking the analytical sample from a large restaurant batch might have compensated the individual variations, giving more representative average concentrations.

Exceptions to the above observation were β-carotene in stir-fried broccoli and boiled green bean and violaxanthin in stir-fried kale. In the case of β-carotene in stir-fried broccoli, that is usually prepared with varying amounts of stalk and small leaves found next to the flowerlets, the difference could be explained by the fact that restaurant E, the samples from which had higher β-carotene content, prepared this vegetable with more leaves than restaurant A. This difference could not be attributed to differences in preparation conditions

because samples obtained from restaurant E always appeared more drastically cooked than those from restaurant A, as seen through the texture of the samples, and would have presented lower carotenoid content than those from restaurant A.

Violaxanthin had previously been reported as a very labile carotenoid (Khachik et al., 1992). The statistically lower concentration of violaxanthin in stir-fried kale samples obtained from restaurant B indicated that more drastic cooking conditions are used in this restaurant. Although not statistically significant, the other carotenoids were also found lower in the samples from restaurant B.

It is also noteworthy that violaxanthin had lower levels than neoxanthin in all of the samples analyzed. In raw green vegetables, violaxanthin usually surpasses neoxanthin. This observation reinforce the great lability of violaxanthin. In mango, violaxanthin, the principal carotenoid of commercial cultivars in Brazil, was not found in three brands of mango juice (Mercadante and Rodriguez-Amaya, 1998). Auroxanthin, a derivative of violaxanthin, was found instead.

Broccoli and green bean were analyzed both boiled and stir-fried. Conditions during stir-frying might be more drastic than those of boiling, thus reducing the carotenoid levels. However, because of water loss, stir-frying may concentrate the carotenoids, giving higher carotenoid content per unit weight of vegetable. In boiling, water is added to the food, causing dilution of the carotenoids. The first observation appeared to predominate in broccoli since all carotenoids had higher concentration in the boiled than in the stir-fried samples. On the other hand, the latter observation might have played a greater role in stir-fried green beans, because they tended to have higher carotenoid concentration than the boiled beans. However, the levels were so low that differences were slight and firm explanations could not be made.

Table 1

Concentration of principal carotenoids in cooked green vegetables from restaurants [a, b]

Food	Restaurant	Carotenoid concentration (μg/g)			
		β-carotene	Lutein	Violaxanthin	Neoxanthin
Broccoli; boiled	A	18,9±5,7a	34,6±8,3a	6,8±1,5a	8,3±2,7a
	B	22,2±4,2a	39,6±5,1a	3,1±4,4a	7,4±2,8a
	E	15,7±3,5a	31,1±5,2a	6,0±2,1a	6,7±1,9a
General average		18,9±5,0	35,1±6,9	5,3±3,2	7,6±2,5
Broccoli; stir-fried	A	11,4±3,7b	27,6±2,7a	5,0±2,0a	7,4±1,5a
	E	20,1±6,5a	37,9±12,9a	4,1±2,6a	6,5±1,5a
General average		15,7±6,7	32,8±10,3	4,5±2,2	6,9±1,5
Endive; stir-fried	C	12,4±3,7	23,4±5,0	6,8±1,5	7,0±2,0
Green bean; boiled	A	1,3±0,2c	2,2±0,5a	tr[c]	tr
	B	1,6±0,3b	2,4±0,4a	tr	tr
	C	2,0±0,3a	2,9±0,7a	tr	tr
General average		1,6±0,4	2,5±0,6	tr	tr
Green bean; stir-fried	A	1,7±0,6a	2,9±0,9a	tr	tr
	B	1,8±0,6a	2,8±0,5a	tr	tr
	C	1,8±0,7a	2,5±1,0a	tr	tr
General average		1,8±0,6	2,7±0,8	tr	tr
Kale; stir-fried	A	22,8±4,2a	35,0±4,5a	8,8±4,4a	7,9±2,4a
	B	22,4±4,2a	28,6±4,9a	2,8±1,6b	4,9±2,1a
	D	24,0±2,3a	31,0±3,7a	5,3±3,7ab	6,3±2,7a
General average		23,0±3.5	31,5±4,9	5,6±4,1	6,4±2,6

[a] mean and standard deviation of five different sample lots.

