UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS
DEPARTAMENTO DE CIÊNCIA DE ALIMENTOS

ESTUDO DA COMPOSIÇÃO DE LARANJAS BRASILEIRAS (*Citrus sinensis*) E SEU USO COMO COMPROVAÇÃO DE AUTENTICIDADE DE SUCO DE LARANJA

Antonio Marcos Pupin

Orientadora: *Prof. Dra. Maria Cecília F. Toledo*
Co-orientador: *Dr. M. John Dennis*

PARECER

Este exemplar corresponde à redação final da tese defendida por ANTONIO MARCOS PUPIN aprovada pela Comissão Julgadora em 15 de dezembro de 1997.

Campinas, 15 de dezembro de 1997.

Profa. Dra. MARIA CECÍLIA F. TOLEDO
Presidente da Banca

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BANCA EXAMINADORA

Profa. Dra. Maria Cecília F. Toledo
(orientadora)

Dr. Alfredo de Almeida Vitali
(membro)

Profa. Dra. Helena Teixeira de Godoy
(membro)

Profa. Dra. Hillary Castle de Menezes
(membro)

Profa. Dra. Isa Beatriz Noll
(membro)

Profa. Dra. Carol Hollingworth Collins
(membro)

Profa. Dra. Heloísa M. Cecchi
(membro)

Campinas, de de 1997

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ABREVIAÇÕES

CCD	Cromatografia em camada delgada
CLAE	Cromatografia líquida de alta eficiência
EMRIE	Espectrometria de massas da razão de isótopos estáveis
FCOJ	Frozen concentrated orange juice
FCOPW	Frozen concentrated orange pulp wash
FG	Flavanonas glicosídicas / flavanone glycosides
FINP-RMN	Fracionamento isotópico natural de posição - ressonância magnética nuclear
FPM	Flavonas polimetoxiladas
FSOJ	Freshly squeezed orange juice
HPLC	High performance liquid chromatography
PDB	Pee Dee Belemnite
PMAO	Padrão médio das águas do oceano
PMF	Polymethoxylated flavones
RMN	Ressonância magnética nuclear
SLC	Suco de laranja concentrado
SIRMS	Stable isotopic ratio mass spectrometry
SLF	Suco de laranja fresco
SNIF - NMR	Site natural isotopic fractionation - Nuclear magnetic resonance
SMOW	Standard mean ocean water
TEA	Trietilamina

THF	Tetrahidrofurano
TMU	Tetrametiluréia
UV	Ultra violeta
Vis	Visível
‰	Partes por mil
$\delta\ ^{13}C$ ‰	Delta carbono 13 por mil
$\delta\ ^{18}O$ ‰	Delta oxigênio 18 por mil
$^{13}C/^{12}C$	Razão entre carbono 13 e carbono 12
$^{18}O/^{16}O$	Razão entre oxigênio 18 e oxigênio 16
$(D/H)_I$	Razão entre deutério e hidrogênio na posição metila da molécula de etanol
$(D/H)_{II}$	Razão entre deutério e hidrogênio na posição metileno da molécula de etanol

RESUMO

Amostras autênticas de suco de laranja (*Citrus sinensis*) das variedades Pera, Natal, Hamlin, Valência, Baía e Lima, amostras autênticas de suco de laranja concentrado (SLC) e água de lavagem da polpa ("pulp wash") e amostras comerciais de suco de laranja fresco (SLF) e congelado foram analisadas quanto à presença dos seguintes compostos: flavanonas glicosídicas (FG), flavonas polimetoxiladas (FPM) e carotenóides e isótopos estáveis de carbono 13, oxigênio 18 e deutério.

As flavanonas glicosídicas narirutina e hesperidina foram analisadas por cromatografia líquida de alta eficiência (CLAE) com detector de UV a 280 nm. Os níveis de narirutina e hesperidina obtidos em amostras autênticas variaram entre 16 e 142 mg/L e 104 e 537 mg/L, respectivamente. A razão hesperidina/narirutina apresentou diferenças de acordo com a variedade. A variedade Pera apresentou a maior razão (média de 8,3) e a variedade Baía a menor (3,6). Suco de laranja concentrado (após diluição para 12 °Brix) apresentou maiores quantidades de FG com teores de narirutina variando entre 62 e 98 mg/L e de hesperidina, entre 531 e 690 mg/L. Em suco de laranja concentrado, preparado a partir de água de lavagem da polpa, o nível de narirutina variou entre 155 e 239 mg/L e o de hesperidina entre 1089 e 1200 mg/L. A análise de amostras de suco de laranja fresco, obtidas junto a supermercados indicou que o conteúdo de FG da maioria delas (13 a 95 mg/L e 106 a 587 mg/L respectivamente, para narirutina e hesperidina) era similar àqueles encontrados para amostras autênticas.

Seis flavonas polimetoxiladas foram determinadas em termos de quantidade relativa e a sinensetina quantificada em amostras de suco de laranja. As FPM foram extraídas com tolueno e analisadas utilizando CLAE com coluna de fase reversa e detecção a 340 nm. A identificação dos picos foi baseada no espectro de UV-visível e na ordem de eluição descrita pela literatura. Sucos de laranja autênticos continham de 0,06 a 0,12 mg/L de sinensetina com teores mais elevados encontrados nas variedades Pera e Natal. Amostras autênticas obtidas junto a indústrias de suco (SLC e água de lavagem da polpa), e amostras de suco de laranja concentrado e de suco de laranja fresco adquiridas no comércio continham, tipicamente, no mínimo 10 vezes mais sinensetina que aquelas obtidas por extração manual do suco. A razão das áreas dos picos das FPM das diferentes variedades foi examinada utilizando análise canônica discriminante. Este procedimento distinguiu as variedades Pera e Hamlin das variedades Natal e Valência, quando obtidas

por extração manual. Da mesma forma, os sucos obtidos por extração manual foram facilmente distinguidos daqueles de origem industrial, ou seja, suco de laranja concentrado, água de lavagem da polpa e de suco de laranja fresco.

Os seguintes carotenóides foram determinados em amostras de suco de laranja: luteína, zeaxantina, β-criptoxantina, α-caroteno e β-caroteno. Os carotenóides foram extraídos com acetato de etila e analisados por CLAE utilizando detector na região do visível a 450 nm. A concentração total de carotenóides em amostras autênticas variou entre 0,11 e 1,21 mg/L, enquanto que β-caroteno, a mais importante fonte de vitamina A, foi detectado em ordem decrescente de concentração nas variedades Pera, Valência, Hamlin, Natal, Lima e Baía. Os carotenóides totais presentes em amostras de suco de laranja concentrado obtidas das fábricas variaram entre 0,26 e 0,48 mg/L, enquanto que as amostras adquiridas no comércio continham quantidades ligeiramente superiores (0,46 - 0,81 mg/L). Suco concentrado de laranja de água de lavagem da polpa apresentou concentrações muito menores variando entre 0,04 e 0,08 mg/L dos carotenóides totais. Amostras de suco de laranja fresco obtidas no comércio continham carotenóides variando entre 0,04 e 0,55 mg/L, com apenas uma amostra apresentando teores fora da faixa encontrada para as amostras autênticas.

Análise isotópica foi a técnica utilizada para caracterizar amostras autênticas de suco de laranja. Fracionamento isotópico natural de posição específica medido por ressonância magnética nuclear (FINPE-RMN) foi empregado para determinar as razões de deutério/hidrogênio, nas posições metil [$(D/H)_I$] e metileno [$(D/H)_{II}$], do etanol produzido pela fermentação do suco de laranja em condições controladas. Espectrometria de massas da razão dos isótopos estáveis (EMRIE) foi utilizada para determinar a quantidade de carbono 13 ($^{13}C/^{12}C$, em partes *per mil*; "‰") no mesmo etanol. EMRIE foi também utilizado para a quantificação de oxigênio 18 ($^{18}O/^{16}O$, ‰) na água do suco da fruta. As razões médias determinadas para $(D/H)_I$, $(D/H)_{II}$, $^{13}C/^{12}C$ e $^{18}O/^{16}O$ em amostras autênticas de suco de laranja foram respectivamente de: 102.3 ppm (DP = 1,7); 126,5 ppm (DP = 1,8), -26,5 ‰ (DP = 0.9) e +2,27 ‰ (DP = 2,5). Estas técnicas isotópicas (FINPE-RMN e RIEEM de carbono-13) podem ser consideradas importantes ferramentas para determinar simultaneamente a adição não declarada de açúcar de cana e milho (RIEEM) e açúcar de beterraba (FINPE-RMN) em suco de laranja. Nenhuma das amostras do varejo foram adulteradas a níveis que pudessem ser detectados por qualquer dos métodos isotópicos empregados.

SUMMARY

Authentic samples of orange juice (*Citrus sinensis*) from the varieties Pera, Natal, Hamlin, Valência, Baía and Lima, authentic samples of frozen concentrated orange juice (FCOJ) and frozen concentrated orange pulp-wash and retail samples of freshly squeezed orange juice (FSOJ) and FCOJ were analysed for the presence of the following compounds: flavanone glycosides (FG), polymethoxylated flavones (PMF) and carotenoids and stable isotopes: carbon 13, oxygen 18 and deuterium.

The flavanone glycosides narirutin and hesperidin were analysed by reversed phase high performance liquid chromatography (HPLC) with UV detection at 280 nm. The juice from hand squeezed fruit gave narirutin and hesperidin concentrations of 16 - 142 mg/L and 104 - 537 mg/L, respectively. The ratio of hesperidin to narirutin showed varietal differences with Pera having the highest ratio (mean 8.3) and Baía the lowest (3.6). Frozen concentrated orange juice (after dilution to 12 °Brix) contained higher quantities of FG with narirutin ranging from 62 to 98 mg/L and hesperidin from 531 to 690 mg/L. In frozen concentrated orange juice pulp-wash the narirutin level ranged from 155 to 239 mg/L and hesperidin from 1,089 to 1,200 mg/L. Retail samples of freshly squeezed juice showed that the FG contents of most samples (13 to 95 and 106 to 587 mg/L respectively, for narirutin and hesperidin) were similar to those found for authentic ones.

Sinensetin was quantified in authentic samples of Brazilian orange juice. In addition, six other polymethoxylated flavones were determined in terms of their relative amounts. The PMF were extracted into toluene and analysed using reversed phase HPLC with detection at 340 nm. Peak identification was based on the UV-visible spectra and the elution order described in the literature. Hand squeezed orange juices contained from 0.06 to 0.12 mg/L sinensetin, with the highest concentrations being found in the Pera and Natal varieties. Commercial samples of frozen concentrated orange juice, frozen concentrated orange pulp-wash, retail FCOJ and retail freshly squeezed orange juice typically contained at least ten times more sinensetin than those found for samples squeezed by hand. The PMF peak area ratios for these sample classes were examined further using canonical discriminant analysis. This procedure could distinguish the hand-squeezed juices of Pera and Hamlin varieties from those of Natal and Valência. Similarly

hand squeezed juices could be readily distinguished from the commercial samples of FCOJ, FCOPW, retail FCOJ and retail FSOJ.

Lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene were determined in samples of orange juice. The carotenoids were extracted with ethyl acetate and analysed by reversed phase HPLC with detection at 450 nm. A concentration range of 0.11 - 1.21 mg/L was determined for total carotenoids in authentic samples with β-carotene, the most important source of Vitamin A, being found in the highest concentration in the Pera variety, followed by Valência, Hamlin, Natal, Lima and Baía varieties. The total carotenoids present in samples of frozen concentrated orange juice obtained from factories ranged from 0.26 - 0.48 mg/L, while retail samples of this product contained slightly more (0.46 - 0.81 mg/L). Frozen concentrated orange pulp-wash presented much lower concentrations ranging from 0.04 to 0.08 mg/L of total carotenoids. Samples of retail freshly squeezed orange juice contained carotenoids ranging from 0.04 to 0.55 mg/L, with only one sample out of the range found for authentic samples.

Stable isotopic analysis was used to characterise authentic samples of orange juice from Brazil. Site Specific Natural Isotopic Fractionation measured by Nuclear Magnetic Resonance (SNIF-NMR) was used to determine deuterium/hydrogen ratios at the methyl [$(D/H)_I$] and methylene [$(D/H)_{II}$] sites of ethanol. This ethanol was produced by fermentation of orange juice under controlled conditions. Stable Isotope Ratio Mass Spectrometry (SIRMS) was used to determine the amount of carbon-13 ($^{13}C/^{12}C$, in parts per mil "‰") in the same ethanol. SIRMS was also used to determine the amount of oxygen-18 ($^{18}O/^{16}O$, ‰) in citrus juice water. The mean ratios determined for $(D/H)_I$, $(D/H)_{II}$, $^{13}C/^{12}C$ and $^{18}O/^{16}O$ in the authentic hand squeezed orange juice samples were respectively: 102.3 ppm (SD = 1.7); 126.5 ppm (SD = 1.8), -26.5 ‰ (SD = 0.9) and +2.27 ‰ (SD = 2.5). These isotopic techniques (SNIF-NMR and carbon 13 SIRMS) are powerful tools to determine simultaneously the undeclared addition of cane and corn sugars (SIRMS) and beet sugar (SNIF-NMR) in orange juice. None of the retail samples analysed was adulterated at levels which could be detected by either isotopic method.

INTRODUÇÃO

Um dos ítems de importância na balança de exportação brasileira é o suco de laranja. Esse produto tem acrescentado às divisas brasileiras bilhões de dólares, sendo exportado para diferentes países incluindo os Estados Unidos, Inglaterra e Japão. Devido ao seu grande valor econômico, Inglaterra e Estados Unidos, entre outros países, têm se mostrado preocupados com possíveis adulterações nesses produtos. Estas adulterações não são necessariamente feitas pelos países exportadores, mas algumas vezes pelos países de destino, que têm sido responsáveis pela adulteração de sucos com água, ácidos orgânicos, açúcares e até misturas sofisticadas de derivados de produtos cítricos.

A adulteração em suco de laranja tem sido relatada desde a década de 30, nos Estados Unidos. Entretanto, o interesse na adulteração deste produto foi despertado somente após o aumento do consumo do suco de laranja industrializado, sendo utilizado para tal fim açúcares, água e ácidos.

O Brasil detém cerca de 80% do mercado internacional de suco de laranja concentrado. Entretanto, o mercado interno se baseia principalmente em suco de laranja fresco, comercializado em diferentes embalagens, e também em suco de laranja concentrado congelado.

De acordo com a legislação brasileira, o suco de laranja deve ser proveniente de frutos da espécie *Citrus sinensis*. A adição de outros sucos não é permitida, com exceção de suco de tangerina (*C. reticulata blanco*), que poderá ser adicionado em um percentual não superior a 10%. Açúcar e conservadores, se adicionados, devem ser declarados no rótulo. Água de lavagem da polpa (também chamada de "pulp wash") e suco de laranja concentrado não podem ser adicionados ao suco de laranja fresco.

Até o presente, existem poucos dados disponíveis na literatura sobre a composição química de laranjas brasileiras e estes, quando relatados, não citam as variedades de maior importância, como a Pera e Natal, responsáveis por cerca de 70% do total das laranjas processadas no Brasil.

O presente estudo teve como objetivo criar um banco de dados sobre os seguintes constituintes em suco autêntico de laranja: a) flavanonas glicosídicas, b) flavonas polimetoxiladas, c) carotenóides, d) isotópos estáveis de carbono 13, oxigênio 18 e deutério. Para fins comparativos, amostras de suco de laranja fresco e congelado, adquiridas no comércio também foram analisadas.

Capítulo 1

REVISÃO BIBLIOGRÁFICA

1.1. Aspectos Mercadológicos do Suco de Laranja

O Brasil é o maior produtor de laranjas do mundo e é responsável por 80% do mercado internacional de suco de laranja congelado (ROBARDS & ANTOLOVICH, 1994). O Estado de São Paulo é responsável por 80% do processamento da laranja nacional e, deste total, 90% são exportados como suco de laranja congelado (FREITAS, 1995).

De acordo com a ABECitrus (Associação Brasileira dos Exportadores de Citrus) o Brasil exporta cerca de hum milhão de toneladas de suco por ano (safra 95/96), totalizando um total de US 1,269,383.00 em divisas para o Brasil.

A produção do suco de laranja envolve diferentes etapas. Inicialmente, ocorre a seleção das laranjas, lavagem e extração. Como resultado da extração são gerados três produtos: suco, óleo essencial e bagaço. O suco passa pelo "finisher", a fim de reduzir o teor de polpa, seguido por centrifugação e aquecimento à 92 °C, quando sofre inibição enzimática e microbiana, seguindo para o processo de concentração (FREITAS, 1995).

A polpa e o bagaço obtidos na etapa de extração, os quais ainda contêm uma certa quantidade de suco, são então submetidos à extração com água em um processo de contra-corrente. O suco obtido é classificado como secundário, e é comumente chamado de "pulp-wash" (água de lavagem da polpa) (FREITAS, 1995).

De acordo com a legislação brasileira (Portaria 371 do Ministério da Agricultura, datada de 09/09/1974) que define suco de laranja (*Citrus sinensis*), este deve ser proveniente de frutas frescas, sãs e maduras, sendo a adição de suco de tangerina (*Citrus reticulata blanco*) permitida em um percentual não superior a 10%. Ainda, de acordo com a legislação vigente, se a adição de açúcar e conservadores ocorrer, os mesmos devem ser

declarados no rótulo. A adição de água de lavagem da polpa e de suco de laranja concentrado ao suco de laranja natural não é permitida.

1.2. Fontes de Adulteração

Os principais componentes do suco de laranja são: água, açúcares (glicose, frutose e sacarose), ácidos orgânicos, sais, vitaminas, essências, pigmentos e substâncias pécticas (CHEN *et al.*, 1992). A adulteração de suco de laranja se torna economicamente vantajosa quando os componentes de maior constituição do suco como os açúcares, água e ácidos ou ainda suco de frutas de menor valor comercial e agentes corantes são adicionados ao suco natural. A literatura cita vários tipos de adulteração de suco de laranja, conforme descritos a seguir.

Existem nos Estados Unidos da América (EUA), desde 1936, relatos de adulteração de suco de laranja pela adição ilegal de óleos essenciais, açúcares e ácidos (JOHNSTON & KAUFFMANN, 1985). Outros episódios envolvendo adulteração de suco de laranja nos EUA também foram citados por BRAUSE (1993). De acordo com os dados apresentados, diretores e presidentes das empresas fraudulentas, além de condenados à prisão, tiveram de pagar multas de até US 480,000.00. As adulterações mais comuns eram a adição de água de lavagem da polpa, suco de grapefruit e açúcar de beterraba ao suco de laranja.

MARTIN (1994) reportou a adulteração de suco de laranja com no mínimo 60% de açúcar, e uma quantidade de ácido D-málico muito maior que a normalmente presente no suco natural. O caso foi para a Corte na Inglaterra e a empresa importadora/distribuidora foi multada em £ 8,000.00. Na Inglaterra, também foi identificada uma outra empresa que adicionava açúcar de beterraba em suco de laranja vendido como 100% puro, em níveis que variavam entre 10 e 20% (O'DONNELL, 1994). Estudos conduzidos pelo MAFF (Ministério da Agricultura e Pesca da Inglaterra)

detectaram adulteração em 16 das 21 marcas principais de suco de laranja comercializados naquele país, envolvendo a adição ilegal de açúcar de beterraba (PATEL, 1994).

1.3. Técnicas Para Verificação de Autenticidade

A verificação da autenticidade de um determinado suco só pode ser feita através do conhecimento prévio da composição química do material genuíno. Entre as diversas análises químicas utilizadas para verificar a autenticidade, podemos citar como exemplo:

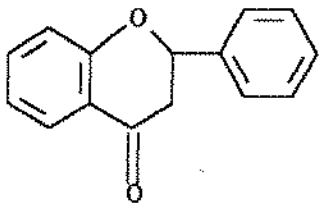
- **composição de flavonóides** (flavanonas glicosídicas e flavonas polimetoxiladas): detectam a adição de sucos não pertencentes à espécie *C. sinensis*.
- **composição de carotenóides**: detecta adição de água de lavagem da polpa.
- **razão isotópica de carbono e deutério** (nos açúcares do suco): detectam a adição de adoçantes como açúcar de cana, milho e beterraba.
- **razão isotópica de oxigênio** (na água do suco): detecta adição de água de torneira, entre outras adições (metais, amino ácidos, proteínas e ácidos orgânicos).

Estas análises são consideradas de grande importância porque fornecem uma impressão digital do material autêntico que, quando comparado com o produto comercial, indicará ou não a sua autenticidade.

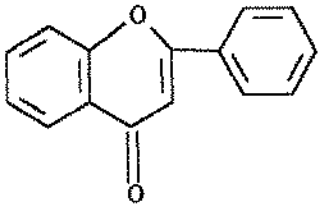
1.3.1. Flavanonas Glicosídicas

Os compostos flavonólicos são caracterizados pela presença do esqueleto C6-C3-C6, enquadrando-se nos seguintes grupos: (a) flavanonas, (b) flavonas e (c) antocianinas (Figura 1). Entre os flavonóides, as flavanonas glicosídicas (FG) têm uma distribuição muito mais restrita e são específicas de sucos cítricos (ATTAWAY *et al.*,

a. flavanona



b. flavona



c. antocianina

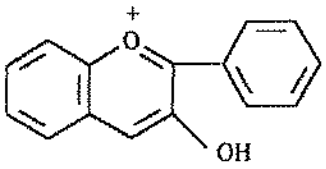


Figura 1. Estrutura química dos flavonóides.

1972). Flavanonas glicosídicas são compostos constituídos de duas frações distintas: um esqueleto C6-C3-C6 e uma molécula de açúcar ligada na posição 7 (RANGANNA *et al.*, 1983).

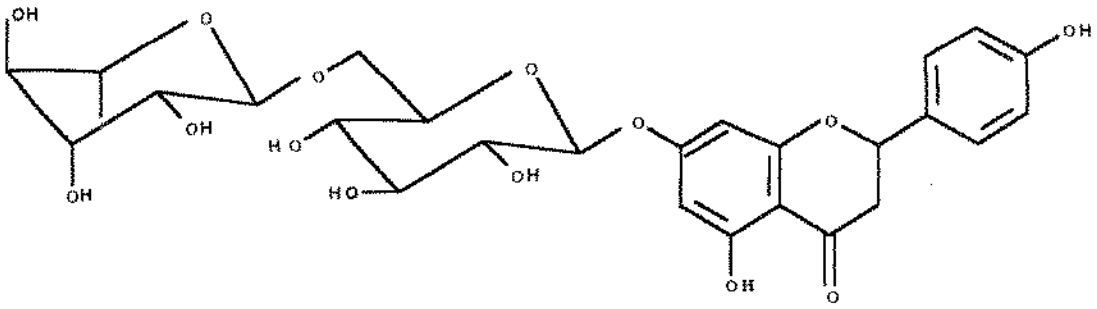
Segundo KEFFORD (1959), o cultivar *Citrus* pode ser dividido em dois grupos, de acordo com a natureza das flavanonas glicosídicas presentes: um grupo contendo hesperidina, substância sem sabor, como a flavanona glicosídica mais importante (laranja doce e limão) e o outro contendo naringina, com sabor amargo, como flavanona glicosídica principal (graprefruit e laranja azeda).

Em *Citrus sinensis*, as flavanonas glicosídicas que têm sido consideradas de maior importância, segundo vários autores, são a hesperidina e a narirutina (Figura 2) (GALENSA & HERRMANN, 1980; SMOLENSKY & VANDERCOOK, 1982; GREINER & WALLRAUCH, 1984; GALENSA *et al.*, 1986; ROUSEFF *et al.*, 1987; MOULY *et al.*, 1994; OOGHE *et al.*, 1994).

MEARS & SHENTON (1973) apresentaram uma revisão bastante completa sobre adulteração e caracterização de sucos de laranja e grapefruit. Nesta revisão, as principais flavanonas glicosídicas citadas em laranja foram: hesperidina, naringenina 7-rutinosídeo, isosakuranetina, 4-β-D glucosídeo naringenina, 4-β-D glucosídeo de naringenina rutinosídeo e neohesperidina. Em grapefruit, os mesmos autores descreveram as seguintes flavanonas glicosídicas: naringina, naringenina 7-rutinosídeo, naringenina 7-rutinosídeo, neohesperidina, hesperidina, poncirina, isosakuranetina, 4-β-D glucosídeo naringenina e 4-β-D glucosídeo de naringenina rutinosídeo. Os autores enfatizaram a utilização destes componentes na distinção entre suco de laranja e grapefruit.

Em revisão publicada em 1975, IRANZO apresentou dados citados na literatura por diversos autores sobre níveis de hesperidina em sucos de laranja, os quais variavam entre 28,0 a 115,0 mg/100 mL. Segundo o autor, estes e outros parâmetros teriam como

a. narirutina



b. hesperidina

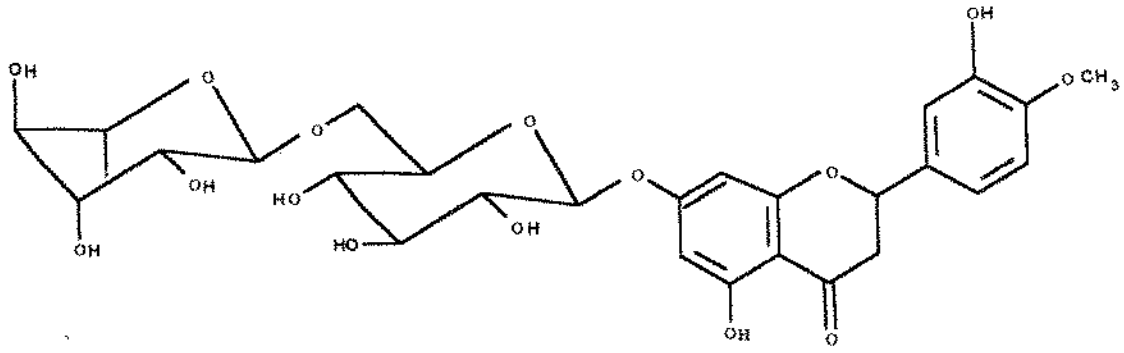


Figura 2. Estrutura química das flavanonas glicosídicas.

principal finalidade criar um banco de dados para detectar possíveis adulterações de suco de laranja.

A fim de detectarem a adulteração de suco de laranja por outro tipo de suco, ROUSEFF *et al.* (1987) analisaram a composição de flavanonas glicosídicas em 52 cultivares de citrus. Para a variedade *C. sinensis* (14 cultivares), os teores de hesperidina e narirutina variaram entre 122 e 254 mg/L e 18 e 65 mg/L, respectivamente, e a relação entre hesperidina e narirutina variou entre 3,1 e 8,3. Não foram detectadas naringina nem neohesperidina nas amostras de *C. sinensis* analisadas. Segundo os autores, a composição de FG em suco de laranja permite a detecção de outros tipos de sucos não pertencentes à espécie *C. sinensis*.

De acordo com ROUSEFF (1988a, b), a adição de 3 % de suco de grapefruit em suco de laranja pode ser detectada pela presença de naringina, a qual está presente em grapefruit mas não em laranja (*C. sinensis*).

A composição de flavanonas glicosídicas em laranja azeda e em grapefruit foi determinada por MOULY *et al.* (1993), como sendo: eriocitrina, neoriocitrina, narirutina, hesperidina, naringina e neohesperidina em concentração de 13, 320; 9,9, nd, 427 e 266 mg/L e de < 1, < 1, 116, 10, 461, 11 mg/L, respectivamente (nd = não detectado). Em função destes resultados, os autores sugeriram que a utilização da razão narirutina/neoeriocitrina poderia ser utilizada como parâmetro de controle de qualidade dos sucos cítricos estudados.

Empregando a análise fatorial discriminante, MOULY *et al.* (1994) utilizaram a composição de flavanonas glicosídicas como diferenciador de sucos cítricos (limão, lima, grapefruit e laranja doce). Os teores médios de hesperidina e narirutina detectados em cinco cultivares diferentes de laranja doce provenientes de cinco países foram 320,8 e 56,8 mg/L, respectivamente (relação hesperidina/narirutina 5,7).

OOGHE *et al.* (1994) analisaram a presença de flavanonas glicosídicas em laranjas de diversas procedências, tendo como principal objetivo criar parâmetros para atestar a autenticidade de *C. sinensis*. Os teores médios de narirutina e hesperidina determinados em 54 amostras autênticas foram 49,0 mg/L e 231,2 mg/L, respectivamente, e a razão hesperidina/narirutina foi 6,0. Os autores concluíram que, como critério de autenticidade de *C. sinensis*, a relação entre as FG hesperidina e narirutina deveria ser no mínimo 3. Também foi concluído que a adição de outros tipos de sucos de fruta (como por exemplo, *C. paradisi*, *C. aurantium* e *C. bergamia*), que não pertencem à espécie *C. sinensis*, poderia ser facilmente detectada pela presença de naringina e de outros FG não presentes em *C. sinensis*. Segundo os mesmos autores, a presença de suco de tangerina (*C. reticulata*) e murcote pode ser detectada em suco de laranja pelo decréscimo da razão hesperidina/narirutina.

ROBARDS & ANTOLOVICH (1994) utilizaram a presença de naringina em suco de grapefruit (como adulterante) conjuntamente com a presença de hesperidina (presente naturalmente em suco de laranja) para a detecção de adulteração em suco de laranja. Estes mesmos autores publicaram em 1995 uma revisão completa dos métodos e das utilidades de cada componente ou classe de compostos (amino ácidos, açúcares, ácidos orgânicos, flavonas polimetoxiladas, flavanonas glicosídicas, análise isotópica de carbono 12 e 13, metais), na detecção de adulteração de suco por água, açúcar, "pulpwash", ácidos orgânicos, entre outros.

