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Departamento de Ciência de Alimentos

Composição e estabilidade de carotenóides em alimentos

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Se, a princípio, a idéia não é absurda,

então não há esperança para ela.

Albert Einstein

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Resumo Geral

Os carotenóides estão entre os componentes de maior interesse em relação aos efeitos benéficos dos alimentos a saúde humana. Entretanto, estas atividades benéficas estão ligadas às suas estruturas e as concentrações presentes nos alimentos. Dados confiáveis de quantificações são necessários para indicar fontes, aprimorar processos, estabelecer melhor a associação entre ingestão/consumo e a incidência/risco de desenvolvimento de doenças e compreender seu mecanismo de ação e degradação. O capítulo 1 apresenta uma revisão bibliográfica das pesquisas realizadas nos últimos 5 anos em relação aos carotenóides em alimentos, descrevendo os principais efeitos benéficos à saúde, a biodiversidade de fontes carotenogênicas, os aspectos analíticos, os processamentos emergentes que buscam preservar e/ou estabilizar estes componentes, e por fim, estudos de degradação.

A alta instabilidade dos carotenóides faz com que sua análise seja um desafio. Para garantir a confiabilidade dos resultados, o Laboratório de Carotenóides da FEA/UNICAMP faz avaliações periódicas do desempenho do método e dos analistas. O capítulo 2 apresenta os resultados mais recentes dessa avaliação, utilizando um material de referência certificado (*baby food composite* 2383). Participaram do estudo, analistas com diferentes tempos de experiência na análise de carotenóides. Houve uma ótima concordância para valores de carotenos. Porém, para xantofilas, o analista com pouca experiência obteve valores inferiores para luteína e zeaxantina.

O capítulo 3 apresenta a reavaliação dos teores de carotenóides em produtos de tomate, que é a principal fonte de licopeno na dieta humana, devido à introdução de

novas variedades de tomate, o desenvolvimento de novos produtos e avanços nas tecnologias de processamento e técnicas analíticas. A faixa de licopeno e β-caroteno total (μg/g) foram, respectivamente, 188-261 e 9,3-13 para extrato, 111-203 e 5,1-7,0 para catchup, 77-117 e 4,4-7,3 para polpa, 93-112 e 5,1-6,4 para molho pronto e 231-471 e 7,0-25 para tomate seco. Tomate seco, que foi analisado pela primeira vez, apresentou os maiores teores de licopeno e luteína.

As folhas também são importantes fontes de carotenóides, no capítulo 4, foram determinados os principais carotenóides em cinco folhas nativas e em duas comumente comercializadas para comparação. A concentração de luteína foi de 119 ± 21, 111 ± 48, 104 ± 44, 87 ± 7 e 34 ± 15 μg/g, e o teor de β-caroteno encontrado foi de 114 ± 22, 97 ± 40, 66 ± 18, 72 ± 9 and 32 ± 14μg/g para caruru, mentruz, taioba, serralha e beldroega, respectivamente. Com exceção da beldroega, todos os valores encontrados foram maiores que das folhas comerciais, salsa e coentro.

No estudo de novas tecnologias, o comportamento dos carotenóides em rúcula, couve e espinafre minimamente processados embalados em atmosfera modificada ativa ou passiva, estocados em diferentes condições de temperatura e luz, foi avaliado e discutido nos capítulos 5, 6 e 7. A qualidade sensorial e a composição gasosa na embalagem também foram avaliadas para verificar o *shelf-life*. De uma forma geral, neoxantina e violaxantina foram mais estáveis nas três verduras folhosas analisadas, com exceção da couve, que na presença de luz, parece ter ocorrido um estímulo do ciclo da violaxantina que envolve a sua de-epoxidação para a formação de zeaxantina. Os teores de luteína e β-caroteno diminuíram em rúcula e couve durante a estocagem, porém, com perdas menores em temperaturas mais baixas. Em espinafre, houve aumento de neoxantina, violaxantina, luteína e β-caroteno durante a estocagem em todas as condições, indicando que em alguns casos, o efeito das enzimas biosintéticas pode

prevalecer em relação às enzimas oxidativas.

Utilizando um Delineamento Composto Central Rotacional, as melhores condições de temperatura e proporção de agente encapsulante e recheio, foram otimizadas para obter uma maior retenção de β-caroteno na microencapsulação de polpa de acerola. As condições ótimas encontradas foram: 30% de maltodextrina ou amido modificado a temperatura de 157ºC e 20% de goma arábica a 175ºC. As retenções de β-caroteno e vitamina C foram, respectivamente, 92 e 106% para maltodextrina, 71 e 109% para o amido modificado, e 76 e 114% para goma arábica. A estabilidade do β-caroteno e da vitamina C da polpa de acerola microencapsulada embalada em sacos de filme flexível aluminizado foi avaliada. A melhor proteção foi verificada para a microencapsulação com goma arábica que obteve 65.4% e 96.7% de retenção de β-caroteno e vitamina C em 4 meses de estocagem, respectivamente. Enquanto que o controle não-encapsulado obteve apenas 26.4% e 79.2% de retenção, respectivamente.

O capítulo 9 estabeleceu uma estratégia para o estudo dos compostos voláteis formados a partir da degradação oxidativa de carotenóides em sistema-modelo de CMC (celulose microcristalina). O experimento foi conduzido com licopeno e os compostos voláteis formados foram tentativamente identificados por GC/MS (Cromatogradia Gasosa com Espectrometria de Massas). Três tipos de revestimento de fibras SPME (Microextração em Fase Sólida) com polaridades diferentes foram estudadas. A fibra mista de DVB/carboxen/PDMS foi a que obteve o maior número de picos e com maior intensidade. Os sete compostos majoritários corresponderam por 78,6% da área total dos picos do cromatograma. Três compostos identificados já foram reportados na literatura como produtos da degradação do licopeno responsáveis pelo aroma de alguns alimentos: o 2-hepten-6-ona, 2-metil, o citral ou geranial (*trans*-2,6-Octadienal, 3,7-dimetil) e o neral (*cis*-2,6-Octadienal, 3,7-dimetil).

Introdução Geral

O consumo de frutas e verduras vem sendo incentivado devido a estudos que apontam uma associação inversa entre a ingestão destes e a incidência ou risco de desenvolver doenças degenerativas, como o câncer e doenças cardiovasculares. Esta proteção tem sido atribuída aos compostos bioativos destes alimentos. Em anos recentes, novas tecnologias vêm sendo desenvolvidas para a produção de alimentos funcionais que preservam melhor seus nutrientes e compostos bioativos.

Dentre os compostos bioativos de maior interesse em relação à saúde humana, estão os carotenóides. A importância destes compostos extrapola seu papel como pigmentos naturais responsáveis pela coloração amarelo-alaranjada de algumas frutas, legumes, raízes, flores, peixes, crustáceos e aves. Os carotenóides apresentam funções ou ações biológicas importantes como atividade pró-vitamínica A, fortalecimento do sistema imunológico e decréscimo do risco de doenças degenerativas como o câncer, doenças cardiovasculares, catarata e degeneração macular. Por se tratar de moléculas altamente insaturadas, os carotenóides são muito instáveis, susceptíveis à oxidação e isomerização. Portanto, o monitoramento dos teores de carotenóides ao longo da cadeia alimentar e a busca de tecnologias emergentes que os preservem melhor são de suma importância.

Este trabalho teve como objetivos: (a) avaliar o desempenho analítico de integrantes do laboratório; (b) reavaliar os teores de carotenóides em produtos de tomate; (c) quantificar os principais carotenóides de folhas nativas brasileiras em

comparação com folhas comercializadas; (d) investigar o comportamento dos carotenóides durante o armazenamento de verduras folhosas minimamente processadas; (e) otimizar as condições de microencapsulação e avaliar a estabilidade de carotenóides durante armazenamento do produto microencapsulado e (f) estabelecer uma estratégia de estudo de compostos voláteis provenientes da degradação do licopeno.

Capítulo 1

Pesquisas recentes sobre carotenóides em alimentos: últimos cinco anos em foco

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Revisão

PESQUISAS RECENTES SOBRE CAROTENÓIDES EM ALIMENTOS: ÚLTIMOS CINCO ANOS EM FOCO

RESUMO

Esta revisão contempla os artigos de pesquisa sobre carotenóides em alimentos publicados entre 2005-2009, selecionados e separados pelos tópicos: saúde, biodisponibilidade, composição, métodos de análise, efeito de processamento e degradação. Vinte e seis por cento das publicações desta seleção foram pesquisas voltadas ao estudo dos efeitos dos carotenóides à saúde, principalmente, associações entre o consumo destes compostos e a incidência de câncer. Houve um aumento notável de estudos sobre a biodisponibilidade dos carotenóides provenientes de alimentos, que também podem ser considerados relacionados à saúde, perfazendo 15% dos trabalhos. A busca por tecnologias alternativas que possam preservar/estabilizar os carotenóides levou a trabalhos que avaliaram os efeitos de processamentos em alimentos (18%). Alguns estudos com levantamentos da composição de carotenóides em alimentos ainda foram publicados (16%), visando complementar banco de dados e/ou avaliar a biodiversidade de países e regiões a fim de minimizar deficiências nutricionais. Trabalhos sobre os métodos analíticos e degradação constituíram 9 e 16%, respectivamente, dos artigos publicados nestes tópicos nos últimos cinco anos.

Palavra-chave: carotenóides, alimentos, saúde, biodisponibilidade, composição, processamento, degradação, método.

INTRODUÇÃO

Os carotenóides estão entre os componentes de maior interesse em relação aos efeitos benéficos dos alimentos a saúde humana. Além da atividade pró-vitamínica A, bem conhecida ao longo dos anos, outras funções biológicas são atribuídas aos carotenóides como a redução do risco de certos tipos de câncer, doenças cardiovasculares, degeneração macular e catarata (Olson, 1999; Moeller et al., 2000; Handelman, 2001; Koh et al., 2004; Gerth et al., 2004; Tapiero et al., 2004; Krinsky and Johnson, 2005; Renzi e Johnson, 2008; Nishino et al., 2009). Essas funções são atribuídas à sua propriedade antioxidante, pela sua capacidade de seqüestrar o oxigênio singleto e reagir com radicais livres (Palace et al., 1999; Young and Lowe, 2001; Stahl and Sies, 2003; Kiokias and Gordon, 2004). Outros modos de ação são: modulação do metabolismo de carcinógenos, inibição da proliferação celular, aumento da diferenciação celular através de retinóides, estimulação da comunicação intercelular, aumento da resposta imunológica e fotoproteção (Olson, 1999; Stahl et al., 2002; Krinsky and Johnson, 2005).

Devido às evidências de suas atividades biológicas benéficas à saúde, também houve um estímulo para as pesquisas sobre a biodisponilidade de carotenóides nos alimentos, avaliando a influência da matriz alimentícia e os efeitos dos diversos processamentos industriais e/ou do preparo para consumo.

Altamente insaturados, os carotenóides são passíveis da degradação durante o processamento e estocagem dos alimentos. Muitos dados sobre a redução das atividades biológicas provocadas pelos processos industriais tradicionais já foram publicados. Em anos recentes, novas tecnologias vêm sendo desenvolvidas, visando preservar os compostos bioativos. Tecnologias não térmicas são especialmente interessantes pela

possibilidade de preservar melhor os componentes dos alimentos. Entre estas tecnologias, está em alta o processamento mínimo, que além de preservar as características naturais dos alimentos, facilita o seu preparo para os consumidores. Buscar alternativas que aumentem a estabilidade de carotenóides durante a estocagem dos alimentos também é primordial, as embalagens com atmosferas modificadas para conservação de frutas e legumes prontos para consumo e os processos de microencapsulação para estabilizar compostos instáveis têm sido investigados.

Na busca de artigos sobre carotenóides em alimentos no banco de dados do *Food Science and Technology Abstracts (Ovid)*, selecionando e separando por tópicos, observou-se que entre estes assuntos, os artigos publicados recentemente pesquisaram: (a) a correlação entre a ingestão de carotenóides e a incidência de algumas doenças (26%); (b) a bioacessibilidade/biodisponibilidade de carotenóides em alimentos (15%); (c) os efeitos dos processamentos industriais e de preparo doméstico (18%), (d) a composição de carotenóides nos alimentos nativos não-comercializados que compõem a biodiversidade e/ou são tradicionalmente consumidos em cada região (16%); (e) a cinética e os mecanismos de degradação (16%) e (f) os métodos de análises (9%). Esta revisão contempla as pesquisas de carotenóides em alimentos sobre estes assuntos publicados nos últimos cinco anos.

Carotenóides e saúde

Estudos epidemiológicos vêm demonstrando que o consumo de frutas e vegetais ricos em carotenóides está associado com uma menor incidência de doenças degenerativas como o câncer, doenças cardiovasculares, degeneração macular relacionada à idade e formação de catarata. Estudos experimentais e observacionais retrospectivos e prospectivos foram realizados em diversos países para correlacionar a

ingestão de carotenóides e a prevenção de câncer. A Tabela 1 apresenta resumidamente alguns dados de pesquisas publicadas entre 2005 e 2009.

Tabela 1. Pesquisas relacionadas ao consumo de carotenóides e a incidência de câncer.

Referência	Estudo	Composto	Tipo de câncer	Conclusões
Kobat et al. (2009)	Longitudinal Concentração sérica	Carotenóides Retinol Tocoferol	Câncer de mama em mulheres na pós-menopausa	- o câncer de mama invasivo foi inversamente associado às concentrações iniciais de α-caroteno e positivamente associado com o licopeno - α-caroteno e β-caroteno foram inversamente associados ao câncer de mama pelas análises de concentrações posteriores
Cha et al. (2008)	Experimental Apoptose em células HCT116	Extrato de *C. ellipsoidea* (violaxantina, anteranxantina, zeaxantina) Extrato de *C. vulgares* (luteína)	Câncer de colo	- os dois extratos realçaram a intensidade fluorescente da população de células apoptosas nas células HCT116 - *C. ellipsoidea* produziu um efeito de apoptose-induzida de aproximadamente 2,5x maior que a *C. vulgares* - os resultados indicam que as xantofilas bioativas da *C. ellipsoidea* podem ser utilizadas como ingredientes funcionais para a prevenção de câncer de colo
Mikhak et al. (2008)	Coorte Gene MnSOD codifica uma enzima antioxidante (SOD2) que protege células contra danos oxidativos	Licopeno	Câncer de próstata	- o gene MnSOD Ala16Val polimorfismo não foi associado ao risco de câncer de próstata total ou agressivo - homens com o genótipo MnSOD Ala/Ala que tiveram baixo consumo de licopeno a longo prazo tiveram um maior risco de câncer agressivo comparado com outros genótipos - quando o nível de antioxidante está baixo, o genótipo MnSOD Ala/Ala pode estar associado com o aumento do risco de câncer de próstata agressivo
Persson et al. (2008)	Caso-controle Concentração plasmática	Carotenóides	Câncer de estômago	- β-caroteno e α-caroteno foram inversamente associados ao câncer de estômago em homens, para mulheres, apenas o β-caroteno - nenhuma associação estatisticamente significativa foi encontrada entre luteína + zeaxantina, licopeno, retinol, α-tocoferol e γ-tocoferol e a incidência de câncer de estômago - os resultados indicaram que aqueles que possuem níveis muito baixos de α-caroteno e β-caroteno no plasma possuem maior risco de desenvolver câncer
Thomson et al. (2008)	Prospectivo	Vitamina C Vitamina E Selênio Carotenóides Vitamina A	Câncer de ovário	- o modelo multivariado de incidência de câncer de ovário não indicou nenhuma relação significativa entre os fatores da dieta e a incidência de câncer de ovário - os resultados indicaram que a ingestão de antioxidantes, carotenóides e vitamina A na dieta não está associado à redução da incidência de câncer de ovário

Key et al. (2007)	Prospectivo	Carotenóides Retinol Tocoferol	Câncer de próstata	- nenhum dos compostos avaliados foi associado significativamente com o risco de desenvolver câncer de próstata - licopeno e carotenóides totais não foram associados com o risco da doença localizada, mas foram inversamente associados com a incidência da doença avançada
Ghosh et al. (2008)	Caso-controle	Vitamina A, C e E α -caroteno β -caroteno Folatos	Câncer cervical	- reduções de incidência de aproximadamente 40-60% foram observadas em mulheres com altas *versus* baixas concentrações dos compostos estudados - os resultados indicam que uma dieta a base de vegetais ricos nesses nutrientes podem ser importantes na redução do risco de desenvolver câncer cervical
Zhang et al. (2007a)	Caso-controle Concentração plasmática	Licopeno Luteína Zeaxantina β-criptoxantina	Câncer de próstata	- sujeitos com maior teor de licopeno no plasma tiveram 55% menos incidência de câncer que os com teores menores - nenhuma associação foi observada para os níveis de α-caroteno e β-caroteno - altos níveis de licopeno, luteína + zeaxantina e β-criptoxantina na circulação foram associados a um baixo risco de desenvolver câncer de próstata
Zhang et al. (2007b)	Caso-controle	Carotenóides	Cancer epitelial de ovário	- uma maior ingestão de carotenóides pode reduzir o risco de câncer epitelial de ovário
Larsson et al (2007)	Coorte	Vitamina A Retinol Carotenóides	Câncer de estômago	- nenhuma associação foi encontrada em relação ao consumo de β-criptoxantina, luteína + zeaxantina ou licopeno - um maior consumo de vitamina A, retinol e carotenóides pró-vitamínicos A pode reduzir a incidência de câncer de estômago
Huang et al. (2007)	Caso-controle	α –caroteno β -caroteno β -criptoxantina Luteína Zeaxantina Licopeno	Câncer de mama	- não foram encontradas associações entre a incidência de câncer de mama e o consumo de α-caroteno e luteína + zeaxantina - Os resultados indicaram que maiores consumos de licopeno, β-caroteno e β-criptoxantina são associados a um menor risco de incidência de câncer de mama
Lunet et al. (2006)	Caso-controle	Carotenóides pró-vitamínicos A Vitamina C	Câncer de estômago	- a ingestão de carotenóides pró-vitamínicos A e vitamina C foram inversamente associados a incidência de câncer gástrico
Kelemen et al. (2006)	Caso-controle	Luteína Zeaxantina	Linfomas não-Hodgkin (Câncer no sistema linfático)	- Alto consumo de vegetais, luteína e zeaxantina e zinco são associados a um menor risco de Linfomas não-Hodgkin
Nkondjock and Ghadiridirian (2005)	Caso-controle	Carotenóides	Câncer de pâncreas	- A ingestão de licopeno proveniente, principalmente, do consumo de tomate, foi associado a uma redução de 31% da incidência de câncer de pâncreas - β-caroteno e carotenóides totais foram associados com uma significativa redução da incidência de câncer em indivíduos não-fumantes - Os resultados indicaram que uma dieta rica em tomates ou produtos de tomate pode ajudar a reduzir a incidência de câncer pancreático

Tang et al. (2005)	Experimental Apoptose em células DU145	Licopeno	Câncer de próstata	- A taxa de apoptose foi reduzida em 42,4% nas células DU145 tratadas com licopeno em comparação com as de controle sem tratamento - Concluíram que o licopeno é um agente preventivo contra o desenvolvimento de câncer de próstata

Dos 15 trabalhos publicados, apenas Thomson et al. (2008) não observaram em seus resultados uma associação entre a ingestão de carotenóides e a incidência de câncer. Os demais trabalhos encontraram, pelo menos, uma associação significativa ou uma evidência do efeito benéfico à saúde de um ou mais carotenóides. O licopeno foi o que obteve maiores correlações benéficas em relação ao seu consumo, sendo associado à redução do desenvolvimento de câncer de mama, próstata e pâncreas. α-Caroteno e β-caroteno foram relacionados à diminuição da incidência de câncer cervical, de mama, estômago e pâncreas. Já as xantofilas, luteína e zeaxantina, foram associadas à redução do risco de desenvolvimento de câncer de colo, próstata e do sistema linfático.

Alguns estudos recentes sobre o efeito dos carotenóides na prevenção de problemas cardíacos também foram realizados. A ingestão de frutas e vegetais ricos em β-caroteno foi inversamente associada ao risco de infarto do miocárdio. Porém, os teores de luteína e zeaxantina no tecido adiposo foram positivamente relacionados a esta incidência (Kabagambe et al., 2005). Em mulheres em pré e pós-menopausa, uma correlação significante entre lipoproteínas de baixa densidade (LDL)-colesterol e ingestão de licopeno proveniente de fontes alimentícias foi encontrada. Em ambos os estágios biológicos e para cada categoria de risco cardiovascular (baixo, moderado e alto), foi observada uma relação inversa com o consumo de licopeno (Torresani, 2009).

Algumas pesquisas estudaram a ação dos carotenóides no tratamento ou prevenção de outras doenças. Vitaglione et al. (2007) desenvolveram um alimento a base de tomate para agir como um coadjuvante no tratamento de pacientes com hepatite C. A

formulação aumentou o nível sérico de carotenóides em sujeitos saudáveis (controle) e, apesar de não ter influência no tratamento, foi eficiente na melhora do estado oxidativo durante a terapia anti-virosa nos pacientes. Já, num estudo prospectivo, o consumo de frutas, vegetais e carotenóides não foi correlacionado à formação de neuroglimas em adultos (Holick et al., 2007). Houve uma pequena evidência para a associação inversa entre a ingestão de licopeno e o risco de desenvolver diabetes tipo 2 (Wang et al., 2006a). Os carotenóides também mostraram uma proteção contra a perda de densidade mineral óssea no trocânter em homens e na espinha lombar em mulheres. Embora a associação não tenha sido consistente em todos os ossos examinados no estudo, os resultados indicam um papel protetor dos carotenóides para densidade mineral óssea em idosos (Sahni, 2009). A luteína demonstrou ter potencial para agir como um importante antioxidante nos eritrócitos e deste modo, contribuir para a prevenção de demência. (Nakagawa et al., 2009)

Em relação ao efeito antioxidante, a luteína mostrou ter maior atividade antioxidante que β-caroteno e o licopeno, além disso, obteve efeito anti-mutagênico (Wang et al., 2006b). A atividade antioxidante da combinação de β-caroteno, vitamina E e vitamina C foi superior a soma dos efeitos antioxidantes individuais, indicando a existência de uma sinergia entre compostos, que pode aumentar a eficácia natural dos antioxidantes. Misturas de luteína ou β-caroteno com isômeros de tocoferol também aumentaram a atividade antioxidante dos carotenóides. Já a mistura com ácido ascórbico apenas exerceu uma tendência de aumento, mas não foi significante (Liu et al., 2008).

Algumas divergências nas pesquisas sobre a eficiência dos carotenóides contra a incidência de doenças foram encontradas. Por exemplo, estudos recentes realizados por Trumbo e Ellwood (2006), Bartlett e Eperjesi (2008) e Cho et al. (2008) não encontraram correlação entre a ingestão de luteína e/ou zeaxantina e a saúde dos olhos tão bem

reportada ao longo dos anos. Os efeitos podem não ser evidentes quando um composto é analisado individualmente, pois há possíveis sinergias ou efeitos aditivos de outros nutrientes. Entretanto, sempre há associações benéficas vinculadas ao consumo de alimentos saudáveis, essencialmente, frutas, legumes e verduras.

Biodisponibilidade dos carotenóides em alimentos

Os carotenóides e seus metabólitos devem ser absorvidos para chegarem aos tecidos e assim, exercer suas atividades biológicas benéficas à saúde. Porém, a absorção de carotenóides no organismo é, geralmente, ineficiente e pode ser influenciada pela matriz alimentícia, pelo tipo de processo, por outros componentes da dieta e pela condição nutricional e fisiológica das pessoas. Portanto, uma estimativa confiável da biodisponibilidade de carotenóides é problemática (Falila et al., 2008). Muitos trabalhos foram publicados nestes últimos cinco anos sobre a biodisponibilidade e/ou bioacessibilidade de carotenóides de alimentos e as influências provocadas pelo preparo para consumo e pelo processamento industrial.

Diversos métodos estão disponíveis para estimar a capacidade do intestino em absorver os carotenóides ingeridos e que chega a ser disponibilizada aos tecidos-alvo (biodisponibilidade). Algumas pesquisas estimam apenas a bioacessibilidade, fração de micronutrientes transferida da matriz alimentícia para as micelas, que é diferente da estimativa da biodisponibilidade, relação da fração ingerida e a recuperada no plasma. Apesar das diferenças, a bioacessibilidade utilizando modelo *in vitro* foi bem correlacionada com dados de biodisponibilidade determinada em humanos (Reboul et al., 2006).

Os carotenóides provenientes de alimentos mais pesquisados em relação a sua bioacessibilidade/biodisponibilidade foram o β-caroteno, licopeno e luteína. Mas, α-

caroteno, zeaxantina e β-criptoxantina também foram estudados. Dos 15 trabalhos apresentados na Tabela 2, oito analisaram a bioacessibilidade *in vitro,* seis avaliaram a biodisponibilidade *in vitro* utilizando células do intestino Caco2 e apenas um realizou o estudo *in vivo*, avaliando a concentração no plasma.

Tabela 2. Bioacessibilidade e biodisponibilidade de carotenóides em alimentos.

Referência	Estudo	Alimento	Interferências	Conclusões
Blanquet-Diot et al (2009)	Biodisponibilidade *In vitro* Modelo do trato gastrointestinal β-caroteno zeaxantina licopeno	Tomates	Sem processamento *in natura*	- zeaxantina e luteína foram estáveis durante toda a digestão *in vitro* - β-caroteno e *trans*-licopeno degradaram nos compartimentos referentes ao jejuno e íleo (30 e 20% no final da digestão, respectivamente) - a micelarização do β-caroteno do tomate vermelho foi menor que a do tomate amarelo - a recuperação de licopeno do tomate vermelho também foi menor que a do suplemento, mostrando o efeito da matriz
Tibaeck et al. (2009)	Bioacessibilidade *In vitro* licopeno	Tomates	Tratamento mecânico e térmico	- a acessibilidade parece aumentar com o tratamento mecânico e térmico - a maceração ou homogeneização sozinha não foram suficientes para aumentar a acessibilidade do licopeno
Granado-Lorencio et al. (2009)	Bioacessibilidade *In vitro* Concentração sérica *In vivo*	Sucos de frutas	Modificadores de absorção (leite e ferro)	- a hidrólise de ésteres das xantofilas e a transferência das xantofilas livre para a fase micelar foram maiores na presença dos modificadores de absorção (*in vitro*) - o consumo de suco de frutas provocou um aumento significante da concentração sérica de alguns carotenóides (*in vivo*) - a concentração de carotenóides aumentou com a ingestão de leite e leite + ferro, porém, não foi significativo
Ryan et al. (2008)	Biodisponibilidade *In vitro* Células Caco2 β-caroteno luteína licopeno β-criptoxantina	*Courgettes* Pimenta vermelha Tomate	Fervura Grelhamento Microondas Vapor	- a concentração de β-caroteno diminuiu na etapa de digestão independente do vegetal ou processo, porém todos os processos de cocção aumentaram a transferência do β-caroteno para as micelas - grelhamento e microondas foram os mais prejudiciais para a micelarização de β-criptoxantina - a micelarização dos carotenóides variou de 1,7 a 100% dependendo do carotenóide, alimento e tipo de cocção - as células Caco2 absorveram a maior quantidade de luteína das micelas em *courgettes* cozidos por microondas

Kean et al. (2008)	Bioacessibilidade *In vitro* Xantofilas Carotenos	Milho	Extrusão Cocção Panificação	- a eficiência de micelarização das xantofilas da farinha milho amarelo foi similar para os extrusados (63%) e pão (69%), mas foi menor para o mingau (48%) - a micelarização das xantofilas da farinha produzida com milho integral foi maior em pão (85%) e similar para *snacks* (46%) e mingau (47%) - a micelarização do β-caroteno foi de 10-23% para o extrusado e pão e 40-63% para o mingau, indicando que o cozimento com água influenciou positivamente a bioacessibilidade dos carotenos apolares
O'Sullivan et al. (2008)	Biodisponibilidade *In vitro* Células Caco2 humana Luteína	Espinafre Fresco Congelado enlatado	Fervura Microondas	- o espinafre enlatado sem cocção obteve o maior teor de luteína que o fresco e o congelado, após a digestão *in vitro* - o cozimento por microondas abaixou significativamente a micelarização da luteína do espinafre enlatado - embora o teor de luteína do espinafre digerido ou das micelas tenha diminuído, não houve diferença significativa na micelarização entre os métodos de cozimento - de forma geral, o transporte celular de luteína foi maior nas micelas de espinafre sem cozimento, independente de ser fresco, congelado ou enlatado
Granado-Lorencio et al. (2007a)	Bioacessibilidade *In vitro* Carotenóides	Verduras folhosas Legumes Frutas	Sem processamento *in natura*	- a estabilidade dos carotenóides em condições similares a digestão foi acima de 75%, independente do alimento analisado - a micelarização variou de 5 a 100% dependendo do carotenóide e do alimento - a hidrólise dos ésteres das xantofilas foi incompleta (<40%) e, ambas as formas, foram incorporadas no sobrenadante, independente da xantofila e alimento analisado - a bioacessibilidade variou bastante entre os diferentes carotenóides em um dado alimento e entre um mesmo carotenóide em diferentes alimentos.
Granado-Lorencio et al. (2007b)	Bioacessibilidade *In vitro* Carotenóides Tocoferol	Nêspera e naranja (xantofilas) Brócolis (carotenóides não esterificados)	Sem processamento *in natura*	- carotenoides e tocoferol obtiveram estabilidade acima de 70% - xantofilas esterificadas foram clivadas pela colesterol esterase, mas não pela lipase pancreática humana - menos de 40% da β-criptoxantina foi hidrolisada e a quantidade de xantofilas livre recuperada no sobrenadante foi maior em sucos que na matriz fresca - xantofilas foram mais transferidas para o sobrenadante que o β-caroteno e o tocoferol
Fernandes-Garcia et al. (2007)	Bioacessibilidade *In vitro* licopeno luteína	Oleoresinas	Adição de óleo e colesterol	- a micelarização da luteína aumentou com a adição de óleo vegetal, porém, a do licopeno diminuiu - colesterol e óleo afetaram significativamente (positivamente ou negativamente) a bioacessibilidade, dependendo das caracterís-ticas lipofílicas do carotenóide

Priyadarshani e Chandrika (2007)	Bioacessibilidade *In vitro* α-caroteno β-caroteno	Cenoura Abóboras Abobrinha Batata doce	Cozido em água Leite de côco e *curry* Salada crua	- o tratamento térmico aumentou a liberação do β-caroteno da cenoura cozida, esta foi 6 vezes maior que na crua - os resultados mostraram que a acessibilidade foi maior quando estes alimentos foram preparados com *curry* e leite de côco - a bioacessibilidade foi influenciada pelo tratamento térmico, tamanho das partículas e adição de gordura - a bioacessibilidade do β-caroteno foi maior em vegetais não-folhosos
Pullakhandam e Failla (2007)	Biodisponibilidade *In vitro* Células Caco2 β-caroteno luteína	Folhas de *Moringa oleifera*	Fresco Liofilizado Adição de óleo de amendoim	- β-caroteno e luteína foram estáveis durante a simulação gástrica e a digestão do intestino - a eficiência de micelarização da luteína foi maior que a do β-caroteno - a adição de óleo de amendoim aumentou a micelarização de ambos, principalmente, do β-caroteno - a biodisponibilidade *in vitro* do β-caroteno e da luteína na fração micelar das folhas foi confirmada pelo acúmulo destes nas células Caco2
Thakkar et al. (2007)	Biodisponibilidade *In vitro* Células Caco2 β-caroteno	Mandioca	10 cultivares Cozimento em água por 30 min	- a recuperação de β-caroteno foi acima de 70% após o processo de digestão - a eficiência de micelarização do β-caroteno foi de 30% (±2) para vários cultivares sem diferença significativa entre os isômeros e linearmente proporcional a concentração da mandioca cozida - a absorção de β-caroteno pelas células Caco2 foi proporcional a quantidade de micelas presentes
Goni et al. (2006)	Bioacessibilidade Fermentação simulando o cólon *In vitro* β-caroteno luteína licopeno	Frutas Vegetais	Sem processamento *in natura*	- a luteína apresentou maior bioacessibilidade (79%) que o β-caroteno (27%) e licopeno (40%) na etapa que simulou a digestão do intestino delgado - quantidades similares de licopeno e β-caroteno foram liberadas da matriz alimentícia (57%) na fase de condições do intestino grosso enquanto que a luteína obteve menor liberação (17%) - os resultados sugerem que 91% do β-caroteno, luteína e licopeno contido nas frutas e verduras estariam disponíveis no intestino durante o processo de digestão - a fermentação relativa ao cólon parece ser importante para a disponibilidade dos carotenóides no intestino
Ferruzzi et al. (2006)	Biodisponibilidade *In vitro* Células Caco2 humana β-caroteno	Compota de maçã, óleo de milho, pérolas aquosolúveis e *Dunaliella salina*	Sem processamento *in natura*	- os isômeros do β-caroteno foram estáveis durante a simulação de digestão - a eficiência de micelarização do isômero *cis* foi maior que na forma *trans* - a isomerização intercelular foi mínima - os *cis*-β-carotenos foram preferencialmente micelarizados e transferidos através da superfície do eritrócito das micelas
Serrano et al. (2005)	Bioacessibilidade Fermentação simulando o cólon *In vitro* β-caroteno luteína	Espinafre *chaya* e *macuy*	Sem processamento *in natura*	- a quantidade de β-caroteno e luteína liberada da matriz alimentícia pela ação das enzimas digestivas variou de 22-67% e 27-77%, respectivamente - os carotenóides liberados pela fermentação relativa ao cólon variaram de 2-11% e parte deles permaneceu intacta na fermentação média, podendo ser potencialmente absorvida no cólon - uma parte dos carotenóides parece ficar indisponível no trato intestinal (16% para o espinafre e 58% para *chaya)*

Composição de carotenóides em alimentos

Pouco se sabe sobre cultivares e variedades de plantas selvagens, não-comercializadas, restritas a pequenos grupos indígenas ou áreas geográficas. Devido à falta de informação sobre a composição nutricional destes vegetais e às influências globais cada vez maiores, a biodiversidade é sub-utilizada (Arora et al., 2008). Com o objetivo de identificar fontes ricas de carotenóides pró-vitamínicos que poderiam ser uma opção de alimento com potencial para diminuir a deficiência de vitamina A, principalmente em países em desenvolvimento, vários autores buscaram alternativas na biodiversidade de cada país ou região, estudando a composição de carotenóides em alimentos nativos.

Pesquisas recentes têm apresentado dados que confirmam a superioridade de micronutrientes presentes em alguns cultivares menos conhecidos e/ou em variedades selvagens em relação aos cultivares mais extensivamente utilizados. As folhas nativas brasileiras não cultivadas (caruru, mentruz, taioba, serralha e beldroega), que são consumidas apenas em algumas regiões rurais do país, apresentaram concentrações de carotenóides maiores que as das folhas comerciais, salsa e coentro, com exceção da beldroega (Kobori e Rodriguez-Amaya, 2008).

Carotenóides pró-vitamínicos A em bananas podem representar menos de 1 µg/100 g para alguns cultivares a até 8500 µg/100 g em outros (Burlingame et al., 2009). Dez cultivares nativos de bananas de polpa amarela ou alaranjada da Austrália obtiveram maiores níveis de carotenóides pró-vitamínicos A que os dois cultivares de polpa creme mais disponíveis comercialmente e consumidos no mundo (Englberger et al., 2006). Em oito cultivares de bananas indianas, a concentração de carotenóides totais foi bem superior na casca que na polpa, indicando que as cascas poderiam ser uma boa alternativa para o combate a deficiência de vitamina A, além de utilizar um sub-produto do

processo de industrialização de frutas (Arora et al., 2008). Resultados similares também foram encontrados em duas variedades de manga, onde a concentração de carotenóides foi maior na casca de frutos maduros que na polpa (Ajila et al., 2006).

A batata-doce apresentou diferenças de concentração de α-caroteno e β-caroteno de quase duas ordens de magnitude (Huang et al., 1999), a variedade alaranjada mostrou ser uma fonte excepcional de pró-vitamina A e pode ter um papel significativo na prevenção da deficiência de vitamina A (van Jaarsveld et al., 2005). Folhas de batata doce também mostraram ser excelentes fontes de luteína, ultrapassando os níveis encontrados em folhas crucíferas (Menelaou et al., 2006). A ingestão de uma variedade ao invés de outra pode ser a diferença entre a deficiência de micronutrientes e a suficiência (Burlingame et al., 2009).

O fato dos teores variarem bastante não apenas entre espécies, mas também com variedades, cultivares e fatores naturais como luz, solo e maturação, torna necessário a análise e inclusão dessas informações em tabelas de composição de alimentos de banco de dados (Rodriguez-Amaya, 2008). Com este mesmo objetivo, vários trabalhos foram publicados avaliando alimentos comumente consumidos em seus países, como a quantificação dos carotenóides em diversos vegetais folhosos de origem indiana (Lakshminarayana et al., 2005; Raju et al., 2007; Bhaskarachary et al., 2008), sete frutas e oito cultivares de *pandanus* (alimento indígena) de Kiribati (Englberger et al., 2005), pistaches da Sicília (Giuffrida et al, 2006), seis diferentes cerejas consumidas na Bulgária (Marinova e Ribarova, 2007), oito cultivares de sorgo maduro consumidos como cereais em regiões da África sub-Saara e Índia (Kean et al., 2007), vinte e um vegetais e doze óleos de origem vegetal comumente consumidos na Índia (Aruna et al., 2009), dez variedades de cinco frutas e cinco variedades de quatro vegetais que fazem parte da dieta dos portugueses (Dias et al., 2009) e em produtos de milho e ovo, frutas e vegetais ricos

em luteína e zeaxantina comprados em supermercados dos Estados Unidos (Perry et al., 2009).

Foi publicada a versão atualizada da database brasileira sobre carotenóides em alimentos, o maior banco de dados de carotenóides em alimentos do mundo, incluindo os *in natura*, processados industrialmente e preparados para consumo (Rodriguez-Amaya, 2008). Além da tabela extensiva, amostragem e preparo das amostras analíticas, fatores que influenciam na composição de carotenóides, efeitos de processamento e estocagem foram demonstrados e discutidos. Foi focalizada também a grande biodiversidade brasileira em fontes de carotenóides, com vários exemplos de frutas e hortaliças nativas tendo maiores concentrações destes compostos que os correspondentes de alimentos comercialmente produzidos.

Ainda são necessários mais esforços e recursos para analisar, compilar e disseminar os dados sobre a composição nutricional da biodiversidade nativa, pouco utilizada e sub-aproveitada dos alimentos. A disponibilidade de dados ajudará os países a promover espécies e variedades locais, além de avaliar e manter os ecossistemas naturais (Burlingame et al., 2009).

Análise de carotenóides

Alguns resultados discrepantes de estudos de biodisponibilidade e de avaliações de dietas ricas em carotenóides, assim como, nos estudos epidemiológicos que correlacionam a ingestão de carotenóides e a incidência de doenças, em parte, podem ser atribuídos aos dados incertos de concentrações de carotenóides em alimentos. As novas tendências das análises de carotenóides em alimentos não refletem apenas os avanços das metodologias e instrumentações analíticas, mas também a maior compreensão do papel dos carotenóides na saúde humana (Rodriguez-Amaya, 2006).

Phillips et al. (2007) reportaram os resultados do *USDA's National Food and Nutrient Analysis Program* sobre um total de 2554 valores obtidos por nove laboratórios para 259 concentrações de nutrientes certificados ou de referência de 26 materiais de referência certificados. Para carotenóides, mais de 20% dos *z-scores* foram acima de ±3,0, que são considerados inaceitáveis. Estes dados reforçaram a dificuldade de quantificar estes analitos em alimentos.

A quantificação precisa e exata de carotenóides é um processo complexo que depende de uma cuidadosa atenção na validação de métodos para matrizes específicas e na execução das análises (Kimura e Rodriguez-Amaya, 1999). Kimura et al. (2007) reportaram um extenso estudo com o desenvolvimento de métodos distintos para análise de carotenóides em batata doce, mandioca e milho por *screening* e cromatografia líquida de alta eficiência (CLAE), a fim de viabilizar a reprodução da análise de carotenóides em laboratórios de países em desenvolvimento com diferentes condições disponíveis. Por outro lado, Akhtar e Bryan (2008) tentaram desenvolver um único método de análise simples, rápido e robusto para determinação de carotenóides majoritários em alimentos processados e em suplementos (tabletes e cápsulas). Porém, o método não foi capaz de separar o licopeno numa mesma corrida e só obteve valores próximos aos da amostra de referência certificada para luteína e β-caroteno. As diferenças dos alimentos em relação à extratabilidade, estabilidade de carotenóides e presença de compostos interferentes, podem facilmente levar a resultados incorretos quando um único método padrão é aplicado para alimentos diferentes (Phillips et al., 2007).