[b] For each vegetable sample, values in the same column bearing different letters are significantly different ($p<0.05$).

[c] tr, trace.

Few data on cooked food is found in the literature, but some can be compared to those obtained in this study (Table 2), although the results of other authors were obtained with samples prepared in the laboratory, simulating home cooking.

The β-carotene content in boiled broccoli was found to be 3,1 μg/g by Lessin, Catigani and Steven (1997), 11,3 μg/g by Hart and Scott (1995) and 14,9 μg/g by Godoy and Rodriguez-Amaya (1998), the latter being a Brazilian data. The value obtained in this study (19,9 μg/g), agrees with the latter data. The discrepancy with the other data, at least in part, can be due to varietal differences.

The concentration of β-carotene in cooked endive reported by Sweeney and March (1971) (9,6 μg/g), agrees with the concentration of β-carotene of 12,4 μg/g from this study.

As in the present paper, other authors demonstrated that boiled green bean is not a good source of carotenoids (Godoy & Rodriguez-Amaya, 1998; Granado, Olmedilla, Blanco & Rojas-Hidalgo, 1992; Hart & Scott, 1995), β-carotene concentration varies from 1.0 to 3.2 μg/g and lutein from 4.8 to 5.5 μg/g. An exception is the paper of Cruz-García, González-Castro, Oruña-Concha, López-Hernández, Simal-Lozano & Simal-Gándara (1997), that reported much higher levels of these carotenoids in boiled green beans, the concentrations being 48,1 μg/g for β-carotene and 16,1 μg/g for lutein. This large difference cannot be easily explained as natural variability.

Table 2

Comparison of the carotenoid content of cooked vegetables with published data

Vegetable/ Reference	Mode of Preparation	Concentration (μg/g)			
		β-Carotene	Lutein	Violaxanthin	Neoxanthin
Broccoli					
Hart and Scott (1995)	Boiled	11.25	19.9	nd[a]	nd
Lessin et al. (1997)	Boiled	3.1	nd	nd	nd
Godoy and Rodriguez-Amaya (1998)	Boiled	14.9	nd	nd	nd
This study	Boiled	18.9	35.1	5.3	7.6
	Stir-fried	15.7	32.8	4.5	6.9
Endive					
Sweeney and March (1971)	Cooked	9.6	nd	nd	nd
This study	Stir-fried	12.4	23.4	6.8	7.0
Green bean					
Godoy and Rodriguez-Amaya (1998)	Boiled	1,0	nd	nd	nd
	Stir-fried	0,8	nd	nd	nd
Cruz-García et al. (1997)	Boiled	48,1	16,1	nd	nd
Granado et al. (1992)	Boiled	2,4	4,8	nd	nd
Hart and Scott (1995)	Boiled	3,2	5,5	nd	nd
This study	Boiled	1,6	2,5	tr	tr
	Stir-fried	1,8	2,7	tr	tr
Kale					
Khachik et al. (1986)	Cooked	126	235	7,7	27,6
This study	Stir-fried	23,0	31,5	5,6	6,4

[a]nd, not determined.

For stir-fried green bean, Godoy and Rodriguez-Amaya (1998) reported a value of 0.8 μg/g for β-carotene, less than half of the value found in this study (1.8 μg/g). The difference may be due to more drastic conditions in the laboratory-simulated stir-frying and/or use of younger beans in Godoy and Rodriguez-Amaya's work. In any case, the levels are low in both studies.

In the case of cooked kale, the concentration of β-carotene, lutein and neoxanthin of 23,0, 31,5 and 6,4 μg/g, respectively, do not agree with those reported by Khachik et al (1986): 126 μg/g for β-carotene, 235 μg/g for lutein and 28 μg/g for neoxanthin. The β-carotene content in raw kale in Khachik et al's work (146 μg/g), also disagrees with previous Brazilian data for β-carotene in raw kale, 26 μg/g (Godoy & Rodriguez-Amaya, 1998) and 38 μg/g (Mercadante & Rodriguez-Amaya, 1991). This difference is too large to be accounted for by varietal differences. Surprisingly, the values reported for violaxanthin in the study of Khachik et al. and in the present study agree, being 7,7 μg/g and 5,6 μg/g respectively.

