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Compostos bioativos em variedades de arroz integral - Caracterização, quantificação e estudo da atividade funcional em adipócitos diferenciados de células tronco mesenquimais

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutor em Patologia.

Orientador (a): Prof(a). Dr(a). Denise Fecchio

Coorientador(a): Prof(a). Dr(a). Camila Renata Côrrea

Botucatu

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Palavras-chave: Adipócitos; Arroz; Células tronco; Compostos bioativos; Gamma Orizanol.

DEDICATÓRIA

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"Meu encanto pela ciência deve-se ao fato de cada pergunta não respondida representar o início de um novo desafio"

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“Se enxerguei mais longe, foi porque me apoiei em ombros de gigantes”
Isaac Newton

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Sumário

CAPÍTULO I: Revisão de Literatura

1. REVISÃO DE LITERATURA

1.1. Consumo e produção mundial de arroz

O arroz (*Oriza sativa* L.) representa um alimento de primeira necessidade e constitui-se o principal componente na dieta de metade da população mundial. Durante séculos o consumo de arroz esteve concentrado principalmente nos países asiáticos, mas o avanço nos processos de produção e novas tecnologias permitem atualmente a produção global e em larga escala do grão[1]. O consumo per capita anual de arroz no Brasil é de aproximadamente 42 kg, enquanto que, o consumo médio mundial é de 66 kg per capita[2]. Em escala global, 20% das calorias obtidas através da alimentação são oriundas do consumo deste grão e segundo estimativa do "International Rice Research Institute", com o crescimento da população mundial, até o ano de 2025 será necessária a produção de 800 milhões de toneladas de arroz ao ano para suprir a demanda de consumo[3]. Atualmente a taxa de consumo aumenta 1,06% ao ano, enquanto que a produção cresce apenas 1%. Deste crescimento da produção, 0,2% correspondem a novas áreas cultivadas e 0,8% a melhorias das técnicas de plantio e sementes. De origem asiática, o arroz é atualmente cultivado em todos os continentes, com produção anual de 738 milhões de toneladas e, analisando a produção mundial de grãos, ocupa a 2ª posição, atrás apenas do milho com produção de aproximadamente 873 milhões de toneladas. Dentre os principais produtores destacam-se China e Índia com 46% da área mundial cultivada e consumo de 50% dos grãos produzidos. No Brasil, a área cultivada é responsável pela produção de 11 milhões de toneladas/ano, ocupando a terceira posição entre os grãos, atrás apenas de soja e milho. O Brasil ocupa a nona posição entre os maiores produtores, sendo o principal produtor fora do continente asiático[4].

1.2. Constituição do grão e processamento pós-colheita

O grão de arroz é formado basicamente por 20% de casca, 6% de cariopse (farelo), 72% de endosperma e 2% de germe (Figura 1). A casca é composta por pálea e lema, estruturas modificadas que conferem proteção ao grão durante seu

desenvolvimento. Na camada de farelo, assim denominada porque é removida durante o processo de polimento, distinguem-se principalmente o pericarpo, tegumento e aleurona. Estas camadas constituem a porção mais externa da cariopse e apresentam uma alta concentração de óleo, proteínas, minerais e compostos lipossolúveis. O endosperma representa a maior parte do grão e consiste de células ricas em amido e proteínas, enquanto que, o embrião ou germe está localizado no lado ventral da base do grão, e armazena basicamente proteínas e lipídeos, que serão utilizados durante o processo de germinação[5].

A escolha do consumidor por determinado tipo de arroz é baseada principalmente em características como aparência após cozimento, odor, consistência e sabor[1]. Diferentemente de outros grãos como milho e trigo que são transformados em outros produtos antes do consumo, o arroz é consumido principalmente na forma de grãos inteiros, com destaque para o arroz polido, parbolizado e integral. O arroz polido (branco) é o mais consumido no mundo e para sua obtenção, o grão deve ser processado para retirada da camada de farelo, através de diferentes graus de polimento que reduzem de 5-10% o peso bruto do grão[6]. O arroz parbolizado é produzido através da imersão e tratamento térmico do grão ainda com casca. Este procedimento é responsável pela liberação de micronutrientes presentes na casca ou cariopse e conseqüente aumento da qualidade nutricional do grão. Após a parbolização, o grão é descascado e comercializado na forma integral ou polida[7]. O grão integral é a apresentação mais nutritiva do arroz, uma vez que, passa apenas pela retirada da casca e conserva a camada de farelo, rica em compostos bioativos[8].

Um dos principais subprodutos do beneficiamento do arroz é o farelo resultante do processo de polimento para obtenção do arroz branco. Especial atenção tem sido direcionada a este subproduto, uma vez que, inúmeros estudos demonstraram neste componente a presença de compostos bioativos com efeitos benéficos para a saúde[9, 10]. Algumas variedades de arroz integral podem apresentar pigmentações, depositadas na cariopse, que conferem um aspecto colorido ao grão e permite classificá-los como arroz vermelho, negro ou roxo[11]. Em comparação com as variedades não pigmentadas, estas espécies podem conter elevadas concentrações de compostos bioativos como carotenóides, vitamina E, γ-orizanol, antocianinas e proantocianinas[8, 12-14].

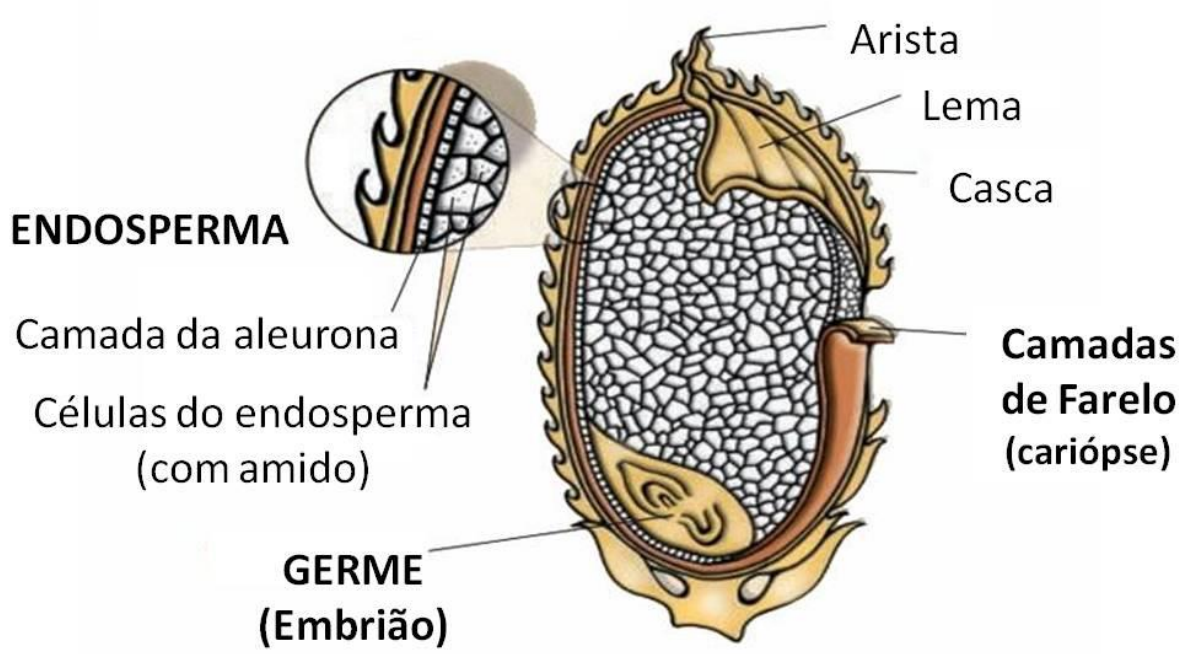


Figura 1: Composição do grão de arroz.
Fonte: Adaptado de: Enciclopédia Brittanica, INC, 2012

1.3. Compostos bioativos do arroz

Entre os principais compostos bioativos encontrados na cariopse dos grãos de arroz, pode-se destacar os carotenóides[15], vitamina E[16] e γ-orizanol[16]. Os carotenóides são um grupo de pigmentos lipossolúveis que ocorrem naturalmente em uma vasta gama de frutas e vegetais, conferindo-lhes as colorações amarelo, laranja e vermelho[17]. Os humanos não possuem a capacidade de sintetizar estes micronutrientes e devem obtê-los através da dieta. Mais de 600 carotenóides foram identificados até o momento. Entretanto, somente 40 deles estão presentes em uma típica dieta ocidental, e destes, apenas 20 foram encontrados na corrente sanguínea ou tecidos. Aproximadamente 90% dos carotenóides presentes na dieta humana são representados por β-caroteno, α-caroteno, licopeno, luteina, β-criptoxantina e zeaxantina (Figura 2). Quanto a estrutura química, os carotenóides são classificados como tetraterpenóides de 40 carbonos, que são formados pela ligação de 8 unidades isoprenóides. A ligação das unidades isoprenóides, grupos metil e inúmeras duplas ligações conjugadas conferem a molécula uma característica simétrica[18]. Diferentes carotenóides são formados essencialmente

por modificações na estrutura molecular, as quais incluem principalmente ciclização, introdução de grupos oxigenados, hidrogenação, desidrogenação e rearranjos. Estas modificações conferem aos carotenóides propriedades bioativas variadas[19], e permitem classificá-los em compostos pró-vitamina A (*e.g*. β-caroteno e β-criptoxantina) ou não pró-vitamina A, pois não são convertidos em retinol (*e.g*. licopeno e luteina)[20].

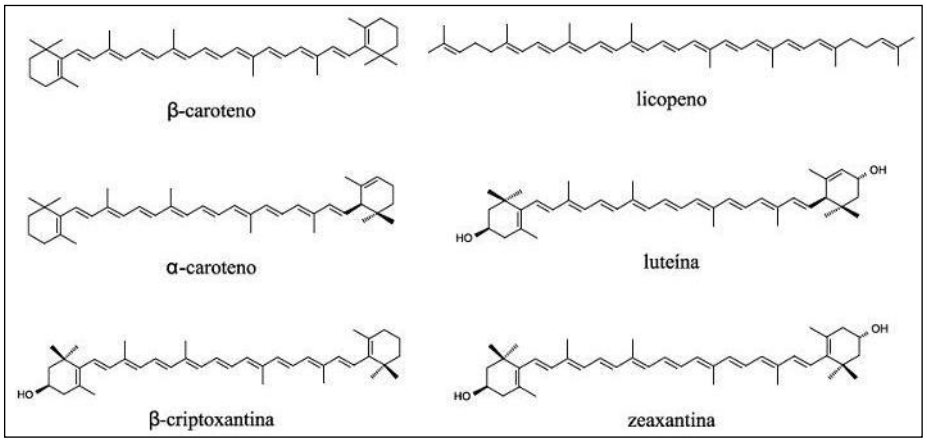
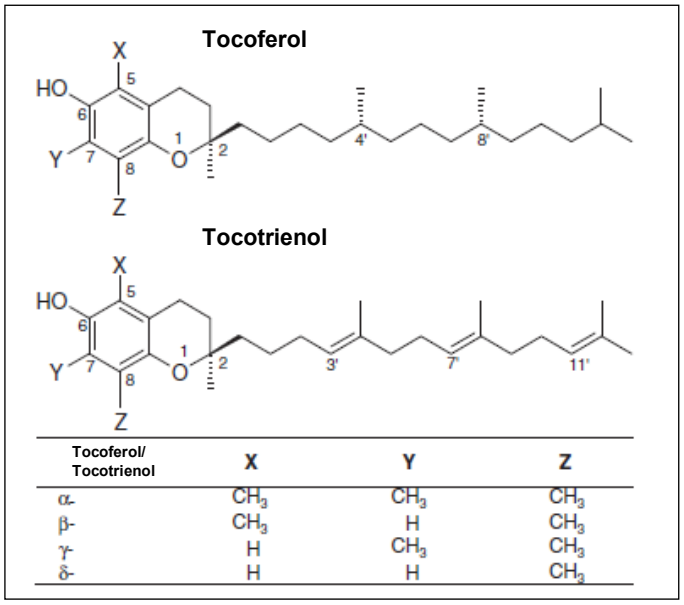


Figura 2: Estrutura química dos principais carotenóides.
Fonte: Adaptado de Rao & Rao, 2007.

Tocoferol e tocotrienol são coletivamente conhecidos como vitamina E e diferem apenas quanto a presença de uma cadeia saturada nos tocoferóis e insaturada nos tocotrienóis. Estes micronutrientes compartilham um estrutura básica comum, formada por um anel 6-cromanol anfifílico, uma cadeia lateral terpenóide localizada no carbono 2 do anel e carbonos quirais nas posições 2-, 4' e 8' da cadeia lateral (Figura 3). A metilação ou hidrogenação do anel, em diferentes posições, é responsável pela formação dos quatro isômeros (α, β, δ e γ) possíveis de tocoferol e trocotrienol[21]. As propriedades antioxidantes atribuídas aos isômeros de vitamina E são resultantes do grupo hidroxila presente no anel cromanol. Alfa-tocoferol é a forma mais abundante de tocoferol em várias espécies de plantas, enquanto que, γ-tocoferol é predominante na maioria das sementes. No farelo de arroz a concentração destes isômeros é influenciada pelo cultivar e condições ambientais como luz e temperatura, portanto, podendo variar entre as espécies[16].