A CLAE é a técnica mais comumente escolhida para separar e quantificar carotenóides. Entretanto, apesar da eficiência desta técnica, há uma etapa crucial para ser avaliada, anterior a separação cromatográfica, que é a extração quantitativa dos carotenóides da matriz do alimento. Para otimizar o procedimento de extração, alguns

fatores que influenciam a extração de carotenóides como tempo, temperatura e permanência das amostras em contato com solvente sob agitação, tamanho das partículas das amostras trituradas e hidratação das amostras antes da extração com solventes foram avaliados (Burkhardt e Bohem, 2007). A hidratação da amostra com água a temperatura ambiente por 5 minutos resultou em valores de carotenóides 1,5 a 2,5 vezes maiores em trigo duro (semolina) e em milho. Kimura et al. (2007) verificaram que a re-hidratação de amostras de milho seco a temperatura ambiente por 30 minutos ou em aquecimento (85℃) por 5, 10 e 15 minutos obtém resultados de extração de carotenóides equivalentes, mas tende a ser maior com o aquecimento por 10 minutos.

A extração também pode ser influenciada pelo solvente utilizado, em casca de tomate em pó, a extração dos carotenóides foi mais eficiente com etanol, comparada com a realizada utilizando acetato de etila (Calvo et al., 2007). Na determinação de carotenóides em milho amarelo, a quantificação foi alterada pela realização ou não da etapa de saponificação (Muzhingi et al., 2008). A recuperação de carotenóides foi maior sem esta etapa, mostrando que deve ser realizada apenas quando há a presença de xantofilas esterificadas.

Avaliação e validação de métodos utilizando materiais de referência certificados é o procedimento preferido para verificar a performance de um método e a capacidade do laboratório em obter resultados confiáveis. Tanto o processo de extração quanto as medidas instrumentais da análise podem ser avaliadas. Dois materiais de referência certificados (SRM) para quantificação de carotenóides estão disponíveis: o *Community Bureau of Reference* BCR485 (*freeze-dried mixed vegetables*) e NIST SRM 2383 (*baby food composite*) (Phillips et al, 2007). Um método também pode ser validado definindo sua linearidade, repetibilidade, precisão, sensibilidade, limites de detecção e quantificação.

Embora a CLAE seja muito exata, os custos elevados e o tempo exigido para realizar a análise, limitam seu uso a um pequeno número de amostras. Portanto, não são aplicáveis quando há a necessidade de analisar muitas amostras, como por exemplo, em programas de melhoramento de produção agrícola onde há milhares de cultivares para serem analisados (Bonierbale et al., 2009). A espectroscopia de reflectância no infravermelho próximo (NIRS) é uma técnica que requer apenas uma etapa de preparação de amostra (triturar e secar), é rápida e relativamente barata, além de facilitar a análise de diversos atributos simultaneamente (Osaki et al., 2006). O potencial do NIRS para estimar os carotenóides já foi demonstrado para milho (Brenna e Berardo, 2004) e recentemente, para batatas *Solanum phureja* (Bonierbale et al., 2009).

A cromatografia contra-corrente de alta velocidade demonstrou ser uma técnica poderosa para isolar compostos bioativos de plantas, como por exemplo, na separação de luteína e zeaxantina de espinafre e milho doce. De acordo com Aman et al. (2005), xantofilas obtidas dos extratos desses alimentos poderiam ser utilizadas como padrões de referência para fins analíticos, sem nenhum processo de purificação adicional.

Um grande progresso foi obtido na garantia de dados confiáveis em análise de carotenóides em alimentos. Etapas críticas e fontes de erros foram identificadas, estudos interlaboratoriais foram conduzidos e materiais referências certificados estão disponíveis. Entretanto, considerando que a análise de carotenóides possui uma dificuldade inerente e requer atenção para muitos detalhes, alguns resultados incorretos ainda podem ser notados (Rodriguez-Amaya, 2008).

Alterações de carotenóides durante processamento e armazenamento de alimentos

Devido a sua alta insaturação, as principais reações que acontecem com os

carotenóides, durante o processamento e armazenamento de alimentos são isomerização geométrica e oxidação, que resultam na perda da cor e alteração da atividade biológica. Já é conhecido que calor, luz e ácidos induzem a isomerização, e que a oxidação depende da disponibilidade de oxigênio e é estimulada pela luz, calor, presença de metais, enzimas e peróxidos (Rodriguez-Amaya, 1999; 2002).

Alguns trabalhos avaliaram a degradação de carotenóides durante a industrialização de alimentos, os processamentos tradicionais que utilizam tratamentos térmicos provocam grandes perdas de carotenóides. Na fabricação de *snacks* por laminação do milho seguida por fritura, houve uma perda média de 36% de carotenóides pró-vitamínicos A (Lozano-Alejo et al., 2007). No processamento de molho de tomates-tangerina, as concentrações de licopeno total e *tetra-cis*-licopeno diminuíram consideravelmente, acompanhado pelo aumento de isômeros *cis* (Ishida et al., 2007).

O processamento doméstico também foi estudado, o brócolis após cozimento tanto em água quanto à vapor obteve maiores teores de β-caroteno e luteína comparado ao brócolis fresco (Gliszczynska-Swiglo et al., 2006). O cozimento de milho por fervura a 100ºC por 30 minutos aumentou a concentração de carotenóides enquanto que o cozimento em forno a 232ºC por 25 minutos degradou em torno de 70% dos carotenóides (Muzhingi et al., 2008).

No preparo para consumo de batata doce *orange-fleshed*, a retenção do todo-*trans*-β-caroteno foi de aproximadamente 77% após cozimento em água, vapor e fritura (Bengtsson et al., 2008). Quando foi assada em estufa, houve perda de 12% e a secagem ao sol resultou em reduções de 9 a 16%. Van Jaarsveld et al. (2006) calcularam a retenção verdadeira, compensando a perda ou ganho de umidade e de sólidos solúveis, no cozimento de batata doce *orange-fleshed* por fervura. Esta retenção variou de 83 a 92% dependendo das condições de cozimento e foi menor (70-80% de retenção) quando

a batata doce foi cozida em pedaços de tamanhos diferentes por mais tempo. O cozimento por fervura parece ser o preparo que mais retém o β-caroteno em batatas doce de diversas variedades laranja ou amarela (Kidmose et al., 2007). O preparo de *chips* por secagem reduziu significativamente o teor de todo-*trans*-β-caroteno (21%) e diminuiu ainda mais quando foi utilizada para fazer farinha.

Entre os novos processamentos, Mclnerney et al. (2007) reportaram algumas evidências dos benefícios do processamento à alta pressão na retenção dos atributos nutricionais de alimentos frescos, pois após a exposição à alta pressão (600 MPa/2 min), não houve alteração do teor de carotenóides e da atividade antioxidante. Além disso, o tratamento a alta pressão aumentou a biodisponibilidade dos carotenóides em vagem, mas teve pouco impacto para os carotenóides de cenoura e brócolis. Outro tratamento não-térmico demonstrou um aumento significativo na concentração de carotenóides de suco de laranja com cenoura, após o processamento com pulso de campo elétrico de alta intensidade, a concentração aumentou com o tempo de tratamento, utilizando 25 e 30 kV/cm (Torregrosa et al., 2005).

A fim de aumentar a vida-de-prateleira de hamburgeres contendo pele de tomate como fonte de licopeno, estes foram produzidos, embalados à vácuo e irradiados com 2 ou 4 kGy. Após tratamento com irradiação e 17 dias de estocagem refrigerada, houve uma perda de 17% de licopeno em relação ao teor inicial (Selgas et al., 2009).

O processamento mínimo é outra tendência para a comercialização de frutas e verduras devido ao aumento da demanda por produtos frescos, com alta qualidade, valor nutritivo e praticidade. Como também não envolve condições drásticas, espera-se que os produtos minimamente processados conservem o frescor e o valor nutricional. Porém, a remoção de partes não comestíveis e injuriadas, o corte e o descascamento resultam em danos físicos, que promovem o crescimento de microrganismos e reações enzimáticas

que tornam o vegetal minimante processado mais perecível que o vegetal intacto *in natura*. Geralmente, ocorre o aumento da respiração e a emissão de etileno (Varoquaux e Wiley, 1994; Martínez et al., 2005).

Orizola-Serrano et al (2008) estudaram a estabilidade do licopeno em seis cultivares de tomates submetidos ao processamento mínimo, embalados com atmosfera modificada e estocados a 4ºC por 21 dias, verificaram que o teor de licopeno foi estável durante este período, com exceção de dois cultivares, Rambo e Bodar. O primeiro manteve o teor por 14 dias e então apresentou uma leve diminuição. Já no Bodar, o teor de licopeno foi decaindo constantemente ao longo do período de estocagem.

Os carotenóides de manga minimamente processada permaneceram estáveis após 9 dias de estocagem quando tratadas com água a 46℃/30 minutos (Djioua et al., 2009). Em outro estudo, houve aumento de β-caroteno durante a estocagem a 5℃ por 10 dias, independente da sanitização utilizada, imersão em ácido ascórbico, ácido cítrico e cloreto de cálcio ou em hipoclorito de sódio (Robles-Sanches et al., 2009). Porém, em jaca minimamente processada houve degradação dos carotenóides durante a estocagem a 6℃ por 35 dias (Saxena et al., 2009). A retenção de carotenóides totais foi de 40 a 57%, variando com o tipo de embalagem utilizada. Nas jacas sem pré-tratamento, a retenção foi de apenas 5 a 39%.

Em cenouras minimamente processadas, estocadas sob vácuo, houve um aumento significativo de 39% no teor de carotenóides totais entre o dia inicial e após 7 de armazenamento a 4ºC (Rocha et al., 2007). Entretanto, cenouras roxas e laranjas embaladas em atmosfera modificada apresentaram perdas de carotenóides gradativas e significantes após 13 dias de estocagem (Alasalvar et al., 2005).

Em verduras minimamente processadas (couve, chicória e espinafre), estocadas

em sacos plásticos por cinco dias a 7-9 ºC, houve perdas de 14-42%, 19-32%, 12-20%, 8-31% para β-caroteno, luteína, violaxantina e neoxantina, respectivamente, sendo que as maiores perdas aconteceram em espinafre, com exceção da neoxantina que degradou mais em couve. (Azevedo-Meleiro e Rodriguez-Amaya, 2005a,b).

Ferrante et al. (2008) estudaram as alterações em folhas inteiras e cortadas de acelga suíça minimamente processada estocadas na ausência e presença de luz até 12 dias a 5ºC. Enquanto o teor de antocianina decaiu fortemente nas folhas cortadas no escuro e na presença de luz, o teor de carotenóides totais não diminuiu significativamente. Estes mesmos autores (Ferrante et al., 2009) reportaram um aumento de antocianinas e uma diminuição de carotenóides de 20 para 16 mg/g após 8 dias de estocagem a 4ºC no escuro em folhas inteiras e cortadas de alface.

Alternativas para o uso de cloro na sanitização foram verificadas em alface minimamente processada estocadas a 4ºC por 10 dias, o efeito da sanitização por imersão em chá verde (Martín-Diana et al., 2008) e por injeção de vapor (Martín-Diana et al., 2007) foram avaliados. O uso de chá verde resultou em teores de carotenóides maiores, comparado com o tratamento com o cloro. Já, utilizando a injeção de vapor, houve redução da concentração de carotenóides totais. Em 2008, estes mesmos autores (Rico et al., 2008) otimizaram o tempo de exposição ao vapor pela metodologia de superfície de resposta e concluíram que a exposição por 10 segundos pode ser considerado o tempo ótimo para manter a vida-de-prateleira do alface por 7-10 dias. No entanto, o uso de vapor mesmo por pouco tempo (5 segundos), reduziu significativamente os teores de ácido ascórbico e carotenóides.

Murcia et al. (2009) concluíram que os vegetais minimamente processados, estocados em embalagens com atmosfera modificada por 0-8 dias num refrigerador doméstico (4ºC), não apresentaram perdas significativas da atividade antioxidante quando

comparados aos vegetais frescos. Granado-Lorencio et al. (2008) reportaram que o processamento mínimo de brócolis embalados com atmosfera modificada não afetou a biodisponibilidade de carotenóides em humanos.

A aplicação da microencapsulação na indústria de alimentos é recente, sendo utilizada principalmente na conservação de compostos responsáveis pelo aroma e sabor. Entretanto, o processo de microencapsulação pode ser utilizado em outras aplicações, como no controle de reações oxidativas e na estabilidade de compostos bioativos. Além disso, pode proporcionar uma liberação controlada do material encapsulado, aumentar a vida-de-prateleira dos produtos, proteger contra perdas nutricionais ou mascarar sabor, odor e cor (Anal e Singh, 2007).

A encapsulação protege os compostos de interesse devido a formação de membranas ou paredes que envolvem as partículas do material encapsulado (Shu et al., 2006). Apesar de existir muitas técnicas de microencapsulação (*spray-drying*, *spray-cooling, spray-chilling*, liofilização, recobrimento por extrusão, extrusão por centrifugação, separação por suspensão rotacional, coacervação, co-cristalização e inclusão por complexação), o processo por *spray-drying* é o mais comumente utilizado pela indústria devido ao menor custo e disponibilidade de equipamentos (Gharsallaoui et al., 2007).

Recentemente, alguns estudos sobre carotenóides microencapsulados pelo processo de *spray-drying* foram conduzidos a fim de avaliar a estabilidade durante o processo e estocagem. No processo de microencapsulação de suco de melancia utilizando maltodextrina como agente encapsulante, a perda de licopeno (24%) em comparação com a do β-caroteno (27%) foi levemente menor (Quek et al., 2006). Em relação à estabilidade, a bixina microencapsulada com goma arábica foi mais estável que a bixina microencapsulada utilizando maltodextrina como material de parede. Além disso, foi confirmado o efeito protetor da microcápsula, pois a bixina encapsulada foi 10x mais

estável que a não-encapsulada (Barbosa et al., 2005).

Diversos materiais de parede podem ser utilizados para encapsular os carotenóides pela técnica de *spray-drying*, porém, cada material confere características morfológicas diferentes que podem influenciar na preservação dos carotenóides. Loksuwan (2007) caracterizou microcápsulas de β-caroteno padrão utilizando amido de mandioca, maltodextrina comercial e amido de mandioca modificado em laboratório. O amido de madioca modificado com ácido após o tratamento a vapor sob pressão obteve a maior eficiência de encapsulação e, conseqüentemente, conferiu maior proteção.

O processo de microencapsulação por liofilização do extrato de licopeno obtido do resíduo do processo da fabricação de suco de tomate, utilizando gelatina e poli-γ-ácido glutâmico como materiais de parede, resultou numa perda de 23,5% de licopeno (Chiu et al., 2007). Blanch et al. (2007) compararam dois processos de microencapsulação utilizando ciclodextrina na estabilização do todo-*trans*-licopeno obtido do tomate, um método convencional por liofilização e um baseado no processo de extração super-crítica. A comparação dos dois métodos mostrou que o método convencional obteve um maior rendimento (93,8% *versus* 67,5%). Porém, com o processo de fluído super-crítico há a vantagem de realizar a extração, fracionamento e encapsulação do licopeno do tomate em uma única etapa, diminuindo o tempo de processo e minimizando o preparo da amostra.

Cinética e mecanismos de degradação de carotenóides

A degradação indesejável dos carotenóides afeta os atributos sensoriais dos alimentos e a atividade biológica destes compostos. Os estudos de cinética de degradação são capazes de determinar parâmetros, como a ordem da reação, a

constante e a energia de ativação, essenciais para prever o impacto da degradação durante a estocagem. Conseqüentemente, os estudos cinéticos são necessários para estabelecer medidas que minimizem mudanças indesejadas e melhorem a qualidade dos alimentos (Koca et al., 2007).

A degradação do licopeno e do β-caroteno foi investigada em sistemas modelos de baixa umidade e aquoso, na presença e ausência de luz. Ambos seguiram uma cinética de primeira ordem em todas as condições estudadas. Porém, a degradação do licopeno foi muito mais rápida que a do β-caroteno em sistemas modelos (Ferreira e Rodriguez-Amaya, 2008). Esta diferença na velocidade de degradação dos dois carotenóides foi muito menor em goiaba liofilizada, indicando que os efeitos da matriz e/ou a concentração inicial superam o da estrutura. Cinéticas de degradação de primeira ordem também foram encontradas para a degradação do licopeno em sistema modelo de oleoresina extraída do tomate (Gunjan et al., 2009), de suco de melancia (Sharma et al., 2008) e de pele de tomate (Kaur et al., 2006); para o β-caroteno, em fatias de cenoura desidratadas (Koca et al., 2007); e para vitamina C e carotenóides totais, em pimentões vermelhos (Di Scala e Crapiste, 2008). Entretanto, curvas de degradação de carotenóides com comportamento bifásico, melhor ajustadas numa equação biexponencial, foram encontradas para a degradação do todo-*trans*-β-caroteno e todo-*trans*-β-criptoxantina em suco de caju (Zepka et al., 2009) e para a bixina em sistema modelo água:etanol (8:2) (Rios et al., 2005). Já em cenouras desidratadas, α-caroteno, β-caroteno e luteína tiveram uma degradação seguindo uma pseudo-cinética de primeira ordem (Lavelli et al., 2007). α- Caroteno e β-caroteno tiveram constantes similares, enquanto que a luteína degradou mais rapidamente.

Apesar da reconhecida conseqüência negativa da degradação de carotenóides, os mecanismos envolvidos não estão elucidados, ao contrário da oxidação de lipídeos, para

a qual as diferentes reações e os produtos iniciais, intermediários e finais são conhecidos. De acordo com Rodriguez e Rodriguez-Amaya (2007), o conhecimento de reações e mecanismos subjacentes da degradação oxidativa dos carotenóides é necessária não somente por evitar a perda destes compostos benéficos durante o processamento e estocagem de alimentos, mas também para avaliar as implicações em processos biológicos. Estudos realizados por King et al. (1997) e Aust et al. (2003) indicam que os produtos da degradação do licopeno aumentam a comunicação célula-célula via *gap junctions*, que parece ser um dos mecanismos de proteção relacionados às atividades preventivas dos carotenóides contra o câncer.

Rodriguez e Rodriguez-Amaya (2007) investigaram a formação de epoxicarotenóides e apocarotenóides durante a oxidação de β-caroteno em sistemas modelos de baixa umidade e em meio aquoso, ambos a temperatura ambiente, simulando alimentos desidratados e sucos, respectivamente, durante a estocagem. Foi demonstrado também a presença de vários dos epoxicarotenóides e apocarotenóides, identificados por CLAE-EM e por comparação com os formados por reações com ácido m-cloroperbenzóico e $KMnO_4$, em alimentos processados contendo β-caroteno como carotenóide majoritário. Os resultados mostraram que a epoxidação e a formação de apocarotenais na autoxidação do β-caroteno ocorrem através da mesma rota em diferentes sistemas e condições. Estes mesmos autores (Rodriguez e Rodriguez-Amaya, 2009) realizaram um estudo similar com licopeno, encontrando um número muito maior de epoxicarotenóides formados pela reação química e nos sistemas modelos e alimentos processados. Isso se deve ao fato que na epoxidação, o oxigênio pode entrar tanto nas duplas ligações isoladas como nas duplas ligações conjugadas terminais da estrutura acíclica de licopeno, enquanto que a entrada de oxigênio no β-caroteno dicíclico ocorre somente nas ligações duplas terminais. Em ambos estudos, esquemas foram propostos

para a epoxidação e a clivagem para apocarotenóis.

Os mecanismos de formação de compostos voláteis também ainda não são totalmente compreendidos. A ciclização intramolecular foi proposta como o principal mecanismo de reação na formação dos compostos voláteis detectados no estudo da degradação térmica de carotenóides em oleoresinas de páprica, tomate e calêndula (*marigold*). De acordo com os autores (Rios et al., 2008), este processo foi ativado pelo impacto térmico gerado durante o processo, seguido pela reação de eliminação na cadeia ou pela reação de fragmentação heterolítica. A presença de outros compostos, como vários metilbelzaldeídos ou isoforona (1,1,3-trimetil-3-ciclohexene-5-ona) também indicou a ocorrência de reações de oxidação de carotenóides que afetaram tanto a cadeia poliênica central quanto os grupos finais.

Ferreira et al. (2008) estudaram os compostos aromáticos majoriotários derivados da degradação de carotenóides formados durante uma simulação do envelhecimento do vinho do porto. Os níveis de β-ionona e β-ciclocitral aumentaram 2,5 vezes nas amostras suplementadas com β-caroteno. O mesmo comportamento foi observado para o teor de β-damascenona com a adição de luteína.

A identificação dos possíveis compostos voláteis formados pela degradação do licopeno também foi descrita em estudos com diferentes variedades e produtos derivados de tomates (Gao et al., 2008; Rios et al., 2008; Davidovich-Rikanati et al., 2009) e melancia (Lewinsohn et al., 2005). Porém, trabalhos que estudaram o mecanismo de degradação utilizando carotenóides puros são raros ou inexistentes.

CONSIDERAÇÕES FINAIS

Apesar da riqueza de conhecimentos atualmente disponíveis sobre os carotenóides, estes compostos continuarão sendo alvos de investigações. As pesquisas

sobre seu papel na saúde certamente serão continuadas com o intuito de esclarecer os pontos controversos. A avaliação da biodisponibilidade junto com a composição deve ser ainda ampliada. A exemplo do Brasil, Europa e Estados Unidos, outros países tentarão obter seus próprios bancos de dados. Os efeitos de processamento, principalmente com tecnologias emergentes, devem ser complementados. Embora a CLAE venha reinando já por duas décadas, a possibilidade de se aplicar outras técnicas analíticas deverá ser estudada.

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Capítulo 2

Intralaboratory assessment of analysts' proficiency for carotenoid analysis using a certified reference material

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INTRALABORATORY ASSESSMENT OF ANALYSTS' PROFICIENCY FOR CAROTENOID ANALYSIS USING A CERTIFIED REFERENCE MATERIAL

ABSTRACT

Carotenoid analysis is inherently challenging, requiring the analyst's expertise and attention to many details. To guarantee the reliability of carotenoid data generated in our laboratory, aside from method development, optimization and validation, periodic evaluation of the analysts' performance is carried out. This paper reports the results of our recent evaluation, using a certified reference material. Five analysts with varying experience in carotenoid analysis participated. The same liquid chromatograph and standard curves were used, restricting the evaluation to the analysts' performance. The HPLC method consisted of extraction with acetone, partition to petroleum ether, saponification with 10% methanolic KOH, washing with water, concentration in a rotary evaporator, drying with nitrogen, dissolving in acetone, separation, identification and quantification. The z-scores were calculated for each carotenoid. There was very good agreement in terms of the carotenes and β-cryptoxanthin for all analysts. For lutein and zeaxanthin, the analyst with little experience in carotenoid analysis obtained lower values but the z-scores were still satisfactory. One analyst who had experience only with carotene analysis also got lower concentrations for the xanthophylls, results traced to the fact that ethyl ether was not used in partitioning the carotenoids from the extracting solvent to petroleum ether.

Running title: Analyt's proficiency for carotenoid analysis

Keywords: analysts' proficiency, carotenoid analysis, certified reference material, intralaboratory evaluation, accuracy, precision

INTRODUCTION

Carotenoids are among the food constituents of major interest in relation to human health. Aside from the well-known vitamin A activity, other biological activities have been attributed to these such as reduction of the risk of developing certain types of cancer, cardiovascular diseases, macular degeneration and cataract (Olson, 1999; Tapiero et al., 2004; Krinsky and Johnson, 2005). These health-promoting actions are widely attributed to the carotenoid's antioxidant activity, by its ability to sequester singlet oxygen and react with free radicals (Palace et al., 1999; Young and Lowe, 2001; Stahl and Sies, 2003; Kiokias and Gordon, 2004). However, other modes of action have been cited: modulation of carcinogen metabolism, regulation of cell growth, inhibition of cell proliferation, enhancement of cellular differentiation, stimulation of cell-to-cell communication, enhancement of the immune system and photoprotection (Olson , 1999; Stahl et al., 2002; Krinsky and Johnson, 2005).

Due to its role in human health and as natural pigments, the need for accurate qualitative and quantitative data on food carotenoids is widely recognized. Because the carotenoids differ in their health-promoting efficacy and coloring property, separation, conclusive identification and individual quantification are necessary. This analysis is inherently difficult, requiring the analyst's expertise, experience and attention to many details. Thus, aside from representative sampling and method validation, the analyst's proficiency should be verified.

Access to interlaboratory evaluation of method and analyst performance, although the preferred procedure, is very limited. Intralaboratory evaluation is needed and standardized protocols have been established (Thompson and Wood, 1993; IUPAC; 1995). Method accuracy can be verified in the laboratory by recovery tests, method comparison and analysis of a certified reference material. Spiked analytes do not behave

in the same way as the endogenous compound, thus the validity of recovery studies of analytes like carotenoids, which are naturally well protected by membranes and cell walls and can be linked to other components in food samples, is questionable. Obtaining comparable results with methods of differing principles/procedure indicate good reliability of the methods. Analysis of a certified reference material is the preferred procedure for verifying method and analyst capability for obtaining accurate results. The analytical process from extraction process to instrumental measurement can be assessed. For carotenoids, two certified reference materials have been developed: Community Bureau of Reference BCR485 (freeze-dried mixed vegetables) and NIST Standard Reference Material (SRM) 2383 (baby food composite) (Phillips et al., 2007; Sharpless et al, 2000).

To ensure the reliability of carotenoid data generated in our laboratory, aside from method development, optimization and validation, periodic evaluation of the analysts' performance is carried out. This paper reports the results of our recent evaluation, using the NIST SRM 2383 certified reference material.

MATERIALS AND METHODS

Experimental

Five analysts, with experience on carotenoid analysis varying from one month to 4 years and one analyst with experience only with carotenes, participated. NIST SRM 2383 Baby Food Composite was used. The same HPLC equipment and standard curves were employed so that the evaluation was restricted to the analyst's performance in preparing the extract for HPLC analysis. Analysis was done in triplicate by each analyst.

Carotenoid analysis

The carotenoids were determined using a method developed and evaluated for

leafy vegetables by Kimura and Rodriguez-Amaya (2002) and validated using a lyophilized vegetable mix certified reference material by Kimura et al. (2007).

A portion of approximately 3g of the homogeneous SRM was weighed and the sample was ground with acetone and Hyflosupercel with a mortar and pestle. The extract was filtered through a sintered glass funnel. Extraction and filtration were repeated until the residue turned colorless (usually 3 times). The carotenoids were transferred to about 50 mL petroleum ether: ethyl ether (2:1) by partition, in a separatory funnel with the addition of water. Saponification of the extract after partition to petroleum ether:ethyl ether was carried out by adding equal volume of 10% KOH metanolic and 0.1% butylated hydroxytoluene to the extract and, after flushing with nitrogen, leaving the stoppered flask in the dark at room temperature overnight (about 16 h) (Kimura et al., 1990). The saponified extract was then washed five times with water, dried with anhydrous sodium sulfate and concentrated in a rotary evaporator, and brought to dryness under nitrogen. Immediately before injection, the carotenoids were dissolved in 2 mL HPLC grade acetone and filtered through 0.22 µm PTFE syringe filter (Millipore, Carrigtwohill, Co Cork, Ireland); a 10 µL aliquot was injected into the liquid chromatograph. All the necessary precautions were taken to avoid alterations or losses of the carotenoids (e.g. exclusion of oxygen, protection from light, avoiding high temperature and contact with acids, use of high-purity, peroxide-free solvents, completion of the analysis within the shortest possible time) and other errors during analysis (Rodriguez-Amaya, 1999).

The HPLC system consisted of a Waters separation module, model 2690 (Waters Corp., Milford, Mass., U.S.A.), equipped with quaternary pump, autosampler injector, degasser, and a photodiode array detector (model 996), controlled by a Millenium workstation (version 3.20). Detection was at the wavelengths of maximum absorption (max plot).

The column was monomeric C_{18} Spherisorb ODS2, 3 μm, 4.6 x 150 mm. The mobile phase consisted of acetonitrile (containing 0.05% of 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.

Identification of the carotenoids was done according to Rodriguez-Amaya (1999), with the combined use of retention time, co-chromatography with standards, and the visible absorption spectra. Quantification was by external standardization. Standards were isolated from roquette leaves (lutein), maize (zeaxanthin), papaya (β-cryptoxanthin), watermelon (lycopene) and carrot (α-carotene and β-carotene) by open column chromatography on MgO:Hyflosupercel (1:1, activated for 4 h at 110 ^{o}C) packed to a height of 20 cm in 2.5 cm i.d. x 30 cm glass column. These columns were developed with increasing amounts of ethyl ether and acetone in petroleum ether; the purity of the carotenoid isolates was monitored by HPLC. The mean purity of the standards was 97% for lutein, 97% for zeaxanthin, 93% for β-cryptoxanthin, 96% for lycopene, 93% for α-carotene and 96% for β-carotene. The concentrations of the standard solutions were corrected accordingly.

The standard curves were constructed by the injection in triplicate of standard solutions at five different concentrations. The curves passed through the origin and were linear at the concentration range expected of the samples, the coefficients of correlation obtained being higher than 0.99.

Calculation of the z-score

The z-score was calculated for each carotenoid for each analyst, as follows: z-

score = $(x - \chi)/\sigma$ (with x being the individual analyst result, χ the NIST mean value, and σ the NIST standard deviation). This calculation of a z-score is suggested by the International Union of Pure Applied Chemistry (IUPAC) and is widely used in laboratory proficiency testing programs. A z-score may be either positive or negative, reflecting either a higher or lower result compared to the assigned value. Generally a z-score less than or equal to 2.0 is considered satisfactory, between 2.0 and 3.0 questionable, and greater than 3.0 unsatisfactory (Thompson and Wood, 1993).

RESULTS AND DISCUSSION

The chromatogram shows that the HPLC column provided baseline separation of the six carotenoids in the SRM Baby Food Composite (Fig. 1).

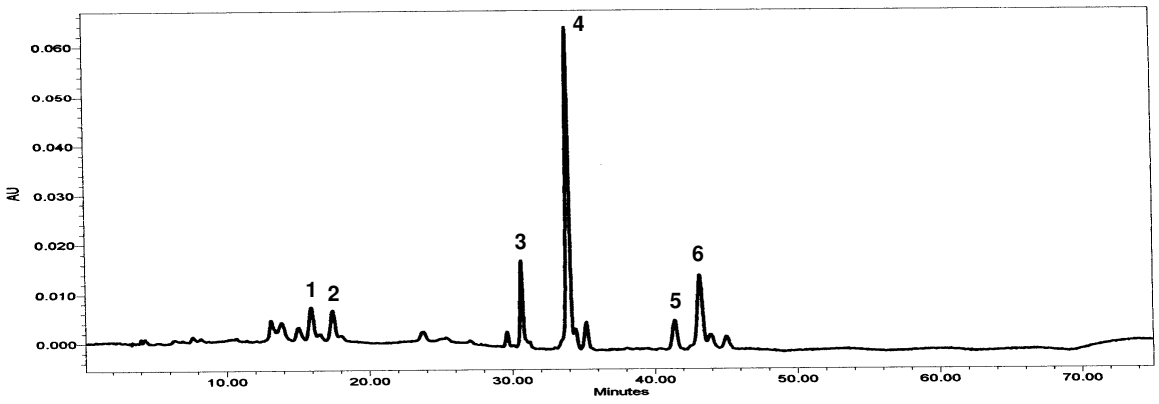

Figure 1. Typical HPLC chromatogram of the carotenoids of SRM Baby Food Composite. Peak identification: 1- lutein; 2- zeaxanthin; 3- β-cryptoxanthin; 4- lycopene; 5- α-carotene; 6- β-carotene.

Figure 2 presents the carotenoid concentrations obtained by the analysts and the certified or reference values (means and standard deviations) furnished by NIST. The

standard score indicates how many standard deviations an observation is above or below the mean. It can be observed that all the analysts obtained good values for the carotenes. For the xanthophylls, the analyst with little experience and the one who had experience only with carotenes, obtained inferior values for lutein and zeaxanthin.

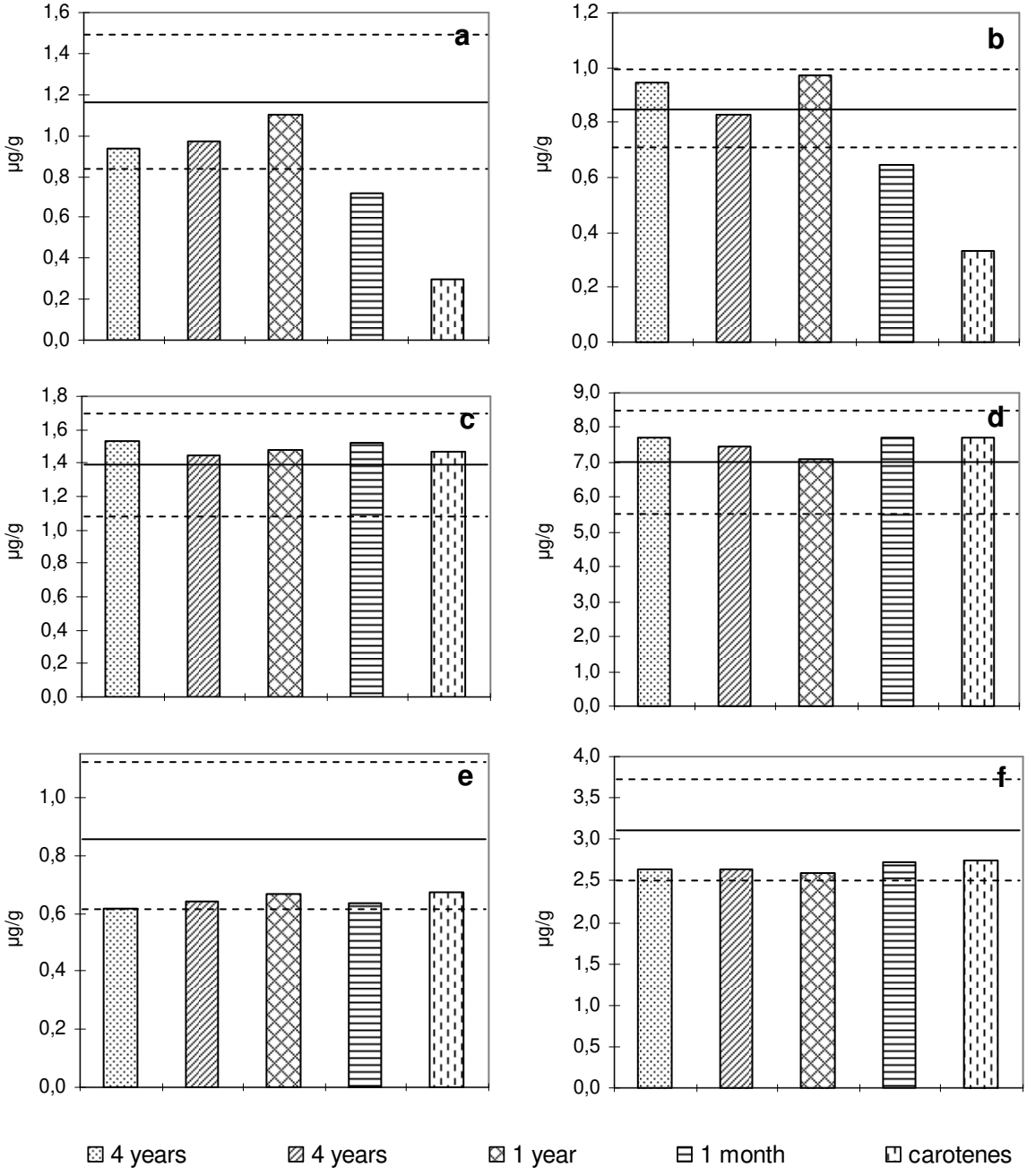

Figure 2. Concentrations of (a) lutein; (b) zeaxanthin; (c) β-cryptoxanthin; (d) lycopene; (e) α-carotene; (f) β-carotene obtained by the analyst. Solid lines indicate means and dashed lines indicate ± standards deviation of certified or reference values furnished by NIST.

Although the concentrations obtained by the analyst with little experience in carotenoid analysis were low for lutein and zeaxanthin, the z-scores were satisfactory (z-score<2). The analyst who had experience only with carotenes obtained results which was questionable for lutein (2<z-score<3) and not satisfactory for zeaxanthin (z-score>3). The other analysts (more than one year of experience in carotenoid analysis) obtained good results for all carotenoids (z-scores less than 1) (Fig. 3).

Table 1. Means and standard deviations of carotenoids content obtained by the analyst, means ± standards deviation of certified or reference values furnished by NIST and z-scores between parentheses.

Experience	Carotenoids (μg/g)[a]					
	lutein	**zeaxanthin**	**β-cryptoxanthin**	**lycopene**	**α-carotene**	**β -carotene**
4 years	0.94 ± 0.07 (-0.67)	0.95 ± 0.02 (0.63)	1.53 ± 0.08 (0.49)	7.70 ± 0.14 (0.47)	0.62 ± 0.02 (-0.97)	2.64 ± 0.06 (-0.77)
4 years	0.97 ± 0.03 (-0.58)	0.83 ± 0.04 (-0.21)	1.44 ± 0.01 (0.20)	7.56 ± 0.20 (0.29)	0.64 ± 0.01 (-0.88)	2.63 ± 0.06 (-0.77)
1 year	1.10 ± 0.06 (-0.18)	0.97 ± 0.05 (0.80)	1.48 ± 0.02 (0.32)	7.07 ± 0.27 (0.05)	0.67 ± 0.01 (-0.75)	2.59 ± 0.08 (-0.84)
1 month	0.72 ± 0.09 (-1.33)	0.65 ± 0.06 (-1.53)	1.52 ± 0.03 (0.47)	7.70 ± 0.29 (0.47)	0.64 ± 0.00 (-0.89)	2.72 ± 0.09 (-0.63)
carotenes	0.30 ± 0.02 (-2.62)	0.33 ± 0.01 (-3.78)	1.46 ± 0.03 (0.27)	7.73 ± 0.18 (0.48)	0.67 ± 0.03 (-0.74)	2.74 ± 0.12 (-0.61)
NIST	1.16 ± 0.33	0.86 ± 0.14	1.38 ± 0.31	7.00 ± 1.5	0.85 ± 0.24	3.12 ± 0.63

[a] means ± standards deviation of triplicate analyses

Reevaluating the analytical results of the analyst who had z-score greater than 3.0, it was discovered that the low levels were due to the fact that he did not utilize ethyl ether (together with petroleum ether) in the partition step. Ethyl ether makes the ether layer more polar, avoiding the loss of xanthophylls (more polar than carotenes because of the presence of hydroxyl groups) to the subsequently discarded water phase. The saponification step might have also influenced the results. Necessary to hydrolyze carotenol esters, saponification is error prone and above analyst did not have experience

in this step either. Repeating the analysis with the addition of ethyl ether, the results obtained by this analyst were satisfactory for both lutein and zeaxanthin, the z-scores being less than 2.0, 1.58 for lutein and 1.86 for zeaxanthin.

