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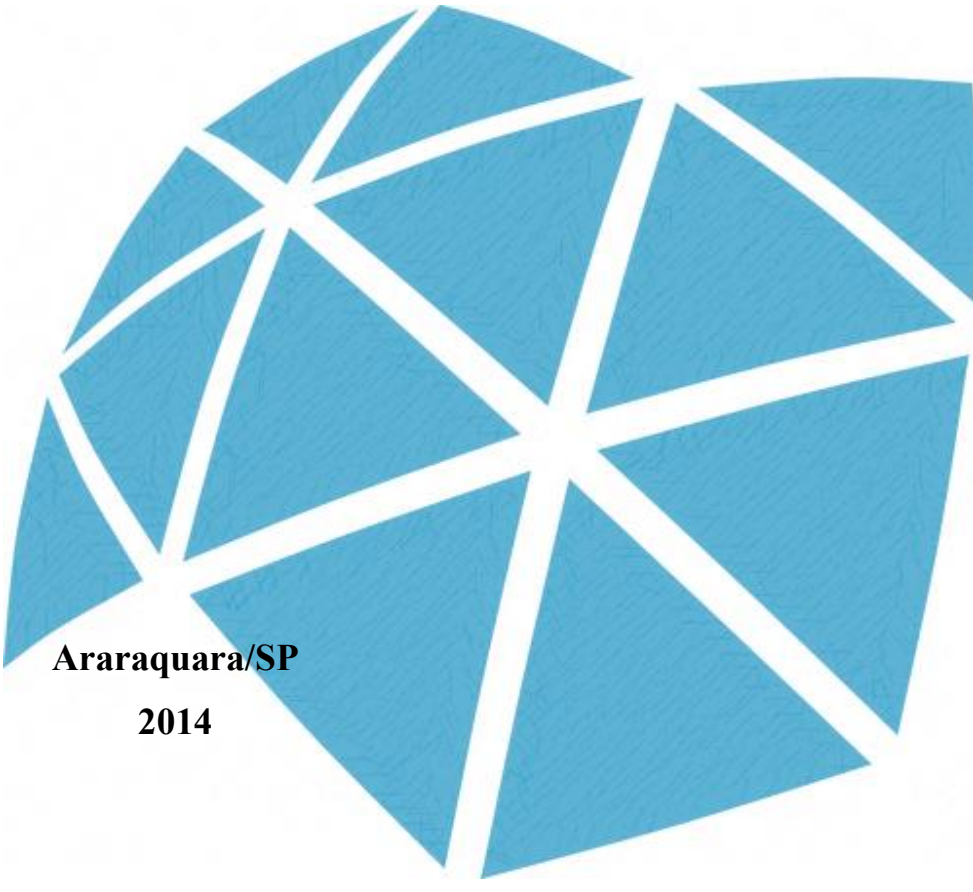
JACQUELINE QUEIROZ DA SILVEIRA

BIODISPONIBILIDADE DE FLAVANONAS E ATIVIDADE ANTIOXIDANTE DO SUCO DE LARANJA FRESCO *VERSUS* SUCO DE LARANJA PASTEURIZADO EM HUMANOS SAUDÁVEIS



Araraquara/SP
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JACQUELINE QUEIROZ DA SILVEIRA

Biodisponibilidade de Flavanonas e Atividade Antioxidante do Suco de Laranja Fresco *versus* Suco de Laranja Pasteurizado em Humanos Saudáveis

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Pais (João Marques e Elza) e Irmã (Kelly Cristina)

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SER FAMOSO OU SER IMPORTANTE...

"Você e eu sabemos que vamos morrer um dia. Desse ponto de vista, não é a morte que me importa, porque ela é um fato. O que me importa é o que eu faço da minha vida enquanto a minha morte não acontece, para que essa vida não seja banal, superficial, fútil, pequena…

A esta hora preciso ser capaz de fazer falta. No dia que eu me for eu quero fazer falta. Fazer falta não significa ser famoso, significa ser importante. Há uma diferença entre ser famoso e ser importante. Muita gente não é famosa e é absolutamente importante. Importar significa levar para dentro. Alguém me importa para dentro, me carrega.

Eu quero ser importante. Por isso, para ser importante eu preciso não ter uma vida que seja pequena. E uma vida se torna pequena quando ela é uma vida que só se apoia em si mesmo, fechada em si. Eu preciso transbordar. Eu preciso me comunicar. Eu preciso me juntar. Eu preciso me repartir nessa hora… Minha vida que, sem dúvida é curta, eu desejo que ela não seja pequena…"

Mário Sérgio Cortella

LISTA DE ABREVIATURAS

AMU	Atomic mass unit
ABTS	2,2 azobis, 3-ethylbenzothiozoline-6-sulphonic acid
AUC	*Area under the curve*
BMI	Body mass index
CMAX	Concentração máxima
DMSO	Dimethyl sulfoxide
DRI	Dietary reference intake
FOJ	Fresh orange juice
GLUC-HESP	Glucuronide hesperitin
GLUC-NAR	Glucuronide naringenina
GLUC-S-HESP	Glucuronide sulfate hesperitin
GLUC-S-HESP	Glucuronide sulfate hesperitin
HMF	Heptamethoxyflavone
HPLC	*High performance liquid chromatography*
HPTN	Hesperitin
HSP	Hesperidin
HSP-S	Hesperitin sulfate
ISR	Isosakuranetin rutinoside
MDA	Malondealdeído
NAR	Naringenin
NR	Narirutin
NOB	Nobelitin
OJ	Orange juice
POJ	Pasteurized orange juice
SIN	Sinensetin
TAN	Tangeretin
TBARS	Thiobarbituric acid-reactive substances
TMAX	Tempo máximo
TMS	Tetramethylscutellarein

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RESUMO

As flavanonas (hesperidina e narirutina) presentes quase exclusivamente em laranjas são associadas a diversos benefícios à saúde. O objetivo deste estudo foi analisar as diferenças dos parâmetros farmacocinéticos dos metabólitos das flavanonas e a atividade antioxidante em humanos, após o consumo de dois tipos de suco de laranja: fresco e pasteurizado. O suco fresco foi extraído com o auxílio de um espremedor *MJ-20 Basic* e o pasteurizado (95ºC/15s) foi fornecido pela Citrosuco, Matão-SP. Ambos os sucos foram obtidos a partir de laranjas Pera Rio do mesmo lote. Participaram 12 homens e 12 mulheres saudáveis, com 27 ± 6 anos, IMC de 24 ± 3 kg/m^2, não-fumantes e que não utilizavam nenhum tipo de hormônio ou suplementos dietéticos. Os voluntários ingeriram 11,5 ml/kg de peso corporal de suco de laranja fresco e, após *washout* de 30 dias, ingeriram a mesma quantidade de suco pasteurizado. Foi solicitado que os voluntários evitassem o consumo de alimentos cítricos nos 3 dias precedentes aos dias experimentais e as refeições do dia (café da manhã, almoço e lanche da tarde) foram fornecidas a fim de assegurar a abstenção de alimentos ricos em flavonoides. A urina foi coletada durante 24 h e o sangue foi coletado em jejum, 3, 4, 5, 6, 7, 8 e 24 h após o consumo do suco. Os metabólitos foram analisados no plasma sanguíneo e na urina por cromatografia líquida de alta performance por ionização electrospray acoplada à espectrometria de massa (HPLC-ESI-MS). A atividade antioxidante foi avaliada no soro sanguíneo por ABTS e TBARS, nos tempos: jejum, 4, 8 e 24 h. As concentrações de hesperidina e narirutina no suco de laranja comercialmente pasteurizado foram 3,2 e 6,6 vezes maiores do que no suco fresco, respectivamente, e estes compostos foram encontrados em grande parte na fração insolúvel (pellet) no suco pasteurizado, enquanto que ocorreram quase completamente na fração solúvel (sobrenadante) no suco fresco. A análise cromatográfica da urina mostrou concentrações de conjugados de ácido glicurônico e de grupos sulfato de hesperitina e naringenina após o consumo de ambos os sucos, sendo que todos os

metabólitos foram significativamente maiores após o suco de laranja pasteurizado. Os resultados dos parâmetros farmacocinéticos mostraram que não houve diferenças significativas na Tmax para os metabólitos de hesperidina e narirutina entre os sucos e, de modo geral, os valores de AUC e Cmax dos metabólitos detectados foram superiores após a ingestão do suco de laranja pasteurizado comparado ao suco de laranja fresco. As diferenças na farmacocinética dos metabólitos de flavanonas não foram substancialmente influenciadas pelas diferentes distribuições nas formas solúveis e insolúveis destes compostos. Os dois tipos de suco de laranja, fresco e pasteurizado, levaram ao aumento da capacidade antioxidante e diminuição da concentração de malondialdeído, sugerindo que o suco de laranja, independente do processamento, pode atuar na prevenção do estresse oxidativo mesmo após uma ingestão aguda (única).

ABSTRACT

Flavanones (hesperidin and narirutin) present almost exclusively in oranges, are associated with various health benefits. The aim of this study was to analyze the differences in the pharmacokinetic parameters of the flavanones metabolites and antioxidant activity in humans after consumption of two types of orange juice: fresh and pasteurized. Fresh orange juice (FOJ) is extracted with the aid of a squeezer MJ-20 Basic and pasteurized (95°C/ 15s) was provided by Citrosuco Matão SP. Both juices were obtained from Pera Rio oranges from the same batch. Participated 12 men and 12 healthy women, 27 ± 6 years, BMI 24 ± 3 kg/m^2, and non-smokers who did not use any hormones or dietary supplements. Volunteers ingested 11.5 ml/kg body weight of fresh orange juice and after washout of 30 days, they ingested the same amount of pasteurized juice. Volunteers were asked to avoid consumption of citrus foods in the preceding three days to the experiment and meals of the day (breakfast, lunch and afternoon snack) were provided to ensure the avoidance of foods rich in flavonoids. Urine was collected during 24 h and the blood was collected from fasting, 3, 4, 5, 6, 7, 8 and 24 h after the consumption of the juice. The metabolites were analyzed in plasma and urine by high-performance liquid chromatography- electrospray ionization- mass spectrometry (HPLC-ESI-MS). The antioxidant activity was evaluated in blood serum by ABTS and TBARS, at fasting, 4, 8 and 24 h. The concentrations of hesperidin and narirutina after the ingestion of commercially pasteurized orange juice were 3.2 and 6.6 times higher than after fresh juice, respectively, and these compounds have been found largely in the insoluble fraction (pellet) in pasteurized juice, while occurred almost entirely in the soluble fraction (supernatant) in fresh juice. The urine chromatographic analysis showed concentrations of conjugated of glucuronic acid and sulfate groups of hesperetin and naringenin after consumption of both juices, with all the metabolites was

significantly greater after the pasteurized orange juice. The results of the pharmacokinetic parameters showed no significant differences in Tmax for metabolites of hesperidin and narirutin between juices and, in general, the AUC and Cmax of the metabolites detected were higher after ingestion of pasteurized orange juice compared to fresh orange juice. The differences in the pharmacokinetics of metabolites of flavanones were not substantially influenced by different distributions in soluble and insoluble forms of these compounds. The two types of orange, fresh and pasteurized juice led to increased antioxidant capacity and decreased concentration of malondialdehyde, suggesting that the orange juice, independent of processing, can act in the prevention of oxidative stress even after an acute ingestion (single).

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INTRODUÇÃO GERAL

As frutas e os sucos cítricos possuem grandes quantidades de vitaminas, minerais, carotenoides e flavonoides, que têm sido associados a benefícios à saúde humana (MARTI et al, 2009; SANCHEZ-MORENO et al, 2003). Os flavonoides cítricos, presentes naturalmente no alimento ou em formas purificadas, têm efeitos biológicos em seres humanos e animais, como antioxidantes, anti-inflamatórios, anti-hipertensivos e redução dos níveis de lipídios sanguíneos que, efetivamente estão relacionados à prevenção de doenças cardiovasculares (RAMPRASATH et al, 2014; ROZA, XIAN-LIU e GUTHRIE, 2007; KUROWSKA e MANTHEY, 2004; KUROWSKA et al, 2000; BOK et al, 1999). Estudos relatam que o consumo regular de suco de laranja aumenta a atividade antioxidante do sangue (VAN ACKER et al, 2000) e reduz o nível de radicais livres (VINSON e JANG, 2001), conferindo proteção contra o câncer e outras doenças crônicas (MANTHEY, GUTHRIE e GROHMANN, 2001; BORRADAILE, CARROLL e KUROWSKA, 1999).

O suco de laranja é fonte dietética de flavonoides cítricos, as flavanonas (KAWAII et al, 1999), hesperidina e narirutina, os quais têm sido associados a atividades hipolipidêmicas (BOK et al, 1999; SANTOS et al, 1999), anti-inflamatórias e anticancerígenas (MANTHEY e BENDELE, 2008; TANAKA et al, 1997; YANG et al, 1997). Nos alimentos, as flavanonas são moléculas glicosiladas que são degradadas pelas enterobactérias presentes no intestino delgado e absorvidas como compostos aglicona (hesperitina e naringenina) (KANAZE et al, 2007). Estes compostos são absorvidos pelo cólon intestinal e fígado como evidenciado em estudos prévios de biodisponibilidade (BRETT et al, 2009; KANAZE et al, 2007; MANACH et al, 2005; FRANKE et al, 2005; ERLUND et al, 2000). Os monoglicuronídeos de hesperitina são os principais metabólitos das flavanonas presentes no plasma após a ingestão de suco de laranja, contudo, após o consumo de 500 a 1000 mL

do suco, foram encontrados níveis baixos de hesperitina (<2 μmol/L), indicando uma biodisponibilidade limitada destes compostos e ainda, a excreção urinária relativa foi semelhante para ambas as flavanonas, independente da dose (MANACH et al, 2003).

O processamento do suco de laranja influencia na concentração dos nutrientes e nas quantidades dos compostos flavonoides nas frações solúvel e insolúvel (TOMÁZ-NAVARRO et al, 2014; GIL-IZQUIERDO, GIL e FERRERES, 2002). Os sucos de laranja prontos para o consumo são divididos em categorias, entre elas encontram-se o NFC e o suco natural/fresco. O suco NFC, termo inglês que significa "non-frozen concentrated", é o suco pasteurizado que não passou pelo processo de concentração ou diluição durante a produção, e ainda, não é acrescido de água ou açúcares. O suco fresco/natural é o suco fornecido imediatamente após a extração, sem pasteurização ou qualquer outro tratamento físico e químico, e por isso, tem curto prazo de validade (CITRUS BR, 2014).

O suco de laranja fresco apresenta maiores quantidades de vitamina C e ácido fólico, 125 mg e 76 μg, respectivamente, enquanto os sucos pasteurizados apresentaram 86 mg de vitamina C e 46 μg de folato. Por outro lado, os sucos integrais e industrialmente pasteurizados apresentaram aproximadamente o dobro da quantidade de flavonoides cítricos quando comparados ao suco fresco (GIL-IZQUIERDO, GIL e FERRERES, 2002; USDA, 2014).

Existem poucos estudos científicos sobre a biodisponibilidade dos compostos flavonoides presentes em laranjas e seus sucos, sendo que, a maioria utilizou o tratamento enzimático dos compostos para melhorar a absorção dos mesmos, e ainda, não há nenhum trabalho mostrando a farmacocinética dos metabólitos conjugados aos ácidos glicurônicos e grupos sulfatos, investigação proposta no presente estudo. Os estudos de biodisponibilidade da hesperidina e narirutina, ocorridos entre os anos de 2000 a 2014, utilizaram diferentes tipos de sucos como o fresco espremido à mão,

sucos comerciais com e sem o enriquecimento com hesperidina, homogeneizado a alta pressão e até mesmo com a fruta in natura. A área sob a curva (AUC) da hesperitina variou entre 0,95 a 10,3 μmol/L/h, a concentração máxima (Cmax) apresentaram uma grande faixa, variando de 96 a 2.176 nmol/h, e o tempo de concentração máxima (Tmax) variou entre 4,4 a 7 horas após a ingestão do suco ou da fruta. A porcentagem de absorção variou entre 0,4 e 2,4% e a excreção urinária relativa foi de 1,7 a 6,6 %, com a dose de hesperitina ingerida variando entre 18.8 a 220 mg. A dose ingerida de narirutina foi menor em comparação à dose de hesperitina, variando entre 3.3 e 45.2, com uma absorção de 0,15 a 3,12% e excreção urinária de 0,7 a 12,5% (TOMAZ-NAVARRO et al, 2014; VALLEJO et al, 2010; BREDSDORFF et al, 2010; BRETT et al, 2009; MULLEN et al, 2008; MANACH et al, 2003; ERLUND et al, 2000).

Baseados nas evidências dos efeitos benéficos dos flavonoides cítricos na prevenção e controle de enfermidades crônicas é de interesse quantificar os metabólitos das flavanonas presentes no sangue e na urina, compreender a diferença dos parâmetros farmacocinéticos e investigar a capacidade antioxidante destes compostos presentes nos sucos de laranja comparando dois tipos de processamento diferentes: espremido fresco e pasteurizado.

CAPÍTULOS

CAPÍTULO 1 – Revisão Bibliográfica

Flavonoides

Polifenóis são compostos fitonutrientes originados do metabolismo secundário dos vegetais, caracterizados por um anel aromático com um ou mais grupos hidroxila. Existem pelo menos 8.000 compostos polifenólicos na natureza, incluindo flavonoides, álcoois fenólicos, lignanas, estilbenos e ácidos fenólicos. Estes compostos agem na defesa das plantas contra a radiação ultravioleta, radicais livres e agentes patogênicos[27] e de acordo com estudos epidemiológicos, quando ingeridos exercem proteção ao organismo humano contra doenças degenerativas (KANAZE et al, 2007; BAHADORAN et al, 2013; SCALBERT et al, 2005).

Os flavonoides compõem um grupo de polifenóis que contém uma estrutura básica comum formada por C_6-C_3-C_6. A estrutura consiste em dois anéis aromáticos (A e B) unidos por três carbonos, formando um anel pirano fechado (C) (Figura 1).