KIRKSEY *et al.* (1995) utilizaram a relação hesperidina/narirutina para detectar a adição de água de lavagem da polpa ("pulp-wash") em suco de laranja, prática proibida em muitos países. De acordo com os autores, se a relação hesperidina/narirutina for menor que 2, o suco deverá ser considerado suspeito, indicando possível adição de "pulp-wash".

1.3.1.1. Métodos de análise

DAVIS (1947) foi um dos pioneiros na análise de flavanonas glicosídicas totais em sucos. Embora o método colorimétrico proposto, que envolve a adição de hidróxido de sódio ao suco de laranja na presença de dietileno glicol, seguida por medição da cor produzida na região do espectro visível, não fosse específico para um tipo de flavonóide, o mesmo foi utilizado até a década de oitenta para quantificação de flavanonas glicosídicas em sucos de frutas. Entretanto, verificou-se posteriormente que este método não fornecia valores corretos, devido à interferência de outros componentes presentes na amostra, nem quantificava separadamente hesperidina ou narirutina ou naringina. Desta forma, metodologias mais específicas para a detecção e quantificação individual de FG começaram a ser introduzidas na década de 60. Citam-se, como exemplo, a espectroscopia no ultra violeta (UV) (HENDRICKSON *et al.*, 1959), a cromatografia em camada delgada (CCD) (NISHIURA *et al.* 1969 e 1971; COFFIN, 1971; BENK, 1972) e a cromatografia líquida de alta eficiência (CLAE) (FISHER & WHEATON, 1976; FISHER, 1978).

Como citado anteriormente, DAVIS (1947) utilizou o método colorimétrico para a quantificação de flavanonas glicosídicas em suco de frutas. Neste estudo foram determinadas as seguintes concentrações de flavanonas glicosídicas (totais) respectivamente para suco de grapefruit, limão, laranja e tangerina: 0,038; 0,046; 0,080 e 0,065 (g/100 mL).

COFFIN (1971) analisou diversas flavanonas glicosídicas em sucos de frutas por cromatografia em camada delgada, tendo como meta principal a separação destes compostos. As seguintes flavanonas glicosídicas foram encontradas em grapefruit: isosakuranetina 7-neohesperidosídeo e isosakuranetina 7-rutinosídeo, hesperetina e naringenina. Em suco de laranja foram detectados os 7-rutinosídeos das mesmas flavanonas. Suco de limão apresentou isosakuranetina 7-rutinosídeo, hesperetina, naringenina e eriodictiol, e um glicosídeo contendo ramnose, glucose e uma flavanona

não identificada, enquanto que suco de lima continha hesperetina 7-rutinosídeo, naringenina e eriodictiol.

O uso da cromatografia líquida de alta eficiência na quantificação individual de flavanonas glicosídicas foi inicialmente descrito por FISHER & WHEATON (1976). Coluna de fase reversa, detector de UV com comprimento de onda ajustado para 280 nm e fase móvel água:acetonitrila (80:20) foram utilizados para separar e quantificar naringina em suco de grapefruit. A concentração média encontrada para naringina foi de 394 mg/L. De acordo com os autores, esta nova metodologia apresentava diversas vantagens em relação ao método de Davis, como por exemplo maior rapidez e especificidade na determinação de FG.

FISHER (1978) utilizou as mesmas condições descritas por FISHER & WHEATON (1976) para quantificar hesperidina em duas variedades de laranja. Os níveis detectados para sucos de laranja variedades Valência e Hamlin foram, respectivamente, 98 a 120 mg/L e 54 a 100 mg/L, dependendo do tipo de extração utilizado.

Esta mesma técnica (CLAE) e o método de Davis foram utilizados por GALENSA & HERRMANN (1980) na determinação dos teores de narirutina e hesperidina em suco de laranja de diversas variedades. Os níveis detectados de narirutina e hesperidina variaram entre 17 e 153 mg/L (CLAE) e 480 e 1180 mg/L (Médodo de Davis), respectivamente. O valor médio apresentado pela relação entre hesperidina e narirutina determinada pelos dois métodos (Davis e CLAE) foi de 2,2.

Mais recentemente, diversos autores têm utilizado a técnica de cromatografia líquida acoplada ao detector de UV e coluna de fase reversa na detecção e análise de flavanonas glicosídicas em diversos tipos de sucos de frutas (ROUSEFF *et al.*, 1987; ROUSEFF 1988 a, b; ROBARDS & ANTOLOVICH, 1994; OOGHE *et al.*, 1994; MOULY *et al.*, 1994; KIRKSEY *et al.*, 1995).

Como mencionado anteriormente, a maioria das análises descritas para a análise de flavanonas glicosídicas em suco de frutas foi conduzida utilizando coluna C18 e detector de ultravioleta. Alternativamente, SONTAG & KRAL (1981) e GAMACHE *et al.* (1993) utilizaram detector amperométrico para a quantificação das flavanonas glicosídicas. Cromatografia gasosa foi utilizada por DRAWERT *et al.* (1980), mas os compostos analisados tiveram que sofrer derivatização com hexametildisilazano (HMDS), uma vez que as FG não são voláteis.

A Tabela 1 apresenta uma compilação da literatura sobre análise de flavanonas glicosídicas em diversos tipos de frutas e sucos.

1.3.2. Flavonas Polimetoxiladas

O termo genérico flavonas polimetoxiladas (FPM) representa o grupo químico das flavonas, as quais possuem mais que quatro grupos metoxilas (CEN, 1991). FPM são encontradas quase que exclusivamente em citrus, com uma distribuição muito específica e característica para cada tipo de fruta (TATUN *et al.*, 1978). Uma vez que a concentração relativa do flavonóide varia de acordo com cada variedade, a composição de FPM em suco de *Citrus* tem sido proposta como medida de sua autenticidade (KEFFORD & CHANDLER, 1970).

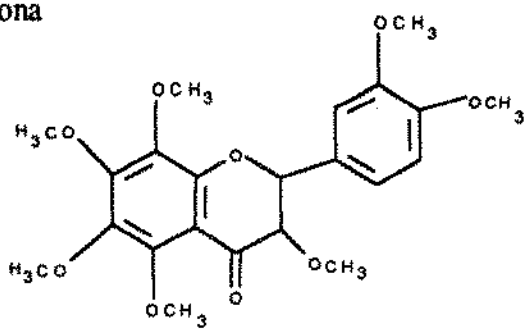
As FPM estão presentes nas folhas e também em todas as partes do fruto como casca, flavedo, albedo, membranas e suco (OOGHE *et al.*, 1994). As maiores concentrações de FPM são encontradas na casca dos citrus e as menores quantidades no suco (VELDHUIS *et al.*, 1970). As principais flavonas presentes em *C. sinensis* são: sinensetina, quercetogetina, nobiletina, heptametoxiflavona, scutelareína e tangeretina (VELDHUIS *et al.*, 1970). As estruturas químicas das FPM estão apresentadas na Figura 3.

Tabela 1. Tipos de coluna e fase móvel utilizados na detecção de flavanonas glicosídicas em frutas cítricas.

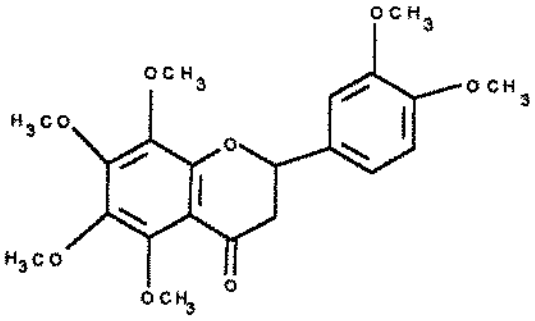
Amostra	Coluna	Fase Móvel	λ (nm)	Gradiente	Referência
grapefruit	μBondapak C18 < 10 μm (300 x 4 mm d.i.)	água : acetonitrila (80:20, v/v)	280	não	FISHER & WHEATON, 1976
suco de laranja	μBondapak C18 < 10 μm (300 x 4 mm d.i.)	água : acetonitrila (80:20, v/v)	280	não	FISHER, 1978
suco de laranja	LiChrosorb Si 60 5 μm (600 x 3 mm d.i.)	benzeno : acetonitrila ou isooctano : etanol : acetonitrila	270	não	GALENSA & HERRMANN, 1980
diversos cultivares de citrus	Zorbax ODS 5 μm (250 x 4,6 mm d.i.)	água : acetonitrila : ácido acético (79,5 :20 : 0,5)	280	não	ROUSEFF *et al.*, 1987
suco de laranja	Zorbax ODS 5 μm (250 x 4,6 mm d.i.)	20 to 50 % B em 10 min. (A = 1 % ácido acético em água; B = 1 % ácido acético em acetonitrila)	280	sim	PERFETTI *et al.*, 1988
suco de laranja	M.S. Gel C18 5 μm (15 x 4,6 mm d.i.)	A = 0,1 mol/L NaH_2PO_4 (pH 3,35) adicionados 10 mg/L SDS, B = 0,1 mol/L NaH_2PO_4 : 50 mg/l SDS : metanol (60:30:10)	a	sim	GAMACHE *et al.*, 1993
grapefruit / suco de laranja	Supelco C18 3 μm (125 x 3.6 mm d.i.)	água : acetonitrila: ácido acético (79,5 : 20 : 0,5)	280	não	ROUSEFF, 1988a, b
grapefruit e laranja azeda	Alltech RP 18 UHS 5 μm (250 x 4,6 mm d.i.)	água : acetonitrila : tetrahidrofurano : ácido acético (80 : 16 : 3 : 1)	280	não	MOULY *et al.*, 1993
sucos cítricos	Alltech RP 18 UHS 5 μm (250 x 4.6 mm d.i.)	água : acetonitrila : tetrahidrofurano : ácido acético (80 : 16 : 3 : 1)	280	não	MOULY *et al.*, 1994
suco de laranja	Waters Nova-Pack C18 4 μm (150 x 3,9 mm d.i.)	tampão fosfato e acetonitrila	280	sim	OOGHE *et al.*, 1994
suco de laranja	Waters Nova-Pack C18 4 μm (150 x 3,9 mm d.i.)	100 % KH_2PO_4 (pH 3,05) para 58 % KH_2PO4 : 42 % acetonitrila (70 : 30; ACN:H_2O) em 38 min.	280	sim	ROBARDS & ANTOLOVICH, 1994

a : detector eletroquímico

a. heptametoxiflavona



b. nobiletina



c. scutelareína

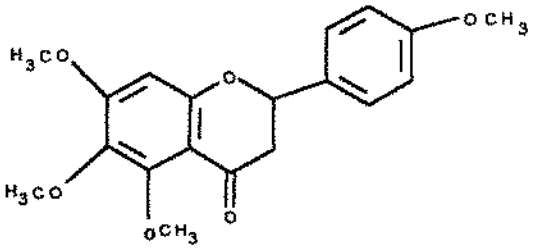
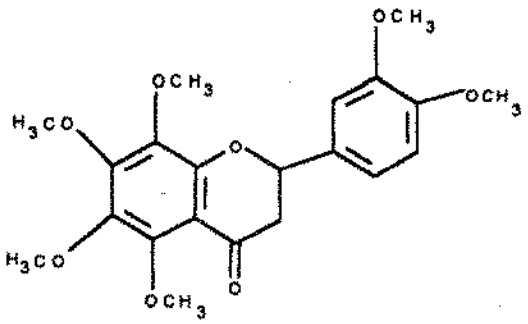
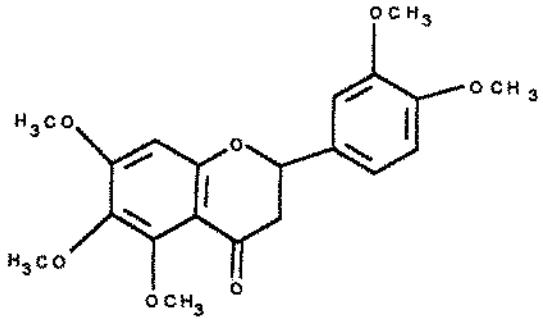


Figura 3. Estrutura química das flavonas polimetoxiladas

d. quercetogetina



e. sinensetina



f. tangeretina

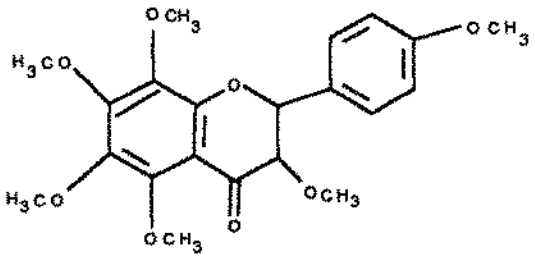


Figura 3. Estrutura química das flavonas polimetoxiladas (continuação).

Além de serem utilizadas para fins de caracterização de sucos ou plantas, os compostos flavonólicos são também citados por sua atividade biológica em mamíferos como por exemplo: dilatação de artérias coronárias, inibição de agregação de plaquetas, retardamento da formação de trombose (LIANG *et al.*, 1996) e propriedades anticancerígenas (KANDASWAMI *et al.*, 1991).

1.3.2.1. Análise de flavonas polimetoxiladas

Cromatografia em camada delgada foi a técnica mais utilizada nos anos 60 e 70 para a análise de flavonas polimetoxiladas. VELDUHIS *et al.* (1970) analisaram cinco FPM em suco de laranja concentrado, produzidos durante o período de 1952 a 1968. O principal objetivo era avaliar o limiar da detecção do gosto básico ("taste threshold") destes compostos em suco comercial.

TING *et al.* (1979) utilizaram pela primeira vez a técnica de cromatografia líquida de alta eficiência para analisar FPM em suco de tangerina e laranja, concluindo que este método poderia ser utilizado para detectar a adição de um suco no outro.

ROUSEFF & TING (1979) também utilizaram a técnica de CLAE para separar e identificar FPM em sucos de frutas. Foram testados diversos solventes de extração com ou sem adição de NaOH. De acordo com os resultados obtidos, o melhor solvente de extração foi o benzeno, enquanto que a adição de NaOH resultou em baixa recuperação e formação de artefatos. Os autores concluíram que este método era mais rápido e preciso que a cromatografia em camada delgada, até então utilizada. Similarmente, GAYDOU *et al.* (1987) diferenciaram óleo de casca de mandarim e de laranja utilizando a composição taxonômica de FPM pela análise discriminante fatorial do conteúdo desses compostos nos dois óleos.

SENDRA *et al.* (1988) desenvolveram um método de extração de FPM em sucos, envolvendo percolação em coluna C18, com eluição seletiva das FPM. As FPMs foram

analisadas por cromatografia em coluna de fase reversa, utilizando detector de arranjo de diodos para confirmação da identidade e pureza dos picos. De acordo com os autores, esta nova metodologia mostrava diversos avanços em relação aos métodos publicados anteriormente, como, por exemplo, simplicidade, segurança e alta recuperação.

HEIMHUBER *et al.* (1988) também descreveram uma outra metodologia para a análise de FPM em suco e casca de laranja. O método baseou-se na eluição seletiva dos compostos de um cartucho C18, utilizando metanol como solvente. Os autores concluíram que a adulteração poderia ser detectada de acordo com a quantidade e distribuição das FPM presentes no suco, casca e água de lavagem da polpa, que variavam consideravelmente. Entretando, os autores não apresentaram resultados quantitativos. Os únicos resultados apresentados foram cromatogramas de amostras de suco de laranja espremido manualmente, suco de laranja comercial e "pulp-wash".

OOGHE *et al.* (1994) analisaram o conteúdo de FPM de sucos de laranja oriundos de diferentes regiões do mundo e também de diversos processos de extração do suco. A análise quantitativa não foi realizada devido a não disponibilidade de padrões comerciais de FPM. Entretanto, foi calculada a quantidade relativa de sete FPM como critério de autenticidade de *C. sinensis*. Desta forma, valores médios da razão das FPM (média ± desvio padrão) foram estabelecidos para suco autêntico de laranja, e a comparação realizada com suco desconhecido, através de um teste estatístico (Teste F). Os autores concluíram que o processo tecnológico parecia não ter influência no padrão de FPM. A adição de suco de tangerina e murcote resultaram em mudanças significativas na composição de FPM, que puderam ser facilmente detectadas mediante o Teste F.

A Tabela 2 mostra dados da literatura sobre o conteúdo de flavonas polimetoxiladas em suco de laranja.

Tabela 2. Níveis (mg/L) de flavonas polimetoxiladas em suco de laranja.

Tipo	I	II	III	IV	V	VI	VII	VIII	IX	Total
Laranja										
SLF[a]	0,007	0,005	0,09	0,001	0,023	0,110	0,029	0,080	0,020	0,37
SLC[a]	0,060	0,033	0,813	0,018	0,0182	1,088	0,260	0,520	0,135	3,11
Mandarim										
SLF[a]	0,007	0,003	0,022	0,007	0,013	0,015	0,018	0,173	0,045	0,40
SLC[a]	0,463	0,271	1,097	0,285	0,766	5,112	0,715	6,822	1,513	17,05
Suco concentrado										
SLC[b]	nd	nd	0,73	nd	nd	1,00	0,27	0,57	0,13	2,70
SLC[c*]	nd	nd	0,90	nd	nd	1,31	0,51	0,81	0,45	4,24

I = isosinensetina, II = hexametil-O-gossipetina, III = sinensetina, IV = tetrametil-O-isoscutelareína, V = hexametil-O-quercetogetina, VI = nobiletina, VII = tetrametil-O-scutelareína, VIII = heptametoxiflavona, IX = tangeretina

SLF = suco de laranja fresco espremido manualmente

SLC = suco de laranja concentrado

a = SENDRA *et al.*, 1988

b = ROUSEFF & TING, 1979

c = VELDHUIS *et al.* 1970

* = cromatografia em camada delgada

nd = não disponível

1.3.3. Carotenóides

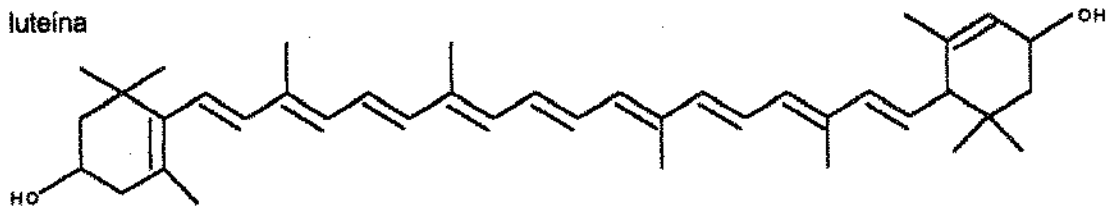
Carotenóides, localizados nos vesículos dos sucos, são uma das fontes de cor no suco de laranja e também um fator importante de qualidade. De acordo com a legislação brasileira, um mínimo de cor é necessário para classificar um suco como sendo de boa qualidade. Suco de laranja também tem sido considerado como uma fonte complementar de carotenóides e, indiretamente, de pró-vitamina A, na dieta, sendo seu consumo correlacionado com a redução da incidência de certos tipos de câncer (COLDITZ *et al.*, 1985; OLSON, 1986; BENDICH, 1989; ZIEGLER, 1989 e 1991; ROUSEFF & NAGY, 1994).

Carotenóides são compostos poliisoprenóides, com 40 átomos de carbono, possuindo um sistema de duplas ligações conjugadas que absorvem fortemente na região do visível (BREEMEN, 1996). Estes compostos são classificados em dois tipos: os carotenos, que são hidrocarbonetos, e as xantofilas, as quais possuem um grupo funcional contendo oxigênio (GOODWIN, 1965 citado por PERFETTI, *et al.*, 1988). Mais de 600 carotenóides já foram identificados, sendo que aproximadamente 50 deles são metabolicamente convertidos para vitamina A, que é essencial para a visão, diferenciação celular e desenvolvimento embrionário (BREEMEN, 1996). A Figura 4 apresenta a estrutura de alguns carotenóides.

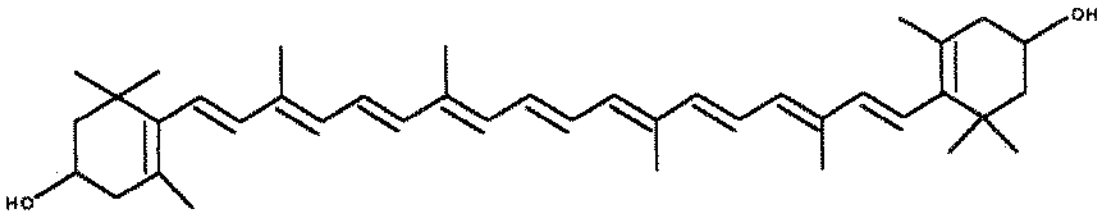
1.3.3.1. Análise de carotenóides

Nas décadas de 60 e 70, os carotenóides de citrus foram extensivamente estudados, tendo sido identificados aproximadamente 115 carotenóides (STEWART, 1973, citado por ROUSEFF *et al.*, 1996). A metodologia utilizada empregava cromatografia em coluna aberta, sendo comumente utilizados os adsorbentes magnésia e uma mistura de magnésia e Hyflo Super-Cell (CURL & BAILEY 1956, 1957, 1961; HIGBY, 1962, 1963; ELAHI & SHAH, 1971; GROSS *et al.*, 1971 e 1972; ROTSTEIN *et al.*, 1972). Os carotenóides foram determinados utilizando-se espectrofotometria ou cromatografia em camada

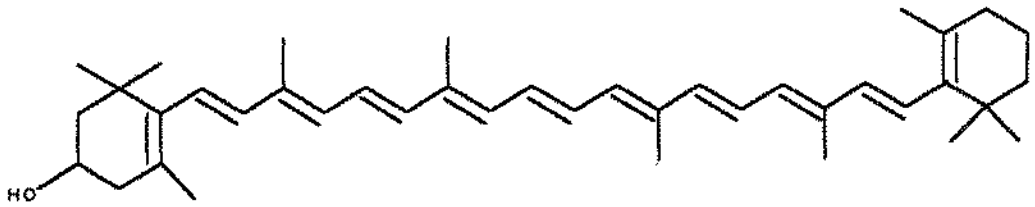
a. luteína



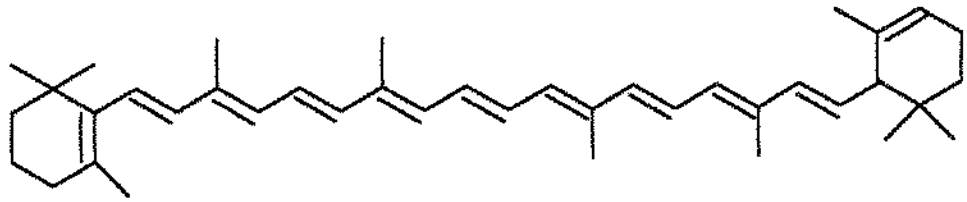
b. zeaxantina



c. beta-criptoxantina



d. alfa-caroteno



e. beta-caroteno

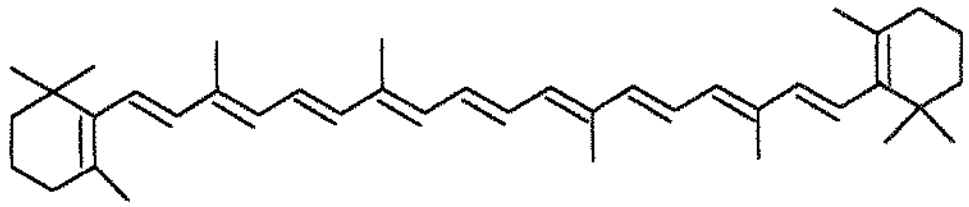


Figura 4. Estrutura química de carotenóides.

delgada. A maioria dos resultados foi apresentada como percentual total dos carotenóides ou como carotenóides totais.

No início dos anos 70, STEWART & WHEATON (1971 e 1973) utilizaram a cromatografia líquida de alta eficiência para a análise de carotenóides em citrus. Os autores concluíram que esta técnica mostrava vantagens em relação à cromatografia em coluna aberta, incluindo simplicidade e rapidez na análise quantitativa.

REEDER & PARK (1975) utilizaram a técnica de CLAE para determinar α-caroteno, β-caroteno e criptoxantina em suco de laranja. Os mesmos autores também utilizaram o método da AOAC (espectrofotométrico) para as mesmas amostras, enfatizando a diferença obtida entre eles. Nas três amostras analisadas, os teores de pró-vitamina A determinados foram de 1,86; 2,14; 1,60 μg/mL e de 1,60; 1,41; 1,33 μg/mL; respectivamente para o método da AOAC e CLAE. A coluna empregada para a análise de carotenos foi a coluna de alumina básica empacotada em laboratório, sendo que para a análise de criptoxantina foi utilizada a coluna Spherisorb, S 10 μm.

Para a quantificação dos carotenóides e do teor de pró-vitamina A em suco de laranja, STEWART (1977a e b) utilizou o sistema de CLAE equipado com colunas de magnésia e sílica e detecção a 440 nm. O autor concluiu que o uso deste sistema reduzia o tempo de residência na coluna, com correspondente diminuição na decomposição e formação de artefatos.

A análise de carotenóides em suco de laranja utilizando cromatografia líquida com coluna de fase reversa (C18) de alta eficiência foi citada pela primeira vez por FISHER & ROUSEFF (1986) e QUACKENBUSH & SMALLIDGE (1986). Ambas as pesquisas usaram coluna C18 para analisar diversos carotenóides, incluindo α-caroteno, β-caroteno, β-criptoxantina e zeinoxantina (o último foi analisado somente por QUACKENBUSH & SMALLIDGE (1986). FISHER & ROUSEFF (1986) concluíram que o método era rápido

e preciso, sendo adequado para a análise de rotina da maioria dos carotenóides não polares em suco de laranja. QUACKENBUSH & SMALLIDGE (1986) excluíram, pela primeira vez, zeinoxantina como contaminação da β-criptoxantina e incluíram a medida da contribuição relativa de α-caroteno e *cis* e *trans*-β-caroteno no teor de carotenóides de alimentos e ração, melhorando substancialmente a confiabilidade dos dados do conteúdo de vitamina A.

PHILIP *et al.* (1989) utilizaram um sistema similar de cromatografia líquida para analisar diversos carotenóides em suco de laranja, com o objetivo principal de determinar adulteração. A fim de criar picos padrões, uma série de 32 picos foi escolhida como representativa de suco de laranja autêntico, para análise padrão por computador.

ROUSEFF *et al.* (1996) publicaram um trabalho onde foi registrada a presença de 39 carotenóides em suco de laranja. Os autores utilizaram uma coluna C30 de fase reversa para separar estes carotenóides, usando como fase móvel um gradiente de água, metanol e *tert*-butil éter. De acordo com os autores, as principais vantagens deste método eram a maior rapidez da análise e resolução do sistema cromatográfico. Não foram, entretanto, apresentados dados quantitativos.

Cromatografia líquida em fase reversa não-aquosa (FRNA) também tem sido utilizada por diversos autores na análise de carotenóides em diferentes substratos (NELIS & DE LEENHEER, 1983; BUSHWAY, 1986; FISHER & ROUSEFF, 1986; HEINONEN, *et al.*, 1989; HANDELMAN, *et al.*, 1992; CHEN, 1992; EPLER *et al.*, 1993; LIN & CHEN, 1995; HART & SCOTT, 1995). No entanto, poucas publicações citam o uso de modificadores na fase móvel para evitar a degradação dos carotenóides na coluna analítica. HANDELMAN *et al.* (1992) utilizaram uma mistura de acetato de amônio (0,01%) na fase móvel e EPLER *et al.* (1993) usaram acetato de amônio 0,05 mol/L dissolvido em metanol e 0,05% de trietilamina em cada solvente. De acordo com os últimos autores, a adição da mistura de acetato de amônio e trietilamina na fase móvel

resultava em 94% de recuperação dos carotenóides estudados. HART & SCOTT (1995) adicionaram um terceiro componente na fase móvel (0,1% BHT, hidroxitolueno butilado), a fim de obter 100% de recuperação dos carotenóides na coluna analítica. Os autores sugeriram que a principal função do acetato de amônio e da trietilamina seria minimizar os efeitos da acidez provocada pelos grupos silanóis livres, acetonitrila e acetato de etila (HANDELMAN *et al.*, 1992; EPLER *et al.*, 1993; HART & SCOTT, 1995).

Outros métodos propostos para a extração de carotenóides em frutas e suco de frutas, utilizaram na sua maioria, o processo de saponificação antes da extração dos carotenóides (GROSS *et al.*, 1971; ROTSTEIN *et al.*, 1972; STEWART, 1977a; BUSHWAY, 1986; QUACKENBUSH & SMALLIDGE, 1986; LIN & CHEN, 1995; HART & SCOTT, 1995; ROUSEFF *et al.*, 1996). Segundo ZAKARIA *et al.* (1979) e De RITTER & PURCELL (1981), o principal objetivo da saponificação é uma purificação dos carotenóides, eliminando principalmente gorduras, ácidos graxos, com liberação dos ésteres de carotenóides e clorofila, além de resultar em um cromatograma simplificado (FISHER & ROUSEFF, 1986).

Entre os métodos que utilizam saponificação na análise de carotenóides, a mistura KOH/MEOH é a mais amplamente usada (REEDER & PARK, 1975; STEWART, 1977a, b; FISHER & ROUSEFF, 1986; HEINONEN *et al.*, 1989; LIN & CHEN, 1995; HART & SCOTT, 1995; ROUSEFF *et al.*, 1996), seguida pela mistura KOH/ETOH (GROSS *et al.*, 1971, 1972; BUREAU & BUSHWAY, 1986; QUACKENBUSH & SMALLIDGE, 1986). Quando a saponificação não foi utilizada, os seguintes solventes de extração foram empregados: tetrahidrofurano (BUREAU & BUSHWAY, 1986; BUSHWAY, 1986), ou uma mistura de hexano, acetona e etanol (ROUSEFF *et al.*, 1992).