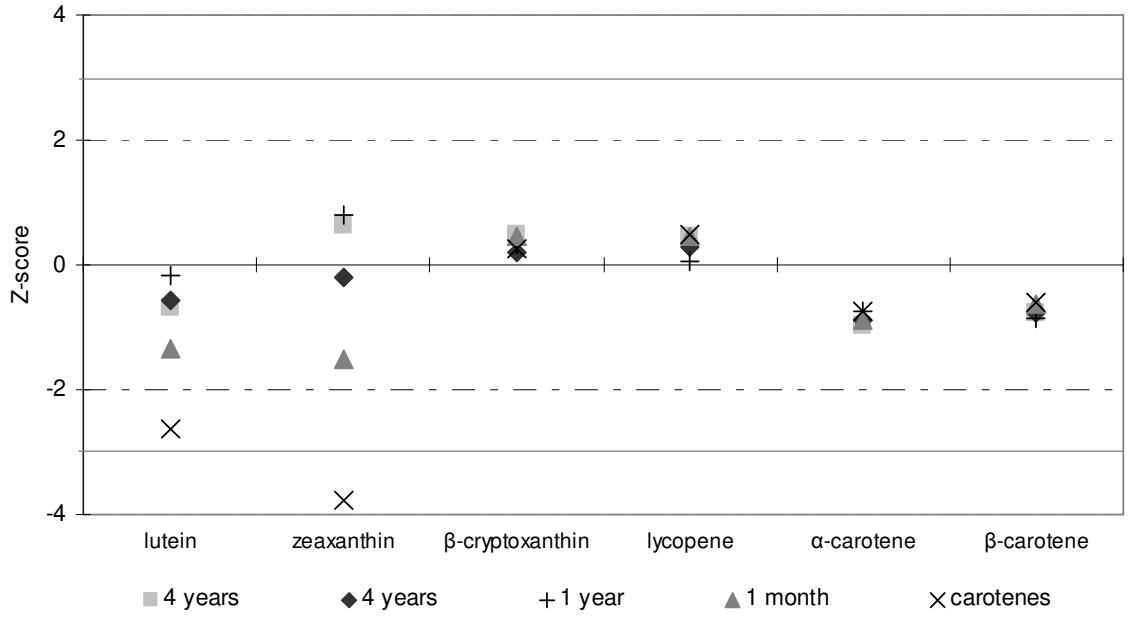

Figure 3. Z-score for lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene concentrations in SRM. Solid lines indicate ±3.0 z-score and dashed lines indicate ±2.0 z-score.

Scott et al. (1996) carried out an interlaboratory study with the participation of 17 European laboratories, using a candidate reference material (lyophilized mixed vegetables). The results indicated that the HPLC systems were not responsible for variations in the analytical data obtained nor was the preparation of the standard solutions a significant problem in the more experienced laboratories. They concluded that the preparation of the extract was the principal factor responsible for the variation of results. This conclusion is reaffirmed in the present study, in which the same HPLC system and

standard curves were used by all participating analysts.

Phillips et al. (2007) reported the results of the USDA's National Food and Nutrient Analysis Program for a total of 2554 values obtained by nine laboratories for 259 certified or reference concentrations of 26 certified reference materials. For total dietary fiber, carotenoids and monounsaturated fatty acids, more than 50% of z-scores were outside ±2.0, demonstrating the difficulty in measuring these analytes in foods.

CONCLUSION

The intralaboratory evaluation of the analyst's performance in determining carotenoids, using a certified reference material, reenforced the importance of the preparation of the carotenoid extract for HPLC analysis and demonstrated once again the inherent difficulty of carotenoid analysis. Even with a previously validated method, the experience of the analyst was a decisive factor in obtaining good results. Evaluation of the analyts' performance is fundamental to ensuring the reliability of analytical results.

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Capítulo 3

Teores de carotenóides em produtos de tomate

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TEORES DE CAROTENÓIDES EM PRODUTOS DE TOMATE

RESUMO

A composição dos carotenóides em produtos de tomate já foi determinada no Laboratório de Carotenóides da FEA/UNICAMP, utilizando cromatografia em coluna aberta. Considerando a introdução de novas variedades de tomate, o desenvolvimento de novos produtos e avanços nas tecnologias de processamento e técnicas analíticas, estes dados necessitavam ser atualizados. Portanto, neste trabalho, determinou-se a composição de carotenóides em produtos de tomates por CLAE. Amostras de extrato, catchup, polpa, molho pronto e tomate seco foram adquiridos em supermercados em Campinas-SP. Para cada produto, cinco lotes diferentes de cada três marcas foram adquiridos, cada lote composto por três embalagens tomadas ao acaso. A faixa de licopeno e β-caroteno total (μg/g) foram, respectivamente, 188-261 e 9,3-13 para extrato, 111-203 e 5,1-7,0 para catchup, 77-117 e 4,4-7,3 para polpa, 93-112 e 5,1-6,4 para molho pronto e 231-471 e 7,0-25 para tomate seco. Tomate seco, que foi analisado pela primeira vez, apresentou os maiores teores de licopeno e luteína. Os teores de licopeno e β-caroteno do extrato de tomate e catchup foram maiores neste estudo comparado com os obtidos anteriormente.

Palavra-chave: carotenóides, licopeno, produtos de tomate, CLAE.

REEVALUATION OF THE CAROTENOID LEVELS IN BRAZILIAN TOMATO PRODUCTS

ABSTRACT

The carotenoid composition of Brazilian tomato products had been determined by our laboratory, using open column chromatography. Considering the introduction of new varieties of the raw material, development of new products, advances in processing technologies and analytical techniques, these data needed to be updated. The objective of this work was to determine the carotenoid composition of processed tomato products by HPLC. Samples of ketchup, sauce, paste, pulp and dried tomato were purchased at supermarkets in Campinas, Brazil. For each product, five different lots of each of three brands, each lot comprising three packages taken at random in the market, were analysed by HPLC. The lycopene and β-carotene ranges (μg/g) were, respectively, 188-261 and 9.3-13 for paste, 111-203 and 5.1-7.0 for ketchup, 77-117 and 4.4-7.3 for pulp, 93-112 and 5.1-6.4 for sauce, 231-471 and 7.0-25 for dried tomato. Dried tomatoes, analyzed for the first time, had the highest lycopene and lutein levels. The lycopene and β-carotene contents of tomato paste and ketchup analysed in the present study were appreciably higher than those obtained previously.

Keywords: carotenoids, lycopene, tomato products, HPLC.

INTRODUÇÃO

O tomate é um produto agrícola importante no mundo inteiro e é o vegetal mais consumido no país. O Brasil lidera a produção de tomate para processamento industrial na América do Sul, sendo o maior mercado consumidor de seus derivados industrializados. Os programas de melhoramento genético de instituições de pesquisas contribuíram para o progresso da cultura no país, priorizando a obtenção de cultivares mais bem adaptadas às condições climáticas das principais regiões produtoras (áreas do Cerrado em GO e MG), resistentes e/ou tolerantes a doenças e pragas limitantes e com melhores características agronômicas e industriais[1].

Ao longo da década de 90, as linhagens foram substituídas por híbridos de alto potencial produtivo e com características que atendiam aos requisitos dos processadores. Assim, as variedades de polinização aberta, que chegaram a ocupar cerca de 75% de toda área cultivada, deixaram praticamente de ser plantadas[2]. O impulso do uso de híbridos começou a se expandir a partir de 1997. Informações obtidas nas indústrias processadoras indicavam que as cultivares híbridas ocupava 45% da área plantada em 1998 e quase a totalidade da área em 2002[3]. De acordo com Melo e Vilela (2004)[2], os principais híbridos plantados eram a Heinz 9992, APT 533, Heinz 9665, APT 529, Heinz 9553, Hypeel 108, Hycolor 312 e RTP 1095.

Há numerosos estudos sobre carotenóides contidos em tomate e seus derivados na literatura. Giovannucci[4] fez uma extensa revisão sobre os trabalhos publicados sobre tomates, produtos atomatados, câncer e estudos epidemiológicos. Concluiu que os tomates e seus produtos industrializados têm sido associados consistentemente com a diminuição do risco de certos tipos de câncer, com evidências fortes para pulmão, estômago e próstata e sugestivas para câncer cervical, de mama, boca, pâncreas, colo e esôfago. Estes benefícios são sempre atribuídos ao licopeno. Porém, um efeito direto do

licopeno ainda não foi comprovado, e outros compostos presentes no tomate também podem ser importantes por suas atividades isoladas ou por interagir com o licopeno.

As atividades biológicas dos carotenóides têm sido relacionadas às suas propriedades antioxidantes, isto é, a sua capacidade de seqüestrar o oxigênio singleto e de interagir com radicais livres[5,6]. O licopeno mostrou ser mais eficiente no seqüestro de oxigênio singleto que o β-caroteno e outros antioxidantes estudados por Di Mascio et al.[7].

De acordo com Bramley[8], 85% do licopeno consumido pelos humanos é obtido do tomate ou de seus derivados e o restante é proveniente da melancia, *grapefruit*, goiaba e mamão. O Brasil também conta com fontes de licopeno, como a pitanga[9], melancia[10], mamão[11] e o caqui[12]. No entanto, pelo alto consumo de tomate e seus produtos, estes continuam sendo as principais fontes deste carotenóide na dieta brasileira.

No Brasil, foram comercializados, em 2000, cerca de 350 mil toneladas de produto atomatados, sendo 41% de extrato simples concentrado, 30% de molhos prontos, 15% de catchup e 14% de polpa de tomate[13]. Em 2006, as companhias apostaram nos molhos prontos, que apresentavam crescimento contínuo de 46% nos últimos quatro anos, enquanto o consumo de extratos e polpas diminuía[14].

A importância dos produtos processados de tomate se torna mais evidente quando a biodisponibilidade é considerada. Estudos indicam que o processamento pode aumentar a biodisponibilidade dos carotenóides pela ruptura da parede celular e pela desnaturação das proteínas complexadas com os carotenóides, facilitando a sua liberação da matriz alimentícia. O licopeno do tomate *in natura* ou do suco de tomate sem processamento foi menos absorvido em comparação com o licopeno de produtos de tomate processados, como o suco de tomate cozido com óleo de milho[15], o tomate cozido com azeite de oliva[16] e o extrato de tomate[17,18, 19].

Bohm e Bitschi[20] verificaram a biodisponibilidade do licopeno em mulheres,

separando-as em três grupos que ingeriram tomate *in natura*, suco de tomate ou cápsulas de licopeno oleaginoso. A menor absorção de licopeno ocorreu em mulheres que ingeriram tomate fresco, pois a matriz alimentícia sem processamento deve ter diminuído a biodisponibilidade do licopeno. A ruptura da matriz pela homogeneização mecânica e/ou o tratamento térmico aumentaram a biodisponibilidade do licopeno e do β-caroteno no estudo realizado por van het Hof et al.[21] com extrato de tomate. O β-caroteno de cenoura e espinafre submetidos ao tratamento térmico também obteve maior biodisponibilidade em comparação com a obtida dessas fontes *in natura* [22].

A biodisponibilidade parece estar afetada também pelas formas isoméricas. Os carotenóides estão presentes predominantemente na natureza na forma *trans*, os isômeros *cis* aparecem em concentrações bem menores, ocorrendo um aumento durante o processamento e estocagem. Já no plasma humano, foram encontrados altos teores de *cis*-licopeno[23,24], indicando uma melhor absorção destes pelo organismo. De acordo com Boileau et al.[25,26], os isômeros *cis* do licopeno são mais biodisponíveis que as formas *trans* por serem mais solúveis nas micelas de ácidos biliares e por serem incorporados preferencialmente pelos quilomicrons. Por outro lado, o *trans*-β-caroteno foi preferencialmente absorvido em comparação com o 9-*cis*-β-caroteno em humanos[27,28]. Portanto, quando há quantidades mensuráveis de isômeros *cis* nos alimentos, a separação e a quantificação dos isômeros são recomendáveis.

Tavares e Rodriguez-Amaya[29] já reportaram a composição de carotenóides de tomates e seus produtos determinada por cromatografia em coluna aberta. Porém, considerando a introdução de novas variedades de matéria-prima[1-3], o desenvolvimento de novos produtos[14] e avanços nas tecnologias de processamento e técnicas analíticas, estes dados necessitavam ser atualizados. Portanto, este trabalho teve como objetivo

avaliar os teores de carotenóides em cinco produtos de tomates industrializados, comercilalizados em Campinas-SP: extrato, catchup, polpa, molho pronto e tomate seco.

MATERIAL E MÉTODOS

Amostras

Foram amostradas três marcas de cada produto (A, B e C), extrato, polpa, molho pronto e catchup, em diferentes supermercados da região de Campinas-SP. Para cada marca, foram analisados cinco lotes diferentes, compostos por três embalagens por lote coletadas ao acaso nos supermercados. As análises foram realizadas dentro das datas de validade dos produtos. Como o tomate seco é mais comumente adquirido e consumido à granel pela população, a amostragem foi composta por cinco amostras de 0,5 Kg, compradas em mercados diferentes. O tomate seco e o molho pronto foram analisados pela primeira vez.

Determinação de carotenóides

A análise de carotenóides foi realizada por cromatografia líquida de alta eficiência (CLAE), baseada na metodologia de Kimura e Rodriguez-Amaya[30], efetuando-se as adaptações necessárias às amostras. Este método foi validado anteriormente por Kimura et al.[31], utilizando um material de referência certifcado.

As amostras das três embalagens de cada lote foram homogeneizadas num processador de alimentos. A extração dos carotenóides foi realizada com 2-3 g de amostra, dependendo do produto, utilizando almofariz e pistilo, misturando celite (hyflosupercel) à amostra e utilizando acetona como solvente de extração. A mistura foi filtrada em funil de Büchner e o resíduo foi levado novamente ao almofariz. A extração e a filtração foram repetidas até que o resíduo se tornasse incolor. Fez-se a partição para éter

de petróleo em um funil de separação e o extrato etéreo foi então concentrado em evaporador rotatório (T≤ 35° C) e seco com N_2. Imediatamente antes da injeção no cromatógrafo, o extrato seco foi redissolvido em acetona de grau cromatográfico e filtrado em filtro PTFE 0,22 μm.

Os padrões foram isolados de melancia (licopeno), rúcula (luteína) e batata doce (β-caroteno) por cromatografia em coluna aberta (CCA) de vidro empacotada com mistura de MgO:hyflosupercel (1:1), previamente ativada por 4 horas à 110°C. As soluções foram quantificadas espectrofotometricamente na região visível e as concentrações foram corrigidas de acordo com a pureza da solução determinada por CLAE. A média da pureza dos padrões obtidos foram 96% para licopeno, 94% para luteína e 96% para β-caroteno.

A identificação dos carotenóides foi realizada de acordo com Rodriguez-Amaya[32], utilizando em conjunto o comportamento cromatográfico e co-cromatografia com padrões de carotenóides, análise dos espectros de absorção (λ_{max} e estrutura espectral fina) obtidos pelo detector de arranjo de diodos (DAD) e espectrofotômetro UV-Visível e reações químicas específicas para grupos substituintes como acetilação, metilação, redução e rearranjo de grupos epóxidos.

A análise por cromatografia líquida de alta eficiência foi conduzida em um módulo de separação Waters (modelo 2690) equipado com bomba quaternária, degasser a vácuo na linha e DAD Waters (Modelo 996), controlados por software Millenium (versão 3.20). Foi utilizada uma coluna monomérica C_{18} Spherisorb ODS2, 3 μm, 4,6 x 150 mm. A detecção dos carotenóides foi feita nos comprimentos de onda de absorção máxima (max plot) e a quantificação foi realizada por padronização externa. A curva foi composta por cinco pontos em triplicata. As curvas passaram pela origem e foram lineares nas faixas de concentração esperadas para as amostras, os coeficientes de correlação obtidos foram

maiores que 0,99.

RESULTADOS E DISCUSSÃO

A Figura 1 apresenta um cromatograma típico dos carotenóides dos produtos industrializados de tomate, demonstrando a predominância de licopeno.

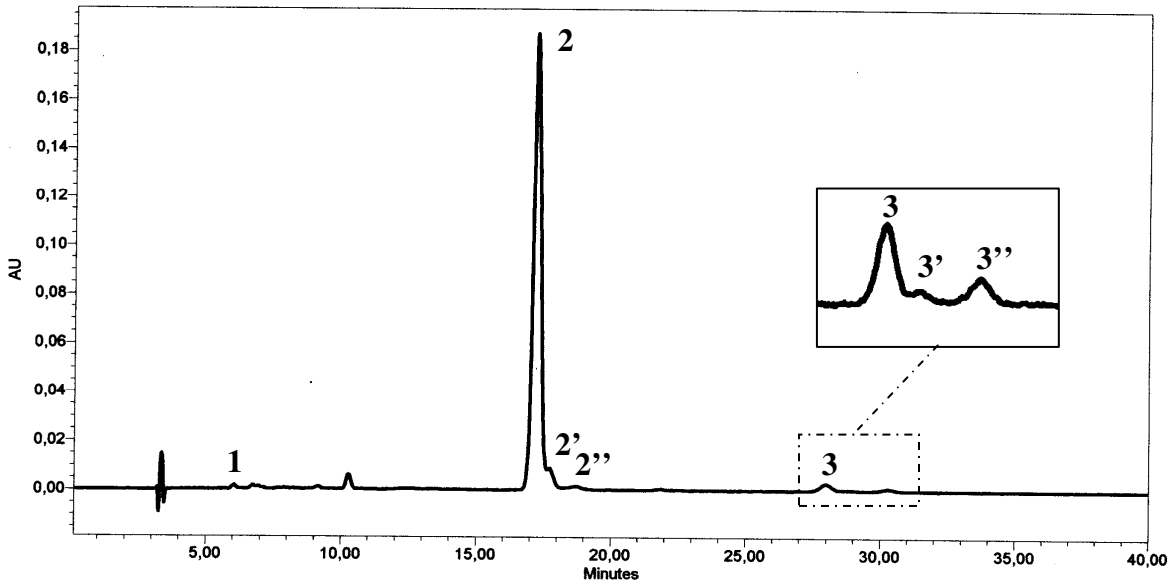

Figura 1. Cromatograma típico obtido por CLAE dos carotenóides de produtos de tomate. Identificação dos picos: 1. luteína, 2. *trans* licopeno, 2' e 2''. isômeros *cis* do licopeno, 3. β-caroteno e 3' e 3''. isômeros *cis* do β-caroteno. Condições cromatográficas estão descritas no texto.

A identificação do pico de licopeno foi realizada pelo seu espectro de absorção na região visível ($\lambda_{máx}$ a 448, 473 e 505 nm na fase móvel; estrutura espectral % III/II = 71), de 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 = 17 minutos). Este carotenóide co-eluiu com o padrão de licopeno

isolado por CCA.

O β-caroteno apresentou $\lambda_{máx}$ a 455 e 482 nm na fase móvel, tendo pouca estrutura espectral (% III/II = 25), refletindo um cromóforo de 11 duplas conjugadas, porém, com duas duplas nos anéis β. O tempo de retenção (t_R = 28 minutos) indica a ausência de substituintes. Além disso, este carotenóide co-eluiu com o padrão de β-caroteno isolado por CCA.

A luteína apresentou $\lambda_{máx}$ a 424, 448 e 476 nm na fase móvel, tendo estrutura espectral intermediária (% III/II = 60), que é típico para um carotenóide com 10 duplas conjugadas, nove na cadeia poliênica e uma no anel β. O comportamento cromatográfico foi de acordo com o característico para carotenóides dihidroxilados (t_R = 6 minutos). Este carotenóide também co-eluiu com o padrão de luteína isolado por CCA.

Observa-se na Tabela 1 que o licopeno foi encontrado em alta concentração em todos os produtos, variando de 77 μg/g em polpa de tomate a 361 μg/g no tomate seco.

Tabela 1. Teores dos principais carotenóides nos produtos de tomate em base úmida.

Marca	Concentração de carotenóides (μg/g)[a]				
	licopeno		β-caroteno		luteína
	trans	*cis*	*trans*	*cis*	total[b]
Extrato					
A	243 ± 43	14 ± 3,2	9,2 ± 2,5	3,6 ± 1,0	tr[c]
B	248 ± 25	13 ± 1,8	9,1 ± 1,5	3,6 ± 0,5	tr
C	179 ± 23	9,6 ± 1,3	6,5 ± 1,1	2,8 ± 0,1	tr
Catchup					
A	104 ± 17	6,6 ± 0,6	3,5 ± 0,7	1,5 ± 0,5	tr
B	192 ± 38	11 ± 1,8	5,2 ± 1,6	1,8 ± 0,6	tr
C	147 ± 33	8,0 ± 0,2	4,7 ± 1,2	1,8 ± 0,2	tr
Polpa					
A	72 ± 12	5,1 ± 1,2	3,0 ± 0,7	1,4 ± 0,4	tr
B	110 ± 30	6,7 ± 2,2	5,1 ± 2,1	2,1 ± 0,7	tr
C	81 ± 23	5,0 ± 0,7	3,2 ±0,5	1,4 ± 0,2	tr
Molho pronto					
A	90 ± 23	6,6 ± 1,4	3,3 ± 0,7	2,1 ± 0,3	tr
B	84 ± 12	8,9 ± 0,9	3,0 ± 0,5	2,1 ± 0,4	tr
C	99 ± 26	13 ± 0,5	4,1 ± 0,5	2,3 ± 0,2	tr
Tomate seco					
à granel	343 ± 96	18,3 ± 5,2	13 ± 6,8	2,7 ± 0,9	4,0 ± 0,9

[a] Média e desvio padrão dos 5 lotes diferentes
[b] total = *trans* + *cis*
[c] tr = traços

Tavares e Rodriguez-Amaya[29] encontraram concentrações de *trans*-licopeno diferentes dos valores obtidos neste estudo; as faixas obtidas foram 158–183, 86–103 e 88–133 μg/g para extrato de tomate, catchup e polpa, respectivamente. Isso se deve, provavelmente, às substituições do cultivar de tomate utilizado pela indústria e/ou as modificações realizadas no processamento pela indústria. A técnica analítica também pode ter influência, pois estes autores utilizaram coluna aberta.

A composição de carotenóides em molho pronto e tomate seco foram avaliadas pela primeira vez. Os molhos de tomate apresentaram teor de licopeno e β-caroteno similares aos da polpa e bem menores que do extrato. É importante lembrar, no entanto, que os molhos prontos de tomate não necessitam ser diluídos durante o preparo para consumo.

O tomate seco, um produto introduzido mais recentemente, possui licopeno, β-caroteno e luteína em concentrações 10 vezes maiores que no tomate fresco. Segundo Melo e Vilela[1], o desenvolvimento de cultivares para o processamento do tomate seco ainda constitui um importante nicho para pesquisas, pois vem apresentando tendências de franca expansão no mercado. Atualmente, não existem cultivares adequadas para a produção de tomate seco, as cultivares utilizadas possuem grande quantidade de água na polpa que exige muito tempo para desidratação completa (em média 12 horas). A fabricação de tomate seco no Brasil tem sido realizada por pequenas empresas que ainda utilizam equipamentos artesanais. Mesmo com sua produção ainda não otimizada pela indústria, o tomate seco apresentou as maiores concentrações de todos os carotenóides avaliados.

Embora os produtos de tomate tenham sido extensivamente analisados em outros países, foi encontrado apenas um trabalho brasileiro, além dos dois trabalhos realizados no nosso laboratório, que avaliou apenas uma marca de polpa e catchup e ainda os resultados de carotenóides individuais foram reportados em base seca. Os teores de carotenóides totais obtidos em base úmida foram bem superiores para polpa (348-355 μg/g) e próximos para catchup (123-131 μg/g)[33] em comparação com os nossos resultados.

Os teores de licopeno e β-caroteno total (μg/g) dos produtos de tomate avaliados

nos Estados Unidos, encontrados no banco de dados do USDA (*United States Departament of Agriculture*)[34], foram respectivamente, 288 e 9,0 para extrato, 167 e 5,6 para catchup, 218 e 3,1 para polpa, 140 e 2,6 para molho pronto. Estes valores foram próximos para o extrato e catchup e maiores para polpa e molho pronto, em relação aos dados do presente trabalho.

CONCLUSÃO

A introdução de novas variedades de matéria-prima e os avanços das tecnologias de processamento refletiu num aumento das concentrações de carotenóides nos produtos de tomates comercializados no Brasil. O desenvolvimento de novos produtos, como os molhos prontos e o tomate seco, também contribui para o aumento da oferta desses alimentos fontes de carotenóides. O tomate seco destacou-se pelos altos teores de licopeno.

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Capítulo 4

Uncultivated Brazilian green leaves are richer sources of carotenoids then are commercially produced leafy vegetables

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Uncultivated Brazilian green leaves are richer sources of carotenoids than are commercially produced leafy vegetables

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Abstract

Background. *With the continuing problem of vitamin A deficiency, the recognition of the role of carotenoids in disease prevention, and international programs promoting biodiversity, determination of the carotenoid content of indigenous Brazilian foods is needed.*

Objective. *To determine the principal carotenoids in native leaves and compare the levels with those in commercially produced leafy vegetables.*

Methods. *The indigenous Brazilian leafy vegetables caruru, mentruz, taioba, serralha, and beldroega were analyzed with the use of a previously developed and validated high-performance liquid chromatography (HPLC) method. Parsley and coriander leaves, which were previously shown to be the richest in carotenoids among commercially produced leaves, were analyzed for comparison. Five sample lots of each vegetable collected at different times during the year were analyzed immediately after harvest.*

Results. *Lutein concentrations were 119 ± 21, 111 ± 48, 104 ± 44, 87 ± 7, and 34 ± 14 μg/g, and β-carotene contents were 114 ± 22, 97 ± 40, 66 ± 18, 72 ± 9, and 32 ± 14 μg/g for caruru, mentruz, taioba, serralha, and beldroega, respectively. Except for beldroega, these values were higher than those for commercial leaves. Parsley had 88 ± 18 μg/g of lutein and 65 ± 13 μg/g of β-carotene. Coriander leaves contained 74 ± 6 μg/g of lutein and 55 ± 5 μg/g of β-carotene. The violaxanthin and neoxanthin concentrations were also higher in the native leaves. Comparison with values for previously analyzed commercial leafy vegetables confirmed the higher carotenoid levels of the native leaves.*

Conclusions. *The indigenous leaves investigated are richer sources of carotenoids than are commercially produced leafy vegetables.*

Key words: β-Carotene, carotenoid, leafy vegetable, lutein

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Background

Vitamin A deficiency continues to be a serious nutritional problem in the developing world, including some parts of Brazil [1]. Because of the generally higher cost of animal sources of preformed vitamin A, provitamin A carotenoids from plant foods are the major dietary sources of this vitamin in developing countries [2]. The consumption of leafy vegetables has been advocated for years, but the role of leafy vegetables in the alleviation of vitamin A deficiency was questioned after they were reported not to improve the vitamin A status in breastfeeding Indonesian women, on the basis of changes in serum retinol concentration [3]. However, with the use of the deuterated-retinol dilution technique, it was shown that green and yellow vegetables could maintain body stores of vitamin A in Chinese children [4], and daily consumption of Indian spinach had a positive impact on the total body vitamin A stores of Bangladeshi men [5]. Employing the same technique, Ribaya-Mercado et al. [6] found that green leafy vegetables, when ingested with minimal fat, enhanced serum carotenoids and the total body vitamin A pool size in Filipino school-aged children and could restore low liver vitamin A concentrations to normal levels.

Moreover, leafy vegetables are widely available, can be grown in home gardens, and provide various nutrients and other health-promoting constituents. They are among the richest sources of the most potent provitamin A carotenoid, β-carotene. A perusal of tables of the carotenoid composition of foods [7–9] reveals that the higher levels of this carotenoid are found in leafy vegetables. The bioavailability of carotenoids in

leafy vegetables can be enhanced by appropriate food-preparation procedures [10, 11].

Carotenoids, including both provitamin A and non-provitamin A carotenoids, are also credited with other beneficial effects on human health, particularly the reduction of the risk of developing degenerative diseases such as cancer, cardiovascular disease, macular degeneration, and cataract [12–17]. With the exception of lettuce [18, 19], the principal carotenoid in leaves is lutein, which, together with zeaxanthin, is considered responsible for the lowering of the risk of macular degeneration and cataract [20–26]. Unlike other leaves, lettuce also has lactucaxanthin (ε,ε-carotene-3,3'-diol) as a major carotenoid, the formation of which partially deviates the carotenoid pathway from hydroxylation of α-carotene to lutein. Thus, in lettuce, the proportion of lutein in relation to the other carotenoids is lower, and β-carotene or violaxanthin surpasses it as the predominant carotenoid [18].

An international, multidisciplinary initiative, led by the Food and Agriculture Organization (FAO) and Bioversity International, under the umbrella of the Convention of Biological Diversity, promotes the sustainable use of biodiversity for food security and nutrition, as a contribution to the achievement of the Millennium Development Goals [27]. A fundamental part of this effort is the determination of the content of nutrients and health-promoting constituents in native species so that their utilization can be enhanced.

In response to the FAO initiative, the objective of the present work was to determine the carotenoid composition of indigenous, uncultivated Brazilian leafy vegetables. The carotenoid composition of these vegetables was determined previously by open-column chromatography, but lutein and violaxanthin were not separated and were quantified jointly [28]. This reevaluation was carried out taking advantage of modern high-performance liquid chromatography (HPLC).

Materials and methods

Samples

The following indigenous Brazilian leafy vegetables were analyzed: caruru (*Amaranthus viridis*), mentruz (*Lepidium pseudodidymum*), taioba (*Xanthosoma sagittifolium*), serralha (*Sonchus oleraceus*), and beldroega (*Portulaca oleracea*). These edible leaves were obtained from two small vegetable farms in Araraquara and Campinas, São Paulo, where the plants grew spontaneously in plots of commercially produced leafy vegetables.

These five vegetables grow quickly without special care and are resistant to pests. Decades ago, they were widely cultivated and used by Brazil's large rural population. Nowadays, with the majority of the Brazilian population concentrated in urban areas, these vegetables are no longer cultivated and are not found in supermarkets, except in the northern and northeastern regions, where caruru and taioba are still widely cultivated and consumed. However, these vegetables can still be encountered growing spontaneously throughout Brazil [29]. These vegetables can also be found in other Latin American countries [30], Asia [31], and Africa [32]. Caruru, mentruz, and beldroega were among the leafy vegetables analyzed for their β-carotene contents in India, Indonesia, and Thailand [31].

Parsley (*Petroselinum hortense*) and coriander leaves (*Coriandrum sativum*), which were shown to be the richest in β-carotene content among commercially produced leaves in a previous study [33], were analyzed for comparison. Although these leaves were obtained from supermarkets, they were delivered and purchased right after harvest.

Five sample lots collected at different times from May to December 2006 were analyzed individually in duplicate for each vegetable immediately after harvest. For each sample lot (weighing about 500 g), the leaves were homogenized in a household food processor.

Carotenoid analysis

The carotenoids were determined by a method developed and evaluated for leafy vegetables by Kimura and Rodriguez-Amaya [34] and validated with the use of a lyophilized vegetable mix certified reference material by Kimura et al. [35].

An aliquot of approximately 3 g of the homogenized sample was weighed, and the carotenoids were extracted with cold acetone in a Polytron MR2100 homogenizer (Kinematica AG, Lucerne, Switzerland) for 1 minute at velocity 11 and filtered through a sintered glass funnel. Extraction and filtration were repeated until the residue turned colorless (usually three cycles of extraction and filtration).The carotenoids were transferred to about 50 mL of petroleum ether:ethyl ether (2:1) by partition in a separatory funnel with the addition of water. The ether solution was washed free of acetone, dried with anhydrous sodium sulfate, concentrated in a rotary evaporator, and brought to dryness under nitrogen. Immediately before injection, the carotenoids were dissolved in 2 mL of HPLC-grade acetone and filtered through a 0.22-μm PTFE syringe filter; a 10-μL aliquot was injected into the liquid chromatograph. All necessary precautions were taken to avoid alterations or losses of the carotenoids (e.g., exclusion of oxygen; protection from light; avoidance of high temperature and contact with acids; use of high-purity, peroxide-free solvents; and completion of the analysis within the shortest possible time) and other errors during analysis [36].

The HPLC system consisted of a Waters separation module, model 2690, equipped with a quaternary pump, an autosampler injector, a degasser, and a photo-

diode array detector (model 996), controlled by a Millennium workstation (version 2010). Detection was at the wavelengths of maximum absorption (max plot).

The column was monomeric C_{18} Spherisorb ODS2, 3 μm, 4.6 × 150 mm (Waters Corporation, Milford, MA). The mobile phase consisted of acetonitrile (containing 0.05% triethylamine), methanol, and ethyl acetate, used at a flow rate of 0.7 mL/min. A concave gradient (curve 10) was applied from 95:5:0 to 60:20:20 in 20 minutes, maintaining this proportion until the end of the run. Reequilibration took 15 minutes.

The carotenoids were identified according to Rodriguez-Amaya [36], with the combined use of retention time, cochromatography with standards, and the visible absorption spectra. The spectral fine structure, expressed as % III/II, was calculated as 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 [37]. For the mono-*cis*-β-carotenes, identification was based on the presence of a *cis* peak at a wavelength about 142 nm below the longest-wavelength absorption maximum (peak III) in the spectrum of the all-*trans*-carotenoid and by slightly lower λ_{max} values (2 to 6 nm) than those of the all-*trans* form. The intensity of the *cis* peak, expressed as % A_B/A_{II}, was calculated as the ratio of the height of the *cis* peak, designated as A_B, and the height of the middle maximum absorption peak, designated as A_{II}, multiplied by 100.

Quantification was by external standardization. Standards were isolated from roquette leaves by open-column chromatography on MgO:Hyflosupercel (1:1, activated for 4 hours at 110°C) packed to a height of 20 cm in a 2.5 cm (inside diameter) × 30 cm glass column [34]. This column was developed with increasing amounts of ethyl ether and acetone in petroleum ether; the purity of the carotenoid isolates was monitored by HPLC. The mean purity of the standards was 95% for neoxanthin, 96% for violaxanthin, 98% for lutein, 95% for β-carotene, and 92% for α-carotene. The concentrations of the standard solutions were corrected accordingly.

The standard curves were constructed by the injection in triplicate of standard solutions at five different concentrations. The curves passed through the origin and were linear at the concentration range expected of the samples, the coefficients of correlation obtained being higher than 0.99.

For the quantitative analysis, saponification was not carried out because this error-prone step was found unnecessary, since the chlorophylls were separated from the carotenoids during chromatography and the hydroxy carotenoids in leaves were not esterified. However, for the qualitative analysis, saponification of the extract after partition to petroleum ether was carried out with an equal volume of 10% metanolic KOH overnight at room temperature in the dark [36]. The saponified extract was then washed with water to remove the alkali.

Results and discussion

Qualitative composition

The principal carotenoids of the leafy vegetables were identified as 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) (**fig. 1** and **table 1**). The minor carotenoids were identified as zeaxanthin (β,β-carotene-3,3'-diol), α-carotene (β,ε-carotene), 9-*cis*-β-carotene,

TABLE 1. Carotenoid concentrations and vitamin A values of native Brazilian leafy vegetables compared to parsley and coriander leaves

Leafy vegetables	Concentration (μg/g)[a]					RAE[b] (μg/100g)
	Neoxanthin	Violaxanthin	Lutein	β-carotene	α-carotene	
Beldroega *Portulaca oleracea*	8.7 ± 5.7	22 ± 13	34 ± 14	32 ± 14	nd[c]	265
Caruru *Amaranthus viridis*	26 ± 6	62 ± 10	119 ± 21	114 ± 22	4.7 ± 1.8	973
Coriander leaves *Coriandrum sativum*	18 ± 2	37 ± 5	74 ± 6	55 ± 5	nd	458
Mentruz *Lepidium pseudodidymum*	31 ± 16	58 ± 23	111 ± 48	97 ± 40	nd	809
Parsley *Petroselium crispum*	22 ± 3	36 ± 5	88 ± 18	65 ± 13	nd	542
Serralha *Sonchus oleraceus*	25 ± 3	53 ± 6	87 ± 7	72 ± 9	nd	600
Taioba *Xanthosoma sagittifolium*	28 ± 17	38 ± 17	104 ± 44	66 ± 18	7.1 ± 4.2	582

a. Means and standard deviations of five samples lots analyzed individually in duplicate.
b. Retinol activity equivalents (RAE): conversion factor of 12:1 for β-carotene and 24:1 for α-carotene.
c. Not detected.

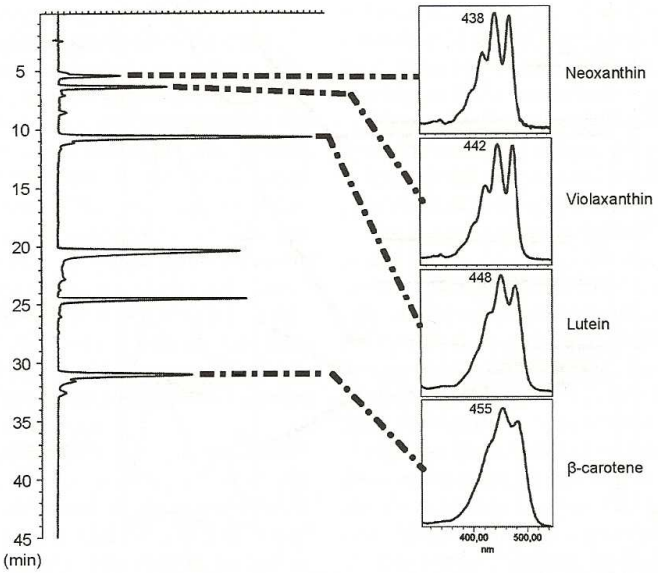

FIG. 1. Typical HPLC chromatogram of the carotenoids of mentruz and photodiode array spectra of the principal carotenoids. Chromatographic conditions are described in the text.

13-*cis*-β-carotene (**fig. 2**), and α-cryptoxanthin (β,ε-carotene-3'-ol) (**fig. 3**). These carotenoids were identified as described below; all of these carotenoids cochromatographed with the respective standards.

Neoxanthin: The λ_{max} at 415, 438, and 467 nm in the mobile phase and the defined spectral fine structure (% III/II=88) were consistent with a carotenoid having eight conjugated double bonds and an allenic group in the polyene chain. Chromatographically, it behaved as a trihydroxy carotenoid.

Violaxanthin: The λ_{max} at 417, 442, and 471 nm in the mobile phase and the high spectral fine structure (% III/II = 96) of the visible spectrum were characteristic of a carotenoid with nine conjugated bonds in the polyene chain. It behaved chromatographically as a dihydroxycarotenoid with other less polar substituents.

Lutein: The absorption spectrum, with λ_{max} at 424, 448, and 476 nm in the mobile phase and less fine structure (% III/II = 60), was typical of a carotenoid with 10 conjugated double bonds: 9 in the polyene chain and 1 in the β-ring. The chromatographic behavior was that of a dihydroxycarotenoid.

β-Carotene: The λ_{max} at 428 (shoulder), 455, and 480 nm in the mobile phase with low spectral fine structure (% III/II = 25) was compatible with a chromophore of 11 conjugated double bonds, 2 of which were situated in the β-rings. The chromatographic behavior was that of a dicyclic carotene.

Zeaxanthin: The absorption spectrum was identical to that of β-carotene, reflecting the same chromophore. The chromatographic behavior was that of a dihydroxycarotenoid.

α-Carotene: Having the same chromophore, the absorption spectrum resembled that of lutein. The hydrocarbon nature was shown by the chromatographic behavior.

9-cis-β-Carotene: The absorption spectrum was characteristic of a *cis* isomer of β-carotene (λ_{max} at wavelengths slightly lower than those of β-carotene, *cis* peak at 342 nm in the mobile phase), and the % A_B/A_{II} was 10. These characteristics were shown by Mercadante et al. [38] to be those of 9-*cis*-β-carotene, the structure of which was confirmed by ^1H nuclear magnetic resonance (NMR), ^{13}C NMR, mass spectrum, and circular dichroism spectrum.

13-cis-β-Carotene: Also exhibiting a spectrum typical of a *cis*-isomer of β-carotene, the *cis* peak was at 338 nm in the mobile phase and the % A_B/A_{II} was 45. These properties are those of a *cis*-β-carotene with the *cis* double bond located at position 13.

α-Cryptoxanthin: This carotenoid was differentiated from β-cryptoxanthin by the spectrum, which resembled that of α-carotene (**fig. 3**) rather than that of β-carotene. It was distinguished from zeinoxanthin (β,ε-caroten-3-ol) by a positive reaction to methylation with acidified methanol, indicating the allylic position of the hydroxyl group.