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CAPÍTULO 4

CAROTENOID DATA FOR COOKED FOODS: DIRECT ANALYSIS VERSUS CALCULATION USING RETENTION PERCENTAGES

Artigo a ser enviado ao Journal of Food Composition and Analysis

Carotenoid Data for Cooked Foods: Direct Analysis Versus Calculation Using Retention Percentages

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Data on cooked vegetables is lacking in food carotenoid databases. Direct analysis is difficult, time-consuming and expensive. Thus, data have been calculated from raw sample results, using retention percentages. This work was carried out to compare values obtained by direct analysis with those obtained by calculation. Percent retention and percent concentration change were obtained for carotenoids of cooked broccoli, endive, green bean and kale. Using percent change, calculation gives the values obtained by direct analysis of cooked samples. Percent retention correctly shows loss of carotenoids during cooking, but does not provide the carotenoid concentrations of cooked vegetables. However, applying percent retention or change on data of raw samples other than those used to obtain the percentages, is confounded by the interplay of factors such as season and maturity and differing cooking conditions, values calculated with percent retention rather than percent change, sometimes coinciding better with those of direct analysis.

Key Words: carotenoid, analysis, retention, cooked vegetables.

INTRODUCTION

The consumption of carotenoid-rich foods has long been recognized as beneficial to health because some of these compounds are transformed to vitamin A in the human organism. More recently, interest in the study of the carotenoids has increased because of their antioxidant and immunoenhancement properties, lowering the risk of degenerative diseases such as cancer, macular degeneration and cardiovascular diseases.

Some vegetables that are carotenoid sources are consumed preferentially or only in cooked form, but most of the published data on carotenoids refer to the composition of raw foods and are limited to β-carotene.

The best way to obtain data on cooked or processed foods is by direct analysis. This approach, however, is very expensive, difficult and time-consuming, requiring considerable material resources and trained human resources. As an alternative, values can be calculated from the data of raw samples corrected by retention percentages obtained during common cooking practices (Rand et al., 1991). However, a detailed literature review shows that retention calculations are frequently subject to errors (Rodriguez-Amaya, 1997).

Heinonen et al. (1997) carried out a study that compared values obtained by direct analysis with calculated values, the only work found in the literature with this objective. Three mixed diets were analyzed in terms of several nutrients, among them β-carotene. The values obtained for β-carotene by analysis was only 50 to 60% of the values calculated for this carotenoid.

In the present study, the concentrations of the principal carotenoids of raw and cooked green vegetables were determined in paired samples, the percentages of retention

and concentration change were calculated and the values obtained by direct analysis and by calculation were compared.

MATERIALS AND METHODS

Sample Preparation

Paired samples of four green vegetables were analyzed: broccoli, endive, green beans and kale. Raw samples were purchased at a local market, each sample being analyzed on the same day it was purchased. Sample preparation was done as follows:

Broccoli: flowerlets and small leaves were separated from the stalks of two bunches, mixed and separated in three portions. One portion was analyzed in the raw state, another was boiled and the other was cut into smaller pieces and stir-fried.

Endive: all the leaves from two heads of endive were cut longitudinally at the middle. Halved leaves from one side were put together and analyzed in the raw state and those from the other side were cut in strips of about 2 cm and stir-fried.

Green beans: 500 g of green beans were cut into pieces of about 4 cm, mixed and separated into three portions. One portion was analyzed raw, one was boiled and the other was stir-fried.

Kale: the leaves from two bunches of kale were cut longitudinally at the middle. Halved leaves from one side were put together and analyzed raw and those from the other side were cut into very narrow strips and stir-fried.

Broccoli and green beans were boiled in water with salt, enough to cover the sample, in a pot without lid, for five minutes counted after the boiling point was reached. Stir-frying was done with a minimum amount of hot oil and the vegetables were seasoned with garlic and salt. A small amount of water was added during stir-frying of broccoli,

green beans and kale just enough to prevent burning. Stir-frying took four minutes for broccoli, endive and kale and ten minutes for green beans.