Tocoferol/ Tocotrienol	X	Y	Z
α-	CH_3	CH_3	CH_3
β-	CH_3	H	CH_3
γ-	H	CH_3	CH_3
δ-	H	H	CH_3

Figura 3: Estrutura química do tocoferol e tocotrienol e os radicais responsáveis pela formação de cada isômero.
Fonte: http://lipidlibrary.aocs.org/topics/tocopherols/index.htm

Gamma-orizanol é um dos principais compostos bioativos presentes no grão de arroz e foi identificado pela primeira vez em 1954 por Kaneko & Tsuchiya[22], a partir do óleo extraído do farelo de arroz, como um composto simples de fitoesteróis. Mais tarde descobriu-se que o γ-orizanol é uma mistura de esteril ferulatos (Figura 4), formados pela esterificação do grupo hidroxila de esteróis (campesteril, β-sitoesteril e estigmasteril) ou álcool triterpeno (cicloartenil, 24-metilenocicloartanil e cicloartanol), com o grupo carboxílico do ácido ferúlico[23]. Com base na absorbância máxima de 330 nm, ao menos 25 componentes do γ-orizanol foram identificados até o momento. Entretanto, 5 destes componentes são responsáveis por cerca de 95% do conteúdo total do composto e, em ordem decrescente de conteúdo são: cicloartenil *trans*-ferulato (34-44%), 24-metilenocicloartenil *trans*-ferulato (19-26%), campesteril *trans*-ferulato (15-23%), β-sitoesteril *trans*-ferulato (7-17%) e estigmasteril *trans*-ferulato (1-7%)[24]. A concentração aproximada de γ-orizanol em grãos de arroz integral varia entre 0,2 e 1g/kg, dependendo do cultivar, subespécie, condições de cultivo (temperatura, luminosidade) e processamento[25-27].

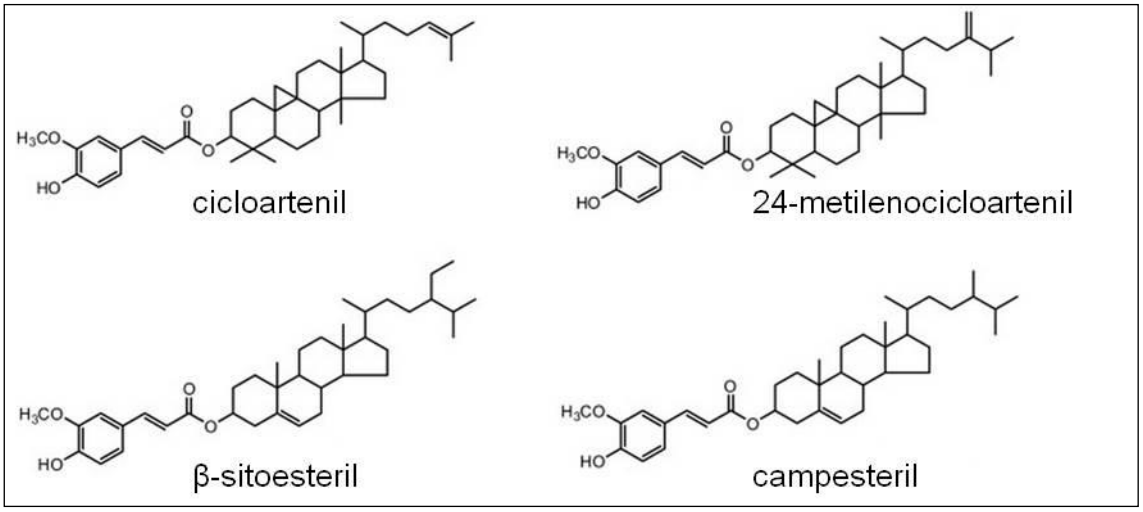


Figura 4: Estrutura química dos principais constituintes do γ-orizanol.
Fonte: Adaptado de Xu & Godber, 2001.

1.4. Bioatividade de Carotenóides, Vitamina E e γ-orizanol

Inúmeras pesquisas demonstraram uma correlação positiva entre o consumo de grãos integrais, incluindo o arroz, e conseqüentes efeitos benéficos ao organismo[1, 28-32]. Em um estudo prospectivo realizado nos Estados Unidos, pesquisadores observaram que o consumo de arroz branco esta associado com elevado risco de desenvolvimento de diabetes mellitus tipo 2 (DM2), enquanto que, o consumo de arroz integral está associado com a diminuição do risco[32]. Neste mesmo estudo, foi observado que a substituição do arroz branco, por quantidade similar de arroz integral, resultou em diminuição do risco, independente do estilo de vida, etnia e outros componentes da dieta.

As análises dos reais efeitos fisiológicos de compostos bioativos como carotenóides, vitamina E e γ-orizanol, na prevenção ou recuperação de doenças, encontra-se em constante crescimento. Contudo, é marcante a dificuldade em analisar, através de testes laboratoriais ou experimentais, o exato mecanismo de ação no organismo humano destas moléculas e de seus metabólitos.

Os carotenóides são potentes antioxidantes que apresentam uma ampla gama de funções biológicas e relacionadas com sua atividade “sequestradora” de

espécies reativas de oxigênio. A presença de uma longa cadeia polieno é responsável pela inativação das espécies reativas de oxigênio, porém, outros fatores como o número de duplas ligações conjugadas, contribuem com a atividade antioxidante. Entre os carotenóides, o licopeno, com onze duplas ligações conjugadas e duas não conjugadas, apresenta o melhor efeito sequestrador de oxigênio singleto. Os carotenóides, como carotenos e luteína, apresentam mecanismos antioxidantes alternativos, e exercem atividade protetora contra o estresse oxidativo, diminuindo a peroxidação lipídica e conseqüentes danos às membranas celulares[33]. O efeito fotoprotetor contra os danos oxidativos à retina, induzidos por radicais livres, é um dos mais estudados papeis antioxidantes dos carotenóides. A ingestão de alimentos ricos em luteína e zeaxantina está diretamente relacionada com proteção contra a catarata e degeneração macular[34]. O exato mecanismo pelo qual ocorre esta fotoproteção dos olhos é desconhecido. Entretanto, além da ação direta sobre o oxigênio singleto e radicais livres, acredita-se que estes pigmentos podem absorver parte da energia luminosa azul, proveniente de ondas curtas, e assim evitar os danos oxidativos[35].

Estudos epidemiológicos também apontam uma relação positiva entre o consumo de dietas baseadas em elevada concentração de carotenóides e a diminuição do risco de câncer, doença cardiovascular e diabetes[36, 37]. Giovannuci (1999), demonstrou que a ingestão regular de tomate ou produtos derivados (molho, suco, etc.), assim como elevados níveis plasmáticos de licopeno, estão inversamente relacionados com o risco de desenvolvimento de câncer de próstata, pulmão e estômago[38]. Outros estudos demonstram que a suplementação ou efeito direto dos carotenóides, ingeridos através de dietas, pode inibir a proliferação de células cancerígenas[39] e reduzir o risco de câncer[40]. A associação dos carotenóides com a diminuição do risco de desenvolvimento de doenças cardíacas e aterosclerose, também vem sendo amplamente estudada[41]. Embora elevadas concentrações plasmáticas de β-caroteno e licopeno tenham sido associadas com risco reduzido de doenças cardíacas[42, 43], outros estudos não observaram resultados semelhantes[44, 45]. A atividade antioxidante dos carotenóides sobre a oxidação de LDL-colesterol, inibindo seu acúmulo na parede arterial, foi o principal fator observado na inferência desta hipótese de prevenção

das doenças cardiovasculares. Entretanto, elevadas concentrações de carotenóides na corrente sanguínea não demonstraram associação com prevenção ou diminuição do risco[45].

O desenvolvimento de DM2 está relacionado principalmente a fatores de risco como obesidade e atividade física reduzida. Contudo, evidências sugerem que o estresse oxidativo pode contribuir com a patobiologia do diabetes[46], e o consumo de dietas baseadas em frutas e vegetais, ricos em carotenóides, pode minimizar seus efeitos deletérios. Múltiplos fatores como a auto oxidação da glicose, que resulta na produção de radicais livres, e a glicação de proteínas, estão envolvidos no estresse oxidativo e consequentes complicações do diabetes. Em indivíduos com elevados níveis séricos de carotenóides, estes efeitos deletérios de oxidação foram ausentes ou reduzidos, permitindo estabelecer uma relação direta entre elevados níveis séricos de carotenóides e diminuição da intolerância a glicose e desenvolvimento de DM2[47].

Todas as isoformas de vitamina E são potentes antioxidantes, que exercem principalmente a função de remover radicais peroxil, através da doação de um hidrogênio presente no anel cromanol. Em função da similaridade molecular dos isômeros de tocoferol e tocotrienol (α, β, δ e γ) acreditava-se que estes compostos possuíssem atividade antioxidante similar. Entretanto, novos estudos, *in vivo*, sugerem que tocotrienóis possuem uma interação maior com os radicais peroxil, quando comparado com tocoferol[48]. Utilizando testes *in vitro,* Müller *et al* (2010), demonstraram que o grupo fenol dos isômeros é um elemento importante na atividade antioxidante[49], porém esta atividade depende dos solventes e agentes oxidantes utilizados nos testes laboratoriais. A abundância de α-tocoferol no corpo humano e o efeito antioxidante relativamente similar dos isômeros de vitamina E, muitas vezes levam a negligência das moléculas de tocotrienol nas pesquisas básicas e clínicas. Alguns estudos relatam um efeito neuroprotetor, anticâncer e depletor de colesterol, por parte dos tocotrienóis, maior que o atribuído ou observado em tocoferóis[48]. Porém, é necessário cuidado ao avaliar o efeito de compostos bioativos na prevenção de doenças, assim como, sua real atividade no organismo humano. Enquanto estudos demonstram uma correlação positiva entre a ingestão de vitamina E, através da dieta ou suplementação, e a prevenção de câncer[50, 51], a observação inversa também é relatada. Em um

grande estudo clínico, avaliando mais de 35 mil pacientes, pesquisadores encontraram uma associação negativa entre a suplementação com vitamina E e risco de câncer de próstata. Indivíduos que consumiam 400 UI/dia de vitamina E apresentaram um aumento de 17% na incidência do câncer[52]. Apesar da dose diária de vitamina E recomendada pelo *U. S. Food and Drug Administration* (FDA), para adultos, ser de 30 UI ou aproximadamente 20 mg, grande parte dos suplementos comerciais fornecem uma concentração ≥ 100 UI de licopeno.

Gama-orizanol é o composto bioativo, presente no arroz integral ou seu subprodutos como o óleo e farelo, que tem recebido maior atenção da comunidade científica nos últimos anos. Isto porque, seus mecanismos de ação no organismo humano não estão totalmente elucidados e vários estudos presentes na literatura destacam sua atividade antioxidante, a ação hipocolesterolêmica (reduzindo os níveis séricos e a absorção de colesterol), inibição da progressão tumoral e o efeito antiadipogênico[53]. Entretanto, a menssuração *in vivo* da atividade antioxidante de um composto é difícil de ser avaliada e direciona muitos estudos a estabelecerem o uso de modelos *in vitro* para especular a ação antioxidante do γ-orizanol[54]. Utilizando diferentes ensaios, Islam *et al.*, (2009), observaram que cicloartenil ferulato, 24-metilenocicloartenil ferulato, β-sitosteril ferulato e ácido ferúlico (todos componentes do γ-orizanol), exerceram uma forte ação antioxidante e protetora da peroxidação lipídica, comparável a do α-tocoferol[55]. Neste mesmo estudo, observou-se que os esteril ferulatos exercem uma importante ação anti-inflamatória, através da contra-regulação da transcrição de NF- κB[55]. Sabe-se que, como efeito secundário da produção de espécies reativas de oxigênio, ocorre a indução de NF-κB, um dos mais importantes fatores transcricionais na inflamação[55, 56]. Entretanto, estudos adicionais são necessários para determinar se γ-orizanol, e seu componentes, atuam inibindo diretamente à sinalização de NF-κB, ou se este efeito ocorre em função da atividade antioxidante.