The chromatograms of saponified and unsaponified samples (**fig. 3**) showed that the hydroxy carotenoids were not esterified, contrary to those in fruits, which

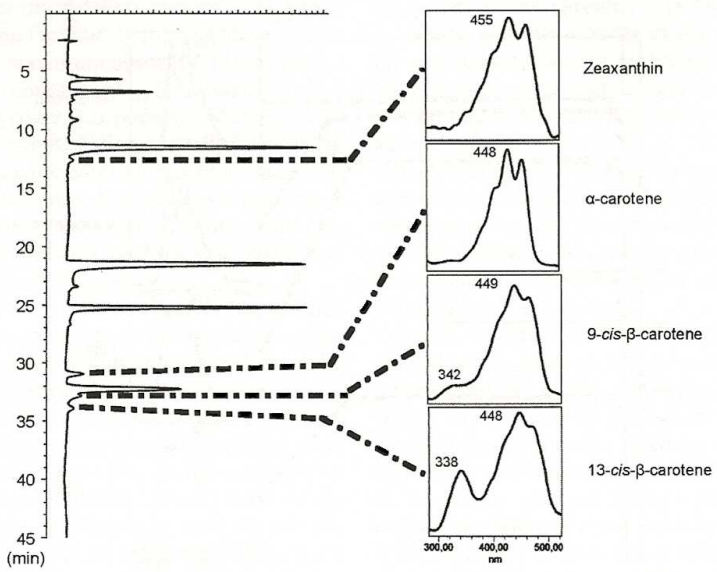

FIG. 2. Typical HPLC chromatogram of the carotenoids of taioba and photodiode array spectra of the minor carotenoids. Chromatographic conditions are described in the text.

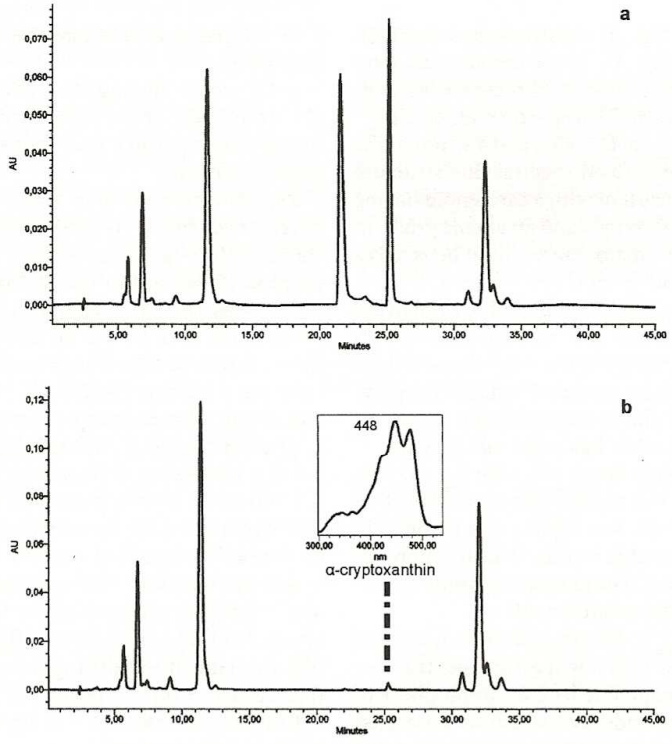

FIG. 3. Typical HPLC chromatograms of the carotenoids of (a) unsaponified and (b) saponified samples of caruru and visible absorption spectrum of α-cryptoxanthin. Chromatographic conditions are described in the text.

are known to be esterified with fatty acids [39], and α-cryptoxanthin was present as a minor carotenoid, masked by chlorophyll in the unsaponified sample.

In contrast to the highly varied and complex carotenoid composition of fruits, leafy vegetables are known to have the same qualitative composition, particularly in terms of the principal carotenoids [39]. The identity of these carotenoids in kale, endive, and New Zealand spinach was confirmed by HPLC–mass spectrometry by Azevedo-Meleiro and Rodriguez-Amaya [40, 41] and by chemical tests such as acetylation with acetic anhydride of secondary hydroxyl groups (as in neoxanthin, violaxanthin, lutein, and zeaxanthin), methylation with acidified methanol of allylic secondary hydroxyl groups (as in lutein and α-cryptoxanthin), and epoxide-furanoid rearrangement of 5,6-epoxides (as in neoxanthin and violaxanthin).

There is some divergence, however, in the identity of the minor carotenoids. α-Carotene is sometimes reported as a minor carotenoid. In the present work, it was found only in taioba and caruru (**fig. 2** and **table 1**). The presence of α-cryptoxanthin instead of β-cryptoxanthin, first reported by Mercadante and Rodriguez-Amaya [42], is confirmed in the present study. The frequent presence of zeaxanthin as a minor carotenoid is justified by its participation in the violaxanthin cycle, which is believed to have a role in photoprotection in plants [43, 44]. This cycle involves the epoxidation of zeaxanthin to violaxanthin under limiting light and the de-epoxidation of violaxanthin to zeaxanthin under light.

Carotenoids are present in nature predominantly in the more stable *trans* configuration. The occurrence of small amounts of *cis* isomers has been increasingly reported, as in the case of leafy vegetables. In terms of human health, differentiation of *cis*- and *trans*-β-carotene is important, because the *cis*-β-carotenes have long been considered to have lower vitamin A activity than their all-*trans* isomers [45]. More recently, all-*trans*-β-carotene was found to be preferentially absorbed [46–48] as compared with 9-*cis*-β-carotene. The amount of *cis*-β-carotene in leafy vegetables, however, has been found to be negligible.

Quantitative composition

The mean lutein concentrations were 119, 111, 104, 87, and 34 μg/g, respectively, for caruru, mentruz, taioba, serralha, and beldroega (**table 1**). The β-carotene levels were 114, 97, 66, 72, and 32 μg/g, respectively. The mean β-carotene values obtained previously by open-column chromatography were 110, 85, 67, 63, and 30 μg/g, respectively [28].

Except for beldroega, the carotenoid concentrations of the uncultivated leaves were higher than those of commercially produced leaves. Parsley had 88 μg/g of lutein and 65 μg/g of β-carotene. Coriander leaves contained 74 μg/g of lutein and 55 μg/g of β-carotene. The violaxanthin and neoxanthin concentrations were also higher in the native leafy vegetables.

The retinol activity equivalents (RAE) of the uncultivated leaves ranged from 265 μg/100 g in beldroega to 973 μg/100 g in caruru (**table 1**). Parsley had 542 μg/100 g and coriander leaves 458 μg/100 g.

The recently recommended RAE values are presented in this paper, but it must be remembered that the RAE conversion factor of 12 μg/g β-carotene to 1 μg/g retinol was based on the bioefficacy of carotenoids in a mixed diet eaten by healthy people in developed countries [49] and may not represent the situation in developing countries. It is half the retinol equivalent (RE) previously recommended by the FAO and the World Health Organization (WHO) [2, 50]. As was done in developed countries, this topic must be thoroughly examined by researchers in the Third World to establish the applicable conversion factors. On the basis of their results showing low bioefficacy of β-carotene from vegetable diets in developing countries [51, 52], West et al. [53] suggested that the conversion factor for β-carotene to retinol should be greater (e.g., 21:1). On the other hand, it has been observed that absorption and bioconversion of plant carotenoids to vitamin A are more efficient in people with deficient vitamin A status [54, 55], indicating higher bioefficacy in populations in developing countries and thus a lower factor. On the basis of data from Bangladeshi men, Haskell et al. [5] estimated a factor of 9.5:1 for Indian spinach.

Parsley and coriander leaves are used as herbs and are therefore consumed sparingly. Thus, for comparative purposes, the concentrations of the principal carotenoids of commercially produced leafy vegetables, as analyzed in previous studies, are shown in **table 2**. The β-carotene concentration varied from 9.9 μg/g in Freelice lettuce to 51 μg/g in New Zealand spinach (corresponding to 82 and 421 μg RAE/100 g, respectively). The corresponding lutein contents were 10 and 72 μg/g, respectively. Notably, the widely consumed lettuce had the lowest carotenoid levels. The native leafy vegetables investigated in the present study, except for beldroega, are richer sources of carotenoids than these commercial leafy vegetables. Thus, commercial production and greater consumption of the native vegetables are recommended.

Acknowledgments

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TABLE 2. Mean carotenoid concentrations of commercially produced leafy vegetables.

Leafy vegetable/Reference	Mean concentration (μg/g)				RAE[a] (μg/100g)
	Neoxanthin	Violaxanthin	Lutein	β-carotene	
Chicory [18] *Chicorium intybus* (Hydroponic)	15	21	57	36	302
Cress [19] *Nasturtium officinalis*	18	26	56	27	227
Cress [18] (Hydroponic)	17	26	75	37	308
Endive [40] *Chicorium endivia*	22	29	62	44	362
Endive [40] (Minimally processed)	16	23	43	31	258
Kale [41] *Brassica oleracea* var *acephala*	26	42	57	42	353
Kale [41] (Minimally processed)	20	27	52	34	285
Curly lettuce [19] *Lactuca sativa*	7.6	19	14	16	129
Curly lettuce [18] (Hydroponic)	6.4	14	15	17	142
Freelice lettuce [18] (Hydroponic)	5.4	8.1	10	9.9	82
French lettuce [18] (Hydroponic)	11	20	23	25	205
Smooth lettuce [18] (Hydroponic)	9.9	19	21	23	190
Smooth lettuce [19]	7.5	18	14	15	124
New Zealand Spinach [40] *Tetragonia expansa*	22	39	72	51	421
New Zealand Spinach [40] (Minimally processed)	22	31	68	55	458
Rucula [19] *Eruca sativa*	18	40	50	28	237
Rucula [18] (Hydroponic)	12	21	52	33	275

a. Retinol activity equivalents (RAE): conversion factor of 12:1 for β-carotene and 24:1 for α-carotene.

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Capítulo 5

Behavior of Flavonols and Carotenoids during Storage of Minimally Processed Roquette Leaves under Passive Modified Atmosphere Packaging

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BEHAVIOR OF FLAVONOLS AND CAROTENOIDS DURING STORAGE OF MINIMALLY PROCESSED ROQUETTE LEAVES UNDER PASSIVE MODIFIED ATMOSPHERE PACKAGING

ABSTRACT

Minimally processed roquette leaves were packed in passive modified atmosphere and stored at three conditions: 1ºC in the dark and 9ºC with or without light (1040 lux) exposure. The products were evaluated during storage in terms of headspace gas composition, sensory attributes, carotenoid and flavonol contents. The sensory quality decreased slightly during 12 days at 1ºC in the dark. At 9ºC, the vegetable shelf-life was predicted to be 6 days in the dark and 4 days with light. Quercetin and kaempferol remained practically stable during 8 days of storage at 1℃ in the dark, increasing slightly after 12 days of storage. At 9℃, in the absence or presence of light, these two flavonols increased during storage, especially kaempferol in the dark. Neoxanthin and violaxanthin did not change significantly. Lutein and β-carotene decreased 6.7% and 5.3%, respectively, after 8 days at 1ºC in the dark, followed by a slight increase after 12 days. At 9ºC in the dark, the reduction of lutein and β-carotene were 9.0% and 7.5%, respectively, in 5 days. The effect of light was evident; from the second day, the levels of lutein and β-carotene were 8.5% and 6.8% lower than those of roquette stored at the same temperature in the dark for the same period.

Key words: flavonols, carotenoids, roquette leaves, minimal processing, modified atmosphere package

COMPORTAMENTO DE FLAVONÓIDES E CAROTENÓIDES DURANTE A ESTOCAGEM DE RÚCULA MINIMAMENTE PROCESSADA EMBALADA EM ATMOSFERA MODIFICADA PASSIVA

RESUMO

Rúcula minimamente processada foi embalada em atmosfera modificada passiva e estocada em três condições: 1ºC no escuro e 9ºC com ou sem luz (1040 lux). Os produtos foram avaliados durante a estocagem em relação à composição do gás dentro da embalagem, atributos sensoriais, concentração de flavonóides e carotenóides. A qualidade sensorial decresceu levemente durante 12 dias a 1ºC no escuro. A vida de prateleira foi prevista para 6 dias a 9 ºC no escuro e 4 dias com luz. Teores de quercetina e kaempferol permaneceram praticamente estáveis durante 8 dias de estocagem a 1ºC no escuro, aumentando levemente após 12 dias. Neoxantina e violaxantina não tiveram alterações significativas. Luteína e β-caroteno diminuíram 6.7% e 5,3%, respectivamente, após 8 dias a 1ºC no escuro, seguido de um leve aumento após 12 dias. A 9ºC no escuro, a redução de luteína e β-caroteno foi de 9.0% e 7.5%, respectivamente, em 5 dias. O efeito da luz foi evidente, a partir do segundo dia, os níveis de luteína e β-caroteno foram 8.5% e 6.8% menores que aqueles da rúcula estocada na mesma temperatura no escuro no mesmo período.

Palavras-chaves: flavonoides, carotenoides, rúcula, minimamente processado, embalagem em atmosfera modificada

INTRODUCTION

Fruits and vegetables are sources of vitamins, minerals and fiber, which are essential for maintaining human health. These foods also contain health-promoting compounds that provide benefits beyond basic nutrition such as prevent or reduce the incidence of degenerative diseases (Steinmetz and Potter, 1996; Ness and Powles 1997; Liu et al., 2000, 2001; Hung et al., 2004). Among these bioactive compounds are carotenoids and flavonoids. Flavonoids has been linked with lower risk of coronary heart disease and cancer (Hertog et al., 1995, 1997; Knekt et al., 1997; Yochum et al., 1999; Neuhouser 2004). Aside from their action against cancer and cardiovascular diseases (Singh and Goyal 2008; Nishino et al., 2009), carotenoids also reduce the risk of macular degeneration and cataract (Moeller et al., 2000; Gerth et al., 2004; Renzi and Johnson 2008) and some carotenoids have provitamin A activity.

Fresh-cut or minimally processed fruits and vegetables have attracted consumers' attention due to the increasing demand for convenience, fresh-like qualities and high quality food products. However, the minimal processing operations of peeling, cutting and shredding result in more rapid decay of the ready-to-use products compared to the original intact produce. Oxidation, enzymatic activity, moisture loss and proliferation of microorganisms are accelerated. Destroying the plant structure increases the rate of senescence of tissues and reduces their resistance to microbial spoilage (Artés et al., 2007). Also, respiratory activity and ethylene emission are generally increased (Varoquaux et al., 1994; Martínez et al., 2005). Low temperature storage and modified atmosphere packaging (MAP) are employed to maintain freshness, extend shelf-life and ensure safety of minimally processed foods (Zagory 1998). Lowering the O_2 level and increasing the CO_2 level suppress respiration, ethylene production, cut-surface browning, senescence and growth of microorganisms (Wang, 2006). Water loss is reduced because of high relative

humidity within MAP.

Minimal processing and MAP are expected to retain the nutrients and other bioactive compounds of vegetables and fruits because thermal processing is not involved. However, studies to demonstrate such expectation are still limited and research on this aspect is urgently needed.

The present study was carried out to monitor the levels of flavonols and carotenoids in minimally processed roquette during storage under passive modified atmosphere packaging. Roquette, a widely consumed leafy vegetable in Brazil, is a rich source of flavonols (quercetin and kaempferol) (Huber et al., 2009) and carotenoids (lutein, β-carotene, violaxanthin, neoxanthin) (Azevedo-Meleiro and Rodriguez-Amaya, 2005a).

MATERIALS AND METHODS

Minimal processing

The roquette leaves were processed in a small scale industry of minimal processing of vegetables located in São Roque, São Paulo. The leaves were washed, selected, trimmed, and washed again with chlorinated water (100 ppm) and water at approximately 6℃. The leaves were then sanitized by immersion in a solution of peracetic acid (0.07%), immersed in a citric acid solution as antioxidant (0.4%) and centrifuged at low speed. These operations were carried out in a refrigerated room maintained at a temperature of about 14℃. Portions of approximately 150 g were packed in heat-sealed plastic bags. The packaging material was a laminated film, bioriented polypropylene (BOPP)/low density polyethylene (LDPE) film; oxygen transmission rate was 1.514 mL (STP)/(m^2.day) at 23ºC under dry condition. Air was injected into the packages to confer mechanical protection, and avoid anaerobiosis during storage. The samples were

immediately transported, in isothermic boxes cooled with ice, to the laboratories for storage and sensory and chemical analyses.

Storage

The samples were stored at the following conditions: 1ºC in the dark and 9ºC with or without light (1040 lux) exposure. The samples exposed to light were in special shelves with two lamps (brand Osram, model 30W/765, daylight) of the same length as the shelves. The intensity of light over the product was measured with a luximeter, model 407026 (Extech Instruments Corp., Waltham, MA, USA) with a resolution of 1 lux. During storage, sensory attributes (overall appearance, overall quality, wilting and senescence) were evaluated. Headspace gas composition, carotenoid and flavonol contents were determined.

The flavonoid and carotenoid analyses were carried out for as long as the sensory analyses indicated that the product was still acceptable. Thus, the leaves stored at 1℃ in the dark were analyzed at 2, 3, 5, 8 and 12 days of storage; those stored at 9℃ in the dark at 2, 3 and 5 days and under light at 2 and 3 days of storage.

Three packages were mixed for each day of analysis and a sub-sample was drawn and homogenized in a food processor. Aliquots were then taken and weighed for flavonoid and carotenoid determinations. All analyses were carried out in triplicate.

Evaluation of the sensory quality

Changes in the sensory quality were evaluated by a panel of 10 untrained panelists. All the attributes were evaluated using a structured scale of five points. The scores for overall appearance and overall quality were: 1 – very bad, 2 – bad, 3 – regular, 4 – good, 5-excellent. For wilting and senescence, the scores were: 1 – very intense, 2 –

intense, 3 – moderate, 4 – slight, 5 – absent. A score of 3 was considered the limit for acceptability of the product for each of the quality attribute evaluated.

Determination of headspace gas composition

The levels of O_2 and CO_2 in the atmosphere of the package were determined using a Shimadzu gas chromatograph model 14A (Shimadzu Corp., Nakagyo-ku, Kyoto, Japan), equipped with a thermal conductivity detector operated at 150ºC, a Porapak N column and molecular sieve 13X (Supelco Inc, Bellefonte, PA, USA) at 50ºC, and an injector set at 70ºC. From each package, an aliquot of 0.5 mL of the headspace gas was hermetically withdrawn through a silicone septum adhered to package's surface. The results were expressed as percentages in volume of gas.

Flavonoid Analysis

A known amount of water (1:2, water:sample) and ascorbic acid (enough to give a final concentration of 0.04%) were added to the weighed sample and homogenization was undertaken for 3 min at a velocity of 25,000 rpm in a Polytron MR2100 homogenizer (Kinematica AG, Littau, LU, Switzerland). Using 7.5 g of the homogenized sample, the flavonols were quantified as aglycones according to a method optimized and validated by Huber et al. (2007). The extraction/hydrolysis was done with 50% aqueous methanol with 1.6 M HCl at 90ºC for 5 h. The optimum hydrolysis condition was determined by a Central Composite Rotational Design (CCRD) and response surface analysis. The extract was cooled and filtered through a glass-sintered funnel, the volume was completed to 50 mL with methanol and the solution was sonicated for 5 min. An aliquot of about 2 mL was filtered through a 0.45 µm PTFE syringe filter (Millipore, Carrigtwohill, Co Cork, Ireland); a 20 µL aliquot was injected into the liquid chromatograph.

A Waters liquid chromatograph model 2690 (Waters Corp., Milford, MA, USA) was used, equipped with a Rheodyne injector (model 7725i), a photodiode array detector (Waters 996) set at 370 nm, and a Nova-Pak C18 column, controlled by Software Millenium 3.20. The mobile phase consisted of methanol:water (both acidified with 0.03% formic acid) in a multilinear gradient, starting with 20:80, changing to 45:55 in 5 minutes, 48:52 in 17 minutes, returning to 20:80 in 20 minutes. The flow rate was 1.0 mL/min.

The identification of the flavonols was based on the retention times, co-chromatography with standards, and the UV spectra obtained with the photodiode array detector. Quantification was by external standardization. The quercetin and kaempferol standards were obtained from Sigma Chemicals Co. (St. Louis, MO, USA).

Carotenoid analysis

The carotenoids were determined according to a method developed and evaluated for leafy vegetables by Kimura and Rodriguez-Amaya (2002) and validated using a lyophilized vegetable mix certified reference material by Kimura et al. (2007).

Using 3 g of the homogenized sample, the carotenoids were extracted with cold acetone in the Polytron MR2100 homogenizer for 1 min at 11,000 rpm, and filtration through a glass-sintered funnel. Extraction and filtration were repeated until the residue turned colorless. The carotenoids were transferred to about 50 mL petroleum ether: ethyl ether (2:1) by partition, in a separatory funnel with the addition of water. The ether solution was washed free of acetone, dried with anhydrous sodium sulfate, concentrated in a rotary evaporator, and brought to dryness under nitrogen. Prior to injection, the carotenoids were dissolved in 2 mL HPLC grade acetone and filtered through a 0.22 µm PTFE syringe filter (Millipore, Carrigtwohill, Co Cork, Ireland); a 10 µL aliquot was injected into the liquid chromatograph. All the necessary precautions were taken to avoid

alterations or losses of the carotenoids and other errors during analysis.

The HPLC system consisted of another Waters separation module, model 2690 (Waters Corp., Milford, MA. USA) equipped with quaternary pump, autosampler injector, degasser and a photodiode array detector (model 996), controlled by a Millenium workstation 3.20. Detection was at the wavelengths of maximum absorption (max plot).

The column was monomeric C_{18} Spherisorb ODS2, 3 μm, 4.6 x 150 mm. The mobile phase consisted of acetonitrile (containing 0.05% of triethylamine), methanol, and ethyl acetate, used at a flow rate of 0.7 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 carotenoids were identified according to Rodriguez-Amaya (1999), with the combined use of retention time, cochromatography with standards and the visible absorption spectra. Leafy vegetables are known to have the same qualitative composition, especially of the principal carotenoids. The identity of these carotenoids in kale, endive and New Zealand spinach was confirmed by HPLC-MS by Azevedo-Meleiro and Rodriguez-Amaya (2005a,b).

Quantification was by external standardization. Standards were isolated from roquette by open column chromatography on MgO:Hyflosupercel (1:1, activated for 4 h at 110 ^{o}C) packed to a height of 20 cm in 2.5 cm i.d. x 30 cm glass column (Kimura and Rodriguez-Amaya, 2002). This column was developed with increasing amounts of ethyl ether and acetone in petroleum ether; the purity of the carotenoid isolates was monitored by HPLC. The mean purity of the standards was 95% for neoxanthin, 92% for violaxanthin, 97% for lutein, and 92% for β-carotene. The concentrations of the standard solutions were corrected accordingly.

In both flavonoid and carotenoid analyses, the standard curves were constructed

by the injection in triplicate of standard solutions at five different concentrations. The curves passed through the origin and were linear at the concentration range expected of the samples, the coefficients of correlation obtained being higher than 0.99.

Statistical analysis

To verify statistically significant differences, the results of the flavonoid and carotenoid analyses were sumitted to analysis of variance ($p<0.05$), the means being compared by Tukey test, utilizing the GraphPad Prism 2.01 program.

RESULTS AND DISCUSSION

Gas composition in the package

Figure 1 presents the gas levels (O_2 and CO_2) in the packages during storage. A passive modification of the atmosphere in the package can be noted, as a function of respiration of the product and permeability of the packaging, but an equilibrium was not reached. CO_2 (initially absent or up to 0.03% in the air) increased to 12% and O_2 was reduced from 21% (in air) to 3-4%, in all of the conditions studied. According to Sigrist (2002), atmospheres containing about 5-7% O_2 and 10-15% CO_2 are better to preserve minimally processed roquette at 5ºC. Development of anaerobiosis was not observed in any of the packages subjected to three conditions during storage.

The effect of storage temperature on gas composition was evident. At 9ºC in the dark, the O_2 level fell to 4% and CO_2 reached 12% after 5 days. At 1ºC in the dark, O_2 decreased to 12% and CO_2 reached only 6% during the same period. This indicates that the temperature has a higher effect on produce respiration rate on the package gas transmission rate accelerating the modification on the atmosphere inside the package.

The effect of light on the package modified atmosphere can be verified in roquette

stored at 9ºC. After three days of storage in the presence of light, O_2 was reduced to 2% and CO_2 increased to 12%. In the absence of light these gases reached 10 e 8%, respectively.

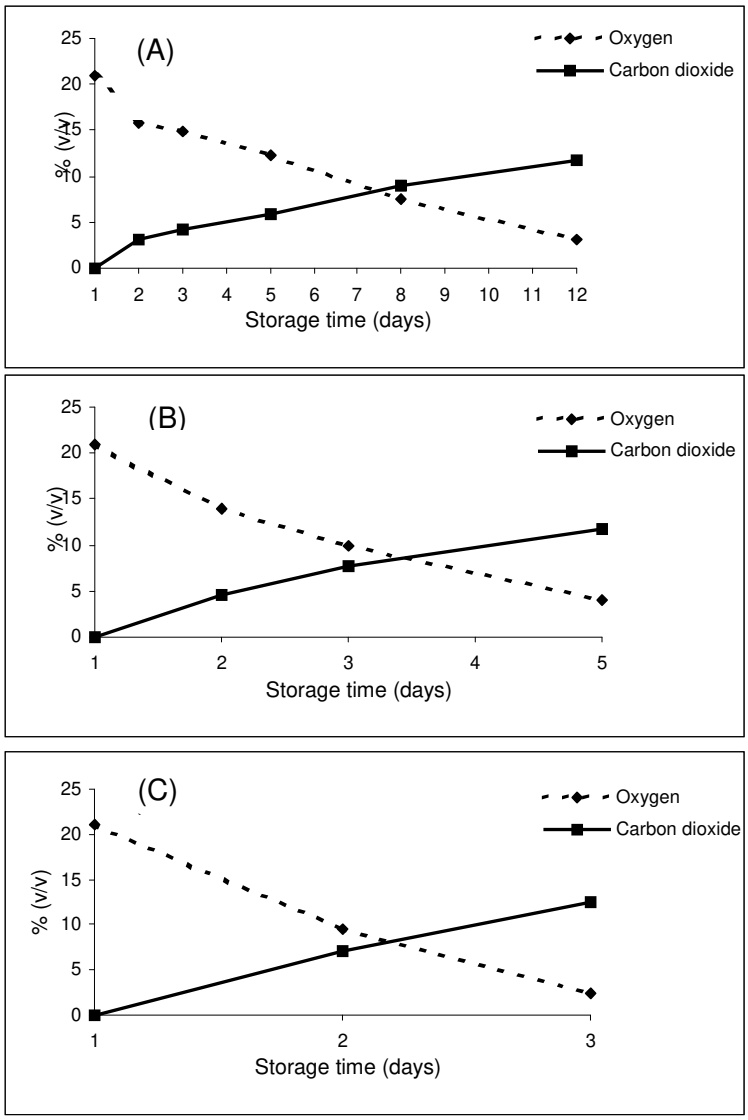

Figure 1. Evolution of the oxygen and carbon dioxide levels in the atmosphere of the packages of minimally processed roquette during storage at (A) 1℃ in the dark, (B) 9℃ in the dark, (C) 9℃ under light.

Sensory quality

Figure 2 shows the results of the sensory evaluation. In 12 days of storage at 1℃ and 9℃ in the dark, the sensory properties evaluat ed remained acceptable, thus it was not possible to estimate the end of the shelf life under these conditions. For the leaves stored at 9℃ exposed to light, the shelf life can be esti mated to be 3 days, based on the overall appearance of the product.

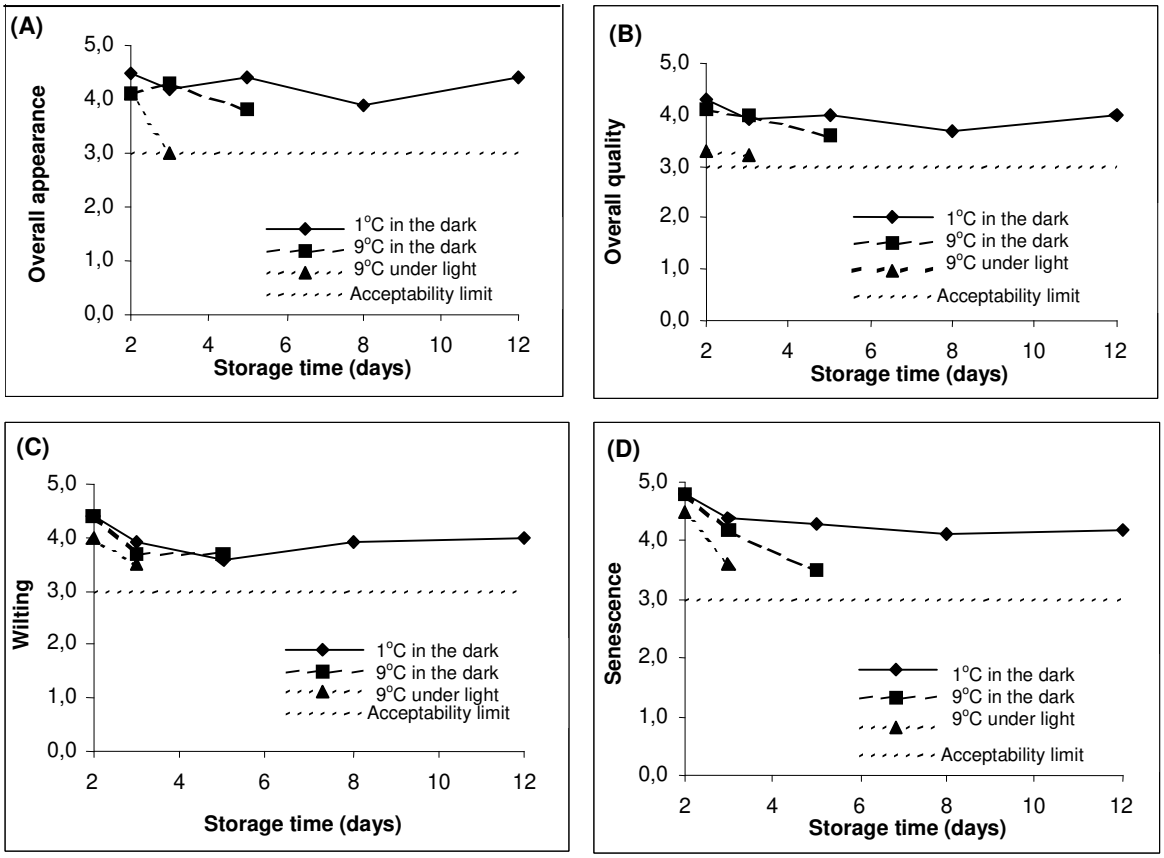

Figure 2. Sensory quality of minimally processed roquette during storage under different lighting condition and temperature (A) overall appearance; (B) overall quality; (C) wilting; (D) senescence. The y axes scores are described in the text.

Flavonol levels during storage

Typical HPLC chromatograms of the flavonols and carotenoids of roquette are in Figure 3. Figure 4 shows the flavonol levels during storage of the minimally processed roquette.

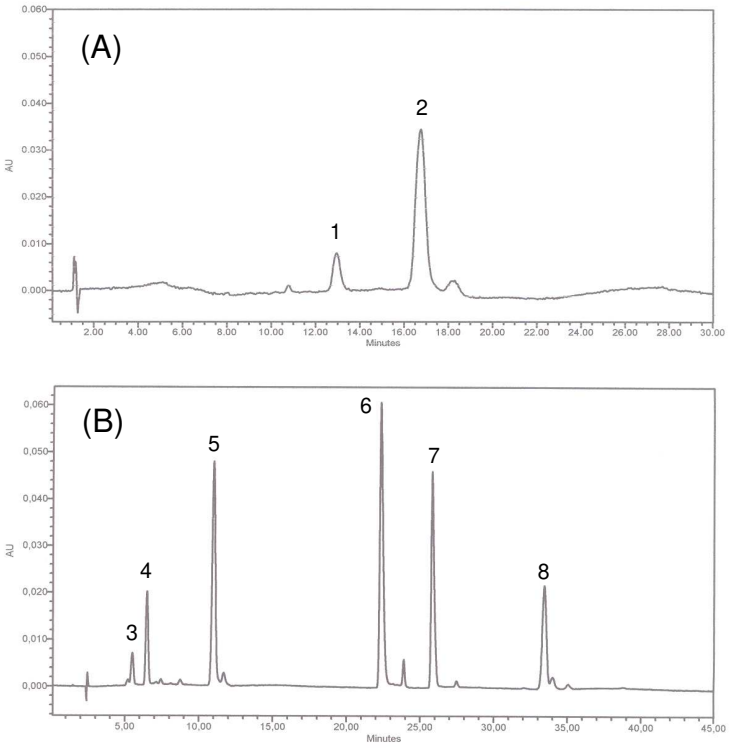

Figure 3. Typical HPLC chromatograms of (A) flavonols and (B) carotenoids of minimally processed roquette. Peak identification: 1. quercetin; 2. kaempferol; 3. neoxanthin; 4. violaxanthin; 5. lutein; 6 and 7. clorophylls; 8. β-carotene. Chromatographic conditions are described in the text.

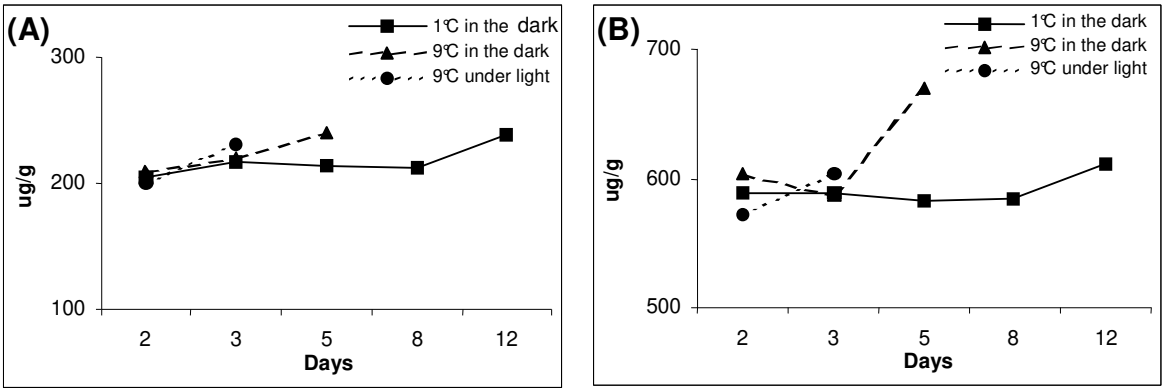

Figure 4. Concentrations of (A) quercetina e (B) kaempferol in minimally processed roquette during storage at 1℃ in the dark and at 9 ℃ without and with light exposure.

At 1℃ in the dark, from initial contents of 203 an d 582 ug/g, respectively, quercetin and kaempferol remained practically stable during 8 days of storage, increasing slightly after 12 days of storage. In samples stored at 9℃, in the absence or presence of light, these two flavonols increased during storage, especially kaempferol in the dark. Thus, loss of flavonols does not appear to be a problem during modified atmosphere storage of minimally processed roquette; in fact, some increase can occur at some points during storage.

Temperature elevation and exposition to light may have two opposing effects on flavonoids and carotenoids: increase biosynthesis or accelerate degradation. The concentrations of these compounds would therefore reflect which of the two effects is predominating. In the case of minimally processed vegetables, because thermal processing is not involved, the biosynthetic enzymes may remain active. On the other hand, cutting the vegetables may destroy compartmentation of degradative enzymes, which can then promote degradation. Enhanced biosynthesis is favored when cellular integrity is preserved, as in the present study, the leaves being left whole.

Gil et al. (1999) reported that the total flavonoid content of minimally processed spinach remained stable during 7 days of storage at 10ºC in packages with air or modified atmosphere. Ferreres et al. (1997) observed that the quercetin glycoside content of minimally processed white and green lettuce packed in perforated polyethylene bags, stored in a small room with humidified air at 5ºC, was stable during 14 days. In red lettuce, where the flavonoid level was more elevated, there was an increase in seven days, declining thereafter up to the 14 day storage period.

Carotenoid levels during storage

Figure 5 presents the carotenoid levels during storage. Neoxanthin and violaxanthin did not change significantly. Lutein and β-carotene, however, decreased 6.7% and 5.3%, respectively, after 8 days at 1ºC in the dark. However, an increase was observed after 12 days, probably because at this time, the biosynthetic enzymes were more active than oxidative enzymes. At 9ºC in the dark, the reduction of lutein and β-carotene were 9.0% and 7.5%, respectively, in 5 days. The effect of light was evident; the levels of lutein and β-carotene, respectively, were 8.5% and 6.8% lower, compared to roquette stored at the same temperature in the dark.

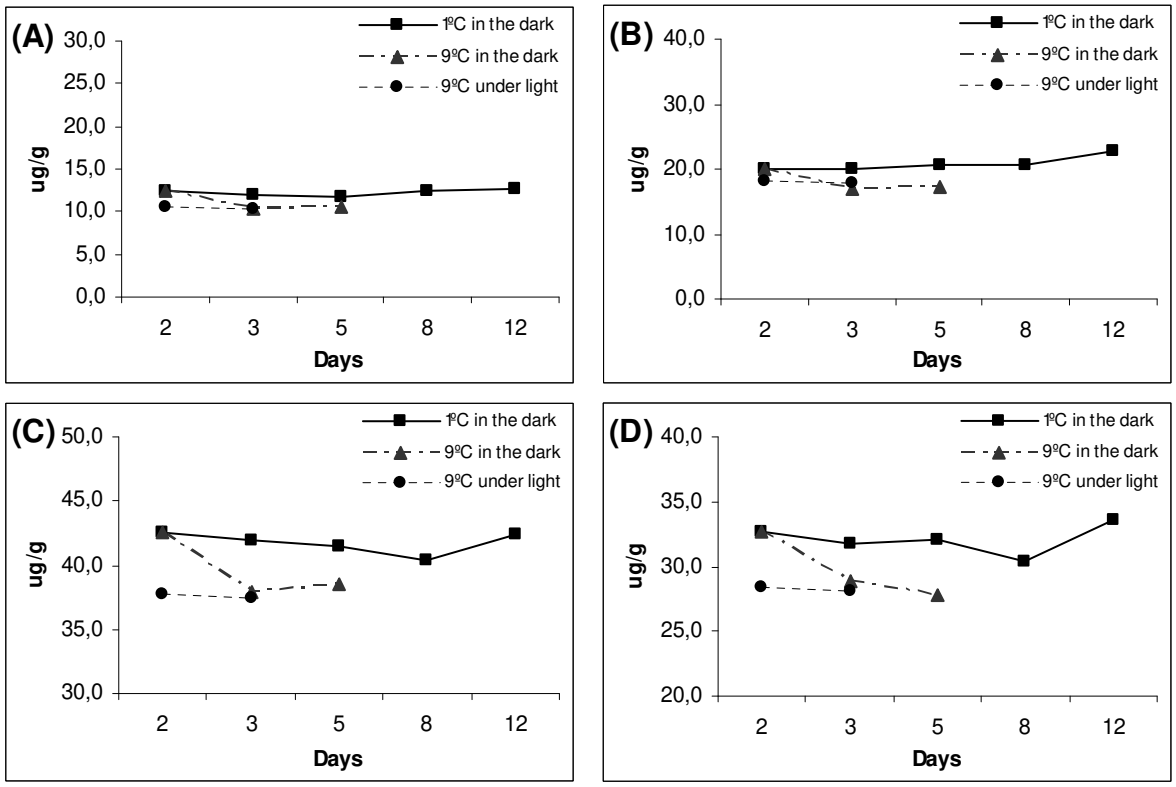

Figure 5. Concentrations of (A) neoxanthin, (B) violaxanthin, (C) lutein and (D) β-carotene of minimally processed roquette during storage at 1ºC in the dark and at 9ºC without and with light exposure.

In minimally processed leafy vegetables (kale, endive and New Zealand spinach) stored in polyethylene bags for five days at 7-9 ºC, losses of 14-42%, 19-32%, 12-20%, 8-31% were observed for β-carotene, lutein, violaxanthin and neoxanthin, respectively, greater losses being observed in New Zealand spinach with the exception of neoxanthin which degraded more in kale (Azevedo-Meleiro et al., 2005a,b). These greater losses were expected in this study since modified atmosphere packaging was not employed.

Carnelossi et al. (2002) investigated the effect of temperature (1, 5 e 10ºC) and

type of packaging (different permeability to O_2 and CO_2 and PET trays) on minimally processed kale stored for 15 days. The total carotenoid content remained stable during the storage period at the three temperatures studied. However, it was less stable when the vegetable was packed in PET trays and there was a slight increase when the high permeability package was used at 1ºC storage.