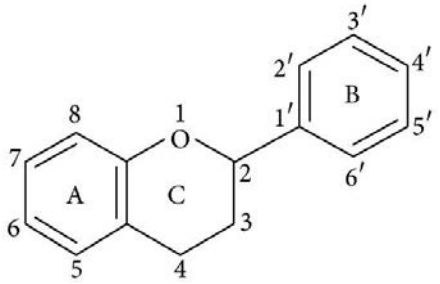
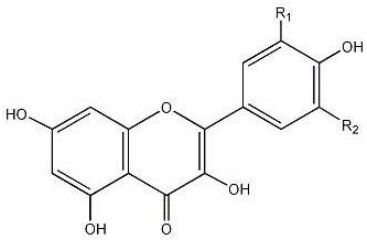


Figura 1 - Estrutura básica dos Flavonoides (Fonte: D'ARCHIVIO et al, 2007)

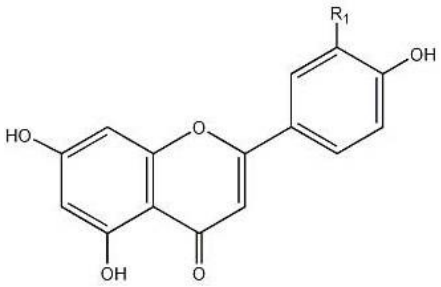
De acordo com o estado de oxidação do anel pirano os flavonoides são divididos em seis subclasses: flavonóis, flavonas, flavanonas, isoflavonas, antocianidinas, flavanóis (Figura 2). Mais de 4.000 flavonoides foram identificados (D'ARCHIVIO et al, 2007).

FLAVONOL



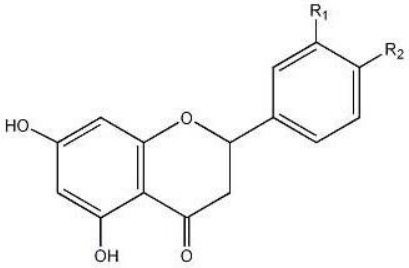
R_1 = H; R_2 = H: Kaempferol
R_1 = OH; R_2 = H: Quercetin
R_1 = OH; R_2 OH: Myricetin
R_1 = OCH_3; R_2 = H: Isorhamnetin

FLAVONA



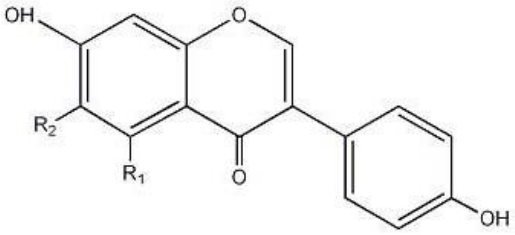
R_1 = H: Apigenin
R_1 = OH: Luteolin

FLAVANONA



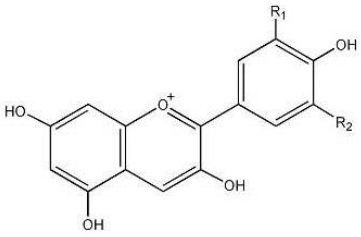
R_1 = H; R_2 = OH; Naringenin
R_1 = OH; R_2 = OH; Eriodictyol
R_1 = OH; R_2 = OCH_3: Hesperetin

ISOFLAVONA



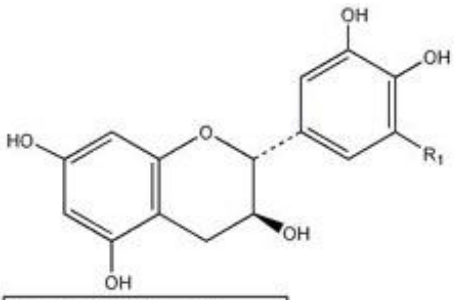
R_1 = H; R_2 = H: Daidzein
R_1 = OH; R_2 = H: Genistein
R_1 = H; R_2 = OCH_3: Glycitein

ANTOCIANIDINA



R_1 = H; R_2 = H: Pelagonidin
R_1 = OH; R_2 = H: Cyanidin
R_1 = OH; R_2 = OH: Delphinidin
R_1 = OCH_3; R_2 = OH: Petunidin
R_1 = OCH_3; R_2 = OCH_3: Malvidin

FLAVANOL



R_1 = H: (+)-Catechin
R_1 = OH: (+)-Gallocatechin

Figura 2. Estruturas químicas das subclasses de flavonoides (Fonte: KUMAR e PANDEY, 2013)

Vegetais, chás e vinhos são as principais fontes alimentares dos flavonoides e os efeitos benéficos do seu consumo sobre a saúde são suportados por evidências epidemiológicas. A relação entre o consumo de flavonoides e a proteção contra doenças cardiovasculares tem sido estudada com muita ênfase (BHULLAR et al, 2014; QURESHI et al, 2013; PFEUFFER et al, 2013) e, além disso, os flavonoides tem mostrado capacidade de ação sobre a eliminação de radicais livres, propriedades anti-inflamatórias, anticarcinogênese, antivirais e prevenção de doenças degenerativas relacionadas à idade (PUSPARINI et al, 2013; MISHRA et al 2013; BEKING e VIEIRA, 2010).

Flavanonas (Hesperidina e Narirutina)

As flavanonas representam um pequeno grupo de compostos derivados dos flavonoides, que incluem a hesperidina e a narirutina, presentes em laranjas, e a eriocitrina, encontrada especificamente em limões. Em frutas cítricas, as flavanonas são responsáveis por aproximadamente 95% do conteúdo total flavonoides (PETERSON et al, 2006). São caracterizadas pela presença de uma cadeia de três carbonos saturados e um átomo de oxigênio no C_4, sendo geralmente glicosiladas por um dissacarídeo na posição C_7 (D'ARCHIVIO et al, 2007). Este dissacarídeo pode ser uma neohesperidose, que confere o sabor amargo, encontrada na naringina em *grapefruit*, ou uma rutinose, insípida, encontrada na hesperidina em laranjas (CHANET et al, 2012).

As flavanonas contém vários glicosídeos de três principais agliconas: hesperitina, naringenina e eridictiol que diferem-se pelas substituições de hidroxil e metoxil nos anéis A e B (Figura 1) (BRETT et al, 2009). Nas laranjas, os principais glicosídeos são hesperidina (hesperitina-7-O-rutinosídeo, hesiperetina-7-O-glicuronídeo) e narirutina (naringenina-7-O-rutinosídeo, naringe-nina-

7-O-glicuronídeo, naringenina-4-O-glicuronídeo) (Figura 3) (D'ARCHIVIO et al, 2007; LEUZZI, 2000).

A distribuição das flavanonas nos alimentos é limitada, ocorrendo teores elevados nas frutas cítricas e seus sucos e sendo encontradas poucas quantidades em tomates e algumas plantas aromáticas como a hortelã. Desta forma, indivíduos que consomem alimentos cítricos regularmente, apresentam uma exposição relativamente alta a esta classe de flavonoides (PANDEY e RIZVI, 2009).

A ingestão diária de flavanonas agliconas em adultos foi estimada entre 2,7 e 78,0 mg (ERDMAN et al, 2007; PEREZ-JIMENEZ, 2011)[3], constituindo a maior parte de flavonoides totais consumidos em diferentes países europeus (KNEKT et al, 2002). As diversas variedades de laranjas e seus sucos tem sido apontados como os principais contribuintes para a ingestão das flavanonas (ZAMORA-ROS et al, 2010).

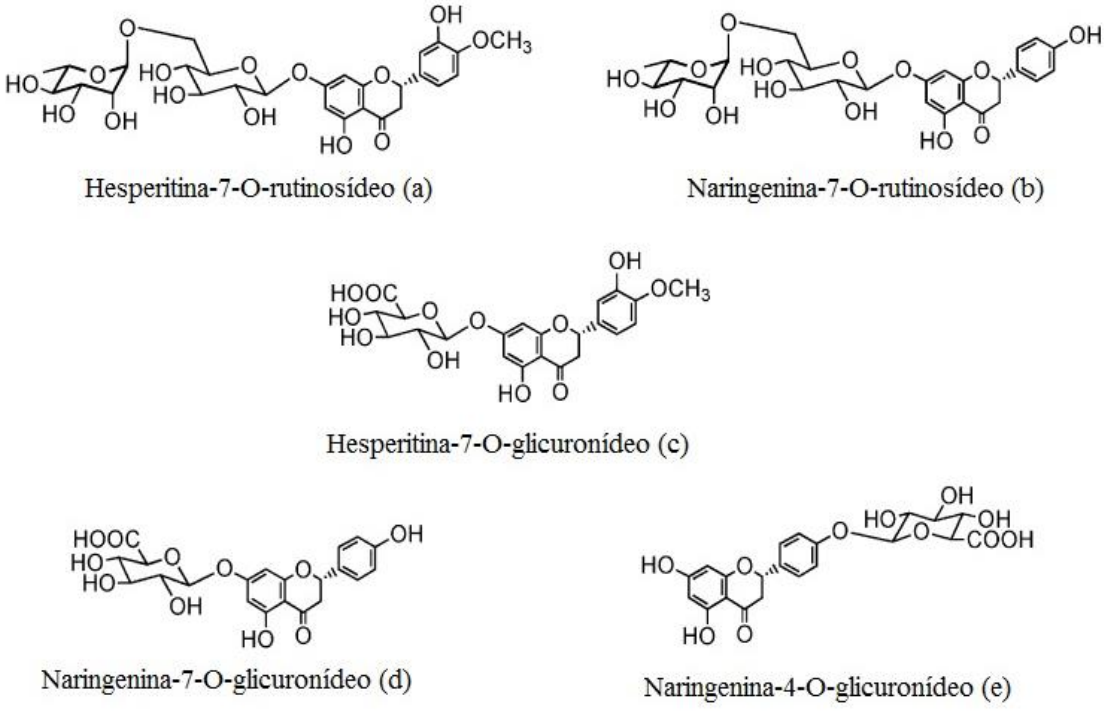


Figura 3- Estruturas glicosiladas das flavanonas (Fonte: MULLEN et al, 2008)

Efeitos Benéficos das Flavanonas sobre a Saúde

Estudos anteriores de suplementação com suco de laranja ou com flavanonas na forma isolada, tem demonstrado várias propriedades de proteção à saúde, como antioxidantes (SNYDER et al, 2011), hipolipidêmicas (APTEKMANN e CESAR, 2013), anti-hipertensivas (MORAND et al, 2011), anti-inflamatórias (RIZZA et al, 2011) e antidiabéticas (YOSHIDA et al, 2013).

Os flavonoides presentes na laranja podem reduzir a oxidação pós-prandial, que é um importante contribuinte para o desenvolvimento do estado de doenças crônicas. Um estudo *crossover*, com 16 indivíduos (homens e mulheres), que utilizou 1) placebo (ácido ascórbico e açúcar, equivalentes ao suco de laranja), 2) placebo mais hesperidina e 3) suco de laranja, mostrou que o placebo com hesperidina e o suco de laranja aumentaram significativamente a capacidade antioxidante no soro sanguíneo evidenciando que os compostos fenólicos da laranja contribuem diretamente para a proteção oxidativa pós-prandial (SNYDER et al, 2011).

Aptekmann e Cesar (2013) compararam o perfil lipídico de homens e mulheres com níveis sanguíneos de colesterol normais e moderadamente elevados, que consumiam suco de laranja por longo prazo (≥ 12 meses) a indivíduos com as mesmas características, porém, não consumidores. Os resultados mostraram que o grupo dos consumidores de suco, com ambos os níveis de colesterol, apresentaram o colesterol total, LDL-c, ApoB e a razão LDL/HDL significativamente menores do que os indivíduos homólogos não consumidores, concluindo que o consumo de suco de laranja a longo termo está associado a baixos fatores de risco para doença cardiovascular.

Estudos sugerem que os efeitos anti-hipertensivos do suco de laranja estão associados às flavanonas. Basile et al (2010) mostraram que o consumo de 500 mL/dia de suco de laranja pasteurizado, durante 8 semanas, diminuiu significativamente a pressão arterial diastólica, em homens saudáveis. Similarmente, estudo realizado por Morand et al (2011) em um período de tempo

menor (4 semanas) em homens de meia-idade e com excesso de peso, também evidenciou a diminuição da pressão arterial diastólica.

Rizza et al (2011) elucidaram os mecanismos de ação da hesperitina na estimulação da produção de NO que pode se opor a ações aterogênicas de citocinas pró-inflamatórias em células endoteliais vasculares. O estudo demonstrou que o consumo oral diário durante três semanas de hesperidina melhora a função endotelial, reduz a circulação de biomarcadores da inflamação e altera favoravelmente o perfil lipídico em indivíduos com síndrome metabólica.

Segundo Yoshida et al (2013), a inflamação induzida pela obesidade contribui para o desenvolvimento da resistência à insulina e do diabetes mellitus do tipo II e, recentemente, foi evidenciado que receptores Toll-like (TLR) estão envolvidos neste processo. Assim, a regulação adequada da expressão ou ativação das TLR's é uma estratégia importante para melhorar a doenças relacionadas à obesidade. Os pesquisadores mostraram que a naringenina, suprime a expressão da TLR durante a diferenciação dos adipócitos e macrófagos e também inibe o fator de necrose tumoral-α (TNF-α). Estes resultados correlacionam-se com a supressão de mediadores inflamatórios e a melhoria da hiperglicemia e tomados em conjunto, estes dados sugerem que a naringenina exibe propriedades anti-inflamatórias e antidiabéticas.

Metabolismo e Biodisponibilidade dos Flavonoides/ Flavanonas

O metabolismo, a biodisponibilidade e a atividade biológica dos flavonoides dependem de fatores como a configuração, o número total de grupos hidroxila e a substituição de grupos funcionais sobre a sua estrutura nuclear (KUMAR e PANDEY, 2013; QURESHI et al, 2013). A biodisponibilidade dos diversos polifenóis difere muito de um para outro, de modo que os mais abundantes na dieta não são necessariamente os que apresentam maiores concentrações de

metabólitos ativos na circulação sanguínea e tecidos alvo. Quando ingeridos, os compostos bioativos são extensivamente metabolizados por enzimas intestinais e hepáticas e pela microbiota presente no cólon intestinal, resultando em metabólitos conjugados diferentes dos compostos nativos (KANAZE et al, 2007).

Como mencionado anteriormente, as flavanonas (hesperidina e narirutina) estão naturalmente presentes nas frutas cítricas ligadas a moléculas ramnoglicosídeas, como os 7-O-rutinosídeos de hesperitina e de naringenina. A molécula de glicose é um dos principais determinantes do local de absorção e da biodisponibilidade dos flavonoides e é reconhecido que a biodisponibilidade dos monoglicosídeos de flavonoides é muito maior do que os rutinosídeos (MANACH et al, 2005; NIELSEN et al, 2006; HOLLMAN et al, 1999). Outro fator que pode ser determinante para os parâmetros farmacocinéticos são as diferenças na microbiota entérica devido a uma grande variabilidade interindividual (ERLUND et al, 2000).

Os rutinosídeos são absorvidos apenas na parte distal do intestino após hidrólise pela microbiota do cólon e este processo é provavelmente o passo limitador da velocidade de absorção. A Cmax da hesperitina tem sido captada em média 6 h após a ingestão do suco de laranja (ERLUND et al, 2000; MANACH et al, 2003; NIELSEN et al, 2006; HOLMANN et al, 1999). Em contraste, as agliconas e glicosídeos de flavanonas são absorvidos no intestino delgado, local onde a absorção ocorre mais rapidamente (Cmax em 1 h para hesperitina) (NIELSEN et al, 2006).

A absorção dos glicosídeos de flavonoides no intestino delgado pode ocorrer de duas maneiras: 1) o glicosídeo é hidrolisado pela lactase-florizina hidrolase, presente na borda em escova, e então a aglicona livre difunde-se através das células epiteliais por transporte passivo ou por difusão facilitada (DAY et al, 2003) e 2) a molécula glicosídica pode ser transportada para o interior do enterócito por meio de um transportador de glicose, o SGLT-1 (*sodium-glucose linked transporter*)

e deglicosilada pelas β-glicosidases (glicocerebrosidases e glicosidases citosólicas) presentes intracelularmente. Ambas as vias de absorção originam agliconas intracelulares que, posteriormente, se tornam conjugados glicurônicos ou sulfatados (Figura 4) (NIELSEN et al, 2006; DAY et al, 2003).

No intestino delgado e no epitélio do cólon, os flavonoides já poderão sofrer reações de conjugação, como a glicuronidação e a metilação (BRAND et al, 2008; SPENCER et al, 1999). Após serem transportados dos enterócitos ao fígado pelo sistema-portal ligados à albumina ou via linfática, os flavonoides sofrem biotransformações por meio das enzimas de conjugação de fase II, UDP's glicuronosiltransferases e sulfotransferases, formando uma variedade de metabólitos com ácidos glicurônicos e grupos sulfatos (SCALBERT et al, 2000; MATSUMOTO et al, 2004). A glicuronidação e a sulfatação são especialmente importantes porque aumentam substancialmente o peso molecular e a solubilidade das agliconas dos polifenóis (WILLIAMSON, 2002; WU et al, 2011; XIAO e HÖGGER, 2013). Finalmente, os metabólitos conjugados aos glicuronídeos e sulfoglicuronídeos podem ser excretados pela bile e/ou urina, dependendo do metabólito formado (MANACH et al, 2003; HEIM, TAGLIAFERRO e BOBILVA, 2002).

Os metabólitos conjugados produzidos endogenamente são responsáveis pelos efeitos sistêmicos dos flavonoides, portanto, o conhecimento sobre a natureza dos metabólitos gerados pelo metabolismo é fundamental para a realização de estudos sobre os supostos efeitos positivos na saúde (BREDSDORFF et al, 2010).