Assim como os carotenóides e a vitamina E, o γ-orizanol apresenta a capacidade de inibir a oxidação de colesterol via redução da peroxidação hepática de lipídeos e aumento dos níveis de glutationa e superóxido dismutase[57]. Outra característica relacionada ao efeito hipocolesterolêmico deste composto bioativo é atribuída à inibição da síntese de novas moléculas de colesterol pelo organismo

humano em função da similaridade molecular entre γ-orizanol e colesterol[58]. Son et al., (2010), demonstraram o efeito hipocolesterolêmico do γ-orizanol em camundongos submetidos a uma dieta hiperlipídica. Os animais que receberam a dieta rica em gordura e suplementada com 0,5% de γ-orizanol não apresentaram níveis menores de colesterol total plasmático e hepático e aumento de HDL colesterol, quando comparados com o grupo controle[59]. Resultados semelhantes foram observados em estudos clínicos ou experimentais que utilizaram diretamente o γ-orizanol ou subprodutos do arroz como o óleo extraído do farelo[60-62].

Os distúrbios do metabolismo estão diretamente relacionados com a obesidade e outras doenças como DM2 e doenças cardiovasculares. Entre os potenciais desencadeadores destes distúrbios metabólicos estão os receptores ativados por proliferadores de peroxissomo (PPARs), que regulam várias vias de sinalização celular. A desregulação do PPAR-γ está particularmente associada ao desenvolvimento da obesidade e distúrbios metabólicos[63]. Ho *et al*., (2012), demonstrou que a utilização de extratos de arroz germinado inibiram a ativação de genes ligados a lipogênese, entre os quais PPAR- γ[64]. Em outro estudo, Imam *et al*., (2013), observou que a contra-regulação de PPAR-γ, e conseqüente efeito anti-obesidade, pode ser exercida por extratos do arroz geminado ou mesmo, pela associação de γ-orizanol e ácido gama aminobutírico[53].

Entre os inúmeros potenciais do γ-orizanol, a sua associação com prevenção do câncer também foi relatada. Vários estudos demonstram que esteril ferulatos possuem atividade anticarcinogênica do cólon[65, 66]. Em modelos experimentais de câncer de cólon induzido por azoximetano, o uso de farelo de arroz reduziu a incidência, iniciação e tamanho do tumor[65, 67]. Entre os componentes do γ-orizanol, observou-se que esteril ferulatos atenuam o crescimento e disseminação do câncer de mama, quando células MDA-MB-231 foram introduzidas em camundongos[68]. O cicloartenil ferulato e 24-metilenocicloartenil ferulato também demonstraram atividade citotóxica contra células MCF-7 de adenocarcinoma mamário[69]. Outros compostos presentes no farelo de arroz, demonstraram grande inibição de câncer de mama, próstata e intestino, em camundongos que receberam uma dieta contendo 30% de farelo de arroz[70].

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2. JUSTIFICATIVA DO ESTUDO

O conceito de que alimentos apenas previnem a fome ou causam efeitos adversos como a obesidade, quando consumidos em excesso, está sendo revisto nos últimos anos. Um crescente corpo de evidências relaciona o consumo de alimentos ricos em compostos bioativos com a redução da morbidade e mortalidade causadas por doenças crônicas e relacionadas com distúrbios metabólicos. Portanto, a ciência dos alimentos funcionais, que atuam em um ou mais alvos no organismo prevenindo doenças, além de combater ou corrigir a deficiência de nutrientes, está se tornando um campo emergente. Estudos epidemiológicos têm sugerido que o consumo de grãos integrais, entre eles o arroz, e seus subprodutos estão associados com reduzido risco de desenvolvimento de doenças crônicas, tais como obesidade, diabetes mellitus tipo II, câncer e doenças cardiovasculares. Estes efeitos são atribuídos à presença de compostos bioativos únicos como os carotenóides, vitamina E e γ-orizanol. Desta forma, quatro novas variedades de arroz desenvolvidas pelo National Institute of Crop Science, Rural Development Administration, Milyang, Korea, foram analisadas para caracterização, quantificação e estudo da atividade funcional dos compostos bioativos, assim como, a estabilidade destes compostos frente variados processos de preparação para consumo e armazenamento.

CAPÍTULO II: Artigo 1

FAT-SOLUBLE BIOACTIVE COMPONENTS IN COLORED RICE VARIETIES

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Fat-Soluble Bioactive Components in Colored Rice Varieties

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RESUMO

Os compostos bioativos do arroz variam de acordo com a variedade e condições de cultivo. Componentes lipossolúvies como γ-orizanol, tocoferóis, tocotrienóis, carotenóides e ácidos graxos foram analisados em variedades de arroz integral, integral açucarado, vermelho e negro, utilizando métodos estabelecidos de cromatografia líquida de alta pressão e cromatografia gasosa. Todas as amostras também foram submetidas a análise por cromatografia líquida acoplada a espectrometria de massa (LTQ-Orbitrap XL), para identificar a abundância iônica $[M-H]^-$ de γ-orizanol, variando de m/z 573.3949 a 617.4211. O maior conteúdo de tocoferóis (α-, 1.5; γ-, 0.5 mg/100 g) e carotenóides (luteína 244; trans-β caroteno 25 μg/100 g) foram observados no arroz negro; tocotrienóis (α-, 0.07; γ-, 0.14 mg/100 g) em arroz vermelho e γ-orizanol (115 mg/100 g) no arroz integral açucarado. Em todas as amostras de arroz integral coloridas, os principais ácidos graxos encontrados foram palmítico (16:0), oleico (18:1n-9) e linoleico (18:2n-6). A análise dos componentes de γ-orizanol por espectrometria de massa permitiu identificar 3, 10, 8, e 8 álcoois triterpenóides ou esteril ferulatos nas amostras integral, integral açucarado, vermelho e negro, respectivamente. Estas identificações dos componentes de γ-orizanol, assim como, a concentração dos compostos bioativos, pode levar a elucidação das funções biológicas de cada componente à nível molecular. O consumo de amostras de arroz integral colorido, ricas em compostos bioativos benéficos, pode representar uma interessante estratégia dietética para melhoria da saúde.

PALAVRAS-CHAVE: carotenóides, informática, luteína, LC-MS/MS, orizanol, tocoferol

ABSTRACT

Bioactive components in rice vary depending on the variety and growing condition. Fat-soluble components such as γ-oryzanol, tocopherols, tocotrienols, carotenoids and fatty acids were analyzed in brown, sugary brown, red and black rice varieties using established high-performance liquid chromatography (HPLC) and GC methodologies. In addition, these colored rice varieties were further analyzed using a high-resolution liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) (LTQ-Orbitrap XL) to identify the $[M-H]^-$ ions of γ-oryzanol, ranging from m/z 573.3949 to 617.4211. The highest content of tocopherols (α-, 1.5; γ-, 0.5 mg/100 g) and carotenoids (lutein 244; trans-β carotene 25 μg/100 g) were observed in black rice; tocotrienols (α-, 0.07; γ-, 0.14 mg/100 g) in red rice, and γ-oryzanol (115 mg/100 g) in sugary brown rice. In all colored rice varieties, the major fatty acids were palmitic (16:0), oleic (18:1n-9), and linoleic (18:2n-6) acids. When the γ-oryzanol components were further analyzed by LC-MS/MS, 3, 10, 8, and 8 triterpene alcohols or sterol ferulates were identified in brown, sugary brown, red, and black rice varieties, respectively. Such structural identification can lead to the elucidation of biological function of each component at the molecular level. Consumption of colored rice rich in beneficial bioactive compounds may be a useful dietary strategy for achieving optimal health.

KEY WORDS: carotenoid, informatics, lutein, LC-MS/MS, oryzanol, tocopherol

1. INTRODUCTION

Rice (*Oryza sativa L.*) is consumed as a staple food by over one-half of the world's population and represents one of the most important food crops worldwide**.** Cultural aspects, flavor and nutritional values are the major aspects influencing consumer preference[1]. A long history of cultivation and selection under diverse environments has lead to the remarkable diversity of rice varieties. The growing interest in functional foods and a high global prevalence of micronutrient deficiency has intensified the research on the potential of rice as a source of bioactive micronutrients[2-5]. In recent years, new rice varieties enriched in micronutrients such as vitamin E, carotenoids and γ-oryzanol have become available. However, since a large proportion of essential micronutrients are lost when outer layers of rice are removed,[6] the amount retained is still unclear.

Carotenoids are a group of important natural antioxidants that act as radical scavengers and singlet oxygen quenchers[7]. In addition to their antioxidative potential, evidence suggests that carotenoids prevents various diseases associated with oxidative stress, such as age-related macular degeneration, cancer, cardiovascular, and other chronic diseases[8]. There is limited information about the content of carotenoids in rice varieties probably due to the low content of this bioactive compound.

Historically, α-tocopherol has been considered the most important vitamin E homologue due to its physiological activity. However, α-tocotrienol has been reported to have more than three times the free radical scavenging activity than α-tocopherol[9]. Although normal phase high-performance liquid chromatography (HPLC) gives a good separation of α-, β-, δ- and γ-tocopherol (T) and tocotrienol (T3) isomers, the simultaneous identification of carotenoids, vitamin E isomers and γ-oryzanol has not been reported.

γ-Oryzanol has been described as the major bioactive compounds in rice[10]. Gamma-oryzanol is a mixture of steryl ferulates with cycloartenol, 24-methylenecycloartenol, β-sitosterol and campesterol being the most predominant[11, 12]. In addition to its potent antioxidant activity, γ-oryzanol has been reported to reduce plasma cholesterol levels[13], and have anti-inflammatory[14] and anticancer effects[15]. Besides human health benefits, γ-oryzanol has been used in food industry

as an additive to improve the storage stability[16] since it can protect against oxidation of fatty acids in grains[17].

Various rice varieties, such as sugary rice with a high sucrose content (11%), red rice with a high antioxidant activity, and black rice with a giant embryo, have been developed by the Rural Development Administration of Korea. With the introduction of new rice varieties, there is a need for additional data on their bioactive micronutrient content. The current study determined the content of carotenoids, vitamin E, γ-oryzanol and fatty acids in brown, sugary brown, red and black rice varieties. Furthermore, identification of γ-oryzanol components was specifically targeted using a high-resolution liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) in these colored rice varieties.

2. MATERIALS AND METHODS

2.1. Reagents

HPLC grade methanol (MeOH), tetrahydrofuran (THF), methyl tert-butyl ether (MTBE), ammonium acetate, ethanol, acetonitrile and fatty acids, lutein and β-carotene standards were purchased from Sigma Co. (St Louis, MO, USA). Gamma-oryzanol standard was obtained from Wako (Osaka, Japan) and γ- and α-tocopherols and tocotrienols were purchased from Cayman Chemical (Ann Arbor, MI, USA).

2.2. Sample preparation

Brown rice (Ilmie), sugary brown rice (Danmi, sugar content 30% higher than brown rice), red rice (Geun kanghongmi), and black rice (Milyang 263; black rice with giant embryo) were grown and harvested at National Institute of Crop Science, Rural Development Administration, Milyang, Korea during the 2010 - 2011 growing season. The grains were dehusked using rice sheller (SY88-TH, Ssangyong Ltd., Incheon, Korea). The moisture contents of brown, sugary brown, red, and black rice varieties were 14.4%, 11.7%, 12.9%, and 12.0%, respectively. The unpolished rice grains were milled to a powder using a 100 mesh sieve (1093 Cyclotec Mill; FOSS Co., Ltd., Hillerød, Denmark), vacuum packed, and shipped to the Jean Mayer

USDA-Human Nutrition Research Center on aging at Tufts University, Boston and University ofMilan, Italy for analysis.

2.3. Fatty acids profile

Lipids were extracted from 50 mg of freeze-dried rice samples[18] after addition of an internal standard (50ug of 17:0). The samples were then subjected to saponification and methylation procedures. The resulting fatty acid methyl esters were quantified using an established gas chromatography method, as previously described[19]. Peaks of interest were identified by comparison with authentic fatty acid standards (Nu-Chek Prep, Inc. Elysian, MN, USA) and expressed as molar percentage (mol%) proportions of total fatty acids. Interassay coefficients of variation (CV) were <4.5% for fatty acids present at levels >1%.

2.4. HPLC analysis for carotenoids, tocopherols, tocotrienols and γ-oryzanol

One hundred milligrams of powdered and dried rice were placed in a 25x165-mm test tube and overlaid with 5 ml of MeOH, vortexed for 30 s and incubated for 1 hr in shaking incubator at 25°C. The test tubes were then centrifuged at 800 g for 10 min and the MeOH layer transferred to 25-mL volumetric flask. The residue was subjected to similar procedures of extraction four more times using THF, and the supernatant obtained from five separate extractions were combined to reach a final volume of 25 ml. Ten milliliters of the combined extract was dried under nitrogen, resuspended in 100 µl of ethanol and from this volume, 20 µl was injected onto HPLC.