Ferrante et al. (2008) studied quality changes during storage of fresh-cut or intact Swiss chard leafy vegetables under dark or lighted storage until 12 days at 5ºC. While anthocyanin content strongly decreased in cut leaves in the dark and under light, total carotenoids did not significantly decline. These same authors (Ferrante et al, 2009) evaluating cut and intact lamb's lettuce leaves, reported an increase in anthocyanins and a decrease in total carotenoids from 20 to 16 mg/g of after 8 days of storage at 4ºC in darkness in both treatments.

CONCLUSION

It can therefore be concluded that passive MAP together with refrigeration can preserve the flavonols and carotenoids of minimally processed roquette leaves during storage. Quercetin and kaempherol were stable during storage, tending to increase at some points, especially under light exposure. Neoxanthin and violaxanthin were also stable at all conditions, but lutein and β-carotene were reduced at 9ºC with and without light exposure. At 1ºC in the dark, both had a slight reduction in the first day and had an increase at the end of the storage.

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Capítulo 6

Behavior of Flavonols and Carotenoids during Storage of Minimally Processed Kale Leaves under Passive Modified Atmosphere Packaging

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BEHAVIOR OF FLAVONOLS AND CAROTENOIDS OF MINIMALLY PROCESSED KALE LEAVES DURING STORAGE IN PASSIVE MODIFIED ATMOSPHERE PACKAGING

ABSTRACT

Minimally processed kale leaves were packed in passive modified atmosphere and stored at three conditions: 1ºC in the dark and 11ºC with or without light exposure. The products were evaluated during storage in terms of headspace gas composition, sensory attributes, flavonol and carotenoid contents. The sensory quality decreased slightly during 17 days at 1ºC in the dark. At 11ºC, the vegetable shelf-life was predicted to be 6 days in the dark and 3 days with light. Quercetin and kaempferol were stable during storage for 15 days at 1°C in the absence of light. At 11°C in the dark, quercetin was stable during 10 days, increasing slightly on the 8th day. Kaempferol decreased up to the 5th day but increased on the 8th day, decreasing again on the 10th day. After 5 days at 11°C under light, the flavonol levels were significantly higher than those of the initial values. Neoxanthin and violaxanthin did not change significantly after 15 days at 1ºC in the dark. Lutein and β-carotene, however, decreased 7.1% and 11.3%, respectively. At 11ºC in the dark, neoxanthin, violaxanthin, lutein and β-carotene decreased 16.1%, 13.2%, 24.1% and 23.7% after 10 days, respectively. At 11°C under light, neoxanthin and lutein had a slight increase while violaxanthin and β-carotene decreased 23.1% and 16.5% after 5 days.

Key words: flavonols, carotenoids, kale leaves, minimal processing, modified atmosphere packaging

INTRODUCTION

Consumption of fruits and vegetables has been widely associated with lower incidence of degenerative diseases (Steinmetz and Potter, 1996; Ness and Powles 1997; Liu and others 2000, 2001; Hung and others 2004). This protection against diseases has been attributed to bioactive compounds found in these plant foods, such as flavonoids and carotenoids. Flavonoids has been linked with lower risk of coronary heart disease and cancer (Hertog and others 1995, 1997; Knekt and others 1997; Yochum and others 1999; Neuhouser 2004). Aside from their action against cancer and cardiovascular diseases, carotenoids also reduce the risk of macular degeneration and cataract (Moeller and others 2000; Gerth and others 2004; Renzi and Johnson 2008; Singh and Goyal 2008; Nishino and others 2009) and some carotenoids have provitamin A activity.

Fresh-cut or minimal processing is the current trend for marketing fruits and vegetables, stimulated by consumer's demand for convenience and for fresh-like qualities. These benefits, however, are offset by the rapid deterioration and short shelf-life of these products and the potential health hazards associated with spoilage (Artés and others 2007). Cutting, peeling or shredding destroys the natural protection of the epidermis and the compartmentation that separates enzymes from substrates, resulting in physical damage, microbial development and enzymatic reactions that can render the processed product more perishable than the original intact produce. Respiratory activity and ethylene emission are generally increased (Varoquaux and others 1994; Martínez and others 2005). To maintain freshness, extend shelf-life and ensure safety, low temperature and modified atmosphere packaging (MAP) are employed (Zagory 1998). Lowering the O_2 level and increasing the CO_2 level suppress respiration, ethylene production, cut-surface browning, senescence and growth of microorganisms (Wang, 2006). Water loss is reduced because of high relative humidity within MAP.

Because thermal processing is not involved, minimal processing and MAP are expected to retain the nutrients and other bioactive compounds of vegetables and fruits. However, studies to demonstrate such expectation are still limited and fragmentary. With the current emphasis on the health-promoting effects of foods, investigations on this aspect are urgently needed.

The present study was carried out to evaluate the stability of carotenoids and flavonols in minimally processed kale during dark and lighted storage under passive modified atmosphere packaging. To the best of our knowledge, this is the first time that individual flavonols and carotenoids had been simultaneously monitored during storage of minimally processed leaves. Kale, a widely consumed leafy vegetable in Brazil, is a rich source of flavonols (quercetin and kaempferol) (Huber and others 2009) and carotenoids (lutein, β-carotene, violaxanthin, neoxanthin) (Azevedo-Meleiro and Rodriguez-Amaya, 2005a).

MATERIALS AND METHODS

Minimal processing

The kale leaves were processed in a small-scale industry located in São Roque, São Paulo, engaged in minimal processing of vegetables. The pre-washed leaves were selected, trimmed, and washed with chlorinated water (100 ppm) and water at approximately 6°C. These leaves were cut into 2 mm strips, sanitized by immersion in a solution of hypochlorite (200 ppm), washed again with water and centrifuged at low speed. These operations were carried out in a refrigerated room maintained at a temperature of about 15°C. Portions of approximately 150 g were packed in expanded polystyrene trays (205 x 140 x 3.5 mm), wrapped with stretched polyvinyl chloride film (mean thickness of 11 μm after stretching); oxygen transmission rate was 12,889 mL (STP)/(m^2.day) at 23ºC

under dry condition. The samples were immediately transported, in isothermic boxes cooled with ice, to the laboratories for storage and sensory and chemical analyses.

Storage

The samples were stored at three different conditions: 1ºC in the dark and 11ºC without or with light (576 lux) exposure. The samples exposed to light were in special shelves with two lamps (brand Osram, model 30W/765, daylight) of the same length as the shelves. The intensity of light over the product was measured with a luximeter, model 407026 (Extech Instruments Corp., Waltham, MA, USA) with a resolution of 1 lux. During storage, headspace gas composition, sensory attributes (overall appearance, overall quality, discoloration, wilting, senescence and undesirable odor), carotenoid and flavonol concentrations were determined.

The flavonoid and carotenoid analyses were carried out while the sensory analyses indicated that the product was still acceptable. Thus, the leaves stored at 1℃ in the dark were analyzed at 1, 3, 5, 8, 10, 12 and 15 days of storage; those stored at 11℃ in the dark at 1, 3, 5, 8 and 10 days and under light at 1, 3 and 5 days of storage.

Three packages were mixed for each day of analysis and a sub-sample was drawn and homogenized in a food processor. Aliquots were then taken and weighed for flavonoid and carotenoid determinations. All analyses were carried out in triplicate.

Evaluation of the sensory quality

Alteration of the sensory quality was evaluated by a panel of 10 untrained panelists, using a structured scale of five points. The scores for overall appearance were: 1 – very bad, 2 – bad, 3 – regular, 4 – good, 5-excellent. For discoloration, wilting, senescence and undesirable odor the scores were: 1 – absent, 2 – slight, 3 – moderate, 4

– intense, 5 – very intense. Each panel member was presented a package of the product for each treatment. A score of 3 was considered the limit of acceptability of the product for each of the quality attribute evaluated.

Determination of gas composition

The O_2 and CO_2 levels in the package's headspace were determined using a Shimadzu gas chromatograph model 14A (Shimadzu Corp., Nakagyo-ku, Kyoto, Japan), equipped with a thermal conductivity detector operated at 140ºC, Porapak Q and molecular sieve 5A (Supelco Inc, Bellefonte, PA, USA) columns at 82ºC, and an injector set at 84ºC. From each package, an aliquot of 0.5 mL of the headspace gas was hermetically withdrawn through a silicone septum adhered to the package's surface. The results were expressed as percentages in volume of gas.

Flavonoid Analysis

A known amount of water (1:1, water:sample) and ascorbic acid (enough to give a final concentration of 0.04%) were added to the weighed sample and homogenization was undertaken for 3 min at a velocity of 25,000 in a Polytron MR2100 homogenizer (Kinematica AG, Littau, LU, Switzerland). Using 7.5 g of the homogenized sample, the flavonols were quantified as aglycones according to a method optimized and validated by Huber and others (2007). Simultaneous extraction/hydrolysis was done with 50% aqueous methanol with 1.0 M HCl at 90ºC for 6 hours. The optimum hydrolysis condition was determined by a Central Composite Rotational Design (CCRD) and Response Surface Analysis. The extract was cooled and filtered through a glass-sintered funnel, the volume was completed to 50 mL with methanol and the solution was sonicated for 5 min. An aliquot of about 2 mL was filtered through a 0.45 µm PTFE syringe filter (Millipore,

Carrigtwohill, Co Cork, Ireland); a 20 µL aliquot was injected into the liquid chromatograph.

A Waters liquid chromatograph model 2690 (Waters Corp., Milford, MA, USA) was used, equipped with a Rheodyne injector (model 7725i), a photodiode array detector (Waters 996) set at 370 nm, and a Nova-Pak C18 column, controlled by Software Millenium 3.20. The mobile phase consisted of methanol:water (both acidified with 0.03% formic acid) in a multilinear gradient, starting with 20:80, changing to 45:55 in 5 minutes, 48:52 in 17 minutes, returning to 20:80 in 20 minutes. The flow rate was 1.0 mL/min.

The identification of the flavonols was based on the retention times, co-chromatography with standards, and the UV spectra obtained with the photodiode array detector. Quantification was by external standardization. The quercetin and kaempferol standards were obtained from Sigma Chemicals Co. (St. Louis, MO, USA).

Carotenoid analysis

Using 3 g of the homogenized sample, the carotenoids were determined according to a method developed and evaluated for leafy vegetables by Kimura and Rodriguez-Amaya (2002) and validated using a lyophilized vegetable mix certified reference material by Kimura and others (2007).

The method consisted of extraction with cold acetone in the Polytron MR2100 homogenizer, for 1 min at 11,000 rpm, and filtration through a glass-sintered funnel. Extraction and filtration were repeated until the residue turned colorless. The carotenoids were transferred to about 50 mL petroleum ether: ethyl ether (2:1) by partition, in a separatory funnel with the addition of water. The ether solution was washed free of acetone, dried with anhydrous sodium sulfate, concentrated in a rotary evaporator, and brought to dryness under nitrogen. Prior to injection, the carotenoids were dissolved in 2 mL HPLC grade acetone and filtered through the 0.22 µm PTFE syringe filter; a 10 µL

aliquot was injected into the liquid chromatograph. All the necessary precautions were taken to avoid alterations or losses of the carotenoids and other errors during analysis.

Another Waters separation module, model 2690 (Waters Corp., Milford, MA. USA) was used, equipped with quaternary pump, autosampler injector, degasser and a photodiode array detector (model 996), controlled by a Millenium 3.20. Detection was at the wavelengths of maximum absorption (max plot).

The column was monomeric C_{18} Spherisorb ODS2, 3 μm, 4.6 x 150 mm. The mobile phase consisted of acetonitrile (containing 0.05% of triethylamine), methanol, and ethyl acetate, used at a flow rate of 0.7 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.

Identification of the carotenoids was done according to Rodriguez-Amaya (1999), with the combined use of retention time, co-chromatography with standards and the visible absorption spectra. Leafy vegetables are known to have the same qualitative composition, especially of the principal carotenoids. The identity of these carotenoids in kale, endive and New Zealand spinach was confirmed by HPLC-MS by Azevedo-Meleiro and Rodriguez-Amaya (2005a,b).

Quantification was by external standardization. Standards were isolated from a leafy vegetable (roquette) by open column chromatography on MgO:Hyflosupercel (1:1, activated for 4 h at 110 °C) packed to a height of 20 cm in 2.5 cm i.d. x 30 cm glass column. This column was developed with increasing amounts of ethyl ether and acetone in petroleum ether; the purity of the carotenoid isolates was monitored by HPLC. The mean purity of the standards was 95% for neoxanthin, 96% for violaxanthin, 97% for lutein, and 95% for β-carotene. The concentrations of the standard solutions were corrected accordingly.

In both flavonoid and carotenoid analyses, the standard curves were constructed by the injection in triplicate of standard solutions at five different concentrations. The curves passed through the origin and were linear at the concentration range expected of the samples, the coefficients of correlation obtained being higher than 0.99.

Statistical analysis

To verify the existence of statistically significant differences, the results of the flavonoid and carotenoid analyses were submitted to analysis of variance ($p<0.05$), the means being compared by the Tukey test, utilizing the GraphPad Prism 2.01 program.

RESULTS AND DISCUSSION

Gas composition in the package

Figure 1 shows the gas levels (O_2 and CO_2) in the packages during storage. A passive modification of the atmosphere inside the package can be noted, as a function of respiration of the product and permeability of the packaging. At 1ºC in the dark, an equilibrium atmosphere was established in the package from the second day (9-15% O_2 and 4-10% CO_2). The effect of storage temperature on gas composition was evident. At 11ºC in the dark, O_2 level fell to 0.2% and CO_2 reached 13% after 10 days. This indicated that the temperature had a greater effect on the produce respiration rate than on the package gas transmission rate, accelerating the modification of the atmosphere inside the package.

The effect of light can also be verified in kale stored at 11ºC. After 4 days of storage in the presence of light, O_2 was reduced to 0.3% and CO_2 increased to 21%. In the dark, modification of the atmosphere was less intense, the levels of O_2 and CO_2 staying at around 7%, in the same period. Exposure of the leaves to light (576 lux) apparently

increased the metabolism of the leaves, accelerating the modification of the passive atmosphere in the package.

Sensory quality

Figure 2 shows the results of the sensory evaluation. Through the overall appearance, development of undesirable odor and discoloration, the shelf-life of the samples stored at 1ºC in the dark was estimated to be 17 days. During this period, wilting and senescence continued to be within the acceptable range. For the leaves stored at 11ºC in the dark, the shelf-life was 6 days, based on the overall appearance and discoloration. At the same temperature in the presence of light, the limit of acceptability for overall appearance and undesirable odor was reached on the third day.

Flavonol levels during storage

Typical HPLC chromatograms of the flavonols and carotenoids of kale are in Figure 3. Figure 4 shows the flavonol levels during storage of the minimally processed kale.

The flavonols quercetin and kaempferol in the kale samples were stable during 15 days of storage at 1ºC in the absence of light, with kaempferol increasing slightly on the 15th day. At 11ºC in the dark, quercetin had a slight increase on the 8th day of storage, but went back to the initial level in 10 days. Kaempferol decreased up to the 5th day, increased on the 8th day and decreased again on the 10th day. The quercetin and kaempferol levels in the samples stored at 11ºC under light remained the same on the 1st and 2nd day, but were statistically higher than the initial concentrations on the 5th day.

To ensure that the changes in the levels of flavonoids and carotenoids during storage was not due to changes in the moisture content of the leaves, the moisture content was determined in the initial samples and during storage. It was maintained at 90 ± 0.5%.

In fresh-cut Swiss chard, the total flavonoid content increased in both MAP (7% O_2 and 10% CO_2) and air-stored samples during 8 days at 6°C, the increase being more significant in the former (Gil and others 1998). In contrast, vitamin C decreased, especially in the MAP-stored leaves, reaching levels 50% lower than the initial content. These same authors found that the total flavonoid content in fresh-cut spinach remained stable during 7 days of storage at 10°C in packages with air or modified atmosphere (Gil and others 1999). Vitamin C was better preserved in MAP-stored spinach.

Investigating spinach, Bottino and others (2009) found that during cold storage, the total flavonoid content remained practically constant in intact leaves but increased slightly in fresh-cut leaves.

Ferreres and others (1997) observed that the quercetin glycoside content of minimally processed red pigmented lettuce packed in perfurated polyethylene bags, stored in a small room with humidified air at 5°C, was stable in the white and green tissues during 14 days. In red tissues, where the flavonoid level was more elevated, there was an increase in 7 days, declining thereafter up to the 14 day storage period. The anthocyanin content decreased in both green and red tissues. In lamb's lettuce, free and total phenols increased in both control (intact) and fresh-cut leaves (Ferrante and others, 2009). The total phenol content increased faster in cut leaves after 5 days, but was 23% higher in the control after 8 days of storage at 4ºC.

Carotenoid levels during storage

Figure 5 presents the carotenoid levels during storage. Neoxanthin and violaxanthin did not change significantly, but lutein and β-carotene decreased 7.1% and 11.3%, respectively, after 15 days at 1ºC in the dark. At 11ºC in the dark, neoxanthin, violaxanthin, lutein and β-carotene decreased 16.1%, 13.2%, 24.1% e 23.7% after 10

days, respectively. At 11°C under light, neoxanthin and lutein had a slight increase, while violaxanthin and β-carotene decreased 23.1% and 16.5%, respectively, after 5 days.

The effect of light can be observed in the 23.1% reduction of violaxanthin in five days and the appearance of zeaxanthin (Figure 3), indicating that the violaxanthin cycle was functioning. This cycle, which is believed to have a role in photoprotection in plants, involves the de-epoxidation of violaxanthin to zeaxanthin under light and the epoxidation of zeaxanthin to violaxanthin under limiting light (Young and others 1997; Demmig-Adams 1990).

In minimally processed leafy vegetables (kale, endive and New Zealand spinach) stored in polyethylene bags for five days at 7-9 ºC, losses of 14-42%, 19-32%, 12-20%, 8-31% were observed for β-carotene, lutein, violaxanthin and neoxanthin, respectively, greater losses being observed in New Zealand spinach with the exception of neoxanthin which degraded more in kale (Azevedo-Meleiro and others 2005a,b). These greater losses were expected in this study since modified atmosphere packaging was not employed.

Carnelossi and others (2002) investigated the effect of temperature (1, 5 e 10ºC) and type of packaging (different permeability to O_2 and CO_2 and PET trays) on minimally processed kale stored for 15 days. The total carotenoid content remained stable during the storage period at the three temperatures studied. However, it was less stable when the vegetable was packed in PET trays and there was a slight increase when the high permeability package was used at 1ºC storage. Vitamin C decreased with storage, especially in more permeable packaging.

Ferrante and others (2008) studied quality changes during storage of fresh-cut or intact Swiss chard leafy vegetables under dark or lighted storage until 12 days at 5°C. While anthocyanin content strongly decreased in cut leaves in the dark and under light, total carotenoids did not significantly decline. These same authors (Ferrante and other

2009) evaluating cut and intact lamb's lettuce leaves, reported an increase in anthocyanins and a decrease in total carotenoids from 20 to 16 mg/g of after 8 days of storage at 4ºC in darkness in both treatments.

With minimally processed lettuce, Martín-Diana and others (2007) verified the effect of steamer jet-injection as an alternative to the usual chlorine sanitizing treatment. Significant reduction of ascorbic acid content and, to a lesser extent, of the total carotenoid concentration during storage for 10 days at 4ºC was observed. In the lettuce treated with chlorine, the carotenoid level was maintained at 13 µg/g whereas the lettuce exposed to vapor had only one-third of this concentration. In 2008, these same authors (Rico and others 2008) optimized the short time blanching (steaming) by Response Surface Methodology. It was concluded that steamer treatment of 10 seconds could be considered the optimum time for maintaining the shelf life (mainly texture and browning) of fresh-cut lettuce for 7–10 days in optimum conditions. However, the use of the steamer even for very short time (5 seconds) significantly reduced the ascorbic acid and carotenoid contents of the samples.

Our results together with those of other authors reveal a tendency of flavonoids to remain stable or increase at some points while carotenoids tend to maintain their levels or decrease during storage of minimally processed leaves. Fluctuations or seemingly inconsistent levels of these phytochemicals can be explained by the possible occurrence of processes with opposing effects on their concentrations. Because thermal processing is not involved, the biosynthetic enzymes may remain active, increasing the phytochemical's level. On the other hand, cutting/shredding the vegetables may destroy compartmentation of oxidative enzymes, which can then promote degradation, especially of the carotenoids. Temperature elevation and exposition to light increase biosynthesis but also accelerate degradation. The concentrations of the phytochemicals at any one time would reflect which

of the two processes is predominating.

Increases in flavonoid content can also be explained by another phenomenon. Wounding brought about by cutting, chopping or shredding induces the synthesis of the enzymes of the phenylpropanoid pathway, subsequent synthesis and accumulation of protective phenolic compounds and tissue browning (Bolin and Hoysoll 1991; Lopez-Galvez and others 1996; Tomás-Barberán and others 1997). Discoloration was not observed in the present study probably because one of the benefits of MAP is to prevent this cut-surface browning.

CONCLUSION

Passive modified atmosphere packaging together with refrigeration extended the shelf-life of minimally processed kale. Quercetin and kaempherol were stable during storage, tending to increase at some points, especially under light exposure. Neoxanthin and violaxanthin were also stable at low temperature (1°C) in the dark, but lutein and β-carotene were slightly reduced. At higher temperature (11°C) in the dark, all four major carotenoids decreased with greater losses of lutein and β-carotene. Under light, violaxanthin loss was greater, followed by β-carotene, while neoxanthin and lutein increased slightly.

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FIGURES

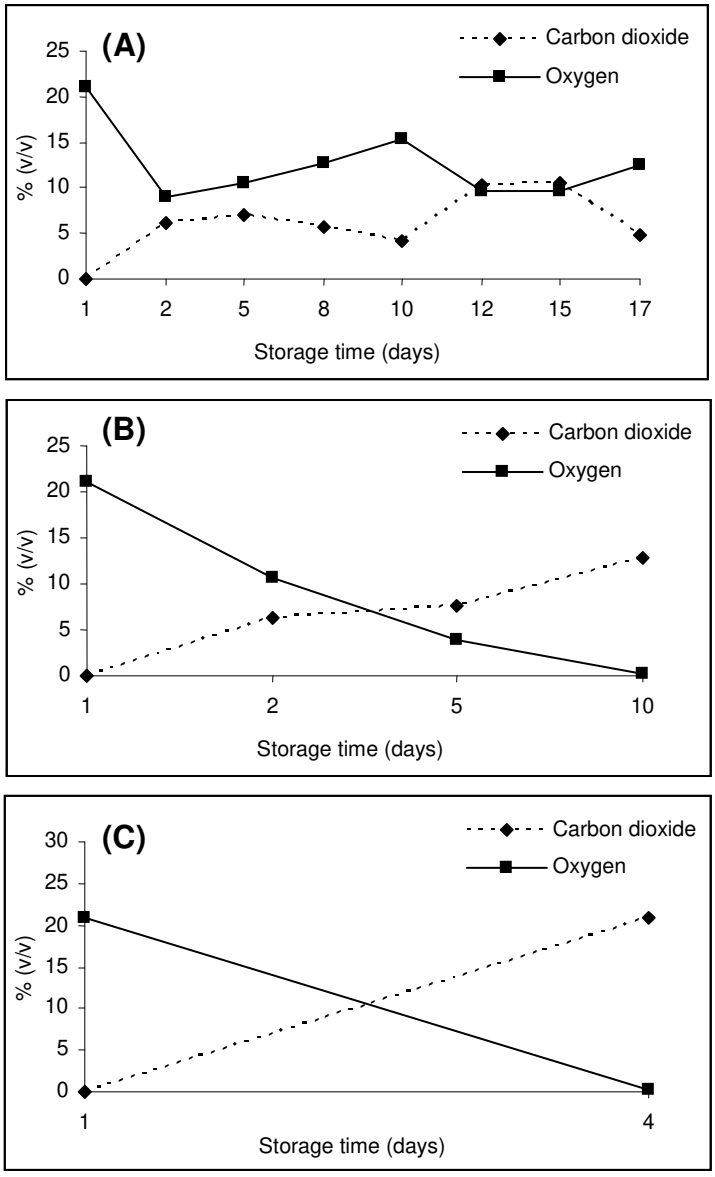

Figure 1. Evolution of the oxygen and carbon dioxide levels in the atmosphere of the packages of minimally processed kale during storage at (A) 1℃ in the dark, (B) 11℃ in the dark, (C) 11℃ under light.

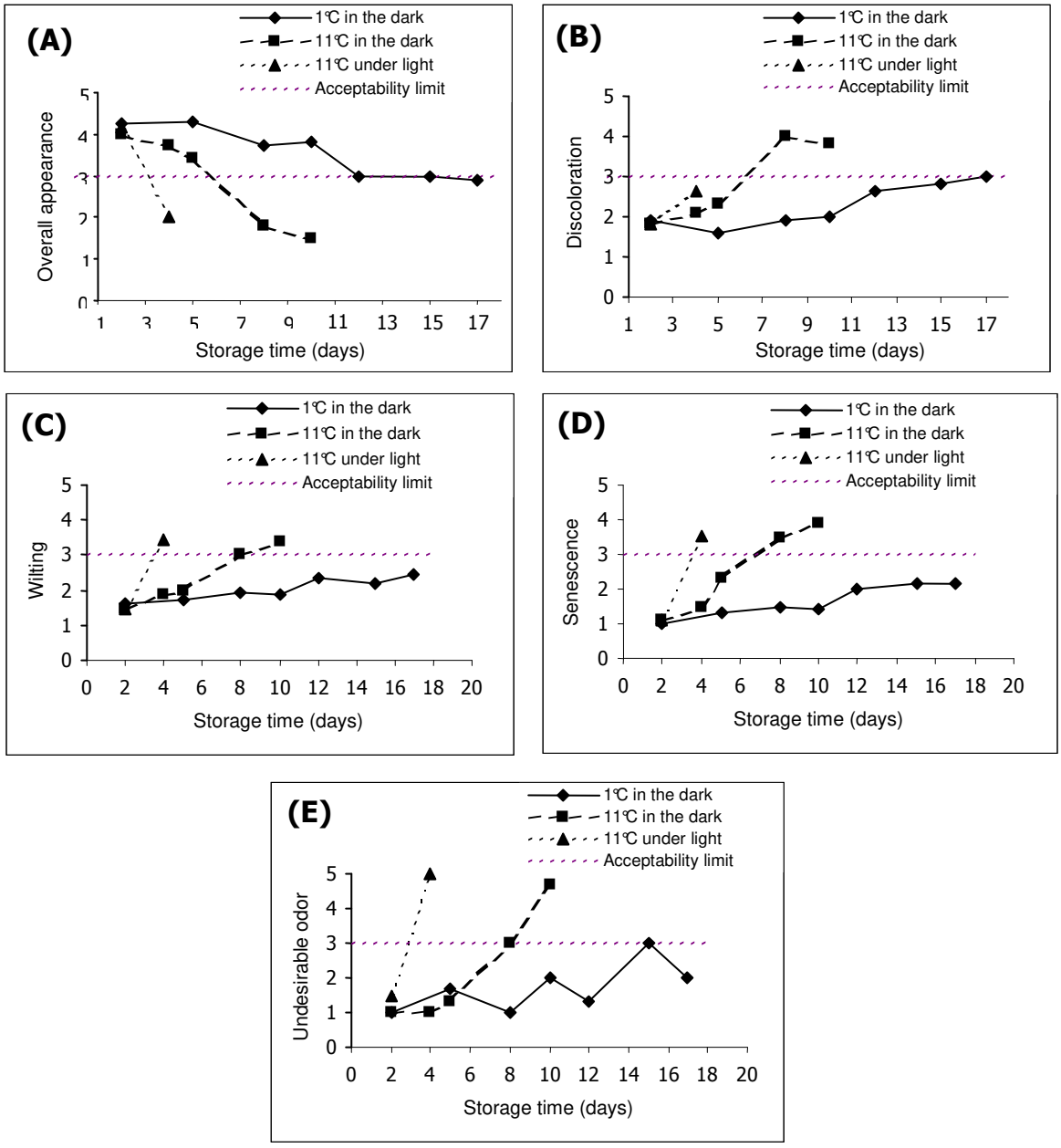

Figure 2. Sensory quality of minimally processed kale during storage under different lighting condition and temperature (A) overall appearance; (B) discoloration; (C) wilting; (D) senescence; (E) undesirable odor. The y axes scores are described in the text.

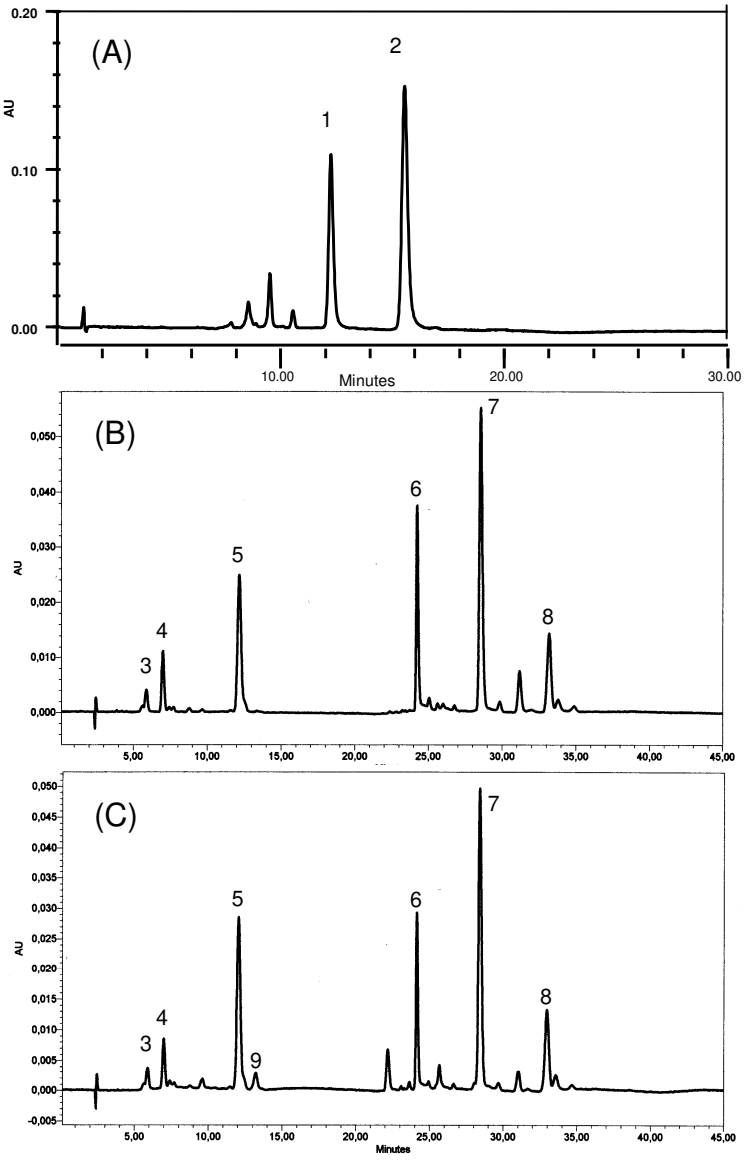

Figure 3. Typical HPLC chromatograms of (A) flavonols and (B;C) carotenoids of minimally processed kale. Peak identification: 1. quercetin; 2. kaempferol; 3. neoxanthin; 4. violaxanthin; 5. lutein; 6 and 7. clorophylls; 8. β-carotene; 9. zeaxanthin. Chromatographic conditions are described in the text.

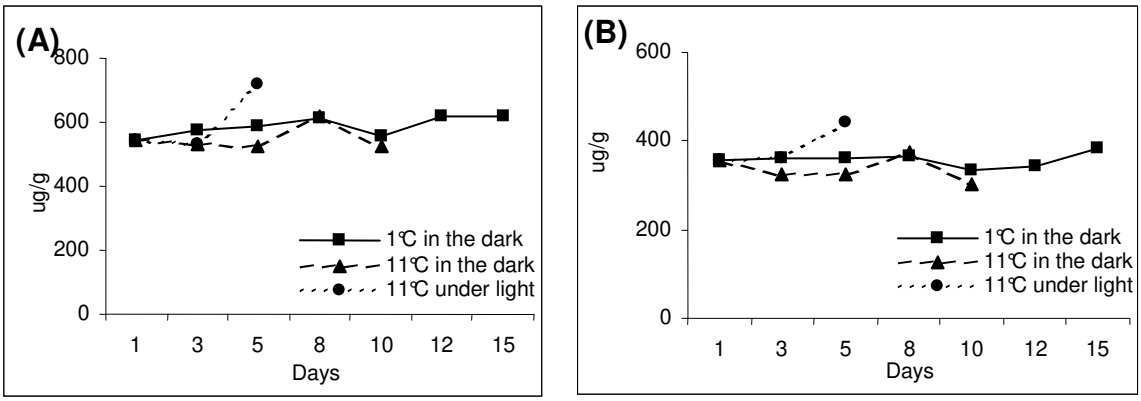

Figure 4. Concentrations of (A) quercetina e (B) kaempferol in minimally processed kale during storage at 1℃ in the dark and at 11℃ witho ut and with light exposure.

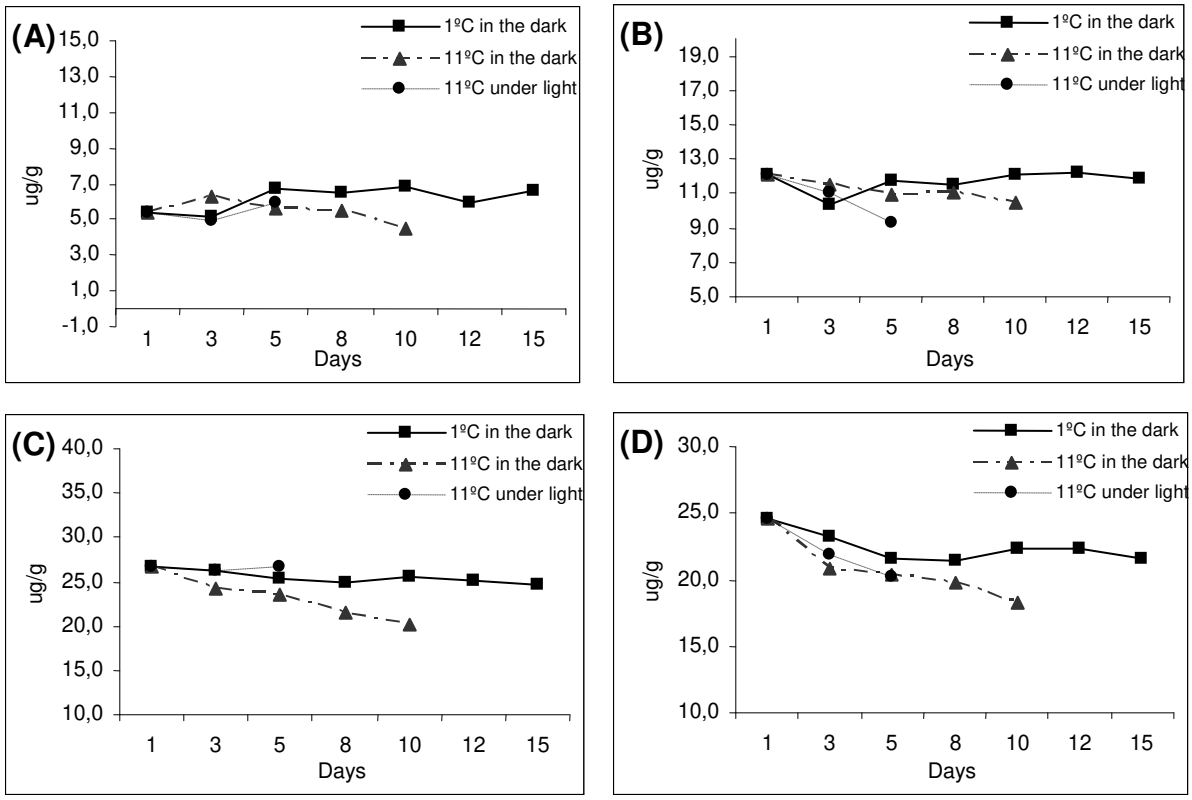

Figure 5. Concentrations of (A) neoxanthin, (B) violaxanthin, (C) lutein and (D) β-carotene of minimally processed kale during storage at 1℃ i n the dark and at 11℃ without and with light exposure.

Capítulo 7

Behavior of Flavonols and Carotenoids during Storage of Minimally Processed New Zealand Spinach Leaves under Modified Atmosphere Packaging

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BEHAVIOR OF FLAVONOLS AND CAROTENOIDS OF MINIMALLY PROCESSED NEW ZEALAND SPINACH LEAVES DURING STORAGE UNDER MODIFIED ATMOSPHERE PACKAGING

ABSTRACT

Fresh-cut minimally processed New Zealand spinach leaves were packed under modified atmosphere and stored at different conditions: 1ºC in the dark and 9ºC with or without light exposure. Headspace gas composition, sensory attributes, carotenoid and flavonol contents were evaluated during storage. The sensory quality decreased slightly during 13 days at 1ºC in the dark. At 9ºC, the vegetable shelf-life was predicted to be 9 days in the dark and more than 7 days with light. Flavonols, lutein and β-carotene decreased significantly during minimal processing. The quercetin and kaempferol levels fluctuated during storage, decreasing or increasing at some points, ending with levels similar to the initial values at 1°C, but higher at 9°C, in the dark. At 9°C under light, the final levels were lower than the original values. Although the carotenoids levels also oscillated, being lower than at the beginning of storage at a few points, neoxanthin, violaxanthin, lutein and β-carotene increased during storage at the three conditions studied.

Key words: flavonols, carotenoids, New Zealand spinach leaves, minimal processing, modified atmosphere packaging

INTRODUCTION

Associated with lower incidence of degenerative diseases (Steinmetz and Potter, 1996; Ness and Powles, 1997; Liu and others 2000, 2001; Hung and others 2004), consumption of fruits and vegetables is being promoted worldwide. The protection against diseases has been attributed to bioactive compounds found in these plant foods, such as flavonoids and carotenoids. Flavonoids has been linked with lower risk of coronary heart disease and cancer (Hertog and others 1995, 1997; Knekt and others 1997; Yochum and others 1999; Neuhouser 2004). Aside from their action against cancer and cardiovascular disease, carotenoids also reduce the risk of macular degeneration and cataract (Moeller and others 2000; Gerth and others 2004; Renzi and Johnson 2008; Singh and Goyal 2008; Nishino and others 2009) and some carotenoids have provitamin A activity.

Minimal processing together with passive or active modified atmosphere packaging are current trends for marketing fruits and vegetables. The modern life style, which diminishes the time available for preparing food, the increase in purchasing power, and greater concern about health are factors which contribute to the significant increase in the demand for minimally processed foods (Baldwin et al., 1995).

Fresh-cut vegetables meet consumers' demand for healthy, palatable and easy to prepare food, but these benefits are offset by the rapid deterioration and short shelf-life of these products. Peeling, cutting, shredding removes the natural protection of the epidermis and destroys the internal compartmentation that separates enzymes from substrates, making minimally processed vegetables much more perishable than the original intact produce. Respiratory activity and ethylene emission are also generally increased (Varoquaux et al., 1994; Martínez et al., 2005). To maintain freshness, extend shelf-life, ensure safety and promote sale, low temperature storage and modified atmosphere packaging (MAP) are used (Zagory, 1998).

Little is yet known on the effects of fresh-cut preparation, handling and treatments such as MAP on the nutritional quality of fresh-cut vegetables. There is increased interest in generating information on nutrients and bioactive compounds of these products (Artés et al., 2007).

The present study was carried out to evaluate the stability of flavonols and carotenoids in minimally processed New Zealand spinach under modified atmosphere packaging. New Zealand spinach, a widely consumed leafy vegetable in Brazil, is a rich source of flavonols (quercetin and kaempferol) (Huber et al., 2009) and carotenoids (lutein, β-carotene, violaxanthin, neoxanthin) (Azevedo-Meleiro and Rodriguez-Amaya, 2005b).

MATERIALS AND METHODS

Minimal processing

The New Zealand spinach leaves were processed in a small-scale industry located in São Roque, São Paulo, involved in minimal processing of vegetables. The pre-washed leaves were selected, trimmed, washed with soap for vegetables followed by plain water at approximately 6℃. The leaves were then sanitized b y immersion in a solution of peracetic acid (0.07%), and centrifuged at low speed. These operations were carried out in a refrigerated room maintained at a temperature of about 15℃. Portions of about 200 g were packed in heat-sealed plastic bags. The packaging material was a laminated film, bioriented polypropylene (BOPP)/low density polyethylene (LDPE) film; oxygen transmission rate was 1.514 mL (STP)/(m^2.day) at 23ºC under dry condition. Nitrogen was injected into the packages to confer mechanical protection, and create a modified atmosphere with low oxygen. The samples were immediately transported, in isothermic boxes cooled with ice, to the laboratories for storage and sensory and chemical analyses.