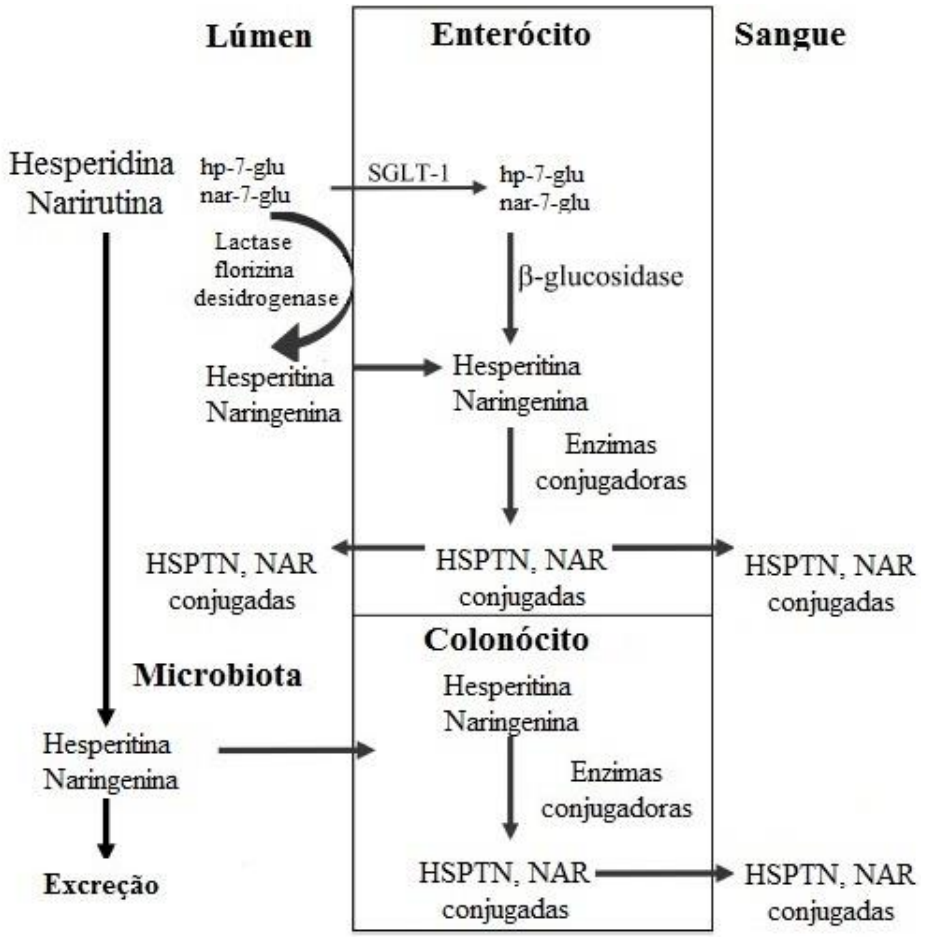


Figura 4. Caminhos metabólicos da Hesperidina e Narirutina (Adaptado de NIELSEN et al, 2006)

Estudos sobre a Biodisponibilidade das Flavanonas

Foi realizado um levantamento de estudos que quantificaram as flavanonas, hesperidina e a narirutina, após ingestão de suco de laranja fresco, pasteurizado, reconstituído de concentrado, homogeneizado a alta pressão e a fruta, no qual foram considerados os seguintes dados: autor/ano, tipo do suco de laranja, quantidade de suco ingerido, de hesperitina e naringenina; e parâmetros farmacocinéticos: área sobre a curva (AUC), concentração máxima (Cmax), tempo da concentração máxima (Tmax), excreção urinária relativa à dose ingerida e a porcentagem de absorção (Tabelas 1 e 2).

Erlund et al (2000) avaliaram as concentrações plasmáticas e urinárias das flavanonas em oito voluntários saudáveis (cinco mulheres e três homens) que ingeriram uma dose de suco de laranja (8 ml/kg de peso corporal). Foram colhidas onze amostras de sangue e urina de 24 h após a administração do suco. As concentrações plasmáticas máximas (Cmax) foram 0,6 μmol/L para naringenina e o valor correspondente para hesperitina foi de 2,2 μmol/L. A excreção urinária relativa foi de 1,1% para naringenina e 5,3% para hesperitina. Os resultados deste estudo mostraram que as concentrações de hesperitina e naringenina são relativamente elevados após a ingestão de suco de laranja e que a variação interindividual na biodisponibilidade é um fator importante. Os autores concluíram que as atividades biológicas atribuídas as flavanonas podem resultar em efeitos positivos à saúde de indivíduos que consomem laranja regularmente (Tabelas 1 e 2).

Manach et al (2003) analisaram a cinética plasmática e a excreção urinária das flavanonas, hesperidina e narirutina. Após o jejum de 12 horas, cinco voluntários saudáveis ingeriram 0,5 mL ou 1 litro de suco de laranja comercial. O sangue foi colhido em 10 tempos diferentes em um período de 24 h. A urina foi coletada durante 48 horas, em cinco frações. Os metabólitos das flavanonas apareceram no plasma 3 h após a ingestão do suco, atingindo o pico entre 5 e 7 h, em seguida, voltou à linha de base em 24 h. O pico de concentração plasmática da hesperitina foi de 0,46 μmol/L e 1,28 μmol/L após a ingestão de 0,5 e 1 L, respectivamente. E o pico de concentração da naringenina foi menor: 0,20 μmol/L após a dose de 1 L. As formas de circulação encontradas da hesperitina foram glicuronídeos (87%) e os sulfoglicuronídeos (13%). Para ambas as flavanonas, a excreção urinária foi quase completa após 24 h da ingestão do suco de laranja. A excreção urinária relativa foi semelhante em ambas as flavanonas, não dependendo da dose. A partir do consumo moderado ou alto de suco de laranja as flavanonas representam uma parte importante do conjunto de polifenóis totais presentes no plasma (Tabelas 1 e 2).

A hipótese de que a biodisponibilidade de fitonutrientes dietéticos é influenciada pela matriz alimentar em que eles são consumidos, fez com que Mullen et al (2008) investigassem o impacto do iogurte integral na biodisponibilidade e no metabolismo das flavanonas do suco de laranja. Plasma e urina foram colhidos durante 24 h após o consumo de 250 ml de suco de laranja, contendo um total de 168 μmol de hesperitina-7-O-rutinosídeo e 12 μmol de naringenina-7-O-rutinosídeo, com e sem 150 ml de iogurte integral. Os resultados revelaram que a hesperitina-7-O-glicuronídeo depois ingestão de suco de laranja teve uma Cmax de 922 nmol/L e um Tmax de 4,4 h. Quando o suco foi consumido com iogurte, nem a Cmax de 661 nmol/L nem o Tmax de 5,1 h foram significativamente diferentes dos obtidos com o suco sem iogurte. O glicuronídeo de hesperitina também foi excretado na urina juntamente com a hesperitina-O-glicuronídeo, hesperitina-O-glicuronídeo-O-sulfato, hesperitina-O-diglicuronídeo, naringenina-O-diglicuronídeo, naringenina-7-O-glicuronídeo e naringenina-4'-O-glicuronídeo. A excreção urinária não foi afetada pela adição de iogurte no suco, assim, houve pouco impacto do iogurte sobre o destino das flavanonas do suco de laranja. Os autores comentaram que são necessárias investigações detalhadas sobre a complexidade dos efeitos da matriz dos alimentos sobre a absorção dos flavonoides quando são consumidos como parte de uma refeição real, em oposição à ingestão com o estômago vazio de um único item alimentar (Tabelas 1 e 2).

A absorção das flavanonas pode ser afetada pelo processamento do suco e a bioatividade dos fitonutrientes depende de como eles são metabolizados após a absorção. Brett et al (2009) realizaram um estudo crossover randomizado com 20 indivíduos que consumiram uma única porção de laranja (150 g) ou suco (300 g) e um adicional de 109 indivíduos de ampla faixa etária (18-80 anos) consumidores de suco e avaliaram a relação da absorção das flavanonas com a idade, sexo e índice de massa corpórea (IMC). Após o consumo da fruta ou do suco, conjugados de flavanonas, mas não as agliconas ou rutinosídeos, foram detectados no plasma e na urina. As flavanonas conjugadas

incluem 7- e 4'-O-monoglicuronídeos de naringenina, 7- e 3'-O- monoglicuronídeos de hesperitina, diglicuronídeos de hesperitina e hesperitina glicuronídeo sulfatado. A análise dos dados da farmacocinética plasmática e da excreção urinária em uma base ajustada da dose não indicou diferenças na absorção ou excreção de qualquer flavanona entre as matrizes: fruta ou suco. No conjunto de dados sobre a excreção urinária a variação individual se mostrou muito grande (de 0 a 59%). Houve uma diminuição significativa na excreção da hesperitina (mas não da naringenina) com o aumento da idade, e com relação ao sexo e IMC foi demonstrado não haver associação com a variação na excreção das flavanonas (Tabelas 1 e 2).

Bredsdorff et al (2010) determinaram a absorção, conjugação e excreção da naringenina-7-O-rutinosídeo (narirutina) em humanos, em comparação com o glicosídeo correspondente em uma matriz de suco de laranja. O estudo crossover e duplo cego contou com oito voluntários saudáveis que consumiram (1) suco de laranja com teor natural de naringenina-7-O-rutinoside e (2) suco de laranja com naringenina-7-O-glicosídeo (obtida por meio de tratamento enzimático com α-ramnosidase). O sangue foi coletado em doze pontos de tempo e três frações de urina foram coletadas em 24 h. A área sob a curva de naringenina no plasma a partir de suco de laranja enriquecido (2) foi aumentada em cerca de 4 vezes, a Cmax foi 5,4 vezes mais elevada e Tmax foi diminuída 311 para 92 min em comparação com o suco de laranja (1), indicando a mudança do local de absorção, a partir do cólon para o intestino delgado. Além disso, a quantidade de metabólitos na urina foi de 7 para 47% da dose, após o consumo do suco de laranja tratado com α-ramnosidase (2). Todas as amostras de urina continham os metabólitos naringenina-7 e 4-O-glicuronídeos. Além disso, para examinar o efeito da dose e o tratamento com ramnosidase sobre os perfis de hesperitina conjugada, um outro tratamento suplementar foi realizado em que o suco de laranja foi fortificado com o conteúdo três vezes maior que o original de hesperitina-7-O-rutinosídeo (3). Foram encontrados cinco metabólitos

de hesperitina (3'-O-glicuronídeo, 7-O-glicuronídeos, 5,7-O-diglicuronídeo, 3',7-O-diglicuronídeo, 3'-O-sulfato), com o mesmo perfil dos conjugados. Os dados deste estudo mostraram que a biodisponibilidade da naringenina é aumentada pela conversão do rutinosídeo em glicosídeo, mas o perfil dos conjugados de flavanonas formados e excretados na urina não foi afetado pelo local de absorção, nem com o aumento de 3 vezes na dose (Tabelas 1 e 2).

Vallejo et al (2010) estudaram os efeitos da concentração e da solubilidade das flavanonas de sucos de laranja reconstituídos sobre a sua biodisponibilidade em um estudo crossover com 10 voluntários saudáveis. Foram avaliadas cinco bebidas com diferentes concentrações de flavanonas. Os sucos de laranja comerciais (29,2-70,3 mg de flavanonas/100 mL) foram comparados a sucos de laranja experimentais enriquecidos com 110,2 mg/100 mL de flavanonas. Glicuronídeos e sulfatos de hesperitina e naringenina foram detectados e quantificados no plasma e na urina. O estudo mostrou que a solubilidade das flavanonas, em particular a da hesperidina, no suco é um fator chave tanto para a biodisponibilidade como para a excreção das flavanonas e a Cmax no plasma, correlaciona-se bem com a concentração da flavanona solúvel no suco, enquanto parece não haver qualquer correlação com a ingestão total das flavanonas. Além disso, foi observada uma grande variação interindividual, sendo consistente para cada indivíduo depois da ingestão das diferentes bebidas, o que sugere que a biodisponibilidade das flavanonas é também dependente da ocorrência da microbiota específica que é capaz de remover os rutinosídeos provenientes do suco, que resulta em agliconas que são, então, absorvidos no intestino (Tabelas 1 e 2).

Tomás-Navarro et al (2014) avaliaram o efeito da homogeneização de alta pressão comparando a pasteurização convencional sobre a excreção urinária das flavanonas em 18 voluntários estratificados em diferentes níveis de absorção/excreção de flavanonas (alta, média e baixa). Os resultados mostraram que o conteúdo de flavanonas e a solubilidade foram similares em ambos os

sucos, enquanto que o suco homogeneizado a alta pressão apresentou partículas (microssuspensões no pellet - parte insolúvel do suco) de tamanhos menores. A ingestão de quantidades semelhantes de flavanonas solúveis levou, também, à excreção urinária relativa semelhante de metabólitos. No entanto, diferenças significativas foram encontradas quando os voluntários foram estratificados por suas capacidades de excreção (maiores após a ingestão do suco homogeneizado a alta pressão). Assim, as diferenças observadas para excreção das flavanonas após a ingestão dos sucos pelos voluntários com alta excreção indicaram claramente que as características do suco, tais como o tamanho das partículas, pode também ser relevante para a absorção. Os autores concluíram que a estratificação dos indivíduos por sua capacidade de excreção é mais relevante do que os tratamentos tecnológicos avaliados em termos de biodisponibilidade das flavanonas, devendo ser considerada em estudos clínicos com sucos da fruta ou com os compostos isolados, uma vez que poderia explicar a grande variabilidade interindividual (Tabelas 1 e 2).

Tabela 1 - Estudos da biodisponibilidade da Hesperitina.

		Quantidade ingerida	Hesperitina (Dose)	Hesperitina (Plasma)			Excreção Urinária Relativa	Absorção
				AUC	Cmax	Tmax		
Fonte	**Suco de laranja**	mL/g	mg	μmol/L/h	nmol/L	h	Dose ingerida %	%
Erlund et al, 2000 (n=8)	SCP	8mL/ kg peso corporal	126 ± 26	10.3 ± 8.2	2176 ± 1591	5.4 ± 1.6	5.30 ± 3.1	2.4
Manach et al, 2003 (n=5)	SCP	500	110 ± 3.7	4.2 ± 1.1	462 ± 69	5.4 ± 0.40	4.13 ± 1.18	1.2
		1000	220 ± 7.4	9.3 ± 1.9	1286 ± 130	5.8 ± 0.37	6.41 ± 1.32	1.3
Mullen et al, 2008 (n=8)	SCP com hesperitina	250	50.7 ± 0.0	4.1 ± 2.9	922 ± 224	4.4 ± 0.5	6.30 ± 2.0	2.4
Brett et al, 2009 (n=20)	SCP	300	71.8 ± 8.1	1.1 ± 1.2	103 ± 126	6.2 ± 2.0	4.63 ± 3.05	0.5
	Fruta/ Laranja	150	79.7 ± 17.7	1.2 ± 1.7	96 ± 124	7.0 ± 4.23	4.53 ± 3.44	0.5
Bredsdorff et al, 2010 (n=16)	SCP	1 mg/kg peso corporal	ND	ND	ND	ND	ND	ND
Vallejo et al, 2010 (n=10)	SCR – A	400	35.05	1.2 ± 0.3	325 ± 65	4.6 ± 0.7	5.4 ± 1.2	1.03
	SCR – B		77.40	0.95 ± 0.23	366 ± 70	6.4 ± 0.7	1.7 ± 0.4	0.40
Tomás-Navarro et al, 2014 (n=18)	SFEM	400	18.8	ND	ND	ND	8.1 ± 1.4 / 8.4 ±1.5	----
	SHAP		51.6	ND	ND	ND	4.8 ± 1.1/ 10.1 ± 2.4	----
	SCP		56.2	ND	ND	ND	3.3 ± 0.5/ 7.7 ± 1.1	----

ND = Não determinado AUC = Área sob a curva; Cmax = Concentração máxima; Tmax = Tempo de concentração máxima
SCP = Suco comercial pasteurizado; SCR = Suco comercial reconstituído (dois tipos: A e B); SFEM = Suco fresco espremido à mão; SHAP = Suco homogeneizado à alta pressão

Tabela 2 - Estudos da biodisponibilidade da Naringenina.

		Quantidade ingerida	Naringenina (Dose)	Naringenina (Plasma)			Excreção Urinária Relativa	Absorção
				AUC	Cmax	Tmax		
Fonte	**Suco de laranja**	mL/g	mg	µmol/L/h	nmol/L	h	Dose ingerida (%)	%
Erlund et al, 2000 (n=8)	SCP	8mL/ kg peso corporal	23 ± 2	2.6 ± 1.6	643 ± 404	5.5 ± 2.9	1.1 ± 0.8	3.12
Manach et al, 2003 (n=5)	SCP	500	22.6 ± 1.9	0.43 ± 0.17	59 ± 18	4.6 ± 0.6	7.1 ± 1.9	0.51
		1000	45.2 ± 4.0	1.29 ± 0.33	199 ± 40	5.0 ± 0.45	7.9 ± 1.7	0.77
Mullen et al, 2008 (n=8)	SCP com hesperitina	250	3.3 ± 0.0	ND	ND	ND	17.7 ± 3.9	ND
Brett et al, 2009 (n=20)	SCP	300	9.4 ± 0.7	0.647 ± 0.625	53 ± 53	4.5 ± 2.5	10.2 ± 6.8	1.87
	Fruta/Laranja	150	11.8 ± 5.5	0.846 ± 0.846	85 ± 118	5.9 ± 1.8	12.5 ± 10.6	1.95
Bredsdorff et al, 2010 (n=16)	SCP	1 mg/kg peso corporal	ND	0.30 ± 0.20	120 ± 140	5.2 ± 3.0	7.0 ± 3.0	ND
Vallejo et al, 2010 (n=10)	SCR – A	400	12.38	0.066 ± 0.027	37 ± 10	4.7 ± 1.1	2.6 ± 0.5	0.15
	SCR – B		15.19	0.735 ± 0.179	443 ± 109	5.7 ± 0.7	0.7 ± 0.2	1.31
Tomás-Navarro et al, 2014 (n=18)	SFEM	400	7.0	ND	ND	ND	11 ± 1.8 / 11.4 ±1.9	----
	SHAP		21.9	ND	ND	ND	7.7 ± 1.9/ 9.3 ± 2.3	----
	SCP		22.5	ND	ND	ND	6.7 ± 1.2/ 8.5 ± 1.6	----

ND = Não determinado AUC = Área sob a curva; Cmax = Concentração máxima; Tmax = Tempo de concentração máxima
SCP = Suco comercial pasteurizado; SCR = Suco comercial reconstituído (dois tipos: A e B); SFEM = Suco fresco espremido à mão; SHAP = Suco homogeneizado à alta pressão

Polimetoxiflavonas

As polimetoxiflavonas (PMFs) são metabólitos secundários de flavonoides que sofreram metilações sucessivas em seus grupos hidroxílicos, sendo exclusivos nas espécies cítricas e encontradas principalmente na casca dos frutos. Assim como os flavonoides, as polimetoxiflavonas também exercem função de defesa contra diversos patógenos causadores de doenças nas plantas (DEL RIO et al, 1998).

