UNIVERSIDADE ESTADUAL DE LONDRINA

CÍNTIA LADEIRA HANDA

OTIMIZAÇÃO DA EXTRAÇÃO DE COMPOSTOS BIOATIVOS E PARÂMETROS DA FERMENTAÇÃO DA FARINHA DE SOJA COM *Aspergillus oryzae* OU *Monascus purpureus* E POTENCIAL ANTI-HIPERTENSIVO DE ALIMENTOS DE SOJA

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Tese apresentada ao Programa de Pós Graduação em Ciência de Alimentos da Universidade Estadual de Londrina como requisito parcial à obtenção do título de Doutora em Ciência de Alimentos.

Orientadora: Dra. Elza Iouko Ida.
Coorientadora: Dra. Sandra Regina Georgetti.

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Londrina, 04 de agosto de 2017.

Dedico

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HANDA, Cíntia Ladeira. **Otimização da extração de compostos bioativos e parâmetros da fermentação da farinha de soja com *Aspergillus oryzae* ou *Monascus purpureus* e potencial anti-hipertensivo de alimentos de soja.** 2017. 182 f. Tese (Doutorado em Ciência de Alimentos) – Universidade Estadual de Londrina, Londrina, 2017.

RESUMO

A soja é fonte de proteínas e óleo e contém compostos bioativos, como peptídeos, vitaminas e fenólicos, principalmente as isoflavonas que apresentam benefícios à saúde humana. Entre as diferentes formas de isoflavonas, as agliconas possuem maior atividade antioxidante e biodisponibilidade. A partir da soja são produzidos alimentos fermentados e não fermentados, sendo que a partir da extração do óleo de soja obtém-se como coproduto a farinha desengordurada de soja (FDS) que contém elevado teor de proteínas e compostos bioativos. A fermentação em estado sólido (FES) é uma estratégia para aumentar o potencial de utilização e valorização de coprodutos da agroindústria, pelo aumento da capacidade antioxidante e liberação de compostos bioativos. Assim, o objetivo deste trabalho foi otimizar a extração de isoflavonas e fenólicos totais das FDS fermentadas (FDSF) com *Monascus purpureus* (Mp) ou *Aspergillus oryzae* (Ao), investigar os efeitos dos parâmetros e do tempo de fermentação das FDSF-Mp e FDSF-Ao sobre os compostos bioativos e avaliar a capacidade anti-hipertensiva *in vitro* de vários alimentos de soja digeridos. A otimização da extração de isoflavonas e compostos fenólicos com atividade antioxidante das FDSF-Mp e FDSF-Ao foi realizada utilizando o delineamento de mistura simplex-centroide com multi-resposta. O efeito dos parâmetros de FES (pH inicial, água adicionada e temperatura de incubação) sobre o conteúdo fenólicos totais (CFT), isoflavonas e atividade antioxidante das FDSF-Mp e FDSF-Ao foi investigado utilizando o delineamento composto central rotacional, otimização multi-resposta e correlação de Pearson. O efeito do tempo de fermentação das FDSF-Mp e FDSF-Ao sobre a atividade da β-glucosidase, teor de isoflavonas, CFT, atividade antioxidante, teor de proteínas totais e açúcares solúveis foi avaliado por 7 dias. O potencial inibitório da enzima conversora de angiotensina (ECA) dos alimentos de soja digeridos *in vitro* (pepsina e pancreatina) foi avaliado a partir de extrato de soja, tofu, broto, iogurte de soja, tempeh, natto e em frações hidrolisadas (pepsina, tripsina e quimotripsina) de proteínas de soja 7S e 11S. A otimização multi-resposta indicou que o solvente extrator mais eficiente foi constituído por água e etanol (0,500:0,500, p/p). O pH inicial, água adicionada e temperatura de incubação da FDSF-Mp influenciaram no teor das diferentes formas de isoflavonas e não apresentaram efeitos sobre o CFT e atividade antioxidante (DPPH e ABTS). Enquanto que para a FDSF-Ao, a água adicionada e temperatura de incubação influenciaram no teor de todas as formas de isoflavonas, CFT e atividade antioxidante (DPPH, FRAP e ABTS). A FES com Mp ou Ao favoreceu a formação de compostos bioativos como as isoflavonas agliconas. O tempo de fermentação da FDSF-Mp e FDSF-Ao influenciou a atividade de β- glucosidase, teor de diferentes formas de isoflavonas, CFT, atividade antioxidante, teor de proteínas totais e açúcares solúveis. Maior potencial de inibição da ECA foi observado no extrato de soja. Natto e iogurte de soja apresentaram maior inibição da ECA do que o tempeh. Brotos germinados por três dias apresentaram maior inibição da ECA do

que os germinados por 5 ou 7 dias. A fração hidrolisada de 11S exibiu maior inibição da ECA do que a 7S e os peptídeos de 1-4,5 kDa apresentaram maior inibição da ECA.

Palavras-chave: Isoflavonas agliconas. Fermentação. Compostos fenólicos. Compostos bioativos. Antioxidante. Anti-hipertensivo .

HANDA, Cíntia Ladeira. **Optimization of bioactive compounds extraction and fermentation parameters of soy flour by *Aspergillus oryzae* or *Monascus purpureus* and antihypertensive potential of soy foods.** 2017. 182 p. Thesis (Doctoral degree in Food Science) – Universidade Estadual de Londrina, Londrina, 2017.

ABSTRACT

Soybean is source of protein and oil, containing bioactive compounds such as peptides, vitamins and phenolics, especially isoflavones known for provide health benefits. Among the different isoflavone forms, the aglycones have higher antioxidant activity and bioavailability. Fermented and non-fermented foods are produced from soybeans. The defatted soybean flour (DSF), which contains high content of proteins and bioactive compounds, is obtained as a co-product of soybean oil extraction. The solid-state fermentation (SSF) is a strategy to increase the potential of utilization and valorization of agro-industry co-products, by increasing the antioxidant capacity and releasing bioactive compounds. The objective of this work was to optimize the extraction of isoflavones and total phenolics from the DSF fermented (DSFF) by *Monascus purpureus* (Mp) or *Aspergillus oryzae* (Ao); to investigate the effects of parameters and fermentation time of DSFF-Mp and DSFF-Ao on bioactive compounds, and to assess the *in vitro* antihypertensive capacity of various digested soy foods. The optimization of the extraction of isoflavones and phenolic compounds with the antioxidant activity of the DSFF-Mp and DSFF-Ao was carried out using the simplexcentroid mixture design with multi-response optimization. The effect of the SSF parameters (initial pH, added water and incubation temperature) on the total phenolic content (TPC), isoflavones and antioxidant activity of the DSFF-Mp and DSFF-Ao was investigated using central composite rotational design, multi-response optimization and Pearson's correlation. The effect of fermentation time of DSFF-Mp and DSFF-Ao on β-glucosidase activity, isoflavone content, TPC, antioxidant capacity, total protein and soluble sugars content was evaluated for 7 days. The inhibitory potential of the angiotensin converting enzyme (ACE) of the *in vitro* digested (pepsin and pancreatin) soy foods was evaluated from soymilk, tofu, sprout, soy yogurt, tempeh, natto and from hydrolysed (pepsin, trypsin and chymotrypsin) soy proteins fractions 7S and 11S. The multi-response optimization indicated that the most efficient solvent extractor consisted of water and ethanol (0.500:0.500, w/w). The initial pH, addition of water and incubation temperature of DSFF-Mp influenced the content of different forms of isoflavones and had no effect on the TPC and antioxidant activity (DPPH and ABTS). While for DSFFAo, the addition of water and incubation temperature influenced the content of all isoflavone forms, TPC and antioxidant activity (DPPH, FRAP, and ABTS). FES with Mp or Ao favoured the formation of bioactive compounds such as aglycone isoflavones. The fermentation time of DSFF-Mp and DSFF-Ao influenced the β-glucosidase activity, content of different forms of isoflavones and TPC, antioxidant activity, total protein and soluble sugars content. The highest potential for ACE inhibition was observed in soymilk. Natto and soy yogurt showed greater inhibition of ACE than tempeh. Sprouts germinated for three days showed greater inhibition of ACE than those germinated for 5 or 7 days. The hydrolysed fraction of 11S exhibited greater inhibition of ACE than 7S and the peptides of 1-4.5 kDa showed greater inhibition of ACE.