A Tabela 3 apresenta dados da literatura sobre a presença de carotenóides em suco de laranja.

Tabela 3. Níveis (mg/L) de carotenóides em laranjas e sucos de laranja.

Variedade/ Tipo	β-Criptoxantina	α-Caroteno	β-Caroteno	Luteína	Referência
Valência	1,08 - 1,22	0,11 - 0,15	0,14 - 0,23	na	REEDER & PARK, 1975
Diversas variedades	0,005 - 4,66	0,006 - 0,11	0,007 - 2,375	na	STEWART, 1977a
SLC	0,10 - 0,70	0,042 - 0,126	0,044 - 0,136	na	STEWART, 1977b
SLC	0,38*	0,064*	0,095*	na	QUACKENBUSH & SMALLIDGE, 1986
Suco de laranja	0,019*	nd	0,029*	0,28(a)*	HEINONEN *et al.*, 1989

SLC = suco de laranja concentrado
nd = não detectado
na = não analisado
a = luteína + zeaxantina
* = assumindo para o suco de laranja densidade de 1,05 g/mL

1.3.4. Análise da Razão de Isótopos Estáveis

Na natureza, os átomos de carbono-12, hidrogênio-1 e oxigênio-16 estão acompanhados de pequenas proporções de seus isótopos estáveis (Tabela 4), e tem sido mostrado que a abundância destes isótopos varia de acordo com a espécie da planta, tornando possível a classificação dos produtos em diversos subgrupos, de acordo com a planta da qual foram obtidos (MARTIN *et al.*, 1991).

1.3.4.1. Carbono e oxigênio

A análise isotópica de açúcares e água de suco de frutas tem sido utilizada como uma técnica para detectar adulteração em diversos tipos de sucos pela adição ilegal de adoçantes e água de torneira. Isto só é possível porque os açúcares e água tem

Tabela 4. Composição isotópica média de produtos orgânicos (MARTIN *et al.*, 1991).

	Hidrogênio			**Carbono**			**Oxigênio**		
Átomo	^{1}H	^{2}H	^{3}H	^{12}C	^{13}C	^{14}C	^{16}O	^{17}O	^{18}O
número atômico	1	1	1	6	6	6	8	8	8
massa atômica	1	2	3	12	13	14	16	17	18
Proporção (%)	99,985	0,015	(a)	98,904	1,096	(b)	99,763	0,037	0,2

(a): radioativo (praticamente inexistente no estado natural)
(b): radioativo (muito pouco abundante)

composições isotópicas que dependem da origem da planta e da origem geográfica, respectivamente (NISSENBAUM *et al.*, 1974; SMITH, 1975). A razão $^{13}C/^{12}C$ do açúcares de frutas, por exemplo, difere daquelas originadas dos açúcares de cana e milho, e esta diferença não é detectada por análises químicas tradicionais, como cromatografia líquida, ou por técnicas enzimáticas. A origem desta diferença se origina da maneira como o açúcar é sintetizado pela planta. Açúcar de cana e milho pertencem ao grupo das plantas que usam o ciclo C4, ou ciclo de Hatch-Slack para a síntese de açúcares. Durante a fotossíntese do ciclo C4, a enzima fosfoenolpiruvato carboxilase é utilizada pela planta. Este sistema enzimático não discrimina significativamente contra o isótopo mais pesado do carbono (composição do ar 98,9% ^{12}C; 1,1% ^{13}C). Açúcar de beterraba e de citrus usam o ciclo Calvin ou C3, que utilizam a enzima ribulose bifosfato carboxilase. Este sistema enzimático discrimina contra o ^{13}C em uma extensão muito maior que o ciclo C4 (O'LEARY, 1988). Como resultado, diferentes quantidades de ^{13}C são encontradas em produtos derivados dos ciclos C3 e C4. Valores típicos de $\delta^{13}C$ ‰ (definido como delta carbono 13 por mil) para suco de laranja e açúcar são de -26,4‰ e -11,4‰, respectivamente (BRICOUT & KOZIET, 1985). Estes valores são expressos em relação ao $\delta^{13}C$ do padrão PDB (Pee Dee Belemnita, formação Pee Dee da Carolina do Sul, E.U.A., CRAIG, 1957), que é de 0,011237. Açúcar de beterraba não pode ser

diferenciado por esta técnica, uma vez que o mesmo possuí um valor de $\delta^{13}C$ de -24,4‰, muito similar ao açúcar de citrus (SMITH, 1975).

A maioria dos vegetais utiliza o ciclo de Calvin e possuí valores de $\delta^{13}C$ de -22 a -33 *per mil*, enquanto as plantas que utilizam o ciclo Hatch-Slack possuí valores entre -10 e -20 *per mil* (BENDER, 1971; SMITH & EPSTEIN, 1971) (Tabela 5).

Tabela 5. Razões isotópicas de carbono em amostras diversas (ROBARDS & ANTOLOVICH, 1995).

Amostra	$\delta^{13}C$ ‰
PDB	0
dióxido de carbono atmosférico	- 7
produtos de plantas C3 (ex.: laranja, açúcar de beterraba)	- 22 a - 33
produtos de plantas C4 (ex.: açúcar de cana ou de milho)	- 9 a - 15
suco de laranja	- 24,5
açúcar de milho invertido	- 9,7

Para a determinação da relação $^{13}C/^{12}C$ em suco de frutas é necessária a transformação dos açúcares, por combustão, em dióxido de carbono (CO_2), e posterior análise dos gases ($^{13}C^{16}O_2$ e $^{12}C^{16}O_2$) em espectrômetro de massas. A quantidade relativa de $^{13}C/^{12}C$ é determinada de acordo com a equação 1:

$$\delta\,^{13}C_{PDB}\,‰ = \left(\frac{R_{amostra}}{R_{padrão}} - 1\right) x1000 \qquad (1)$$

onde o padrão é o dióxido de carbono formado da rocha calcária chamada PDB, e R é a razão entre o $^{13}C/^{12}C$ da amostra e padrão. Valores negativos (ie, inferiores ao do padrão) significam que as plantas possuem uma quantidade menor de $\delta^{13}C$ em relação ao padrão PDB.

Água (H_2O) de suco de citrus, como citado anteriormente, também possui propriedades isotópicas particulares. O isótopo mais pesado do oxigênio (^{18}O), que representa cerca de 0,2 % do oxigênio total do ar , torna-se mais enriquecido em água de plantas devido ao fenômeno da evapotranspiração. Portanto, é possível distinguir entre água de torneira e água de suco de fruta. Similarmente à análise de $^{13}C/^{12}C$, a quantidade relativa de $^{18}O/^{16}O$ é determinada de acordo com a equação 2:

$$\delta\ ^{18}O_{SMOW}\ ‰ = \left(\frac{R_{amostra}}{R_{padrão}} - 1\right) x1000 \qquad (2)$$

onde o padrão é o SMOW ("Standard Mean Ocean Water" = 0,00015576, padrão da média das águas do oceano) definido por CRAIG (1961) e R é a razão entre $^{18}O/^{16}O$ entre amostra e padrão.

A análise de isótopos estáveis tem sido usada desde o início dos anos 70 para a detecção de adulteração em suco de fruta. BRICOUT (1971 e 1973), BRICOUT *et al.* (1972) propuseram o uso do isótopo leve (^{16}O) e pesado (^{18}O) de oxigênio para a distinção entre suco de laranja reconstituído e concentrado. NISSENBAUM *et al.* (1974) estudaram a presença de deutério (D), ^{18}O e ^{13}C em sucos de frutas (suco de laranja e grapefruit), a fim de se detectar a adição de água corrente e açúcar (açúcar de beterraba ou cana) em suco natural. Os autores concluíram que a razão $^{18}O/^{16}O$ poderia ser utilizada para detectar a adição de água corrente ($\delta^{18}O$ = -4,5 ‰) em suco de laranja fresco ($\delta^{18}O$ = +3,28 ‰) enquanto que açúcar de cana ($\delta^{13}C$ = -12,2 ‰) poderia ser detectado pelo valor de ^{13}C no açúcar de laranja ($\delta^{13}C$ = -24,3 ‰). Açúcar de beterraba ($\delta^{13}C$ = -24,3 ‰) não pode ser detectado por este método devido à similaridade dos valores obtidos de $\delta^{13}C$ entre os dois açúcares.

DONNER & BILLS (1981) determinaram a razão entre ^{13}C e ^{12}C em 42 amostras de suco de laranja. Os autores concluiram que esta era a menor variação encontrada

naquela época para qualquer componente ou propriedade física do suco de laranja. Os valores obtidos ($\delta^{13}C$) variaram entre -23,4 a -25,6 ‰, desvio padrão de 0,591. No ano seguinte, o método de espectrometria de massas de $^{13}C/^{12}C$ foi adotado como oficial para a detecção de açúcar invertido de xapore de milho em suco de laranja (DONNER & BILLS, 1982).

BRAUSE *et al.* (1984) analisaram diversos componentes de suco de frutas, a fim de verificar a autenticidade do suco de laranja. A análise isotópica realizada incluia a determinação de ^{13}C e ^{18}O em inúmeros produtos. De acordo com os autores, os valores de $\delta^{13}C$ deveriam ser menores que -22 ‰ e os de $\delta^{18}O$ deveriam ser maiores que +1 ‰ para que o suco pudesse ser considerado autêntico.

BRICOUT & KOZIET (1985) analisaram diversos produtos que poderiam ser utilizados para adulterar suco de laranja, como por exemplo, açúcar de cana e beterraba e açúcar de milho invertido. De acordo com os autores a análise isotópica de deutério (em ésteres de nitrato) poderia ser utilizada para detectar a adição de açúcar de milho invertido e de açúcar de cana em suco de laranja.

Em trabalho similar, os mesmos autores (BRICOUT & KOZIET, 1987) analisaram diversos lotes de açúcar de beterraba produzida em diversas regiões (Israel, Canadá, E.U.A. e França) e amostras de açúcar de cana (proveniente da França, E.U.A. e Israel) para caracterizar estes produtos e possibilitar sua detecção em suco de laranja. Os resultados mostraram a influência da região e, consequentemente, do clima, na quantidade de deutério (em ésteres de nitrato) em açúcar de beterraba. Conforme relatado, os valores encontrados variaram de acordo com a latitude. No Canadá por exemplo o valor foi de $\delta D = -160$ ‰, enquanto que no Texas o δD determinado foi de -109 ‰. A quantidade de δD (em ésteres de nitrato) em amostras de suco de laranja variou entre -49 a -2 ‰, com uma média de -22,1 ‰ (DP = 10) e o valor de $\delta^{13}C$ variou entre -28,1 e -23,8 ‰, com uma

média de -25,1 ‰ (DP = 0,9). Açúcar de cana também foi analisado e os seguintes valores foram obtidos: $\delta^{13}C$: -11,7 a -11,2 ‰ e de -41 ‰ para δD.

No mesmo ano, trabalho similar foi publicado por DONER *et al.* (1987). Os autores determinaram a faixa de $\delta^{18}O$ e de δD para açúcar de beterraba e laranja. Os valores médios encontrados foram de +27,8 ‰ e -143 ‰ e de +34,9 ‰ e -27 ‰, respectivamente. Com base nos valores de $\delta^{18}O$ e δD em açúcar genuíno de suco de laranja, uma fórmula discriminatória foi desenvolvida, a fim de detectar com 99,99% de confidência a adição ilegal de açúcar de beterraba em suco de laranja.

Em 1993 KOZIET *et al.* publicaram um trabalho colaborativo para a determinação de C13 em açúcares de frutas e sucos vegetais. Os resultados mostraram boa reprodutibilidade e repetibilidade entre os 15 laboratórios participantes, e concluíram que a introdução desta técnica na área de controle de qualidade de suco de frutas aumentaria o controle de autenticidade dos mesmos.

Além de detectar adulteração em suco de laranja esta técnica também tem sido utilizada em outros alimentos e bebidas como por exemplo suco de maçã (DONER *et al.*, 1980; DONER & PHILLIPS, 1981; BRAUSE & RATERMAN, 1982; LEE & WROLSTAD, 1988, PILANDO & WROLSTAD, 1992; ROßMANN & TRIMBORN, 1996); aromas (CULP & NOAKES, 1992); mel (WHITE & WINTERS, 1989; WINKLER & SCHIMIDT, 1980); azeite de oliva (BIANCHI *et al.*, 1993); suco de tomate (MARELL *et al.*, 1978); vinagre (SCHMID *et al.*, 1978; SCHMID *et al.*, 1981); vinhos (ROßMANN *et al.*, 1996); saquê (MARTIN *et al.*, 1983); uísque (SIMPKINS & RIGBY, 1982); alimentos e bebidas diversas (WINKLER & SCHIMIDT, 1980) e diferentes tipos de frutas (KRUEGER *et al.*, 1986).

1.3.4.2. Deutério (em etanol)

No início dos anos 80, MARTIN *et al.* (1982 e 1983) propuseram uma nova metodologia para a distinção de determinados tipos de açúcares. A análise utiliza o etanol obtido da fermentação de várias fontes de carboidratos, como milho, açúcar de cana, beterraba, arroz, batata e maçã entre outros. O método se baseia na medida da razão entre deutério e hidrogênio (D/H) no grupo metil [definido como $(D/H)_I$] e metileno [definido como $(D/H)_{II}$] do etanol originado da fermentação dos açúcares. A medida é realizada por espectroscopia de Ressonância Magnética Nuclear (RMN) de deutério. Os autores descobriram que as razões $(D/H)_I$ do etanol refletiam a origem dos açúcares e eram características da planta de origem. Por exemplo, etanol de maçã tem um $(D/H)_I$ de 100,9 ppm e etanol de açúcar de beterraba tem um $(D/H)_I$ de 94,1 ppm (MARTIN *et al.*, 1982). Portanto, se açúcar de beterraba for adicionado a suco de maçã ou suco de laranja (plantas C3) este método, denominado Fracionamento Isotópico Natural de Posição Específica por Ressonância Magnética Nuclear (FINPE-RMN), detectará esta adulteração (MARTIN *et al.*, 1996a). As proporções relativas de $(D/H)_I$ e $(D/H)_{II}$ são determinadas pelas equações 3 e 4 (MARTIN *et al.*, 1996b), conforme apresentadas a seguir:

$$\left(\frac{D}{H}\right)_I = 1{,}5866 \times T_I \times \frac{m_{st}}{m_A} \times \frac{\left(\frac{D}{H}\right)_{st}}{t_m^D} \qquad (3)$$

$$\left(\frac{D}{H}\right)_{II} = 2{,}3799 \times T_{II} \times \frac{m_{st}}{m_A} \times \frac{\left(\frac{D}{H}\right)_{st}}{t_m^D} \qquad (4)$$

onde:

T_I = [altura do sinal I (CH_2DCH_2OH) / altura do sinal da TMU]

T_{II} = [altura do sinal II (CH_3CHDOH) / altura do sinal da TMU]

$(D/H)_{st}$ = razão isotópica da TMU fornecida pela fabricante (ex. 132,83 ppm)

m_{st} = massa da TMU

m_A = massa da amostra

t_m^D = teor alcoólico em massa do destilado

TMU = tetrametiluréia

Na literatura existe um número restrito de trabalhos publicados que utilizaram a técnica de FINPE-RMN para a detectação ilegal de açúcar em sucos de frutas. TATEO & MARTIN (1991) analisaram diversos tipos de suco de frutas italianas, incluindo suco de laranja, e os resultados das análises isotópicas mostraram que as amostras de suco de laranja tinham sido adulteradas com adição de açúcar externo, não sendo especificado qual tipo de açúcar.

MARTIN *et al.* (1996a) analisaram diversas amostras de suco de laranja natural e concentrado (de diferentes variedades e safras), originárias da Flórida, Brasil, Israel e algumas de origem desconhecida. Os resultados médios obtidos para $\delta^{13}C$ (em etanol) foram de -26,8; -26,5; -26,6; -26,5 ‰, e de $(D/H)_I$ (em etanol) foram de 104,8; 104,3; 106,9; 105,0 ppm, respectivamente. No mesmo ano, este método foi adotado pela AOAC como método padrão para detecção de açúcar de beterraba em suco de fruta (MARTIN *et al.*, 1996b).

A Tabela 6 apresenta dados citados pela literatura na análise de ^{13}C e $(D/H)_I$ e ^{18}O em suco de laranja.

Tabela 6. Valores médios de razões isotópicas em suco de laranja.

Amostra	$\delta^{13}C‰$ média (DP[a])	$(D/H)_I$ média (DP)	$(D/H)_{II}$ média (DP)	$\delta^{18}O‰$ média (DP)	Referência
SLC autêntico:	- 26,4 (0,21)	104,3 (0,59) (‡)	125,9 (0,85) (‡)	na	MARTIN *et al.*
espremido no laboratório	- 26,6 (0,45)	104,2 (0,73) (‡)	125,0 (1,37) (‡)	na	(1996a)*
açúcar	- 28,1 a - 26,3	nd	nd	nd	BRICOUT &
polpa	- 28,1 a - 26,6	nd	nd	nd	KOZIET
					(1987)*
suco de laranja	- 26,4	nd	nd	nd	BRICOUT & KOZIET (1985)*
suco de laranja	- 25,7 (1,0)	nd	nd	nd	KRUEGER & REESMAN (1982)**
suco de laranja	- 25,6 a - 23,4	nd	nd	nd	DONER & BILLS (1981)**
suco de laranja	nd	nd	nd	+ 3,28 (0,85)	NISSENBAUM *et al.* (1974)**
suco de laranja	nd	nd	nd	(~) + 5,0	BRICOUT (1973)*
suco de laranja	nd	nd	nd	+ 5,0 a + 6,8	BRICOUT *et al.* (1972)*

a = desvio padrão; * = suco de laranja do Brasil; ** = suco de laranja de outros países; ‡ = resultados normalisados versus SMOW (standard mean ocean water); nd = não disponível; SLC = suco de laranja concentrado.

1.4. Referências Bibliográficas

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Capítulo 2

FLAVANONE GLYCOSIDES IN BRAZILIAN ORANGE JUICE

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FLAVANONE GLYCOSIDES IN BRAZILIAN ORANGE JUICE

SUMMARY

Authentic samples of oranges, frozen concentrated orange juice and pulp-wash, and retail samples of freshly squeezed orange juice and frozen concentrated orange juice have been collected in Brazil and analysed for the flavanone glycosides (FG) narirutin and hesperidin by reversed phase HPLC with UV detection at 280 nm. The juice from hand squeezed fruit gave narirutin and hesperidin concentrations of 16-142 mg/l and 104-537 mg/l, respectively. The ratio of hesperidin to narirutin showed varietal difference with Pera having the highest ratio (mean 8.3) and Baía the lowest (3.6). Frozen concentrated orange juice (after dilution to 12 °Brix) contained higher quantities of FG with narirutin ranging from 62 to 98 mg/l and hesperidin from 531 to 690 mg/l. In frozen concentrated orange juice pulp-wash the narirutin level ranged from 155 to 239 mg/l and hesperidin from 1,089 to 1,200 mg/l. The analysis of 23 samples of freshly squeezed juice from the Brazilian market place showed that the FG content of most samples (13 to 95 and 106 to 587 mg/l respectively for narirutin and hesperidin) was similar to those found for authentic ones, indicating that these orange juices were not adulterated.

INTRODUCTION

The fruit juice industry is one of the biggest agricultural businesses in the world, with a trade exceeding 10 billion (US) dollars (Patel, 1994). Brazil is the largest orange producer and is responsible for 80% of the international market for frozen concentrated orange juice (Robards & Antolovich, 1994). Most Brazilian orange juice is exported as concentrate but the Brazilian retail market is largely based on freshly squeezed orange juice (FSOJ) sold in various packaging and on fresh oranges which are available at relatively cheap prices in local markets. The Brazilian orange juice industry also recovers juice from the orange pulp by an aqueous extraction procedure. The material recovered, called pulp-wash, is widely used in drink manufacture. As might be expected, FSOJ commands higher prices than frozen concentrated orange juice (FCOJ) and pulp-wash. Therefore there is always the possibility of pulp-wash and/or FCOJ being substituted for FSOJ. Marketing this product as 100% pure orange juice, obtained from freshly squeezed oranges without the addition of sugar, water, acids or preservatives represents a fraud against the consumer and an economic loss for the honest processor.

At present there is little data available for flavanone glycosides (FG) in Brazilian orange juice. Mouly *et al.* (1994) have analysed FG in several samples of orange juice but only three were from Brazil. Other published data (Rouseff *et al.*, 1987) relate to varieties which are not produced in significant quantities in Brazil. The varieties Pera and Natal account for 71% of the oranges produced in Brazil (Steger, 1990). Data on the FG content of the Pera variety is available for oranges grown in Florida (Rouseff *et al.*, 1987). However, to our knowledge there is no data available on the levels of FG in the Natal variety or Brazilian Pera. In addition, data from the commercial samples produced in Brazil such as commercial frozen concentrated orange juice and pulp-wash and from retail market has not been previously reported.

FG have been used to detect admixtures of citrus juices such as adulteration of orange juice by grapefruit (Rouseff, 1988) or vice-versa. Mouly *et al.* (1994) used

factorial discriminant analysis to differentiate varieties of *Citrus sinensis* according to the amount of FG and through the presence of three unknown peaks. Kirksey *et al.* (1995) have used the ratio narirutin/hesperidin to detect the addition of pulp-wash in orange juice, a practice prohibited in several countries.

The current study provides FG data from authentic samples of the most important varieties of oranges produced and processed in Brazil. It has been used to asses the authenticity of orange juice samples from the Brazilian market place.

MATERIAL AND METHODS

Standards

Naringin was obtained from Sigma, hesperidin from Aldrich and narirutin from Apin Chemicals (Oxon, UK). Narirutin and naringin were dissolved in the mobile phase (water:acetonitrile:tetrahydrofuran:acetic acid, 80:16:3:1, v/v/v/v). Hesperidin was dissolved in dimethylformamide:water (2:1, v/v). Standards were prepared weekly by appropriate dilutions with chromatographic mobile phase to give concentrations ranging from 5.0 to 85.0 mg/l for hesperidin, 2.5 to 20.0 mg/l for narirutin, 4.0 to 20.0 mg/l for naringin.

Recovery studies

Reagent blanks were analysed for the presence of possible interferents and no extraneous peaks were observed. In order to ensure that the FG were correctly identified and quantified, samples were spiked with different concentrations of narirutin (26.4 to 76.8 mg/l), hesperidin (149.0 to 403.5 mg/l) and naringin (20.2 to 161.6 mg/l).

Quality control

An In-House Reference Material (IHRM) was prepared from a sample of well mixed orange juice (1l) divided into portions of 12 ml and stored in a domestic freezer until required. Initially 10 samples of the IHRM were analysed and the average and standard deviation (SD) were obtained for hesperidin and narirutin so as to establish the repeatability of the method. Subsequently, the IHRM was analysed with each batch to ascertain that the method was under control. To accept the batch, the value obtained for the IHRM had to be between the average ± 2 SD, otherwise the batch was rejected.

Samples

Authentic samples of oranges from different varieties as well as commercial concentrated orange juice and pulp-wash were collected from processing plants in the State of São Paulo (Brazil). Retail samples (frozen concentrated orange juice and freshly squeezed orange juice) were purchased from supermarkets in the metropolitan area of Campinas (State of São Paulo, Brazil), during the years of 1995/1996.

Sample preparation

The citrus fruits were hand squeezed and the juices filtered through a stainless steel sieve (1.25 mm). Frozen concentrated orange juice and frozen concentrated pulp-wash were diluted to 12 °Brix with Millipore water. Retail freshly squeezed orange juices were sieved before use. All of the samples were stored at frozen.

Sample analysis

The analyses were conducted according to Mouly *et al.* (1994), as follows.

The juice samples (5ml) were mixed with dimethylformamide (DMF) (10 ml) and ammonium oxalate (0.05 mol/l, 10 ml) and placed in a steam bath at 90 °C for 10 minutes. The samples were then cooled and the volume made up to 50 ml with Millipore water. The solutions were centrifuged at 10 °C until clear. The clarified juice was then filtered through Acrodisc filters (0.45 µm nylon, Gelman Sciences), and placed in a 2 ml ambered flask for liquid chromatography analysis.

High performance liquid chromatography (HPLC)

A C18 Nucleosil 5 µm (250x4.6 mm) column was used with a guard-column (Alltima C18 5µm, 7.5x4.6 mm) at room temperature. 20 µl injections were made using a solvent of water:acetonitrile:tetrahydrofuran:acetic acid (80:16:3:1, v/v/v/v) and a flow of 1 ml/min. FG were detected using UV at 280 nm.

The FG were identified in the samples by comparing the retention time with that of standards and quantified by comparing the integrated peak areas (Spectra Physics Integrator) with that of an external standard. Peak identity was confirmed by using a Spectra Focus Scanning Detector (Spectra Physics). This equipment takes spectra from 3 points at different times across the HPLC peak and compares these spectra. If these spectra are identical, the peak is considered pure; i.e. no interferents are present. A peak purity index is calculated automatically where 100% is perfectly pure. Typically in this work, peak purity indices of 97 -100% were achieved.

RESULTS AND DISCUSSION

Quality control

The repeatability of the method has been determined using data from the IHRM. 26 measurements gave means of 54.4 mg/l (SD = 2.2), CV = 4.0 % and 262.7 mg/l (SD = 6.7), CV = 2.6 % for narirutin and hesperidin, respectively. Table 1 shows the recoveries obtained for spiked samples. Means of 112.1 % (SD = 9.1), 106.5 % (SD = 6.6), 103.0 % (SD = 8.3) were established for narirutin, hesperidin and naringin respectively. No correction for recovery was therefore necessary. These recoveries are in accordance with those cited by Mouly *et al.* 1994. Reagent blanks demonstrated that no background interferents were present.

Table 1. Recovery of flavanone glycosides added to orange juice.

Flavanone glycosides	**n**	**Amount added (mg/l)**	**Amount found (mg/l)**	**Recovery (%)**
Narirutin	16	26.4 - 76.8	27.2 - 88.8	97.1 - 122.0 $\overline{X}$ = 112.1 (SD = 9.1)
Hesperidin	16	149.0 - 403.5	148.9 - 476.4	96.0 - 118.1 $\overline{X}$ = 106.5 (SD = 6.6)
Naringin	14	20.2 - 161.6	20.5 - 177.6	98.8 - 109.9 $\overline{X}$ = 103.0 (SD = 8.3)

n = number of spiked samples
SD = standard deviation

Authentic Samples

Freshly squeezed orange juice

The concentration of FG found for authentic samples of hand squeezed juice are shown in Table 2. The results proved variable. Narirutin and hesperidin concentrations ranged from 16.1 to 141.8 mg/l and 103.7 to 537.1 mg/l, respectively. The ratio hesperidin/narirutin varied from 3.2 to 11.3. As anticipated from previous literature, neither naringin nor neohesperidin were detected in any of the samples analysed (Galensa & Herrmann, 1980; Greiner & Wallrauch; 1984; Galensa *et al.*; 1986; Rouseff *et al.*, 1987; Mouly *et al.*, 1994; Ooghe *et al.*, 1994). The concentrations of the FG were similar to those cited by several authors for narirutin (18 to 120.7 mg/l) and hesperidin (122 to 379.3 mg/l) (Rouseff *et al.*, 1987; Mouly *et al.*, 1994; Ooghe *et al.*, 1994). Previous work has emphasised the differences which may occur due to variety and also to different extractor pressures (Fisher, 1978; Rouseff *et al.*, 1987; Kirksey *et al.*, 1995).

The following average ratios of hesperidin/narirutin were obtained for the varieties Pera, Natal, Valência, Hamlin, Baía and Lima: 8.3; 5.5; 5.5; 4.2.; 3.6; 6.4, respectively. The Pera variety presented the highest ratio among the samples analysed and proved sufficiently different from the rest of the samples to suggest that this represented a genuine varietal difference. The lowest hesperidin/narirutin ratio was found for the Baía variety. Ooghe *et al.* (1994) also reported low ratios from Baía although no information on the geographic origin of the samples was provided.

Rouseff *et al.* (1987) also provided information on the concentrations of FG in different orange varieties. Pera, Baía, Hamlin and Valência contained 41, 31, 27, 27 mg/l of narirutin and 208, 135, 122, 151 mg/l of hesperidin, respectively. These values are in good agreement with our own. No literature data are available for the Natal variety.

Table 2. Concentrations of flavanone glycosides in authentic samples of hand squeezed orange juice from Brazil.