A tangeritina (TAN) e a nobelitina (NOB) constituem as principais PMFs, sendo encontradas em maiores quantidades nos frutos cítricos, a isosakuranetina (ISR), a sinensetina (SIN), a tetrametilescutelareina (TMS) e a heptamethoxiflavona (HMF) representam o grupo em menor quantidade (MANTHEY et al, 2011). As polimetoxiflavonas têm sido relacionadas a uma variedade de eventos bioquímicos que beneficiam a saúde, particularmente nas vias associadas ao câncer (LI et al, 2007); à biossíntese de lipídeos (KUROWSKA e MANTHEY, 2004), à inflamação (AKAO et al, 2008) e à tolerância à glicose (LI et al, 2008).

Suco de Laranja Fresco x Pasteurizado

A extração e o processamento do suco de laranja alteram as propriedades físico-químicas gerando diferenças na proporção dos nutrientes do alimento, sendo encontrados conteúdos distintos de ácido ascórbico, compostos fenólicos totais, carotenoides e flavonoides. Estas diferenças na produção podem ser impactantes sobre os parâmetros de qualidade e podem influenciar nas características nutricionais e no sabor destes sucos (BAI et al, 2013; BALDWIN et al, 2014; PEREZ-CACHO e ROUSEFF, 2008). A Tabela 3 mostra um comparativo entre o conteúdo dos nutrientes entre os sucos fresco e o comercialmente pasteurizado (USDA, 2008; TACO, 2011).

Sucos de laranja contêm de 470 a 761 mg/L de hesperidina e de 20 a 86 mg/L de narirutina. As partes sólidas das laranjas como a porção branca esponjosa (albedo) e as membranas que separam os segmentos, tem um teor muito elevado de flavanonas; esta é a razão do fruto conter até cinco vezes mais flavonoides do que o suco de laranja (GIL-IZQUIERDO et al, 2003; BAI et al, 2013; BALDWIN et al, 2014).

Tabela 3 - Comparação da composição nutricional dos sucos de laranja: Fresco e Pasteurizado.

Composição Nutricional - Suco de Laranja (250mL)			
Fonte	USDA, 2008*		TACO, 2011**
Tipo de suco	**Fresco**	**Pasteurizado**	**Fresco**
Energia (kcal)	112	117	82,5
Carboidrato (g)	25,8	27,4	19,0
Proteína (g)	1,7	1,7	1,7
Gordura total (g)	0,5	0,4	0,3
Fibra dietética (g)	0,5	0,7	Tr
Potássio (mg)	496	458	373
Sódio (mg)	2	10	Tr
Vitamina A (μg)	25	22	ND
Vitamina C (mg)	124	75	183
Folato (μg)	74	60	ND

Tr = traço; ND = não determinado
*USDA, National Nutrient Database for Standard Reference, 2008
**TACO, Tabela de Composição dos Alimentos, 2011

Estudo verificou que o suco fresco apresenta um teor 30% maior de vitamina C do que o suco pasteurizado. Por outro lado, o suco pasteurizado possui aproximadamente 3,3 vezes maior concentração de compostos fenólicos (GIL-IZQUIERDO, GIL e FERRERES, 2002).

Os principais glicosídeos de flavanonas foram encontrados em concentrações duas vezes mais elevadas no suco de laranja pasteurizado quando comparado ao fresco, indicando que o processamento comercial leva a maior extração das cascas no suco pasteurizado do que os métodos de extração de suco fresco. O teor de compostos fenólicos totais nos sucos seguiu um padrão semelhante ao conteúdo de flavonoides. As polimetoxiflavonas (PMFs), associadas com óleo da casca, ocorreram nos níveis 2,5 vezes mais altos no suco de laranja fresco, em contraste, os limonoides e alcaloides ocorreram em níveis mais elevados no suco pasteurizado (MANTHEY et al, 2011).

OBJETIVOS

- Avaliar os parâmetros farmacocinéticos dos compostos do suco de laranja fresco e comercialmente pasteurizado (flavanonas e metabólitos) em humanos saudáveis;
- Avaliar o índice do estresse oxidativo dos indivíduos por meio de análise de hidroperóxidos e capacidade antioxidante no soro sanguíneo;
- Determinar a composição nutricional e o perfil dos compostos fitoquímicos ativos (fenólicos totais e flavonoides cítricos) no suco fresco e comercialmente pasteurizado;
- Caracterizar o estado nutricional dos indivíduos por meio da avaliação antropométrica, bioquímica, dietética e hemodinâmica.

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CAPÍTULO 2 – Pharmacokinetic of flavanones glycosides after ingestion of fresh squeezed versus commercially processed orange juice in healthy humans

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ABSTRACT

Orange juice is a rich source of flavonoids known to be beneficial to cardiovascular health in humans. The objective of this study was to analyze the pharmacokinetics of the main flavanone glycosides, hesperidin and narirutin, in humans after the consumption of two types of orange juice, fresh squeezed (FOJ) and commercially processed (POJ), which differed in their amounts of soluble (supernatant) and insoluble (pellet) forms of these compounds. Healthy subjects, 12 men and 12 women, aged 27 ± 6, with BMI of 24 ± 3 kg/m2 consumed 11.5 mL kg-1 body weight of FOJ, and after an interval of 30 d, consumed the same quantity of POJ. Both juices were extracted from oranges of *Pera Rio* variety from the same batch of fruits. Plasma samples were collected at specific time points and urine was collected over a 24 h period after juice consumption. The concentrations of hesperidin and narirutin in the POJ were 3.2 and 6.6 times higher than in the FOJ, and while these compounds occurred largely in pellet of the POJ, they were nearly completely soluble in the FOJ. Metabolites in the urine samples showed high concentrations of glucuronic acid and sulfate conjugates after the consumption of both juices. Concentrations in the blood plasma after ingestion of POJ were higher than in the plasma of subjects after FOJ, with the exception for the metabolite identified as a naringenin glucuronic acid. The results showed that there were no significant differences in the Tmax of the pharmacokinetic curves for the metabolites of hesperidin and narirutina. In general, the AUC and Cmax values of these compounds were higher after ingestion of POJ comparing to FOJ, and after corrected in doses of soluble and insoluble forms of juices was found that did not affect the pharmacokinetic parameters.

Key words: Bioavailability, pharmacokinetics, hesperidin, narirutin, metabolites, orange juice.

INTRODUCTION

The benefits of the flavanone glycosides in orange juice to human health are attributed to increased antioxidant capacity[1,2], decreased hypertension[3], blood serum cholesterol[4,5], and inflammation[6], thus protecting against chronic diseases, such as cancer and cardiovascular diseases. Recent studies have now also shown hesperidin to directly contribute to protective vascular effects and to anti-inflammatory genomic profiles in humans following orange juice consumption[3,7]. In animal studies hesperidin and other citrus flavanones exert potent hypotensive[8,9] and anti-inflammatory effects[10,11,12,13], significantly lowered serum levels of triglycerides and low density lipoprotein- and very low density lipoprotein-cholesterol[14,15], blood glucose and insulin resistance[16,17], and decreased bone loss[19,19, 20].

Yet, the low bioavailabilities of citrus juice flavonoids, particularly hesperidin, limit the efficacies of these compounds[21], and so attempts have been made to enrich juices with hesperidin by direct supplementation or by enzymatically-modifying hesperidin to the more water-soluble analog, hesperetin-7-O-glucoside[21,22]. Subjects that consumed orange juice containing hesperetin-7-O-glucoside experienced 4-fold higher Cmax and a much shorter Tmax for the appearance of hesperetin metabolites than subjects consuming conventional orange juice[21]. Similarly higher total absorption, Cmax, and lower Tmax values occurred with the consumption of an α-rhamnosidase-treated orange juice containing naringenin-7-O-glucoside rather than the originally present narirutin[23]. Other factors, particularly solubility, also influence the bioavailability of citrus compounds[22,24,25,26]. Many citrus phenolics exist both soluble in the juice serum and precipitated in the juice cloud[27], and compounds associated with the juice cloud are thought to be available to enzyme actions in the gastrointestinal tract at different rates than the soluble forms[26]. These

differences may influence the pharmacokinetics of these compounds, as well as their actions in humans.

Orange juice processing methods are major influences on the total concentrations and solubilities of the citrus flavanones, and therefore are likely influences on the levels of bioavailable compounds following juice consumption. A number of studies have shown that total concentrations of hesperidin and narirutin and their distributions into the juice cloud are higher in commercially processed orange juice than in fresh squeezed orange juice[25,28,29]. In the present study, we measured the pharmacokinetics of hesperidin and narirutin after single doses of two contrasting styles of orange juice, FOJ and POJ, in healthy humans. Blood plasma and urine concentrations of these compounds and their metabolites were monitored by high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS).

SUBJECTS AND METHODS

Subjects

Healthy subjects, 12 men and 12 women, were selected to participate in this study. Subjects' characteristics were 27 ± 6 y old, weight 68.3 ± 11.7 kg, and body mass index (BMI) 24 ± 3 kg/m2. The subjects were nonsmokers and non-vegetarians, and were not currently using hormones or nutritional supplements or taking medication for any gastrointestinal or metabolic diseases. The subjects did not regularly consume alcohol or perform intensive physical exercise. Systolic and diastolic blood pressures were 118 ± 11 and 70 ± 10 respectively, and the levels of triglycerides, total cholesterol, high-density lipoprotein cholesterol, glucose and insulin were within normal ranges. The study was approved by the Ethics Committee of the School of Pharmaceutical Sciences, São Paulo State University, and an informed written consent was

obtained from each participant before entering the study (protocol # 00558712.5.0000.5426, The Ministry of Health, Brazil).

Study design

The study was a crossover trial where the subjects received two types of orange juice: 1) (FOJ) and, 2) (POJ), on two different days separated by a washout period of 30 days. The subjects agreed to refrain from consuming citrus fruits in any form for 3 days before each treatment and to follow a citrus flavonoid free diet. They were given a list of allowed and prohibited foods. Clinical data (anthropometric and blood pressure) were collected on the day before the first treatment.

The fasted subjects arrived at 8 a.m. on each day of the experiment, and a blood sample of 10 mL was collected (time point zero). The subjects were given 11.5 mL kg-1 body weight of orange juice, FOJ or POJ, in a 10 min period, and after 30 min, they had a flavonoid-free standard breakfast (coffee, skim milk, bread with 3 slices of turkey breast light, and light cheese). Blood samples of 10 mL were collected at 3, 4, 5, 6, 7, 8, and 24 h after each treatment with a catheter installed in a vein of the arm, and blood was sampled into heparinized vacuum tubes. At 4 h after ingestion of the orange juice, the subjects were provided a lunch consisting of skinless chicken breast, rice and beans. After collection of the 7 h blood sample, the subjects were provided with a snack similar to breakfast. Water was freely available during the entire day.

The first urine sample was discarded, and the subsequent urine samples were stored into plastic bottles containing 1 g ascorbic acid, and kept at 4ºC. The subjects were asked to avoid eating any foods that contained polyphenols until the last time point blood sampling. The subjects returned the following morning and the last blood sample was collected 24 h after ingestion of the orange juice.

Orange juices

Two standard boxes of fresh oranges (Pera Rio sp) and 20 L of commercially processed orange juice (POJ), made from the same batch of fruits, were provided by Citrosuco, Matao, Brazil. The fresh squeezed orange juice (FOJ) was prepared from fruit extracted with a commercial fresh fruit juicer (MJ-20 Basic, Mulligan Associates, Inc., Jupiter, FL, USA) in the morning 2 h before starting the procedure for each subject. The POJ was pasteurized at 95ºC/15s, stored in 1 L bottles at -20 ºC, and thawed under refrigeration the day before the experiment.

Extraction of flavonoids in orange juice

Triplicate samples of FOJ and POJ (35 mL) were centrifuged at 27,000 x g at 4 ºC for 30 min. Supernatants were collected and pellets were suspended with 35mL deionized water and centrifuged. The final pellets were vacuum dried for 24 h at 55 °C. Prior to analysis, supernatant samples (1.0 mL) were spiked with 5.4µg mangiferin as an internal standard and analyzed without further processing. The vacuum dried samples were ground to fine powder under liquid nitrogen for sample preparation. The ground pellet (100 mg) was extracted with 3mL dimethyl sulfoxide by shaking for 18h with a platform shaker at 110 rpm at 25°C. The extracts were centrifuged at 7500 x g for 15 min to remove any solid particulates. The supernatant (1.0 mL) was placed in a vial containing 5.4 µg mangiferin (internal standard) prior to analysis by high-performance liquid chromatography-mass spectrometry (HPLC-MS).

Analyses of the orange juice flavonoids were done with a Waters 2695 Alliance HPLC (Waters, Medford, MA) connected in parallel with a Waters 996 PDA detector and a Waters/Micromass ZQ single-quadrupole mass spectrometer equipped with an electrospray

ionization source. Compound separations were achieved with a Waters XBridge C18 column (5µm, 4.5 x 150mm) with linear gradients of acetonitrile and 0.5% aqueous formic acid with a flow rate of 0.75 mL min-1. Identifications of compounds were done by UV and mass spectrometry, and by comparison of retention times of samples and authentic standards. MS parameters were as follows: ionization mode, positive electrospray; capillary voltage 3.0 kV; extractor voltage 5 V; source temperature 100ºC; desolvation temperature 225ºC; desolvation gas flow 465 L h-1; cone gas flow 70 L h-1; scan range m/z 100-900; rate 1 scan sec-1; cone voltages 20 and 40 V. Quantification of flavonoids was done by external calibration curves obtained by injecting different amounts of stock solution containing the internal standard and all the compounds of interest.

Plasma and urine samples preparation

Blood samples were collected in heparinized tubes and stored for a maximum of 15 min before centrifugation at 10,000 × g for 4 min. Plasma was recovered and stored at -80º C until processed. Serum samples (200 µL) were combined with methanol (600 µL), thoroughly mixed, and centrifuged at 10,000 × g for 4 min at room temperature. The supernatants were collected and reduced to dryness under vacuum with a Speedvac centrifugal evaporator (Savant, Holbrook, NY). Total urine samples collected in the flasks were homogenized and a 200 mL sample was withdrawn in each bottle. Five C18 Sep Pak (360 mg of resin/cartridge) (Waters, Milford, MA) were series connected and were preconditioned with methanol and water. The metabolites were absorbed onto the Sep Pak cartridges, and then eluted with 80% methanol. The recovered metabolites were dried under vacuum with a Speedvac. Plasma and urine metabolite samples were dissolved in 1.0 mL methanol/dimethyl sulfoxide (1/1, v/v), and 5.4 µg mangiferin as internal standard were added.

Analyses of flavanone metabolites in human plasma and urine

The flavanone metabolites in human plasma and urine samples were analyzed by HPLC-ESI-MS, with a Waters 2695 Alliance HPLC connected in parallel with a 996 photodiode array (PDA) detector and a Micromass ZQ single-quadrupole mass spectrometer equipped with an electrospray ionization source. Post column split to the PDA and mass ZQ detector was 10:1. Compound separations were achieved with a Waters Atlantis dC18 column (2.1 x 100 mm) using linear gradients of aqueous 0.5% formic acid/acetonitrile, initially composed of 90:10 (v/v), and increased in acetonitrile content to 85:15, 80:20, 70:30, 30:70, 30:70 and then decreased to 90:10 (v/v) at 5, 7, 12, 20, 23, and 25 min, respectively, at a flow rate of 0.45mL min-1. Data handling was done with Mass Lynx software ver. 4.1 (Micromass, Division of Waters Corp., Beverly, MA). MS parameters were as follows: ionization mode, negative ESI-; capillary voltage, 3.0 kV; extractor voltage, -4 V; source temperature, 100ºC; desolvation temperature, 250ºC; desolvation N2 flow, 550 L h-1; cone N2 flow, 100 L h-1; cone voltage, 40 V.

Detection and identifications of the metabolites were based on their characteristic UV spectra, molecular masses, and fragmentation patterns as described previously[22,30]. Hesperetin-glucuronides were identified by ions at 477 and 301 m/z, hesperetin-sulfates at 381 and 301 m/z, mixed sulfo/glucurono-hesperetin at 301, 477, and 557 m/z, and naringenin glucuronides with m/z ions at 477 and 271. Quantifications the metabolites in the plasma and urine samples were made with peak area (PA)/µg conversion factors of authentic metabolite standards using integrated mass-extracted peak areas (PA) obtained either in the scan mode (100-900 amu) or in the single ion response (SIR) mode at the [M-H]- m/z for each compound. Standards for hesperetin-7-O-β-glucuronide (product n.: HD322), hesperetin-3′-O-β-glucuronide (HD324), hesperetin-7, 3′-di-O-

β-glucuronide (HD326) and naringenin-7-O-β-glucuronide (ND329) were obtained from LC Scientific Inc. (Concord, Ontario, Canada). Conversion factors (SIR PA µg-1) of the metabolite standards were linear over the concentrations in the test urine samples.

Plasma metabolites analysis

The plasma metabolites' data were plotted versus time (24 h) and the area under curve was calculated by the trapezoidal rule (AUC):

$$AUC = \frac{1}{2}\sum_{i=1}^{n-1}(m_{i+1} - m_i)(H_{i+1} + H_i)$$

where mi is the ith hour, Hi is the Hth available concentration value, and n is the number of hours. Plasma bioavailability of metabolites was from 0 to 24 h, and the secondary outcomes were Cmax i.e., maximum concentration, and Tmax i.e., time after treatment when reaching Cmax. The pharmacokinetic parameters (AUC, Cmax and Tmax) were calculated using Microcal Origin (version 6.0).