Keywords: Aglycone isoflavones. Fermentation. Phenolic compounds. Bioactive compounds. Antioxidant. Antihypertensive.

SUMÁRIO

1 INTRODUÇÃO

A soja é a oleaginosa mais importante do mundo, sendo fonte de óleo e proteína de alta qualidade, além de conter isoflavonas, diversos ácidos fenólicos e outros flavonoides (MESSINA, 2014) considerados como compostos bioativos. Diferentes tecnologias, como físicas, químicas, biológicas ou uma combinação destas têm sido utilizadas para produção de vários alimentos de soja tradicionais, tais como, o extrato de soja, tofu, broto de soja, natto, tempeh e iogurte de soja.

No Brasil, a soja é principalmente processada para obtenção de óleo, com geração do farelo e farinha desengordurada de soja (FDS). As proteínas da FDS podem ser extraídas para produção de concentrados e isolados proteicos que podem ser adicionados em produtos cárneos, panificação, molhos, massas, bebidas, sopas e alimentos dietéticos (BARNES, 2010; PAN; TANGRATANAVALEE, 2003). Uma grande quantidade de FDS é produzida durante a extração de óleo de soja (CHEN et al., 2013; HASSAAN; SOLTAN; ABDEL-MOEZ, 2015; JONG, 2007; MUTTAKIN; KIM; LEE, 2015).

A fermentação em estado sólido (FES) é considerada uma estratégia efetiva de valorização e utilização de coprodutos da agroindústria, uma vez que promove a liberação de compostos com atividade antioxidante pelo rompimento do vínculo entre fenólicos e outros substituintes em moléculas conjugadas (DULF; VODNAR; SOCACIU, 2016; MARTINS et al., 2011; ZHANG et al., 2017), sendo que na FES os fungos são os micro-organismos mais utilizados (RAIMBAULT, 1998). As mudanças na composição do substrato durante a fermentação ocorrem principalmente devido a produção microbiana de enzimas como amilases, xilanases, glucosilases e proteases (ABD RAZAK et al., 2015).

As alterações na composição do substrato e a produção de compostos de interesse durante a FES dependem fortemente do tipo de substrato, do fungo utilizado e da condição de fermentação (MARTINS et al., 2011). Assim, para aumentar o rendimento do processo de FES é importante otimizar as condições de fermentação estabelecendo adequadamente os parâmetros físicos e químicos (FRANCIS et al., 2003). E a etapa de extração dos produtos desejados é muito importante em FES, sendo que o rendimento da extração é influenciado pelo tipo de

solvente e sua concentração, composição do substrato fermentado, proporção de sólido e solvente e pH (MARTINS et al., 2011).

As práticas de processamento de alimentos de soja empregadas não só afetam a qualidade sensorial, mas também as propriedades nutritivas e funcionais. Os compostos bioativos como peptídeos e compostos fenólicos podem ser liberados ou degradados durante o processamento de alimentos e/ou na digestão gastrointestinal e geralmente apresentam natureza multifuncional, incluindo o potencial antioxidante, imunomodulador, antimicrobiano, antitrombótico, hipocolesterolêmico e anti-hipertensivo (ERDMANN; CHEUNG; SCHRÖDER, 2008; HERNÁNDEZ-LEDESMA et al., 2014).

Os peptídeos com atividade anti-hipertensiva foram caracterizados a partir de hidrolisados proteicos de soja (GIBBS et al., 2004), produtos fermentados (GIBBS et al., 2004; LI et al., 2013) e não fermentados de soja (ALAUDDIN et al., 2015; CAPRIOTTI et al., 2015; PUCHALSKA; MARINA; GARCÍA, 2014; TOMATSU et al., 2013). No entanto, a avaliação da atividade anti-hipertensiva da soja e seus derivados após a digestão simulada ainda não foi caracterizada e pode trazer uma nova compreensão e importância prática para a promoção da saúde, uma vez que os peptídeos anti-hipertensivos devem permanecer ativos na digestão e absorção gastrointestinal e atingir o sistema cardiovascular (GU; WU, 2013).

Durante a fermentação da soja, as isoflavonas glicosiladas podem ser hidrolisadas para as respectivas formas agliconas. Além disso, o processo de fermentação também pode reduzir o teor de oligossacarídeos rafinose e estaquiose, que podem causar flatulência em humanos (MA et al., 2016; WANG et al., 2014).

Dessa forma, o objetivo deste trabalho foi otimizar a extração de isoflavonas e fenólicos totais das farinhas desengorduradas de soja fermentadas com *Monascus purpureus* ou *Aspergillus oryzae,* investigar os efeitos dos parâmetros e do tempo de fermentação das farinhas desengorduradas de soja fermentadas com estes fungos sobre os compostos bioativos e avaliar a capacidade anti-hipertensiva *in vitro* de vários alimentos de soja digeridos.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Otimizar a extração de isoflavonas e fenólicos totais das farinhas desengorduradas de soja fermentadas com *Monascus purpureus* ou *Aspergillus oryzae,* investigar os efeitos dos parâmetros e do tempo de fermentação sobre os compostos bioativos e avaliar a capacidade anti-hipertensiva *in vitro* de vários alimentos de soja digeridos.

2.2 OBJETIVOS ESPECÍFICOS

- Otimizar a extração de isoflavonas e compostos fenólicos com atividade antioxidante de farinhas desengorduradas de soja fermentadas com *Monascus purpureus* ou *Aspergillus oryzae* utilizando o delineamento de mistura simplex-centroide com multi-resposta.
- Investigar os efeitos dos parâmetros de fermentação em estado sólido (pH inicial da FDS, água adicionada em 10 g de FDS e temperatura de incubação) das farinhas desengorduradas de soja fermentadas com *Monascus purpureus* ou *Aspergillus oryzae* sobre o teor de isoflavonas, fenólicos totais e atividade antioxidante (DPPH, ABTS e FRAP) utilizando o delineamento composto central rotacional, a otimização multi-resposta e a correlação de Pearson.
- Avaliar o efeito do tempo de fermentação das farinhas desengorduradas de soja fermentadas com *Monascus purpureus* ou *Aspergillus oryzae* sobre a atividade da β-glucosidase, teor de isoflavonas, fenólicos totais, capacidade antioxidante, teor de proteínas totais e açúcares solúveis.
- Avaliar o potencial inibitório da enzima conversora de angiotensina (ECA) de vários alimentos de soja digeridos e de frações hidrolisadas de proteínas de soja 7S e 11S.

3 REVISÃO BIBLIOGRÁFICA

3.1 Origem, Aspectos Econômicos e Composição Química da Soja

A soja [*Glycine max* (L.) Merrill] teve origem no continente asiático (GOLBITZ; JORDAN, 2006). É uma das culturas agrícolas mais antigas e principais do extremo oriente e desde o início de sua civilização os orientais consumiam a soja como principal fonte de proteína e óleo (LULE et al., 2015).

Foi introduzida no Brasil em 1882 no estado da Bahia por Gustavo D`utra. No Paraná, o cultivo da soja ocorreu em 1936, mas somente em 1955 o seu cultivo foi efetivado e recomendado para minimizar as consequências da geada nos cafezais. Em 1949, o Brasil participou pela primeira vez nas estatísticas internacionais como produtor de soja. Sendo que, em 1942, os Estados Unidos já ocupavam o primeiro lugar na produção mundial (BONATO; BONATO, 1987), posição na qual se mantém até hoje. Atualmente, o Brasil é o segundo produtor mundial de soja, cuja produção concentra-se na Região Centro-Oeste e Região Sul que juntas, correspondem a 80,0% da produção brasileira (CONAB, 2017).