Storage

The samples were stored under the following conditions: 1ºC in the dark and 9ºC without or with light (299 lux) exposure. The samples exposed to light were placed in special shelves with two lamps (brand Osram, model 30W/765, daylight) of the same length as the shelves. The intensity of light over the product was measured with a luximeter, model 407026 (Extech Instruments Corp., Waltham, MA, USA) with a resolution of 1 lux. During storage, headspace gas composition, sensory attributes (overall appearance, overall quality, discoloration, wilting, senescence and undesirable odor), carotenoid and flavonol concentrations were determined.

The flavonoid and carotenoid analyses were carried out while the sensory analyses indicated that the product was still acceptable. Thus, the leaves stored at 1℃ in the dark were analyzed at 2, 4, 7, 10, 15 and 18 days of storage; those stored at 11℃ in the dark at 2, 4, 7, 10 and 15 days and under light at 2, 4 and 7 days of storage.

Three packages were mixed at each day of analysis and a sub-sample was drawn and homogenized in a food processor. Aliquots were then taken and weighed for flavonoid and carotenoid determinations. All analyses were carried out in triplicate.

Evaluation of the sensory quality

Sensory quality was evaluated by a panel of 14 untrained panelists. All the attributes were evaluated using a structured scale of five points. The scores for overall appearance were: 1 – very bad, 2 – bad, 3 – regular, 4 – good, 5-excellent. For discoloration, wilting, senescence and undesirable odor the scores were: 1 – absent, 2 – slight, 3 – moderate, 4 – intense, 5 – very intense. Each panel member was presented a package of the product for each treatment. A score of 3 was considered the limit of acceptability of the product for each of the quality attribute evaluated.

Determination of gas composition

The O_2 and CO_2 levels in the package's headspace were determined using a Shimadzu gas chromatograph model 14A (Shimadzu Corp., Nakagyo-ku, Kyoto, Japan), equipped with a thermal conductivity detector operated at 140ºC, Porapak Q and molecular sieve 5A (Supelco Inc, Bellefonte, PA, USA) columns at 82ºC, and an injector set at 84ºC. From each package, an aliquot of 0.5 mL of the headspace gas was hermetically withdrawn through a silicone septum adhered to the package's surface. The results were expressed as percentages in volume of gas.

Flavonoid Analysis

A known amount of water (1:1, water:sample) and ascorbic acid (enough to give a final concentration of 0.04%) was added to the weighed sample and homogenization was undertaken for 3 min at a velocity of 25,000 rpm in a Polytron MR2100 homogenizer Kinematica AG, Littau, LU, Switzerland). Using 7.5 g of the homogenized sample, the flavonols were quantified as aglycones according to a method optimized and validated by Huber et al. (2007). The extraction/hydrolysis was done with 50% aqueous methanol with 1.0 M HCl at 90ºC for 6 hours. The optimum hydrolysis condition was determined by a Central Composite Rotational Design (CCRD) and response surface analysis. The extract was cooled and filtered through a glass-sintered funnel, the volume was completed to 50 mL with methanol and the solution was sonicated for 5 min. An aliquot of about 2 mL was filtered through a 0.45 µm PTFE syringe filter (Millipore, Carrigtwohill, Co Cork, Ireland); a 20 µL aliquot was injected into the liquid chromatograph.

A Waters liquid chromatograph model 2690 (Waters Corp., Milford, MA, USA) was used, equipped with a Rheodyne injector (model 7725i), a photodiode array detector (Waters 996) set at 370 nm, and a Nova-Pak C18 column, controlled by Software

Millenium 3.20. The mobile phase consisted of methanol:water (both acidified with 0.03% formic acid) in a multilinear gradient, starting with 20:80, changing to 45:55 in 5 minutes, 48:52 in 17 minutes, returning to 20:80 in 20 minutes. The flow rate was 1.0 mL/min.

The identification of the flavonols was based on the retention times, co-chromatography with standards, and the UV spectra obtained with the photodiode array detector. Quantification was by external standardization. The quercetin and kaempferol standards were obtained from Sigma Chemicals Co. (St. Louis, MO, USA).

Carotenoid analysis

Carotenoids were determined according to a method developed and evaluated for leafy vegetables by Kimura and Rodriguez-Amaya (2002) and validated using a lyophilized vegetable mix certified reference material by Kimura et al. (2007).

Using 3 g of the homogenized sample, carotenoids were extracted with cooled acetone in the Polytron MR2100 homogenizer for 1 min at 11,000 rpm, and the extract was filtrated through a glass-sintered funnel. Extraction and filtration were repeated until the residue turned colorless. The carotenoids were transferred to about 50 mL petroleum ether: ethyl ether (2:1) by partition, in a separatory funnel with the addition of water. The ether solution was washed free of acetone, dried with anhydrous sodium sulfate, concentrated in a rotary evaporator, and brought to dryness under nitrogen. Prior to injection, the carotenoids were dissolved in 2 mL HPLC grade acetone and filtered through a 0.22 µm PTFE syringe filter (Millipore, Carrigtwohill, Co Cork, Ireland); a 10 µL aliquot was injected into the liquid chromatograph. All the necessary precautions were taken to avoid alterations or losses of the carotenoids and other errors during analysis.

Another HPLC system consisted of a Waters separation module, model 2690 (Waters Corp., Milford, MA. USA) equipped with quaternary pump, autosampler injector,

degasser and a photodiode array detector (model 996), controlled by a Millenium 3.20. Detection was at the wavelengths of maximum absorption (max plot).

The column was monomeric C_{18} Spherisorb ODS2, 3 µm, 4.6 x 150 mm. The mobile phase consisted of acetonitrile (containing 0.05% of triethylamine), methanol, and ethyl acetate, used at a flow rate of 0.7 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.

Identification of the carotenoids was done according to Rodriguez-Amaya (1999), with the combined use of retention time, cochromatography with the standards and the visible absorption spectra. The identity of these carotenoids in kale, endive and New Zealand spinach was confirmed by HPLC-MS by Azevedo-Meleiro and Rodriguez-Amaya (2005a,b).

Quantification was by external standardization. Standards were isolated from a leafy vegetable (roquette) by open column chromatography on MgO:Hyflosupercel (1:1, activated for 4 h at 110 °C) packed to a height of 20 cm in 2.5 cm i.d. x 30 cm glass column. This column was developed with increasing amounts of ethyl ether and acetone in petroleum ether; the purity of the carotenoid isolates was monitored by HPLC. The mean purity of the standards was 99% for neoxanthin, 96% for violaxanthin, 99% for lutein, and 94% for β-carotene. The concentrations of the standard solutions were corrected accordingly.

In both flavonoid and carotenoid analyses, the standard curves were constructed by the injection in triplicate of standard solutions at five different concentrations. The curves passed through the origin and were linear at the concentration range expected of the samples, the coefficients of correlation obtained being higher than 0.99.

Statistical analysis

To verify the existence of statistically significant differences, the results of the flavonoid and carotenoid analyses were submitted to analysis of variance ($p<0.05$), the means being compared by Tukey test, utilizing the GraphPad Prism 2.01 program.

RESULTS AND DISCUSSION

Gas composition in the package

Figure 1 shows the gas levels (O_2 and CO_2) in the packages during storage. Injection of nitrogen initially reduced the oxygen level to 5 – 10%. After this initial active atmosphere modification, passive modification appeared to occur, as a function of respiration of the product and permeability of the packaging. At 1ºC in the dark, an equilibrium atmosphere was established in the package from the 7th day (<0,5% O_2 and 2-3% CO_2). The effect of storage temperature on gas composition was evident. At 9ºC in the dark, O_2 level fell to 0.2% and CO_2 reached 3% after 2 days. This indicates that the temperature has a greater effect on the produce respiration rate than on the package gas transmission rate, accelerating the modification of the atmosphere inside the package.

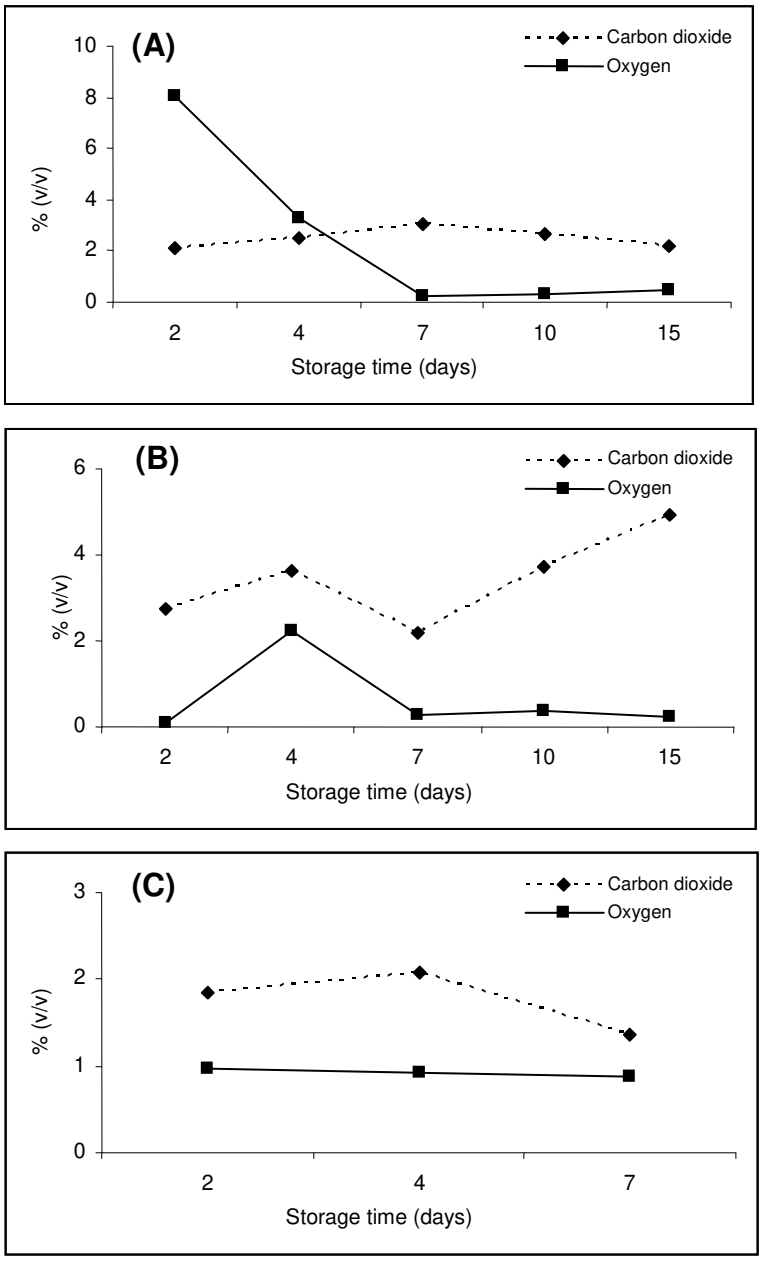

Figure 1. Evolution of the oxygen and carbon dioxide levels in the atmosphere of the packages of minimally processed New Zealand spinach during storage at (A) 1℃ in the dark, (B) 9℃ in the dark, (C) 9℃ under light.

Sensory quality

Figure 2 shows the results of the sensory evaluation. Through the overall appearance, the shelf-lives of the samples stored at 1ºC and 9ºC in the dark were estimated to be 13 days and 9 days, respectively. The sensory properties of the New Zealand spinach stored at 9ºC under light remained acceptable after the 7 days of storage studied, thus it was not possible to estimate the end of shelf-life under this condition.

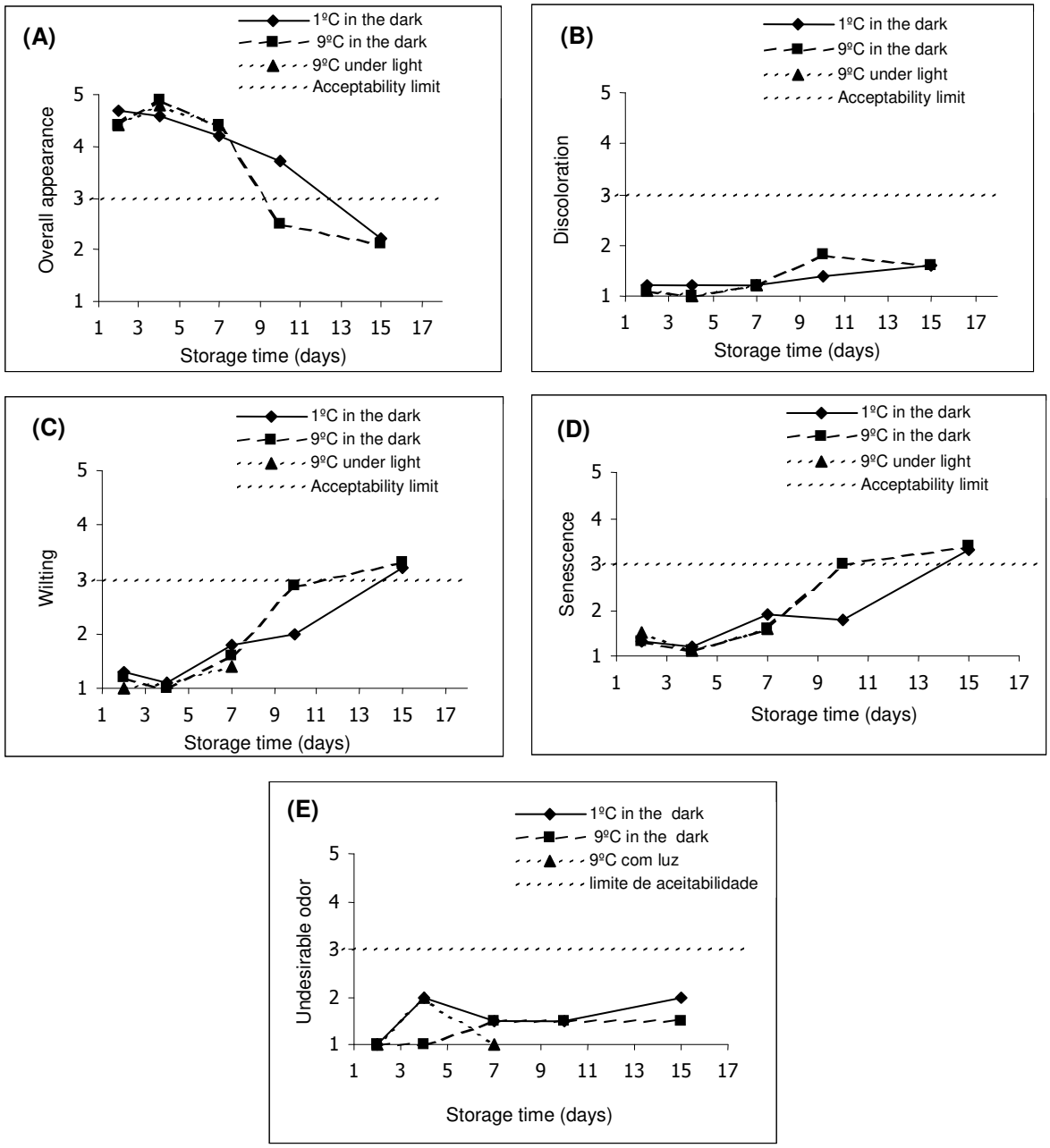

Figure 2. Sensory quality of minimally processed New Zealand spinach during storage under different lighting condition and temperature (A) overall appearance; (B) discoloration; (C) wilting; (D) senescence; (E) undesirable odor. The y axes scores are described in the text.

Effects of minimal processing in flavonols and carotenoids

Typical HPLC chromatograms of the flavonols and carotenoids of New Zealand spinach are shown in Figure 3.

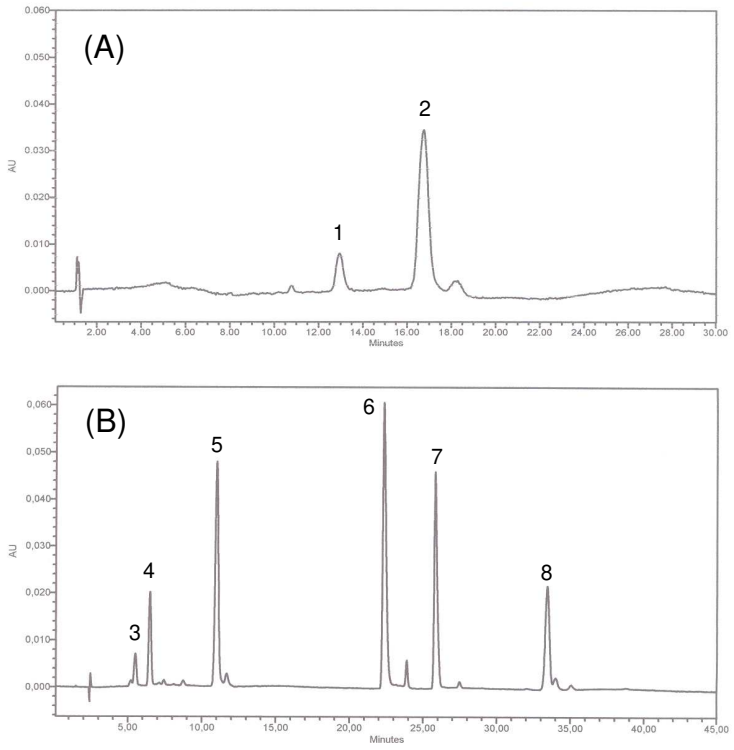

Figure 3. Typical HPLC chromatograms of (A) flavonols and (B) carotenoids of minimally processed New Zealand spinach. Peak identification: 1. quercetin; 2. kaempferol; 3. neoxanthin; 4. violaxanthin; 5. lutein; 6 and 7. clorophylls; 8. β-carotene. Chromatographic conditions are described in the text.

Table 1 presents the flavonoid and carotenoid concentrations before and after minimal processing. All of the bioactive compounds analyzed decreased after processing. The reduction was not significant for neoxanthin and violaxanthin, and significant for flavonols, lutein and β-carotene.

Table 1. Flavonoids and carotenoids contents* (µg/g) before and after the processing.

Compound	Minimally processing	
	Before	After
Quercetin	29.7 ± 0.6a	21.9 ± 0.7b
Kaempferol	170 ± 1.2a	146 ± 4.6b
Neoxanthin	8.3 ± 1.0a	8.2 ± 0.3a
Violaxanthin	20.7 ± 2.1a	20.3 ± 0.5a
Lutein	40.2 ± 1.5a	37.1 ± 0.8b
β-carotene	36.7 ± 1.4a	32.4 ± 0.5b

*Values in the same line with different letters are significantly different ($p < 0.05$)

Flavonol levels during storage

Figure 4 shows the flavonol levels during storage of the minimally processed New Zealand spinach.

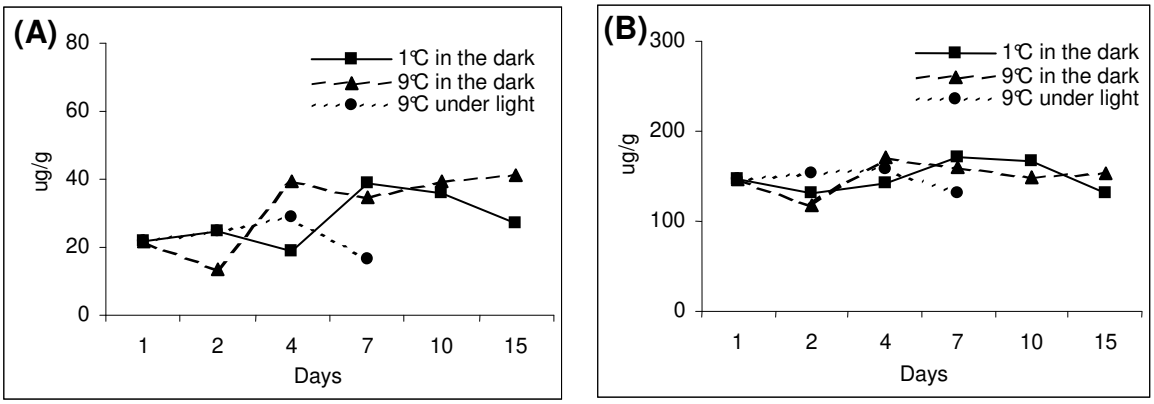

Figure 4. Concentrations of (A) quercetina e (B) kaempferol in minimally processed New Zealand spinach during storage at 1℃ in the dark and at 9℃ without and with light exposure.

At 1°C without light, quercetin decreased slightly on the 4th day and increased on

the 7th day, remaining at levels higher than the initial value up to the 10thday, but decreasing near to the initial value on the 15th day. Kaempferol decreased on the 2nd day, but increased up to the 7th day, decreasing again on 15th day. In samples stored at 9ºC in the dark, quercetin decreased on the 2nd day, but increased on the 4th day, remaining at levels much higher than the initial value up to the 15th day. Kaempferol also decreased on the 2nd day and increased on the 4, but thereafter decreased gradually to a value equivalent to the initial level in 15 days. At 9ºC under light, the flavonols presented increasing values from the 1st to the 4th day, subsequently decreasing on the 7th day.

Also investigating spinach, Bottino et al. (2009) found that the most important flavonoids did not change upon storage in intact leaves and some of them increased significantly during storage in fresh-cut samples.

Gil et al. (1999) reported that the total flavonoid content of minimally processed spinach remained stable during 7 days of storage at 10ºC in packages with air or modified atmosphere.

Carotenoid levels during storage

Figure 5 presents the carotenoid levels of the minimally processed New Zealand spinach. Although the carotenoids levels also oscillated, being lower than those at the beginning of storage at a few points, neoxanthin, violaxanthin, lutein and β-carotene concentrations tended to increase during storage at the different conditions.

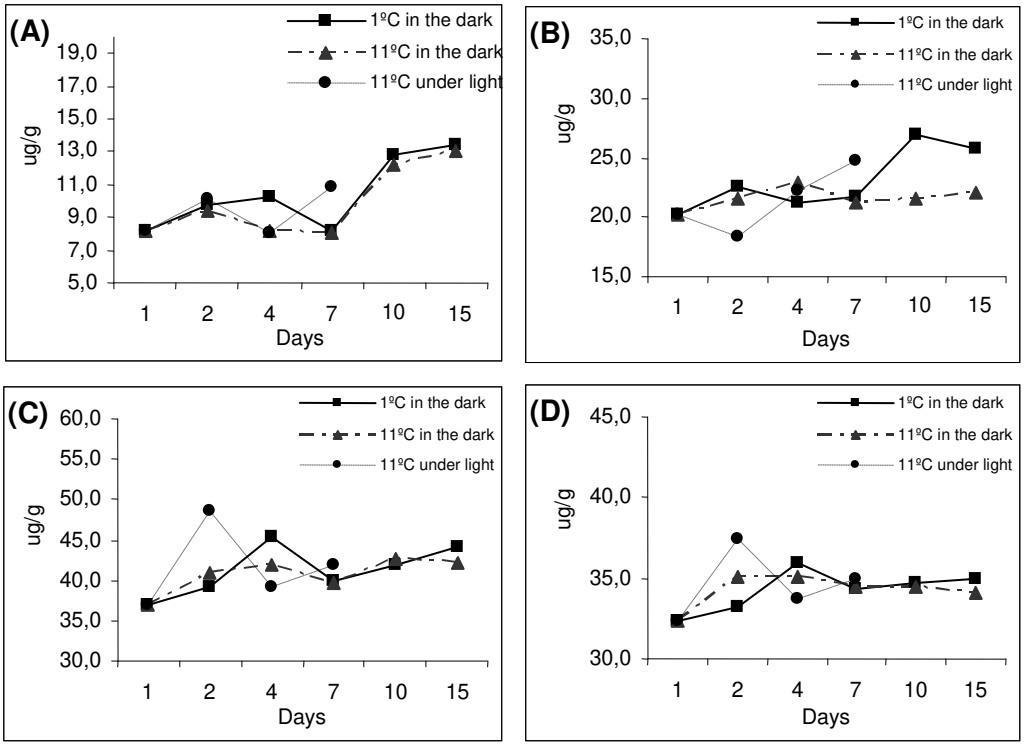

Figure 5. Concentrations of carotenoids: (A) neoxanthin, (B) violaxanthin, (C) lutein and (D) β-carotene of minimally processed New Zealand spinach during storage at 1℃ in the dark and at 9℃ without and with light exposure.

In minimally processed leafy vegetables (kale, endive and New Zealand spinach) stored in polyethylene bags for five days at 7-9 ºC, losses of 14-42%, 19-32%, 12-20%, 8-31% were observed for β-carotene, lutein, violaxanthin and neoxanthin, respectively, greater losses being observed in New Zealand spinach with the exception of neoxanthin which degraded more in kale (Azevedo-Meleiro and Rodriguez-Amaya, 2005a,b). The

carotenoids were expected to decrease in this case because MAP was not employed.

Ferrante et al. (2008) studied quality changes during storage of fresh-cut or intact Swiss chard leafy vegetables under dark or lighted storage until 12 days at 5°C. While anthocyanin content strongly decreased in cut leaves in the dark and under light, total carotenoids did not significantly decline. These same authors (Ferrante et al, 2009) evaluating cut and intact lamb's lettuce leaves, reported an increase in anthocyanins and a decrease in total carotenoids from 20 to 16 mg/g of after 8 days of storage at 4ºC in darkness in both treatments.

Fluctuations or seemingly inconsistent levels of these phytochemicals can be explained by the possible occurrence of processes with opposing effects on their concentrations. Cutting the vegetables may destroy compartmentation of oxidative enzymes, which can then promote degradation. On the other hand, because thermal processing is not involved and the leaves were not shredded, the biosynthetic enzymes may remain active, increasing the phytochemical's level. Enhanced biosynthesis is favored when cellular integrity is preserved, as in the present study, the leaves being left whole. The concentrations of the phytochemicals at any one time would reflect which of the two processes is predominating.

CONCLUSION

Modified atmosphere packaging together with refrigeration extended the shelf-life of minimally processed New Zealand spinach. Flavonoids and carotenoids degraded during minimal processing. However, they increased during the storage at different conditions.

Acknowledgments

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Capítulo 8

Optimization of microencapsulation by spray drying. Stability of β-carotene and vitamin C in microencapsulated acerola

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ABSTRACT

Acerola (*Malpighia glabra* L. or *Malpighia punicifolia* L), a native fruit of tropical America well appreciated for its very high vitamin C content, is also rich in other health promoting compounds such as β-carotene. Microencapsulation can protect these unstable food components during storage. However, degradation can occur during the microencapsulation process itself. Central Composite Design and Response Surface Analysis were used to investigate the effects of the concentration of the encapsulating agent (5.9 to 34%) and the spray dryer inlet temperature (150 to 200ºC). The encapsulating agents studied were: maltodextrin (20 DE), corn starch octenyl succinate (Capsul®) and gum Arabic. The optimum conditions were found to be: 30% maltodextrin or modified starch at an inlet temperature of 157ºC and 20% gum Arabic at 175ºC. Under these conditions, retentions of β-carotene and vitamin C were, respectively, 92 and 106% in maltodextrin, 71 and 109% in modified starch, and 76 and 114% in gum Arabic. The microcapsules were characterized and the stability of β-carotene and vitamin C of the microencapsulated pulp, packed in aluminum foil bags and stored at ambient conditions, was verified. The best protection was shown by gum Arabic, with 65.4% and 96.7% retention of β-carotene and vitamin C, respectively, after four months of storage, compared to only 26.4% and 79.2% retention, respectively, in lyophilized acerola pulp used as control.

Key words: β-carotene, vitamin C, microencapsulation, spray-drying, acerola pulp

INTRODUCTION

Acerola (*Malpighia glabra* L, *Malpighia punicifolia* L ou *Malpighia emarginata* DC), also known as Antilles cherry, Barbados cherry or West Indian cherry, is native of tropical America (Marino Netto, 1986). Known for its very high vitamin C content (Asenjo and Moscoso, 1950; Vendramini and Trugo, 2000; Batista et al., 2000; Assis et al., 2001), it is also rich in other health-promoting compounds such as carotenoids (Porcu and Rodriguez-Amaya, 2006; de Rosso and Mercadante, 2005) and flavonoids (Lima et al., 2005; Hoffmann-Ribani et al., 2009). These antioxidants have been associated with the reduction of the risk of developing chronic diseases such as cancer (Mayne, 1996; Nishino et al., 1999; Gonzáles et al., 2005; Knekt et al., 1997; de Stefani et al., 1999; Garcia-Closas et al., 1999; Yang et al., 2001) and cardiovascular diseases (Hertog et al., 1993, 1997; Knekt et al., 1996; Yochum et al., 1999). Consumption of acerola has increased because of these beneficial effects on health, together with its pleasant flavor.

Brazil is the major producer and exporter of acerola (Mezadri et al., 2006), especially in the form of frozen pulp and juice (Yamashita et al., 2003). The Brazilian climate, with high humidity and temperature, does not favor commercialization of the fresh fruit because of its high moisture content (average of 91%), rapid post-harvest deterioration being commonly observed. As an alternative to preserve the fruit, special attention is being given to the development of adequate drying techniques. Aside from aggregating commercial value, drying reduces port-harvest losses and permits commercialization for a longer time and during the off-season (Marques et al., 2007). Although frequently considered only as a drying process, spray-drying can also be used for microencapsulation when a protective matrix is incorporated (Ré, 1998).

Microencapsulation is a relatively new technology in the food sector, used for the protection, stabilization and controlled release of compounds of interest. The

encapsulating materials generally employed are starches and starch derivatives, proteins, gums, lipids and combinations thereof. The techniques utilized for microencapsulation are spray-drying, freeze-drying, fluidized bed-coating, extrusion, cocrystalization, molecular inclusion e coacervation (Dziezak, 1988; Shahidi and Han, 1993; Popplewell et al., 1995; Ré, 1998). Compared with other methods of microencapsulation, spray-drying has the advantage of being a relatively simple and continuous process (Ré, 1998).

Optimization of processing and storage conditions for greater retention of carotenoids and vitamin C is necessary because these compounds so important to human health are prone to degradation. Considerable losses can occur, depending on such factors as the availability of oxygen, temperature, light, water activity and pH. Porcu and Rodriguez-Amaya (2006) determined the carotenoid composition of fresh and commercially processed acerola and observed that the levels of the principal carotenoids were much lower in the processed products. The highest β-carotene concentration found in the frozen pulp was only half that of the fresh fruit.

Microencapsulation has been carried out with synthetic or isolated carotenoids (Desobry et al., 1997; Higueira-Ciapara et al., 2004), carotenoid extracts (Matioli and Rodriguez-Amaya, 2002) and food (Wagner ans Warthesen, 1995; Selim et al., 2000). Azeredo et al. (2007) reported that anthocyanins and ascorbic acid were stable during the microencapsulation of acerola juice using maltodextrin as encapsulating agent. This indicated that in spite of the high temperature used in the drying process, the time of exposure was short, minimizing the degradation of these heat-sensitive compounds.

The present work had the objetive of optimizing the conditions for the microencapsulation of acerola pulp to obtain the best retention of carotenoids and vitamin C. Additionally, the stability of these bioative substances in the microcapsules, packed in aluminum foil bags, stored at ambient conditions for four months was also investigated.

MATERIALS AND METHODS

Materials

Acerola pulp was obtained from Carbonari Ltda., Jundiaí, São Paulo, immediately after pulping at low temperature. The encapsulating agents used were: maltodextrin 20 DE of Corn Products (Mogi-Guaçu, Brazil), instant gum Arabic of Colloides Naturels International (Rouen, France) and corn starch modified with octenyl succinate (Capsul®) of National Starch Food Innovation (São Paulo, Brazil).

Spray drying

Spray drying was performed in a laboratory scale spray dryer LabPlant SD-05 (Huddersfield, England), with a 2.0 mm diameter nozzle and main spray chamber of 500 mm x 215 mm. The mixture of acerola pulp and encapsulating materials, previously homogenized in a Polytron MR2100 (Kinematica AG, Lucerne, Switzerland) for 2 minutes at 11,000 rpm, was fed into the main chamber through a peristaltic pump at a feed rate of 7.5 mL/min. The drying air flow rate was 73 m^3/h and compressor air pressure was 0.06 MPa. Inlet air temperature varied from 150 ºC to 200 ºC, according to an experimental design, described in the item Experimental design.

Powder recovery (process yield) was calculated as the relationship between total solid content in the resulting powder and total solid content in the feed mixture. Assessment of the retention of carotenoids and vitamin C during microencapsulation was based on their contents on a dry acerola basis, disregarding encapsulating materials and moisture.

Analytical methods

Feed mixtures and spray-dried powders were analysed for moisture, carotenoid

and vitamin C content. Moreover, the powders produced with different encapsulating materials were analysed with respect to their morphology, by means of scanning electron microscopy, and particle size distribution.

Moisture content

Moisture in the powders and feed mixtures was determined gravimetrically by drying in a vacuum oven at 70ºC until constant weight (AOAC, 1990).

Carotenoid analysis

Carotenoid analysis was carried out according to Kimura and Rodriguez-Amaya (2002), with the necessary adaptations to the samples analyzed. Guidelines given by Rodriguez-Amaya (1999) on how to prevent isomerization and degradation during analysis were rigorously followed.

An aliquot of approximately 3 g of the homogenized acerola pulp or 0.5-1.0 g of the homogenized acerola powder was weighed, the carotenoids were extracted with cooled acetone with a mortar and pestle, and the extract was filtered through a sintered glass funnel. Extraction and filtration were repeated until the residue turned colorless (usually 2 times). The carotenoids were transferred to about 50 mL petroleum ether: ethyl ether (2:1) by partition in a separatory funnel with the addition of water. The ether solution was washed free of acetone, dried with anhydrous sodium sulfate, concentrated in a rotary evaporator, and brought to dryness under nitrogen. Immediately before injection, the carotenoids were dissolved in HPLC grade acetone and filtered through a 0.22 µm PTFE syringe filter; a 10 µL aliquot was injected into the liquid chromatograph.

The HPLC system consisted of a Waters separation module, model 2690 (Waters

Corp., Milford, Mass., U.S.A.), equipped with a quaternary pump, autosampler injector, degasser, and a photodiode array detector (model 996), controlled by a Millenium workstation (version 3.20). Detection was at the wavelengths of maximum absorption (max plot). The column was monomeric C_{18} Spherisorb ODS2, 3 μm, 4.6 x 150 mm. Isocratic elution was employed with the mobile phase consisting of acetonitrile (containing 0.05% of triethylamine), methanol, and ethyl acetate 60:20:20, at a flow rate of 0.5 mL/min.

Quantification was by external standardization. β-carotene standard was isolated from sweetpotato by open column chromatography on MgO:Hyflosupercel (1:1, activated for 4 h at 110 ^{o}C) column packed to a height of 20 cm in 2.5 cm i.d. x 30 cm glass column. This column was developed with petroleum ether; the purity of the β-carotene isolated was monitored by HPLC. The purity of the standard was 96%. The standard curve was constructed by the injection in triplicate of standard solutions at five different concentrations. The curve passed through the origin and was linear at the concentration range expected of the samples, the coefficient of correlation obtained being higher than 0.99.

The carotenoids of acerola had been identified by the visible absorption spectra, chemical reactions, co-chromatography with standards, and confirmed by mass spectrometry by Azevedo-Meleiro and Rodriguez-Amaya (2004).

Determination of vitamin C

Vitamin C was determined using a standard AOAC (1984) method, which consist of extracting with 2% oxalic acid (modified by Benassi and Antunes, 1988) and titrating with a solution of sodium 2,6-dichlorofenol indofenol. All the analyses were done in triplicate.

Experimental design

Optimization of the spray-drying process was carried out by response surface analysis. A statistical design, Central Composite Rotational Design (CCRD) (Rodrigues and Iemma, 2005) was chosen to investigate the lack of linearity of the effects of variables (temperature and percentage of encapsulating agent) in the retention of β-carotene and vitamin C, and powder recovery. Each factor was tested in three levels (Table 1).

To establish the range of encapsulant level to be utilized in the experimental design, preliminary tests were carried out with the three encapsulating agents. The percentages of encapsulant evaluated were 25%, 50% and 75%, the morphology of the microcapsules was verified by SEM to find out if there was formation of microcapsules under these conditions. Since the microcapsules obtained were intact at the three concentrations studied for each encapsulant, it was decided that optimization be studied at a range close to the minor concentration of the encapsulant (25%), that is, with the greater concentration of the pulp.

The independent variables (temperature and percentage of encapsulant), their levels and real values are presented in Table 1 and the experimental design is shown in Table 2.

Table 1. Variables and levels for Central Composite Rotational Design.

Variable	Coded variable levels				
	-1.41	**-1**	**0**	**+1**	**+1.41**
x_1 (Temperature)	150	157	175	193	200
x_2 (% encapsulant)	5.9	10	20	30	34.1

Table 2. Experimental conditions of the statistical design of CCRD (factors with coded values) and response.

Variables			Experimental results								
			Maltodextrin			Modified starch (Capsul®)			Gum Arabic		
Trials	x_1	x_2	B-carotene retention	Vitamin C retention	Powder recovery	β-carotene retention	Vitamin C retention	Powder recovery	β-carotene retention	Vitamin C retention	Powder recovery
1	-1	-1	98.7	100.1	25.4	81.1	101.3	19.0	80.0	101.0	18.4
2	1	-1	80.9	108.4	25.2	70.7	113.8	21.6	69.8	109.7	23.8
3	-1	1	91.9	108.7	28.9	71.1	109.3	27.6	72.0	111.7	15.4
4	1	1	78.6	108.5	18.6	57.5	107.6	30.2	69.4	115.6	9.7
5	-1.41	0	99.8	109.9	22.7	72.2	107.3	17.5	86.7	109.4	12.5
6	1.41	0	79.6	106.8	43.5	60.0	108.7	20.2	72.2	114.8	14.7
7	0	-1.41	86.1	101.2	26.3	71.2	91.2	21.4	84.7	96.5	22.3
8	0	1.41	74.8	101.0	21.1	65.9	111.8	14.7	55.6	122.8	10.2
9	0	0	86.7	105.1	26.9	67.4	106.7	24.2	79.8	120.5	16.1
10	0	0	96.2	110.8	25.4	66.2	102.7	23.7	72.0	110.9	18.6
11	0	0	93.6	110.2	24.6	68.9	106.8	27.5	76.5	111.0	15.8

The following polynomial equation was fitted to the data:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2$$

The analysis of variance (ANOVA), test for the lack of fit, determination of the regression coefficients and the generation of three dimensional graphs were carried out using the Statistica 5.5 software (StatSoft, Tulsa, USA).

Characterization of the microcapsules

The powders produced with different encapsulating materials were analysed with respect to their morphology, particle size distribution, solubility and microencapsulation efficiency. Moreover, feed mixtures and spray-dried powders were analysed for moisture, carotenoid and vitamin C content. The assessment of retention of carotenoids and vitamin C during storage was based on their contents on a dry acerola basis, disregarding encapsulating materials and moisture.

Morphology

The microcapsules were observed using a Jeol scanning electron microscope (SEM) model JSM – T300, according to Rosenberg and Young (1993). Powders were attached to a duble-sided metallic adhesive tape mounted on SEM stubs (10 mm). The specimen was subsequently coated with gold in a Balzers evaporator model SCD 050 for 75 seconds with a current of 40 mA, and analysed using SEM operated at 15 kV.

Particle size distribution

Distribution of particle size was measured using a Laser Scattering Spectrometer Mastersizer S model MAM 5005 (Malvern Instruments Ltd., Worcestershire, UK). A small powder sample was suspended in isopropanol and the particle size distribution was

monitorated during each measurement until successive readings became constant. Five readings in duplicate were taken for each wall material studied. The particle size was expressed as *D[4,3]*, the mean diameter over the volume distribution.

Solubility

Solubility of the microcapsules in water was determined in triplicate according to Santos and Fávaro-Trindade (2005). The powder was added to water (0.4% w/v) and gently stirred until complete solubilization. The time necessary for complete microcapsule solubilization was recorded. The powder was considered soluble when the time of solubilization was not more than 5 min. This determination was conducted in triplicate.