Statistical analysis

Clinical characteristics were documented by descriptive statistics. All results are expressed as mean ± SD or SEM. The data distributions were tested for normality, and subsequently, a paired t-test or Wilcoxon test was applied using Sigma Stat version 3.11 (Systat Software Inc., USA).

RESULTS

Phenolic compounds in FOJ and POJ

The flavonoids in orange juice occur mainly as flavanone and polymethoxylated flavones[31,32], although other phenolic compounds such as hydroxycinnamates and phenolic alkaloids also occur[6,33,34]. The concentrations of the main flavonoids in the juices used in this study are summarized in Table 1. Three of the main flavanone glycosides, hesperidin, narirutin, and isosakuranetin rutinoside (didymin) (Figure 1) were identified by their elution times and characteristic UV and mass spectra matching those of authentic standards. Trace levels of other minor flavanones were also observed, but not analyzed.

The results show that 3.53 times higher total flavanone glycoside concentrations occurred in the POJ than in the FOJ. The average POJ and FOJ samples (786 ± 135 mL) consumed in this study contained 121.5 ± 37.1 mg and 37.1 ± 3.1 mg hesperidin, respectively. For narirutin the average doses contained 28.7 ± 4.3 mg and 4.3 ± 0.71 mg in the POJ and FOJ, respectively. In sharp contrast, the polymethoxylated flavones occurred at concentrations 2-3 times higher in the FOJ than in the POJ. In the average sample of FOJ (786 ± 135 mL) the total polymethoxylated flavone content was 4.73 ± 0.18 mg, but only 1.76 ± 0.30 mg in the average sample of POJ. The polymethoxylated flavones occur in orange juice as constituents of the peel oil introduced into the juice by the fruit extraction methods, and consistent with this were the measured differences in the peel oil levels (estimated by limonene content) of the FOJ (0.080 ± 0.004%) and POJ (0.028 ± 0.037%).

The levels of additional juice compounds in the POJ and FOJ were also analyzed. Concentrations of vicenin-2 (6,8-di-C-glucosylapigenin) and feruloylputrescine were 4.5 and 3.3 times higher in the POJ than in the FOJ, respectively (data not shown). Concentrations of the total

hydroxycinnamic acids, ferulic, p-coumaric, and sinapinic acids in the POJ and FOJ released after saponification of the juices are shown in Table 2. These compounds occur in orange juice mainly as hydroxycinnamates with glucaric acids[35]. The levels of ferulic and sinapinic acids were nearly the same in POJ compared to FOJ, while the levels of p-coumaric acid were higher in FOJ (20.5 ± 0.6 ppm) than in the POJ (13.1 ± 1.0 ppm).

In addition to the differences in total concentrations of the main flavanone glycosides and polymethoxylated flavones in the POJ compared to the FOJ, there were also important differences in the distributions of these compounds occurring soluble in the juice serum (supernatant) or precipitated in the juice cloud (pellet). The average percent total flavonoids in the pellet and supernatant of the POJ and FOJ are listed in Table 3. In the FOJ 98.6 % of the hesperidin occurred soluble in solution, whereas in the POJ only 28.6 % of the total hesperidin remained soluble. The remaining 71.4 % of the hesperidin in the POJ was precipitated in the pellet. Similar properties were observed for isosakuranetin rutinoside and narirutin. The polymethoxylated flavones also occurred in the supernatant and precipitated in the pellet. Unlike the flavanone glycosides, there were no clear trends in these distributions for these compounds resulting from the different juice extraction methods. An exception was sinensetin, which occurred mainly soluble in the FOJ (63.3%), but was predominantly in the pellet of the POJ (62.8%).

Plasma and urine metabolites

Previous reports have shown that metabolites of hesperidin and narirutin in humans occur as glucuronic acid and/or sulfate conjugates of the hesperetin and naringenin aglycones[22,30]. In the present study, the relative bioavailabilty and uptake of hesperidin and narirutin in the POJ and FOJ were studied by HPLC-ESI-MS analyses of extracts of blood plasma and urine samples obtained over a 24 h period for each human subject. In plasma, two main metabolites with elution times of

13.1 and 13.6 min exhibited mass ions at 301 m/z and 477 m/z (Table 4), while in urine two additional minor metabolites with elution times of 11.6 and 15.7 min also exhibited these mass ions (Table 5). The metabolites at 13.1 and 13.6 min were identified as hesperetin-7-O-glucuronide and hesperetin-3'-O-glucuronide, respectively, by comparisons with authentic standards. The neutral losses of 176 atomic mass units (amu) associated with these metabolites are attributed to cleavages of glucuronic acid units from the 477 m/z deprotonated molecular ions. Two other metabolites with elution times of 10.3 and 11.2 min also exhibited the 477/301 m/z ions, but also exhibited a mass ion at 557 m/z. The 80 amu neutral losses between the 557 and 477 m/z ions are attributed to cleavages of sulfate units from these metabolites. An additional metabolite detected at 15.0 min exhibited mass ions at 301 and 381 m/z, suggestive of a hesperetin-sulfate conjugate. Two main metabolites with [M-H]- ions at m/z 447 and fragment ions at m/z 271 were observed at 10.6 and 12.3 min in the plasma samples, along with two other minor metabolites at 9.6 and 12.8 min detectable in the urine samples (Table 5), and are tentatively attributed to naringenin-glucuronide conjugates. The metabolite eluting at 12.3 min was identified as naringenin-7-O-glucuronide, based on peak overlaps and spectroscopic comparisons with an authentic standard.

Plasma kinetic data

The pharmacokinetic parameters, area under the curve (AUC), maximum concentration (Cmax), and time of Cmax (Tmax) for the hesperidin and narirutin metabolites in the human plasma are listed in Table 4, and the average kinetic curves of the plasma metabolites obtained from the intake of the FOJ and POJ samples are presented in Figures 2A and 2B, respectively. Concentrations of the flavanone metabolites in the blood plasma of subjects consuming POJ were

higher than in the plasma of subjects consuming FOJ, with the exception for the metabolite tentatively identified as a naringenin glucuronic acid with an elution time of 10.6 min (Table 4). In addition, the tentatively identified hesperetin-sulfate metabolite with an elution time of 15.0 min was detected only after POJ intake. Plasma concentrations of the flavanones metabolites started to increase after the ingestion of orange juice and reached maximum concentrations between 3.7 to 6.3 h (Tmax), and then returned to base values 24 h after orange juice intake.

The mean plasma maximum concentrations (Cmax) were higher after the intake of POJ than after the intake of FOJ for the majority of metabolites, reaching 9 times higher for the naringenin-glucuronide metabolite with an elution time of 12.3 min; 4 and 1.8 times higher for hesperetin-7-O-glucuronide and hesperetin-3'-Oglucuronide (13.1 min and 13.6 min), respectively); and 3.1 times higher for hesperetin-sulfo-glucuronide metabolite with m/z 557 (11.2 min) (Table 4). The areas under the concentration-time curve (AUC 0 – 24), calculated using the trapezoidal method for the plasma metabolites, were higher for three metabolites from POJ than the areas obtained after intake of the FOJ, and reached 2.4 times higher for naringenin-7-O-glucuronide, 2.6 times higher for the hesperetin-sulfo-glucuronide metabolite (11.2 min), and 4.1 times higher for hesperetin-7-O-glucuronide (13.1 min). Only one metabolite, a tentatively identified naringenin-glucuronide (10.6 min), was 2.4 times higher in the plasma measurements following intake of FOJ than following the intake of POJ.

Urine metabolites

Analyses of the flavonoid metabolites in the urine samples obtained from the subjects after the consumption of both types of juices showed high concentrations of glucuronic acid and sulfate conjugates (Table 5), and numerous similarities occurred between the plasma and urine metabolite

profiles. The 10.3 min 557 m/z metabolite was detected in the urine of subjects after consuming POJ, but not after consuming FOJ. The 10.9 and 11.2 min 557 m/z metabolites were detected at levels 11 and 2.4 times higher in the urine of subjects consuming the POJ compared to subjects consuming FOJ. The 15.0 min 381 m/z metabolite was detected at 2.5 times higher values in the urine of subjects consuming the POJ compared to subjects consuming FOJ. The 9.6, 10.6, 12.3, and 12.8 min 447 m/z naringenin glucuronide metabolites were present respectively at 1.7, 1.7, 2.3 and 2.7 times higher after ingestion of POJ compared to the ingestion of FOJ. The naringenin glucuronide metabolite at 11.6 min was detected in the urine after intake of POJ, but not after intake of the FOJ. In a manner similar to the naringenin glucuronide metabolites, hesperetin-7-O-glucuronide and hesperetin-3'-O-glucuronide at 13.1 min, 13.6 min, and the 15.7 min 477 m/z metabolites were present, respectively, at 2.3, 2.5 and 1.9 times higher after ingestion of the POJ than after the ingestion of the FOJ.

DISCUSSION

Orange juice has long been known to play important roles in healthy diets, contributing to daily intakes of folic acid, vitamin C, potassium, calcium and other minerals. Particular benefits of orange juice consumption now focus on cardiovascular health, where orange juice has recently been shown to improve lipid profiles in humans by decreasing low-density lipoprotein cholesterol in hypercholesterolemic subjects, and by improving lipid transfer to high-density lipoprotein[5,36,37]. Additionally, in spite of its high sugar content, orange juice triggers no inflammatory oxidative responses in human, rather, the consumption of orange juice has been shown to prevent the induced oxidative and inflammatory responses caused by high-fat meal[38,39]. Although complete understandings of the modes of action for these beneficial effects are lacking, results from in vitro

and animal trials suggest that the flavonoids in orange juice are at least partly responsible for these effects. Preclinical studies with the metabolites of the main orange juice flavonoids, hesperidin and narirutin, also provide evidence for the roles of these compounds in cardiovascular protective effects[40,41,42,43].

Many of the orange juice flavonoids, including hesperidin and narirutin, occur both soluble in supenatant and precipitated in the pellet, and a number of studies have now shown that these distributions are influenced by commercial juice processing and storage techniques[25,28]. In commercially processed orange juice the majority of the flavanone glycosides occur precipitated in the juice cloud (pellet)[24]. Also associated with the juice cloud are major portions of the polymethoxylated flavones[28]. Under the physiological conditions of the gastrointestinal tract, a high percentage of the flavanones occurs as precipitated chalcones[26], and the uptake and pharmacokinetics of these compounds are thought to be influenced by the relative distributions of soluble and precipitated forms. Hand squeezing of the fruit provides a higher content of soluble and permeable hesperidin than industrial extraction, while freezing and cold storage of processed juice decreases hesperidin solubility[26]. No effects on hesperidin solubility were seen with juice pasteurization and concentration.

The orange juices in our study were prepared by commercial industrial processing techniques and by a commercial fresh-squeezed method, and the differences in the flavonoid contents of the juices were consistent with earlier studies. The POJ contained substantially higher total concentrations of the flavanone glycosides than the FOJ, and most of these occurred precipitated in the cloud fraction (pellet). In contrast, the flavanone glycosides in the FOJ occurred nearly all in the soluble form. Preparation of the FOJ introduced a high content of peel oil with a resultant higher polymethoxylated flavone content than in the POJ. These two styles of juices

provided very different doses of flavonoids to the human subjects, and were valid systems to test for the influences of insoluble and soluble states of the flavanone glycosides on their absorption and pharmacokinetics in humans.

The metabolites of hesperidin and narirutin detected in the blood plasma and urine of the subjects that consumed the FOJ and POJ agreed with those detected in previous studies. Vallejo et al (2010) reported the detection of naringenin-7-O-glucuronide, naringenin-4'-O-glucuronide, naringenin and hesperetin sulfates, and hesperetin-7-O-glucuronide and hesperetin-3'-O-glucuronide as major constituents in human plasma. Other minor-occurring naringenin and hesperetin diglucuronides and sulfoglucoronides were also detected in human plasma[22,23,30]. Similar metabolites have been reported in urine of human subjects post orange juice consumption. We report the detection of hesperetin-7-O-glucuronide, hesperetin-O-3'-glucuronide, three hesperetin-sulfoglucuronides, a hesperetin sulfate, and naringenin-7-O-glucuronide in the human plasma extracts (Table 4). Additional sulfo/glucurano-metabolites were detected in the concentrated extracts of the human urine samples (Table 5). The above metabolites have also been reported elsewhere[23,29,30,44]. Further metabolism of these compounds by colon microbiota leads to the ring fission of the main flavanone structures, leading to conjugated forms of ferulic and m-coumaric acids, phenyl propionic acids, benzoic acids, hippuric acid, and others[45]. Glucuronic acid conjugates of these metabolites were detected, but not quantified, by Vallejo et al (2010). Similar compounds were detected in our current study, and further analyses of these compounds in the plasma and urine samples are in progress. It is also possible that some of these metabolites are also produced from the hydroxycinnamates in the juices consumed in this study.

The pharmacokinetics show that the AUC, Tmax, and Cmax, when corrected for differences in dose amounts, were not influenced by the soluble/ insoluble ratios. This is in

contrast to the study of Vallejo et al (2010) where the influences of hesperidin and narirutin solubility on the bioavailabilities of these compounds consumed in orange juice were analyzed. Orange juices were tested with varying levels of total, precipitated, and soluble flavanones. Their study demonstrated a direct link between the bioavailabilities in plasma and urinary excretions of orange juice flavanones and their solubility, irrespective of contrasting total hesperidin levels in the test juices, hence indicating that precipitation renders hesperidin non-biologically available. Yet, this conclusion is based on the detection and quantitation of intact flavanone metabolites, and yet, hesperidin metabolism by the colon microbiota to render the ring-fission products at the exclusion of intact flavanone glucuronide/sulfate metabolite formation cannot be ruled out as a means of rendering precipitated hesperidin bioavailable. The Tmax values of flavanone metabolites in the plasma (4-7 h) are indicative that these compounds are passed to the colon, where bacterial enzymes hydrolyze the glycosides to aglycones, which are subsequently glucuronidated and sulfated by both colon and liver enzymes. This is in contrast to flavanone monoglucosides, which are absorbed in the small intestine where they are released as aglycones by β-glucosidase and lactate phloridzin hydrolase enzymes[46,47].

Our study also analyzed juices with contrasting flavanone profiles, where the POJ used in this study appeared to potentially match several of the commercial juices and pulp-enriched juice investigated by Vallejo et al (2010). The hesperidin in the latter juices contained 78% in the precipitated form in comparison to 71% in our POJ (Table 3). However, the FOJ, containing less than 2% precipitated hesperidin contrasted sharply with any of the juices analyzed by Vallejo et al (2010). Levels of soluble hesperidin in the FOJ and POJ were nearly identical at 46.5 and 44.2 µg mL-1, respectively. The total of the AUC values for hesperetin-3-O-glucuronide, the hesperetin glucuronide sulfate metabolite detected at 11.2 min, and naringenin-7-O-glucuronide in the plasma

of subjects consuming POJ totaled 848 nmol x h/L in contrast to the AUC value of 255 nmol x h/L for the subjects consuming FOJ. These relative values reflect the differences in total flavanones in these juices rather than the very similar soluble flavanone levels occurring in these juices. These correlations hold also for differences in the Cmax values (Table 4).

CONCLUSION

From these findings, it is concluded that for the two styles of juices used in this study, differences in the total metabolite concentrations, and the pharmacokinetic parameters of the flavanone metabolites were not substantially influenced by the differences in the distributions of the soluble and insoluble forms of these compounds. The POJ provided a much higher dose of the flavanones, but the FOJ provided much higher doses of the polymethoxylated flavones, consenquentely the AUC and Cmax values of the flavanones in the blood plasma were higher after ingestion of POJ comparing to FOJ.

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Table 1. Concentrations of flavanone glycosides and polymethoxylated flavones (μg mL-1) in pasteurized orange juice (POJ) and fresh orange juice (FOJ).

Flavonoids	**POJ**	**FOJ**
Flavanone Glycosides		
Narirutin (NR)	36.5 ± 5.5	5.5 ± 0.91
Hesperidin (HSP)	154.6 ± 47.2	47.2 ± 4.03
Polymethoxylated Flavones		
Isosakuranetin Rutinoside (ISR)	10.5 ± 4.3	4.27 ± 1.34
Sinensetin (SIN)	0.60 ± 0.03	1.77 ± 0.07
Nobiletin (NOB)	0.84 ± 0.04	2.36 ± 0.19
Tetramethylscutellarein (TMS)	0.35 ± 0.03	0.86 ± 0.07
3,5,6,7,8,3′,4′-Heptamethoxyflavone (HMF)	0.35 ± 0.03	1.18 ± 0.10
Tangeretin (TAN)	0.11 ± 0.02	0.45 ± 0.04

Table 2. Contents of hydroxycinnamic acids (μg mL-1) in pasteurized orange juice (POJ) and fresh orange juice (FOJ).

Hydroxycinnamic Acids	**POJ**	**FOJ**
p-Coumaric acid	13.1 ± 1.0	20.5 ± 0.6
Ferulic acid	66.2 ± 3.1	42.1 ± 2.1
Sinapinic acid	15.1 ± 0.6	16.0 ± 0.3

Table 3. Percent of average total flavonoid content of insoluble precipitated pellets and soluble serum supernatant of pasteurized orange juice (POJ) and fresh orange juice (FOJ).

Flavonoids (%)	**POJ**		**FOJ**	
Flavanone Glycosides	**Pellet**	**Supernatant**	**Pelle t**	**Supernatant**
Narirutin (NR)	37.6	62.4	1	99
Hesperidin (HSP)	71.4	28.6	1.4	98.6
Polymethoxylated Flavones				
Isosakuranetin Rutinoside (ISR)	78	22	4.1	95.9
Sinensetin (SIN)	62.8	37.2	36.7	63.3
Nobiletin (NOB)	51.1	48.9	33.3	66.7
3,5,6,7,8,3′,4′-Heptamethoxyflavone (HMF)	51.5	48.5	37.2	62.8
Tangeretin (TAN)	71.4	28.6	72.2	27.8

Table 4. Pharmacokinetics parameters for hesperidin and narirutin metabolites after ingestion of fresh squeezed (FOJ) and commercially processed orange juice (POJ) metabolites in human blood plasma.