A soja tem um papel importante no atual desenvolvimento da economia brasileira, gerando 1,5 milhões de empregos em 17 Estados (Associação Brasileira das Indústrias de Óleos Vegetais - ABIOVE, 2017). Na safra 2016/17 a produção brasileira está estimada em 113,92 milhões de toneladas, totalizando 33.889,9 mil hectares de área plantada, com produtividade prevista de 3.362 kg/ha (CONAB, 2017). Entretanto, a produção mundial está estimada em 351,31 milhões de toneladas e os Estados Unidos, Brasil e Argentina são responsáveis por 82,0% da produção mundial da soja em grão (USDA, 2017). Com o aumento na produção de soja, o Brasil ampliou suas exportações em quase 4 milhões de toneladas, alcançando o recorde de exportação de 34,8 milhões de toneladas entre janeiro e maio de 2017, representando 41,2% do valor total exportado pelo Brasil como produtos do agronegócio (MAPA, 2017).

A soja tornou-se um importante produto agrícola devido à sua capacidade de adaptação geográfica, composição química única como leguminosa, elevado valor nutritivo e aos benefícios funcionais tanto para a saúde como para finalidade de elaboração de produtos industriais (CIABOTTI et al., 2006). O grão de

soja convencional possui composição química quase completa, incluindo proteínas, lipídeos, carboidratos e diversos minerais. É uma leguminosa cujo grão é constituído de 90,0% de cotilédones, 2,0-3,0% de hipocótilo e 6,0% de tegumento (TSUKAMOTO et al., 2001; ALI, 2010). A composição química da soja depende de fatores genéticos e ambientais, maturidade da planta e condições de plantio (MEDIC; ATKINSON; HURBURGH, 2014). Contém, em base úmida, 35,0 a 40,0% de proteínas, 15,0 a 20,0% de gordura, 30,0% de carboidratos, 10,0 a 13,0% de umidade e 5,0% de minerais e cinzas (GOLBITZ; JORDAN, 2006; LIU, 1997).

Os carboidratos da soja são constituídos por duas frações: carboidratos solúveis (não estruturais) e insolúveis (estruturais). Os carboidratos insolúveis, ou fibras alimentares, provêm principalmente da tegumento e parede celular e são compostos de celulose, hemicelulose e pectina (GOLBITZ; JORDAN, 2006). Os principais carboidratos ou açúcares solúveis presentes na soja são: sacarose (>55,0%), estaquiose (>30,0%), rafinose (7,0-8,0%) e verbascose (1,0-2,0%) (FAN; ZANG; XING, 2015; HOU et al., 2009). A rafinose, estaquiose e verbascose contém uma, duas e três moléculas de galactose, respectivamente, ligadas a uma molécula de sacarose via ligação glicosídica α1→6 e pertencem à família de oligossacarídeos de rafinose (RFOs) (HAGELY; PALMQUIST; BILYEU, 2013; MEDIC; ATKINSON; HURBURGH, 2014). Esses oligossacarídeos representam 5,0% da matéria seca da soja (CHEN; VADLANI; MADL, 2014). Os RFOs não são digeridos ou utilizados como nutrientes diretamente pelo humano, mas são utilizados como nutrientes pelas bifidobactérias no intestino delgado. Dessa forma, são considerados potenciais prebióticos que podem ser utilizados para melhorar a função imune no organismo humano (GOLBITZ; JORDAN, 2006; MA et al., 2016; ŠVEJSTIL; MUSILOVÁ; RADA, 2015). No entanto, quando as bactérias hidrolisam esses açúcares, há formação de gás no intestino fazendo com que os RFOs sejam descritos como os responsáveis pela flatulência após a ingestão de soja e outras leguminosas, o que tem restringido a sua aceitação pelo consumidor (DE FÁTIMA VIANA et al., 2005; HAGELY; PALMQUIST; BILYEU, 2013; VIANA et al., 2007). Em alimentos de soja fermentados, como tempeh e natto, a concentração de RFOs é baixa devido à hidrólise realizada pela enzima α-galactosidase secretada pelos micro-oganismos (KUMAR et al., 2010).

Os lipídeos de armazenamento da soja são encontrados principalmente na forma de triacilglicerídeos. Uma molécula de triacilglicerídeo é composta de três moléculas de ácidos graxos, podendo possuir cadeia saturada e insaturada esterificados em um esqueleto de glicerol. Dentre os lipídeos da soja, o ácido graxo linoleico (18:2) é o mais abundante e representa 50,0-55,0% do conteúdo total dos ácidos graxos, seguido dos ácidos oleico (18:1), 23,0-25,0%; palmítico (16:0) 10,0-11,0%; linolênico (18:3), 6,0-9,0% e esteárico (18:0), 4,0-6,0% (MEDIC; ATKINSON; HURBURGH, 2014; MIHAIL; ZORAN, 2011). O óleo de soja tem um alto valor nutritivo porque é uma fonte rica de ácidos graxos insaturados, como o ácido oleico, ácido linoleico e ácido linolênico, sendo estes dois últimos os ácidos graxos considerados essenciais, pois não são produzidos pelo organismo humano. No entanto, o número de insaturações está diretamente relacionado à instabilidade do óleo. Assim, o elevado teor de ácidos graxos insaturados faz com que o óleo de soja seja relativamente instável e propenso à oxidação e ao desenvolvimento do sabor indesejável (MEDIC; ATKINSON; HURBURGH, 2014; LI, 2006).

As principais proteínas de soja são as globulinas que podem ser classificadas de acordo com os seus coeficientes de sedimentação da centrifugação e cujas frações são denominadas de 2S, 7S, 11S e 15S. As frações 7S (β-conglicinina) e 11S (glicinina) são os principais componentes das proteínas de soja e representam 40,0% e 30,0% do total de proteínas no grão, respectivamente (XU et al. 2011). A glicinina (11S) é um hexâmero com uma massa molar entre 320-380 kDa, composta por subunidades de polipeptídeos ácidos (A1a, A1b, A2, A3, A4, A5) e básicos (B1a, B1b, B2, B3, B4) ligados via ligação dissulfeto e não são glicosiladas (FUKUSHIMA, 2001; MARUYAMA et al., 2003). A β-conglicinina (7S) é uma glicoproteína trimérica contendo 4,0% de carboidratos, massa molar de 180 kDa e constituída por três subunidades (α’, α e β) associadas por ligações hidrofóbicas e de hidrogênio (MARUYAMA et al., 2001; THANH; SHIBASAKI, 1978).

As proteínas da soja apresentam vantagens nutricionais, funcionais e tecnológicas, tais como: i) proporcionam um bom equilíbrio na composição de aminoácidos e contém todos os aminoácidos essenciais, ii) possuem componentes fisiologicamente benéficos que têm sido relacionados com a redução do colesterol e do risco de hiperlipidemia e doenças cardiovasculares, iii) apresentam excelentes propriedades tecnológicas como gelificação, emulsão e capacidade de retenção de

óleo e água (NISHINARI et al., 2014). Os produtos proteicos comerciais à base de soja estão disponíveis em três formas principais, conforme o seu teor de proteínas: FDS (50,0-59,0% de proteínas), concentrado de proteínas de soja (65,0-72,0% de proteínas) e isolado de proteínas de soja (>90,0% de proteínas) (XU et al., 2011). A FDS é um produto gerado na extração do óleo de soja que tem sido utilizada para alimentação animal ou como fonte de proteínas (VILLALOBOS et al., 2016). Sua obtenção envolve a trituração fina de flocos de soja desengordurados resultantes do esmagamento do grão em moinho de rolo seguido da extração do óleo com solvente e posterior remoção do solvente residual por evaporação (ALI, 2010). Assim, a FDS tem maior teor de proteína em comparação à farinha de soja integral contendo 54,0% de proteínas, 0,5 a 1,0% de lipídeos, 17,0 a 18,0% de fibra alimentar e 30,0 a 35,0% de carboidratos totais, além de apresentar maior teor de isoflavonas do que o grão de soja ou produtos derivados (GOLBITZ; JORDAN, 2006). A farinha desengordurada de soja comercial apresenta predominância de isoflavonas β-glicosídeos e malonil-β-glicosídeos e são similares aos grãos de soja (GENOVESE et al., 2007). A farinha de soja tem sido utilizada como ingrediente para uma variedade de produtos alimentícios incluindo sopas, bebidas, sobremesas, produtos de panificação, cereais matinais e produtos cárneos (DURAZZO; GABRIELLI; MANZI, 2015; HETTIRACHCHY; KALAPATH, 1997; XU et al., 2011).