A previously reported HPLC method[20] was used with slight modification for simultaneous analysis of carotenoids, tocopherols, tocotrienols and γ-oryzanol. A reverse phase HPLC system consisting of a Waters 2695 Alliance system (Waters Co., Milford, MA), C18 guard column (3µm, 33 x 4.6 mm, Perkin-Elmer, Norwalk, CT), C30 semibore carotenoid column (3 µm, 150 x 3.0 mm, YMC, Wilmington, NC) and Waters 2996 photodiode array detector. The HPLC mobile phase solvent A was MeOH/MBTE/water (85:12:3, v/v/v, with 1.5% of ammonium acetate in the water), and the mobile phase solvent B was MeOH/MBTE/water (8:90:2, v/v/v, with 1% of

ammonium acetate in the water). The gradient was set up for 0.4 mL/min begin at 100% solvent A followed by 45% solvent A over a 21 min linear gradient and hold at 45% A for 1 min. This is followed by an 11 min linear gradient to 5% solvent A and 4 min hold at 5% solvent A, finally a 2 min linear gradient back to 100% solvent A. The system is held at 100% solvent A for 8 min for equilibration back to initial conditions.

Carotenoids, tocopherols and tocotrienols, and γ-oryzanol were quantified at 450, 292 and 330 nm, respectively, by determining peak areas under the curve in the HPLC calibrated against known amounts of standards. Identification of each peak in unknown peak was confirmed by the retention time and characteristic spectra of the standards. The interassay coefficient of variation (CV) was 4% (n = 25) and the intra-assay CV was 4% (n = 9).

2.5. LC-MS/MS analysis of γ-oryzanol components.

The MeOH/THF extracts of rice prepared as described above were dissolved in 1 ml of acetonitrile:water (90:10 v/v) and centrifuged at 16000 g at room temperature for 10 min. Aliquots of the supernatant (20 µL) were then analyzed by LC-ESI-MS/MS as follows: chromatographic separation was performed on a Dionex Ultimate 3000 RSLCnano system interfaced to a LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer, equipped with an ESI source. Chromatographic separations were done by reversed-phase elution with a Phenomenex Synergi MAX-RP column (150 x 2.0 mm, 4 micron, 80 Å, Torrance, CA, USA). The mobile phase consisted of a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) and the flow rate was set at 200 µL/min.

The gradient was increased linearly from 90% to 100% B over 50 min and then held at 100% B for 20 min. The composition of the eluent was then restored to 90% B within 2 min, and the system was re-equilibrated for 5 min. The mass spectrometer was equipped with an electrospray interface, which was operated in negative-ion mode and controlled by the Xcalibur software (version 2.0) under the following parameters: capillary temperature 275°C, capillary voltage -45 eV.

During the analysis, the mass spectrometer continuously performed scan cycles in which a high-resolution (resolving power r = 60,000 at m/z 400) full scan (80–1500

m/*z*) in the profile mode was first made by the orbitrap, after which the MS^2 spectra were recorded in the centroid mode and in both CID and HCD for the three most intense ions (isolation width, *m*/*z* 3; normalized collision energy, 35 collision-induced dissociation arbitrary units). For real time internal mass calibration, a list of common contaminants was used according to Keller *et al*[21]. Dynamic exclusion was enabled (repeat count, 3; repeat duration, 10 s; exclusion list size, 25; exclusion duration, 120 s; relative exclusion mass width, 5 ppm). Data were acquired and analyzed using Qual Browser 2.0.7 software (Xcalibur package, from Thermo Fisher Scientific). The relative content of the oryzanol components for each rice variety was calculated by considering that the ionization efficiencies for all the identified oryzanol components are similar as by considering the following equation:

Relative content (%) oryzanol A = (Area A/Area T) x100

Where area A is the area of the peak relative to the oryzanol A and reconstituted by setting as filter ion the monoisotopic mass of the oryzanol A with a 5 ppm of mass tolerance. Area T is the sum of the areas of all the oryzanol species identified in the chromatographic run and calculated for each of them as reported for oryzanol A.

2.6. Statistical analysis

The results are reported as mean ± standard deviation. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Holm-Sidak test to multiple comparisons using SigmaStat 3.5 (Systat Software, San Jose, Ca, USA). Statistical significance was set at $p< 0.05$.

3. RESULTS

3.1. Fatty acid profile

Table 1 presents the fatty acid profiles (in moles %) of colored rice varieties. In the colored rice varieties analyzed, monounsaturated oleic acid and polyunsaturated linoleic acid were the major unsaturated fatty acids. The most abundant saturated fatty acid in these colored rice varieties was palmitic acid (C16:0) followed by stearic acid (C:18:0).

Table 1. Fatty acid profile of the colored rice varieties.

	Rice Varieties			
Fatty acid (mol %)	**Brown**	**Sugary brown**	**Red**	**Black**
SFA (total)	**21.27**	**28.84**	**22.13**	**23.97**
14:0, Myristic	0.57	0.70	0.62	0.70
16:0, Palmitic	17.71	23.85	18.34	19.12
18:0, Stearic	1.79	2.69	1.83	2.56
20:0, Arachidic	0.48	0.72	0.53	0.61
22:0, Behenic	0.25	0.32	0.29	0.33
24:0, Tetracosanoic	0.48	0.56	0.53	0.63
MUFA (total)	**40.69**	**26.89**	**38.58**	**40.27**
16:1n-9, cis-7-Hexadecenoic	0.05	0.08	0.05	0.07
16:1n-7, Palmitoleic	0.16	0.29	0.14	0.15
18:1n-9, Oleic	38.85	25.24	36.85	38.53
18:1n-7, cis Vaccenic	1.19	0.80	1.12	1.11
20:1n-9, Gadoleic	0.39	0.44	0.37	0.37
22:1n-9, Erucic	0.04	0.03	0.03	0.04
PUFA (total)	**38.04**	**44.27**	**39.29**	**35.76**
18:2n-6, Linoleic	36.85	42.52	38.04	34.21
18:3n-3, Alpha-Linolenic	1.19	1.75	1.24	1.55

Analysis was done in duplicate.
SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.

3.2. Carotenoids, vitamin E isomers and γ-oryzanol in colored rice varieties

The amount of carotenoids, vitamin E and γ-oryzanol in the four colored-rice varieties were quantified using a HPLC system. As shown in **Table 2**, lutein and β-carotene were the only carotenoids identified in detectable quantities. Lutein contents ranged from 5.1 to 243.7 μg/100g, and β-carotene was found only in sugary brown and black rice at 12.1 and 24.6 μg/100g, respectively. Small amount of zeaxanthin were detected only in the black rice (data not shown).

Tocopherols were the most prevalent vitamin E isomers in the four colored-rice varieties (**Table 2**). Contents of α-tocopherol and γ-tocopherol ranged from 0.9 to 1.5 mg/100g and 0.15 to 0.7 mg/100g, respectively. The highest content of total (α + γ) tocopherol was observed in black rice at 1.97 mg/100g.

Significant variations in γ-oryzanol were observed in the four colored rice varieties. Red, brown, black and sugary brown rice presented 45.2, 46.4, 80.1 and 115.3 mg of γ-oryzanol per 100 g of dried rice powder, respectively. The highest content of γ-oryzanol was found in sugary brown rice (115.3 mg/100g), a new rice variety developed by the Korean National Institute of Crop Science. In our analysis, this variety contained a 2.5-fold higher γ-oryzanol content than the other colored rice varieties.

Table 2. Fat soluble micronutrient contents in colored rice varieties.

	Rice Varieties			
	Brown	**Sugary brown**	**Red**	**Black**
Carotenoids (µg/100g)				
Lutein	5.1 ± 0.25[b]	9.0 ± 0.88[b]	6.1 ± 0.19[b]	243.7 ± 5.44[a]
trans β-Carotene	ND	12.1 ± 0.32[b]	ND	24.6 ± 0.21[a]
Vitamin E (mg/100g)				
α-Tocopherol	0.90 ± 0.96[b]	0.94 ± 0.07[b]	1.0 ± 0.005[b]	1.51 ± 0.06[a]
γ-Tocopherol	0.32 ± 0.02[c]	0.70 ± 0.05[a]	0.15 ± 0.02[d]	0.46 ± 0.02[b]
α-Tocotrienol	0.05 ± 0.01[b]	0.04 ± 0.002[b]	0.07 ± 0.002[a]	0.06 ± 0.004[ab]
γ-Tocotrienol	0.13 ± 0.01[a]	0.11 ± 0.01[b]	0.14 ± 0.004[a]	0.13 ± 0.003[a]
γ-Oryzanol (mg/100g)	46.4 ± 2.56[c]	115.3 ± 8.58[a]	45.2 ± 0.60[c]	80.1 ± 2.73[b]

Means ± SD;
[a-d]Different letters in the same line are significantly different ($p < 0.05$).
ND, not detected.

3.3. Identification of γ-oryzanol components using a LC-MS/MS.

The colored rice varieties were further analyzed by the LC-ESI-MS/MS system to identify the γ-oryzanol constituents and to determine their relative abundance. **Table 3** presents the γ-oryzanol components identified in the four colored rice varieties. **Figure 1** shows the total ion currents chromatograms for the four colored-rice varieties, which were reconstituted by setting a mass range of *m/z* 570-620. Overall, the LC-ESI-MS analyses of all extracts analyzed revealed 10 mass values and 18 peaks (**A**-**L**) attributed to γ-oryzanol components, thus revealing the presence of different isobaric compounds. Compounds **C**, **D**, and **F** are the most abundant species in all the analyzed samples and they were identified as follows: (**C**) cycloartenol ferulate, (**D**) 24-methylenecycloartanol ferulate and (**F**) sitosterol ferulate.

Different isomers were also identified on the basis of their different retention times and order of chromatographic elution. In the current study, the Δ7-isomer of campesterol *trans*-ferulate (**E_1**) was identified in the ion traces relative to sugary brown, red and black rice varieties (**Figure 1**). In contrast, sitosterol *trans*-ferulate (**F_1**) was not detected when the current ions were reconstituted by setting the mass range *m/z* 570-620. However, it was easily detected in sugary brown and black rice varieties when the current ions were reconstituted by setting the ion at the monoisotopic mass value of the target ion. The C18 reverse-phase HPLC is able to separate *trans*- and *cis*-ferulate isomers and *cis*-isomers have a longer retention time than their corresponding *trans*-isomers. Based on these chromatographic properties, we easily identified three pairs of *trans*- and *cis*- ferulate isomers and, in particular, the cycloartenol *trans*- (**C_1**) and *cis*- (**C_2**) ferulate, 24-methylenecycloartanol *trans*- (**D_1**) and *cis*- (**D_2**) ferulate and sitosterol *trans*- (**F_2**) and *cis*- (**F_3**) ferulate. Overall, in the four colored rice varieties analyzed, we determined 10 of the 11 known molecular mass values associated with ferulates structures (nominal mass values (Da): 574, 576, 578, 588, 590, 592, 602, 604, 616, and 618). The relative content of the different γ-oryzanol components for each rice variety was then calculated and the results are summarized in **Table 4**.

Table 3. Identified γ-oryzanols in colored rice varieties by a the liquid chromatography-mass spectrometry/mass spectrometry.

Peak	Experimental mass $[M-H]^-$	Formula[a]	Identification	Theoretical mass $[M-H]^-$	Accuracy (ppm)	Average RT (min.)	MS/MS fragment ions			
							$[M-H-Me]^-$	$[M-H-2Me]^-$	$[feruloyl]^-$	feruloyl related$^-$
A	573.39499									
	A	$C_{38}H_{54}O_4$	24-methylenecholesterol *trans*-ferulate	573.39	0.10	**31.37**	558.39	543.39	193.21	178.08
B	587.41125									
	B1	-	unknown, possible isomer of B2			**35.18**	572.39	557.67	193.15	177.24 - 175.04
	B2	$C_{39}H_{56}O_4$	Stigmasterol *trans*-ferulate	587.41	1.14	**42.57**	572.37	557.51	193.11	178.10
C	601.4273									
	C1	$C_{40}H_{58}O_4$	Cycloartenol *trans*-ferulate	601.42	1.78	**36.64**	586.39	571.46	193.20	177.04 - 75.13
	C2	$C_{40}H_{58}O_4$	Cycloartenol *cis*-ferulate	601.42	1.78	**37.79**	586.42	571.67	193.13	178.01 - 175.12
D	615.44235									
	D1	$C_{41}H_{60}O_4$	24-methylenecycloartanol *trans*-ferulate	615.44	0.76	**40.39**	600.42	575.47	193.29	177.09 - 175.16
	D2	$C_{41}H_{60}O_4$	24-methylenecycloartanol *cis*-ferulate	615.44	0.76	**44.18**	600.44	575.55	193.28	177.19 - 175.01
E	575.41176									
	E1	$C_{38}H_{56}O_4$	Δ7-campesterol *trans*-ferulate	575.41	2.05	**39.66**	560.41	-	193.33	-
	E2	$C_{38}H_{56}O_4$	Campesterol *trans*-ferulate	575.41	2.05	**41.20**	560.42	545.45	193.12	178.13 - 175.15
F	589.42709									
	F1	$C_{39}H_{58}O_4$	Δ7-sitosterol *trans*-ferulate	589.42	1.46	**44.33**	574.42	559.53	193.10	178.13 - 177.11 - 175.15
	F2	$C_{39}H_{58}O_4$	Sitosterol *trans*-ferulate	589.42	1.46	**45.80**	574.40	559.41	193.06	178.02 - 177.13 - 175.11
	F3	$C_{39}H_{58}O_4$	Sitosterol *cis*-ferulate	589.42	1.46	**47.34**	574.46	559.78	193.13	175.07 - 178.02
G	577.42722									
	G	$C_{38}H_{58}O_4$	Campestanol *trans*-ferulate	577.42	1.71	**46.33**	562.42	-	193.06	175.10
H	603.44303									
	H	$C_{40}H_{60}O_4$	Cycloartanol *trans*-ferulate	603.44	1.91	**47.13**	588.45	-	193.12	174.98
I	591.44359									
	I	$C_{39}H_{60}O_4$	Stigmastanol *trans*-ferulate	591.44	2.89	**51.30**	576.46	-	193.13	177.05
L	617.41997									
	L1	$C_{40}H_{57}O_5$	(24S)-Cycloart-25-ene-3β,24-diol-3β-*trans*-ferulate	617.42	1.91	**11.93**	602.39	587.43	193.31	175.14 - 177.05 - 177.97
	L2	$C_{40}H_{57}O_5$	(24R)-Cycloart-25-ene-3β,24-diol-3β-*trans*-ferulate	617.42	1.91	**13.24**	602.39	587.36	193.28	175.08 - 177.27
	L3	-	Cycloart-23Z-ene-3β,25-diol-3β-*trans*-ferulate	617.42	1.91	**17.27**	802.39	587.45	-	175.11

[a]Theoretical formula associated to the precursor mass (M) as generated by Qual Browser (Xcalibur, Thermo Fisher Scientific) from high-resolution spectra using the following constrains: #C atoms 30–50, #H atoms 20–80, #O atoms 0–10, and 5 ppm mass tolerance.
RT, retention time.