Flavanone Metabolites	**Molecular Ion (amu)**	**RT[a] (min)**	**AUC[b] (nmol x h/L)**		**Cmax[c] (nmol/L)**		**Tmax[d] (h)**	
			FOJ	**POJ**	**FOJ**	**POJ**	**FOJ**	**POJ**
Hesperetin glucuronide	477/301	13.1[e]	138 ± 42.3	560 ±195*	22.0 ± 5.7	87.2 ± 37.3*	5.00 ± 1.55	4.67 ± 1.03
		13.6[f]	53.5 ± 18.4	67.1 ±21.8	3.1 ± 1.0	5.7 ± 1.5	3.67 ± 1.21	4.50 ± 2.43
Hesperetin glucuronide sulfate	557/477/301	10.3	33.6 ±11.1	25.3 ± 4.8	2.2 ± 0.9	2.2 ± 1.1	5.00 ± 1.90	4.33 ± 1.37
		11.2	36.6 ±11.3	95.9 ± 66.4*	7.0 ± 4.5	21.5 ± 16.0*	4.33 ± 1.03	5.17 ± 0.75
Hesperetin sulfate	381/301	15.0	nd	15.0 ± 13.4	nd	6.6 ± 2.4	nd	5.17 ± 1.17
Naringenin glucuronide	447/271	10.6	129 ± 30.4*	53.0 ± 27.5	15.4 ± 2.5	15.2 ± 4.7	6.33 ± 0.52	5.67 ± 1.21
		12.3[g]	80.3 ±77.6	192 ± 91.9*	14.1 ± 8.3	127 ± 15.4*	4.67 ± 2.73	5.00 ± 1.26

Values are expressed as mean ± standard deviation. * Values are significantly different between juices/ paired t-test ($P < 0.05$)
a RT, Retention Time; b AUC, area under the curve; c Cmax, maximum concentration; d Tmax, time at Cmax.
e Hesperetin-7-O-glucuronide; f Hesperetin-3'-O-glucuronide; g Naringenin-7-O-glucuronide

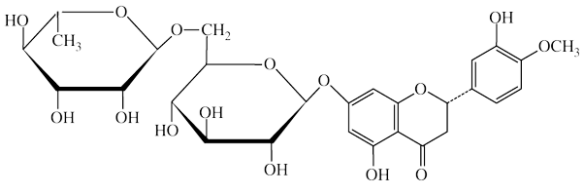
Table 5. Hesperidin and narirutin metabolites excreted in human urine 24 h after consumption of fresh squeezed (FOJ) and commercially processed (POJ) orange juice.

Flavanone Metabolites	Molecular Ion (amu)	RT[a] (min)	Urine Metabolites (μmol/L)	
			FOJ	POJ
Hesperetin glucuronide	477/301	11.6	---	7.09 ± 2.35*
		13.1[b]	28.7 ± 10.7	65.8 ± 9.2*
		13.6[c]	91.0 ± 27.0	226 ± 29*
		15.7	4.05 ± 0.71	7.71 ± 1.66*
Hesperetin glucuronide sulfate	557/477/301	10.3	---	3.14 ± 1.42*
		10.9	6.45 ± 1.42	72.2 ± 24.2*
		11.2	31.1 ± 24.6	72.9 ± 11.7*
Hesperetin sulfate	381/301	15.0	162 ± 29	404 ± 75*
Naringenin glucuronide	447/271	9.6	5.15 ± 0.58	8.90 ± 1.03*
		10.6	21.6 ± 2.0	37.6 ± 4.7*
		12.3[d]	22.3 ± 8.1	51.9 ± 8.7*
		12.8	20.7 ± 6.7	56.2 ± 8.9*

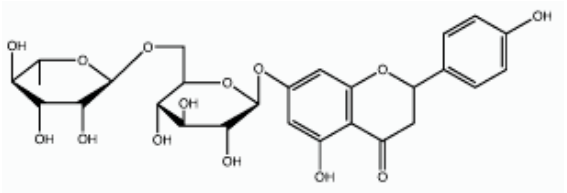
Values are expressed as mean ± standard deviation
* Values are significantly different ($P < 0.001$)
a RT, Retention Time; b Hesperetin-7-O-glucuronide
c Hesperetin-3'-O-glucuronide; d Naringenin-7-O-glucuronide

Figure 1. The main flavanone glycosides: hesperidin (A), narirutin (B), and isosakuranetin rutinoside (didymin) (C).

A)



B)



C)

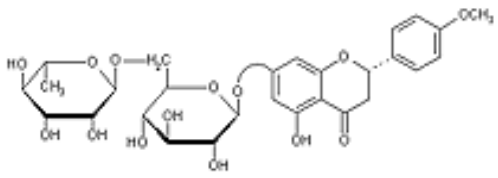
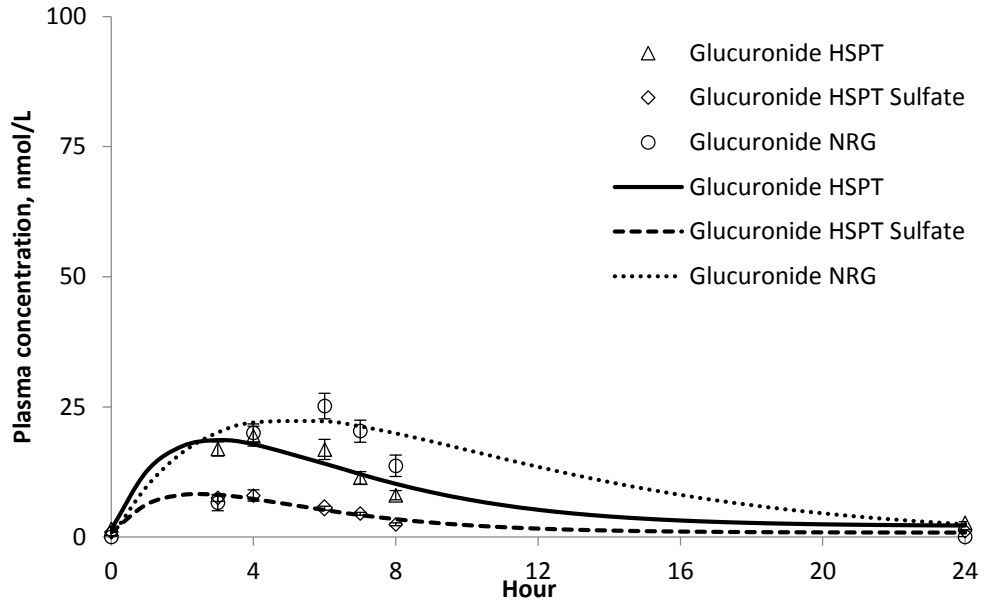
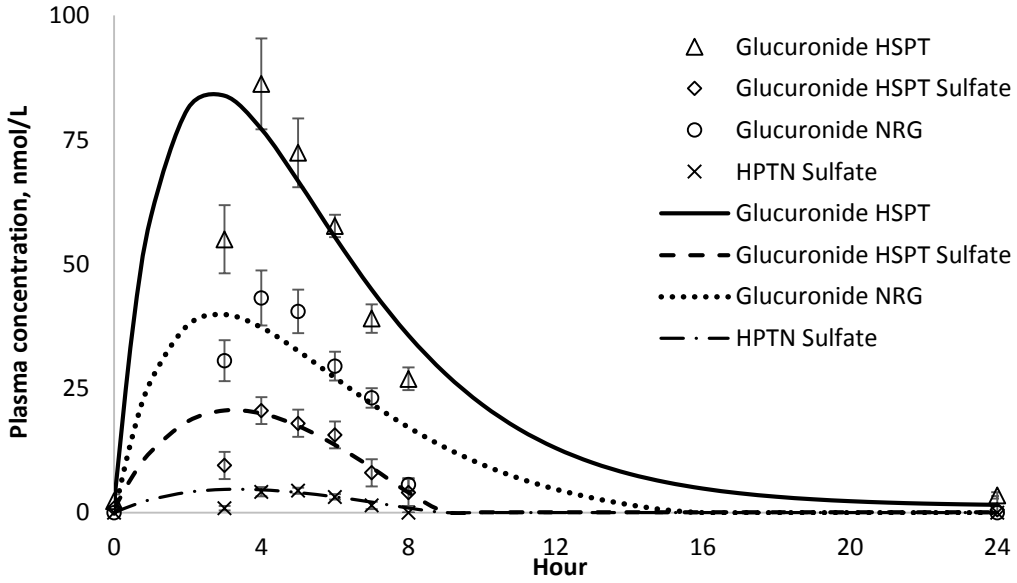


Figure 2. Plasma concentration versus time of flavanones metabolites: glucuronide hesperetin (HSPT), glucuronide hesperetin sulfate, glucuronide naringenin (NRG), and hesperetin sulfate, after ingestion of fresh squeezed (A) and commercially processed (B) orange juice. Values are expressed as mean with standard error (n=24).

A)



B)



CAPÍTULO 3 – A single dose of fresh squeezed and commercially processed orange juices improved biomarkers of oxidative stress in humans

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ABSTRACT

Orange juice is a natural source of vitamins and flavonoids and regular consumption has been suggested to increase blood antioxidant activity. The objective of this study was to investigate the effect the ingestion of a single oral dose of orange juice on the markers of oxidative stress comparing two types of extraction: fresh squeezed and commercially pasteurized, analyzed by ABTS and TBARS. Twenty-four subjects participated in this study 24 subjects of both genders, 27 ± 6 years old, weight 68.3 ± 11.7 kg. The fresh squeezed orange juice (FOJ) was extracted with a commercial juicer, and the processed juice (ready-to-drink) was pasteurized at 95ºC/15 s. Oranges and juices were provided by Citrosuco, Brazil. Subjects ingested a dose of 11.5 mL/ body weight of fresh squeezed orange juice and, after a 30 d washout, they ingested the same quantity of commercially processed juice. A fasting blood sample was collected, and following the ingestion of orange juice, blood samples were withdrawn at 4, 8, and 24 h. It were found higher amounts of hesperidin and narirutin were present in processed juice than in fresh juice, while fresh juice had higher content of ascorbic acid compared to processed juice. Malondialdehyde concentration showed a significant reduction in blood plasma and there was an increase in antioxidant capacity after consumption of fresh and commercially processed orange juices. The results showed that a single ingestion of orange juice improves the stress oxidative markers in blood plasma, demonstrating the high antioxidant capacity of vitamin C and citrus flavonoids of both juices.

KEY WORDS: antioxidant capacity, fresh, orange juice, oxidative stress, processed

INTRODUCTION

Citrus fruits and juices are natural sources of vitamins and flavonoids, and their consumption have been associated with human health benefits such as improvement of antioxidant status, anti-inflammatory and hypocholesterolemic properties, which are related to the prevention of chronic degenerative diseases[1,2,3,4,5,6]. It has been suggested that regular consumption of orange juice increases blood antioxidant activity[7] and reduces the level of free radicals[8], which may protect against the development of cardiovascular diseases[9,10].

The antioxidant capacity property of orange juice is related to the increased efficiency of antioxidant enzymes present in blood plasma and liver, which reduce tissue lipid peroxidation[11]. This has significant implications for heart disease, considering that previous studies have shown that oxidation of low-density lipoproteins (LDLs) leads to inflammation that has a key role in atherogenesis, a risk factor for cardiovascular events[11,12,13].

There are different models of LDL oxidation and inflammation, and the myeloperoxidase (MPO) is regarded to be more physiopathologically important. MPO is an innate immunity enzyme that adsorbs at the surface of LDL, promoting oxidation of amino acid residues and formation of oxidized lipoproteins that is not recognized by the LDL receptor and is accumulated by macrophages leading to foam cell formation. The oxidized lipoproteins activate endothelial cells, monocytes and macrophages, inducing proinflammatory molecules such as TNF-α and IL-8. This process may also inhibit fibrinolysis mediated via endothelial cells and consecutively increase the risk of thrombus formation[14].

Orange juice extraction can influence the concentration of the nutrients and phytonutrients citrus bioactive compounds. Flavanones are prevalent in certain parts of the fruit, the tissues such as the albedo and the segment membranes separating have more flavanones than the pulp[15].

Additionally, it has been reported that the pasteurized orange juice has more flavanones compared to fresh juice. On the other hand, fresh orange juice has higher amounts of vitamin C than the pasteurized juice[16,17,18]. Thus, it is possible that differences in the composition of the two types of juices may lead to different antioxidant properties.

The objective of the present study was to compare the effect of ingesting a single oral dose of fresh squeezed orange juice and commercially pasteurized orange juice on the markers of oxidative stress index in healthy subjects, by essays for radical ABTS and the Thiobarbituric acid reactive substances (TBARS).

CASUISTIC AND METHODOLOGY

Subjects

Healthy subjects, 12 men and 12 women, were selected among adults to participate in this study. Subjects' characteristics recorded age (27 ± 6 years old), weight (68.3 ± 11.7 kg), and body mass index (BMI, 24 ± 3 kg/m^2). The subjects were nonsmokers and non-vegetarians, and they were not using hormones or nutritional supplements and were not taking medication for gastrointestinal or metabolic disease. They did not regularly consume alcohol or perform intensive physical exercise. The study was approved by the Ethics Committee of the School of Pharmaceutical Sciences, Sao Paulo State University (nº 129.084/ 2012).

Orange juices

Before extraction of fresh juice, the oranges were properly washed with soap and water, and then they were sanitized with alcohol 70%. Fresh squeezed orange juice (FOJ), extracted with a commercial juicer (MJ-20 Basic, Mulligan Associates, Inc., Jupiter, FL, USA), was prepared in the

morning, 2 h before starting the clinical trial. The processed juice (ready-to-drink) was pasteurized at 95 ºC/ 15 s, stored in 1 L bottles at -20 ºC, and thawed under refrigeration the day before the experiment. Two standard boxes of oranges (*Pera Rio sp*) and 20 L of commercially extracted and processed orange juice (POJ), made from the same batch of fruits, were provided by Citrosuco, Brazil.

Study design

Three days before the each treatment, the subjects avoided consumption of citrus fruits in any form. Subjects ingested a dose of 11.5 mL/ body weight of FOJ and, after a washout of 30 days, they ingested the same quantity of commercially processed orange juice. In the morning of the experimental day, a fasting blood sample (8 mL) was collected from a large antecubital vein. Following, these subjects ingested the orange juice, and blood samples (8mL) were withdrawn at 4, 8, and 24 h. Serum was isolated from blood samples by centrifugation.

Anthropometric, biochemical, hemodynamic, and dietetic parameters

Body weight, height, skinfold thicknesses (triceps, biceps, subscapular, and suprailiac), and waist circumference were measured[19,20]. The BMI was used to determine the nutritional status of the subjects. The percentage of body fat was determined by adding the skinfold thicknesses as recommended and the equations were used to estimate body fat percentage[19,21]. Commercial kits were used for determination of biochemical parameters (triglycerides, total cholesterol, HDL-C, glucose, and insulin), and LDL-C was calculated[22]. Blood pressure was taken twice using the automatic blood pressure monitor ReliOn® (HEM-741 CRELN, USA). Food intake was assessed by 24-hour recalls. Energy, macronutrients (carbohydrates, protein, lipids), and micronutrients (vitamin A, C, folate,

potassium and sodium) intakes were determined by the software Nutwin, version 3.1, Paulista School of Medicine, UNIFESP, SP, Brazil.

Orange juice analysis (flavanones and ascorbic acid)

Analysis of orange juice flavonoids was done in triplicate with a Waters 2695 Alliance HPLC (Waters, Medfor, MA) connected in parallel with a Waters 996 PDA detector and a Waters/Micromass ZQ single-quadrupole mass spectrometer equipped with an electrospray ionization source. Compound separations were achieved with a Waters XBridge C18 column (5µm, 4.5mm x 150mm) with a gradient run of acetonitrile and 0.5% formic acid and a flow rate of 0.75 $mL.min^{-1}$. Identification of compounds were done by mass spectra and comparison of retention times of the sample and authentic standards. MS parameters were as follows: ionization mode, electrospray positive; capillary voltage 3.0 kV; extractor voltage 5 V; source temperature 100˚C; desolvation temperature 225˚C; desolvation gas flow 465 L h-1; cone gas flow 70 $L.h^{-1}$; scan range m/z 100-900; rate 1 scan sec-1; cone voltages 20 and 40 V. Quantification of flavonoids was done by external calibration curves obtained by injecting different amounts of stock solution containing internal standard (mangiferin) and all the compounds of interest.

Ascorbic acid content analysis was performed in triplicate according to AOAC (2005)[23].

Malondialdehyde (TBARS assay)

The thiobarbituric acid-reactive substances (TBARS) assay was used as an indicator of lipid peroxidation in subject serum[24,25]. The 1,1,3,3-tetraethoxypropane (TEP) was used as standard for malondialdehyde (MDA) equivalents (1 mol TEP = 1 mol MDA in reacting with thiobarbituric acid - TBA). Two hundred μL of MDA standard (0; 1.25; 1.88; 2.50; 3.13; 3.75; 6.25, and 12.50 μM) and 200 μL of each serum sample were mixed with 200 μL of sodium dodecyl sulfate (SDS) and then

500 µL of staining reagent (5.3 mg/mL of TBA diluted in 20% acetic acid, pH 3.5) were vortexed, incubated at 100ºC for 60 min, and cooled on ice for 10 min. The standards and samples were centrifuged at 10,000 rpm for 10 min, and the absorbance of the supernatant was determined at 532 nm in a microplate reader (Epoch, Biotek). TBARS concentration was based on the molar extinction coefficient of MDA obtained by an analytical curve.