3.2 A SOJA COMO ALIMENTO

A utilização de soja como alimento para consumo humano varia conforme o país. Na Ásia, a soja tem sido utilizada para produção de alimentos tradicionais como o extrato de soja, tofu e produtos fermentados, enquanto que nos países ocidentais a soja tem sido mais utilizada como ingrediente no processamento de alimentos, como óleo e as proteínas de soja (HYMOWITZ, 2008; RIAZ, 2006). A fabricação dos vários alimentos de soja, fermentados e não fermentados, pode envolver a utilização de várias etapas de processamento como limpeza, hidratação, trituração, filtração, tratamento térmico, coagulação, fermentação, germinação etc. Todas essas etapas, individualmente ou em combinação, podem causar mudanças físicas, químicas e nutricionais nos produtos finais. Os alimentos de soja não

fermentados incluem o extrato de soja, tofu, broto e outros, enquanto que os fermentados incluem principalmente natto, tempeh, iogurte de soja entre outros.

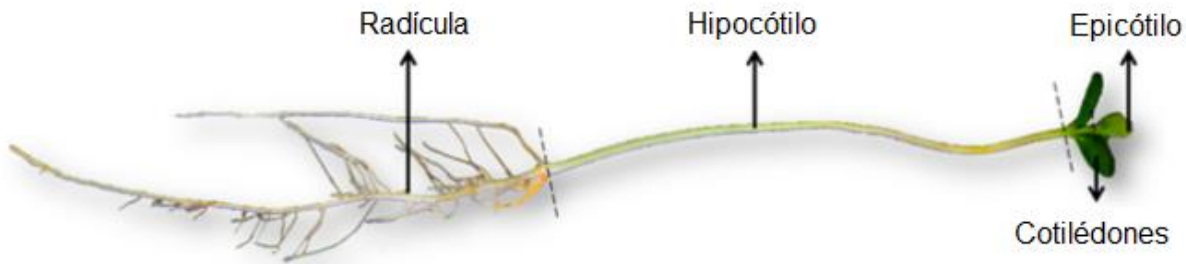
O extrato de soja se assemelha ao leite de vaca na aparência física e as seguintes etapas são utilizadas para fabricação: seleção do grão, maceração e moagem úmida, separação do extrato aquoso com obtenção do resíduo denominado de okara (fibra), cozimento para inativar as enzimas lipoxigenases e inibidores de tripsina, formulação e embalagem (GIRI; MANGARAJ, 2012). Para produção do extrato de soja, no método chinês o cozimento é realizado após a filtração, enquanto que no método japonês o cozimento é realizado antes da trituração ou antes da filtração (VONG; LIU, 2016). O extrato de soja possui 3,0% de proteínas, 2,0% de lipídeos e 2,0% de carboidratos que são constituídos principalmente por sacarose, rafinose e estaquiose (PENG et al., 2017).

Para produzir o tofu utiliza-se o extrato de soja no qual se adiciona um agente coagulante, sendo o *nigari* o coagulante tradicional usado no Japão que contém principalmente o cloreto de magnésio. Também podem ser utilizados como agentes coagulante o cloreto de cálcio, sulfato de cálcio, glucona-delta lactona, suco de limão ou vinagre. Para obtenção do tofu prensado, o coalho é cortado e depois pressionado, produz-se o soro, o conteúdo de água no tofu é reduzido e a textura torna-se firme. Assim, a quantidade de soro formado influencia na firmeza do tofu (LIU, 1997; USDA, 2013). O tofu prensado pode apresentar vários graus de firmeza, tais como *soft*, regular, firme, extra firme e outros que refletem a quantidade de água pressionada do coalho formado. O “filled” ou tofu seda é um tipo de tofu não prensado, sem produção de soro e o processo de coagulação das proteínas para formar o coalho ocorre na embalagem (GOLBITZ; JORDAN, 2006; LIU; CHANG, 2004). O tofu típico contém entre 10,0 e 15,0% de proteínas e entre 5,0 a 9,0% de gordura e baixo conteúdo de carboidratos e fibras, podendo variar dependendo da perda de soro (GOLBITZ; JORDAN, 2006).

Os brotos de soja são obtidos a partir da soja germinada e são constituídos pela radícula, hipocótilo, epicótilo e cotilédones (Figura 1). Os brotos de soja são consumidos frescos e apresentam crocância e seu processo de produção inclui assepsia dos grãos, maceração em água e germinação, sendo que o tempo de germinação depende da finalidade e do seu consumo. Os brotos de soja devem ser lavados e o tegumento é removido e são consumidos crus ou preparados na

elaboração de sopas ou saladas (GAN et al., 2017; GOLBITZ; JORDAN, 2006; KUMARI; CHANG, 2016; LIU, 1997). Os brotos de soja germinados por 7 dias apresentam um aumento de 4,0% no teor de proteínas e uma redução de 5,0-6,0% no teor de carboidratos e lipídeos (SHI; NAM; MA, 2010). Além disso, germinação da soja afeta o teor de isoflavonas totais e de suas diferentes formas. Essas mudanças no teor e perfil de isoflavonas dependem do estágio de germinação e do metabolismo fisiológico das sementes, sendo que o teor total de isoflavonas aumenta nos cotilédones e diminuiu nas radículas com o processo de germinação (RIBEIRO et al., 2006). Em brotos germinados por 168 h, as isoflavonas totais apresentam maior concentração nos cotilédones e as agliconas são encontradas principalmente no hipocótilo e na radícula (QUINHONE JÚNIOR; IDA, 2014).

Figura 1 - Componentes de sementes de soja germinadas (broto)



Fonte: Quinhone Júnior e Ida, (2014)

O natto é um alimento de soja fermentado por bactéria, cuja produção envolve a lavagem dos grãos, maceração, cozimento a vapor, drenagem, arrefecimento, inoculação com *B. natto*, incubação por 16-20 h à 38 °C e maturação por 1-2 dias a temperatura de refrigeração (LIU, 1997). O aroma, sabor e a textura viscosa de natto são considerados como um desafio quanto a sua aceitação pelos consumidores de países ocidentais (HE; CHEN, 2013).

O tempeh é produzido por fermentação de soja macerada, cozida e descascada no qual utiliza o *Rhizopus oligosporus* à temperatura ambiente até que os grãos estejam unidos por micélio branco. Pode ser cozido, grelhado ou frito (GOLBITZ; JORDAN, 2006; LIU, 1997) Os tempos de maceração, cozimento e fermentação alteram o teor e distribuição das diferentes formas de isoflavonas no tempeh. Durante a etapa de fermentação ocorre bioconversão das isoflavonas glicosiladas em agliconas. As condições de produção recomendadas para obtenção

de um tempeh com maior teor de isoflavonas agliconas são: maceração por 6 h, cozimento dos cotilédones por 15 min e tempo de fermentação de 18 h (BORGES et al., 2016).

O iogurte de soja é obtido a partir da fermentação do extrato de soja pasteurizado e inoculação com bactérias produtoras de ácido láctico (GOLBITZ; JORDAN, 2006; HE; CHEN, 2013; LIU, 1997). Na fermentação, ocorre a hidrólise das proteínas do extrato de soja, redução do pH, aumento da viscosidade e produção de metabólitos bacterianos que contribuem com o sabor (FARNWORTH et al., 2007).

O consumo direto da soja na alimentação humana ainda é baixo no ocidente, no entanto, os seus efeitos benéficos têm sido divulgados amplamente e impulsionado o consumo de produtos à base de soja (REETZ et al., 2012). Os alimentos de soja, principalmente os fermentados, têm apresentado contribuições significativas para a saúde e bem-estar do humano. No entanto, há necessidade de mais investigações sobre a segurança dos micro-organismos, análise econômica das inovações no setor, regulamentos governamentais como rotulagem e experimentos clínicos que identifiquem os principais benefícios destes alimentos de soja fermentados (CHEN et al., 2012).

3.3 Benefícios dos Compostos Bioativos da Soja

Os compostos bioativos de alimentos são componentes naturais que possuem atividade biológica e, em alguns casos, apresentam valor nutritivo. Estes quando consumidos desempenham um papel importante na saúde, crescimento e desenvolvimento humano, além de contribuir na redução dos riscos de certos tipos de doenças (LAGOS et al., 2015).