Table 4. Relative contents of γ-oryzanol components in colored rice varieties

	Rice varieties			
Peak	**Brown**	**Sugary brown**	**Red**	**Black**
A	0.0%	3.1%	0.2%	0.4%
B1	0.0%	4.0%	0.0%	0.0%
B2	0.0%	3.4%	4.0%	3.6%
C1	14.7%	16.6%	17.1%	16.6%
C2	0.0%	12.7%	4.2%	7.6%
D1	53.1%	15.1%	22.5%	28.5%
D2	1.6%	5.6%	2.1%	1.1%
E1	0.0%	0.3%	0.6%	0.6%
E2	10.6%	7.0%	17.4%	10.8%
F1	0.0%	1.5%	0.0%	1.3%
F2	12.8%	10.5%	15.4%	15.6%
F3	1.1%	1.2%	2.3%	2.9%
G	1.9%	2.7%	4.8%	4.7%
H	1.2%	1.5%	2.6%	2.0%
I	1.6%	1.3%	3.1%	3.6%
L1	0.8%	5.8%	1.8%	0.3%
L2	0.6%	5.7%	1.9%	0.4%
L3	0.0%	2.0%	0.0%	0.0%

Relative content (%) oryzanol A = (Area A/ Area T) x100;
Area A is the area of the peak relative to the oryzanol A; Area T is the sum of the areas of all the oryzanol components identified.

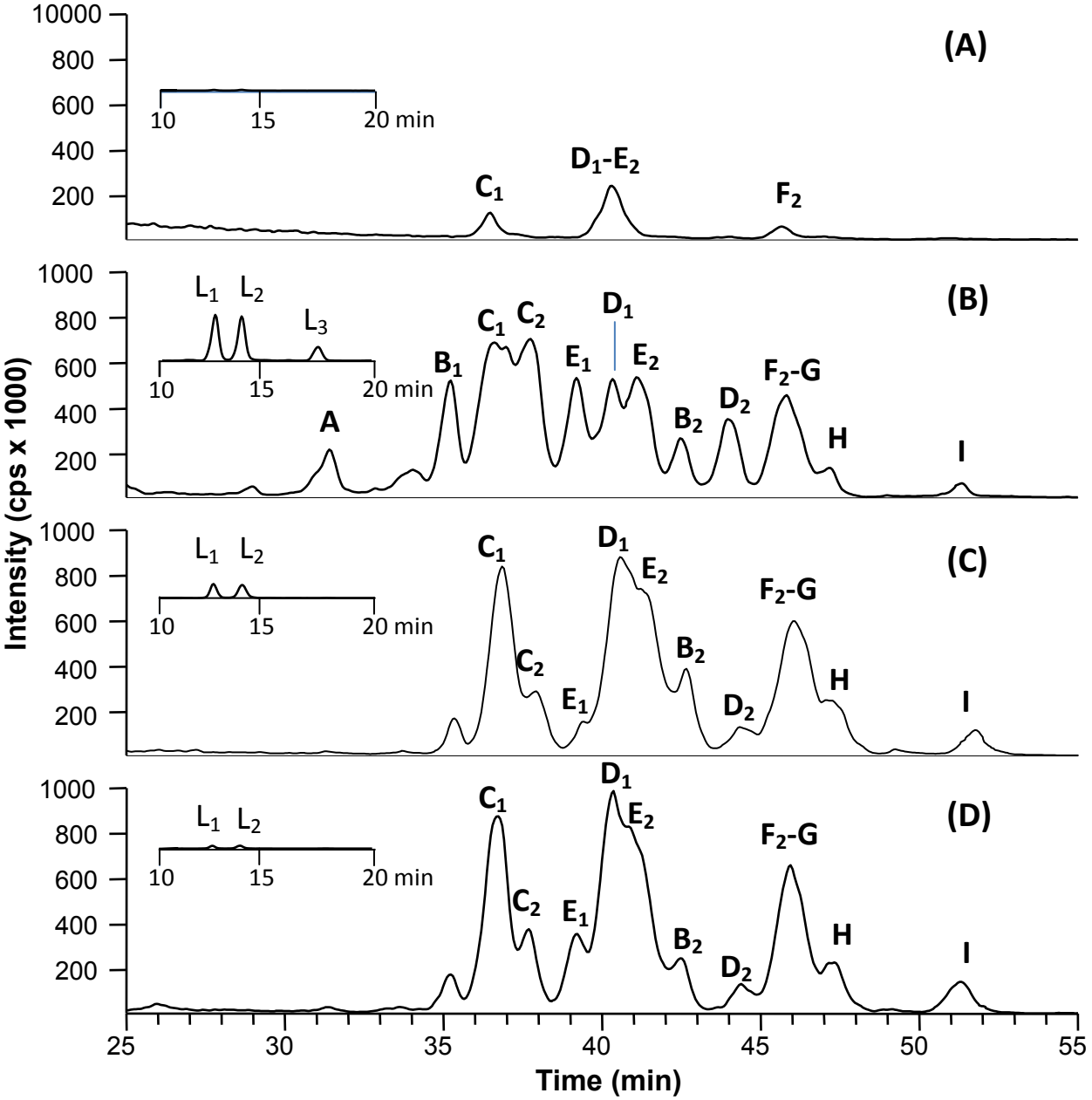

Figure 1. Typical mass range (m/z 570–620) ion current chromatograms of γ-oryzanol components in brown (**A**), sugary brown (**B**), red (**C**) and black (**D**) rice varieties.

4. DISCUSSION

In the current study, fatty acids, carotenoids, vitamin E isomers, and c-oryzanol contents were determined in brown, sugary brown, red, and black rice varieties. The information obtained was important because it identified the varied and high contents of fat-soluble bioactive components in colored rice providing useful information for selecting rice varieties according to bioactive compounds for achieving optimal health.

Unsaturated fatty acids accounted for the largest lipid fraction in rice varieties, consistent with previous reports[22-24]. The fatty acids are reported to be homogenously distributed in rice kernel; thus, the milling process only decreases the amount of these compounds not altering their profiles[25]. Although it has been suggested that rice containing high β-carotene may contain a higher proportion of saturated fatty acids in their lipid fraction[22], the sugary brown rice and black rice containing β-carotene also had higher unsaturated fatty acids than saturated fatty acids. Sugary brown rice had less MUFA and more SFA and PUFA when compared with those of the other three colored rice varieties. Black rice contained less linoleic acid than other colored rice varieties. The content of linoleic acid may be increased by the germination process, as reported previously[26].

Interestingly, black rice had a 27-48 folds higher content of lutein than the other three varieties. Small amount of lutein (5-9 μg/100g) were also found in the brown, sugary brown and red rice varieties. The reason that we were able to detected β-carotene and lutein in rice varieties unlike the previous study might be due to the less polished rice varieties and higher sensitivity of the analytical method[27].

The content of total γ-oryzanol in red, brown, and black rice varieties was similar to those of previous reports[11, 28, 29]. Phytochemical contents are variable among rice fractions and the best source of c-oryzanol is the rice bran followed by unpolished rice and polished rice. It has been reported that the coryzanol content in brown rice is also cultivar dependent reports [11, 28,30].

In the current study, mass analyses were performed by using a LTQ Orbitrap XL, which is a hybrid mass spectrometer, combining extremely high mass accuracy (<5 ppm with external calibration) and resolution with the capability of multiple levels of fragmentation[31], for further analysis of γ-oryzanol in colored rice. We have recently applied such instrumental features to set-up an integrated high resolution mass

spectrometric and informatics approach for the rapid identification of phenolic compounds in plant extract[32], which was applied for the identification of γ-oryzanol components in this study. Compounds were identified by searching the experimental monoisotopic masses (tolerance 5 ppm) in a database generated by including all the known structures of γ-oryzanol components so far reported in the literature[33-35]. The structure confirmation of γ-oryzanol components was then achieved on the basis of the elemental composition as determined by the accurate mass value, by matching the experimental isotopic pattern with that calculated and on the basis of the collision induced dissociation (CID) experiments showing the diagnostic fragment ions of γ-oryzanol components, as previously reported[33], and, in particular, the deprotonated ferulic acid at *m/z* 193 and the related $[M-H-Me]^-$ and $[M-H-2Me]^-$ fragment ions. In addition, the Δ7-isomers of two major γ -oryzanol components, campesterol *trans*-ferulate and sitosterol *trans*-ferulate, were confirmed by shorter retention times than their Δ5-isomers on the C18 reverse-phase HPLC, as reported previously[34]. Given the overall data, it can be considered that compounds cycloartenol *trans*-ferulate, 24-methylenecycloartanol *trans*-ferulate, campesterol *trans*-ferulate, and sitosterol *trans*-ferulate represent the basic γ-oryzanol components in the brown rice. On the other hand, sugary brown rice contained the highest variety of γ-oryzanol components since all the 10 mass values and 18 peaks were found in significant amounts and where cycloartenol *trans*-ferulate, cycloartenol *cis*-ferulate, 24-methylenecycloartanol *trans*-ferulate, and sitosterol t*rans*-ferulate represented the most abundant (>10%). Interestingly, 24-methylenecholesterol *trans*-ferulate was present at relevant concentrations only in the sugary brown rice. The profile of γ-oryzanol components in red and black rice varieties was similar and both characterized by high relative amounts of cycloartenol *trans*-ferulate, 24-methylenecycloartanol *trans*-ferulate, campesterol *trans*-ferulate, and sitosterol *trans*-ferulate. The most relevant difference between red rice and black rice consists of the higher content of (24S)-cycloart-25-ene-3β,24-diol-3β-*trans*-ferulate and (24R)-cycloart-25-ene-3β,24-diol-3β-*trans*-ferulate in the red rice and of the presence of Δ7-sitosterol *trans*-ferulate only in the black rice.

In contrast to a previous study reporting a higher content of tocotrienols than tocopherols in white and brown rice varieties[33], the current study found higher content of tocopherols than tocotrienols in all four rice varieties. β and δ isomers of

tocopherol and tocotrienols were not detected using MeOH/THF solvent extraction, consistent with the previous report using methanol extraction[29]. The vitamin E content of rice varieties is reported to be variable, probably due to the different methods of quantification and solvents employed in the extraction process. The growing conditions can also affect the content of bioactive components in rice varieties.

This study indicates that the colored rice varieties are rich sources of fat-soluble bioactive components, in particular, various γ-oryzanols, vitamin E isomers, and carotenoids. In addition, it provides a structural basis for studying the biological functions of these bioactive components at molecular levels. A higher priority may be given to the development of rice varieties that contain high amounts of various bioactives without altering their agronomic performance as well as preserving the cultural and socially acceptable organoleptic qualities.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interest exist.