Antioxidant capacity (ABTS assay)

The 2,2´-azinobis (3-ethylbenzthiazoline sulfonate) (ABTS) assay was used to determinate the antioxidant capacity of subjects serum[26]. Trolox was used as standard. Five μl of a 7 mM solution of ABTS was added along with 88 ml of a 140 mM potassium persulfate solution and the mixture left at room temperature, in the dark. Before use, the solution was diluted (1:88) with a 10 mM sodium phosphate buffer, pH 7.4 (initial absorbance at 734 nm of 0.7). Five μL of Trolox standard (0, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00 mM) and 5 µL of each serum sample were mixed with 300 μL of $ABTS^{\cdot+}$ solution. After 5 min, absorbance at 734 nm was measured in a microplate reader (Epoch, Biotek). Antioxidant concentration was based on the molar extinction coefficient of Trolox obtained by an analytical curve.

Statistical analysis

Clinical characteristics were documented by descriptive statistics. All results are expressed as mean ± SD. The data distributions were tested for normality, and subsequently, a t-test was applied using Sigma Stat version 3.11 (Systat Software Inc., USA). One way anova was applied to the juice data. $P < 0.05$ was considered statistically significant.

RESULTS

Assessment of anthropometric, metabolic and blood pressure data

Twenty-four subjects successfully completed the study and none reported undesirable or adverse effects after intake of fresh or processed orange juice. Descriptive statistics of the baseline data are summarized in Tables 1, 2 and 3. All subjects had normal clinical parameters, BMI, and waist circumference. As expected, body fat was higher in women than men. Both systolic and diastolic blood pressure levels were normal and did not exhibit differences between genders during this intervention (Table 1).

Table 1. Anthropometric and hemodynamic parameters of subjects.

Anthropometric Parameters	**Men (n= 12)**	**Women (n = 12)**
Age (y)	27.7 ± 6.7	26.8 ± 5.0
Body Weight (kg)	74.5 ± 10.1	62.2 ± 10.2*
BMI (kg/m^2)	24.5 ± 2.7	23.6 ± 3.3
Waist Circumference (cm)	85.5 ± 10.2	73.7 ± 7.6*
Body Fat (%)	21.5 ± 6.3	31.6 ± 4*
Blood Pressure (mmHg)		
Systolic	122 ± 10	112 ± 12
Diastolic	67.1 ± 8.5	72.3 ± 10.3

Values are expressed as mean ± standard deviation
Values are significantly different ($P < 0.05$)

Nutritional assessment of subjects calculated from three 24-hour recalls applied before the experiments (two weekdays and one day on the weekend), revealing no differences between fresh and processed OJ treatments regarding daily intake of food energy, protein, carbohydrate, dietary fiber, total lipid, cholesterol, vitamin C, folate, potassium, and sodium ($p>0.05$) (Table 2). Regarding

the Dietary Recommended Intake (DRI)[27], the intake of protein, carbohydrate and lipids were acceptable (Acceptable Macronutrient Distribution Ranges - AMDR = 10-35%, 45-65%, 20-35% Total Energy Expenditure -TEE, respectively), although the intake of fibers (Adequate Intake - AI = 25-30 g/d), vitamin C (Recommended Dietary Allowance - RDA = 75-90 mg/d), folate (RDA = 400 μg/d) and potassium (AI= 4.7 g/d) were insufficient. On the other hand, the men's average daily intake of dietary cholesterol was higher than the maximum intake recommended (250 mg), while both gender had higher sodium intake than the recommended (AI = 1.5 g/d).

Table 2. Dietary intake one week before the experiment with fresh squeezed and commercially processed orange juice.

	Men (n=12)		**Women (n=12)**	
	Fresh OJ	**Processed OJ**	**Fresh OJ**	**Processed OJ**
Food Energy (kcal/d)	2562 ± 623	2353 ± 863	1787 ± 411	1839 ± 431
Protein (g/d)	126 ± 535	117 ± 43.3	81 ± 35	82.8 ± 29.5
Carbohydrate (g/d)	320 ± 98.4	278 ± 78	238 ± 55.9	229 ± 68
Fibers (g/d)	18.5 ± 7.9	15.6 ± 10.3	16.5 ± 10.1	13.1 ± 7.4
Total Lipids (g/d)	81.3 ± 29.2	94.1 ± 39.7	54.9 ± 16.8	63.7 ± 22.8
Cholesterol (mg/d)	310 ± 146	362 ± 178	228 ± 165	267 ± 159
Vitamin C (mg/d)	68.0 ± 62.9	36.5 ± 27.1	59.5 ± 50.7	43.3 ± 35.3
Folate (μg/d)	130 ± 96	139 ± 117	91.7 ± 62.5	103 ± 63
Potassium (g/d)	4.1 ± 1.1	3.6 ± 1.0	3.7 ± 1.5	3.8 ± 1.3
Sodium (g/d)	2.3 ± 1.2	2.4 ± 1.0	1.9 ± 0.9	2.1 ± 1.0

Average of three 24-hour recalls (two weekdays and one weekend)
Values are expressed as mean ± standard deviation
Values are significantly different between juices ($P < 0.05$)

Biochemical data were analyzed independent of gender, since there is no specific biochemical references for man or woman, excepted for HDL, as it was shown on Table 3. All biochemical parameters measured on the first blood sample (fasting), before the intake of fresh or processed orange juice, and were below the reference values ($p > 0.05$). Therefore, triglycerides, total cholesterol, LDL-C, HDL-C, glucose, insulin, leucocytes and hemoglobin of all subjects were inside the normal range.

Table 3. Biochemical parameters of subjects immediately before (time 0) the intake of fresh squeezed and commercially processed orange juice.

		Subjects (n=24)		
		Fresh OJ	**Processed OJ**	**Reference**
Triglycerides (mg/dL)		89.6 ± 41.4	90.4 ± 58.9	< 150
Total Cholesterol (mg/dL)		176 ± 27.5	175.5 ± 31.4	< 200
LDL-C (mg/dL)		104 ± 28	106 ± 29	< 110
HDL-C (mg/dL)	men	45.7 ± 8.8	43.4 ± 10.0	≥ 40
	women	63.2 ± 9.5	58.6 ± 12.2	≥ 50
Glucose (mg/dL)		82.9 ± 6.2	78.8 ± 6.7	70–99
Insulin (μM/mL)		9.6 ± 8.2	8.9 ± 6.3	< 25
Blood Cells				
Leucocytes (mm^3)		6.9 ± 1.8	6.5 ± 1.5	4.0 – 11
Hemoglobin (g/dL)		14.8 ± 1.1	14.5 ± 1.1	12.8 – 17.8

Values are expressed as mean ± standard deviation
Values are significantly different ($P < 0.05$)

From the composition analysis of the flavanones in the fresh squeezed and commercially processed orange juices by HPLC-ESI-MS, we observed significant differences in the quantities of

hesperidin and narirutin. The processed orange juice presented high amounts of both flavanones compared to fresh juice. The amount of hesperidin was 3.2 times higher and the narirutin was 6.6 times higher in the processed juice. The ascorbic acid content was 24.7% higher in fresh than in processed juice (Table 4).

Table 4. Composition of the main flavanones (hesperidin and narirutin) and ascorbic acid in the orange juices.

	Orange juices	
	Fresh squeezed	**Commercially processed**
Narirutin ppm (ug/mL)	5.5 ± 0.9	36.5 ± 1.2*
Hesperidin ppm (ug/mL)	47.2 ± 4.1	154.6 ± 6.9*
Ascorbic acid (mg/100mL)	38.9 ± 0.59*	29.3 ± 0.45

Triplicate samples of fresh squeezed and commercially processed orange juices
Values are expressed as mean ± standard deviation
Values are significantly different ($P < 0.01$)

Blood serum antioxidant capacity showed some differences over time when analyzed for each type of orange juice. There was an increase in antioxidant capacity 4 h after ingestion of fresh orange juice which remained at the 8 h sampling, but returned to baseline by 24 h. Meanwhile, there was an increase in the antioxidant capacity only 8h after ingestion of processed orange juice, which also returning to baseline by 24 h. The comparison between the two juices for evaluation of ABTS, showed a statistical difference 4 hours after ingestion; showing that the antioxidant capacity of fresh juice was greater than that of processed juice (Table 5).

There was a decrease in MDA concentration observed after the ingestion of both juices. A significant decrease occurred at 4 hours after ingestion of fresh orange juice with no differences at 8

and 24 h. A decrease in MDA with the processed juice occurred 24 h after ingestion. Further, no statistically differences were observed in the MDA concentration between the two types of juices (Table 5).

Table 5. Biomarkers of oxidative stress at baseline, 4, 8 and 24 h post orange juice ingestion by healthy subjects.

Time Course	**Total antioxidant capacity (mM)**		**Malondialdehyde (μM)**	
hours	Fresh OJ	Processed OJ	Fresh OJ	Processed OJ
Baseline	1.40 ± 0.006^{a}	1.40 ± 0.02^{a}	4.03 ± 0.63^{a}	3.86 ± 0.84^{a}
4	1.42 ± 0.007^{b}	1.40 ± 0.03^{a}*	2.99 ± 1.33^{b}	3.59 ± 0.77^{ab}
8	1.41 ± 0.005^{b}	1.42 ± 0.02^{b}	3.12 ± 1.13^{ab}	2.89 ± 1.31^{ab}
24	1.41 ± 0.05^{ab}	1.41 ± 0.03^{ab}	3.23 ± 0.98^{ab}	2.73 ± 1.12^{b}

Values are expressed as mean ± standard deviation
T-test was applied to data points and one way anova was applied to the juice data
Values are significantly different ($P < 0.05$)
*Letters represents differences between data points and * represents differences between juices*

DISCUSSION

A single dose of orange juice can increase the antioxidant capacity in the human body, and the result does not differ between the two types of juice process: fresh squeezed and commercially processed. The anthropometric, hemodynamic, and biochemical results showed that the subjects presented similar health conditions and that they were within the reference standards. The dietetic evaluation applied a week before each experiment showed low intake of vitamin C, suggesting that the subjects abstained from citrus foods as requested previously.

Prior studies have linked the antioxidant capacity of foods with vitamin C and flavonoids. Guimarães et al (2010)[28] quantified the antioxidant molecules in orange and other citrus juices in order to understand their contribution to overall bioactive properties, and revealed that antioxidants were more strongly correlated with ascorbic acid followed by reducing sugars and phenolic compounds. Stella et al (2011)[29] quantified the acid ascorbic and the total phenolic compounds in ready-to-drink Brazilian orange juice and verified that the juices showed high levels of total phenolic compounds and the authors correlated these results positively and strongly with the total antioxidant activity of ready-to-drink orange juice samples.

In this study, we found higher amounts of both flavanones, hesperidin and narirutin, in processed juice than the fresh juice. Conversely, the ascorbic acid evaluation showed that fresh juice had high levels of ascorbic acid than processed juice, which can be attributed to the method of extraction. This suggests a balance of antioxidants and nutrients between the differently extracted juices, leading to additive and synergistic effects. In agreement with to our data, Baldwin et al (2012)[16] analyzed the effect of processing techniques on quality of orange juices and verified that the fresh juice presented about 30% more ascorbic acid content compared to commercially processed juice.

The results of MDA concentration showed a significant reduction in plasma levels, indicating that bioactive compounds from orange juice decreased some of the harmful reactions on cellular structures, which can lead to atherosclerosis. MDA is produced as a common product after oxidative stress, which disrupts the cell membrane integrity among other deleterious effects[30].

Studies have shown that regular consumption of orange juice effectively reduces the marker of lipid peroxidation (MDA) and increases antioxidant activity in the blood. Sanchez-Moreno et al (2003)[2] studied the properties of ascorbic acid after two distinct interventions, with a dose-response

single intake of 500 ml of orange juice; and another multiple-dose response with two doses of 250 mL per day for two weeks. It was observed that orange juice consumption increased plasma concentrations of ascorbic acid by 40-64% after 3 hours ingestion, and this increase was inversely related to concentrations of prostaglandin 8-epi-$PGF_{2\alpha}$. Snyder et al (2011)[31] also reported that consumption of orange juice increased the antioxidant capacity and reduced lipid oxidation in plasma after a postprandial period. Consistent with these reports, Foroudi et al (2014)[11] indicated that daily consumption of 750 mL of orange juice increased total antioxidant status and reduced plasma concentrations of MDA.

This study showed that flavonoid compounds (especially high in commercially processed orange juice) and ascorbic acid (especially high in fresh squeezed juice) can protect lipids against oxidative damage after only dose of orange juice in healthy human.

CONCLUSION

Several studies have shown that the regular consumption of orange juice effectively reduces the MDA concentration, an indicator of lipid peroxidation, and increases the antioxidant activity in plasma. In this study, we found that a single oral dose of fresh squeezed or commercially pasteurized orange juice also showed a favorable effects on stress oxidative markers, however, the process of juice extraction had no different effects on these markers.

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Author disclosure Statement: The authors declare that they have no competing interests.

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ANEXOS

Anexo 1. Aprovação Comitê de Ética

FACULDADES DE CIÊNCIAS FARMACÊUTICAS DO CÂMPUS DE ARARAQUARA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Biodisponibilidade de fitonutrientes e atividade antioxidante do suco de laranja fresco versus suco de laranja pasteurizado em humanos saudáveis

Pesquisador: Thais Borges Cesar

Área Temática: Área 8. Pesquisa com cooperação estrangeira.

Versão: 3

CAAE: 00558712.5.0000.5426

Instituição Proponente: Faculdades de Ciências Farmacêuticas do Câmpus de Araraquara da UNESP

DADOS DO PARECER

Número do Parecer: 129.084

Data da Relatoria: 30/10/2012

Apresentação do Projeto:

O projeto encontra-se bem redigido e contempla os itens necessários ao Protocolo de Pesquisa.

Objetivo da Pesquisa:

O objetivo é claro e encontra-se bem delimitado.

Avaliação dos Riscos e Benefícios:

A análise crítica dos riscos e desconfortos está bem detalhada.

Comentários e Considerações sobre a Pesquisa:

A pesquisa pretende compreender a diferença entre a biodisponibilidade dos fitonutrientes e a capacidade antioxidante do suco de laranja fresco em comparação ao suco de laranja comercialmente pasteurizado.

Considerações sobre os Termos de apresentação obrigatória:

Adequados.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Protocolo adequado.

Situação do Parecer:

Aprovado

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Anexo 2. Certificate of Approval - Western Institutional Review Board

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THE FOLLOWING WERE APPROVED

INVESTIGATOR: John A. Manthey Ph.D.
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BOARD ACTION DATE: 01/25/2013
PANEL: 2
STUDY APPROVAL EXPIRES: 01/25/2014
STUDY NUM: 1137031
WIRB PRO NUM: 20130070
INVEST NUM: 180135
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CONTINUING REVIEW: Annually
SITE STATUS REPORTING: Annually

SPONSOR: Thais B. Cesar
PROTOCOL NUM: 100558712.5.0000.5426
AMD. PRO. NUM:
TITLE:
Bioavailability and antioxidant activity of fresh versus pasteurized orange juice in healthy humans

APPROVAL INCLUDES:
Investigator
Protocol (05-31-2012)

WIRB APPROVAL IS GRANTED SUBJECT TO:

WIRB HAS APPROVED THE FOLLOWING LOCATIONS TO BE USED IN THE RESEARCH:
USDA/Agricultural Research Service/U.S. Horticultural Research Laboratory, 2001 S. Rock Rd., Fort Pierce, Florida 34945

If the PI has an obligation to use another IRB for any site listed above and has not submitted a written statement from the other IRB acknowledging WIRB's review of this research, please contact WIRB's Client Services department.

ALL WIRB APPROVED INVESTIGATORS MUST COMPLY WITH THE FOLLOWING:

1. Conduct the research in accordance with the protocol, applicable laws and regulations, and the principles of research ethics as set forth in the Belmont Report.

2. Although a participant is not obliged to give his or her reasons for withdrawing prematurely from the clinical trial, the investigator should make a reasonable effort to ascertain the reason, while fully respecting the participant's rights.

IF YOU HAVE ANY QUESTIONS, CONTACT WIRB AT 1-800-562-4789
This is to certify that the information contained herein is true and correct as reflected in the records of the Western Institutional Review Board (WIRB), OHRP/FDA parent organization number IORG 0000432, IRB registration number IRB00000533. WE CERTIFY THAT WIRB IS IN FULL COMPLIANCE WITH GOOD CLINICAL PRACTICES AS DEFINED UNDER THE U.S. FOOD AND DRUG ADMINISTRATION (FDA) REGULATIONS, U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES (HHS) REGULATIONS, AND THE INTERNATIONAL CONFERENCE ON HARMONISATION (ICH) GUIDELINES.

Full AAHRPP Accreditation

APÊNDICES

Apêndice 1. Termo de Consentimento Livre e Esclarecido (TCLE)

Nome.....,
RG....., estado civil....., idade.....,
residente à rua .....
.....,
bairro....., cidade....., telefones de contato ....., declaro ter sido orientado e esclarecido sobre o protocolo de pesquisa a seguir:

O senhor(a) está sendo convidado(a) a participar de uma pesquisa que pretende verificar se a ingestão de suco de laranja comercial pasteurizado e de suco de laranja fresco, tomado em duas ocasiões diferentes com um intervalo mínimo de 30 dias, contribui para melhorar a capacidade de neutralizar compostos nocivos produzidos pelo organismo (capacidade antioxidante), de reduzir o colesterol e o açúcar do sangue, prevenindo contra as doenças do coração e o diabetes.

Ao participar desta pesquisa, o senhor(a) será submetido(a) a avaliação física e nutricional em duas ocasiões distintas, no início do estudo e após 30 dias, e que será perguntado sobre sua saúde pessoal e sua alimentação usual. Também participará de duas avaliações bioquímicas, que incluem a colheita de sangue e urina em duas ocasiões distintas. O pesquisador responsável irá orientá-lo sobre os procedimentos que serão realizados durante a pesquisa e que são descritos a seguir, e também poderá orientá-lo sobre uma dieta adequada.

O senhor(a) deverá beber em duas ocasiões um total de aproximadamente 6 copos de suco de laranja, sendo na primeira ocasião oferecido 3 copos de suco de laranja fresco, e na segunda ocasião 3 copos de suco de laranja integral pasteurizado, havendo um período não inferior a 30 dias entre as ingestões de suco. Também é necessário para evitar interferência nos resultados que o senhor(a) se abstenha de consumir suco de laranja ou a laranja durante os 3 dias que antecedem o dia do consumo de suco de laranja fresca ou do suco de laranja pasteurizado do experimento.