A soja é conhecida principalmente devido ao seu alto teor de proteína e óleo. No entanto, há um crescente interesse entre pesquisadores de diversas áreas, como a ciência de alimentos, nutrição, saúde e medicina, que procuram elucidar os benefícios da soja como alimento para a saúde do humano. Entre os vários componentes da soja, as proteínas, os peptídeos bioativos, os compostos fenólicos como principalmente as isoflavonas são os mais investigados devido aos seus efeitos benéficos à saúde humana (GU; WU, 2013; NGUYEN et al., 2016; MAMILLA; MISHRA, 2017; KUMARI; CHANG, 2016; XU; CHANG, 2008).

3.3.1 Peptídeos Bioativos

Os peptídeos na sequência da proteína original de origem animal ou vegetal são inativos. No entanto, os peptídeos tornam-se bioativos, quando liberados após hidrólise, processamento de alimentos, digestão gastrointestinal ou proteólise microbiana. Estes peptídeos apresentam cadeias contendo de 2 a 20 aminoácidos e sua bioatividade depende do comprimento da cadeia, composição e sequência de aminoácidos. Os benefícios desses peptídeos para a saúde humana podem ser diversos, tais como anti-hipertensivos, antioxidantes, antiobesidade, imunomoduladores, antidiabéticos, hipocolesterolêmicos e anticancerígenos (SANJUKTA; RAI, 2016; SINGH; VIJ; HATI, 2014).

A hipertensão é diagnosticada quando as pressões arteriais sistólica e diastólica são maiores do que 140 e 90 mmHg, respectivamente. O sistema renina-angiotensina (SRA) é a principal via que leva à contração do vaso sanguíneo. Durante a regulação normal da pressão arterial, a enzima renina converte o angiotensinogênio em um fragmento peptídico inativo denominado de angiotensina I. A enzima conversora de angiotensina (ECA) (peptidil-dipeptidase, E.C. 3.4.15.1) converte a angiotensina I em angiotensina II, que se liga aos receptores da parede vascular para causar contrações dos vasos sanguíneos. No entanto, durante os distúrbios metabólicos, a atuação excessiva do SRA induz a elevação do nível de angiotensina II, que é a principal causa de hipertensão arterial. Além disso, a ECA também contribui para a hipertensão por meio da degradação e inativação da bradicinina que é um vasodilatador (ALUKO, 2015).

Um dos mecanismos mais estudados para verificar a atividade anti-hipertensiva dos peptídeos bioativos é por inibição da ECA (BARBANA; BOYE, 2011; SANJUKTA; RAI, 2016). Os peptídeos inibidores de ECA são vistos como uma alternativa às drogas sintéticas devido ao crescente interesse por segurança, economia e redução de efeitos colaterais (SINGH; VIJ; HATI, 2014). Dessa forma, os hidrolisados de proteínas de soja e produtos de soja fermentados e não fermentados têm sido investigados devido ao efeito anti-hipertensivo por inibir a atividade da ECA.

A capacidade de inibição da ECA, geralmente é expressa pelo valor de IC_{50}, que indica a concentração necessária para inibir em 50,0% a atividade da

ECA. O baixo valor de IC_{50} indica uma atividade inibitória maior da ação da ECA, pois necessita um teor de peptídeos menor para inibir 50,0% da atividade da ECA (MARGATAN et al., 2013).

A hidrólise enzimática de proteínas da soja tem sido utilizada como uma estratégia para obtenção de peptídeos que inibem a ECA. No entanto, a atividade de inibição da ECA pelos hidrolisados proteicos dependem de vários fatores, tais como, o tipo e concentração da enzima, tempo de hidrólise e hidrólise sequencial por diferentes enzimas. As frações de proteínas de soja, β-conglicinina e glicinina, hidrolisadas com uma protease ácida de *Monascus purpureus* apresentaram valores de IC_{50} de 0,126 e 0,148 mg/mL, respectivamente (KUBA et al., 2005). Os hidrolisados de isolado proteico de soja (IPS) e fração de β-conglicinina obtidos com papaína apresentaram o valor de IC_{50} de 0,177 e 0,170 mg/mL, respectivamente. Estes valores foram o dobro (0,361 e 0,588 mg/mL) da atividade inibidora da ECA quando comparado com os hidrolisados obtidos com pepsina de IPS e fração de glicinina, respectivamente (MARGATAN et al., 2013).

Os IPS hidrolisados obtidos com alcalase (endopeptidase alcalina) produzida por *Bacillus licheniformis* apresentaram atividade inibitória de ECA que variaram de 33,6% (1,0 UA, 30 min) a 66,6% (1,0 UA, 120 min). A atividade inibitória da ECA do IPS hidrolisado aumentou nos primeiros 120 minutos (66,60%) e diminuiu para 40,4% após 1440 min de incubação, sendo que os valores de IC_{50} foram de 30,40 e 62,30 mg/mL, respectivamente. O longo tempo de hidrólise pode causar uma degradação excessiva das proteínas e consequente redução da atividade inibitória da ACE (NGUYEN et al., 2016).

O hidrolisado de proteínas de soja obtido com termolisina apresentou inicialmente um valor de IC_{50} de 53,60 µg/mL, após a adição de pepsina diminuiu para 51,80 µg/mL e após a adição de tripsina aumentou para 115,60 µg/mL. Estes resultados indicaram que a posterior digestão com tripsina (enzima gástrica) influenciou a atividade inibitória da ECA de hidrolisados de proteínas de soja (GU; WU, 2013).

O extrato de soja após tratamento com proteinase PROTIN SD-NY10 (produzida por *Bacillus amyloliquefaciens*) apresentou maior atividade inibitória da ECA e foi dose dependente, ou seja, extrato de soja com 0,0% de SD-NY10, o valor de IC_{50} foi igual a 8,75 µg/mL; 0,01% SD-NY10, o valor de IC_{50} foi igual a 1,55 µg/mL;

0,1% de SD-NY10, o valor de IC_{50} foi igual a 0,26 µg/mL; e 1,0% de SD-NY10, o valor de IC_{50} foi igual a 0,22 µg/mL (TOMATSU et al., 2013). Em estudos *in vivo* com ratos espontaneamente hipertensos (REH), via administração oral, o extrato de soja hidrolisado, também tratado com PROTIN SD-NY10 (0,05%, m/m; por 16 h a 50-55 °C), apresentou uma redução na pressão sanguínea dos REH que foi atribuída à inibição da atividade da ECA e consequente diminuição da angiotensina II (ALAUDDIN et al., 2015).

Os peptídeos extraídos do grão de soja e de brotos germinados na ausência de luz por 5 dias a 30 e 40 °C, apresentaram valores de IC_{50} de 0,174; 0,098 e 0,025 mg/mL, respectivamente. Portanto, observou-se que no processo de germinação da soja ocorreu a promoção e liberação de peptídeos de baixa massa molar com atividade inibitória da ECA, via ativação de proteinases naturais (MAMILLA; MISHRA, 2017).

Os produtos fermentados de soja também apresentam atividade inibitória da ECA. Os produtos tradicionais de soja fermentados, como o natto e tempeh apresentaram atividade de inibição da ECA com IC_{50} de 0,100 e 0,510 mg/mL, respectivamente (OKAMOTO et al., 1995). Segundo Ibe et al. (2009), o natto apresentou IC_{50} de 0,270 mg/mL. Neste estudo o inibidor de ECA foi extraído do natto, purificado parcialmente e administrado por via oral com dose única (1 mg, 10 mg e 100 mg/kg de peso corporal) em REH. A pressão arterial foi medida a cada hora e até 5 h após a administração. Os resultados indicaram que mesmo na dose mais baixa, o inibidor de ECA apresentou uma diminuição significativa da pressão arterial após 4 h de administração.

Os extratos de soja fermentados com lactobacilos (*L. acidophilus* BT 1088, *L. fermentum* BT 8219, *L. acidophilus* FTDC 8633 e *L. gasseri* FTDC 8131) a 37 °C por 24 h apresentaram IC_{50} de 2,110; 2,740; 1,010 e 1,510 mg/mL, respectivamente. Estes resultados evidenciaram que o aumento de atividade inibitória da ECA de extratos de soja fermentados foi influenciado pelo tipo de micro-organismo utilizado (EWE et al., 2011).