Carotenoid Determination

The analytical method was based on the procedure established by Kimura and Rodriguez-Amaya (2001), with some modifications to adapt it to the samples analyzed. Standards were obtained from leafy vegetables, the carotenoids being extracted with cold acetone, partitioned to petroleum ether and concentrated in a rotary evaporator. Carotenoid standards were then isolated by open column chromatography (OCC). Samples were analyzed by high performance liquid chromatography (HPLC). The samples were ground in a food processor and subsamples were weighed (36-38 g for green beans and 2.5-4.0 for other samples) for analysis. The carotenoids were extracted with cold acetone and partitioned to petroleum ether. The extract was saponified (adding equal volume of 10% KOH in methanol to the petroleum ether extract containing 0.1% of BHT, the mixture being left at room temperature in the dark) and washed when necessary, concentrated in a rotary evaporator and dried under nitrogen. Injection into liquid chromatograph was done soon after the carotenoids were redissolved in HPLC grade acetone (2 ml) and filtered through a 0.22 μm PTFE filter.

Raw samples, in which carotenoids were more difficult to extract, were shaken in an ultrasonic bath with acetone for 20 minutes before extraction. Acetone extracts of stir-fried broccoli, endive and kale, that contained excess oil, were left in a freezer for two hours to solidify the oil and then filtered inside the freezer through a cold glass sintered funnel. Partition then followed. One-hour saponification was necessary for boiled green beans

extracts to remove a chlorophyll degradation product that eluted close to β-carotene. In stir-fried green bean extracts, overnight saponification was required to remove both the degradation product and part of the oil. Oil was completely removed by keeping the carotenoids, after redissolving in acetone for injection, in the freezer for 30 minutes, followed by filtration, before injecting.

HPLC conditions previously established for leafy vegetables (Kimura and Rodriguez-Amaya, 2001) were used. The analysis was performed on a Waters separation module (model 2690) equipped with an automatic injector, controlled by a Millenium workstation (version3.10). Separation of the carotenoids was carried out with a C18 monomeric column (Spherisorb S3 ODS2), 3μm, 4.6 x 150 mm and the mobile phase, at a flow rate of 0.5 ml/min, was composed of acetonitrile:methanol:ethyl acetate in a gradient from 95:5:0 to 60:20:20 in 20 minutes, the latter proportion being maintained until the end of the run. Triethylamine (0.05%) was added to acetonitrile, as recommended by Hart and Scott (1995), to improve the recovery of carotenoids from the chromatographic column. Detection was at the wavelengths of maximum absorption (max plot), using a UV-Visible photodiode array detector (Waters model 996).

Calculation of percent retention and percent change

The percentage of true retention of the carotenoids during cooking was calculated according to Murphy et al (1975), using the equation:

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

To calculate the percentage change of the carotenoid concentration during cooking, the following equation was used:

$$\% \text{ concentration change} = \frac{\text{carotenoid content per g of cooked food}}{\text{carotenoid content per g of raw food}} \times 100$$

RESULTS AND DISCUSSION

The carotenoid contents of the raw and cooked vegetables analyzed, the percent retention, the percent concentration change and the calculated values for carotenoids in the cooked vegetables are shown in Table 1. The carotenoid concentrations of boiled vegetables are lower than those of the corresponding raw samples because of the absorption of water during boiling, which decreases the carotenoid content per unit weight of sample. On the other hand, there is no general trend with stir-fried vegetables. Higher carotenoid concentration can be noted in some cases, reflecting the loss of water during stir-frying, which increases the amount of carotenoid per unit weight of sample. Lower values can be observed in other cases, indicating appreciable degradation of carotenoids.

Comparing values obtained through calculation with the values obtained by analysis, it can be clearly observed that values obtained using retention percentages do not agree with results obtained by analysis, while values obtained using percent concentration change are the same as those obtained by analysis.