O senhor(a) deverá doar um total de 160mL de sangue em duas ocasiões (80 mL por vez) para a determinação dos lípides (colesterol e triglicérides), glicemia e insulina, e para avaliar a atividade contra compostos nocivos no sangue (atividade antioxidante). A colheita de sangue será feita durante 24 h, sendo que a primeira colheita, de 15 mL, antecede a ingestão do suco de laranja. As demais

colheitas, de 10 mL cada, serão feitas após ingestão do suco, nos tempos de 4 h, 5 h, 6 h, 7h e 8 h. Haverá ainda uma colheita de sangue de 15 mL 24h após a ingestão do suco. A colheita de sangue será realizada em sala específica para exames clínicos laboratoriais, utilizada para os exames de longa duração, com cadeiras reclináveis, ambiente com temperatura confortável, com televisão e revistas para entretenimento. O local de colheita será o Laboratório de Análises Clínicas São Lucas, Avenida Feijó 1013, Centro, Araraquara, SP.

O senhor(a) deverá realizar colheitas de urina nos dias relativos a ingestão do suco de laranja fresco e pasteurizado. A urina será colhida espontaneamente durante o período de 24 h após a ingestão do suco de laranja fresco ou pasteurizado, em local privado, com material descartável e após a higienização corporal, a ser instruída pelo pesquisador.

A sua participação na pesquisa será voluntária e livre de qualquer ônus, inclusive receberá ressarcimento para o deslocamento visando à colheita de dados dietéticos, físicos e exames clínicos, e também receberá alimentação (café da manhã, almoço e lanche da tarde) nas ocasiões em que efetuará a colheita de sangue e de urina.

É necessário informá-lo que durante a pesquisa o senhor(a) não poderá estar sob tratamento medicamentoso para controle do colesterol ou de triglicérides, ou fazendo suplementação com vitaminas, minerais ou bioflavonoides, e se necessitar de medicamentos ou suplementos, o senhor(a) deverá informar imediatamente o pesquisador sobre esta nova condição de saúde.

Os riscos que o senhor(a) será submetido(a) ao participar desta pesquisa são mínimos, apenas terá o desconforto das colheitas de sangue que serão feitas em local confortável, com material descartável para a retirada do sangue, buscando o menor desconforto possível com equipamentos que minimizem a punção venosa, como por exemplo agulhas ultrafinas e sistema de escalpo para redução do número de punções, sendo necessária três punções venosas para a colheita de 8 amostras de sangue durante um intervalo de 24 h. No total, contabilizando as duas etapas experimentais, a primeira com o suco de laranja fresco e a segunda com o suco de laranja pasteurizado, deverão ser realizadas 6 punções venosas (bem sucedidas) para a retirada do sangue.

O senhor(a) deverá retornar ao laboratório caso seja solicitado pelos pesquisadores, com ressarcimentos de despesas com transporte.

Para assegurar sua privacidade, o seu nome será mantido em sigilo antes, durante e após a pesquisa, e se desejar, receberá informações sobre o resultado da pesquisa.

O senhor(a) poderá desistir da pesquisa em qualquer momento, sem nenhum prejuízo ou penalização, mas que avisará os pesquisadores se isto ocorrer.

O senhor(a) deverá notificar qualquer situação de anormalidade relacionada à pesquisa e para tanto deverá entrar em contato com o pesquisador responsável pelos telefones: (16) 3301-6927 e/ou (16) 3301-4690. Para outros esclarecimentos e reclamações, o senhor(a) poderá entrar em contato com o Comitê de Ética em Pesquisa da Faculdade de Ciências Farmacêuticas da UNESP, Rodovia Araraquara-Jaú, km 1, Araraquara, São Paulo, telefone: (16)3301-6897.

Pelo presente esclarecimento, o senhor(a) concorda em participar do estudo: "Biodisponibilidade de fitonutrientes e atividade antioxidante do suco de laranja fresco versus suco de laranja pasteurizado em humanos saudáveis", sob responsabilidade da pesquisadora Profª. Dra. Thaïs Borges Cesar.

Araraquara, ____/____/____

_____

Assinatura do Voluntário

_____

Assinatura do Pesquisador

Apêndice 2. Recordatório de 24 horas (Rec24h)

Nome:_____
Data: _____/_____/_____ Dia da semana ref. ao consumo: _____

Café da Manhã horário:	
Lanche da Manhã horário:	
Almoço horário:	
Lanche da Tarde horário:	
Jantar horário:	
Ceia horário:	

Apêndice 3. Biodisponibilidade da Hesperitina e Naringenina

	Qtide	Hesperitina (dose)	Hesperitina (Plasma)			Excreção Urinária Relativa	Absorção
			AUC μmol/L/h	Cmax nmol/L	Tmax h	Dose ingerida %	%
SLP	786 ± 135	47.5 ± 8.2	1.23 ± 0.08	190 ± 18	4.7 ± 0.8	4.13 ± 3.33	0.8
SLF		14.4 ± 2.5	0.43 ± 0.02	53.5 ± 3.0	5.0 ± 1.5	3.76 ± 2.19	0.9
	Qtide	**Naringenina (dose)**	**Naringenina (Plasma)**			**Excreção Urinária Relativa**	**Absorção**
			AUC μmol/L/h	Cmax nmol/L	Tmax h	Dose ingerida %	%
SLP	786 ± 135	10.5 ± 1.8	0.40 ± 0.03	77.8 ±7.6	5.2 ± 1.2	3.81 ± 2.13	1.05
SLF		1.57 ± 0.27	0.34 ± 0.03	45.8 ± 3.3	5.3 ± 1.0	8.65 ± 4.85	5.95

SLP = Suco de Laranja Pasteurizado; SLF = Suco de Laranja Fresco
AUC = Área sob a curva; Cmax = Concentração máxima; Tmax = Tempo de concentração máxima

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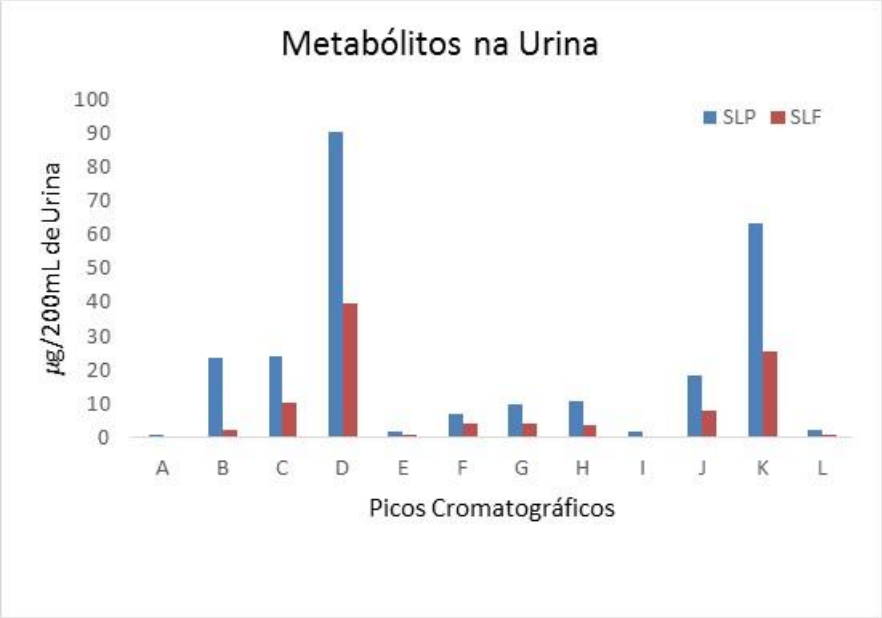
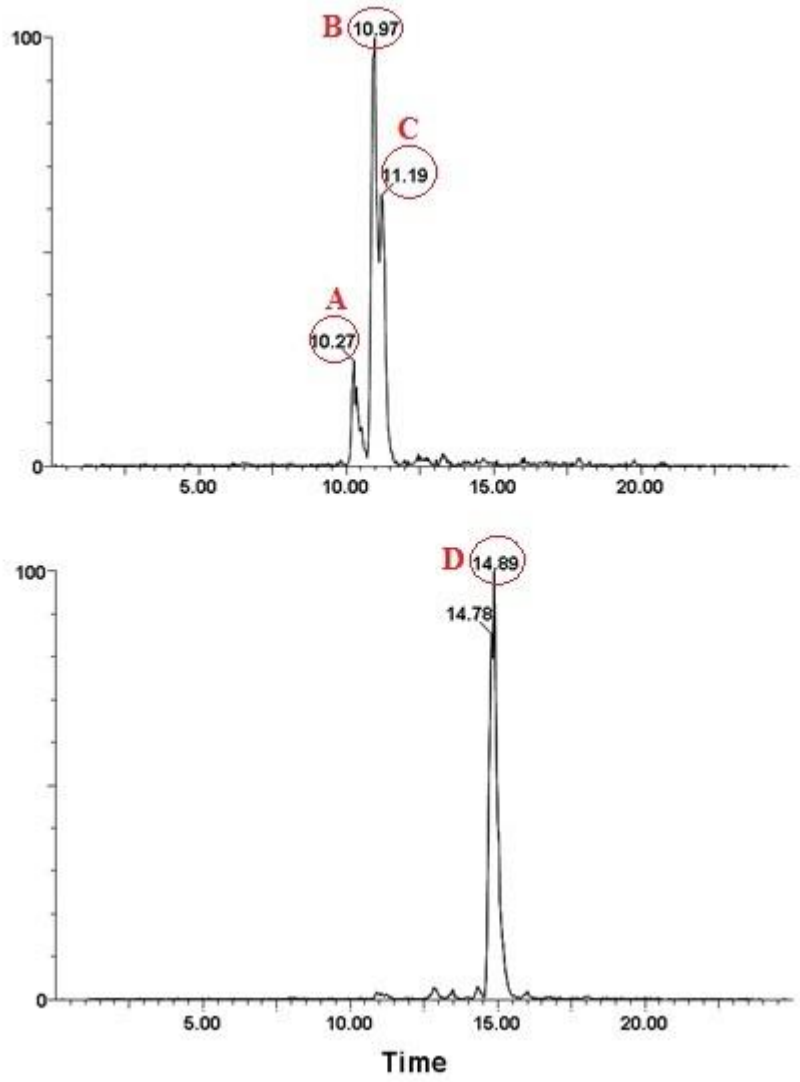
Apêndice 4. Gráfico comparativo da excreção urinária dos metabólitos após a ingestão de suco de laranja fresco e pasteurizado.

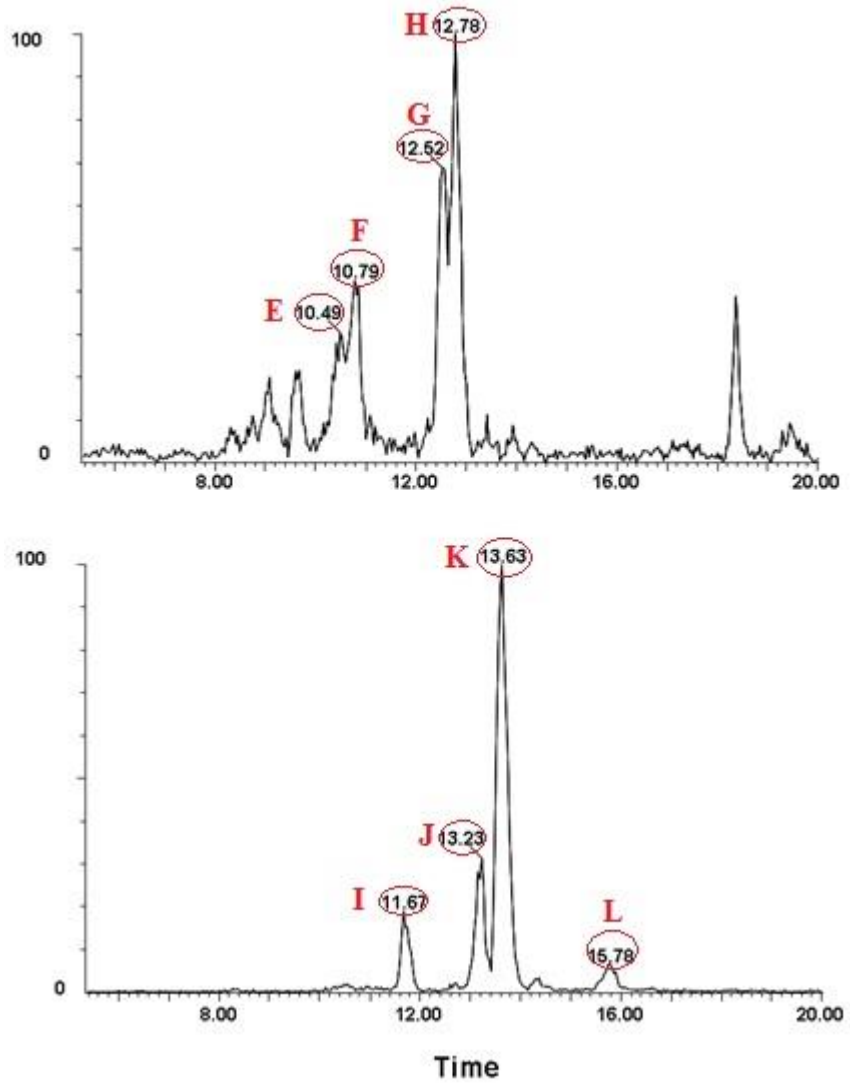


Comparação da concentração de flavanonas e seus metabólitos na urina dos voluntários após a ingestão de 11,5 mL/kg de peso corporal de suco de laranja fresco e pasteurizado. **A, B, C** = Glucuronídeo Sufatado de Hesperitina**; D** = Hesperitina Sulfatada**; E, F, G, H** = Glucuronídeo de Naringenina**; I, J, K, L** = Glucuronídeo de Hesperitina.

Apêndice 5. Cromatogramas representativos dos metabólitos de hesperitina e naringenina detectados na urina, após ingestão do suco de laranja.

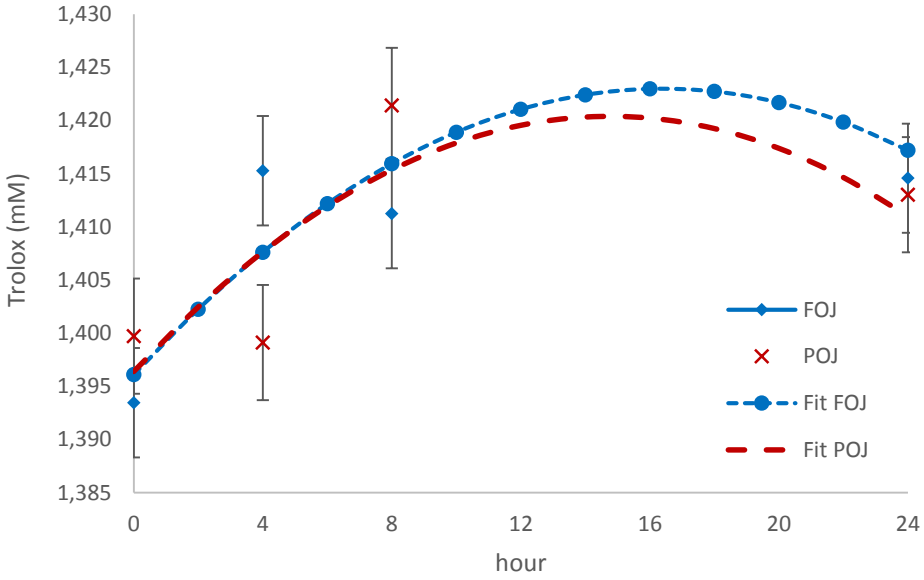


Picos A, B e C = 557 uma (superior) e pico D = 381 uma (inferior).

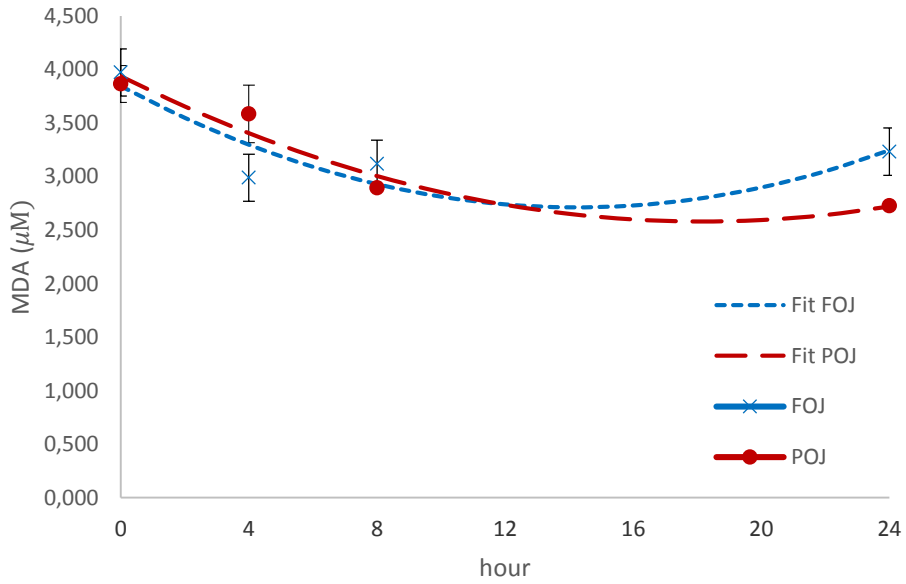


Picos E, F, G, H = 447 uma (superior) e picos I, J, K e L = 477 uma (inferior).

Apêndice 6. Gráficos comparativos da atividade antioxidante e concentração de MDA entre os sucos de laranja: fresco e pasteurizado.



Atividade antioxidante após a ingestão do suco de laranja fresco e pasteurizado, avaliado por ABTS.



Concentração de malondialdeído (MDA), após a ingestão do suco de laranja fresco e pasteurizado, avaliado por TBARS.