Embora, o processamento de soja possa melhorar a capacidade inibitória da ECA pela liberação de peptídeos, é importante ressaltar que para o peptídeo exercer qualquer função no organismo, há necessidade de passar intacto ou ser liberado no trato gastrointestinal, pois somente poderá exercer um efeito benéfico

quando absorvido no intestino humano (PUCHALSKA, MARINA, & GARCÍA, 2014). Assim, a hidrólise gastrointestinal é de particular importância na biodisponibilidade destes peptídeos inibidores da ECA, pois após a ingestão as enzimas gastrointestinais podem hidrolisar os peptídeos, aumentando ou diminuindo a sua atividade (BARBANA; BOYE, 2011).

3.3.2 Compostos Fenólicos e Isoflavonas

Os compostos fenólicos estão presentes em frutas e alguns vegetais comestíveis, incluindo oleaginosas como soja, canola e semente de linhaça que são utilizados como alimentos ou fontes de ingredientes de alimentos. No entanto, os tipos, quantidades e propriedades de compostos fenólicos presentes nestes alimentos e seus derivados variam consideravelmente (ESCARPA; GONZÁLEZ, 2001; SIGER; NOGALA-KALUCKA; LAMPART-SZCZAPA, 2008).

A dieta rica em compostos fenólicos tem sido relacionada com efeitos benéficos à saúde humana. Em abril de 2016, o governo japonês aprovou o uso de compostos fenólicos como ingredientes funcionais de 7 das 10 categorias de alimentos para uso específico na saúde. Esta aprovação reconhece que os compostos fenólicos podem ser utilizados como ingredientes com potencial para o desenvolvimento de produtos alimentícios funcionais (SHIMIZU, 2017). Os compostos fenólicos são capazes de inibir o crescimento de células cancerosas atuando em múltiplas vias de sinalização, reduzindo a inflamação e modulando a resposta imune (BENVENUTO et al., 2016). Assim, os compostos fenólicos, dentre eles as isoflavonas, têm sido investigados amplamente em relação aos efeitos anti-inflamatórios e anticancerígenos (GUO; KONG; MEYDANI, 2009; VALERIO et al., 2009; VERRI et al., 2012).

A inflamação é um mecanismo de defesa que permite que o organismo humano responda à injúria e ao ataque de patógenos. Uma das formas de defesa das células contra patógenos é a geração de espécies reativas de oxigênio (EROs), a qual é seguida por uma infiltração de leucócitos para remover os patógenos remanescentes (GUZIK; KORBUT; ADAMEK-GUZIK, 2003; REUTER et al., 2010). Estas espécies podem ser geradas por todos os tipos de células vasculares, as quais incluem células endoteliais, células musculares lisas, fibroblastos adventícios e

adipócitos perivasculares (TOUYZ et al., 2011). Em contraste ao mecanismo protetor, a inflamação crônica pode promover dano substancial ao tecido e favorecer as condições pró-carcinogênicas (KUNDU; SURH, 2012).

O estresse oxidativo, o qual é definido como um desequilíbrio entre sistemas pró-oxidante e antioxidante, está associado a muitas complicações vasculares (KIM; BYZOVA, 2015). As enzimas redutoras são capazes de sequestrar as EROs e produtos de oxidação lipídica e, assim, proteger as células e tecidos dos danos potenciais oriundos do estresse oxidativo (CHEN et al., 2010). A enzima superóxido dismutase (SOD, EC 1.15.1.1) converte os radicais superóxidos em oxigênio molecular e peróxido de hidrogênio (H_2O_2). Em seguida, a catalase (EC 1.11.1.6) decompõe o H_2O_2 a oxigênio molecular e água, ou a glutationa peroxidase (GSH peroxidase, EC 1.11.1.9) pode decompor o H_2O_2 em água (EL-BELTAGI; MOHAMED, 2013). Se o excesso de EROs não for removido por essas enzimas, uma série de alterações celulares e enzimáticas serão desencadeadas e assim causar uma inflamação excessiva que pode contribuir para várias doenças agudas e crônicas caracterizadas por produção não controlada de citocinas pró-inflamatórias e EROs (VERNAZA et al., 2012).

A resposta inflamatória pode causar mais dano do que a própria infecção e tem sido demonstrada a importância do equilíbrio dessa resposta imune (GARCÍA-LAFUENTE et al., 2009). Dessa forma, existe um grande interesse na produção de ingredientes alimentares que são capazes de regular as respostas inflamatórias e oxidativas (VERNAZA et al., 2012). Tem sido demonstrado que os compostos fenólicos podem atuar diretamente nas cascatas de sinalização envolvidas na inflamação e desenvolvimento de câncer (KANG et al., 2011). Assim, os compostos fenólicos têm sido amplamente investigados por causa de seus efeitos antioxidantes, anticancerígenos, anti-inflamatórios e anti-hiperglicêmicos (CROZIER; JAGANATH; CLIFFORD, 2009).

Os principais mecanismos da atividade antioxidante dos compostos fenólicos foram descritos como: (i) eliminação de radicais livres; (ii) quelação de metais; (iii) inibição de vários tipos de oxidases (como ciclo-oxigenase) e (iv) estimulação de enzimas com propriedades antioxidantes (superóxido dismutase, catalase etc) (NIJVELDT et al., 2001; XYNOS et al., 2012).

Os ensaios de capacidade antioxidante *in vitro* são importantes para verificar se há ou não correlação entre antioxidantes e os níveis de estresse oxidativo, contudo, são limitados e, às vezes, pode não haver similaridade com sistemas biológicos reais. Para avaliar a capacidade antioxidante existem métodos diretos baseados em estudos de cinética química e ensaios mediados pela transferência de elétrons denominados de métodos indiretos. (HUANG; OU; PRIOR, 2005). Os métodos diferem quanto à duração, modo de mensuração e condições de temperatura, oxigenação e o meio (lipídico, emulsionado ou aquoso) (CUVELIER; BONDET; BERSET, 2000).

Os ensaios de capacidade antioxidante *in vitro* podem ser classificados como de: transferência de elétrons (ET) e transferência de átomo de hidrogênio (HAT) com base nas reações químicas envolvidas. Os ensaios que tem como base a cinética de HAT envolvem um esquema de reação competitivo no qual o antioxidante e o substrato concorrem pelos radicais peroxil termicamente gerados pela decomposição de compostos. São exemplos o ensaio ORAC (Oxigen Radical Absorbance Capacity), que visa medir a capacidade de absorção do radical oxigênio, o sistema β-caroteno/ ácido linoleico, e o método DPPH (2,2-difenil-1-picrilhidrazil). Os de ET são ensaios que medem a capacidade de um antioxidante de reduzir um oxidante, que muda de cor quando reduzido e incluem os ensaios de ABTS (2,2', azinobis (3-etilbenzotiazolina-6-ácido sulfônico)), Folin-Ciocalteu e FRAP (Poder Antioxidante de Redução do Ferro), cada um ultiliza diferentes reagentes cromogênicos com diferentes potenciais redox (APAK et al., 2007; HUANG; OU; PRIOR, 2005).

Nem todos os antioxidantes se comportam da mesma forma para diferentes fontes de radicais, portanto, um único ensaio não pode ser considerado como parâmetro de capacidade antioxidante total, o que torna necessário o desenvolvimento de diferentes métodos específicos para cada fonte de radical (PRIOR; WU; SCHAICH, 2005).

Os compostos fenólicos são produtos do metabolismo secundário de plantas, sintetizados a partir das vias do chiquimato e do acetato, formando um grande e complexo grupo de fitoquímicos. Podem apresentar moléculas simples, como os ácidos fenólicos ou moléculas altamente polimerizadas, como os taninos. A maioria dos fenólicos ocorre como glicosídeos, com um açúcar (monossacarídeo,

dissacarídeo ou polissacarídeo) ligado ao grupo hidroxil da estrutura química, sendo a glicose o mais comum. Além disso, pode estar associado a outros compostos como as proteínas (ANGELO; JORGE, 2007; BRAVO, 1998; OZDAL; CAPANOGLU; ALTAY, 2013; TSAO, 2010). Apresentam importância na conservação de alimentos, atuando como antioxidantes, prevenindo ou retardando a deterioração e mantendo o valor nutritivo (OOMAH; SITTER, 2009). Ainda, podem ser aplicados em indústrias de corantes naturais, tintas, papel e cosméticos (IGNAT; VOLF; POPA, 2011).