Variety/ Sample			Narirutin (mg/l)	Hesperidin (mg/l)	Ratio (Hesperidin/Narirutin)
Pera	1		52.9	269.1	5.1
	2		19.7	212.1	10.8
	3		31.1	227.8	7.3
	4		30.8	252.7	8.2
	5		16.1	182.4	11.3
	6		20.0	132.9	6.6
	7		30.9	350.4	11.3
	8		62.4	398.6	6.4
	9		39.8	303.9	7.6
	10		26.4	228.2	8.6
		$\bar{x}$	**33.0**	**255.8**	**8.3**
Natal	1		43.7	294.7	6.7
	2		35.1	188.7	5.4
	3		24.0	103.7	4.3
	4		38.4	148.6	3.9
	5		33.5	243.0	7.3
		$\bar{x}$	**34.9**	**195.7**	**5.5**
Valência	1		79.7	291.4	3.7
	2		35.4	193.6	5.5
	3		40.4	218.7	5.4
	4		42.9	321.4	7.5
		$\bar{x}$	**49.6**	**256.3**	**5.5**
Hamlin	1		69.5	321.2	4.6
	2		72.9	342.3	4.7
	3		71.9	252.6	3.5
	4		141.8	537.1	3.8
		$\bar{x}$	**89.0**	**363.3**	**4.2**
Baia	1		68.8	264.9	3.9
	2		135.2	427.1	3.2
		$\bar{x}$	**102.0**	**346.0**	**3.6**
Lima	1		21.7	110.8	5.1
	2		29.4	223.1	7.6
		$\bar{x}$	**25.6**	**167.0**	**6.4**
			range: 16.1 - 141.8	**range**: 103.7 - 537.1	**range**: 3.2 - 11.3
			$\bar{x}$ **= 48.7**	$\bar{x}$ **= 260.8**	$\bar{x}$ **= 6.3**
			SD = 31.7	SD = 97.9	SD = 2.3

Frozen concentrated orange juice (FCOJ) and frozen concentrated orange pulp-wash (FCOPW)

Table 3 shows the results for FG in FCOJ. Narirutin and hesperidin concentrations were found to be much higher than those in hand pressed orange juice. The mechanical pressure exerted by industrial plant is greater than that which can be achieved manually and this leads to greater extraction of FG from the wall membrane and albedo (Fisher, 1978; Kirksey *et al.*, 1995). The amount of FG in FCOJ ranged from 62.4 to 97.8 (mg/l) and from 531.0 to 689.9 (mg/l) for narirutin and hesperidin, respectively. The ratios of hesperidin/narirutin (6.3 to 9.5) were within the upper range found for hand pressed orange juice, which was 3.2 to 11.3. The average ratio for hand pressed orange juice was 6.3 (SD 2.3) but for FCOJ it was 8.2 (SD = 1.2). The lower standard deviation found for the industrial product may be because this material is produced from a large amount of oranges of different varieties and origins. The composition of the final juice will therefore reflect the average composition. Similarly, the use of industrial extractors provides a consistent juicing pressure which will prevent variability occuring from this factor.

In FCOPW (Table 4) the amounts of FG found were much higher than those detected in FCOJ and in hand squeezed orange juice. The narirutin ranged from 154.8 to 239.1 mg/l and hesperidin from 1,089.3 to 1,200.4 mg/l. However the ratios were basically the same (5.0 to 7.1). Similar results were recorded by Greiner and Wallrauch (1984) for hesperidin but they did not report results for narirutin. They found that hesperidin concentrations of 6 samples of pulp-wash ranged from 624 to 1,031 mg/l with an average of 904 mg/l. They also determined hesperidin in FCOJ finding an average of 388 mg/l (range 211 to 642 mg/l).

Table 3. Concentrations of flavanone glycosides in frozen concentrated orange juice (FCOJ) from Brazil, diluted to 12 °Brix.

Variety		Narirutin (mg/l)	Hesperidin (mg/l)	Ratio (Hesperidin/Narirutin)
FCOJ	Hamlin	97.8	646.4	6.6
	Pera	62.4	591.2	9.5
	Pera	72.4	687.1	9.5
	Pera	71.2	613.1	8.6
	Pera	83.1	658.8	7.9
	Pera	79.8	689.9	8.6
	Pera	75.3	656.4	8.7
	*	84.0	531.0	6.3
		range: 62.4 - 97.8	range: 531.0 - 689.9	range: 6.3 - 9.5
		$\bar{x}$ = **78.3**	$\bar{x}$ = **634.2**	$\bar{x}$ = **8.2**
		SD = 10.6	SD = 53.6	SD = 1.2

* mixture of several varieties not identified by the producer

Table 4. Concentrations of flavanone glycosides in frozen concentrated orange pulp-wash (FCOPW) from Brazil, diluted to 12 °Brix.

	Narirutin (mg/l)	Hesperidin (mg/l)	Ratio (Hesperidin/Narirutin)
FCOPW 1*	239.1	1192.7	5.0
2*	180.5	1200.4	6.7
3*	175.0	1089.3	6.2
4*	154.8	1093.2	7.1
	range: 154.8 - 239.1	range: 1,089.3 - 1,200.4	range: 5.0 - 7.1
	$\bar{x}$ = **187.4**	$\bar{x}$ = **1,143.9**	$\bar{x}$ = **6.3**
	SD = 36.2	SD = 60.9	SD = 0.9

* varieties not identified by the producer

It has been postulated that the ratio hesperidin/narirutin in pulp-wash differs from that in orange juice, and this ratio could be used as a reference value to detect the addition of pulp-wash in orange juice (Kirksey *et al.*, 1995). According to these authors a ratio less than 2 should be considered suspect. Ooghe *et al.* (1994) stated that if the ratio hesperidin/narirutin is low, ranging from 2.52 to 3.93, the orange juice may contain pulp-wash. However, for Brazilian orange pulp-wash this method cannot be applied because the ratio between hesperidin and narirutin in orange juice and pulp-wash is similar. Nevertheless, the absolute concentration of FG present are much higher in pulp-wash than in orange juice (1,089.3 to 1,200.4 and 103.7 to 537.1 mg/l; 154.8 to 239.1 and 16.1 to 141.8 mg/l, respectively for hesperidin and narirutin). These relatively high levels of FG detected in pulp-wash could provide an indicator of addition of pulp-wash to freshly squeezed orange juice. Where the levels of narirutin and hesperidin are found in higher concentrations than for authentic samples, there is an indication that either pulp-wash, FCOJ or a mixture of both has been added to the freshly squeezed orange juice.

Retail samples

Retail frozen concentrated orange juice (RFCOJ)

Table 5 shows the results for FG in RFCOJ. The samples analysed showed a similar range of FG concentration (57.3 to 64.7 mg/l and 439.2 to 520.2 mg/l for narirutin and hesperidin, respectively) when compared to authentic FCOJ. Similarly, the ratio hesperidin/narirutin (7.5 to 9.1) was similar to those from authentic hand squeezed orange juice and commercial FCOJ. These figures suggest that these samples have not been adulterated with pulp-wash in amounts that could be detected by this method.

Table 5. Concentrations of flavanone glycosides in retail frozen concentrated orange juice (RFCOJ), from Brazil, diluted to 12 °Brix.

RFCOJ	Narirutin (mg/l)	Hesperidin (mg/l)	Ratio (Hesperidin/Narirutin)
1	58.6	439.2	7.5
2	64.7	511.3	7.9
3	57.3	520.2	9.1

Freshly squeezed orange juice (FSOJ)

Table 6 shows a summary of the results obtained for FG in retail FSOJ. 23 of the 24 samples analysed fell within the range established for authentic samples (narirutin 16.1 to 141.8 mg/l and hesperidin 103.7 to 537.1 mg/l). Similarly, the ratio hesperidin/narirutin found for 21 FSOJ fell within the range of authentic samples (3.2 to 11.3) while three others were just outside (11.4, 11.7, 11.9). One sample proved interesting. Although the ratio was within the authentic range, the concentration of both narirutin and hesperidin were both higher than the authentic range (94.8 and 586.6 mg/l respectively). This data suggest that this sample may contain either FCOJ or FCOPW or both. Other samples of the same brand were also analysed and the FG were within the authentic range. However all samples of this brand, and another brand showed the presence of an unknown peak eluting after hesperidin and a further investigation of these samples was conducted.

Identification of an unknown peak in two brands

The Scanning UV Spectra of this unknown peak showed a maximum absorption at approximately 260 nm which is completely different from those observed for the FG standards and authentic samples. It has been suggested by Robards & Antolovich (1995) that some preservatives such as sorbate, methyl and propylparabens could be added to orange juice. Several preservatives were therefore examined by LC and sorbic acid

matched the retention time. This preservative is used as a mold and yeast inhibitor (The Merck Index, 1989).

Table 6. Concentrations of flavanone glycosides in Brazilian retail freshly squeezed orange juice (FSOJ).

Retail FSOJ	**Narirutin (mg/l)**	**Hesperidin (mg/l)**	**Ratio (Hesperidin/Narirutin)**
n = 24	**range**: 13.1 - 94.8	**range**: 105.8 - 586.6	**range**: 2.6 - 11.9
	$\bar{x}$ **= 35.0**	$\bar{x}$ **= 266.3**	$\bar{x}$ **= 8.3**
	SD = 17.6	SD = 137.1	SD = 3.0

n = number of analysed samples

Sorbic acid was therefore chromatographed under the same conditions as used for FG analysis. The retention time matched that of the unknown peak and the scanning UV spectra also showed good agreement. To support this identification, sorbic acid and the sample were run on a second column (Hicarbosphere ODS, 3 µm, 150 x 4.6 mm) and the peak showed the same retention time for standard and sample. Finally the sample and a standard of sorbic acid was analysed by LC-APcI-MS (liquid chromatography - atmospheric pressure chemical ionisation - mass spectrometry) (VG Platform I). Both standard and sample provided a spectra which was predominantly a single ion mass 111.05 corresponding to the deprotonated molecular ion ($[M-H]^-$) confirming the identity as sorbic acid. The amount of sorbic acid detected in the samples ranged from 111.0 to 130.0 mg/l.

Although the Brazilian legislation (Act number 55871/65) allows the addition of 1,000 mg/l of sorbic acid to fruit juices this preservative must be declared on the label. In both cases neither of the two brands declared the addition of sorbic acid. The juices have been sold as 100 % pure orange juice obtained from fresh oranges.

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Capítulo 3

POLYMETHOXYLATED FLAVONES IN BRAZILIAN ORANGE JUICE

Trabalho submetido para publicação na revista Food Chemistry

POLYMETHOXYLATED FLAVONES IN BRAZILIAN ORANGE JUICE

SUMMARY

Sinensetin has been quantified in authentic samples of Brazilian orange juice. In addition the relative amounts of six further polymethoxylated flavones (PMFs) have been determined. The PMFs were extracted into toluene and analysed using reversed phase HPLC with detection at 340 nm. Peak identification was based on the UV-visible spectra and the elution order described in the literature. Hand squeezed orange juices contained from 0.06 to 0.12 mg/l sinensetin with the highest concentrations found in Pera and Natal varieties. Commercial samples of frozen concentrated orange juice (FCOJ), frozen concentrated pulp-wash (FCOPW), retail FCOJ and retail freshly squeezed orange juice (FSOJ) typically contained at least ten times more sinensetin than those found for samples squeezed by hand. The PMFs peak area ratios for these sample classes were examined further using canonical discriminant analysis. This procedure could distinguish the hand-squeezed juices of Pera and Hamlin varieties from those of Natal and Valência. Similarly hand squeezed juices could be readily distinguished from the commercial samples of FCOJ, FCOPW, retail FCOJ and retail FSOJ.

INTRODUCTION

Authentic orange juices are produced exclusively from the fleshy part of the orange, with no pulp-wash, sugar, preservatives, or other ingredients added (Vogels *et al.*, 1996). Brazil is the most important producer of orange juice in the world and is responsible for 80% of the international market in frozen concentrated orange juice (FCOJ) (Robards & Antolovich, 1994). FCOJ is not widely consumed in Brazil, due to the availability of fresh oranges at affordable prices. However, the retail market for freshly squeezed orange juice (FSOJ) has grown considerably in the last few years with many brands now available. The prices of FCOJ and pulp-wash are lower than FSOJ which makes them potentially applicable to the fraudulent extension of FSOJ. Similarly, other fruit juices may be potential adulterants when the season or market price offers the possibility of economic advantage.

Polymethoxylated flavones (PMFs) have been used in the past to identify and characterise several kinds of juices. Thin layer chromatography was the preferred technique in the early seventies. Velduhis *et al.* (1970) analysed five PMFs in FCOJ produced from 1952 to 1968. The main objective was to evaluate the taste threshold of these compounds in commercial FCOJ. Ting *et al.* (1979) used the technique of high performance liquid chromatography (HPLC) to analyse PMFs in tangerine and orange juices, and it was concluded that this method could be used to detect the presence of one juice in the other. Similarly Gaydou *et al.* (1987) differentiated orange and mandarin peel oil using PMFs. Ooghe *et al.* (1994) measured the composition of PMFs in several varieties of orange juice and used the relative peak area and the correspondent standard deviation to detect juice adulteration.

Data from PMFs in Brazilian orange juice are scarce. Heimhuber *et al.* (1988) analysed one sample of pulp-wash from Brazil. They indicated that adulteration of orange juice with peel or pulp-wash could be detected from differences in the amount and distribution of PMFs. Details of the country of origin of the samples analysed by Ooghe *et*

al. (1994) are not described although it is likely that some came from Brazil. Similarly, little data is available concerning orange juice in the retail market place.

The objective of this work was to determine the relative amounts of PMFs (as peak area ratios) for the most important varieties of oranges available in Brazil. In addition, commercial FCOJ, pulp-wash and samples from the Brazilian market place were analysed for comparative purposes.

MATERIAL AND METHODS

Standard

Sinensetin (Apin Chemicals, Oxon, UK) (5 mg) was dissolved in 50 ml of methanol. Chromatographic standards (prepared weekly) were obtained by diluting appropriate amounts of the concentrated solution. The concentrations for sinensetin ranged from 0.2 to 4.0 mg/l.

Quality control

An In-House Reference Material (IHRM) was analysed throughout the period of study to ascertain that the method was in control and to determine the reproducibility of the method. The IHRM was prepared from a sample of orange juice (1l) which was well mixed and divided in vials containing 12 ml each and these were frozen at - 20 °C until analysis. Initially eight analyses of the IHRM were performed and the average and standard deviation (SD) were obtained for the ratio of the six PMFs analysed (total of 10 results). For each batch of samples (usually 12 samples) a sample of IHRM was analysed together. To accept the batch, the value obtained for the IHRM should be between the average ± 2 SD, otherwise the analysis was rejected.

Samples

Authentic samples of oranges from different varieties and commercial concentrated orange and pulp-wash were obtained from producers in the State of São Paulo (Brazil). Retail samples (frozen concentrated orange juice and freshly squeezed orange juice) were purchased from supermarkets in the metropolitan area of Campinas (State of São Paulo, Brazil), during the years 1995/1996.

Sample preparation

The citrus fruits were hand squeezed and the juices filtered through a stainless steel sieve (1.25 mm). Frozen concentrated orange juice and frozen concentrated pulp-wash were diluted to 12 °Brix with Millipore water. FSOJ was sieved before using. All of the samples were stored at - 20 °C.

Sample analysis

The analyses of the samples were conducted following the principles of the method described by Ooghe *et al.* (1994) with modifications as described below.

Samples of orange juice (5 ml) were transferred to a 25 ml centrifuge tube. Five ml of toluene were added and the solution mixed in a Vortex at high speed for 10 seconds, following centrifugation at 3000 rpm for 10 min at 10 °C. The upper phase was pipetted off and the extraction step was repeated twice. The organic fractions were pooled and evaporated under a nitrogen stream at approximately 50 °C. The residue was dissolved in 1 ml methanol and injected in the chromatographic system.

Samples of concentrated juice were diluted to 12° Brix and then treated as for single strength juice. The concentration of sinensetin was recorded after dilution to permit comparison between concentrated and single strength juices.

High Performance Liquid Chromatography (HPLC)

The HPLC apparatus consisted of a Waters 625 LC System, autosampler Gilson 231 XL and a Spectra Focus UV-Vis detector (Spectra Physics). A 20 µl loop was used for injection. Solvents were HPLC grade. The mobile phase was a ternary mixture of water:acetonitrile:tetrahydrofuran (53:43:4, v/v/v). The column was a C18 Nucleosil 5µm (250x4.6 mm id., Alltech) with a guard-column Alltima C18 5µm (7.5x4.6 mm id., Alltech). The column was kept at room temperature (± 22 °C) and the flow rate was 0.7 ml/min. The wavelength was adjusted to 340 nm. The peak areas were determined using a

Spectra Physics integrator. Peak identities were confirmed by a Spectra Focus Scanning Detector (Spectra Physics), under the same conditions described above.

Peak identification was based on the elution order cited by the literature (Ting *et al.*, 1979; Rouseff & Ting, 1979; Ooghe *et al.*, 1994), and by comparing the UV spectra of PMFs with those in the literature (Sendra *et al.*, 1988).

RESULTS AND DISCUSSION

Sample preparation and high performance liquid chromatography

The procedure used in this work showed some improvements in relation to those published previously (Veldhuis *et al.*, 1970; Rouseff & Ting, 1979; Ting *et al.*, 1979; Ooghe *et al.*, 1994). PMFs have been extracted from orange juice using benzene (Veldhuis *et al.*, 1970; Rouseff and Ting, 1979; Ooghe *et al.*, 1994) and chloroform (Ting *et al.*, 1979). However it is desirable to use extraction solvents of low toxicity where possible. In the present study toluene was used because it is less toxic than benzene and chloroform (The Sigma Aldrich Library of Chemical Safety Data, 1985). Secondly, the chromatographic separation was conducted using an isocratic system that separated all PMFs (Figure 1), which is much simpler than using a gradient system.

Peak purity and identification

The peak identities were based on the elution order cited by several authors (Ting *et al.*, 1979; Rouseff & Ting, 1979; Ooghe *et al.*, 1994). A scanning UV detector (Spectra Physics) was used to characterise and identify the PMFs (Sendra *et al.*, 1988). The order of elution was: peak I: sinensetin, II: quercetogetin, III: nobiletin, IV: heptamethoxyflavone, V: scutellarein, VI: tangeretin. When samples were analysed, five UV spectra were taken across each peak. The good agreement of these spectra indicated that no UV absorbing species were coeluting with the species of interest.

Quantitation

Sinensetin showed a linear response within the range studied: 0.2 to 4.0 mg/l ($r = 0.999$ or better). Samples were quantified against this external standard. Unfortunately the absence of available standards for other PMFs prevented their quantitative measurement. Ooghe *et al.* (1994) overcome this problem by describing the PMF content of his samples using their relative peak areas and we have adopted the same practice in order to permit a comparison of data.

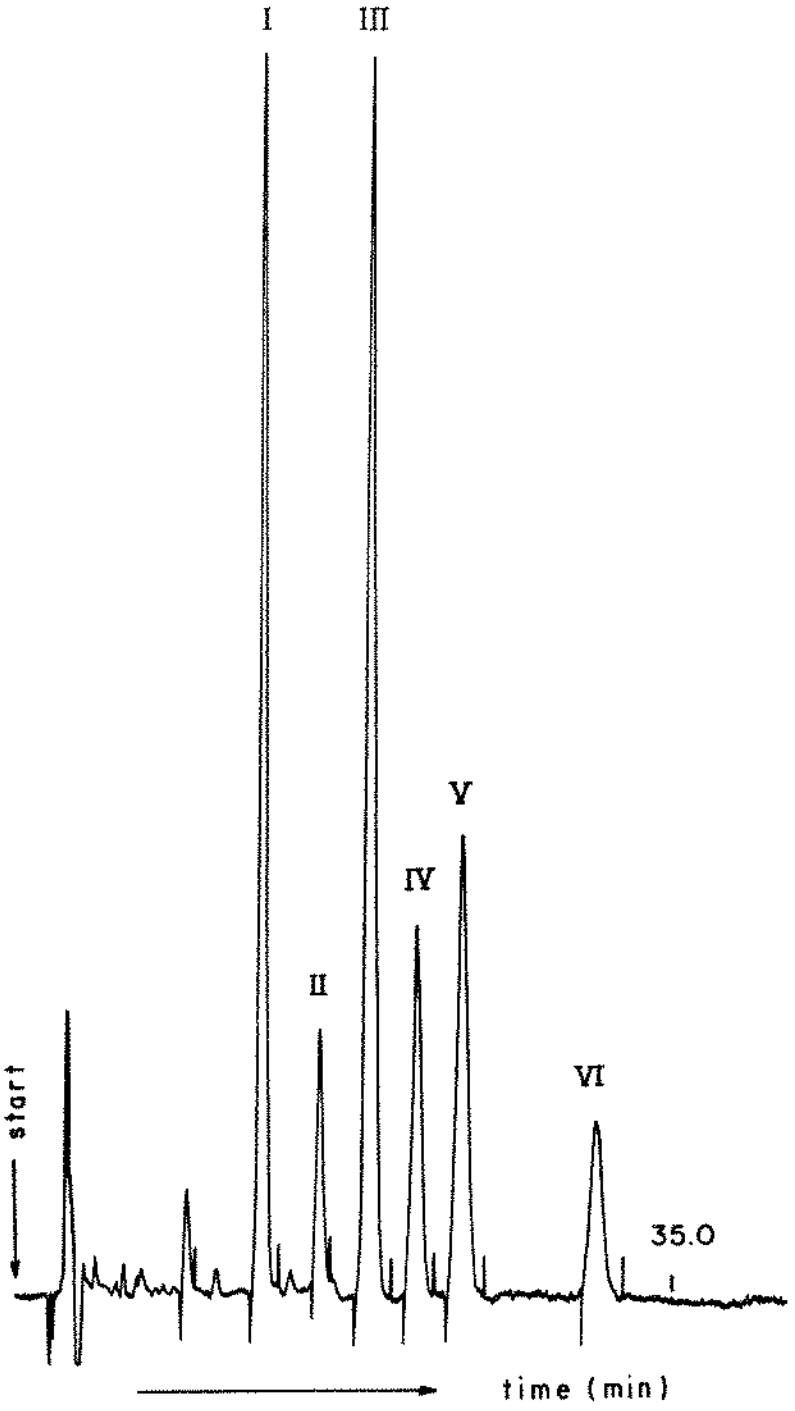

Figure 1. HPLC of PMFs from an orange juice extract. Chromatographic conditions: Column C18 Nucleosil 5 µm (250x4.6 mm id, Alltech), pre-column C18 5µm (7.5x4.6 mm id. Alltech), mobile phase water:acetonitrile:tetrahydrofuran (53:43:3), flow 0.7 ml/min., UV 340 nm. Peak I sinensetin, II quercetogetin, III nobiletin, IV heptamethoxyflavone, V scutellarein, VI tangeretin.

Quality control

Table 1 provides data on the precision of the analytical method from 18 determinations of an IHRM measured throughout the period of study. The SD ranged from 0.06 to 0.12 for the ratios measured and was considered satisfactory. This range no doubt reflects the differences in concentration of the individual compounds. No background interference was observed from reagent blanks.

Table 1. Relative peak areas of PMFs and level of sinensetin (mg/l) for the IHRM.

	n	I / II	I / IV	I / VI	III / I	III / IV	III / V	III / VI	IV / V	IV / VI	VI / II	Sinensetin
$\bar{x}$	18	3.69	2.91	3.60	1.39	4.08	1.99	4.95	0.49	1.22	1.03	1.96
SD		0.13	0.11	0.14	0.02	0.09	0.02	0.04	0.02	0.04	0.02	0.07

n= number of samples analysed

peak I: sinensetin, II: quercetogetin, III: nobiletin, IV: heptamethoxyflavone, V: scutellarein, VI: tangeretin

Authentic, commercial and retail samples of orange juice

Table 2 provides data from hand squeezed oranges. The levels of sinensetin ranged from 0.04 to 0.18 mg/l. Similar quantities were found by Sendra *et al.* (1988) who reported a mean value of 0.09 mg/l. It is apparent from the data that sinensetin concentrations differ with plant variety. Pera, Natal, Baía and Lima varieties gave almost double the concentration of sinensetin when compared with Valência and Hamlin. Varietal differences in peak ratios were also observed. For example, the peak ratio VI/II for variety Pera was lower than that of Natal, Valência, Hamlin and Baía varieties.

Table 2. Relative peak areas of PMFs and sinensetin levels (mg/l) in authentic hand squeezed samples of orange juice (see Table 1 for peak identification).

		I / II	I / IV	I / VI	III / I	III / IV	III / V	III / VI	IV / V	IV / VI	VI / II	Sinensetin
Pera n = 6	$\bar{x}$	**2.07**	**4.06**	**6.20**	**1.49**	**6.00**	**1.46**	**9.34**	**0.24**	**1.53**	**0.34**	**0.12**
	SD	0.27	0.59	1.25	0.26	1.15	0.48	3.22	0.04	0.20	0.06	0.04
Natal n = 4	$\bar{x}$	**2.71**	**2.02**	**2.24**	**2.13**	**4.20**	**1.65**	**4.67**	**0.41**	**1.11**	**1.32**	**0.12**
	SD	0.50	0.64	0.73	0.20	0.86	0.43	1.01	0.15	0.07	0.51	0.06
Valência n =3	$\bar{x}$	**2.54**	**2.54**	**3.36**	**1.68**	**4.25**	**1.20**	**5.70**	**0.28**	**1.36**	**0.81**	**0.07**
	SD	0.82	0.30	0.68	0.09	0.28	0.18	1.49	0.03	0.45	0.36	0.02
Hamlin n = 3	$\bar{x}$	**4.62**	**1.85**	**2.46**	**2.80**	**5.12**	**1.59**	**6.63**	**0.32**	**1.33**	**1.85**	**0.06**
	SD	1.99	0.19	0.64	0.66	0.83	0.59	0.42	0.14	0.28	0.47	0.03
Baia n = 1		**4.19**	**3.09**	**5.10**	**1.25**	**3.86**	**1.65**	**6.37**	**0.43**	**1.65**	**0.82**	**0.11**
Lima n = 1		**1.90**	**3.79**	**5.26**	**1.11**	**4.20**	**1.06**	**5.82**	**0.25**	**1.39**	**0.36**	**0.10**

n = number of samples analysed

Table 3 indicates that FCOJ, after dilution to 12° Brix, contained considerably more sinensetin than was found in the hand squeezed juices with concentrations ranging from 1.27 to 2.36 mg/l. This effect is undoubtedly due to the increased extraction pressure which commercial equipment is able to exert. Several authors have emphasised the influence of extraction pressure on juice composition including the hesperidin content, pectin content and UV polyphenolic absorption (Petrus & Dougherty, 1973; Gherardi *et al.*, 1980; Cohen *et al.*, 1984; Kirksey *et al.*, 1995). Kanes *et al.* 1993 have indicated that PMFs are found in high concentration in the peel and in low concentration in the juice.

Table 3. Relative peak areas of PMFs and sinensetin levels (mg/l) in authentic samples of frozen concentrated orange juice (FCOJ) and in authentic samples of frozen concentrated pulp-wash (FCOPW), both diluted to 12 °Brix (see Table 1 for peak identification).

		I / II	I / IV	I / VI	III / I	III / IV	III / V	III / VI	IV / V	IV / VI	VI / II	Sinensetin
FCOJ n = 3	$\bar{x}$	**4.69**	**2.59**	**3.39**	**1.58**	**4.06**	**2.67**	**5.31**	**0.66**	**1.32**	**1.40**	**1.84**
	SD	0.36	0.36	0.40	0.13	0.39	0.19	0.21	0.06	0.15	0.26	0.55
FCOPW n = 2	$\bar{x}$	**4.47**	**3.21**	**5.07**	**1.51**	**4.79**	**2.78**	**7.61**	**0.58**	**1.60**	**0.88**	**1.30**
	SD	0.03	0.58	0.34	0.17	0.31	0.02	0.37	0.03	0.18	0.05	0.19

n = number of samples analysed

The concentrations of sinensetin given in Table 3 are in agreement with previous reports for FCOJ. Veldhuis *et al.* (1970) found levels varying from 0.50 to 2.05 mg/l of sinensetin using thin layer chromatography. Sendra *et al.* (1988) determined sinensetin concentrations in composite samples of FCOJ. A mean value of 0.8 mg/l was obtained for samples from two factories having a "low" level of PMFs and a mean value of 2.9 mg/l from those having a "high" level of PMFs. According to the authors this difference was due to the type of extractor which the factories employed to produce the juice. The peak ratios for FCOJ showed fair agreement when compared to authentic samples of hand squeezed orange. However peak area ratios I/II was lower for Pera, Natal and Valência hand squeezed juices and ratio III/V was consistently lower for all hand squeezed juices.

PMFs in FCOPW (Table 3) proved very similar to FCOJ with, for instance, no significant observable differences in sinensetin concentrations. However some differences in relative amounts of the PMFs are apparent from the calculated ratios, with I/VI and III/VI, for example, higher in the two samples of pulp-wash analysed. Ooghe *et*

al. (1994) found that samples of orange juice containing pulp-wash could not be distinguished from authentic samples on the basis of PMF data.

Retail FCOJ (Table 4) showed similar amounts of sinensetin and peak ratios when compared to authentic FCOJ (i.e. that collected directly from factories, Table 3). Some differences can be observed in peak ratios I/II and VI/II. Veldhuis *et al.* (1970) found similar values of sinensetin in retail (0.80 to 1.45 mg/l) and commercial samples of FCOJ (0.50 to 2.05 mg/l).

Table 4. Relative peak areas of PMFs and sinensetin levels (mg/l) in retail samples of frozen concentrated orange juice (RFCOJ), diluted to 12 °Brix (see Table 1 for peak identification).

		I / II	**I / IV**	**I / VI**	**III / I**	**III / IV**	**III / V**	**III / VI**	**IV / V**	**IV / VI**	**VI / II**	**Sinensetin**
RFCOJ n = 3	$\bar{x}$	**3.89**	**2.99**	**4.12**	**1.38**	**4.11**	**2.19**	**5.67**	**0.53**	**1.38**	**0.95**	**2.03**
	SD	0.11	0.17	0.32	0.07	0.05	0.08	0.21	0.02	0.03	0.05	0.24

n = number of samples analysed

Retail freshly squeezed orange juice (RFSOJ) (Table 5) gave concentrations of sinensetin between those found in hand squeezed orange juices and FCOJ. Only one sample showed a concentration which reached the levels found in the FCOJ samples. Admixture of a product such as FCOJ or pulp-wash high in PMFs might be one reason for this occurrence. However it is not possible to rule out other reasons including use of fruit variety (e.g. Pera) having a higher PMF content or the use of extraction equipment having a high operating pressure. Ooghe *et al.* (1994) found relatively high levels of sinensetin and tangeretin, and a relatively low scutellarein value in Pera oranges from Brazil. They therefore excluded this sample from their statistical evaluation.