Microencapsulation efficiency

The microencapsulation efficiency (ME) was calculated as follows (Wagner and Warthesen,1995; McNamee et al., 1998; Barbosa et al., 2005):

% ME = [(β-carotene total – surface β-carotene)/ β-carotene total] x 100

To determine the amount of surface β-carotene on encapsulated powder, triplicate samples of the acerola powder were accurately weighed (~1g) into 50 mL centrifuge tubes and extracted with 5 mL dichloromethane in a Vortex for 30 seconds, followed by centrifugation at 3000 rpm for 10 min at 4ºC. After phase separation, the liquid phase was collected and filtered through a Millipore 0.22 µm PTFE syringe filter and analyzed for β-carotene by HPLC to determine the fraction of carotene not encapsulated but retained on powder surfaces.

Stability

The packaging chosen for the stability study was that most utilized by the

powdered fruit juice industry, consisting of flexible aluminized film with the following layers: polyester (PET), adhesive, aluminum, adhesive and polyethylene (PE), according to the information given by the manufacturer, presented in Table 3.

Table 3. Composition of the aluminized flexible filme of the packaging used in the stability study.

Material	Gramature minimum (g/m^2)	Gramature nominal (g/m^2)	Gramature maximum (g/m^2)
Polyester (PET)	16.0	16.8	17.6
Adhesive	1.8	2.5	3.2
Aluminum	19.4	21.6	23.8
Adhesive	1.8	2.5	3.2
Polyethylene (PE)	31.5	35.0	38.5
Total (g/m^2)	72.0	81.0	90.0

PET confers rigidity to the packaging, aluminum forms a barrier to gases, water vapor and light, and PE is added to seal the packaging (Sarantopoulos et al., 2002). About 10 g of the microencapsulated acerola pulp was packed in each packaging with an area of 60 cm^2, thermosealed in the three lateral sides.

The storage study was done for a period of 4 months under ambient condition, the temperature and the relative humidity being monitored by a DataLogger HT-500 version 2.0, which registered these parameters every 6 h. The carotenoid and vitamin C concentration were determined at 0, 15, 30, 60, 90 and 120 days of storage.

RESULTS AND DISCUSSION

Optimization of microencapsulation

Table 4 presents the coeffcients of regression for the responses retention of carotenoids, vitamin C and powder yield for maltodextrin, modified starch and gum Arabic. For the elaboration of the models, as a function of the variables studied, 5-10% of

significance was adopted in the statistical evaluation of the coefficients of regression obtained.

Table 4. Estimates of the coefficients of regression of the quadratic polynomial model and significance (p-valor), for the responses analyzed in the microencapsulation process.

	β-carotene retention		Vitamin C retention		Powder recovery	
	Coefficient	**p-valor**	**Coefficient**	**p-valor**	**Coefficient**	**p-valor**
Maltodextrin						
β_0	92.17	<0.0001	108.70	<0.0001	25.65	0.0013
β_1	-7.46	0.0022	0.45	0.7197	2.36	0.3751
β_{11}	-0.63	0.7003	0.24	0.8697	2.77	0.3825
β_2	-3.14	0.0599	1.06	0.4097	-1.31	0.6128
β_{22}	-5.23	0.0195	-3.39	0.0601	-1.96	0.5285
β_{12}	1.11	0.5715	-2.14	0.2548	-2.53	0.4940
Modified starch (Capsul®)						
β_0	67.49	<0.0001	105.41	<0.0001	25.10	0.0007
β_1	-5.16	0.0072	1.60	0.4227	0.63	0.7773
β_{11}	-0.01	0.9952	2.10	0.3804	-1.34	0.6168
β_2	-3.84	0.0227	3.88	0.0879	0.47	0.8318
β_{22}	1.23	0.4236	-1.15	0.6225	-1.74	0.5189
β_{12}	-0.81	0.6494	-3.57	0.2276	1.00	0.7500
Gum Arabic						
β_0	76.12	<0.0001	114.13	<0.0001	16.83	<0.0001
β_1	-4.17	0.1073	2.53	0.2066	0.35	0.5716
β_{11}	1.17	0.6633	-1.35	0.5446	-1.14	0.1603
β_2	-6.20	0.0332	6.72	0.0119	-4.28	0.0007
β_{22}	-3.50	0.2263	-2.59	0.2676	0.19	0.7938
β_{12}	1.93	0.5495	-1.21	0.6444	-2.78	0.0196

Table 5 shows the regression coefficients for the coded second order polynomial equation, the *F* values and the determination coefficients (R^2). Some non-significant terms were eliminated and the resulting equations were tested for adequacy and fitness by the analysis of variance (ANOVA). By the R^2 and F calculated, it can be observed that the experimental results had good fit with the models obtained for the responses retention of β-carotene with the use of maltodextrin and modified starch and powder yield for gum Arabic (R^2 greater than 0.80), and low but acceptable R^2 for the retention of β-carotene e vitamin C for gum Arabic.

Table 5. Equations that represent the responses as function of temperature (X_1) and amount of encapsulating agent (X_2) in the microcapsules studied.

	Response	Equação Y = (mean) (values of X_1 and X_2 codified)	R^2	F_{CAL}	F_{TAB} (α, v_R, v_r)
Maltodextrin	β-carotene	$Y = 91.6 - 7.5\ X_1 - 5.0\ X_2^2$	0.80	15.70	4.46
Capsul®	β-carotene	$Y = 68.4 - 5.2\ X_1 - 3.8\ X_2$	0.83	19.53	4.46
Gum Arabic	β-carotene	$Y = 77.4 - 4.2\ X_1 - 6.2\ X_2$	0.60	6.09	3.11*
	Vitamin C	$Y = 111.3 + 6.7\ X_2$	0.62	6.63	5.12
	Powder Recovery	$Y = 16.1 - 4.3\ X_2 - 2.8\ X_1\ X_2$	0.88	17.65	4.46

R^2: coefficient of determination;
$F_{CAL} = (QM_{Regression}/QM_{Residue})$;
α: level of significance (5%); v_R: degree of liberty of regression; v_r: degree of liberty of residue;
* α: level of significance (10%); v_R: degree of liberty of regression; v_r: degree of liberty of residue.

Thus, it was possible to generate five response surfaces presented in Figure 1. It can be noted that the axes of the responses are presented in increasing order.

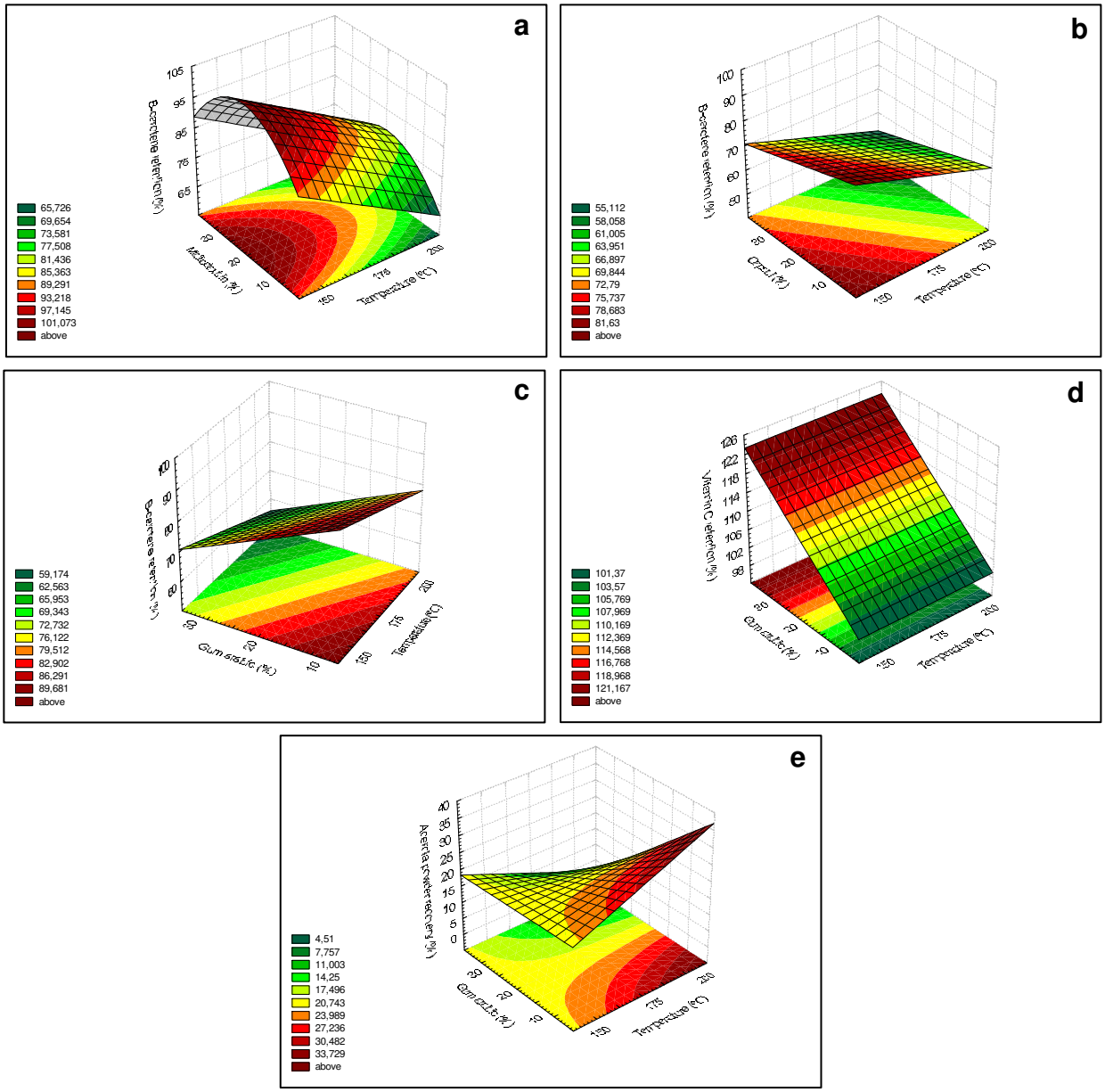

Figure 1. Response surfaces for the retention of β-carotene in the microencapsulation of acerola pulp by spray-drying, using maltodextrin (a), modified starch - Capsul® (b) and gum Arabic (c); retention of vitamin C (d) and powder recovery (e) for gum Arabic.

Although it was not possible to generate surfaces for some responses, the effect of the variables studied can be evaluated.

Maltodextrin

For maltodextrin, the surface response (*Figure 1a*) showed that the higher the temperature, the lower the retention of β-carotene, coherent with the expected effect of temperature on the degradation of carotenoids. Optimum percentages of encapsulating agent was close to the central point, at a wide range of 10 a 30% of encapsulant. For the retention of vitamin C, the variables did not significantly influence the response in the range studied, demonstrating the robustness of the process for this vitamin since all the tests obtained 100% recovery. Evaluating the effects of the variables on powder recovery (*Tabela 2*), it can be observed that the response varied from 18.6 to 43.5%. Although the yield was higher in trial 6, a lower retention was obtained (79.6%). Thus, the condition of trial 3 was chosen as the one that obtained a set of more favorable responses: powder recovery of 28.9% with good retention of β-carotene (91.9%) and vitamin C (108.7%).

Desobry et al. (1997) reported 11% loss of β-carotene in encapsulation with maltodextrin 25 DE and Azeredo et al. (2007) 12.2% of degradation of vitamin C in microencapsulation of filtered acerola juice with maltodextrin 10DE by spray-drying. Finotelli and Rocha-Leão (2005) found 100% retention of vitamin C during microencapsulation utilizing different proportions of maltodextrin and modified starch (Capsul®) by spray-drying.

Studying the effects of conditions in spray-drying açaí pulp encapsulated with maltodextrin, utilizing DCC with response surface analysis, Tonon et al. (2008) concluded that the higher the inlet air, the greater the powder recovery of the process, but the retention of anthocyanin is lower. The same trend was seen in this study but with β-

carotene.

Modified starch (Capsul®)

The surface generated for the response retention of β-carotene for the modified starch (*Figure 1b*) also showed lower retention with an increase in temperature and with lower concentration of the encapsulant. There was a slight increase with lower concentration of the encapsulant. The retention of vitamin C (*Table 2*) was good in all trials (91-114%). In relation to powder recovery, the response varied from 14.7 to 30.2%. The trial with the highest powder recovery also had lower retention of β-carotene, only 57.5%. Thus, trial 3 was also selected because of the combination of responses, obtaining powder recovery of 27.6%, with retention of 71.1% of β-carotene and 109.3% of vitamin C.

In the microencapsulation of the oleoresin of *Rosa mosqueta* using starch or gelatin, the retention of *trans*-rubixanthin was 60% (Robert et al., 2003). For *trans*-lycopene and *trans*-β-carotene, retentions were lower with starch (54 and 71%, respectively) than with gelatin (72 and 99%, respectively).

Gum Arabic

For gum arabic, it was possible to generate three response surfaces. The temperature did not have much effect on the retention β-carotene (*Figure 1c*) and the optimum range for the encapsulant's concentration was near 10-20%. The retention of vitamin C increased with higher concentration of gum arabic and the temperature did not have a significant effect at the range studied (*Figure 1d*). The encapulant's concentration influenced only the response powder recovery at the highest temperature (*Figure 1e*). Evaluating the three response surfaces, the conditions at the central point were chosen to obtain good retention of β-carotene (76.1%) and vitamin C (114.1%) with powder recovery

of 16.8%.

Leach et al. (1998) studied, by experimental design, the influence of temperature on the retention of β-carotene in the microencapsulation of *Dunaliella salina* with maltodextrin:gum arabic (3.5:1). The retention varied from 57 to 91%; of all the conditions studied, the best retention was obtained at the lowest temperatures.

Vitamin C recovery after spray drying was 99.7% and 97.6% for the starch coverings containing 1 and 2% of gelatin as binding agent, respectively, both containing 10% of ascorbic acid, and 98.8% for the gum arabic, containing 30% of ascorbic acid (Trindade and Grosso, 2000).

Characteristics of the microcapsules

Morphology

Figure 2 shows the SEM microphotographs of acerola powders produced with 30% of maltodextrin and modified starch at inlet temperature of 157ºC and 20% of gum arabic at 175ºC. The microcapsules had different shapes, depending on the encapsulant. Those of maltodextrin were spherical with smooth surface and those of modified starch and gum arabic were irregular with indented surface. These properties are characteristic of the encapsulant materials (Rosenberg et al., 1985; Figueiredo, 1998; Bertolini, 2001). The indented surface of microcapsules made with gum arabic could be attributed to the spray-drying process, which caused shrinkage of particles during the drying and cooling stages (Pedrosa-Islas et al., 1999).

According to Sheu and Rosenberg (1998) spray-dryed microcapsules with wall material consisting of polysaccharides exhibit notable surface indentations and the formation of indentations has been attributed to effects of wall composition, atomization and drying parameters, uneven shrinkages at early stages of drying, and to the effects of a

surface tension-driven viscous flow. The thermal expansion of air or water vapour inside the drying particles (“ballooning”, associated with high drying rates) can smooth out dents (to a varying extent). The effectiveness of dent smoothing is dependent on the drying rate and viscoelastic properties of the wall matrix.

The microcapsules were also submitted to cutting with a blade to evaluate the wall and the interior. The figures (original magnification 7000x) showed that the microcapsules did not present cracks or porosity. It was observed that the microcapsules had continuous wall, rounded, without cracks or breakage, which was fundamental to guarantee the low permeability to gases, greater protection and retention of the core ingredient. The images also confirmed that microcapsules produced by spray-drying could be void (Rosenberg et al. 1985), the encapsulated material being retained on the wall and not at the center (Trindade and Grosso, 2005). Rodriguez-Huezo (2004) also reported center voids in microcapsules when mixtures of gellan gum, mesquie gum, gum arabic and/or maltodextrin 10 DE were used to encapsulate carotenoids. Emulsions with high solid contents (35%) produced microcapsules with large central voids and particle sizes, whereas emulsions with low solid contents rendered microcapsules with smaller central voids and particle size. Central void size seems to depend on the viscosity exhibited by the multiple emulsions. Formation of the central void is related to the expansion of the particles during the latter stages of the drying process (Verhey, 1973).

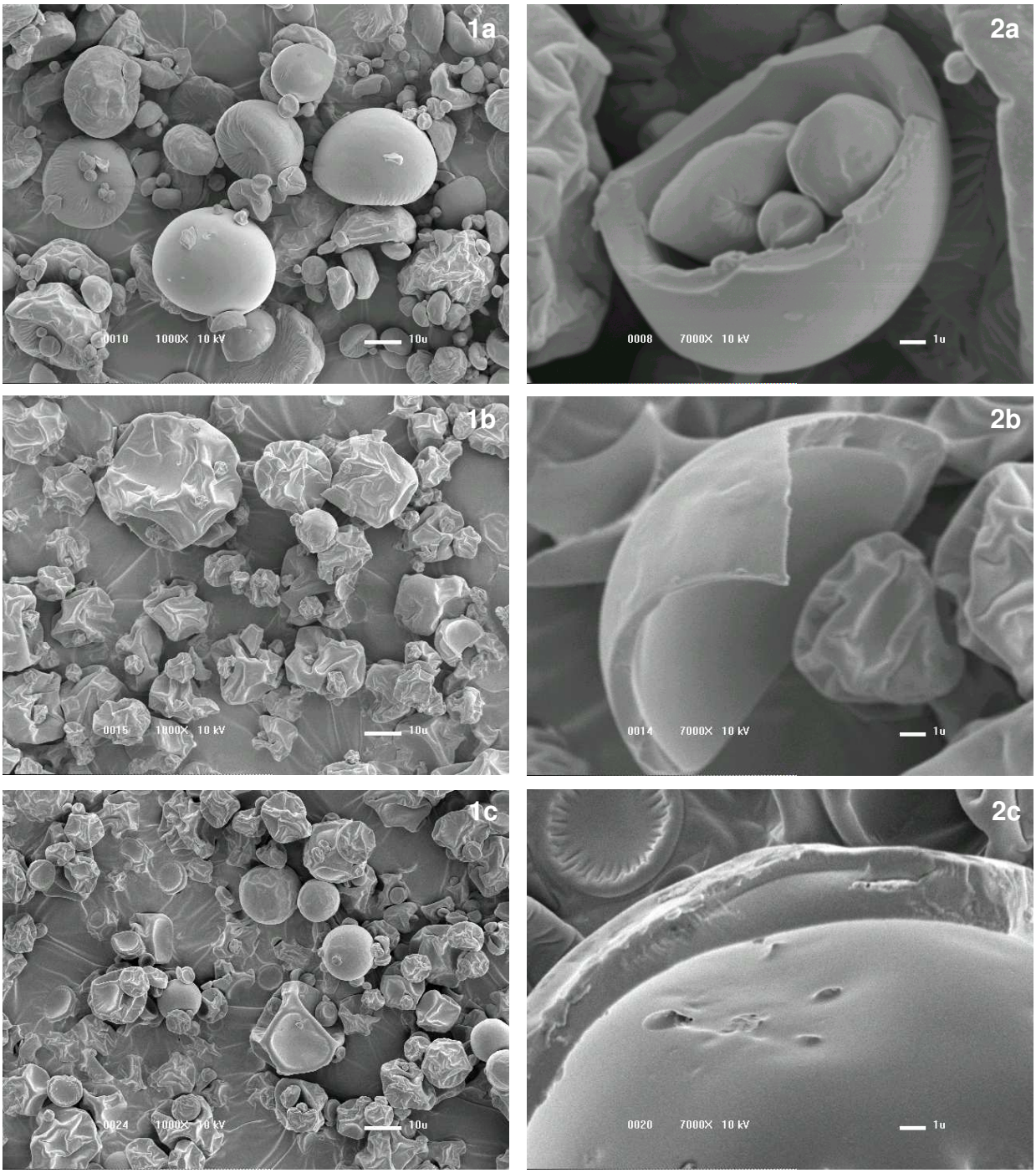

Figure 2. Micrographs of microcapsules of acerola pulp: (1) microcapsules of different sizes (x 1000) and (2) cut microcapsules (x 7000) encapsulated with (a) maltodextrina, (b) modified starch and (c) gum Arabic.

Particle size distribution

According to the graphs in Figure 3, the particles had bimodal distribution, i.e., two distinct peaks, each representing a predominant size. This same distribution was reported by Tonon et al. (2008) for the microencapsulation of açaí pulp with maltodextrina. The presence of large particles can be attributed to an agglomeration process, in which the formation of irreversible bonds led to the formation of bigger particles.

The means of five readings in duplicate of the *D[4,3]* values (mean diameter over the volume distribution) for the microcapsules produced under the optimum conditions were 18.1 ± 0.3, 22.7 ± 0.2 and 17.2 ± 0.7 for maltodextrin, modified starch and gum Arabic, respectively.

According to Jones (1988), the droplet size of an atomized emulsion and, hence, powder particle size are influenced by the nozzle port size, position of the spray nozzle, liquid delivery rate, atomizing air pressure, and solution concentration (viscosity).

This analysis confirms that there was wide variation in the particle size as observed by the analysis by SEM. These sizes are within the range of sizes for particles produced by spray-drying, which vary from 5 to 150 μm (Chang et al., 1988; Southwest Research Institute, 1991; Onwulata et al., 1994).

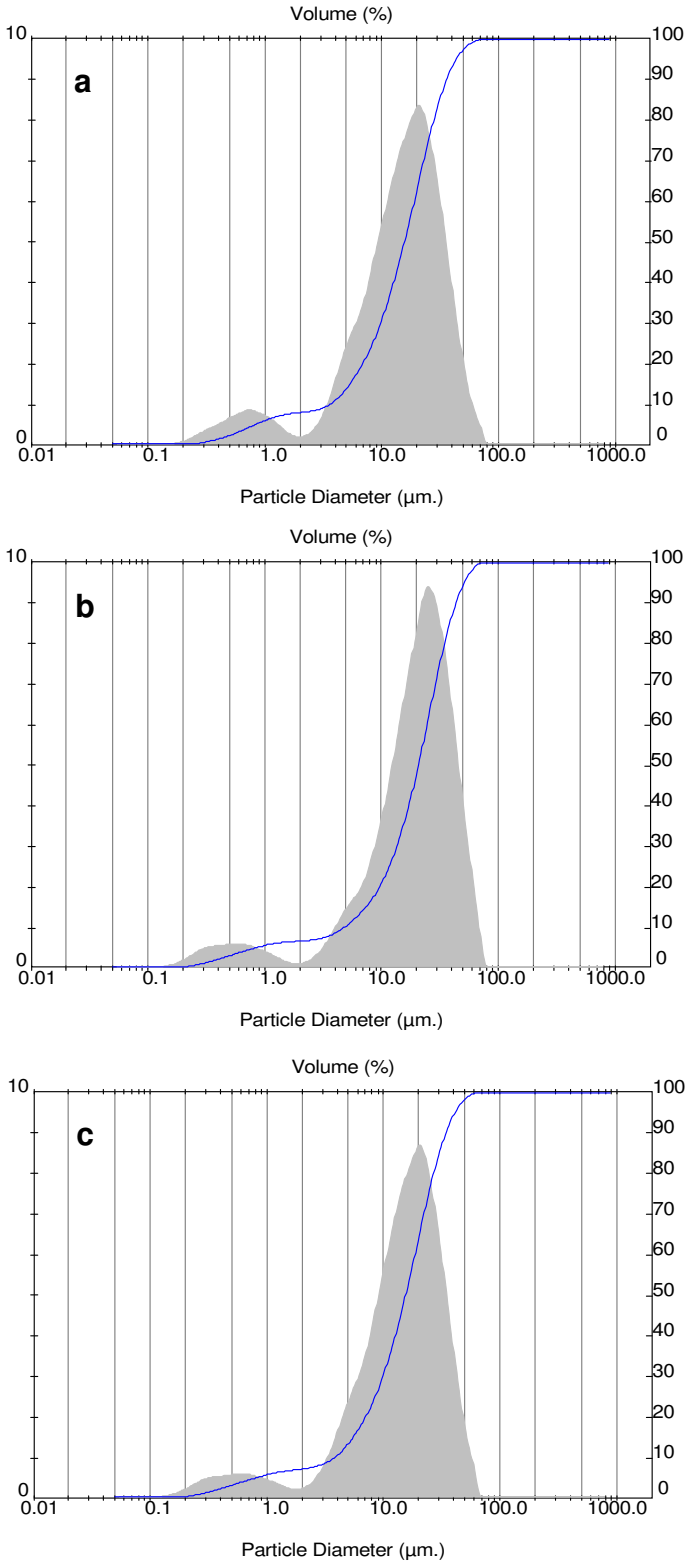

Figure 3. Particle size distribution of powders produced with maltodextrin (a), modified starch (b) and gum Arabic (c).

Solubility

The powders obtained with the three wall materials in the microencapsulation process dissolved completely in less than 2 min, the modified starch microcapsules taking more time (105 sec) to dissolve than those of maltodextrin (68 sec) and gum Arabic (70 sec). These results can be considered good; according to Meyers (1995), microcapsules have good performance when they release 60-70% of the core ingredient in less than 15 min with stirring.

The time needed for solubilization in water of microcapsules of bixin with mixtures of gum Arabic, maltodextrin and sucrose varied from 64 to 183 sec (Barbosa et al., 2005). For passionfruit juice microencapsulated by co-crystallization with sucrose, Astolfi-Filho et al. (2005) took 36.7 and 60.0 sec for solubilization.

Microencapsulation efficiency

In the present study, the efficiency of microencapsulation (ME) in relation to β-carotene under the optimized conditions was 86.8, 85.5 and 89.4% for maltodextrin, modified starch (Capsul®) and gum Arabic, respectively.

In the microencapsulation of emulsions of carotenoids by spray-drying with various mixtures of biopolymers (gellan gum, mesquite gum, gum arabic and maltodextrin 10 DE), Rodríguez-Huenzo et al. (2004) verified ME of 25.6 to 87.5%, the highest ME being obtained for a mixture with greater concentration of total solids (35%). According to Wagner and Warthesen (1995) and McNamee et al. (2001), increasing the proportion of the wall material and the diameter of the nozzle reduced degradation of carotenoids and the amount of surface carotenoids. McNamee et al. (1998) also reported that ME decreased from 100 to 48% when the ratio of soybean oil:gum Arabic was changed from 1:4 to 5:1.

Wagner and Warthesen (1995) obtained 11, 23, 29 e 41% of β-carotene in the surface of microcapsules of carrot with maltodextrin of 4, 15, 25 e 36.5 DE, respectively. The ME did not appear to influence stability since the microcapsule with maltodextrin15 DE had greater retention of carotenoids during storage and higher percentage of carortenoids on the surface than the microcapsules with maltodextrin 4 DE.

The addition of emulsifying agent (Tween 80) in the microencapsulation of bixin increased ME, Barbosa et al. (2005) obtaining 75% and 86% for maltodextrin and gum Arabic, respectively. Nunes and Mercadante (2007) reported MEs greater then those of other studies, between 94 and 96% for the microencapsulation of lycopene by spray-drying utilizing β-cyclodextrin as encapsulating agent.

Temperature and relative humidity

The temperature and relative humidity were monitored daily and the graph in Figure 4 presents the mean data obtained monthly during the storage period. It can be noted that there was an increase in both parameters because the experiment was carried out in spring and summer.

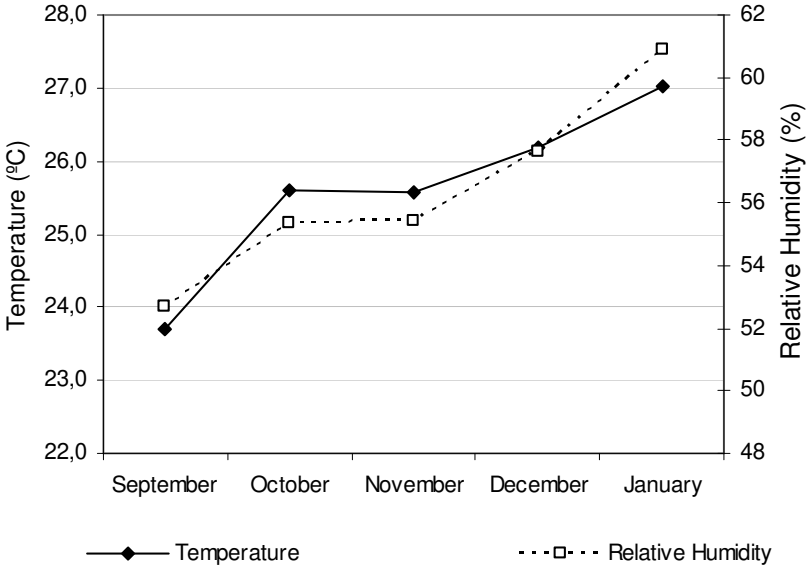

Figure 4. Temperature and relative humidity during 4 months of storage.

Stability of carotenoids

Table 6 shows the carotenoid concentrations and the ratio of *cis* and *trans*-isomers obtained at 0, 15, 30, 60, 90 e 120 days of storage The acerola pulp contained before the spray-drying process 20.8 µg/g of *trans*-β-carotene and 1.6 µg/g of *cis*-β-carotene, i.e., 93% of *trans*- and 7% of *cis*-isomer. The control sample (lyophilized) did not suffer isomerization. The powders obtained by spray-drying had 9.4 to 11.4% of *cis*-β-carotene immediately after the drying process. The proportion of *cis*-isomer increased during storage, reaching 15.7% *cis*-β-carotene.

Table 6. *Trans*- and *cis*-β-carotene (µg/g)* levels and proportion (%) of the total in parenthesis during storage.

Days of storage	Maltodextrin		Modified starch		Gum Arabic		Control	
	trans	***cis***	***trans***	***cis***	***trans***	***cis***	***trans***	***cis***
0	61.0 ^{a}	7.6 ^{a}	55.5 ^{a}	5.8 ^{a}	88.7 ^{a}	11.4 ^{a}	583.7 ^{a}	34.5 ^{a}
	(88.9)	(11.1)	(90.6)	(9.4)	(88.6)	(11.4)	(94.4)	(5.6)
15	48.1 ^{b}	6.2 $^{b, c}$	45.6 ^{b}	4.7 ^{b}	76.1 ^{b}	10.4 $^{a, b}$	419.9 ^{b}	31.2 $^{a, b}$
	(88.5)	(11.5)	(90.7)	(9.3)	(88.0)	(12.0)	(93.1)	(6.9)
30	42.0 ^{c}	6.3 ^{b}	42.0 ^{b}	5.7 $^{a, c}$	63.9 ^{c}	10.2 $^{a, b}$	303.7 ^{c}	27.8 $^{b, c}$
	(86.9)	(13.1)	(88.1)	(11.9)	(86.2)	(13.8)	(91.6)	(8.4)
60	36.7 ^{d}	6.7 ^{b}	30.3 ^{c}	4.8 ^{b}	58.1 ^{d}	9.6 $^{b, c}$	194.0 ^{d}	23.5 $^{c, d}$
	(84.6)	(15.4)	(86.3)	(13.7)	(85.8)	(14.2)	(89.2)	(10.8)
90	34.2 $^{d, e}$	6.4 $^{b, c}$	27.8 $^{c, d}$	5.0 $^{b, c}$	53.6 ^{e}	8.0 ^{c}	162.9 ^{e}	20.2 ^{d}
	(84.3)	(15.7)	(84.7)	(15.3)	(87.0)	(13.0)	(88.9)	(11.1)
120	33.2 ^{e}	5.4 ^{c}	24.7 ^{d}	4.0 ^{b}	56.9 $^{d, e}$	7.8 ^{c}	143.1 ^{e}	17.7^{d}
	(86.0)	(14.0)	(86.0)	(14.0)	(88.0)	(12.0)	(89.0)	(11.0)

Values in the same column with different superscript letters are significantly different ($p < 0.05$)
* Means of triplicate analyses

Robert et al. (2003), Leach et al. (1998) and Wagner and Warthesen (1995) did not verify the formation of *cis*-isomers. The latter authors believed that in a dry system the degradation of α-carotene and β-carotene occurs by direct oxidation, without isomerization *trans-cis*. Isomerization was physically inhibited in dry conditions, considering that a certain amount of solubility is required for molecular mobility. However, Tang and Chen (2000) reported that the all-*trans* forms decrease with an increase in storage temperature or luminosity in freeze-dried carotenoid powder of carrot pulp waste, the formation of 13-*cis*- being favored in the dark and the 9-*cis*- under light.

According to Figure 5, degradation as shown by the total β-carotene content occurred rapidly at the beginning of storage. Matioli and Rodriguez-Amaya (2002) reported that lycopene encapsulated with maltodextrin and gum Arabic had two periods of degradation, both showing first order kinetics, kinetics, faster in the beginning and slower

after 15 days of storage.

The microcapsules with all three encapsulating materials retained more β-carotene than the control which had 26.4% retention in four months, demonstrating the protective effect of microencapsulation. Powders of *Dunaliella salina* that were not microencapsulated had 90% degradation in 7 days under natural light and oxygen while those microencapsulated with a mixture of maltodextrin and gum Arabic were much more stable, showing a first order kinetic model with degradations constants of 0.06 day^{-1} for 200ºC inlet temperature and 0.10 day^{-1} for 265 ºC (Leach et al., 1998).

At the end of the storage period in the present study, gum Arabic showed better performance with 65.4% retention of β-carotene, followed by maltodextrin (57.3% retention) and the modified starch (47.2% retention). For microencapsulated bixin, stability with gum Arabic was also greater than with maltodextrin. Bixin was 10 times more stable in the microcapsules than in the non-encapsulated system in the dark (Barbosa et al., 2005). Comparing four wall materials (maltodextrina 15 DE gum Arabic, gelatin, and sodium caseinate), Beatus and Raziel (1985) found maltodextrin to be the most efficient in protecting the carotenoids of oleoresin of paprika.

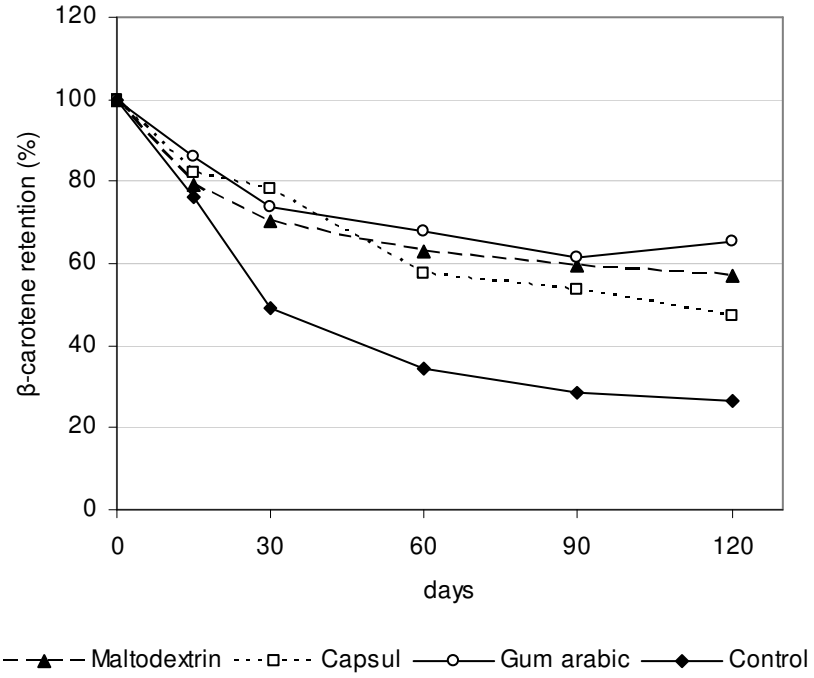

Figure 5. Retention of β-carotene (total) during storage of lyophilized (control) and microencapsulated acerola pulp with maltodextrin, modified starch amido (Capsul®) and gum Arabic.

The beneficial effect of encapsulation as a means of preventing degradation of spray-dried carotenes during storage is believed to be due to the physical barrier against oxidation, the primary mode of degradation of the carotenes in encapsulated carrot powder appeared to be the autoxidation (Wagner and Wathesen. 1995).

Stability of vitamin C

Figure 6 shows that vitamin C was much more stable than β-carotene. There was greater degradation between 15 and 30 days of storage of the uncapsulated lyophilized

acerola pulp (control), which had 79.2% β-carotene retention after 120 days. In a study evaluating the degradation of uncapsulated ascorbic acid at ambient temperature, there was 10% degradation in 30 days, 15% in 45 days and 20% in 60 days (Margolis et al., 2001).

In the present study, the microencapsulated acerola pulp had retention of vitamina C superior to that of the control, having 96.7, 94.0, and 85.9% retention in the microcapsules of gum Arabic, maltodextrin, and modified starch, respectively, indicating better protective effect of microencapsulation on vitamin C.

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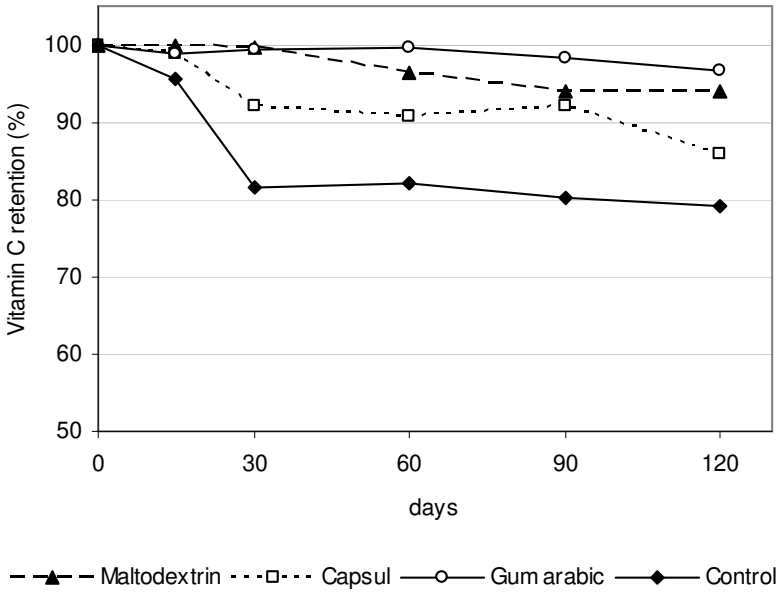

Figure 6. Retention of vitamin C during storage of lyophilized (control) and microencapsulated acerola pulp with maltodextrin, modified starch (Capsul®) and gum Arabic.

During storage of powdered acerola, obtained by drying 90% acerola pulp and 10% maltodextrin in a dryer of the spouted bed type, packed in polyethylene bags stored under

ambient conditions, there was greater reduction than those in our work, 29.7% degradation of ascorbic acid in 60 days (Gomes et al., 2004). On the other hand, Finotelli and Rocha-Leão (2005) observed 7% degradation in microcapsules of maltodextrin:Capsul® (1:1) containing 20% of this vitamin, in 60 days of storage in plastic bags with barrier to gas and light, at a temperature of 28ºC. When only maltodextrin or Capsul® was utilized, loss of vitamin C was 12%, indicating possible synergistic effect between maltodextrin and Capsul® due to some structural interactions.

Utilizing rice starch (with 1 and 2% gelatin as binding agent) and gum Arabic containing 10 and 30% ascorbic acid, respectively, Trindade and Grosso (2000) also verified greater degradation of vitamin C in the starch microcapsules. There was good stability of vitamin C in the gum Arabic microcapsules stored at ambient temperature for 90 days, but reductions of 9 to 19% occurred in the starch microcapsules. After 37 days of storage at 45ºC, 16 and 63% losses of vitamin C were observed in the gum Arabic and rice starch microcapsules, respectively.

Vitamin C in pasteurized frozen acerola, stored at -12 and -18ºC had approximately 3% degradation after 4 months (Yamashita et al., 2003). On the other hand, unpasteurized frozen acerola had 43% and 19% degradation at -12 and -18ºC, respectively, during the same period. The pasteurized bottled juice stored at ambient temperature had 32% loss of vitamin C. Thus, the acerola pulp microencapsulated with gum Arabic, stored at ambient condition in the present work, had vitamin C retention similar to that of frozen acerola pulp.

CONCLUSIONS

Central Composite Design and Response Surface Analysis were effective in determining the optimum conditions for the microencapsulation of acerola pulp. Maltodextrin appeared to protect β-carotene better during the drying process while gum

Arabic was more efficient for vitamin C. Greater losses of β-carotene occurred when modified starch was used as wall material.

The higher the drying temperature, the greater the loss of β-carotene. For vitamin C, the temperature had no significant effect at the range studied (150 to 200ºC). There was a positive effect on powder recovery when gum Arabic was used as microcapsulant.