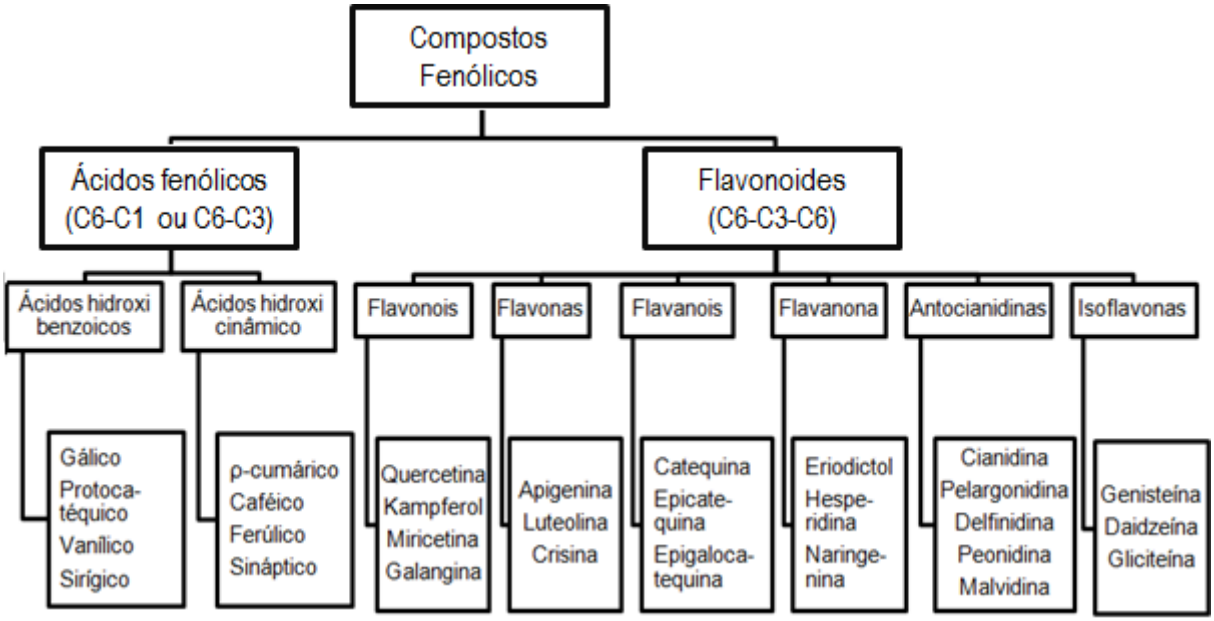
A estrutura química dos fenólicos é composta por hidroxilas e anéis aromáticos nas formas simples ou de polímeros que conferem o poder antioxidante (ANGELO; JORGE, 2007; NACZK; SHAHIDI, 2004). Dessa forma, atuam eliminando os radicais livres por transferência de elétrons ou doação de um átomo de hidrogênio de um grupo hidroxila da sua estrutura aromática, que possui capacidade de suportar um elétron desemparelhado ou quelando os metais de transição, como o Fe^{2+} e Cu^{+}, e interrompendo a reação de propagação de radicais livres na oxidação lipídica (MIN; EBELER, 2008; PODSEDEK, 2007). A atividade antioxidante é influenciada pela posição e grau de hidroxilação, polaridade, solubilidade e estabilidade do radical fenólico (MECCLEMENTS; DECKER, 2010). Destacam-se como antioxidantes fenólicos mais comuns de fonte natural os flavonoides, ácidos fenólicos, taninos e tocoferóis (ANGELO; JORGE, 2007; NACZK; SHAHIDI, 2004).

Dependendo da sua estrutura química, os fenólicos podem ser divididos em diferentes classes e as principais (Figura 2) incluem os ácidos fenólicos e flavonoides (GUO; KONG; MEYDANI, 2009; KARAKAYA, 2004). Estas duas classes de compostos fenólicos são as mais abundantes em alimentos e portanto, são as mais consumidas (BRAVO, 1998; FOWLER; KOFFAS, 2009). Em um estudo realizado na Europa verificou-se que 52,6% da ingestão total de compostos fenólicos foi constituída por ácidos fenólicos e 42,4% por flavonoides (ZAMORA-ROS et al., 2016).

Dentre os ácidos fenólicos, destacam-se a estrutura contendo C6-C1 que incluem os ácidos gálico, *p*-hidroxibenzoico, protocatecuico, vanílico e siríngico; enquanto que os ácidos hidroxicinâmicos são os compostos aromáticos com uma cadeia lateral de três carbonos (C6-C3), sendo os mais comuns os ácidos cafeico, ferúlico, *p*-cumárico e sinápico (BRAVO, 1998). Os flavonoides estão amplamente distribuídos nos alimentos vegetais e apresentam a estrutura química descrita como C6-C3-C6 com mais de 4000 variações e estão presentes principalmente como

glicosídeos (TERAO, 1999). A maioria dos flavonoides possui características biológicas e químicas em comum, tais como a capacidade antioxidante, capacidade de quelar metais (Fe^{2+} e Cu^{+}) e capacidade de modular a atividade de algumas enzimas celulares (HO; RAFI; GHAI, 2010).

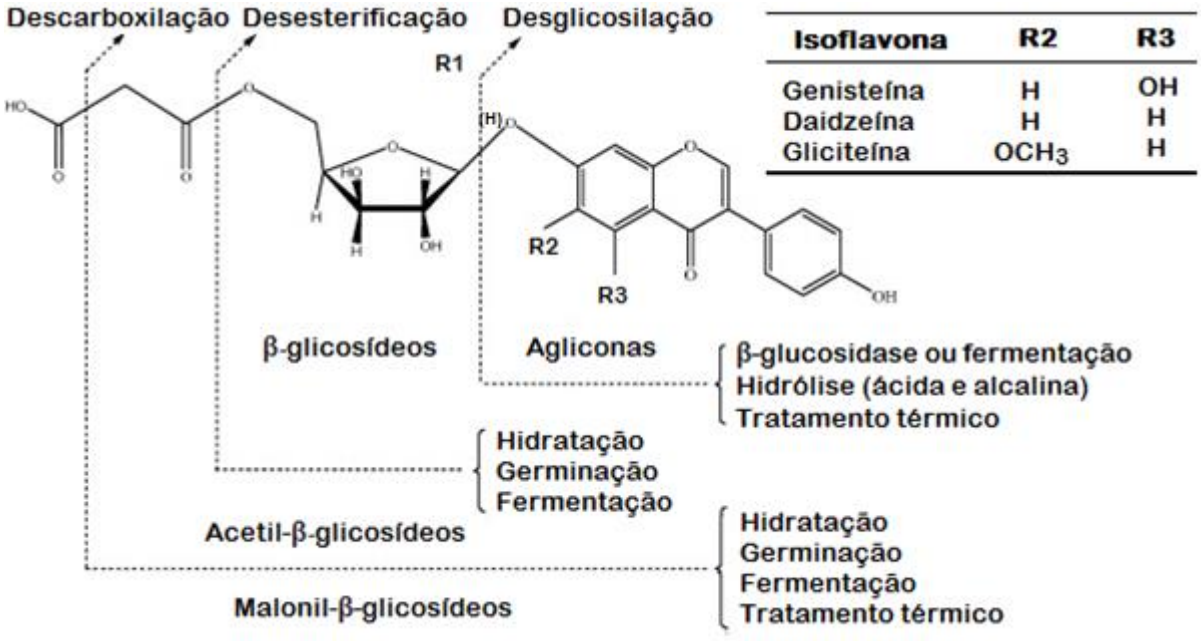
Figura 2 - Classificação dos compostos fenólicos



Fonte: Adaptado de Karakaya (2004).