Table 5. Relative peak areas of PMFs and sinensetin levels (mg/l) in retail samples of freshly squeezed orange (see Table 1 for peak identification).

FSOJ n = 13	I / II	I / IV	I / VI	III / I	III / IV	III / V	III / VI	IV / V	IV / VI	VI / II	Sinensetin
$\bar{x}$	**3.75**	**3.06**	**4.81**	**1.31**	**3.99**	**2.30**	**6.29**	**0.56**	**1.58**	**0.79**	**1.02**
SD	0.49	0.36	0.60	0.10	0.39	0.20	0.70	0.09	0.18	0.15	0.46

n = number of samples analysed

The majority of the peak ratios found in RFSOJ were similar to the ones found for hand squeezed samples. Some differences were observed for peak ratio III/V.

From the results obtained for FSOJ and FCOJ it can be observed that industrial processing has some influence on the peak ratios I/II and III/V (i.e., sinensetin/quercetogetin; nobiletin/scutellarein) when compared to hand squeezed orange juice. Ooghe *et al.* (1994) compared authentic orange juices with their corresponding concentrates. No differences in most of the PMFs were observed except for tangeretin which increased after the concentration process.

From Table 6, it can be seen that authentic hand squeezed samples showed a much higher SD than the retail samples of FSOJ. This reflects the fact that industrial processing involves the mixing of large quantities of juice and hence observed values will tend towards the mean. This effect is also apparent from the data presented by Ooghe *et al.* (1994) in which composite samples were analysed and resulted in data with a much smaller standard deviation. A similar observation was made by Martin *et al.* (1996) on ^{2}H SNIF/NMR and ^{13}C SIRA/MS data from samples of industrial processed and hand squeezed orange juice. Typically, standard deviations were 50% higher in the latter.

Table 6. Summary for peak ratio in authentic (hand squeezed) and in authentic samples of orange juice from literature (see Table 1 for peak identification).

		I / II	I / IV	I / VI	III / I	III / IV	III / V	III / VI	IV / V	IV / VI	VI / II	Sinensetin*
Authentic samples (n = 18)	$\bar{x}$	**2.82**	**2.91**	**4.11**	**1.85**	**4.94**	**1.47**	**6.88**	**0.31**	**1.37**	**0.92**	**0.10**
	SD	1.25	1.05	1.93	0.60	1.15	0.42	2.67	0.11	0.28	0.65	0.04
Ooghe *et al.* (1994)	$\bar{x}$	**4.50**	**2.20**	**4.17**	**1.30**	**2.85**	**2.94**	**5.32**	**1.08**	**1.96**	**1.15**	**n.a.**
	SD	0.71	0.42	0.94	0.13	0.55	0.38	0.97	0.32	0.54	0.38	n.a.

* = mg/l
n.a. = not available, n = number of samples analysed

An appreciation of the tabulated data is difficult to achieve by visual inspection. Canonical discriminant analyses (CDA), using the statistical software package from SPSS (Release 5.0.2, Jan. 1993, SPSS Inc.), was therefore investigated in order to classify the sample groups (DEFERNEZ & KEMLEY, 1997). As can be seen from Figure 2 (varieties Lima and Baía were excluded due the low number of samples) the method separated varieties Pera and Hamlin from the remaining varieties but was not able to distinguish between Natal and Valência. Commercial FCOJ, FCOPW and retail samples of freshly squeezed orange juice were distinct from hand squeezed orange juice. This no doubt reflects the greater concentration of PMFs in these samples.

Heimhuber *et al.* (1988) discussed the possibility of using the variability in the amount and distribution of PMFs in juice, pulp-wash and peel to detect adulteration. They observed visual differences in the PMF chromatograms of hand pressed orange juice, juice diluted commercially from concentrate, and pulp-wash, albeit no quantitative data was given. Our data does not lead to the same conclusions. Although the amount of PMFs vary for each kind of juice analysed, insufficient differences were observed to detect the addition of pulp-wash to orange juice. As Ooghe *et al.* (1994) suggest, the method is best applied to detecting the addition of non-*Citrus sinensis* juices such as tangerine to orange juice.

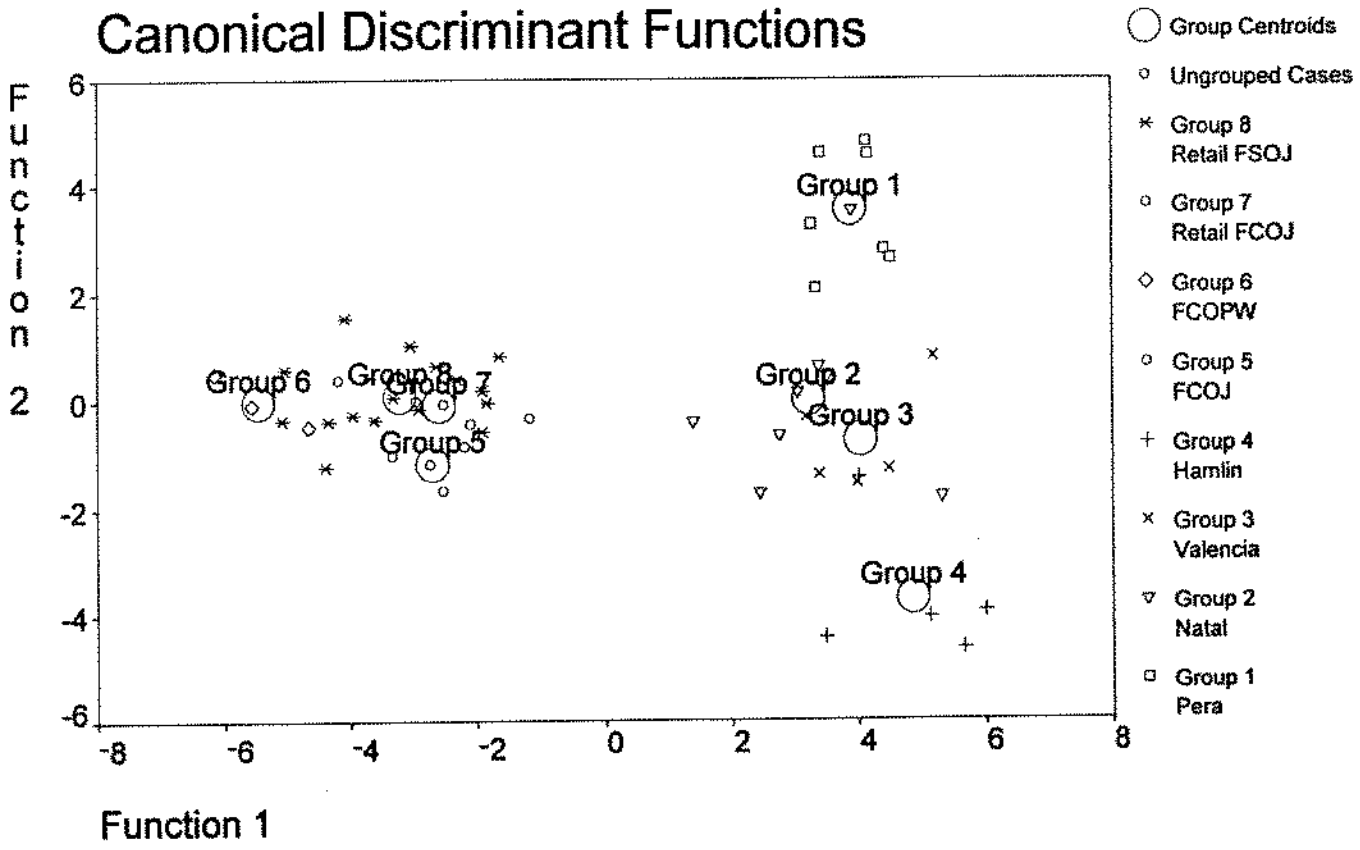

Figure 2. Canonical Discriminant analysis of several kinds of orange juice using PMF data (FCOJ, frozen concentrated orange juice; FCOPW, frozen concentrated orange pulp-wash; FSOJ, freshly squeezed orange juice).

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Capítulo 4

HPLC ANALYSIS OF CAROTENOIDS IN ORANGE JUICE

Trabalho submetido para publicação na revista Food Chemistry

HPLC ANALYSIS OF CAROTENOIDS IN ORANGE JUICE

SUMMARY

Lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene have been determined in samples of Brazilian orange juice (*Citrus sinensis*). The concentrations found in factory produced concentrates have been compared with those obtained on the Brazilian retail market and with authentic hand squeezed juices. Analysis of the latter enabled a comparison of varieties to be made. A concentration range of 0.11 - 1.21 mg/l was determined for total carotenoids with β-carotene, the most important source of Vitamin A, being found in the highest concentration in the Pera variety followed by Valência, Natal, Lima and Baía varieties. The total carotenoids present in samples of frozen concentrated orange juice (FCOJ) obtained from factories ranged from 0.26 - 0.48 mg/l, while retail samples of this product contained slightly more (0.46 - 0.81 mg/l). Frozen concentrated orange pulp-wash presented much lower concentrations ranging from 0.04 to 0.08 mg/l of total carotenoids. Fourteen samples of retail freshly squeezed orange juice contained carotenoids ranging from 0.04 to 0.55 mg/l, with only one sample out of the range found for authentic samples. This could be due to the addition of pulp-wash to this sample, in which undeclared sorbic acid was also detected.

INTRODUCTION

Brazil provides 80% of the world market of frozen concentrated orange juice (FCOJ) (Robards & Antolovich, 1994). Although there is a retail market for FCOJ in Brazil, freshly squeezed orange juice (FSOJ) sold in various packaging is gaining market share and various brands are now available. Pulp-wash is an industrially traded commodity. It is prepared, as the name suggests, as an aqueous extract of the pulp and enzymes are sometimes used to increase the effectiveness of this process. It is used in the formulation of soft drinks but is not normally sold directly to consumers. FSOJ commands a higher price than FCOJ or pulp-wash, and is retailed as a 100% natural juice obtained from fresh oranges without the addition of any preservatives, sugars, colorants or water.

Orange juice may be included among the dietary sources of carotenoids and, indirectly, pro-vitamin A. Consumption of this vitamin has been correlated to a reduction in the incidence of certain cancers (Colditz *et al.*, 1985; Olson, 1986; Bendich, 1989; Ziegler, 1989 and 1991).

Fisher & Rouseff (1986) described the analysis of carotenoids (including α-carotene, β-carotene, and β-cryptoxanthin) in orange juice using HPLC. Quackenbush & Smallidge (1986) extended this work to include zeinoxanthin and separated it from β-cryptoxanthin. They measured α-carotene and *cis* and *trans*-β-carotene which substantially improved the reliability of information on vitamin A content in food. Philip *et al.* (1989) used a similar system to analyse several carotenoids in unsaponified orange juice with the objective of determining adulteration. In order to create peak patterns for computer pattern analysis, a series of 32 peaks were chosen as representative of authentic orange juice.

Recently, Rouseff *et al.* (1996) reported the use of a unique C-30 reversed phase column to separate thirty-nine carotenoids, using a gradient of water, methanol and *tert*-

butyl ether. According to the authors the main advantage of using this method was the improvement in resolution of the chromatographic system and the speed of analysis.

A limited amount of data is available concerning the analysis of carotenoids in orange juice and as is often the case the varieties widely used in Brazil are not represented. Pera Rio (40.4%) is the most widely grown Brazilian variety with Natal (30.6%), Valência (17.3%), Hamlin (7.8%) and others (3.9%) also grown (Steger, 1990). Data on the carotenoid content of Brazilian orange varieties either from the fruit or as the processed product is limited. This paper reports the concentrations of α-carotene, β-carotene, lutein, zeaxanthin and β-cryptoxanthin in these products.

MATERIAL AND METHODS

Standards

Sudan I (dye), α-carotene, β-carotene and lutein were obtained from Sigma. Zeaxanthin and β-cryptoxanthin were obtained from Apin Chemicals (Oxon, UK).

Lutein, α-carotene, β-carotene, zeaxanthin and β-cryptoxanthin (5 mg each) were dissolved in chloroform containing 0.1% butylated hydroxytoluene (BHT) and the volume made up to 25 ml (ambered flask). This solution was prepared monthly. A working standard was prepared by dilution of the stock standard (2.5 ml) with acetonitrile (100 ml). Chromatographic standards (prepared daily) were obtained by diluting appropriate amounts of the working standards and adding 100 µl of Sudan I (50.0 mg/l dissolved in acetonitrile). The concentrations for the carotenoid standards ranged from 0.03 to 0.4 mg/l.

To calculate the concentration of each carotenoid standard solution, 5 ml were evaporated under nitrogen and dissolved in the appropriate solvent according to described by Hart & Scott (1995) and the absorbance measured. The concentration was determined using the specific extinction coefficient of each carotenoid.

Quantitative analysis

The concentration of carotenoids in the samples was determined by comparison with external standards taking account of the response from the injection standard (Sudan I). No correction for recovery was applied to the data.

Recovery studies

Recovery of carotenoids was measured for quality control purposes. Samples were spiked with several concentrations of each carotenoid in the range of 0.07 to 0.53 mg/l.

Quality control

An In-House Reference Material (IHRM) was used during the analysis to ascertain that the method was under control and to determine the repeatability of the method. A sample of orange juice (1l) was well mixed and divided in vials containing 12 ml each and frozen at - 20° C until analysis.

Samples

Authentic samples of oranges from different varieties as well as commercial concentrated orange juice and pulp-wash were collected from processing plants in the State of São Paulo (Brazil). Retail samples (frozen concentrated orange juice and freshly squeezed orange juice) were purchased from supermarkets in the metropolitan area of Campinas (State of São Paulo) during the years of 1995/1996.

Sample preparation

Citrus fruit were hand squeezed and the juices filtered through a stainless steel sieve (1.25 mm). No allowance was made for differences in °Brix of these samples. Frozen concentrated orange juice and frozen concentrated pulp-wash were diluted to 12 °Brix with Millipore water and results are expressed on this basis. Retail freshly squeezed orange juices were sieved before analysis. All of the samples were stored at - 20 °C.

Sample analysis

Samples of orange juice (5 ml) were extracted with ethyl acetate (3 x 50 ml) containing BHT (0.004%). The organic phase was transferred through anhydrous sodium sulphate (50 g) and collected in an ambered round bottom flask. To the aqueous residue 50 ml of methanol was added (containing 0.004% BHT) followed by 100 ml of 1 mol/l NaCl. The solution was well mixed and further extracted with ethyl acetate (75 ml and 25 ml, containing 0.004 % BHT). The ethyl acetate fractions were then transferred through the sodium sulphate and combined with the previous extracts. Finally the sodium sulphate was washed with a further 50 ml of ethyl acetate (0.004 % BHT). The pooled ethyl acetate

was evaporated to dryness in a rotary evaporator at 40 °C. The extract was transferred quantitatively to a 10 ml volumetric flask using portions of 1.5 ml of mobile phase (acetonitrile:methanol:1,2-dichloroethane, 60:35:5, v/v/v). The internal standard (100 µl, Sudan I, 50 mg/l in acetonitrile) was added and the volume was made up to 10 ml.

High Performance Liquid Chromatography (HPLC)

The HPLC apparatus consisted of a Waters 625 LC System, equipped with an autosampler Gilson 231 XL and a Spectra Focus UV-Vis detector (Spectra Physics). A 100 µl loop was used for injection. Solvents were HPLC grade. The mobile phase was a ternary mixture of acetonitrile:methanol:1,2-dichloroethane (60:35:5, v/v/v) to which 0.1 % BHT, 0.1% triethylamine and 0.05 M of ammonium acetate (in methanol) was added (Hart & Scott, 1995). The column was a C18 Vydac 201TP54 5µm (250 x 4.6 mm id., Vydac) with a guard-column Alltima C18 5 µm (7.5 x 4.6 mm id., Alltech). The column was kept at room temperature (about 22 °C) and the flow rate was 1 ml/min. The wavelength was adjusted to 450 nm. The peak areas were measured using a Millennium Software v. 2.0 (Waters). Peak identity was confirmed by a Spectra Focus Scanning Detector (Spectra Physics). This equipment takes spectra (380-520 nm) from 3 points at different times across the HPLC peak and compares these spectra. If these spectra are identical, the peak is considered pure; i.e. no interferents are present.

Peak purity and identification

A Scanning UV detector was used to identify and characterise the carotenoids determined in this study. The peaks were identified by comparison of the spectra with standards and literature values and by the retention time standards.

RESULTS AND DISCUSSION

Sample preparation and high performance liquid chromatography

In order to avoid possible degradation and/or formation of artefacts the samples were extracted directly with ethyl acetate without saponification. The use of ethyl acetate prevented the problem of emulsion formation which may occur when using other solvents. According to De Ritter & Purcell (1981) the main reason for saponification is a gross purification of the carotenoids, which removes neutral fats, fatty acids and esters present in the juice, besides simplifying the chromatogram (Fisher & Rouseff, 1986). This approach has been adopted by others analysing citrus juices e.g. Rouseff *et al.* (1992), who analysed carotenoids in red grapefruit cultivars, without saponification, using a mixture of hexane, acetone and ethanol, as extraction solvent.

The liquid chromatographic procedure uses a non-aqueous reverse phase (NARP) system which includes triethylamine, ammonium acetate and BHT to prevent the degradation of carotenoids on column. NARP chromatography has been used by several authors to analyse carotenoids in different foods (Nelis & De Leenheer, 1983; Bushway, 1986; Fisher & Rouseff, 1986; Heinonen *et al.*, 1989; Handelman *et al.*, 1992; Chen 1992; Epler *et al.*, 1993; Lin & Chen, 1995; Hart & Scott, 1995). However, the use of modifiers in the mobile phase to avoid degradation of the carotenoids is a recent innovation. Handelman *et al.* (1992) used ammonium acetate (0.01%) in the mobile phase while Epler *et al.* (1993) included both ammonium acetate and triethylamine in their solvent mixture. This gave a 94% recovery for the carotenoids studied. Hart & Scott (1995) added a third component to the mobile phase (0.1% BHT) in order to achieve approximately 100% recovery. It has been suggested that the main function of ammonium acetate and triethylamine in the mobile phase is to minimize the effects of the acidity provided by free silanol groups on the HPLC column (Handelman, *et al.*, 1992; Epler *et al.*, 1993; Hart & Scott, 1995).

In order to avoid degradation of carotenoids during extraction, a maximum of two samples were extracted simultaneously in subdued lighting and immediately injected in the HPLC system. Tests conducted extracting more than 2 samples at once resulted in lower recovery levels.

Peak purity and identification

The spectra obtained showed no indication of coeluting and all agreed with those reported in the literature (De Ritter & Purcell, 1981). This technique has been used by several authors to identify carotenoids in orange juice, grapefruit and in green vegetables (Fisher & Rouseff, 1986; Rouseff *et al.*, 1992; Chen, 1992; Rouseff *et al.*, 1996). A typical chromatogram of carotenoids in orange juice is shown in Figure 1.

Internal standard

There are no carotenoid-like materials commercially available which would be suitable for use as an internal standard. It was therefore decided to use a dye (Sudan I) for this purpose. Since this material does not have the same susceptibility to oxidation as carotenoids, it was used solely to quantify the injection and so was added at the end of the extraction process. Sudan I [1-(phenylazo)-2-naphthalenol] has previously been used as an internal standard by Quackenbush & Smallidge (1986) to determine pro-vitamin A in food and by Philip *et al.* (1989) to detect adulteration of orange juice with added carotenoids.

Linearity of response and limit of detection

All carotenoids showed a linear response within the range studied: 0.03 to 0.4 mg/l ($r = 0.999$ or better). The following limits of detection were estimated using a signal to noise ratio of 3: lutein, zeaxanthin β-cryptoxanthin 0.01 (mg/l) α and β-carotene 0.02 (mg/l).

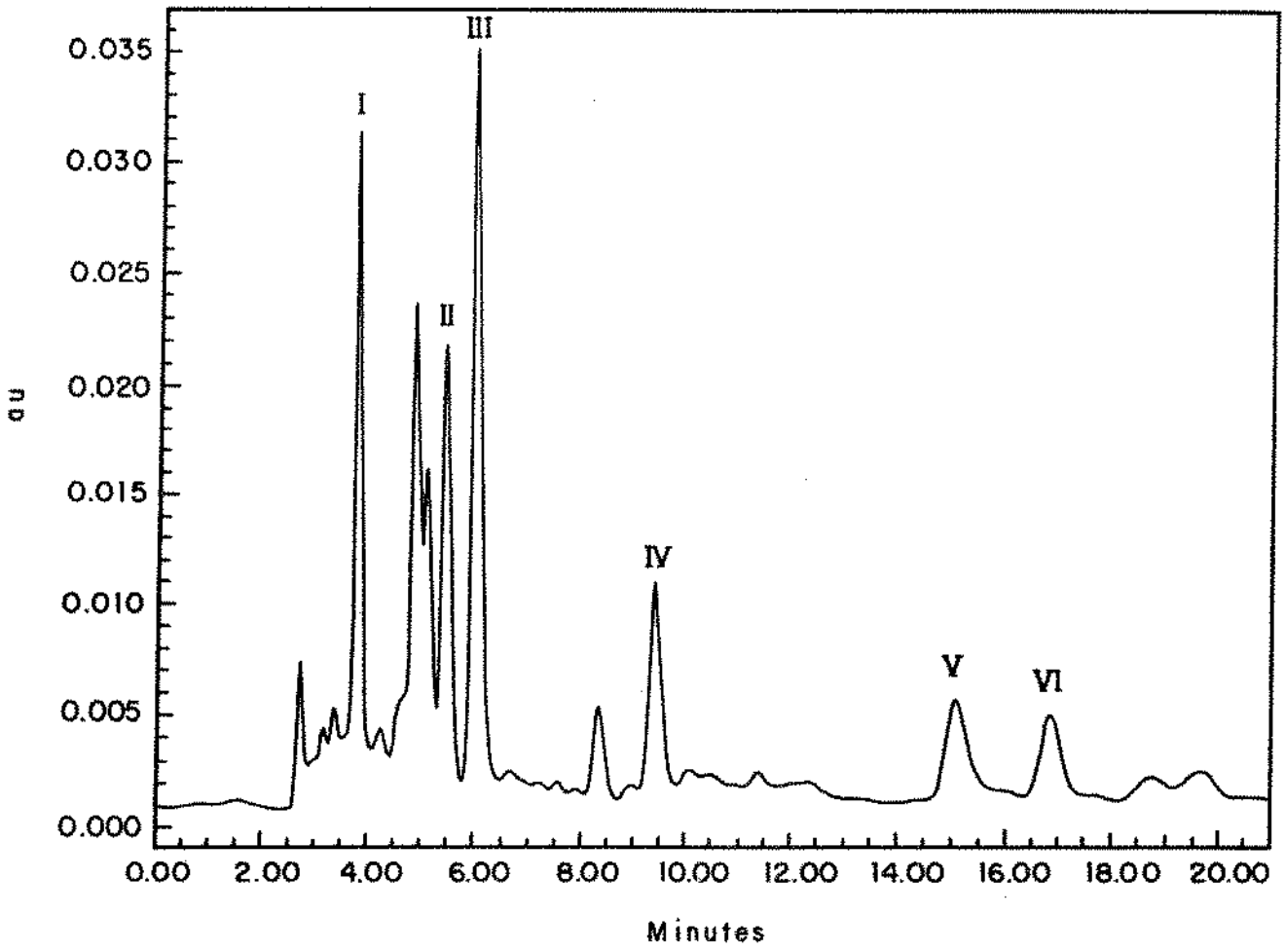

Figure 1. HPLC chromatogram of carotenoids in an authentic sample of orange juice: I Sudan I, II lutein, III zeaxanthin, IV β-cryptoxanthin, V α-carotene, VI β-carotene. Column C18 Vydac 5 μm (250x4.6 mm i.d.), pre-column C18 Alltech 5 5 μm (7.5x4.6 mm i.d.), mobile phase acetonitrile:methanol:1,2 diclorethane (60:35:5, v/v/v) added with 0.1% BHT, 0.1% TEA, 0.05 M ammonium acetate in methanol. Flow 1.0 ml/min, Vis 450 nm.

Quality control

Table 1 shows the precision achieved from 21 analyses of an IHRM determined over the course of the study. An average coefficient of variation (CV) of 16.9 % was obtained for the 5 carotenoids considered. Hart & Scott (1995) determined a CV ranging from 5.6 to 11.8% and 4.9 to 10.8%, respectively, for short term and long term reproducibility in a mixture of vegetables. Epler *et al.* (1993) used a similar system to ascertain that the method was "under control" and to determine the reproducibility of the method to quantify carotenoids in human serum and food. In their analyses the CV for the "low quality control" and "normal quality control" ranged from 3.1 to 14.0 % and 2.1 to 15.2%, respectively. Heinonen *et al.* (1989) obtained an average of 10 % in the CV of triplicate analyses with values ranging from 0.8 to 44% depending on the amount and complexity of the carotenoids in the food item.

Table 1. Mean and coefficient of variation of the IHRM for the determination of carotenoids.

	Lutein	**Zeaxanthin**	**β-Cryptoxanthin**	**α-Carotene**	**β-Carotene**
Mean (mg/l)	0.06	0.10	0.03	0.02	0.03
CV (%)	19.8	17.2	13.4	17.7	16.6

21 samples were analysed.

Table 2 shows the recoveries obtained from the analysis of fortified reagent blanks. The average value was 71 % for the 5 carotenoids studied. Limited information is available on recovery studies for carotenoids in orange juice. Fisher & Rouseff (1986) obtained a mean recovery of 80% for β-carotene and Quackenbush & Smallidge (1986) recovered 96% of β-carotene added.

Table 2. Recoveries levels for carotenoids in orange juice.

Compound	n	Amount added (mg/l)	Mean recovery % (SD)
Lutein	10	0.09 - 0.53	78.1 (11.2)
Zeaxanthin	10	0.07 - 0.43	70.8 (12.8)
β-Crypthoxantin	10	0.10 - 0.46	66.9 (11.6)
α-Carotene	10	0.09 - 0.47	77.6 (11.2)
β-Carotene	10	0.10 - 0.53	61.3 (13.3)

n = number of samples

Authentic samples

Hand squeezed orange juice

The carotenoid content of authentic samples of hand squeezed orange juices is given in Table 3. The different varieties investigated showed some variation in carotenoids concentrations. The total amount of carotenoids ranged from 0.11 to 1.21 mg/l. The variety Pera Rio contained the highest amount of carotenoids ranging from 0.63 to 1.21 mg/l, while concentrations in the other varieties were fairly similar. In all samples zeaxanthin was present in the highest concentration. β-carotene, the main source of pro-vitamin A, was found in higher concentrations in the Pera variety.

Table 3. Levels of carotenoids (mg/l) in authentic (hand squeezed) orange juice.

Variety		Lutein	Zeaxanthin	β-Crypthoxanthin	α-Carotene	β-Carotene	Total
Pera Rio (n = 5)		0.14 - 0.23	0.30 - 0.53	0.10 - 0.19	0.04 - 0.18	0.05- 0.08	**0.63 - 1.21**
	$\bar{x}$	0.18	0.39	0.13	0.09	0.07	**0.86**
Natal (n = 4)		0.04 - 0.06	0.06 - 0.12	0.01 - 0.03	0.03 - 0.04	0.02 - 0.03	**0.18 - 0.28**
	$\bar{x}$	0.05	0.09	0.02	0.03	0.03	**0.22**
Valência (n = 3)		0.06 - 0.07	0.04 - 0.10	0.02	0.03 - 0.07	0.02 - 0.05	**0.17 - 0.31**
	$\bar{x}$	0.07	0.08	0.02	0.05	0.04	**0.25**
Hamlin (n = 3)		0.03 - 0.08	0.06 - 0. 27	0.02 - 0.06	nd - 0.02	nd - 0.02	**0.11 - 0.45**
	$\bar{x}$	0.05	0.16	0.04	0.01	0.01	**0.27**
Baía (n=1)		0.05	0.04	0.08	nd	0.02	**0.19**
Lima (n=1)		0.08	0.10	0.01	0.02	nd	**0.21**
Pera Coroa (n=1)		0.08	0.15	0.05	0.05	0.04	**0.37**

nd = not detected (< 0.02 mg/l)

n = number of analysed samples

Results not corrected for recovery

These results are in reasonable agreement with those described by Heinonen *et al.* (1989) and Stewart (1977a), although the levels found were lower than the ones found by Reeder & Park (1975). As demonstrated by Stewart (1977a) the amount of carotenoids increases with the fruit maturity and vary according to the variety. For example, in his study the content of β-carotene in the Valência variety ranged from 0.008 (7 October) to 0.089 mg/l (30 March) and, for the same period, the Murcott variety ranged from 0.027 to 2.38 mg/l.

Frozen concentrated orange juice (FCOJ) and frozen concentrated orange pulp-wash (FCOPW)

The carotenoid content of FCOJ and of FCOPW is detailed in Table 4. Again the variety Pera Rio (in FCOJ) contained higher amounts of carotenoids when compared to the other two samples. The results for total carotenoids in FCOJ (0.26 to 0.48 mg/l) are in reasonable agreement with those previously described for commercial FCOJ (Stewart, 1977b; Quackenbush & Smallidge, 1986).