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CAPÍTULO III: Artigo 2

GAMMA-ORYZANOL AS A POTENTIAL ANTIOBESITY AGENT: BIOACTIVITY AND STABILITY IN RICE VARIETIES

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Artigo nas Normas do periódico: **Food Chemistry**

RESUMO

O arroz integral é uma rica fonte de γ-orizanol e outros compostos bioativos com potenciais benefícios para a saúde. Um crescente acúmulo de evidências tem sugerido um efeito antiobesidade do γ-orizanol, principal fitosterol do farelo de arroz. Entretanto, as concentrações necessárias para exercer este efeito e a quantidade residual deste composto após cozimento ou armazenamento não estão bem estabelecidas. O efeito antiobesidade do γ-orizanol foi testado em células tronco mesenquimais derivadas de tecido adiposo humano (ASCs) e pré-adipócitos 3T3-L1, após diferenciação em adipócitos imaturos. Além disso, a estabilidade do γ-orizanol foi analisada em quatro variedades de arroz após preparo (cozimento e torra) ou armazenamento (temperatura ambiente, 4º e -80ºC, por 22 semanas). γ-Oryzanol significantemente diminuiu o acúmulo de lipídeos pelos adipócitos durante a diferenciação de ambas as células, ASCs e 3T3-L1. Os métodos de preparo, cozimento e torra aumentaram o conteúdo de γ-orizanol em todas as variedades, quando comparado com as amostras cruas. À temperatura ambiente o γ-orizanol foi estável por até 16 semanas, enquanto que, nenhuma diminuição foi observada quando as amostras de arroz foram armazenadas a 4º ou -80ºC. Nossos resultados demonstraram que o γ-orizanol diminuiu o acúmulo de lipídeos e pode ser considerado um composto bioativo com potencial efeito antiobesidade. Os métodos de cozimento e torra aumentaram as concentrações de γ-orizanol em todas variedades de arroz.

PALAVRAS-CHAVE: γ-Orizanol, arroz integral, efeito antiobesidade, células-tronco,

ABSTRACT

Whole grain rice is a rich source of γ-oryzanol and others bioactive compounds with potential health benefits. Increasing evidences has been suggested a antiobesity effect of γ-oryzanol, the major phytosterol of rice bran. However, required concentrations to exert this effect and the amounts remaining in grains after cooking or storage, are not well established. The antiadipogenic effect of γ-oryzanol was tested in human adipose tissue-derived mesenchymal stem cells (ASCs) and 3T3-L1 preadipocytes, after differentiation in immature adipocytes. In addition, the stability of γ-oryzanol was analyzed, in four rice varieties, after cooking (steam and roast) or storage (room temperature, 4º and -80ºC, over 22 weeks). γ-oryzanol significantly decreased lipid accumulation by adipocytes during differentiation of both cells, ASCs and 3T3-L1. Steam and roast methods of cooking increased γ-oryzanol content, in all varieties, as compared to raw samples. At room temperature γ-oryzanol was stable up to 16 weeks, and any lost was observed when rice varieties were stored at 4º and -80ºC for 22 weeks. Our results showed that γ-oryzanol suppressed lipid accumulation and may be considered a bioactive compound with potential antiobesity effect. Steaming and roasting process increased the concentrations of γ-oryzanol in all rice varieties.

KEY WORDS: γ-Oryzanol, brown rice, antiobesity effect, stem cells

1. INTRODUCTION

Bioactive micronutrients are abundant in whole grain rice and has a great potential to improve health in individuals consuming a brown rice (unmilled) based diet[1, 2]. The milling process is done to give grains a finer texture and avoid rapid degradation, but also removes bioactive compounds as carotenoids, vitamins and γ-oryzanol (Orz). The bran layer removed is the major source of Orz, a mixture of ferulic acid (4-hydroxy-3-methoxycinnamic acid) esters of triterpene alcohols[3], that has received special attention because its health benefits. Its concentration is variable in rice varieties and dependent mainly of cultivar, milling degree, storage conditions and extraction methods[4, 5].

A great variety of biological effects are attributed to Orz, such as anti-diabetic[2], antioxidant[6, 7] and anti-inflammatory activities[8]. However, recently the most studied activity of Orz is its hypolipidemic and antiobesity effects[9]. Obesity is considered the leading metabolic disease and a risk factor for development of type II diabetes mellitus, dyslipidemia and cardiovascular disease. The intake of diets based mainly in fat promotes obesity as a result of a imbalance between energy intake and expenditure[10, 11]. Several studies have been shown that regular consumption of whole grain cereals, legumes, fruit and vegetable provide benefits in prevention of obesity and its associated health risks[12-15]. These benefits are associated mainly to bioactive compounds[16-18]. However, the mechanism of action and the bioactive compound responsible for this effect remains unclear. Anti-obesity effects of brown rice seems to be related to micronutrients and not to other components as fiber[19]. Several studies, *in vitro and in vivo*, have found that brown rice or Orz can suppress 3T3-L1 lipid accumulation and body weight gain[2, 6, 20-23]. This effects are carried out by diverse mechanisms as lowering lipid and cholesterol levels[18], influence in adipocytes microenvironment[24, 25] and down regulation of several factors involved in adipocytes differentiation[21]. Therefore, a better understanding of Orz effects in adopogenesis is necessary and reasonable with a global wave of obesity and consequent increase in associated diseases.

Rice is usually cooked before consume, although a small amount of cooked or raw rice are used in processed foods as cereal and cereal bars or additive in dairy products to improve nutritional values. However, the bioavailability of

these micronutrients is directly affected by cooking and storage conditions[26, 27]. Micronutrients may be destroyed or lost during storage because of its sensitivity to factors as temperature, light and oxygen[26, 28]. Among rice varieties, the most commonly consumed is white rice, whereas brown and colored varieties (black, red or purple) consumption has increased. Studies have reported that colored rice present antioxidant properties related to a well-known class of fat-soluble micronutrients: Orz, phenolic acids, carotenoids and vitamins[29-31]. Ageing of rice stored at different temperatures is responsible by changes like pasting properties, flavor and color[32, 33], whereas data about changes in Orz are scarce. Micronutrients naturally occurring in rice have variable distribution[34], and the same properties that make them useful to improve human health, create challenges in prevent the degradation.

In the present study, we investigate the possible effect of γ-oryzanol on lipid accumulation by immature adipocytes. In addition, we investigated the effects of different rice-cooking methods, time and temperature of storage on total γ-oryzanol concentration in four colored brown rice varieties.

2. MATERIAL AND METHODS

2.1 Cells and reagents

Human adipose tissue-derived mesenchymal stem cells (ASCs) (cat. PCS-500-011), 3T3-L1 preadipocytes (cat. CL-173) and adipocyte differentiation toolkit (cat. PCS-500-050) were purchased from the ATCC® (Manassas, VA, USA). Oil red O and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Antibiotic/antimycotic solution was purchased from Gibco (Life Technologies Inc., Gaithersburg, MD).

Analytical grade solvent and chemicals such as ethanol, tetrahydrofuran (THF), methyl tert-butyl ether, ammonium acetate, and methanol were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Fatty acids standards were obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA), gamma-oryzanol from Wako (Osaka, Japan) and α- and γ- tocopherols and tocotrienols from Cayman Chemical (Ann Arbor, MI, USA).

2.2 Cell culture and treatment

Cells were cultured in 24 wells plates containing basal medium (DMEM), until reach 70% of confluence. After confluence, cells were induced to differentiate for 4 days using initiation medium (ATCC®, PCS-500-050). On day 4 and thereafter, the cells were cultured in maintenance medium (ATCC®, PCS-500-050), which was freshly changed every 3 days until day 15. To maintenance medium was add Orz (dissolved in acetone) at final concentrations of 5, 1, 0.5 and 0.1 μM. In treated and control cells, the final concentration of acetone was <0,01%. Citotoxicity was evaluated by MTT colorimetric assay[35].

2.3 Oil red O staining

To assess lipid accumulation, on day 15, cells were fixed with 10% formaldehyde and then stained for 2 h with Oil red O solution. Fat droplets stained by Oil red O were solubilized with isopropanol and the absorbance read at 490 nm.

2.4 Glycerol-3-phosphate dehydrogenase (GPDH) activity

Differentiated cells were washed twice with ice-cold PBS and GPDH buffer (20 mM Tris–HCl, 1 mM EDTA, and 1 mM β-mercaptoethanol, pH 7.3) was added. Cell lysates were homogenized and centrifuged at 4ºC for 5 min, 13.000 g. The supernatant was mixed to GPDH reaction buffer (0,1M triethanolamine, 2mM EDTA, 0,2mM NADH and 0,1mM β-mercaptoethanol and 0,2mM dihydroxyacetone phosphate, pH 7.5). GPDH activity was measured at 340 nm. The protein concentration was determined using the Bradford method. Results were expressed as mU/mg protein where, one unit of enzyme activity corresponds to the oxidation of 1 μmol of NADH/min[36].

2.5 Rice samples

Four samples of "brown" japonica rice (*i.e*., whole kernel rice) were grown and harvested at the National Institute of Crop Science, Rural Development Administration, Miryang, Korea, during the 2010-2011 growing season. Brown rice, sugary brown rice (containing high amounts of sugar content than brown rice by 30%), red rice, and black rice with giant embryo were dehusked, as previous report[37], using rice sheller (SY88-TH, Ssangyong Ltd., Incheon, Korea), grinded into powder using 100 mesh sieve (1093 Cyclotec Mill, FOSS Co., Ltd, Denmark) and stored at -80ºC.

The mean moisture content of dehusked rice was 12% (Precisa HA60 IR moisture analyser, Precisa instruments, Germany). All the analyses were carried out in triplicate.

2.6 Storage and cooking process

The four rice varieties were stored at controlled temperature (-80 ºC, 4 ºC and room temperature (RT) (*i.e.* 25 ºC ± 1ºC), in polypropylene tubes under the dark and assessed for 22 weeks.

For steaming, 200 g of rice samples were measured, washed twice with tap water and cooked with a basic rice cooker (an electric rice cooker which is not controlled with pressure during cooking in order to keep the cooking temperature ≤100 ºC) followed by the standard cooking process. After the cooking cycle was completed, the rice was put to rest for 5 minutes for post-absorption and then cooled with ambient temperature.

For roasting, rice samples were placed in a roasting pan and cooked at high temperature from 150 ~ 170 ºC (157 ºC in black rice with giant embryo and 167 ºC in brown rice). Brown rice is missing the moisture for puffing, but black rice with giant embryo was able to puff by starch inside the kernel and a hard shell to contain the pressure.

After cooling to room temperature, cooked rice samples were transferred into a freeze dryer (Ilsin Lab. Lyoph-Pride system) for drying.

2.8 Fatty acids profile

After freeze-drying all rice varieties, internal standard (50 µg of 17:0) was add to 50 mg of each rice sample to extract lipids[38]. The samples were then saponified and methylated[39]. The quantity of obtained fatty acid methyl esters were analyzing using an previously published gas chromatography method[40], and peaks of samples were estimated by curves of authentic fatty acid standards (Nu-Chek Prep, Inc. Elysian, MN). Final results were expressed as molar percentage (mol %) of total fatty acids. Interassay coefficients of variation (CV) were <4.5% for fatty acids present at levels >1%.

2.9 Extraction and HPLC analysis of γ-oryzanol

Powdered and dried rice samples (100 mg) were placed in 25 x 165 mm test tubes and overlaid with 5 ml of MeOH, vortexed for 30s and incubated for 1 h in shaking incubator at 25°C. The test tubes were then centrifuged at 3500 rpm for 10 min and the MeOH layer transferred to a 25 mL volumetric flask. Similar procedures were conducted four more times using THF to reach a combined final volume of 25 ml. Ten milliliters of the combined extract was dried under nitrogen, resuspended in 100 µl of ethanol, and from this volume, 20 µl was injected onto HPLC.

A reverse phase HPLC system consisting of a Waters 2695 Alliance system (Waters Co., Milford, MA), C18 guard column (3µm, 33 x 4.6 mm, Perkin-Elmer, Norwalk, CT), C30 semibore carotenoid column (3 µm, 150 x 3.0 mm, YMC, Wilmington, NC) and Waters 2996 photodiode array detector, was used to quantify γ-oryzanol. The HPLC mobile phase solvent A was MeOH/MBTE/water (85:12:3, v/v/v, with 1.5% of ammonium acetate in the water), and the mobile phase solvent B was MeOH/MBTE/water (8:90:2, v/v/v, with 1% of ammonium acetate in the water). The gradient was set up for 0.4 mL/min begin at 100% solvent A followed by 45% solvent A over a 21 min linear gradient and hold at 45% A for 1 min. This is followed by an 11 min linear gradient to 5% solvent A and 4 min hold at 5% solvent A, finally a 2 min linear gradient back to 100% solvent A. The system was held at 100% solvent A for 8 min for equilibration back to initial conditions.

Orz was quantified at 330 nm by determining peak areas under the curve in the HPLC calibrated against known amounts of standards. Identification of each peak in unknowns was confirmed by the retention time and characteristic spectra of the standards. The inter-assay coefficient of variation (CV) was 4% (*n* = 25) and the intra-assay CV was 4% (*n* = 9).

2.9 Statistical analysis

The results are reported as mean ± standard deviation. Differences between cooking methods, storage temperature and lipid assimilation by differentiated cells were calculated using analysis of variance (ANOVA), with a $P < 0.05$ being considered statistically significant. Post-hoc tests were carried out using Holm-Sidak correction for multiple comparisons. A linear regression analysis was used to analyze correlations between Oil red O and GDPH. Data were analyzed using SigmaStat 3.5 (Systat Software, San Jose, Ca, USA).