The percent retention is a useful index for showing the occurrence of carotenoid degradation during cooking or processing of food. Since weight changes are compensated for, only the retention or loss of carotenoids is monitored. For calculating the carotenoid concentration of cooked food from raw sample data, however, the appropriate index is the

percent concentration change because it reflects the alterations that occur during cooking, which include weight changes.

The lower retention percentages of violaxanthin in all samples, except one, show that it is more prone to degradation than the other carotenoids. In stir-fried endive, β-carotene markedly suffered more degradation than the xanthophylls. This unexpected result was confirmed in two trials. Comparing the percentages obtained for boiled and stir-fried broccoli, there appears to be a greater tendency for degradation in stir-frying. The more than 100% values obtained for boiled and stir-fried green beans does not indicate true increases of carotenoid content; there is no possibility that carotenoids can be produced during cooking. These values merely reflect the difficulty in appraising retention of carotenoids during cooking when the carotenoid levels are so low.

In order to compare published results obtained by direct analysis and calculated values obtained by the different possible ways available, as done in compiling published data for databases, Tables 2 and 3 are presented. To minimize effects of factor other than cooking (e.g. variety, climate), only Brazilian data are included. Although done in different years, by different analytical methods (OCC and HPLC), in home-cooked and restaurant samples, the analytical data obtained by Godoy and Rodriguez-Amaya (1998) and de Sá and Rodriguez-Amaya (2001) are comparable, except for stir-fried green bean, which contains very small amounts of carotenoids.

TABLE 1

Carotenoid concentrations in raw and cooked vegetables obtained by direct analysis and calculated carotenoid concentrations in cooked vegetables [1]

Cooked food/ Carotenoid	Carotenoid concentration (μg/g) by direct analysis		Percent retention	Percent change	Calculated concentration (μg/g) in cooked vegetables	
	raw	cooked			by % retention	by % change
Broccoli, boiled						
Neoxanthin	6.3	5.3	91%	84%	5.7	5.3
Violaxanthin	10.3	5.3	56%	52%	5.7	5.3
Lutein	26.6	24.5	99%	92%	26.4	24.5
β-Carotene	15.2	12.9	92%	85%	13.9	12.9
Broccoli, stir-fried						
Neoxanthin	6.3	5.9	84%	93%	5.3	5.9
Violaxanthin	10.3	6.8	60%	66%	6.2	6.8
Lutein	26.6	26.9	91%	101%	24.2	26.9
β-Carotene	15.2	15.4	92%	102%	13.9	15.4
Endive, stir-fried						
Neoxanthin	12.9	14.2	97%	110%	12.5	14.2
Violaxanthin	17.5	14.9	75%	85%	13.2	14.9
Lutein	43.7	43.4	87%	99%	38.2	43.4
β-Carotene	28.8	22.6	69%	78%	19.9	22.6
Green bean, boiled						
Lutein	2.3	2.6	108%	113%	2.5	2.6
β-Carotene	1.1	1.8	153%	160%	1.7	1.8
Green bean, stir-fried						
Lutein	2.3	2.9	101%	126%	2.3	2.9
β-Carotene	1.1	1.9	137%	170%	1.5	1.9
Kale, stir-fried						
Neoxanthin	14.6	19.2	89%	131%	13.0	19.2
Violaxanthin	23.6	11.4	33%	48%	7.7	11.4
Lutein	48.0	69.2	98%	144%	47.1	69.2
β-Carotene	43.8	59.8	93%	137%	40.7	59.8

[1] Means of duplicate analyses.

TABLE 2

Comparison of β-carotene concentration obtained by direct analysis

Vegetable/mode of preparation	Concentration (μg/g)	
	From Godoy and Rodriguez-Amaya (1998)	From de Sá and Rodriguez-Amaya (2001)
Broccoli, boiled	14.9±1.1[1]	18.9±5.0[4]
Broccoli, stir-fried	nd	15.7±6.7[3]
Endive, stir-fried	nd	12.4±3.7[2]
Green bean, boiled	1.0±0.2[2]	1.6±0.4[4]
Green bean, stir-fried	0.8±0.2[2]	1.8±0.6[4]
Kale, stir-fried	nd	23.0±3.5[4]

[1] Means and standard deviation of four sample lots

[2] Means and standard deviation of five sample lots

[3] Means and standard deviation of ten sample lots

[4] Means and standard deviation of fifteen sample lots

nd: not determined.