Low concentrations of total carotenoids were found in FCOPW with a maximum observed of 0.08 mg/l. Neither α nor β-carotene was detected in the samples analysed. These levels are much smaller than those found in hand squeezed juices or FCOJ. As FCOPW is depleted in colour its addition to orange juice will result in a reduction in the colour intensity. It is possible that colorants such as synthetic β-carotene might be added to mask this adulteration. If this is the case, the differences in the ratio of the individual carotenoids might provide an indication of this practice.

Retail samples

Retail frozen concentrated orange juice (RFCOJ)

The amount of carotenoids ranged from 0.46 to 0.81 mg/l in RFCOJ (Table 5). These concentrations are, if anything, larger than those obtained for the factory samples (FCOJ). Stewart (1977b) and Quackenbush & Smallidge (1986) found similar

concentrations in commercial samples of FCOJ. As there was no evidence of a reduction in the total amount of carotenoids it can be assumed that the retail samples analysed here were not adulterated with pulp-wash.

Table 4. Levels of carotenoids (mg/l) in authentic samples of frozen concentrated orange juice (FCOJ) and in frozen concentrated orange pulp wash (FCOPW), both diluted to 12 °Brix.

Samples		Lutein	Zeaxanthin	β-Crypthoxanthin	α-Carotene	β-Carotene	Total
FCOJ	1[a]	0.06	0.09	0.05	0.02	0.04	**0.26**
	2[b]	0.10	0.22	0.08	0.03	0.05	**0.48**
	3[c]	0.09	0.14	0.08	0.02	0.04	**0.37**
	$\bar{x}$	0.08	0.15	0.07	0.02	0.04	**0.37**
	SD	0 02	0.07	0.02	0.01	0.01	**0.11**
FCOPW * (n = 2)		0.01 - 0.03	0.02 - 0.04	0.01- 0.01	nd	nd	**0.04 - 0.08**

Results not corrected for recovery

nd = not detected (< 0.02mg/l)

[a]: variety Hamlin

[b]: variety Pera Rio

[c]: mixture of several varieties

n: number of analysed samples

*: varieties not specified by the producer

Table 5. Levels of carotenoids (mg/l) in retail samples of frozen concentrated orange juice (FCOJ), diluted to 12 °Brix.

	Lutein	Zeaxanthin	β-Crypthoxanthin	α-Carotene	β-Carotene	Total
n = 3	0.09 - 0.12	0.19 - 0.45	0.08 - 0.11	0.04 - 0.05	0.06 - 0.08	**0.46 - 0.81**
$\bar{x}$	0.11	0.30	0.10	0.04	0.07	**0.62**

Results not corrected for recovery

n = number of samples analysed

Retail freshly squeezed orange juice (RFSOJ)

The amount of carotenoids ranged from 0.04 to 0.55 mg/l in RFSOJ (Table 6). With the exception of one sample, the concentrations observed were in accordance with those anticipated from authentic samples (Table 3) and gave no indication of the addition of pulp-wash or other diluents. The profile of the carotenoids matched that found for authentic samples with zeaxanthin found in the highest concentration and β-carotene present in similar amounts to the authentic hand squeezed juice. It might be anticipated that the variability of industrially processed product would be less than that found for hand squeezed samples because the industrial product will contain a mixture of a large number of fruits prepared in a highly controlled procedure. This expectation proves to be correct. The average concentration of carotenoids in the hand squeezed juice was 0.42 mg/l (SD = 0.30) whereas the retail FSOJ (even including the anomalous result for one sample) gave 0.32 (SD = 0.13)

Table 6. Levels of carotenoids (mg/l) in retail samples of freshly squeezed orange juice.

	Lutein	**Zeaxanthin**	**β-Crypthoxanthin**	**α-Carotene**	**β-Carotene**	**Total**
n = 14	0.02 - 0.13	0.02 - 0.20	nd[a] - 0.13	nd[b] - 0.04	nd[b] - 0.05	**0.04 - 0.55**
$\bar{x}$	0.08	0.11	0.06	0.03	0.04	**0.32**

Results not corrected for recovery

nd[a] = not detected (< 0,01 mg/l)

nd[b] = not detected (< 0,02 mg/l)

n = number of samples analysed

CONCLUSION

The usefulness of the procedure in determining adulteration of retail samples of orange juice can be seen from the data obtained for one sample. The concentration of carotenoids in this sample was considerably less than that found for hand squeezed samples and from the vast majority of industrially produced samples. It is apparent therefore that the sample has been extended, perhaps with a material such as pulp-wash. Further evidence that this sample was not an authentic pure orange juice was provided by the discovery of sorbic acid in it.

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Capítulo 5

THE USE OF ISOTOPIC ANALYSES TO DETERMINE THE AUTHENTICITY OF BRAZILIAN ORANGE JUICE (*Citrus sinensis*)

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THE USE OF ISOTOPIC ANALYSES TO DETERMINE THE AUTHENTICITY OF BRAZILIAN ORANGE JUICE (*Citrus sinensis*)

SUMMARY

Isotopic analysis was used to characterise authentic samples of orange juice (*Citrus sinensis*) from Brazil. Site Specific Natural Isotopic Fractionation measured by Nuclear Magnetic Resonance (SNIF-NMR) was used to determine deuterium/hydrogen ratios at the methyl [$(D/H)_I$] and methylene [$(D/H)_{II}$] sites of ethanol. This ethanol was produced by fermentation of orange juice under controlled conditions. Stable Isotope Ratio Mass Spectrometry (SIRMS) was used to determine the amount of carbon-13 ($^{13}C/^{12}C$ ‰) in the same ethanol. SIRMS was also used to determine the amount of oxygen-18 ($^{18}O/^{16}O$ ‰) in citrus juice water. The mean ratios determined for $(D/H)_I$, $(D/H)_{II}$, $^{13}C/^{12}C$ and $^{18}O/^{16}O$ in the authentic hand squeezed orange juice samples were respectively: 102.3 ppm (SD = 1.7); 126.5 ppm (SD = 1.8), -26.5 ‰ (SD = 0.9) and +2.27 ‰ (SD = 2.48). These isotopic techniques (SNIF-NMR and carbon-13 SIRMS) are powerful tools to determine simultaneously the undeclared addition of cane and corn sugars (SIRMS) and beet sugar (SNIF-NMR) in orange juice. Samples from the Brazilian retail market (frozen concentrated orange juice and freshly squeezed orange juice) were also analysed in order to verify if any of these sweeteners had been added to the juice. None of the retail samples was adulterated at levels which could be detected by either isotopic method. Similarly, the $\delta\ ^{18}O$ ‰ data obtained for retail freshly squeezed orange juice exhibited a range of values similar to authentic juices. None of the retail FSOJ's were extended with ground water at levels that could be detected by this method.

INTRODUCTION

The internal retail market for orange juice in Brazil is mainly based on freshly squeezed orange juice (FSOJ). Because certain sweeteners such as cane, corn and beet sugars cost less than FSOJ they could be added to the juice to illegally extend it in conjunction with the addition of ground water. Juice which was adulterated with these sweeteners and sold as 100% pure without the declared addition of these compounds in the label may result in a prosecution by regulatory agencies.

The isotopic composition of sugars and water depend on both plant variety and geographic origin. Thus addition of these materials can often be detected by measuring isotope ratios of the fruit juice sugars and water. The $^{13}C/^{12}C$ ratio of orange juice sugars differ from those derived from cane and corn sugars and this difference is not detected by traditional chemical analyses such as high performance liquid chromatography or by enzymatic techniques.

The difference originates from the way the sugars are synthesised by the plant. Sugar cane and corn belong to a group of plants which use a C_4 Hatch-Slack pathway to synthesise sugars. Beet sugar and citrus plants use the C_3 Calvin cycle. As a result, different amounts of ^{13}C are found in products derived from C_3 and C_4 plants. Typical values of $\delta^{13}C‰$ (defined as delta carbon-13 per mil) for orange juice and sugar cane are -26.4‰ and -11.4‰, respectively (Bricout and Koziet, 1985). A review of these cycles can be found in work published by O'leary (1988).

At the beginning of the 1980's Martin *et al.* (1982 and 1983) proposed a new method to distinguish between certain types of sugars. The analysis measures ethanol obtained from fermentation sugars and starches. The method is based on the measurement of the ratio of deuterium/hydrogen (D/H) at the methyl position [defined as $(D/H)_I$] and methylene position [defined as $(D/H)_{II}$] of ethanol originating from fermentation of those sugars. The measurements are performed by ^2H Nuclear Magnetic Resonance

spectroscopy (NMR). They have found that the $(D/H)_I$ ratios of the ethanol reflected those of the sugars of origin and are characteristic of the plant origin. For example, ethanol from apple has a $(D/H)_I$ of 100.9 ppm and ethanol from sugar beet has a $(D/H)_I$ of 94.1 ppm (Martin *et al.*, 1982). Therefore, if sugar beet has been added to apple juice the ^2H NMR analysis will detect this adulteration. This method is called Site Specific Natural Isotopic Fractionation - Nuclear Magnetic Resonance (SNIF-NMR) and has been adopted by the AOAC as the standard method for detecting beet sugar addition to fruit juice (Martin *et al.*, 1996a).

Water (H_2O) from citrus juice also has particular isotopic properties and stable isotope analysis has been used since the early 1970's to detect adulteration in fruit juice. The heavier isotope of oxygen (^{18}O), which accounts for 0.2 % of all oxygen in the air, becomes slightly more concentrated in the water of growing plants due to the phenomena of evapotranspiration. As a consequence, it is possible to distinguish between ground water and fruit juice water. Bricout (1971 and 1973) and Bricout *et al.* (1972) proposed measuring the oxygen isotopic ratio ($^{18}O/^{16}O$) to distinguish natural orange juice from juices reconstituted from ground water and concentrate. Bricout (1973) found that water of orange juice from Brazil has a $\delta^{18}O‰ = + 5$ (defined as delta oxygen 18 per mil) and ground water has a $\delta^{18}O‰ = - 8$.

Data for the analysis of $^{13}C/^{12}C$ and D/H in orange juice are available in several works published in the literature (Bricout 1971 and 1973; Bricout *et al.*, 1972; Nissenbaun *et al.*, 1974; Donner and Bills, 1981 and 1982; Brause *et al.*, 1984; Bricout and Koziet, 1985 and 1987; Doner *et al.*, 1987; Tateo and Martin, 1992; Martin *et al.*, 1996a,b). Nevertheless just a few have used the technique of SNIF-NMR to verify the authenticity of orange juice (Tateo and Martin, 1992; Martin *et al.*, 1996a,b). The majority of the works cited involved either the analysis of frozen concentrated orange juice or in a small number of cases, freshly squeezed orange juices from different countries including Brazil. However the varieties tested were not specified. $^{18}O/^{16}O$ ratios of freshly squeezed orange

juice from Brazil were only reported by Bricout *et al.* (1972) and Bricout (1973). Also there have been no studies published on varieties that are most commonly processed in Brazil. According to Steger (1990), Pera and Natal varieties constitute 71% of the total orange production in Brazil. At present, to our knowledge there are no data available for the isotopic ratios of the most important citrus varieties used for producing freshly squeezed orange juice or the resulting retail samples on the Brazilian market. Without monitoring the retail market using $^{18}O/^{16}O$, $^{13}C/^{12}C$, D/H analyses it is possible that the addition of ground water, cane, corn and beet sugars is going undetected.

Both the SIRMS and SNIF-NMR methods rely on comparison with authentic samples. Therefore, a database of isotopic measurements from authentic juices is required.

The main objective of this work was to determine the isotopic ratios of $^{13}C/^{12}C$, $(D/H)_I$, $(D/H)_{II}$ and $^{18}O/^{16}O$ in authentic samples of orange juice from Brazil. These authentic sample data were then used to assess the authenticity of samples of orange juice from the Brazilian retail market.

MATERIAL AND METHODS

Samples

Authentic samples of different varieties of oranges as well as commercial concentrated orange juice and pulp-wash were collected from orange processing plants in the State of São Paulo (Brazil). Retail samples (frozen concentrated orange juice and freshly squeezed orange juice) were purchased from supermarkets in the metropolitan area of Campinas (State of São Paulo, Brazil), during the years of 1995/1996.

Sample preparation

The citrus fruits were hand squeezed and the juices filtered through a stainless steel sieve (1.25 mm). All juices were stored at - 20 °C until required for analysis. Before analysis frozen concentrated orange juice and frozen concentrated pulp-wash were diluted to 12 °Brix with tap water from Norwich city which has a known D/H value of 148.8 ppm.

SNIF-NMR

Fermentation, distillation and sample preparation

The analysis of samples was conducted according to the method described by Martin *et al.* (1996a).

Equipment

A Bruker ARX 500 NMR (Spectra Spin, Switzerland) fitted with a specific 10 mm "deuterium" probe was used. The ^{2}H spectra were recorded at 76.77 MHz with proton decoupling and a fluorine (^{19}F) lock. A 90° pulse was used and an acquisition time of at least $5xT_1$.

Calculations

The isotopic ratios were determined from the methyl $(D/H)_I$ and methylene $(D/H)_{II}$ sites of the ethanol molecule using deuterium NMR. The quantitative data were obtained by direct comparison of the sample peak with the internal standard tetramethylurea (TMU) of known D/H value. The isotopic ratios at the two ethanol sites were determined according to equations 1 and 2 (Martin *et al.*, 1996a).

$$\left(\frac{D}{H}\right)_I = 1.5866 \times T_I \times \frac{m_{st}}{m_A} \times \frac{\left(\frac{D}{H}\right)_{st}}{t_m^D} \qquad (1)$$

$$\left(\frac{D}{H}\right)_{II} = 2.3799 \times T_{II} \times \frac{m_{st}}{m_A} \times \frac{\left(\frac{D}{H}\right)_{st}}{t_m^D} \qquad (2)$$

Where:

T_I = [height of signal I (CH_2DCH_2OH) / height of TMU signal]

T_{II} = [height of signal II (CH_3CHDOH) / height of TMU signal]

$(D/H)_{st}$ = isotope ratio of TMU provided by the supplier (e.g. 132.83 ppm)

m_{st} = mass of TMU

m_A = mass of sample

t_m^D = alcoholic strength by mass of the distillate from ADCS

Stable Isotopic Ratio Mass Spectrometry (SIRMS) $^{13}C/^{12}C$

The carbon isotope measurements were done by combustion of the ethanol obtained from the distillation process.

Equipment

An elemental analyser EA 1108 (Carlo Erba Instruments, Italy) was coupled to a Delta-S Isotope Ratio Mass Spectrometer (IRMS) (Finnigan MAT, Germany) via a Conflo

II open split interface. The quartz reactor tube was filled with tungstic oxide [Tungsten (VI) oxide, WO_3] and pure reduced copper wires and was mantained at 1020 °C.

Stable Isotopic Ratio Mass Spectrometry (SIRMS) $^{18}O/^{16}O$ Analysis

The oxygen isotope ratios were measured by equilibration of orange juice samples with a CO_2 standard for 8 hours in a thermostated shaking water bath device.

Equipment

The equilibration device was connected to the dual inlet system of the Finnigan MAT Delta S SIRMS via a transfer line that incorporated a cold trap (-70 °C), which was used to remove water vapour from the sample gas.

Calculation

The amount of $^{13}C/^{12}C$ and $^{18}O/^{16}O$ in the samples of ethanol and water, respectively was determined according to equation 3:

$$\delta sample‰ = \left(\frac{R_{sample}}{R_{standard}} - 1 \right) x 1000 \qquad (3)$$

where R is the ratio $^{13}C/^{12}C$ or $^{18}O/^{16}O$

The ratios were reported against the following standards: PDB (Pee Dee Belemnite) for $^{13}C/^{12}C$ and SMOW (standard mean ocean water) for $^{18}O/^{16}O$.

Quality Control

According to the analysis (SNIF-NMR, SIRMS $^{13}C/^{12}C$ and SIRMS $^{18}O/^{16}O$) one type of In-House Reference Material (IHRM) was used during the analysis to ascertain that the method was "in control" and to determine the repeatability of the method. For SNIF-NMR a sample of orange juice with a nominal $(D/H)_I$ value of 103.0 ppm

(SD = 0.51) was analysed with every batch of samples. In order to make sure that the absolute values obtained by the NMR were correct three BCR (Community Bureau of Reference) certified reference ethanols from different sources (beet, cane and grape) of known D/H content were acquired before each batch analysis. For SIRMS of ^{13}C a sample of ethanol (obtained in the laboratory) with a nominal $\delta^{13}C‰$ value of -25.1‰ (SD = 0.5) was analysed with every batch of samples. The analytical precision (absolute difference between two measurements on one sample) was ± 0.5‰ or better. For SIRMS of ^{18}O a sample of orange juice with a nominal $\delta^{18}O‰$ value of +2.21 was analysed with every batch of samples. If the values obtained for the IHRM fell outside of the average ± 2 SD the batch was rejected.

RESULTS AND DISCUSSION

CARBON-13

Quality Control

The mean value determined for the IHRM was -25.4 $\delta^{13}C‰$ (SD = 0.2‰; n = 11). The data demonstrates the good repeatability of the method.

Authentic Samples

Table 1 gives the $^{13}C/^{12}C$, D/H and $^{18}O/^{16}O$ results from all of the samples analysed. A summary of the results is given in Table 2.

Table 1, Column 3 presents the $\delta^{13}C‰$ results. The $\delta^{13}C‰$ ratio of ethanol fermented from laboratory squeezed samples varied between -29.2 to -25.5‰ with a mean of -26.6‰ (SD = 0.9) (Table 2). Our results are similar to those published by Martin *et al.*, 1996a (mean = -26.6‰; SD = 0.45). These values are in general slightly lower than those obtained by measuring the $\delta^{13}C$ value directly from the sugar. According to Martin *et al.* (1996b) this is due to the limited fractionation which occurs during the fermentation (usually slightly higher than 1‰). Other authors measured juices directly (i.e. containing other components such as acids, amino acids, pulp) to obtain the $\delta^{13}C‰$ ratio in the samples. Therefore small variations in the results should be expected when comparing whole sample data, sugar data and ethanol data.

From this study there is no evidence that the ratio of carbon-13 is related to variety, as only one variety of orange (Cravo) fell outside of the mean ± 2 SD (97.5% confidence limit) (-26.6‰ ± 1.8). As only one sample of this variety was available for analysis, it is not possible to reach a firm conclusion on this issue. The small seasonal effect observed between samples from 1995 and 1996 is not statistically significant. (Mean $\delta^{13}C‰$ values for 1995 -26.6‰ and 1996 season -26.1‰, respectively (Table 2). Martin *et al.* (1996b) did not find any significant seasonal effect or varietal effects on carbon isotopic ratios of Floridian samples.

Table 1. Levels of $^{13}C/^{12}C$, $(D/H)_I$, $(D/H)_{II}$ and $^{18}O/^{16}O$ in authentic samples of orange juice, authentic frozen concentrated orange juice (FCOJ), frozen concentrated orange pulp-wash (FCOPW), retail FCOJ and retail freshly squeezed orange juice (FSOJ).

Type	Date of collection	$^{13}C/^{12}C$ (δ‰ PDB)	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	$^{18}O/^{16}O$ (V. SMOW)
Authentic Samples (laboratory squeezed orange juice)					
Pera	05/95	-26.1	100.6	123.4	-1.25
	09/95	-26.3	104.5	127.7	3.73
		-26.6	103.3	127.2	3.24
		-26.1	103.7	128.0	3.32
		-26.1	na	na	2.91
		-26.3	104.2	128.3	6.76
	02/96	-26.7	103.6	126.1	-0.54
Valência	09/95	-26.6	101.0	126.4	3.54
		-28.0	103.0	127.1	4.13
		-26.9	103.3	126.7	0.62
Hamlin	09/95	-25.8	100.9	128.8	4.13
		-26.4	100.9	128.7	3.57
		-26.1	101.5	129.5	na
		-25.8	103.7	126.2	na
Natal	09/95	-26.6	101.7	126.9	4.66
	01/96	-25.5	103.8	125.4	na
		-26.0	104.8	125.7	na
		-26.5	103.1	126.3	-0.42
Lima	05/95	-26.9	99.8	122.7	-0.90
Cravo	05/95	-29.2	99.9	124.3	na
Baía	05/95	-27.9	99.2	124.7	-1.12

na = not available
PDB (Pee Dee Belemnite)
SMOW (standard mean ocean water)

Table 1. Levels of $^{13}C/^{12}C$, $(D/H)_I$, $(D/H)_{II}$ and $^{18}O/^{16}O$ in authentic samples of orange juice, authentic frozen concentrated orange juice (FCOJ), frozen concentrated orange pulp-wash (FCOPW), retail FCOJ and retail freshly squeezed orange juice (FSOJ) (Continuation).

Type	Date of collection	$^{13}C/^{12}C$ (δ‰ PDB)	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	$^{18}O/^{16}O$ (V. SMOW)
Authentic Samples (from factory)					
FCOJ					
	09/95	-26.5	101.3	121.9	na
		-26.8	101.1	121.5	na
		-27.0	101.1	122.8	na
FCOPW					
	09/95	-26.9	101.6	122.8	na
Retail Samples					
FCOJ					
	09/95	-26.5	103.1	121.9	na
	01/96	-26.3	102.8	120.1	na
		-26.3	104.3	121.5	
FSOJ (n = 20)	09/95 to 09/96	-29.0 to -25.7	102.8 to 105,9	121.1 to 127,9	-1.44 to + 6.67*

na = not available

* n = 18

Table 2. Mean isotopic values for Brazilian samples of orange juice.

Type	Year of harvesting	Number of samples	$^{13}C/^{12}C$ (δ‰ PDB)	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	$^{18}O/^{16}O$ (V. SMOW)
Authentic Freshly Squeezed	1995	16	-26.6 SD = 0.9	102.0* SD = 1.7	126.7* SD = 2.0	+2.82 SD = 2.4 (n =13)
	1996	4	-26.1 SD = 0.5	103.8 SD = 0.7	125.9 SD = 0.4	-0.11 SD = 2.0 (n=3)
Authentic FCOJ	1995	3	-26.8 SD = 0.3	101.3 SD = 0.1	121.8 SD = 0.6	na
Retail FSOJ	1995	2	-26.8 SD = 0.6	103.1 SD = 0.4	125.8 SD = 1.6	+6.67 (n = 1)
	1996	18	-26.7 SD = 0.9	104.5 SD = 0.8	125.8 SD = 1.6	+3.26 SD = 2.5
Retail FCOJ	1995	1	-26.5	103.1	121.9	na
	1996	2	-26.3 SD = 0	103.6 SD = 1.1	120.8 SD = 1.0	na

* = average of 15 samples
na= not applicable.
n = number of analysed samples
FCOJ = frozen concentrated orange juice
FSOJ = freshly squeezed orange juice
PDB (Pee Dee Belemnite)
SMOW (standard mean ocean water)

Frozen concentrated orange juice and frozen concentrated orange pulp-wash

Commercial frozen concentrated orange juice (FCOJ) and frozen concentrated orange pulp-wash (FCOPW) (Table 1) showed similar $^{13}C/^{12}C$ ratios when compared to authentic samples of orange juice. The values were -26.9‰ for FCOPW and ranged from -27.0 to -26.5‰ for FCOJ. This indicates that the concentration process did not alter the carbon-13 isotope ratio.

Retail Samples

Frozen concentrated orange juice

The values of $\delta^{13}C$‰ of ethanol fermented from retail samples of FCOJ detected varied between -26.5 to -26.3‰ (Table 1). The values obtained suggest that none of the samples analysed was adulterated with sugar cane at levels which can be detected by this method.

Freshly squeezed orange juice

The mean value of $^{13}C/^{12}C$ for all the samples analysed was -26.7‰ (SD = 0.8), similar to that for authentic samples (mean = -26.6‰, SD = 0.9) (Table 1). Only one sample had a value (-29 ‰) outside of the mean ± 2 SD for authentic samples (mean = -26.6‰; SD = 1.8). This should be considered unusual (but not impossible) because normally orange juice processed in a factory is a result of a mixture of several varieties and oranges from different regions. Therefore, the values obtained should tend to the mean. This is supported by the fact that authentic and retail samples of FCOJ had $\delta^{13}C$‰ values which were close to each other. In these circumstances the best approach is to obtain further analytical data. Most authentic orange juices with depleted $\delta^{13}C$‰ values also have low values of $(D/H)_I$. Thus the elevated value of $(D/H)_I$ is a further suggestion of a potential problem with the sample. This could be confirmed or excluded on the basis of further compositional data.

SNIF-NMR

Quality Control

The mean value of $(D/H)_I$ determined for the IHRM for seven analyses was 103.2 ppm (SD = 0.18 ppm). This demonstrates that the analysis was in control and indicates the good repeatibility of the method.

Authentic Samples

Table 1, columns 4 and 5, show the $(D/H)_I$ and $(D/H)_{II}$ ratios for the authentic samples of freshly squeezed orange juice. The values obtained varied from 99.2 to 104.8 ppm and from 122.7 to 129.5 ppm, respectively for $(D/H)_I$ and $(D/H)_{II}$. As can be observed from Table 2 there is a slight increase in the mean value of $(D/H)_I$ for samples from the year 1996 compared to 1995, although the numbers of the samples analysed were not large enough to give a final conclusion. Martin *et al.* (1996b) concluded that the seasonal effect in Floridian samples for 3 consecutive seasons is around 1 ppm for $(D/H)_I$ and 2 ppm for $(D/H)_{II}$ even in extreme drought.

In general there were no varietal trends in the data. However single samples of Baia, Cravo and Lima oranges showed relatively low values of $(D/H)_I$ and $(D/H)_{II}$ (99.2 to 99.9 ppm; 124.3 to 124.7 ppm, respectively) when compared to the other varieties collected in the same year (100.9 to 104.5 ppm; 123.4 to 129.5 ppm, respectively). However the small number of samples available do not permit an evaluation of the whether a seasonal or varietal effect was occuring. Martin *et al.* (1996b) have correlated certain varieties with specific origins, climates or harvest times with small variations in the isotope ratios (usually less than 1 ppm).

The data presented in our work were similar to those found by Martin *et al.* (1996b) analysing authentic freshly squeezed oranges from Brazil. The ratios of $(D/H)_I$ and $(D/H)_{II}$ for authentic hand squeezed orange juice in the seasons 1992 and 1993 ranged from 104.2 to 105.8 ppm and from 121.7 to 128.0 ppm, respectively.

Frozen concentrated orange juice and frozen concentrated orange pulp-wash

Factory samples of FCOJ (Table 1) gave isotope ratios [$(D/H)_I$ 101.1 to 101.3 ppm and $(D/H)_{II}$ 121.4 to 122.5 ppm] within the range found for authentic samples of hand squeezed orange juice [$(D/H)_I$ 99.2 to 104.8 ppm and $(D/H)_{II}$ 122.7 to 126.5 ppm]. Martin *et al.* (1996b) analysed authentic samples of Brazilian FCOJ during the seasons 1992 and 1993. The ratios for $(D/H)_I$ and $(D/H)_{II}$ ranged from 103.0 to 105.8 ppm and 121.7 to 128.0 ppm (n = 73), respectively. FCOPW, [$(D/H)_I$ 101.6 ppm and $(D/H)_{II}$ 122.8 ppm] (Table 1) showed no isotopic differences from FCOJ and authentic hand squeezed orange juice.

Retail Samples

Frozen concentrated orange juice

The ratios measured varied between 102.8 to 104.3 ppm and 120.1 to 121.9 ppm, respectively for $(D/H)_I$ and $(D/H)_{II}$ (Table 1). The ratios of $(D/H)_I$ are within the range found for authentic samples of hand squeezed orange juice (99.2 to 104.8 ppm) and higher than those found for commercial samples of FCOJ (101.1 to 101.3 ppm). The results obtained do not indicate the addition of cane, corn or beet sugars at levels which can be detected by these methods. The $(D/H)_{II}$ ratios were similar to those found for factory authentic samples of FCOJ (121.4 to 122.5 ppm).

Freshly squeezed orange juice (FSOJ)

The values found for $(D/H)_I$ and $(D/H)_{II}$ in FSOJ ranged from 102.8 to 105.9 ppm (mean = 104.4 ppm) and 121.1 to 127.9 ppm (mean = 125.2 ppm), respectively (Table 1). The $(D/H)_I$ ratios were in general higher than those found for authentic samples. Only two samples fell outside of the mean ± 2 SD (97.5% confidence limit) for authentic samples of hand squeezed orange juice. One possible explanation for these relatively high values of $(D/H)_I$ in these two samples could be the addition of sugar cane. As sugar cane has typical $(D/H)_I$ ratio of around 109 ppm its addition to the orange juice would increase the ratio of $(D/H)_I$. However, the results obtained for carbon-13 analysis for these samples

(Table 1, retail samples) did not support any indication of the addition of sugar cane in the orange juice.

OXYGEN-18

Quality Control

The mean value determined for the IHRM was 2.21 $\delta^{18}O‰$ (SD = 0.14‰; n = 17). The data demonstrates the good repeatability of the method.

Authentic Samples

Table 1, column 6, presents the $\delta^{18}O‰$ results. The $\delta^{18}O‰$ ratio of the laboratory squeezed samples ranged from -1.25 to +6.76‰ with a mean of +2.27‰ (SD = 2.48). As can be seen a wide range of values were obtained. Comparing with literature data (Bricout *et al.*, 1972; Bricout, 1973; Nissenbaum *et al.*, 1974) our results showed a much wider variation, probably due to climate effects. As the results presented showed larger variations for individual samples, it is difficult to make any conclusions related to varietal or seasonal effects.

Retail Samples

The data obtained for ^{18}O in retail samples of FSOJ (-1.44 to +6.67‰) showed a similar range to that of authentic samples of freshly squeezed orange juice (-1.25 to +6.76‰) (Table 1). This indicates that these samples were not adulterated with ground water (-5.1‰) at such levels that could be detected by this method.