3. RESULTS

3.1 Lipid accumulation in adipocytes

Oil red O staining was used to analyze the lipid accumulation by 3T3-L1 and ASCs after 11 days of treatment (from day 4 to day 15) with Orz. As shown in **Figure 1 A** and **B**, a different response to treatment was observed between cells. Lipid accumulation in 3T3-L1 was significantly reduced by 5, 1 and 0.5 µM concentrations when compared to untreated control cells. The highest inhibitory effect, in 3T3-L1, was induced by 1µM of Orz, representing 41% less lipid accumulation. Five, 0.5 and 0.1 µM concentrations reduced lipid content by 23, 30 and 15%, respectively. Despite reducing effect of 0.1 µM, no statistically difference to control was observed.

In ASCs adipocytes, the Orz concentrations of 1, 0.5 and 0.1µM significantly decreased lipid accumulation by 35, 41 and 59%, when compared to control, and a slightly reduction was observed in 5 µM treatment. Differently to observed in 3T3-L1 cells, 0.1 µM was the most efficient concentration of Orz to inhibit lipid accumulation by ASCs differentiated into adipocytes.

In order to confirm the Orz effect on lipid accumulation during differentiation, GPDH activity was assessed. As shown in **Figure 2 A** and **B**, GPDH activity in 3T3-L1 and ASCs, respectively, was significantly decreased by different Orz concentrations. The lowest level of GPDH activity was 29 ± 10 and 24 ± 5 mU/mg protein when 3T3-L1 and ASCs were treated with 1 and 0.1 μM of Orz, respectively. Decreased activity of GPDH in response to treatment was significantly correlated with lipid accumulation observed by Oil red O staining (*r* = 0.62 to 3T3-L1 and r = 0.7 to ASCs, p<0.05). Treatments did not affect differentiation and anti-adipogenic effect was achieved at concentration that not caused cell toxicity according to the MTT assay (data not shown).

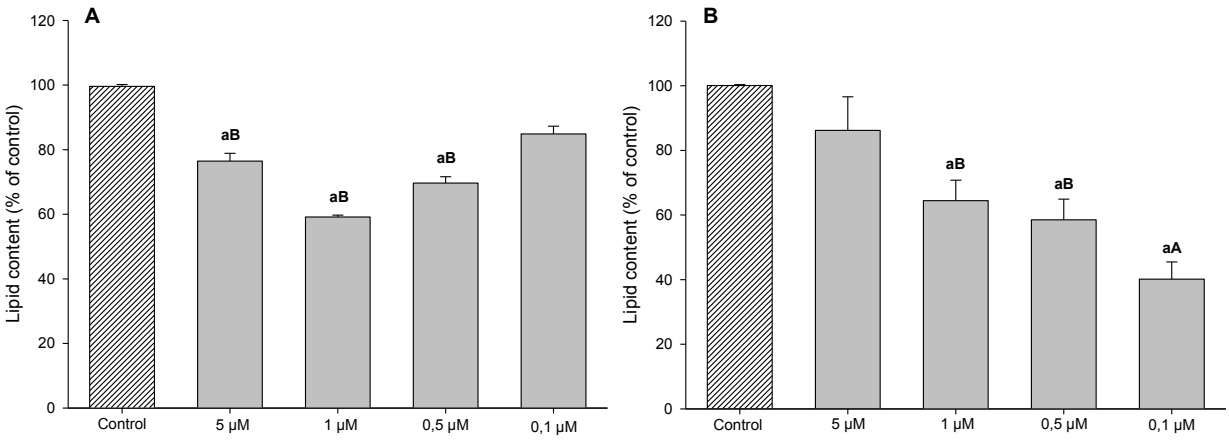

Figure 1: Effects of γ-oryzanol on lipid accumulation in differentiated, **A)** 3T3-L1 and **B)** ASC adipocytes. Intracellular lipid accumulation was measured by Oil red O staining. Results are presented as mean ± SD of triplicate experiments. Different letters indicate difference from control (lowercase) or among treatments (uppercase). *P < 0.05.*

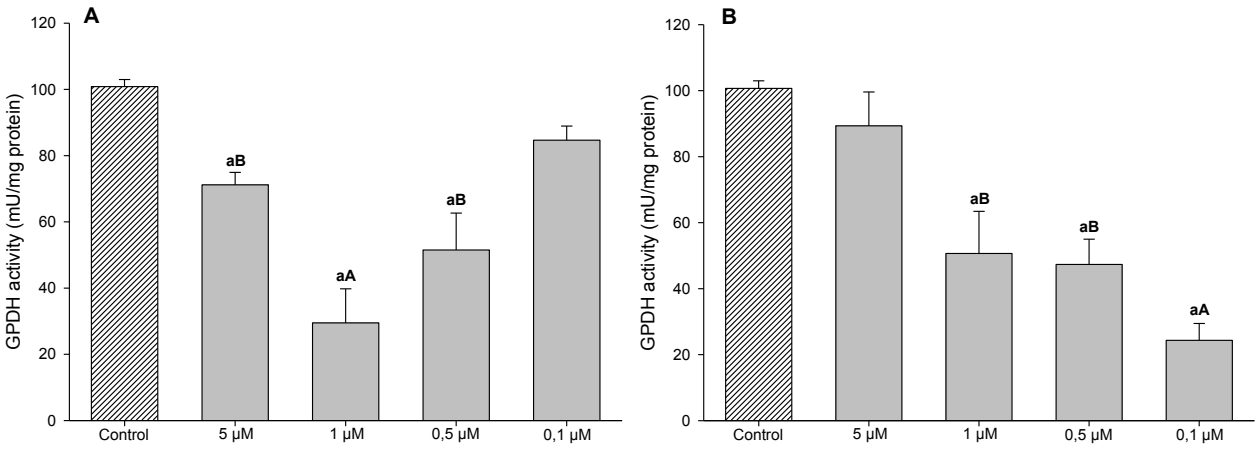

Figure 2: Effects of γ-oryzanol on Glycerol-3-phosphate dehydrogenase (GPDH) activity in **A)** 3T3-L1 and **B)** ASC adipocytes. Results are presented as mean ± SD of triplicate experiments. Different letters indicate difference from control (lowercase) or among treatments (uppercase). *P < 0.05.*

3.2 Effects of cooking and storage on γ-oryzanol content

The changes in the concentration of Orz in rice varieties are shown in **Table 1**. The total Orz in raw samples ranged from 45 to 115 mg/100g (dry weight) with the highest concentration present in sugary brown, followed by black, brown and red rice, respectively. As compared to raw rice, steaming and roasting process increased Orz content in all rice varieties by 2 - 16%. However, steaming process was more effective in release Orz, significantly increasing content in four samples analyzed. Roasting process, equally to steaming, increased Orz in all samples, but significantly only in black rice.

To analyze the effects of time and temperature of storage, as a control data to verify losses of Orz after harvest, samples were stored at -80 ºC, 4 ºC and RT (25 ± 1ºC) during 22 weeks. Rice samples were stable up to 16 weeks at RT (**Fig. 3**). Low temperatures (4 ºC and -80 ºC) were efficient to prevent losses up to 22 weeks (**Fig. 3**).

Table 1: Gamma-oryzanol content in raw and cooked samples (mg/100g dry weight).

	Raw	Steamed	Roasted
Brown	$46 \pm 2^{b,C}$	$51 \pm 1^{a,C}$	$50 \pm 8^{a,b,C}$
Sugary Brown	$115 \pm 8^{b,A}$	$133 \pm 9^{a,A}$	$128 \pm 5^{a,b,A}$
Red	$45 \pm 1^{b,C}$	$50 \pm 1^{a,C}$	$46 \pm 2^{b,C}$
Black	$80 \pm 3^{c,B}$	$89 \pm 3^{a,B}$	$85 \pm 2^{b,B}$

Mean ± SD.
Means with different letters (a–c) in the same line or (A-C) in the same column are significantly different . *P < 0.05.*

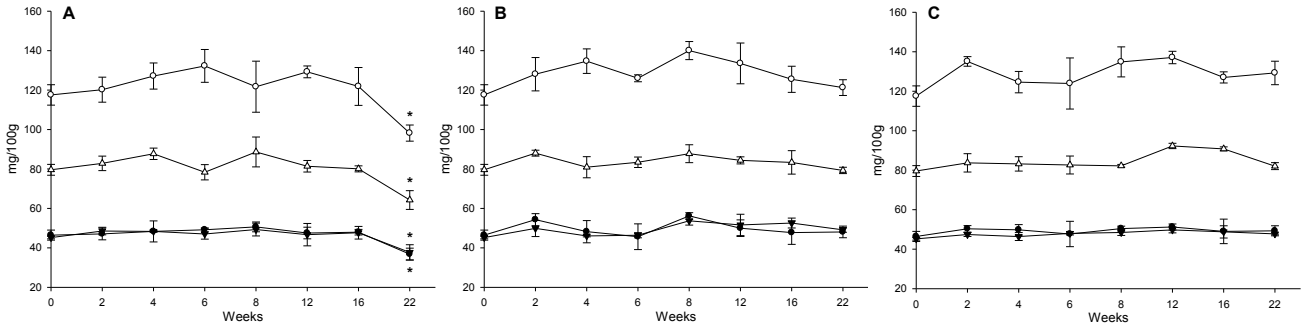

Figure 3: Gamma-oryzanol content over 22 weeks at different storage temperatures. The symbols ●, ○, ▼ and Δ correspond to brown, sugary brown, red and black rice, respectively. A) room temperature (25 ± 1ºC); B) 4ºC; C) -80ºC. The data are expressed as mean ± SD. *Significantly different from the baseline. *P < 0.05.*

3.3 Fatty acids profile

Free fatty acids (FA) were analyzed in raw samples and after cooking methods (**Table 2)**. No significant difference was found in FA composition due to preparation, indicating that the cooking methods used did not interfere in fatty acid composition.

Table 2. Changes in fatty acid profile of rice grains after cooking

Fatty Acid (mol %)	Rice Varieties											
	Brown			Sugary brown			Red			Black		
	Raw	Steamed	Roasted	Raw	Steamed	Roasted	Raw	Steamed	Roasted	Raw	Steamed	Roasted
SFA (total)	**21.27**	**20.96**	**20.91**	**28.84**	**28.71**	**28.69**	**22.13**	**21.75**	**21.60**	**23.97**	**22.55**	**23.38**
14:0, Myristic	0.57	0.56	0.51	0.70	0.66	0.69	0.62	0.55	0.48	0.70	0.64	0.67
16:0, Palmitic	17.71	17.76	17.59	23.85	24.01	23.85	18.34	18.32	18.16	19.12	18.20	18.80
18:0, Stearic	1.79	1.75	1.75	2.69	2.67	2.68	1.83	1.82	1.77	2.56	2.39	2.48
20:0, Arachidic	0.48	0.47	0.47	0.72	0.72	0.72	0.53	0.53	0.54	0.61	0.63	0.63
22:0, Behenic	0.25	0.16	0.20	0.32	0.26	0.29	0.29	0.20	0.23	0.33	0.26	0.29
24:0, Tetracosanoic	0.48	0.26	0.38	0.56	0.39	0.47	0.53	0.33	0.42	0.63	0.43	0.51
MUFA (total)	**40.69**	**41.37**	**41.06**	**26.89**	**27.08**	**26.83**	**38.58**	**39.03**	**39.09**	**40.27**	**41.81**	**40.91**
16:1n-9, cis-7-Hexadecenoic	0.05	0.05	0.05	0.08	0.08	0.08	0.05	0.05	0.05	0.07	0.07	0.07
16:1n-7, Palmitoleic	0.16	0.16	0.16	0.29	0.30	0.30	0.14	0.14	0.13	0.15	0.15	0.15
18:1n-9, Oleic	38.85	39.56	39.23	25.24	25.47	25.23	36.85	37.33	37.39	38.53	40.05	39.15
18:1n-7, cis Vaccenic	1.19	1.16	1.18	0.80	0.75	0.74	1.12	1.11	1.10	1.11	1.08	1.10
20:1n-9, Gadoleic	0.39	0.40	0.40	0.44	0.45	0.45	0.37	0.37	0.38	0.37	0.40	0.39
22:1n-9, Erucic	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.04	0.05	0.04
PUFA (total)	**38.04**	**37.67**	**38.03**	**44.27**	**44.21**	**44.48**	**39.29**	**39.22**	**39.31**	**35.76**	**35.64**	**35.71**
18:2n-6, Linoleic	36.85	36.52	36.82	42.52	42.48	42.68	38.05	37.98	38.04	34.21	34.10	34.18
18:3n-3, Alpha-Linolenic	1.19	1.15	1.21	1.75	1.73	1.78	1.24	1.25	1.27	1.55	1.54	1.53

SFA, satured fatty acids; MUFA, monounsatured fatty acids; PUFA, polyunsatuired fatty acids.