TABLE 3

Comparison of calculated β-carotene concentrations

Vegetable/mode of preparation	Calculated β-carotene concentration (μg/g)				
	a	b	c	d	e
Broccoli, boiled	14.9	15.4	16.6	nd	nd
Broccoli, stir-fried	nd	18.4	16.6	nd	nd
Endive, stir-fried	nd	nd	nd	13.5	11.9
Green beans, boiled	1.0	1.6	1.5	nd	nd
Green beans, stir-fried	0.8	2.0	1.2	nd	nd
Kale, stir-fried	nd	35.2	24.0	47.9	32.6

a - Calculated from raw sample data of Godoy and Rodriguez-Amaya (1998) and % concentration change estimated from the data for raw and cooked vegetables of Godoy and Rodriguez-Amaya (1998).

b - Calculated from raw sample data of Godoy and Rodriguez-Amaya (1998), using the % concentration change obtained in the present study.

c - Calculated from raw sample data of Godoy and Rodriguez-Amaya (1998), using the % retention obtained in the present study.

d - Calculated from raw sample data of Ramos and Rodriguez-Amaya (1987), using the % concentration change obtained in the present study.

e - Calculated from raw sample data of Ramos and Rodriguez-Amaya (1998), using the % retention obtained in the present study.

nd, not determined.

The calculated values in Table 3 are also in fairly good agreement. Applying the percent concentration change estimated from the results of Godoy and Rodriguez-Amaya (1998) (82% for boiled broccoli, 83% for boiled green bean and 67% for stir-fried green bean), on the data of the raw samples obtained by the same authors, gave exactly the concentrations obtained by direct analysis, as in the present study (Table 1). However, the differences in using percent retention and percent concentration change, when applied to samples other than that for which the percentage were obtained, is not as straightforward. The calculated values are closer to those obtained by direct analysis when the percent retention is used in various samples. Since the raw samples in this case came from lots different from those submitted to direct analysis, the interplay of such factors as season and maturity, as well as differing cooking conditions (conditions to which the analyzed samples were submitted and those existing when the retention and change percentages were computed) must have affected the results.

The percent concentration change obtained in the present study for β-carotene in boiled broccoli (92%) differs with that estimated from Godoy and Rodriguez-Amaya (1998) for the same vegetable (82%), both being Brazilian samples, cooked and analyzed on the same day of purchase and prepared by similar conditions (boiling for five minutes). In any case, calculation of the carotenoid concentrations in cooked foods from the raw sample data is a viable alternative to direct analysis, provided that more representative percentages of change are obtained and accurate raw sample data are available.