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CONCLUSÕES

Os resultados obtidos no presente estudo permitem as seguintes conclusões:

- A razão entre as principais flavanonas glicosídicas presentes em suco de laranja da espécie *Citrus sinensis*, ou seja, hesperidina/narirutina não pode ser utilizada como indicador de adição de água de lavagem da polpa ("pulp-wash") em suco de laranja fresco. Entretanto, o teor individual de cada flavanona glicosídica pode ser utilizado para detectar a possível adição de água de lavagem da polpa em suco de laranja fresco, devido à elevada concentração destes compostos neste produto quando comparado com o suco natural.

- Da mesma forma que em outras variedades de *C. sinensis*, as flavonas polimetoxiladas sinensetina, quercetogetina, nobiletina, heptametoxiflavona, scutelareína e tangeretina são encontradas nas variedades Pera, Natal, Hamlin, Valência, Baía e Lima, podendo sua quantidade relativa ser utilizada para caracterizar suco de laranja nacional.

- Os teores de carotenóides totais em água de lavagem da polpa são em geral relativamente inferiores aos apresentados por amostras autênticas e podem ser utilizados, juntamente com a análise de flavanonas glicosídicas, como indicadores da adição deste produto em suco de laranja.

- As razões isotópicas de $^{13}C/^{12}C$, D/H (dos açúcares do suco e etanol, respectivamente) e $^{18}O/^{16}O$ (da água do suco) obtidas para suco de laranja nacional podem ser utilizadas para detectar a adulteração deste suco pela adição de açúcar de cana, milho e beterraba e água de torneira.

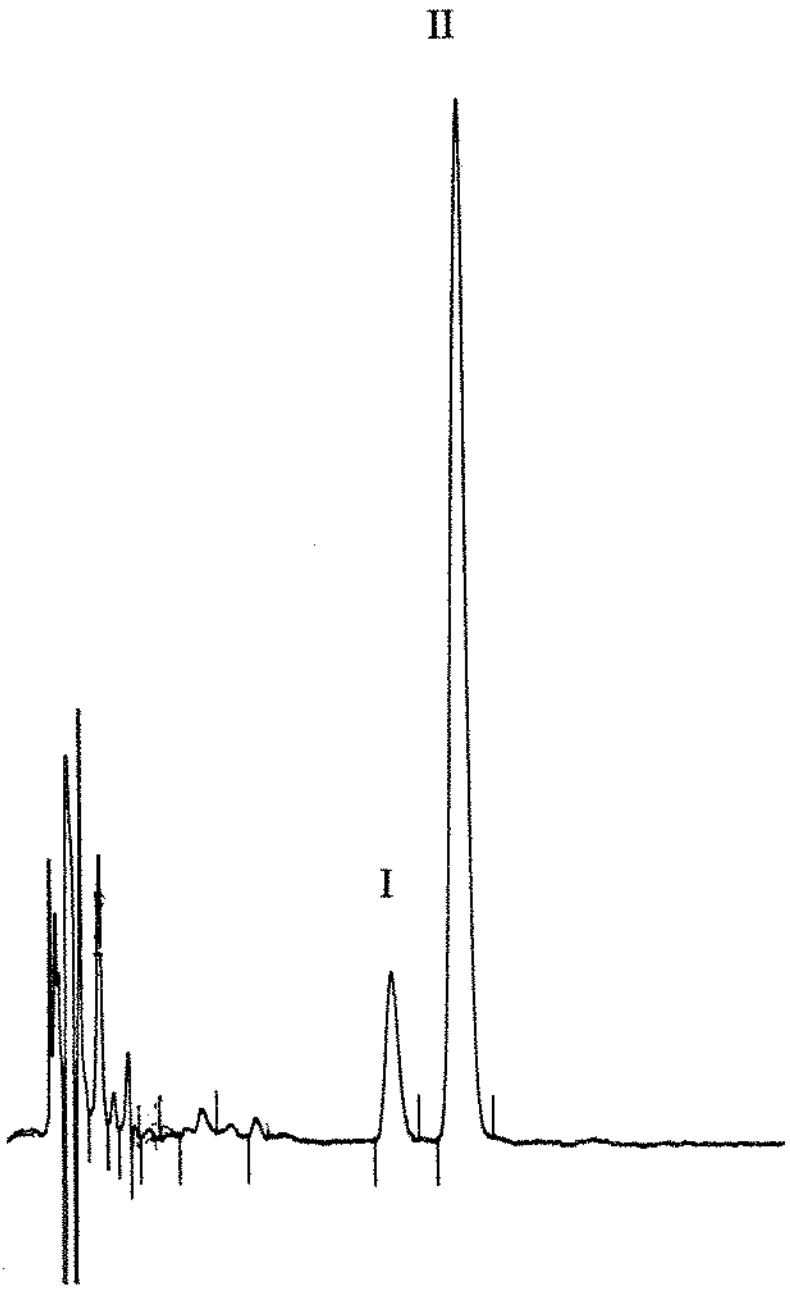
ANEXOS

Anexo I. Níveis de flavanonas glicosídicas em suco de laranja fresco.

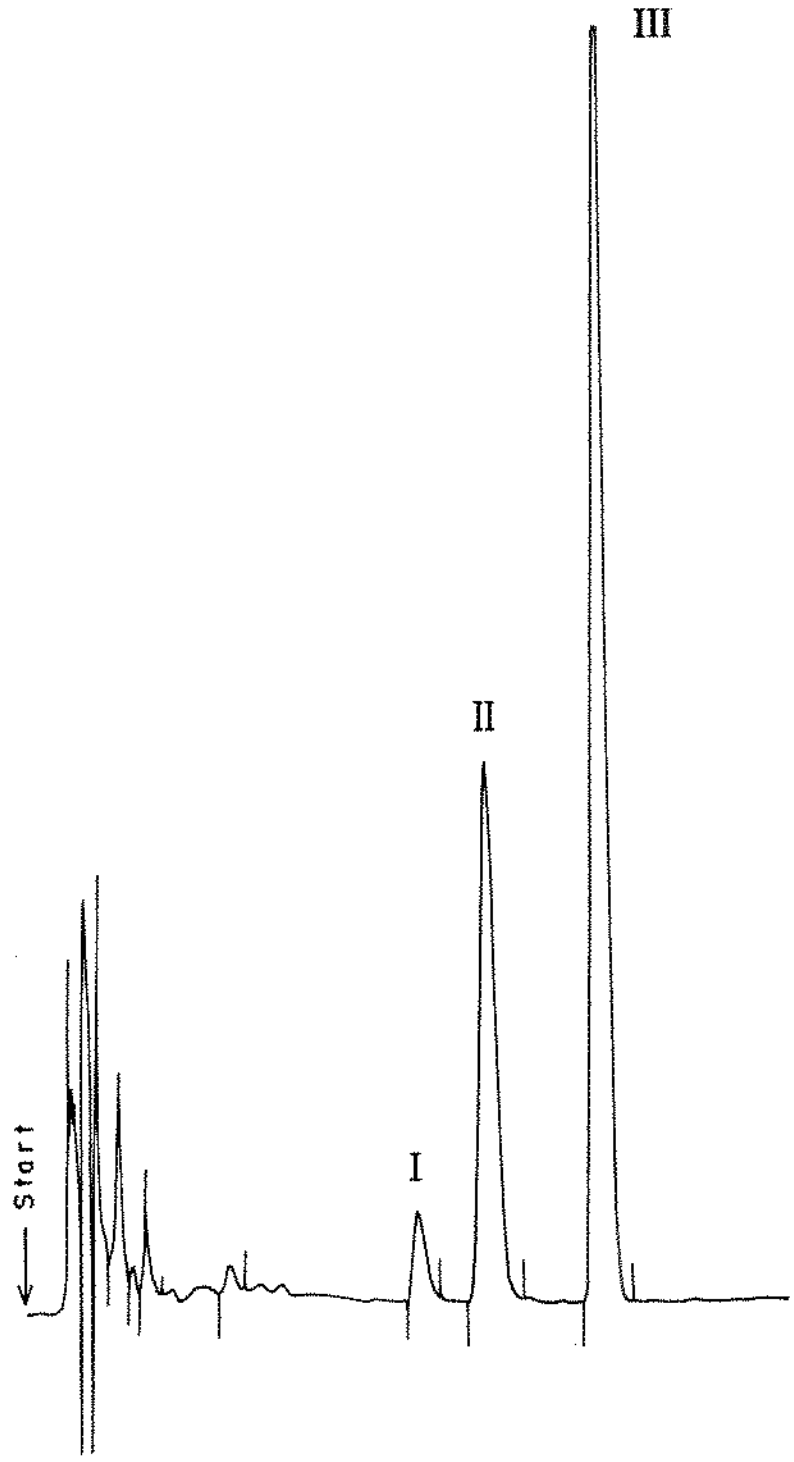
Suco de laranja comercial		Narirutina (mg/L)	Hesperidina (mg/L)	Razão (Hesperidina/Narirutina)
Marca				
	1a	44,9	364,1	8,1
	b	41,9	379,3	9,1
	c	34,1	388,2	11,4
	d	40,6	105,8	2,6
	e	30,8	116,1	3,8
	2a	28,0	300,8	10,7
	b	42,2	401,7	9,5
	c	46,1	393,8	8,5
	d	41,8	209,8	9,4
	3	40,5	481,1	11,9
	4a	16,3	164,1	10,1
	b	24,7	207,8	8,4
	5	14,0	140,9	10,1
	6a*	44,7	319,1	7,1
	b*	94,8	586,6	6,2
	c*	30,2	311,8	10,3
	d*	24,2	106,8	4,4
	e*	21,3	118,3	5,6
	7a	42,7	175,8	4,1
	b	30,9	138,8	4,5
	8*	26,6	265,6	10,0
	9a	13,1	141,0	10,8
	b	13,7	160,3	11,7
	10	56,1	421,4	7,5
		faixa: 13,1 - 94,8 $\bar{x}$ = **35,0** DP = 17,6	faixa: 105,8 - 586,6 $\bar{x}$ = **266,3** DP = 137,1	faixa: 2,6 - 11,9 $\bar{x}$ = **8,3** DP = 3,0

1, 2, 3, etc são marcas
a, b, c, etc são diferentes amostras da mesma marca
* contém ácido sórbico

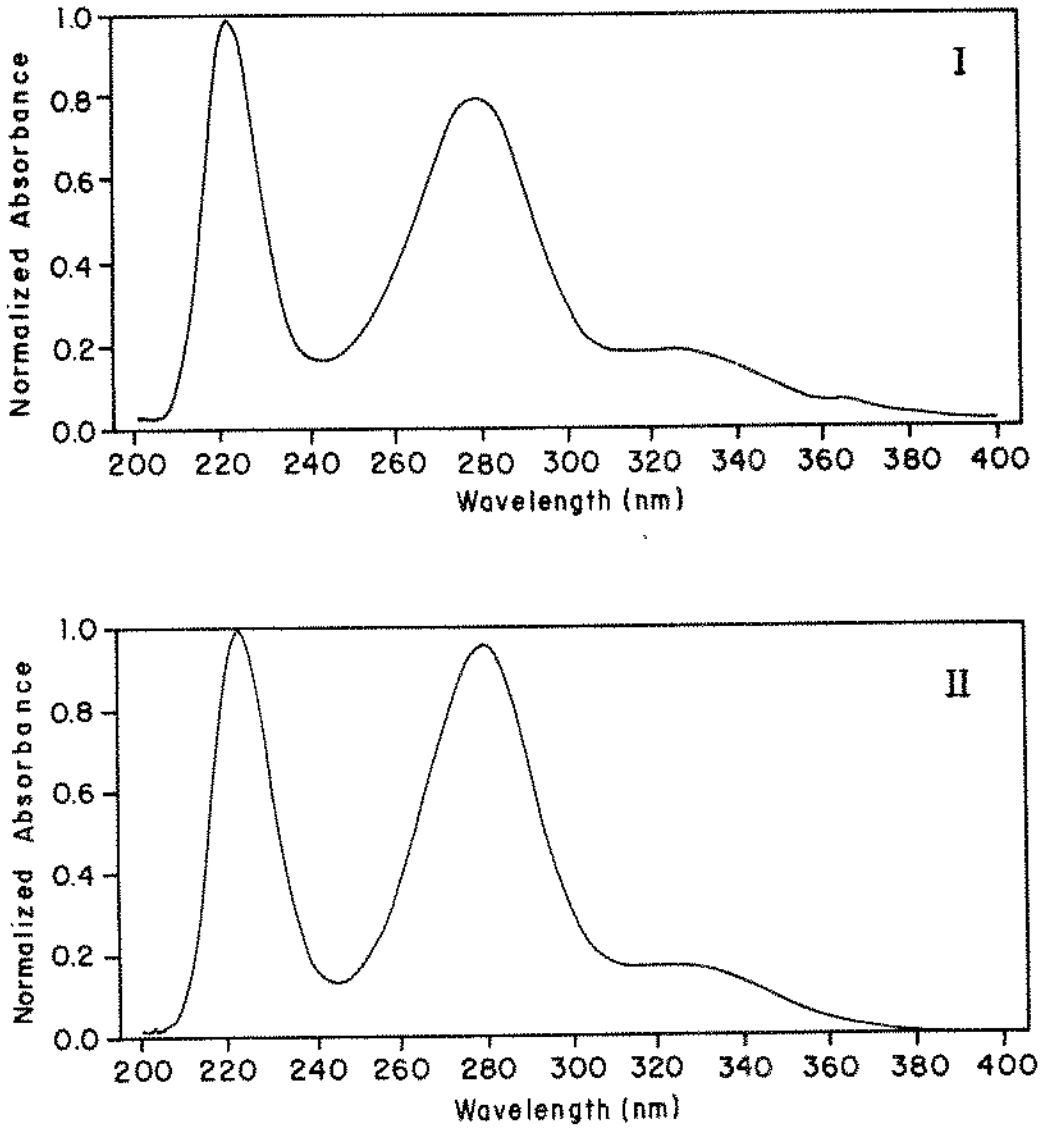
Anexo II. Cromatograma por CLAE de flavanonas glicosídicas em amostra autêntica de suco de laranja. I: narirutina, II: hesperidina. Condições cromatográficas: coluna C18, 5 μm (250 x 4.6 mm d.i.), fase móvel água:acetonitrila:THF:ácido acético (80:16:3:1, v/v/v/v), fluxo 1 mL/min., λ = 280 nm.



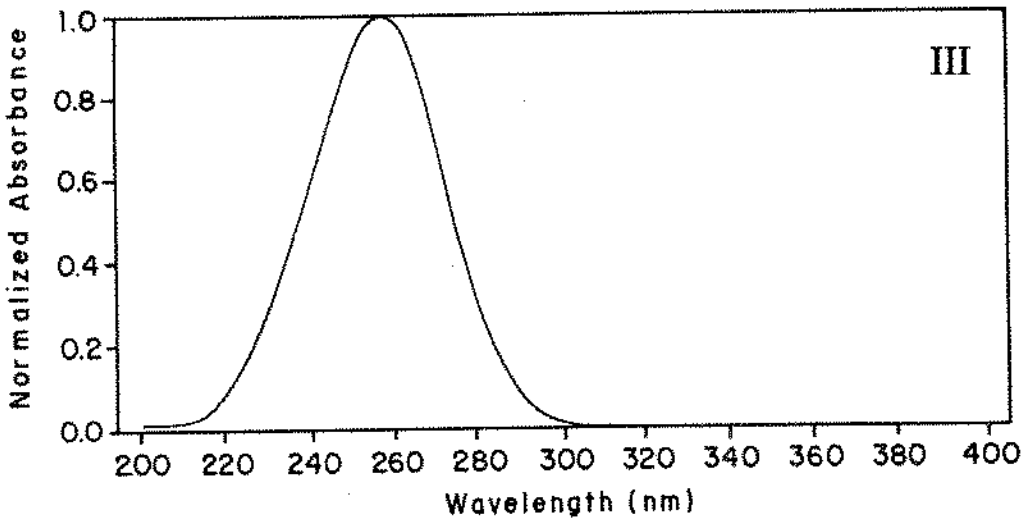
Anexo III. Cromatograma por CLAE de flavanonas glicosídicas em amostra comercial de suco de laranja adulterado com ácido sórbico. I: narirutina, II: hesperidina, III: ácido sórbico. Condições cromatográficas: coluna C18, 5 µm (250 x 4.6 mm d.i.), fase móvel água:acetonitrila:THF:ácido acético (80:16:3:1, v/v/v/v), fluxo 1 mL/min., λ = 280 nm.



Anexo IV. Espectro de UV das flavanonas glicosídicas (solvente água:acetonitrila:THF:ácido acético, 80:16:3:1, v/v/v/v). I: narirutina, II: hesperidina.



Anexo V. Espectro de UV do pico desconhecido (solvente água:acetonitrila:THF:ácido acético, 80:16:3:1, v/v/v/v) (Pico III ácido sórbico) detectado em amostra comercial de suco de laranja fresco.



Anexo VI. Áreas relativas de picos de flavonas polimetoxiladas e níveis de sinensetina (mg/L) em amostras comerciais de suco de laranja fresco.

FSOJ	I / II	I / IV	I / VI	III / I	III / IV	III / V	III / VI	IV / V	IV / VI	VI / II	Sinensetina
1a	3,57	2,98	4,36	1,25	3,71	2,26	5,43	0,61	1,46	0,82	0,92
b	3,45	3,49	5,49	1,24	4,33	2,27	6,81	0,52	1,57	0,63	0,82
c	4,83	3,26	4,59	1,38	4,49	2,55	6,32	0,57	1,41	1,05	1,16
2a	3,66	2,76	4,90	1,27	3,52	2,29	6,24	0,65	1,77	0,75	2,19
b	3,56	2,60	4,06	1,33	3,46	2,16	5,42	0,62	1,57	0,88	1,51
3	4,14	3,64	4,41	1,20	4,38	2,11	5,31	0,48	1,21	0,94	0,81
4	4,11	3,54	5,56	1,21	4,30	2,12	6,74	0,49	1,57	0,74	0,80
5a	3,41	2,95	4,57	1,44	4,24	2,22	6,57	0,52	1,55	0,74	1,01
b	3,01	3,09	5,81	1,34	4,14	1,90	7,78	0,46	1,88	0,52	0,92
6	3,85	2,64	3,97	1,56	4,11	2,26	6,18	0,55	1,50	0,97	0,32
7a	4,11	2,70	4,83	1,24	3,35	2,58	6,00	0,77	1,79	0,85	0,94
b	3,34	3,05	5,19	1,28	3,90	1,98	6,64	0,51	1,70	0,64	0,78
8	4,43	3,02	4,89	1,30	3,91	2,55	6,34	0,65	1,62	0,90	1,07
$\bar{x}$	**3,75**	**3,06**	**4,81**	**1,31**	**3,99**	**2,30**	**6,29**	**0,56**	**1,58**	**0,79**	**1,02**
DP	0,49	0,36	0,60	0,10	0,39	0,20	0,70	0,09	0,18	0,15	0,46

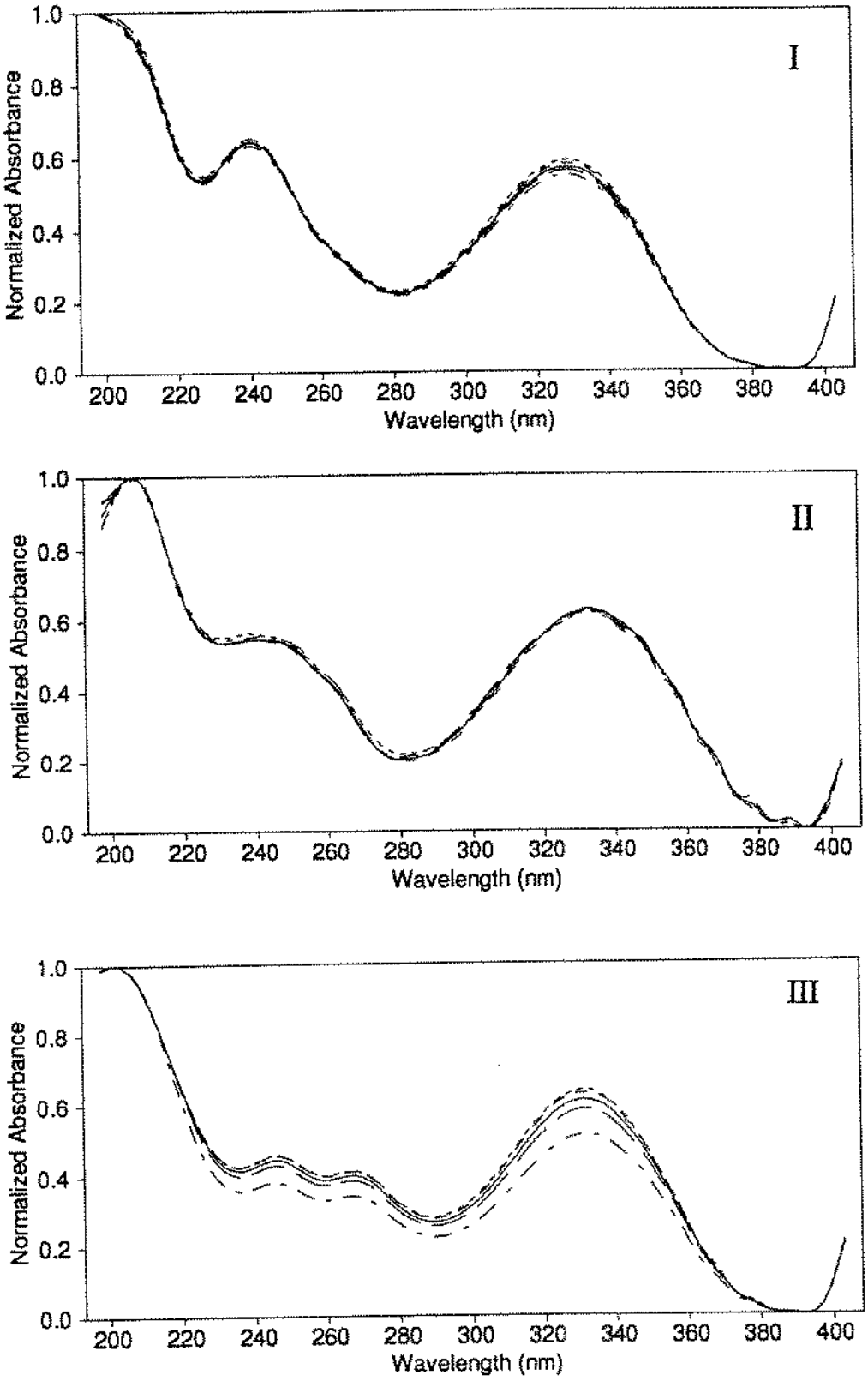
I = sinensetina, II = quercetogetina, III = nobiletina; IV = heptametoxiflavona; V = scutelareína; VI = tangeretina

1, 2, 3, etc = são marcas

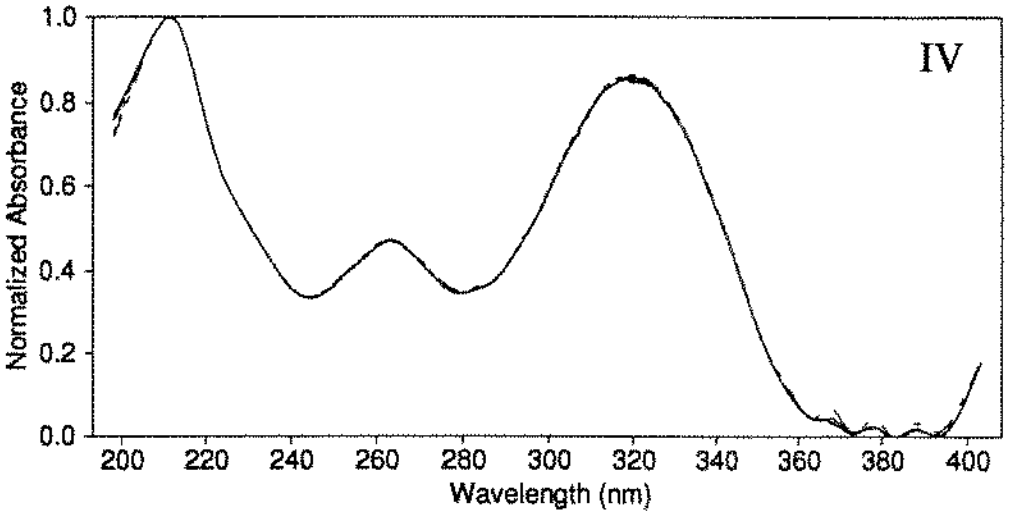
a, b, c = são diferentes lotes da mesma marca

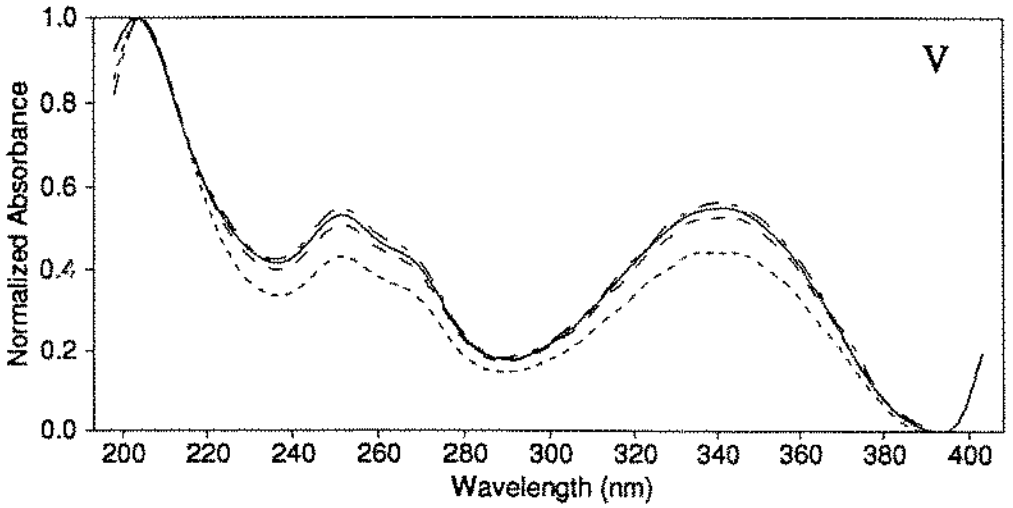
DP = desvio padrão

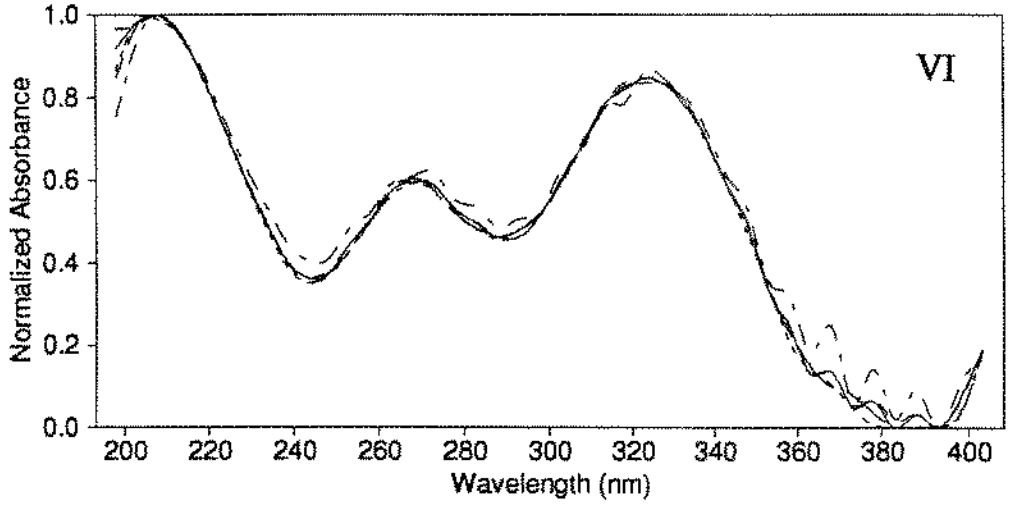
Anexo VII. Espectro de UV das flavonas polimetoxiladas (solvente água:acetonitrila:tetrahidrofurano 53:43:3, v/v/v). I: sinensetina, II: quercetogetina, III: nobiletina, IV: scutellareina, V: heptametoxiflavona, VI: tangeretina.



Anexo VII. Espectro de UV das flavonas polimetoxiladas (solvente água:acetonitrila:tetrahidrofurano 53:43:3, v/v/v). I: sinensetina, II: quercetogetina, III: nobiletina, IV: scutellareina, V: heptametoxiflavona, VI: tangeretina (continuação).