4. DISCUSSION

There are little information available on the Orz effects into adipogenic process and the pre-adipocytes culture model offers a valuable tool to study the role of this bioactive compound in human organism. In recent years, there has been a growing interest in brown rice based diets, fact that may be explained by its bioactive micronutrients with antiobesity[9], antidiabetic[2], antioxidant[6, 7] and anti-inflammatory effects[8]. The consumption of whole grains has been associated with low body weight and adiposity[41]. However, to prevent or treat obesity and related diseases, making healthy food choices, is necessary to know the effects, types and amounts of micronutrients present in food. In a previously report, we showed that Orz, the major bioactive compound in bran layer of rice grain, is variable among rice varieties[37].

In the USA and most of the rest of the world, obesity and associated metabolic disorders have increased to epidemic proportions over the past few decades[42]. Diets based primarily in fat intake are responsible for obesity and over-accumulation of subcutaneous and/or abdominal adipose tissue. Imbalance between energy intake and expenditure, results in fat accumulation mainly by adipocytes. It is known that adipocyte differentiation and adipogenesis are closely related to obesity and occurrence of various metabolic diseases[43]. Adipocytes derive from multipotent mesenchymal stem cells present in adipose tissue and takes on the characteristic of mature adipocyte after lipid accumulation. Is difficult to distinguish cellular intermediates between stem cell and immature adypocytes, so most researches in the field describe two phases of adipogenesis[43, 44]. In the first phase, initiation, stem cells or preadipocytes (*e.g.,* 3T3-L1 cells) are differentiated in immature adipocytes after 4 days in culture with specific requirements. On the second phase, maintenance, immature adipocytes activate its machinery necessary for lipid transport and synthesis until reach full maturity around day 15-17 after the beginning of differentiation.

To evaluate the anti-adipogenic effects of Orz in immature adipocytes, 3T3-L1 and ASCs were treated during all maintenance phase (day 4 to 15) with 5, 1, 0.5 and 0.1 μM of Orz and lipid accumulation was measured by Oil red O. Different concentrations of Orz significantly suppressed lipid accumulation by

adipocytes during differentiation of both cells, 3T3-L1 and ASCs. The highest inhibition in 3T3-L1 cells was induced by an intermediate dose (1 μM), whereas in ASCs 0.1 μM of Orz had the higher inhibition. Based in these results, we can suggest that antiobesity effect of Orz is dose-specific and variable between species. Ho and co-workers (2013), showed that extract of germinated brown rice down-regulated expression, by 3T3-L1 cells, of C/EBP-β, C/EBP-α, PPARγ, and SREBP-1c, important factors involved in adipocytes differentiation. However, the bioactive compound present in the extract and accounted for anti-adipogenic effect have not been studied[21]. Regulation of PPAR-γ and C/EBPs gave a considerable starting point to understand the antiobesity effect of Orz, but the exactly participation of this bioactive compound in adipocyte differentiation is not fully explain[43]. Using animal model, Kozuka *et al*. (2012), demonstrated that Orz isolated or brown rice attenuated mice preference for an high fat diet via the inhibition of hypothalamic ER stress[22]. In a study with metabolic syndrome subjects, researchers observed that when a white rice based diet was switched for brown rice led to a decrease in body weight, systolic blood pressure, HOMA-IR, total cholesterol and LDL-cholesterol levels[2]. In addition to reported effect in gene expression, hypothalamic regulation and metabolic parameters exerted by Orz or brown rice, in this study, we demonstrated that Orz may directly influence in adipocytes lipid accumulation.

Glycerol-3-phosphate in adipose tissues is produced reversibly from dihydroxyacetone phosphate by GPDH and combined with fatty acyl-CoA produce triglyceride. GPDH is highly expressed in mature adipocytes and an indicator of adipocyte late differentiation[45]. Activity of this enzyme is therefore routinely measured to assess differentiation in *in vitro* studies. In the present study, GPDH activity was significantly decreased by Orz treatment in 3T3-L1 and ASCs after 15 days of differentiation. These results confirm the response observed in Oil red O staining, indicating that Orz exhibit an antiadipogenic effect though the inhibition of GPDH activity and consequent lipid accumulation. A similar result was observed by Ho *et al*. (2013), where GPDH activity was decreased in 3T3-L1 cells treated with extract of germinated brown rice[21].

Whole grains such as brown rice and wheat are nutritionally superior to milled ones[46]. However, post harvest rice is stored for long periods and typically

consumed after different cooking processes. During storage and cooking the bioactive compounds may be destroyed by direct action of temperature, oxygen and light[27]. In the present study, we conducted in four rice varieties an analysis of stability under different cooking methods and temperature of storage. As compared to raw samples, both methods of cooking increased Orz content, whereas steaming is a more interesting method, once that higher concentrations were observed after this process. Both cooking process seems release γ-oryzanol-protein complex present in bran layer. These results are in concordance to a recent report[47], where parboiling methods increased Orz content in different brown rice samples.

Brown rice is usually stored for less time than white rice. It is due to more moisture determined by bran layer and consequent elevated susceptibility to fungal contamination and oxidative degradation. Our results showed that Orz was stable over 22 weeks of storage at -80ºC and 4ºC in all four samples. At room temperature, all samples presented a significant decrease in Orz content after 16 weeks. Humidity was not controlled during the experiment, but samples were quickly processed and placed at polypropylene tubes after harvest. Our results are in agreement whit a previously report[27], that showed in brown rice, a 20% reduction on total Orz stored for 6 months. The antioxidant properties of Orz and its relative stability at room temperature has been employed in industrial process to improve the storage time of foods[48].

Almost all Orz present in brown rice is found in bran layer. Rice bran constitutes about 10% of rough rice grain and contains 18%–22% oil. When rice is milled, endogenous lipases responsible by degradation of lipids into free fatty acids, are released to bran layer and may affect FA levels. Normal levels of FA in brown rice are, oleic acid (38.4%), linoleic acid (34.4%) and α-linolenic acid (2.2%) as unsaturated fatty acids, and palmitic (21.5%) and stearic (2.9%) acids as saturated fatty acids[49, 50]. In the present study, we found a similar concentration of FA in brown, sugary brown, red and black rice. Cooking process did not affect free fatty acids levels. Degradation of FA is a important factor to be observed during rice preparation, since they may affect fat-soluble micronutrients and taste.

5. CONCLUSION

Our findings shown that Orz may be considered a bioactive compound with potential antiobesity effects which were performed through the inhibition of adipocytes differentiation *via* reduction in lipid accumulation and as well as through GPDH activity. Significant amounts of Orz were found in brown rice varieties and steaming or roasting process increased the concentrations of γ-oryzanol in all rice cultivars, indicating that these cooking process do not interfere in Orz bioavability. Gamma-oryzanol was stable for 16 weeks at room temperature storage. However, the mechanisms of oxidative damage induced by temperature, oxygen and light may induce its degradation after this period. Therefore, when there is necessity of storage rice grains for a long period, it should be made at low temperatures to avoid losses in Orz content and prevent the quality of food.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interest exist.

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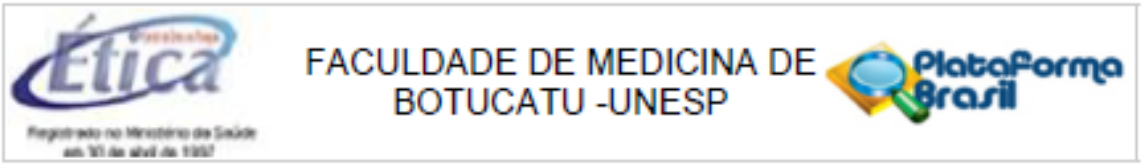
ANEXOS

1. PARECER COMITÊ DE ÉTICA



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Caracterização e atividade funcional de compostos bioativos
Pesquisador: Igor Otavio Minatel
Área Temática:
Versão: 1
CAAE: 31685614.7.0000.5411
Instituição Proponente: Departamento de Patologia
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 712.196
Data da Relatoria: 07/07/2014

Apresentação do Projeto:
Nos últimos anos um crescente interesse por hábitos alimentares mais saudáveis proporcionou a introdução de novos alimentos a dieta básica da população mundial. Entre os alimentos básicos podemos citar os grãos, principalmente arroz, milho e soja. O arroz (Oryza sativa) é um dos grãos produzidos em maior quantidade, sendo consumido com componente básico da dieta, por mais da metade da população mundial. Melhorias dos cultivares e conseqüente geração de variedades mais ricas em compostos com funções bioativas permitem a substituição do arroz normalmente consumido por uma variedade mais nutritiva. As propriedades nutricionais destas novas variedades podem ser quantificadas através da determinação de seus componentes nutricionais. Desta forma, a justificativa para a realização deste projeto, é a necessidade de quantificar os compostos bioativos presentes em quatro variedades novas de arroz integral. Os compostos de interesse serão carotenóides, vitamina E, -oryzanol e ácidos graxos. Após quantificação dos compostos bioativos, será testado, in vitro, o efeito do -oryzanol, sobre a assimilação de lipídios, por células multipotentes diferenciadas em adipócitos.
Trata-se de estudo que visa obtenção de título acadêmico de Igor Otavio Minatel, sob a orientação das profas. Denise Fecchio e Camila Corrêa, do Depto de Patologia.

Objetivo da Pesquisa:
Quantificar isoladamente carotenoides, vitamina E, -oryzanol e os ácidos graxos presentes em

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quatro variedades de arroz integral colorido. Avaliar os efeitos do -oryzanol sobre a assimilação de lipídios, em células tronco mesenquimais derivadas de tecido adiposo e que serão diferenciadas em adipócitos.

Avaliação dos Riscos e Benefícios:

Sem riscos associados ao desenvolvimento do projeto.

A identificação dos componentes bioativos nas diferentes amostras de arroz, irá fornecer valores reais que permitem sugerir a substituição das variedades consumidas atualmente por novas variedades com maior poder nutricional. Não há na literatura estudos demonstrando o efeito do - oryzanol sobre assimilação de lipídios por adipócitos, ou mesmo, a concentração necessária deste composto para exercer atividade depletora de

lipídios. A demosntração dos possíveis efeitos deste composto podem beneficiar indivíduos com problemas de hiperlipidêmia.

Comentários e Considerações sobre a Pesquisa:

Pesquisa de bancada, com cultura padronizada de células. Não envolve sujeitos.

Dados avaliados por espectrometria de massa e HPLC.

As variedades de arroz utilizadas neste estudo serão: integral (Ilmie), integral "açucarado" (Danmi, - possui um conteúdo 30% maior de açúcar que o arroz integral Ilmie), integral vermelho (Geunkanghongmi) e integral preto (Milyang 263 – com embrião "gigante"). Todas as amostras serão provenientes do National Institute of Crop Science, Rural Development Administration, Milyang, Coréia do Sul.

As células tronco mesenquimais utilizadas neste estudo serão adquiridas junto ao American Type Culture Collection (ATCC).

Custeio estimado em R$ 30.000,00, sem referência à origem.

Considerações sobre os Termos de apresentação obrigatória:

Todas as autorizações necessárias são apresentadas. O pesquisador propõe dispensa do TCLE.

Recomendações:

Nenhuma em específico.

Conclusões ou Pendências e Lista de Inadequações:

Recomendo aprovação sem envio a CONEP.

Situação do Parecer:

Aprovado

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Necessita Apreciação da CONEP:
Não

Considerações Finais a critério do CEP:
Projeto de Pesquisa aprovado em reunião do CEP de 07 de Julho de 2.014, sem necessidade de envio à CONEP.

Ao Final deste projeto os pesquisadores devem encaminhar ao CEP o respectivo Relatório Final de Atividades.

BOTUCATU, 08 de Julho de 2014

Assinado por:
SILVANA ANDREA MOLINA LIMA
(Coordenador)

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Campus de Botucatu

Fls. .....
Proc. .....
Rub. .....

MUDANÇA DE TÍTULO EM PROJETO DE PESQUISA*

Objetivo Acadêmico: Tese de Doutorado

Título constante no parecer inicial de aprovação: **Caracterização e atividade funcional de compostos bioativos**

Título final: **Compostos bioativos em variedades de arroz integral - Caracterização, quantificação e estudo da atividade funcional em adipócitos diferenciados de células tronco mesenquimais**

Data da reunião do CEP que aprovou o parecer inicial: 07/07/2014

Declaramos que o trabalho não sofreu alterações nos objetivos e/ou conteúdo metodológico da época de apresentação para análise do CEP.

Denise Fecchio

Nome/assinatura do Orientador(a)

Igor Otavio Minatel

Nome/assinatura do Orientado(a)

13/01/2015 0000000 COMITE DE ETICA EM PESQUISA HC - UNESP

*** Projetos submetidos via plataforma brasil: preencher o formulário, digitalizar e postar no sistema Plataforma Brasil (vide instruções contidas no Of. 06/2014-CEP);**
*** Projetos submetidos anteriormente a Plataforma Brasil: preencher o formulário em duas vias e protocolar no CEP que emitiu o Parecer inicial de aprovação**