ACKNOWLEDGEMENTS

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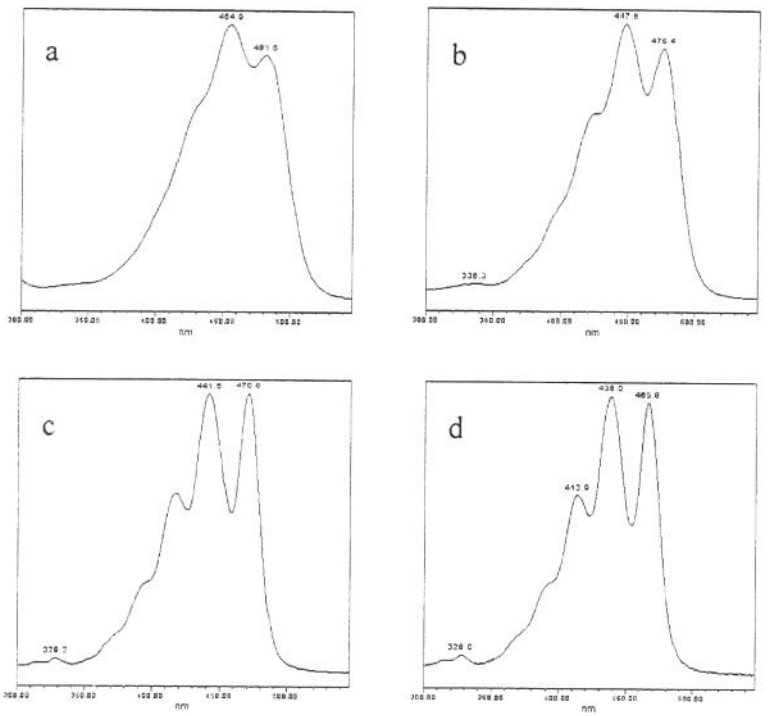
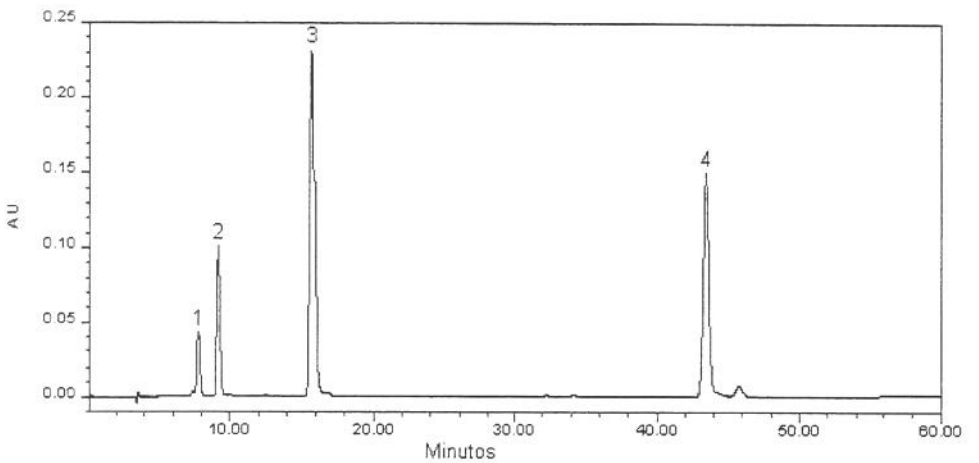
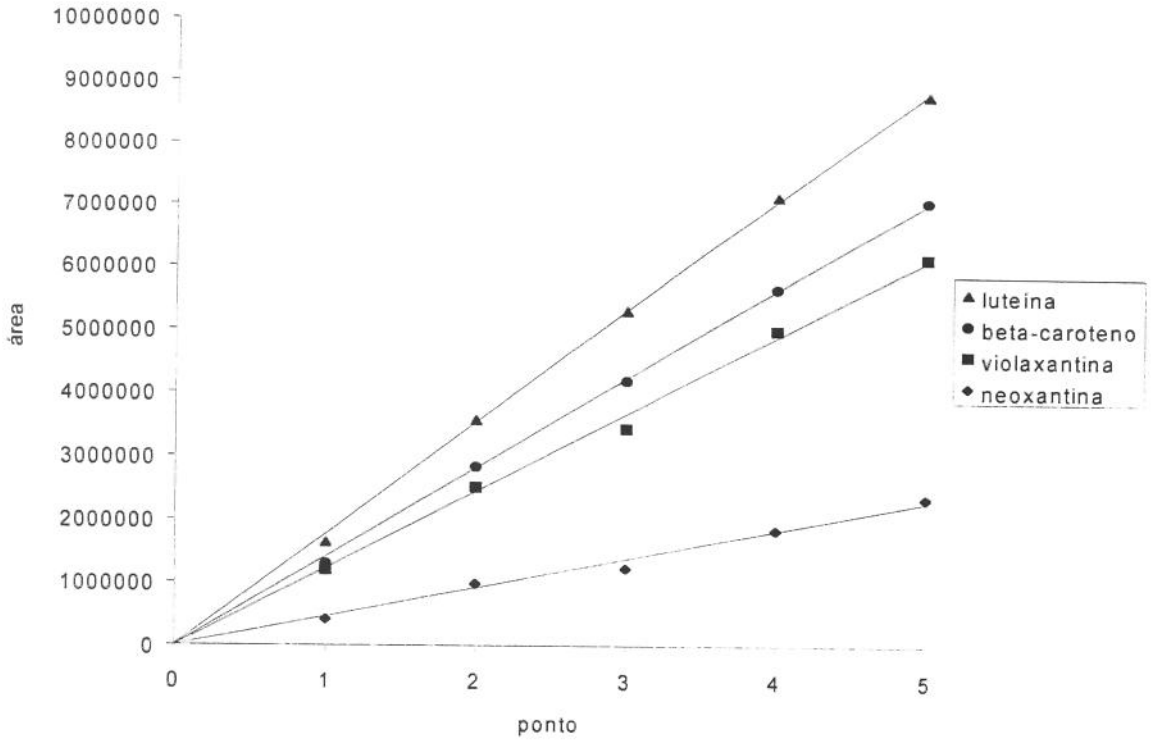
ANEXOS