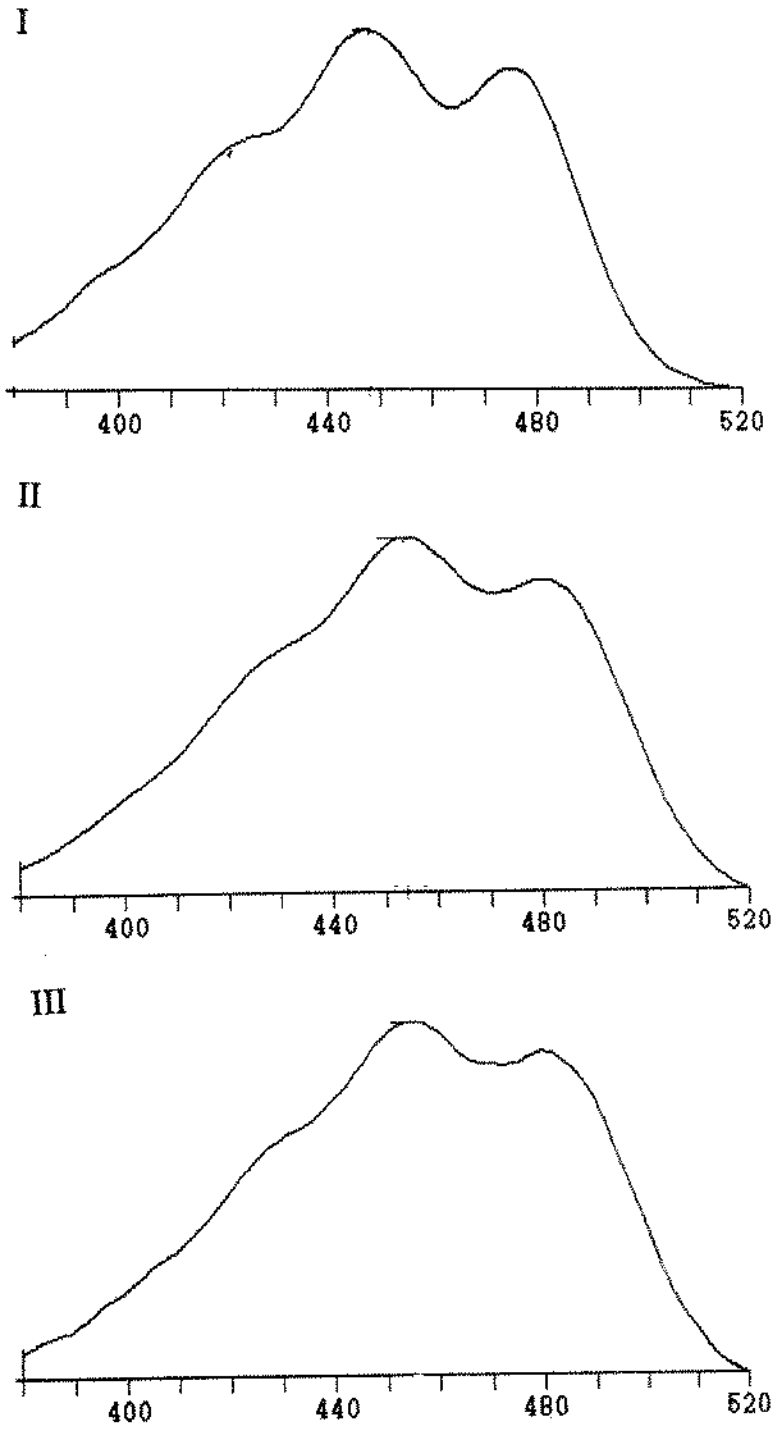


Anexo VIII. Níveis de carotenóides (mg/L) em amostras comerciais de suco de laranja fresco.

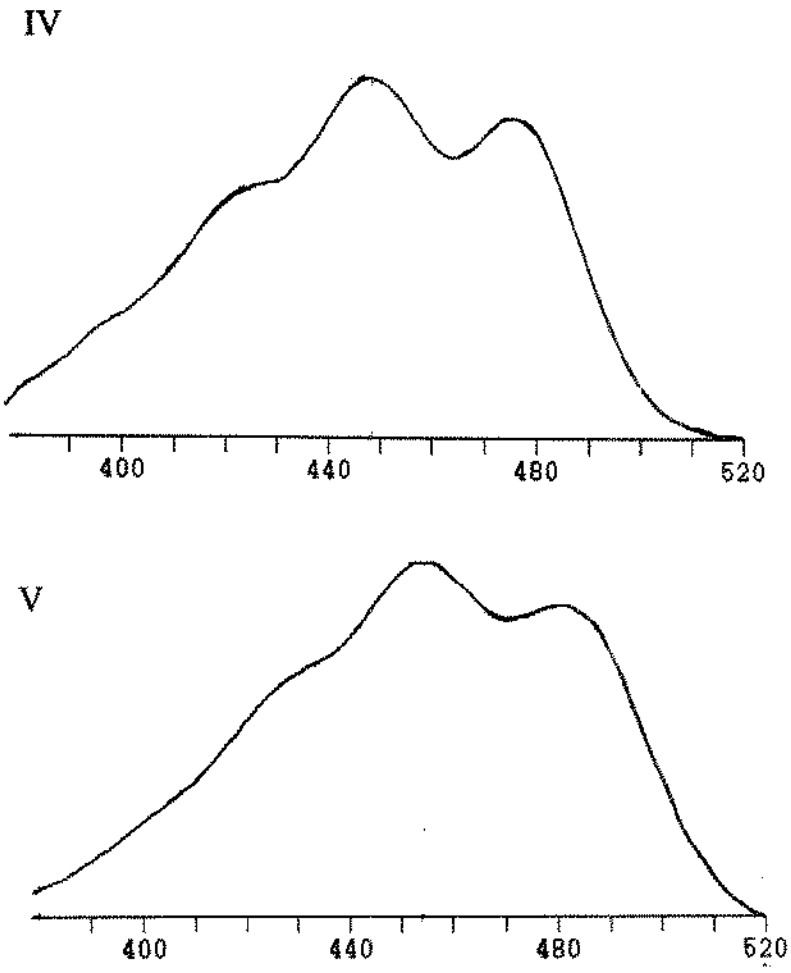
Marcas	Luteína	Zeaxantina	β-criptoxantina	α-caroteno	β-caroteno	Total
1a	0.06	0.10	0.03	0.02	0.03	**0.24**
b	0.10	0.10	0.08	0.02	0.04	**0.34**
c	0.12	0.20	0.08	0.03	0.05	**0.48**
2a	0.08	0.10	0.04	0.03	0.04	**0.29**
b	0.09	0.09	0.05	0.02	0.05	**0.30**
3	0.13	0.20	0.08	0.03	0.05	**0.49**
4	0.12	0.20	0.13	0.04	0.04	**0.53**
4b	0.05	0.09	0.03	nd‡	0.02	**0.19**
5	0.09	0.13	0.07	0.02	0.05	**0.36**
6*a	0.07	0.10	0.04	0.02	0.03	**0.26**
b	0.02	0.02	nd†	0.02	nd‡	**0.06**
c	0.07	0.06	0.03	0.03	0.04	**0.23**
7*	0.08	0.09	0.04	0.04	0.05	**0.30**
8	0.09	0.10	0.12	0.03	0.05	**0.39**
$\bar{x}$	**0.08**	**0.11**	**0.06**	**0.03**	**0.04**	**0.32**
DP	0.03	0.05	0.04	0.01	0.01	0.13

Resultados não corrigidos pela recuperação
† = não detectado (< 0,01mg/L)
‡ = não detectado (< 0,02mg/L)
* = adulterado com ácido sórbico

Anexo IX. Espectro visível de carotenóides [solvente acetonitrila:metanol:1,2 dicloretano (60:35:5, v/v/v) com 0,1% BHT, 0,1% TEA e 0,05 mol/L acetato de amônia em metanol]. I: luteína, II: zeaxantina, III: criptoxantina, IV: α-caroteno, V: β-caroteno.



Anexo IX. Espectro visível de carotenóides [solvente acetonitrila:metanol:1,2 dicloretano (60:35:5, v/v/v) com 0,1% BHT, 0,1% TEA e 0,05 mol/L acetato de amônia em metanol]. I: luteína, II: zeaxantina, III: criptoxantina, IV: α-caroteno, V: β-caroteno (continuação).



Anexo X. Coeficientes de extinção de carotenóides (HART & SCOTT, 1995*).

	Solvente	λ	$E^{1\%}_{1cm}$	E^{mM}_{1cm}	P.M.
Luteína	etanol	445	2550	145,1	569
Zeaxantina	hexano	451	2480	141,1	569
Criptoxantina	hexano	451	2460	141,1	553
α-Caroteno	hexano	444	2800	150,3	537
β-Caroteno	hexano	450	2560	137,4	537

* HART, D. J.; SCOTT, K. J. Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. **Food Chemistry**, *54*, p.101-111, 1995.

Anexo XI. Níveis de $^{13}C/^{12}C$, $(D/H)_I$, $(D/H)_{II}$ e $^{18}O/^{16}O$ em amostras comerciais de suco de laranja fresco.

Amostra		Data da coleta	$^{13}C/^{12}C$ (δ‰ PDB)	$(D/H)_I$ (ppm)	$(D/H)_{II}$ (ppm)	$^{18}O/^{16}O$ (V. PMAO)
SLF						
	1a	01/96	-29,0	104,4	123,8	+0,96
	b	02/96	-27,9	103,3	121,6	- 1,16
	c	02/96	-26,1	103,2	121,1	- 1,44
	d	09/96	-26,1	104,0	124,9	+4,34
	e	09/96	-26,3	104,1	125,5	+4,64
	2a	01/96	-26,9	105,0	125,1	+2,35
	b	02/96	-27,0	105,1	126,7	nd
	c	09/96	-26,4	104,4	124,7	nd
	d	09/96	-26,1	104,2	122,6	-1,15
	3a	01/96	-27,1	105,9	127,9	+5,96
	b	01/96	-26,8	105,2	125,7	+5,12
	c	09/96	-26,3	104,5	127,4	+5,60
	d	09/96	-28,1	104,4	126,5	+5,91
	4a	09/95	-27,2	103,4	126,0	+6,67
	b	01/96	-25,8	105,9	124,5	+1,61
	5a	09/96	-26,1	104,8	126,5	+5,25
	b	09/96	-26,4	105,5	125,8	+5,51
	6	09/96	-25,7	104,4	125,4	+5,78
	7	01/96	-26,8	103,3	125,0	+1,95
	8	09/95	-26,4	102,8	126,2	+4,91
		$\bar{x}$	**-26,7**	**104,4**	**125,2**	**+3,5**
		DP	0,8	0.9	1,8	2,7

nd = não disponível
1, 2, 3, etc = diferentes amostras
a,b,c, etc = diferentes amostras da mesma marca
PDB = padrão Pee Dee Belemnite
PAMO = padrão médio das águas do oceano

Anexo XII. Metodologia para Fracionamento Isotópico Natural de Posição por Ressonância Magnética Nuclear (FINP-RMN) (MARTIN *et al.*, 1996*).

Fermentação e destilação

Um volume de quinhentos mililitros de suco de laranja foi fermentado em um frasco de 1 L pela adição de 0,5 g de levedura desidratada (*Saccharomyces bayanus cerevisiae*). A amostra foi incubada por 3 a 5 dias em incubadora a temperatura de 23 °C. Durante o decorrer da fermentação o nível de açúcar era monitorado utilizando um teste para açúcar (Clinitest, Ames), e quando o teor de açúcar era menor que 0,25%, a amostra era centrifugada a 2000 rpm. Uma alíquota de 400 mL do sobrenadante foi destilada em um aparelho com um sistema automático de controle de destilação (SACD) com coluna Cadiot. O teor de etanol obtido do destilado foi medido utilizando um titulador Karl Fisher (Metler DL 18). Uma segunda alíquota da amostra fermentada foi destilada por arraste a vapor (Chenard, França). A quantidade de etanol foi medida utilizando densitômetro (Paar DMA 58). As medidas do Karl Fisher e Paar foram utilizadas para calcular o rendimento da destilação, que deveria ser maior que 95%, a fim de assegurar que nenhum fracionamento ocorreu com a amostra do material fermentado.

Análise de RMN

Padrões

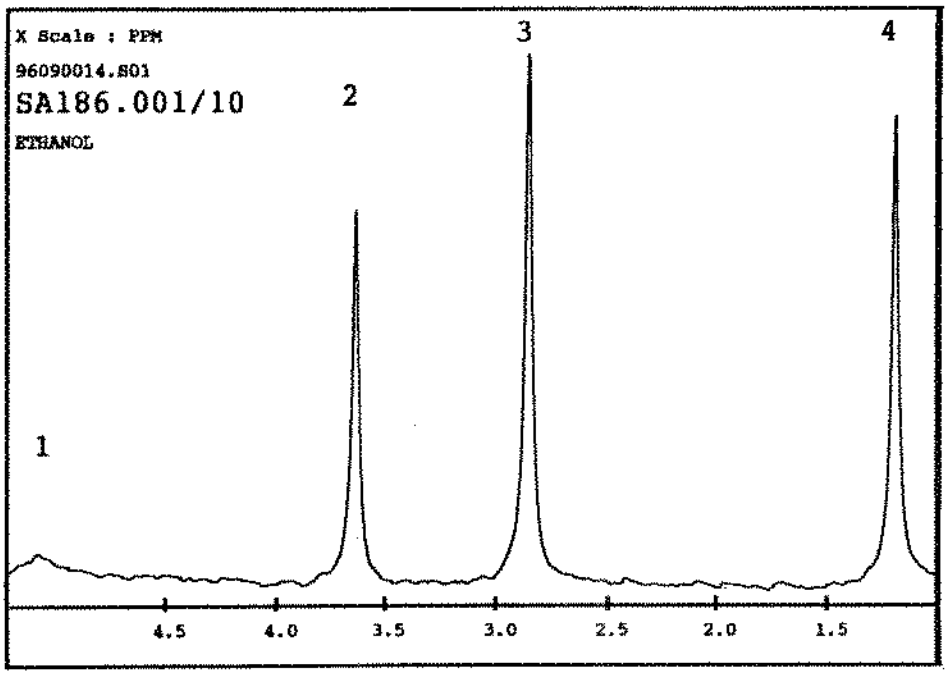
N, N tetrametiluréia (TMU) e hexafluorbenzeno grau RMN foram obtidos da "Community Bureau of Reference" (BCR, Belgium) e Aldrich Chemicals Co, respectivamente.

* MARTIN, G.G.; WOOD, R.; MARTIN, G.J. Detection of added beet sugar in concentrated and single strength fruit juices by deuterium nuclear mangnetic resonance (SNIF-NMR Method): Collaborative study. **Journal of the Association of Official Analytical Chemistry International**, v.79, n.4, p.917-928, 1996.

Preparação das amostras

Em um frasco previamente pesado, 3,2 mL de etanol (do SACD) foram adicionados e a massa de etanol registrada (m_A). No mesmo frasco 1,3 mL de TMU foi adicionado e a massa registrada (m_{st}). Finalmente adicionaram-se 150 μL de hexafluorobenzeno e a solução homogeneizada. A amostra foi transferida para um tubo de RMN de 10 mm (Gold Label, Aldrich) e os dados de deutério da molécula de etanol foram obtidos no equipamento de RMN ajustado para 76,77 MHz.

Anexo XIII. Espectro de ^{2}H RMN do etanol utilizando tetrametiluréia (TMU) como padrão interno. Pico 1: $CH_3CH_2O\mathbf{D}$, Pico 2: $CH_3CH\mathbf{D}OH$; Pico 3: TMU; Pico 4: $CH_2\mathbf{D}CH_2OH$



Anexo XIV. Espectrometria de massas da razão de isótopos estáveis (EMRIE) $^{13}C/^{12}C$

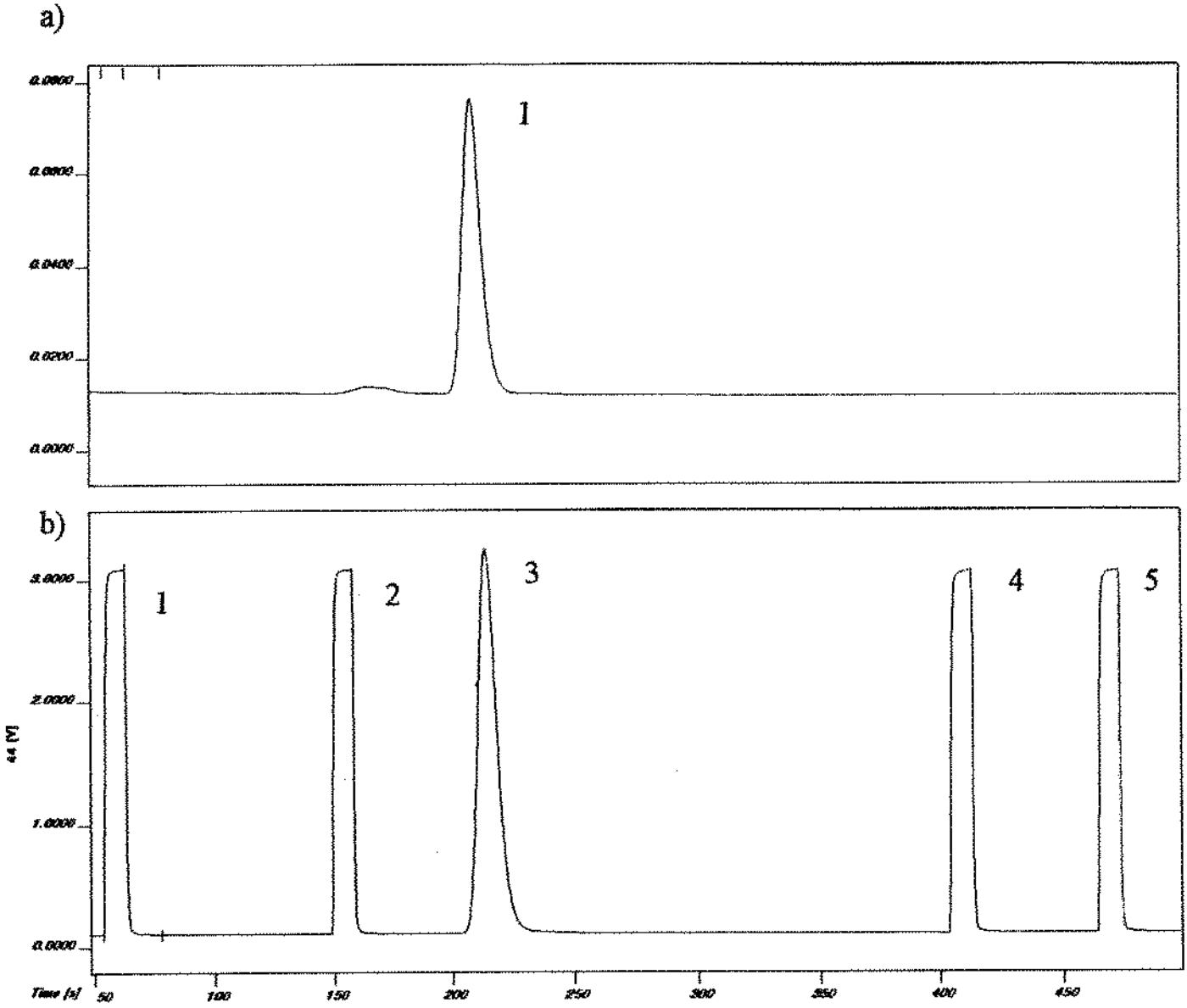
Padrão $^{13}C/^{12}C$

Devido a não disponibilidade comercial de Pee Dee Belemnite (PDB), um material de referência padrão de carbonato, NBS-19 (TS limestone), foi utilizado para calibrar o cilindro de gás de referência (CO_2) utilizado no espectrômetro de massas. NBS-19 tem uma valor preciso *versus* PDB ($\delta^{13}C‰ = + 2.0$), que é a base da escala $\delta^{13}C‰$. O dióxido de carbono foi produzido a partir do NBS-19, usando-se ácido fosfórico a uma temperatura controlada. O CO_2 foi capturado, purificado e usado para a determinação precisa do valor do gás de referência CO_2, relativo ao PDB.

Preparação da amostra e análise

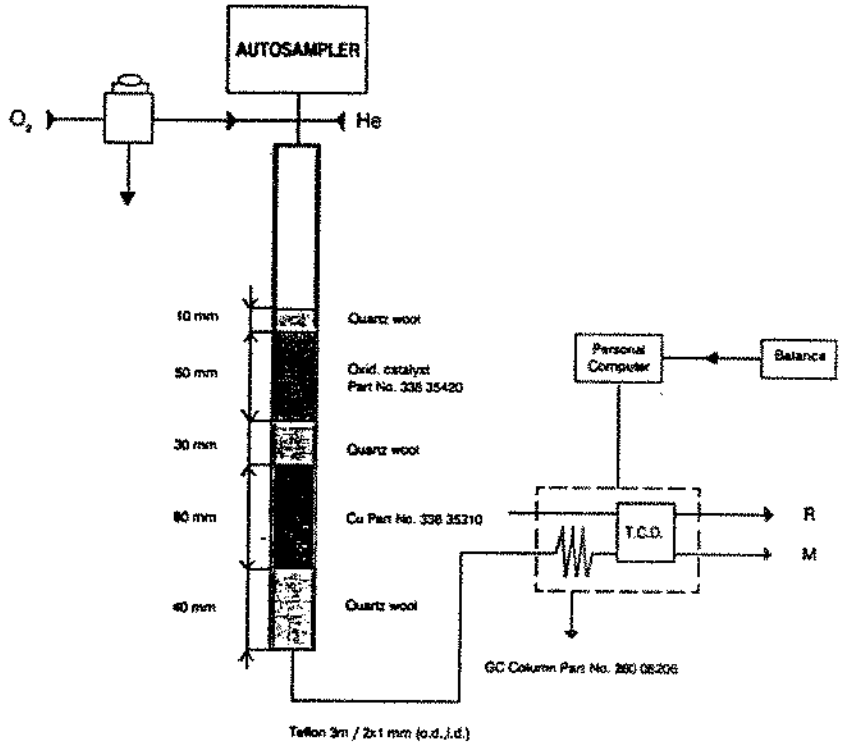
Em uma cápsula de estanho (2,0 mm di; 5,0 mm comprimento) 0,25 µL de etanol (obtido do suco de laranja fermentado) foi colocado e selado usando um alicate. A amostra foi colocada em um amostrador automático (AS-200LS, Carlo Erba) por 15 segundos antes de ser introduzida no sistema de combustão previamente enriquecido com oxigênio. Os gases resultantes da combustão foram carregados por um fluxo de He em uma coluna recheada de cromatografia gasosa (Porapak QS, 2m) a qual separou CO_2 da H_2O, sendo que uma porção de 0,1% do efluente foi para o espectrômetro de massas. Pulsos do gás de referência de dióxido de carbono calibrado foram superimpostos no efluente da coluna cromatográfica antes e depois do pico da amostra. Isto permitiu que a razão dos isótopos de carbono da amostra fosse determinado contra o gás de referência, que tinha uma quantidade precisamente conhecida da razão $^{13}C/^{12}C$ *versus* PDB.

Anexo XV. Cromatograma gasoso/espectro de massas da razão de isótopos de CO_2. (a) cromatograma do gás carbônico com detector de condutividade térmica; Pico 1: CO_2 da amostra; (b) cromatograma do gás carbônico com detector de massas; os picos 1, 2, 4 e 5 são os gases de referência, o pico 3 é o CO_2 da amostra.

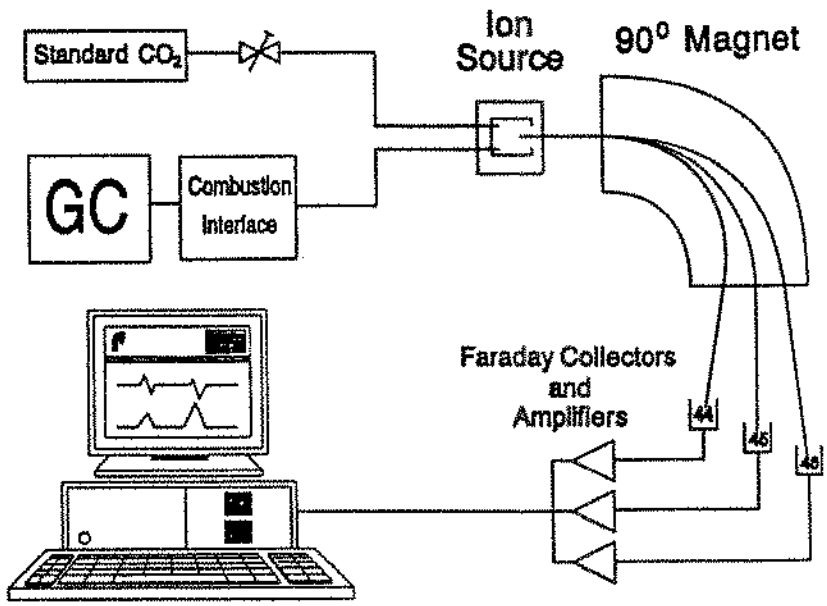


Anexo XVI. Esquema do equipamento utilizado para análise de carbono 13. (a) sistema de injeção automática de amostras, tubo de reação e separação cromatográfica dos gases; (b) espectrômetro de massas para análise de isótopos de carbono.

a)



b)



Anexo XVII. Espectrometria de massas da razão de isótopos estáveis (EMRIE) $^{18}O/^{16}O$

Padrão de $^{18}O/^{16}O$

O Padrão da Média das Águas do Oceano - PMAO ("Standard Mean Ocean Water", SMOW), na qual a escala de $\delta^{18}O‰$ é baseada, foi utilizado para calibrar um padrão local (Água de Torneira de Norwich, ATN). ATN tem um valor de -7,34 $\delta^{18}O‰$. O gás carbônico de referência foi medido contra o padrão NBS-19, que tem um valor bem caracterizado de $\delta^{18}O‰$ versus PMAO.

Preparação da amostra e análise

O método para a determinação de $\delta^{18}O‰$ para amostras líquidas envolve o evacuamento de pequenos frascos que contêm a água ou o suco da fruta e o preenchimento do espaço livre ("head space") com dióxido de carbono, quando se inicia o equilíbrio entre o isótopo de ^{18}O da água, dióxido de carbono (CO_2) e o ácido carbônico (H_2CO_3). Em situações nas quais grandes volumes de água e pequenos espaços livres são utilizados, como neste caso, praticamente todo o oxigênio do espaço livre do dióxido de carbono é trocado durante o equilíbrio e efetivamente se torna idêntico ao $\delta^{18}O‰$ da água. O valor de $\delta^{18}O‰$ do espaço livre do dióxido de carbono é então medido contra o dióxido de carbono de referência, que tem um valor exatamente conhecido de $\delta^{18}O‰$.

As amostras de suco (previamente congeladas) foram colocadas em banho de água até atingir a temperatura ambiente e o sobrenadante, que estava isento de sólidos, foi coletado para análise.

Termoestabilização

As amostras (5 mL) foram termoestabilizadas em conjunto de 24. Cada conjunto era composto de: um padrão externo com valor conhecido exato de $\delta^{18}O‰$, um material de referência interno, analisado em duplicata (suco de laranja fresco), e oito amostras (também analisadas em duplicata).

Os experimentos foram conduzidos utilizando um sistema automático de equilíbrio, o qual permitiu analisar 24 frascos de uma única vez. O sistema era composto de uma bomba de vácuo, que permitia que os frascos fossem evacuados, e de gás carbônico de referência (que possui uma razão isotópica conhecida relativa ao PMAO), o

qual foi utilizado para preencher os frascos com CO_2 que tinham sido previamente evacuados. Este sistema estava acoplado a um banho de água, utilizado para o equilíbrio das amostras (20 °C por oito horas).

O sistema de equilíbrio foi conectado à entrada dupla do Finnigan MAT Delta S EMRIE via linha de transferência que incorporava um "ladrão" a - 70 °C, o qual foi utilizado para remover o vapor de água das amostras gasosas. A operação do sistema de equilíbrio foi controlada via o sistema de dados, que automaticamente evacuava as linhas de gases e permitia as amostras dos gases expandirem em qualquer um dos frascos de amostras para o "pulmão" do sistema duplo de entrada do espectrômetro de massas. Um sistema de "pulmão" idêntico já continha o gás de referência de composição isotópica conhecida. O gás do "pulmão" de referência foi analisado no espectrômetro de massas e a razão $^{18}O/^{16}O$ medida. Em seguida, os gases da amostra do "pulmão" foram analisados no espectrômetro de massas, a razão de isótopos medida, e o valor de delta da amostra gasosa determinanda. Este procedimento, alternando-se amostra e referência foi repetido 10 vezes para cada amostra. O teste de Dixons (Taylor, 1990*) para "outliers", com um limite de confidência de 80%, foi aplicado pelo software para a remoção automática dos "outliers" dos 10 valores medidos de $\delta^{18}O‰$ das amostras. Subsequentemente, o software registrou a média de $\delta^{18}O‰$ das amostras dos gases após a remoção dos "outliers".

Cálculos

Os resultados obtidos foram expressos em valores relativos ao padrão internacional PMAO de acordo com a equação 1:

$$\delta\ ^{18}O_{PMAO}\ ‰ = \left(\frac{R_{amostra}}{R_{padrao}} - 1 \right) x1000 \qquad (1)$$

onde R é a razão $^{18}O/^{16}O$.

* TAYLOR, J.K. Statitistical techniques for data analysis. Lewis Publisher, Inc. Chelsea, MI, 200 p., 1990.

Os dados inicialmente obtidos pelo sistema de dados foram normalizados versus a escala PMAO utilizando o padrão de ATN, de acordo com a equação 2:

$$\delta^{18}O\,‰\ _{(versus\ PMAO)} = \left[\delta^{18}O^{e}_{H_2O}\right]_{amostra} - \left[\left[\delta^{18}O^{e}_{H_2O}\right]_{ATN\ medido} - \left[\delta^{18}O^{e}_{H_2O}\right]_{ATN\ conhecido}\right] \quad (2)$$

onde:

$\left[\delta^{18}O^{e}_{H_2O}\right]_{amostra}$ é o valor 'bruto' de $\delta^{18}O‰$ da amostra calculado através da equação (1),

$\left[\delta^{18}O^{e}_{H_2O}\right]_{ATN\ medido}$ é o valor 'bruto' de $\delta^{18}O‰$ para o padrão de ATN (também calculado através da equação (1) e,

$\left[\delta^{18}O^{e}_{H_2O}\right]_{ATN\ conhecido}$ é o valor exato conhecido de $\delta^{18}O‰$ para o padrão da ATN da escala PMAO.