The percentage of encapsulating agent influenced the retention of β-carotene with all three encapsulating agents; retention of vitamina C and powder recovery were influenced by gum Arabic.

Microencapsulation by spray-drying of acerola pulp can be an alternative for the food industry in preserving acerola and its bioactive compounds such as carotenoids and vitamin C. The wall materials utilized resulted in powders of facile solubility in water, which is extremely convenient for the food industry and consumers in preparing juices.

Gum Arabic appeared to be the material with the best retention for carotenoids and vitamin C. However, maltodextrin had similar performance and can be a practical and inexpensive option. Modified starch showed the worst protection for the bioactive compounds evaluated. However, more research are needed to investigate possible synergistic effects among the wall materials.

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gum Arabic and Capsul®, respectively.

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Capítulo 9

Esquema para o estudo de compostos voláteis provenientes da oxidação de carotenóides

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ESQUEMA PARA O ESTUDO DE COMPOSTOS VOLÁTEIS PROVENIENTES DA OXIDAÇÃO DE CAROTENÓIDES

RESUMO

Os carotenóides são pigmentos naturais altamente insaturados que são passíveis da degradação durante o processamento e estocagem dos alimentos. Apesar da reconhecida conseqüência negativa da degradação de carotenóides, os mecanismos envolvidos ainda não estão elucidados. O objetivo deste trabalho foi desenvolver um método para estudar a degradação oxidativa dos carotenóides em sistema-modelo, pela captura dos compostos voláteis formados, utilizando a técnica de microextração em fase sólida (SPME). O experimento foi conduzido com licopeno e a identificação dos voláteis foi realizada por cromatografia gasosa com espectrometria de massas (GC/MS) e por comparação dos índices de Kovats. Três tipos de revestimento de fibras SPME com polaridades diferentes foram estudadas. A fibra mista de DVB/CAR/PDMS obteve o maior número de picos no cromatograma e com maior intensidade. Os sete principais compostos corresponderam à aproximadamente 78,6% da área total dos picos dos voláteis obtidos nas amostras. Três compostos identificados já foram reportados na literatura como produtos da degradação do licopeno responsáveis pelo aroma de alguns alimentos: o 2-hepten-6-ona, 2-metil, o citral ou geranial (*trans*-2,6-Octadienal, 3,7-dimetil) e o neral (*cis*-2,6-Octadienal, 3,7-dimetil).

Palavras-chaves: degradação, voláteis, licopeno, SPME, GC/MS

INTRODUÇÃO

Os carotenóides são pigmentos naturais e estão entre os componentes de maior interesse em relação aos efeitos benéficos dos alimentos à saúde humana. Entretanto, devido a sua alta insaturação, são compostos passíveis de degradação durante o processamento e estocagem dos alimentos. Tanto a isomerização como a oxidação ocorrem, mas esta última é a principal causa da perda de carotenóides.

Apesar da reconhecida conseqüência negativa da degradação de carotenóides, os mecanismos envolvidos não estão elucidados, ao contrário da oxidação de lipídeos, para a qual as diferentes reações e os produtos iniciais, intermediários e finais são conhecidos. De acordo com Rodriguez e Rodriguez-Amaya (2007), o conhecimento de reações e mecanismos subjacentes da degradação oxidativa dos carotenóides é necessário não somente por evitar a perda destes compostos benéficos durante o processamento e estocagem de alimentos, mas também para avaliar as implicações na saúde humana. Estudos realizados por King et al. (1997) e Aust et al. (2003) indicam que os produtos da degradação do licopeno aumentam a comunicação célula-célula via *gap junctions*, que parece ser um dos mecanismos de proteção relacionados às atividades preventivas dos carotenóides contra o câncer. Por outro lado, produtos da clivagem do β-caroteno mostraram ser altamente reativos (Young e Lowe, 2001; Siems et al., 2002), podendo ser potencialmente tóxicos (Sommerburg et al., 2003).

Os produtos voláteis formados a partir da degradação dos carotenóides podem ser desejáveis e foram reportados por fazerem parte do aroma característico de chá preto (Ravichandran, 2002), vinho (Mendes-Pinto, 2009), tabaco (Davis et al., 1976; Enzell, 1985) e de frutas como o maracujá (Engel e Tressl, 1983; Parliment, 1972) , goiaba (Idstein e Schreier, 1985), uvas (Schreier et al., 1979; Strauss et al., 1986, 1987), tomate (Butery et al., 1988; Stevens, 1970) e marmelo (Lutz e Winterhalter, 1992). Em alguns

alimentos processados, a degradação e a quebra dos carotenóides são indesejáveis, pois além da perda da cor e do valor nutricional, são responsáveis pelo aparecimento de odor estranho, como o que acontece com cenouras desidratadas (Falconer et al.; 1964) e em alguns tipos específicos de vinhos (Rapp e Marais, 1993).

Os mecanismos de formação de compostos voláteis não são totalmente compreendidos. Segundo Enzell (1985), os apocarotenóides são importantes precursores dos compostos voláteis. A formação de apocarotenóides bem como os epoxicarotenóides como produtos iniciais da oxidação de β-caroteno e licopeno foi extensamente estudada por Rodriguez e Rodriguez-Amaya (2007, 2009).

A ciclização intramolecular foi proposta como o principal mecanismo de reação na formação dos compostos voláteis detectados no estudo da degradação térmica de carotenóides em oleoresinas de páprica, tomate e calêndula (Rios et al., 2008). Este processo foi ativado pelo impacto térmico, seguido pela reação de eliminação na cadeia ou pela reação de fragmentação heterolítica. A presença de outros compostos, como vários metilbenzaldeídos ou isoforonas (1,1,3-trimetil-3-ciclohexeno-5-ona) também indicou a ocorrência de reações de oxidação de carotenóides que afetaram tanto a cadeia poliênica central quanto os grupos finais.

Os compostos voláteis formados a partir da degradação do β-caroteno foram os mais estudados devido a sua importância e maior facilidade na obtenção do pigmento puro. Já a identificação dos possíveis compostos voláteis formados pela degradação do licopeno foi descrita apenas em estudos com diferentes variedades e produtos derivados de tomate (Buttery et al., 1987; 1988; 1990; Ishida et al., 1998; Tandon et al., 2001; Simkin et al., 2004; Gao et al., 2008; Rios et al., 2008; Davidovich-Rikanati et al., 2009) e melancia (Kemp et al., 1974; Kemp, 1975; Yajima et al., 1985; Pino et al.; 2003; Lewinsohn et al., 2005). As dificuldades encontradas na análise em alimentos, em que a

composição dos carotenóides é complexa e há interações com os demais constituintes, tornam difícil o estudo dos voláteis da degradação de carotenóides. O objetivo deste trabalho foi estabelecer uma estratégia para estudar a degradação de carotenóides em sistema-modelo simulando alimentos desidratados, capturar os compostos voláteis formados pela técnica de microextração em fase sólida (SPME) e identificar por cromatografia gasosa – espectrometria de massas (GC/MS).

MATERIAIS E MÉTODOS

Material

O sistema-modelo foi preparado utilizando-se licopeno extraído de melancia, isolado por cromatografia em coluna aberta (CCA) ou licopeno comercial (Sigma Chemical Company, St. Louis, MO, EUA) incorporados em celulose microcristalina (CMC) Sigmacell 50 (Sigma Chemical Company, St. Louis, MO, EUA). Foram estudados três tipos de revestimento de fibras de SPME: polidimetilsiloxano (PDMS), poliacrilato (PA) e a fase mista de divinilbenzeno, carboxen e polidimetilsiloxano (DVB/CAR/PDMS), todas da marca Supelco (Sigma-Aldrich Co., Bellefonte, PA, EUA).

Obtenção do licopeno

Os carotenóides de melancia (aproximadamente 300 g) foram extraídos com almofariz e pistilo, misturando celite (hyflosupercel) à amostra e utilizando acetona como solvente de extração, baseada na metodologia de Rodriguez-Amaya (1999) e Kimura e Rodriguez-Amaya (2002). A mistura foi filtrada em funil de Büchner e o resíduo foi levado novamente ao almofariz. A extração e a filtração foram repetidas até que o resíduo se tornasse incolor. Fez-se a partição para éter de petróleo em um funil de separação e o extrato etéreo foi então concentrado em evaporador rotatório (T≤ 35°C).

O licopeno foi isolado por CCA de vidro de 30 cm x 2,5 cm d.i., empacotada com mistura de MgO:hyflosupercel (1:1), previamente ativada por 4 horas à 110° C. Esta coluna foi eluída com o aumento da proporção de éter etílico e acetona em éter de petróleo. A solução de licopeno foi quantificada espectrofotometricamente na região visível e as concentrações foram corrigidas de acordo com a pureza da solução determinada por cromatografia líquida de alta eficiência (CLAE). A pureza dos padrões de licopeno obtidos variou de 94 a 96%.

Preparação do sistema-modelo

O sistema-modelo foi preparado combinando-se licopeno em cloreto de metileno e CMC, de acordo com o procedimento utilizado por Padula (1999). A CMC foi previamente seca em estufa a vácuo a temperatura de 75ºC por 24 horas. A mistura de CMC e licopeno foi homogeneizada em almofariz e pistilo. O cloreto de metileno e o oxigênio adsorvido neste processo foram removidos mantendo o sistema-modelo preparado sob vácuo por 2 horas. Todas as etapas foram conduzidas protegidas da luz. O sistema-modelo continha, ao final, aproximadamente 1 mg de licopeno por grama de sólido seco.

Porções de aproximadamente 1 g do sistema-modelo (CMC + licopeno) foram adicionadas em frascos de 20 mL e imediatamente tampadas com septos de silicone, com parte interna de PTFE. A atmosfera do frasco foi modificada com a injeção de fluxo de oxigênio por 5 minutos. Os frascos foram armazenados em estufa a temperatura de 32 ± 2ºC protegidos da luz na posição horizontal, para aumentar a área de superfície em contato com oxigênio.

Extração dos compostos voláteis

A extração dos compostos voláteis provenientes da degradação do licopeno foi

realizada utilizando a técnica de SPME no espaço confinado (*headspace*) entre a amostra e o frasco vedado. Foram estudados três tipos de revestimento de fibras SPME, com polaridades diferentes, para verificar qual obteria o maior número de picos e/ou maior intensidade dos picos no cromatograma. Antes da adsorção dos compostos, as fibras SPME foram pré-condicionadas de acordo com as instruções do fabricante: 250ºC por 30 minutos para PDMS, 300ºC por 2 horas para a de PA e 270ºC por 1 hora para a de DVB/CAR/PDMS.

Para a escolha do melhor tipo de revestimento da fibra SPME, os frascos contendo o sistema foram estocados em estufa por 4 e 7 dias. Cada fibra foi introduzida no frasco através do septo e exposta no *headspace* a 32 ± 2ºC por 20 minutos para a extração dos compostos voláteis formados. Após o término do tempo de exposição das fibras, estas foram removidas do frasco e inseridas no injetor do cromatógrafo gasoso à 200ºC por 10 minutos para a dessorção térmica dos analitos.

A Figura 1 esquematiza o método proposto para a obtenção dos compostos voláteis formados a partir da oxidação dos carotenóides.

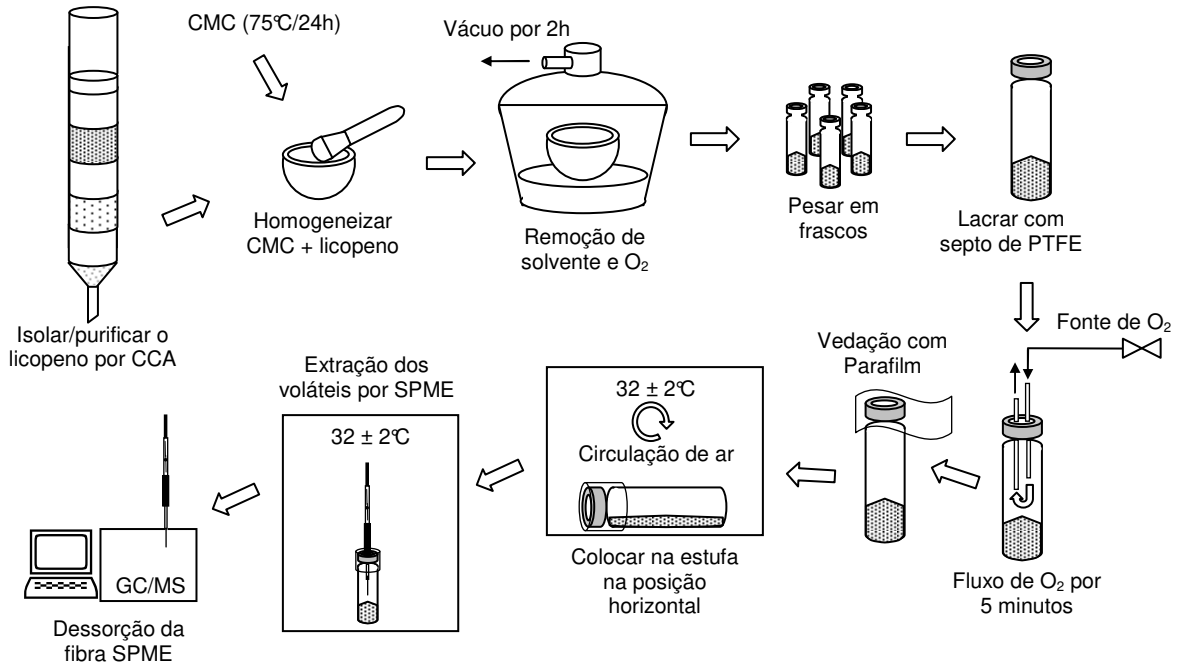

Figura 1. Esquema proposto para a obtenção dos compostos voláteis provenientes da oxidação de carotenóides.

Condições cromatográficas do CLAE

A análise por CLAE para verificar a pureza do licopeno foi conduzida em um módulo de separação Waters, modelo 2690 (Waters Corp., Milford, MA, USA) equipado com bomba quaternária, injetor automático, degasser a vácuo na linha e detector de arranjo de diodos, modelo 996, controlados por software Millenium (versão 3.20). Foi utilizada uma coluna monomérica C_{18}, Spherisorb ODS2, 3 µm, 4.6 x 150 mm. A fase móvel isocrática foi composta por acetonitrila (contendo 0,05% de trietilamina), metanol e acetato de etila (60:20:20) num fluxo de 0,5 mL/min.

Condições cromatográficas do GC/MS

Os compostos voláteis foram analisados em um cromatógrafo gasoso Hewlett

Packard, modelo HP 6890 acoplado a um espectrômetro de massas Hewlett Packard, modelo HP 5973 (Agilent Technologies Inc., Palo Alto, CA, USA). A dessorção térmica da fibra foi realizada no injetor do cromatógrafo a 200ºC, utilizando o modo *splitless,* com a abertura da válvula de divisão em 4 minutos na razão de 1:25 e insersor específico para SPME de 0,8 mm de diâmetro interno. Foi utilizada uma coluna capilar de sílica fundida HP-5 de 30 m x 0,32 mm d.i.x 0,25 µm de espessura de fase estacionária (Agilent Technologies Inc., Palo Alto, CA, USA). Gás hélio foi utilizado como gás de arraste, a uma vazão constante de 1,0 mL/min. O espectrômetro de massas foi operado utilizando uma fonte de ionização por feixe de elétrons a 70 eV e analisador tipo quadrupolo simples com varredura entre 35 a 350 m/z. A interface GC/MS e a fonte de íons foram mantidas a 230ºC. A programação de temperatura do forno do cromatógrafo gasoso foi iniciada a 50ºC, permanecendo nesta temperatura por 10 minutos, em seguida aquecendo com uma taxa de 3ºC/min até atingir 180ºC, a qual foi mantida por 10 minutos.

Identificação dos compostos voláteis da degradação

Os compostos voláteis foram, primeiramente, tentativamente identificados pela comparação dos espectros de massas obtidos com aqueles fornecidos pela biblioteca do *National Institute of Standards and Technology* (NIST 98). Posteriormente, foram comparados os índices de retenção relativa (Índice de Kovats) obtidos experimentalmente com valores encontrados na literatura. Para cálculo do IK, uma mistura de padrões de alcanos (C9 a C17) foi injetada no GC/MS nas mesmas condições cromatográficas acima citadas, aplicando-se a seguinte equação:

$$IK = 100\,Z + \frac{100\,[(\log t'_R\,X) - (\log t'_R\,Z)]}{[(\log t'_R\,Z{+}1) - (\log t'_R\,Z)]}$$

Onde X é o composto de interesse, $t'_R\,X$ é o tempo de retenção ajustado de X, Z é

o número de átomos de carbono do hidrocarboneto com o tempo de retenção imediatamente anterior ao tempo de retenção de *X*, t'_R *Z* é o tempo de retenção ajustado de *Z* e t'_R *Z+1* é tempo de retenção imediatamente posterior ao tempo de retenção de *X* (Jennings e Shibamoto, 1980). Visto que não foram utilizados padrões para a confirmação positiva da identidade dos compostos, estes foram considerados tentativamente identificados.

RESULTADOS E DISCUSSÃO

Otimização das condições de extração dos voláteis

A Figura 2 mostra os cromatogramas dos compostos voláteis extraídos do *headspace* das amostras após 4 dias de estocagem com os três tipos de revestimento de fibra SPME testados. Os cromatogramas obtidos após 7 dias apresentaram a mesma quantidade de picos, porém, com menor intensidade.

Os cromatogramas continham 6 picos principais, independente da fibra utilizada, com diferenças apenas em relação à proporção de intensidade. O maior diferencial foi o número de picos do cromatograma obtido utilizando a fibra mista de DVB/CAR/PDMS, que apresentou 73 picos. Os cromatogramas obtidos pelas fibras de PDMS e PA apresentaram 58 e 32 picos, respectivamente. Como a fibra de DVB/CAR/PDMS foi a que obteve mais picos com maior intensidade, esta foi escolhida para os demais experimentos.

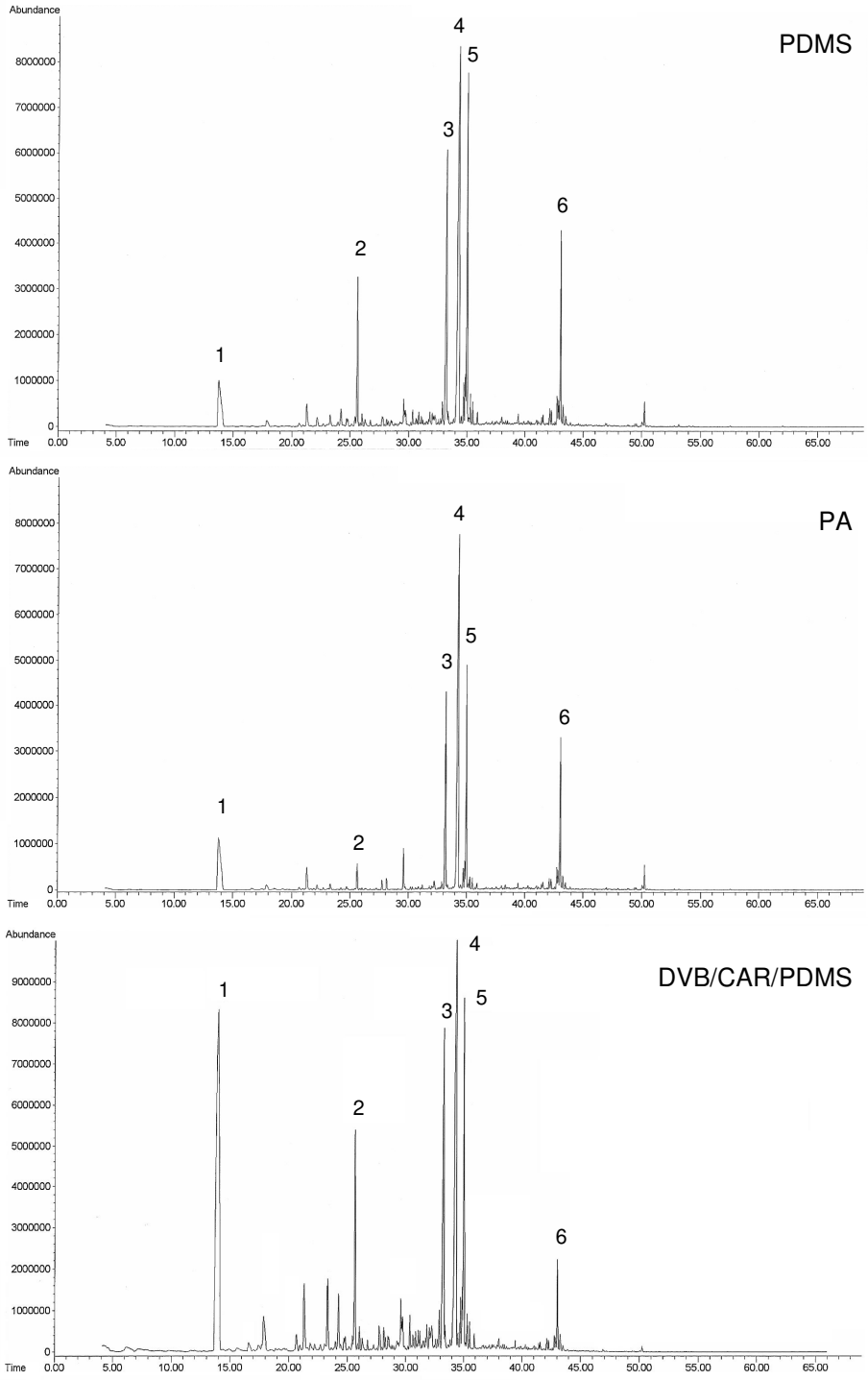

Figura 2. Cromatogramas obtidos dos voláteis da degradação do licopeno em 4 dias com as fibras SPME revestidas por PDMS, PA e DVB/CAR/PDMS. Condições cromatográficas descritas no texto.

O tempo de 10 minutos de extração com dessorção de 20 minutos no compartimento do injetor, foi suficiente para resultar em cromatogramas com picos de boa intensidade e resolução. Tempos maiores de extração, resultaram em alargamento de banda, picos com caudas e/ou sobrepostos. Não houve diferença entre os cromatogramas obtidos com 10 e 20 minutos de dessorção. Portanto, foi escolhido o tempo de 20 minutos para uma melhor dessorção dos compostos da fibra.

Beaulieu e Lea (2006) obtiveram 59 picos estudando os voláteis de 5 variedades de melancia sem semente, utilizando a mesma fibra de DVB/CAR/PDMS. Neste trabalho, foram detectados 73 picos de compostos possivelmente formados a partir da degradação do licopeno, dos quais 26 foram tentativamente identificados.

Identificação dos compostos voláteis

Os cromatogramas dos compostos voláteis da degradação do licopeno após a exposição do sistema-modelo CMC + licopeno extraído da melancia a temperatura de 32 ± 2ºC com presença de oxigênio foram semelhantes para os 1, 2, 4 e 7 dias avaliados, com diferenças apenas em relação à proporção e intensidade dos picos.

A Figura 3 mostra os cromatogramas obtidos para os quatro dias. Nas condições deste estudo, observa-se que a maior quantidade de voláteis gerados pela degradação do licopeno foi obtida já no primeiro dia. Nos demais, a intensidade dos compostos encontrados apenas diminuiu ao longo do tempo e não foi observada a formação de novos. Porém, observa-se que esta diminuição não foi proporcional. O pico 13 é mais intenso apenas no dia 1, houve 77,8% de perda de área após 7 dias. A Tabela 1 apresenta os valores da área dos picos majoritários e/ou dos tentativamente identificados.

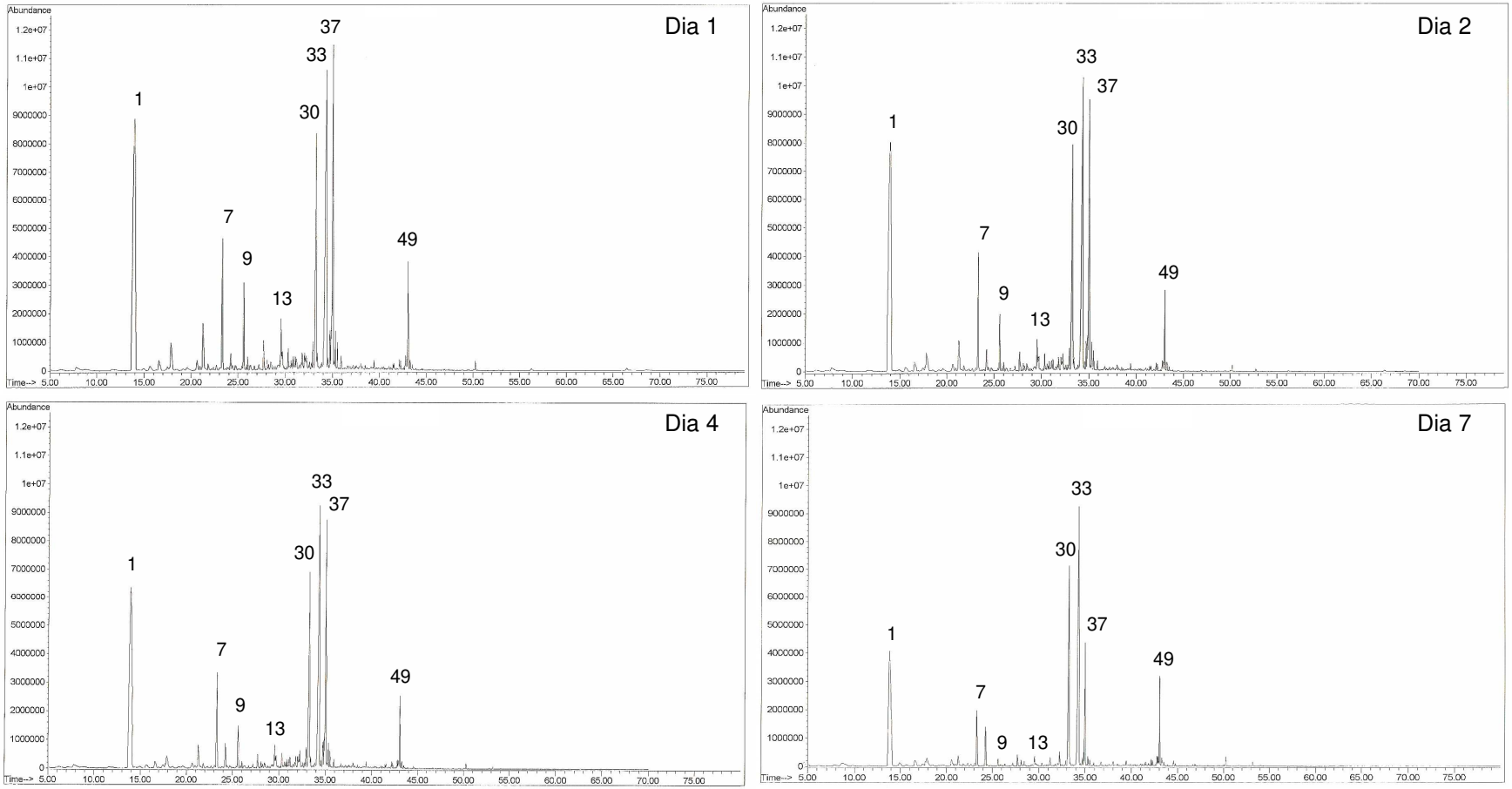

Figura 3. Cromatogramas dos voláteis obtidos do *headspace* com a fibra SPME de DVB/CAR/PDMS após a degradação do licopeno a temperatura de 32 ± 2ºC com presença de oxigênio durante 1, 2, 4 e 7 dias.

Tabela 1. Principais compostos voláteis provenientes da degradação do licopeno a 32 ± 2ºC (identificação tentativa).

Pico	Composto	Q	IK-MS	I	Área (x 10^6)				
					Dia 1	Dia 2	Dia 4	Dia 7	Com
1	2-Hepten-6-ona, 2-metil-	93	1028	a	1699	1531	1200	734	1545
5	3,5,5-Trimetilciclohex-2-en-1-ona	91	1124	a	14	15	12	0	0
7	(NI)	-	1153	-	303	251	192	111	166
9	1-Dodeceno	96	1193	a	157	99	73	11	81
10	Dodecano	95	1200	a	20	14	10	0	10
11	2,6-Octadienal, 3,7-dimetil-, (z)	94	1245	a	15	12	0	0	0
13	2,6-Octadienal, 3,7-dimetil-	97	1274	a	81	52	38	18	48
14	4-Dodeceno	93	1276	b	21	19	16	0	16
17	Ciclopropano, 1-(2-metilbutil)-1-(1-metilpropil)-	91	1295	b	12	9	8	0	9
19	Tridecano	97	1299	a	17	13	12	0	14
27	3-Tetradeceno	94	1340	b	11	9	8	0	12
29	Cicloundecano, 1,1,2-trimetil-	93	1348	b	41	32	29	7	39
30	(NI)	-	1355	-	616	568	478	478	908
31	Ciclotetradecano	91	1358	b	23	19	16	0	0
32	7-Tetradeceno	91	1368	a	12	11	10	0	14
33	(NI)	-	1379	-	1154	1036	864	793	1545
35	7-Tetradeceno (z)	98	1387	b	56	40	36	11	66
36	5-Tetradeceno	96	1390	a	67	51	47	18	70
37	3-Tetradeceno (z)	97	1394	a	715	508	450	173	895
38	Tetradecano	96	1400	a	45	32	29	12	49
40	5-Tetradeceno (z)	98	1415	b	17	13	10	5	20
42	2,5-Ciclohexadieno-1,4-diona,2,6-bis(1,1-dimetiletil)-	99	1466	a	13	10	8	7	15
43	Pentadecano	97	1477	b	0	4	0	0	0
44	Ciclopentano,1-butyl-2-propil	90	1553	b	6	5	4	4	6
45	Ciclooctane, metil	90	1570	b	13	10	8	9	16
47	3-Hexadeceno	99	1586	a	19	13	11	13	24
48	7-Hexadeceno, (z)	98	1589	a	19	13	11	14	25
49	1-Heptadeceno	95	1593	b	161	114	96	125	214
50	1-Octadeceno	99	1789	a	12	8	6	13	14

Q: Qualidade da concordância do espectro de massas com a biblioteca NIST 98
IK-MS: Índice de Kovats experimentais para espectrometria de massas (coluna capilar HP-5)
I (Confiabilidade da identificação): a. Índice de Kovats em concordância com a literatura; b. Índice de Kovats não encontrado na literatura ou encontrado em condições cromatográficas diferentes
Com: Experimento utilizando licopeno comercial após 4 dias de degradação
(NI): Composto não identificado

Para confirmar que os compostos voláteis obtidos eram provenientes apenas da degradação do licopeno e não voláteis do *flavor* da melancia que poderiam ter co-eluido com a fração do licopeno na etapa de purificação, o experimento foi repetido utilizando licopeno comercial padrão com 94% de pureza. Esta amostra obteve um cromatograma similar aos obtidos utilizando o licopeno isolado/purificado da melancia.

Os sete compostos voláteis majoritários corresponderam por aproximadamente 78,6% da área total dos voláteis: 2-hepten-6-ona, 2-metil (27,8%); pico 7 - não identificado (5,0); 1-Dodeceno (2,6%); pico 30 - não identificado (10,1%); pico 33 - não identificado (18,9%); 3-Tetradeceno (11,7%) e; 1-Heptadeceno (2,6%).

Não foi possível identificar compostos intermediários que ajudariam a elucidar a formação destes compostos tentativamente identificados. Embora o método adotado não os tenha encontrado, não significa necessariamente que tais produtos não tenham sido formados. Eles podem ter se transformado tão rapidamente que a quantidade momentânea acumulada foi abaixo do limite de detecção do método utilizado.

Yajima et al. (1985) identificaram sete hidrocarbonetos na composição do *flavor* de melancia: pentadecano, 1-pentadeceno, hexadecano, heptadecano, octadecano, nonadecano e heneicosano. O pentadecano também foi encontrado neste estudo e, apesar de ainda não conseguirmos explicar a rota de formação, isto indica que os hidrocarbonetos de cadeias lineares sem ramificações também podem ser derivados da degradação do licopeno.

Apenas três compostos encontrados já foram reportados na literatura (Stevens, 1970; Kanasawud e Crouzet, 1990; Caris-Veyrat et al., 2003) como produtos da degradação do licopeno, o 2-hepten-6-ona, 2-metil, o citral ou geranial e o neral. Os possíveis caminhos para a formação destes compostos estão esquematizados na Figura 4.

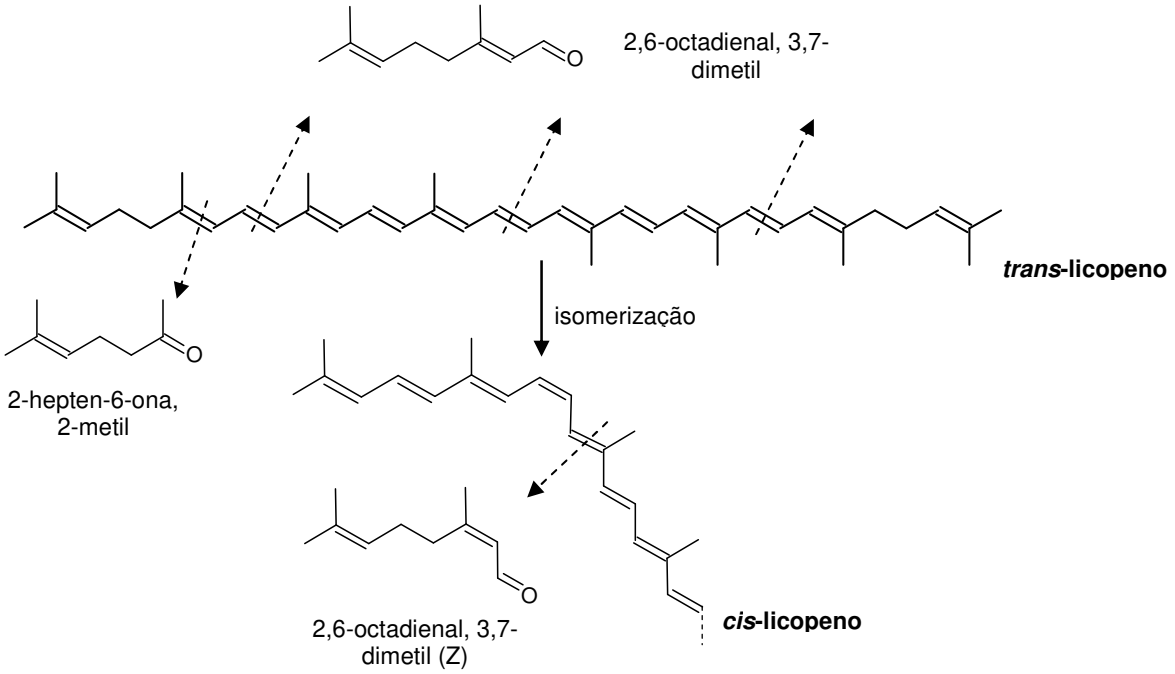

Figura 4. Possível esquema da formação do 2-hepten-6-ona, 2-metil, geranial (*trans*-2,6-octadienal, 3,7-dimetil) e neral (*cis*-2,6-octadienal, 3,7-dimetil), provenientes da degradação do licopeno.

Dados sobre a formação de compostos voláteis do licopeno são escassos, apenas o 2-hepten-6-ona, 2-metil e o geranial também foram encontrados por alguns autores como produtos de degradação do licopeno em tomates (Cole e Kapur, 1957; Schreier et al., 1979; Coulibaly et al., 1979; Kanasawud e Crouzet, 1990; Ishida et al., 1998; Lewinsohn et al., 2005) e melancia (Kemp et al., 1974; Kemp, 1975; Yajima et al., 1985; Pino et al.; 2003; Lewinsohn et al., 2005; Beaulieu e Lea, 2006). De acordo com Kanasawud e Crouzet (1990), rotas térmicas ou enzimáticas estão envolvidas na formação destes compostos.

O geranial (pico 13) foi um dos compostos que apresentou maior redução na intensidade do pico ao longo do tempo, chegando quase a ficar inexistente. Kanasawud e Crouzet (1990) reportaram fenômeno parecido ao estudar degradação do licopeno a temperatura de 30 a 97ºC

na presença de ar ou oxigênio por 3 horas. O isômero neral só foi formado em temperaturas acima de 50ºC, enquanto que os teores de geranial reduziram nesta mesma condição. Na presença de oxigênio, a quantidade de voláteis obtida da degradação do licopeno aumentou significativamente e o 2-hepten-6-ona, 2-metil foi sempre o principal composto formado. Rios et al. (2008) reportaram outros compostos voláteis gerados pela degradação de carotenóides, além do 2-hepten-6-ona, 2-metil e o 2,6-octadienal, 3,7-dimetil, em oleoresinas de tomate após a degradação térmica a 50ºC, 100ºC e 150ºC. Estes encontraram compostos como o tolueno, *m*-xileno, 3,5-heptadien-2-ona, 6-metil e etanona, 1-(metilfenil). Porém, as temperaturas estudadas foram superiores a utilizada neste estudo.

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Conclusões Gerais

1. A literatura nos últimos cinco anos demonstra que os efeitos dos carotenóides na saúde humana continuam sendo a maior preocupação de pesquisadores na área de alimentos.

2. A avaliação intralaboratorial utilizando material de referência certificado mostrou que a experiência do analista na quantificação de carotenóides foi um fator decisivo para obtenção de bons resultados. A avaliação periódica da proficiência dos analistas é necessária para garantir a confiabilidade dos dados gerados pelo laboratório.

3. Entre os produtos processados de tomate analisados (extrato, polpa, catchup, molho pronto e tomate seco), o tomate seco que é um produto pronto para consumo, apresentou os maiores teores de licopeno e luteína.

4. A composição de carotenóides em caruru, mentruz, taioba, serralha e beldroega, segue o perfil que vem sendo constatado em verduras folhosas, com a predominância de luteína, β-caroteno, violaxantina e neoxantina. Com exceção da beldroega, os valores encontrados foram maiores que os da salsa e coentro, previamente demostradas como as folhas comerciais com maiores concentrações de carotenóides

5. De forma geral, neoxantina e violaxantina foram mais estáveis nas três verduras folhosas minimamente processadas, com exceção da couve, que na presença de luz, parece ter ocorrido um estímulo do ciclo da violaxantina, indicado pela formação de zeaxantina.

6. Os teores de luteína e β-caroteno diminuíram em rúcula e couve durante a estocagem, com perdas menores em temperaturas mais baixas. Em espinafre, houve aumento de neoxantina, violaxantina, luteína e β-caroteno durante a estocagem em todas as condições, indicando que em alguns casos, a ação das enzimas biosintéticas pode prevalecer em relação às enzimas oxidativas.

7. As condições ótimas de temperatura e a proporção de agente encapsulante e recheio para obter uma maior retenção de β-caroteno e vitamina C na microencapsulação por *spray-dryer* de polpa de acerola são 30% de maltodextrina ou amido modificado a temperatura de 157ºC e 20% de goma arábica a 175ºC.

8. Uma retenção total de vitamina C durante o processo de microencapsulação por *spray-dryer* pode ser conseguida com os três agentes encapsulantes. Já para o β-caroteno, a retenção é maior para a maltodextrina (~92%), seguida pela goma arábica (~76%) e Capsul® (~71%).

9. Na estocagem de polpa de acerola microencapsulada, a goma Arábica oferece maior proteção, ~65,4% de retenção para o β-caroteno e ~96,7% de retenção para a vitamina C. Na polpa de acerola liofilizada a retenção é menor (~26,4% de retenção para β-caroteno e ~79,2% de retenção para vitamina C).

10. Um esquema para o estudo dos voláteis provenientes da degradação de carotenóides foi proposto, consistindo de um sistema-modelo de CMC, simulando alimentos

desidratados, captura dos voláteis utilizando a técnica de SPME com fibra mista de DVB/CAR/PDMS e identificação por GC/MS e cálculo de índice de Kovats.

11. Sete principais compostos voláteis da degradação do licopeno corresponderam por ~79% da área total dos voláteis. Três compostos identificados já foram reportados na literatura como produtos responsáveis pelo aroma de alguns alimentos: 2-metil-2-hepten-6-ona, o citral ou geranial (*trans*-2,6-Octadienal, 3,7-dimetil) e o neral (*cis*-2,6-Octadienal, 3,7-dimetil).