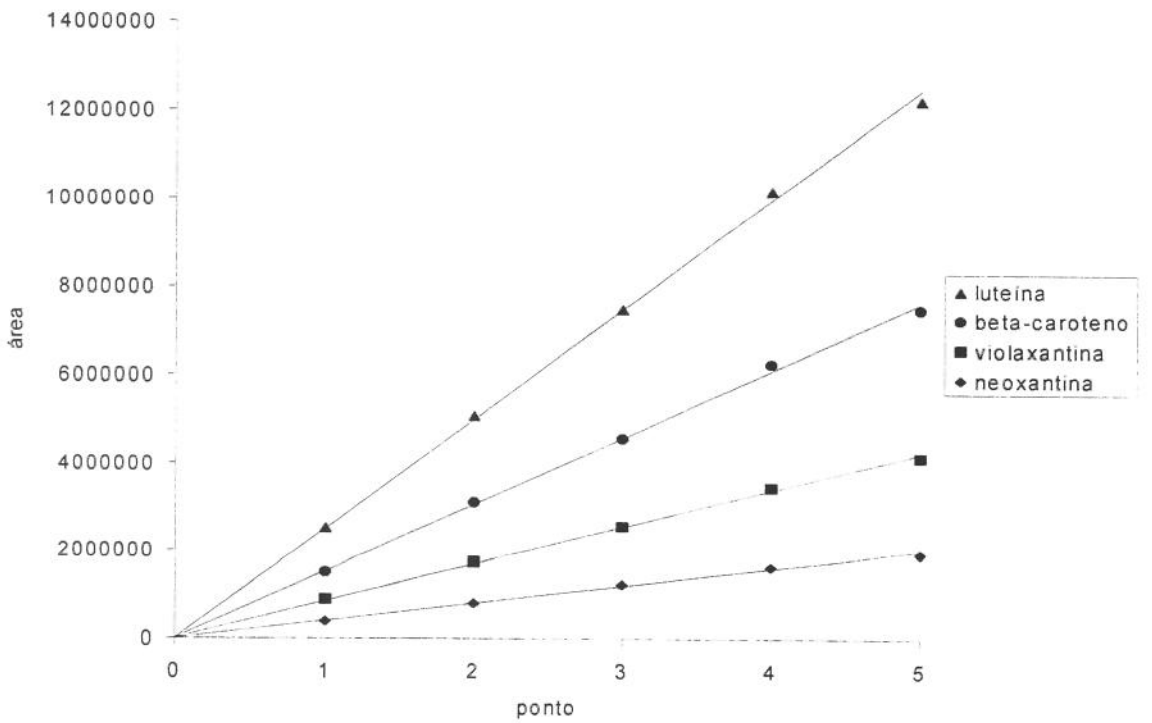
ANEXO I. Espectros visíveis dos carotenóides investigados, obtidos por detector de arranjo de diodos. (a) β-caroteno; (b) luteína; (c) violaxantina; (d) neoxantina



ANEXO II. Cromatograma típico da mistura de padrões de carotenóides. 1 - neoxantina; 2 - violaxantina; 3 - luteína; 4 - β-caroteno.



ANEXO III. Curva de calibração obtida no início das análises



ANEXO IV. Curva de calibração obtida após troca da lâmpada do detector