As isoflavonas pertencem ao grupo dos flavonoides e são os principais metabólitos secundários da soja. Existem principalmente três formas moleculares básicas denominadas de genisteína, daidzeína e gliciteína. Cada forma básica possui quatro derivados, denominados de agliconas, β-glicosídeos, malonil-β-glicosídeos e acetil-β-glicosídeos (Figura 3), totalizando 12 diferentes formas de isoflavonas (MORAS et al., 2017; LIU, 1997). No entanto, pode ocorrer a conversão entre as diferentes formas de isoflavonas devido as várias etapas do processamento da soja e seus derivados, conforme pode ser observado na Figura 3 (CHEN et al., 2015a).

Figura 3 - Estrutura química das isoflavonas



Isoflavona	R2	R3
Genisteína	H	OH
Daidzeína	H	H
Gliciteína	OCH_3	H

Fonte: Adaptado de Chen et al. (2015a)

A concentração de compostos fenólicos e de isoflavonas em produtos de soja, bem como a capacidade antioxidante, dependem de fatores genéticos do grão, local de plantio, ambiente em que foi cultivado e do processamento empregado. O conteúdo de fenólicos totais (CFT) variou de 654,6 a 5219,6 µg/g de soja em base seca de 204 linhagens de soja provenientes dos Estados Unidos, China e Coreia. As concentrações médias do CFT foram de 2729,1 µg/g de sementes de soja americanas, 1680,4 µg/g de sementes de soja chinesas e 1977,6 µg/g de sementes de soja coreanas. As sementes de soja pequenas apresentaram maior CFT (2241,7 µg/g de sementes), enquanto que as sementes de tamanho médio e grande apresentaram menor CFT ou seja, de 1926,8 e 1949,9 µg/g, respectivamente (KIM et al., 2012). Ainda, os mesmos autores observaram que o teor de isoflavonas totais variou de 682,4 a 4778,1 µg/g de sementes. Dentre os três países, as sementes de soja da Coreia apresentaram a maior concentração de isoflavonas totais (2252,6 µg/g de sementes). Em relação ao tamanho, as sementes pequenas apresentaram a maior concentração total de isoflavonas (2520,0 µg/g de sementes). O teor médio de isoflavonas totais em sementes médias e grandes foi de 1904,4 e 1777,5 µg/g de sementes, respectivamente.

Segundo Genovese, Hassimotto e Lajolo (2005), o conteúdo e o perfil de isoflavonas de 14 variedades de soja brasileira produzidas pela Embrapa variaram

significativamente (570-1880 µg de isoflavonas/g de grão de soja), sendo que 90 à 95,0% estavam nas formas glicosiladas. As isoflavonas β-glicosídeos representaram as principais formas encontradas (50,0 a 59,0% do total) seguidas de malonil-β-glicosídeos (28,0 a 39,0% do total). Observaram também que o CFT variou de 1430 a 2250 µg/g de grão de soja.

Conforme observado por Cho et al. (2013) o conteúdo de isoflavonas individuais e totais de 13 cultivares de soja coreana de quatro cores diferentes e plantadas em duas safras apresentaram diferenças consideráveis entre os anos de safra e as cultivares. A soja de cor verde apresentou maior teor de isoflavonas totais com 3079,42 µg/g de soja, comparada com a soja de cor amarela (2393,41 µg/g de soja), preta (2373,97 µg/g de soja) e marrom (1821,82 µg/g de soja). Além disso, observaram que o grupo malonil-β-glicosídeo apresentou maior teor de isoflavonas, seguido de isoflavonas β-glicosídeos e agliconas, enquanto que o menor conteúdo foi observado no grupo acetil-β-glicosídeos.

As diversas operações e tipos de processamento utilizados na manufatura dos alimentos de soja podem influenciar o conteúdo e o perfil dos compostos fenólicos e isoflavonas, bem como a capacidade antioxidante dos produtos finais. Em geral, o processamento da soja (Tabela 1), reduz o teor das isoflavonas nos produtos finais, com exceção da FDS, na qual devido à extração do óleo, há uma concentração das isoflavonas.

Os efeitos da hidratação da soja, como uma etapa prévia ao processamento do extrato de soja, sobre as propriedades dos grãos e perda de isoflavonas foram descritos por De Lima, Kurozawa e Ida (2014) e De Lima e Ida, (2014). Os autores observaram que a hidratação prolongada da soja não promoveu um amolecimento adicional dos grãos e que quando a soja foi hidratada a 55 °C em tempo reduzido ocorreu uma maior conversão de isoflavonas β-glicosídeos em agliconas. Portanto, recomendaram que a hidratação da soja seja realizada a 55 °C por 5 h, uma vez que nesta condição, foi atingida a umidade de 120,0% e ocorreu aumento de 6 vezes no teor de agliconas sem afetar negativamente a dureza dos grãos. Além disso, nesta condição de hidratação da soja, o balanço de massa de isoflavonas totais indicou que houve 80,7% de retenção, 6,8% de lixiviação e 12,6% de degradação de isoflavonas. Após a hidratação dos grãos de soja também foi observado o aumento no teor de isoflavonas totais que foi atribuído à eficiência da

extração de isoflavonas decorrente do amolecimento do tecido dos grãos (DE LIMA; KUROZAWA; IDA, 2014; MALAYPALLY; ISMAIL, 2010).

Tabela 1 - Teor de isoflavonas em alimentos de soja (mg/100 g)

Alimentos de soja	Daidzeína	Genisteína	Gliciteína
Soja crua	62,07	80,99	14,99
Farinha desengordurada de soja	64,55	87,31	15,08
Soja germinada cozida no vapor	5,00	6,70	0,80
Soja germinada crua	12,86	18,77	2,88
Natto	33,22	37,66	10,55
Bebida de soja	2,75	5,10	-
Isolado proteico de soja	30,81	57,28	8,54
Iogurte de soja	13,77	16,59	2,8
Extrato de soja	4,84	6,07	0,93
Tempeh	22,66	36,15	3,82
Tofu firme preparados com nigari	12,31	16,1	2,75
Tofu regular preparado com sulfato de cálcio	8,56	12,99	1,98
Tofu seda	9,15	8,42	0,92

Fonte: Adaptado e reorganizado a partir do banco de dados do USDA (Departamento de Agricultura dos EUA) para o conteúdo de Isoflavonas de alimentos selecionados (BHAGWAT; HAYTOWITZ; HOLDEN, 2011).

O cozimento da soja com vapor e pressão promoveu um aumento no CFT, taninos condensados, ácido gálico, ácido 2,3,4-trihidroxibenzóico e na atividade antioxidante que foi medida pelos métodos de eliminação de radicais livres DPPH, redução férrica (FRAP) e capacidade de absorver radicais de oxigênio (ORAC). Observou-se que todas as técnicas utilizadas de cozimento converteram em grande parte as isoflavonas malonil-β-glicosídeos para as suas formas β-glicosídeos correspondentes. Em adição, os grãos de soja cozidos com vapor e pressão apresentaram maior teor de isoflavonas totais em relação à soja crua, possivelmente devido aos danos nas estruturas dos polímeros ligados (ligninas) com liberação de ácidos fenólicos e isoflavonas da matriz proteica durante o cozimento com vapor e pressão (XU; CHANG, 2008). O processamento térmico do extrato de soja de variedades de soja amarela por ultra alta temperatura (UHT) reduziu o CFT e teor de malonil-β-glicosídeos e agliconas, enquanto que aumentou o teor de flavonoides totais, de isoflavonas β-glicosídeos e acetil-β-glicosídeos. Além disso, aumentou a atividade antioxidante de eliminação de radicais livres, poder de redução do Fe^{3+} e de

absorção de radicais de oxigênio de ambas as variedades de soja amarela (XU; CHANG, 2009).

O processo de germinação da soja também altera o teor das diferentes formas de isoflavonas. Assim, Quinhone Júnior e Ida (2015) observaram que o teor de daidzina, genistina e β-glicosídeo total da soja germinada por 168 h reduziu em 1,1, 1,9 e 1,8 vezes, respectivamente, em relação a soja não germinada. Entretanto, o teor de isoflavonas malonil-β-daidzina e malonil-β-genistina aumentou em 2,5 e 1,6 vezes, respectivamente, enquanto que o teor de malonil-β-glicitina diminuiu em 1,5 vezes. Em contrapartida, nesta soja germinada por 168 h, observaram que o conteúdo total de agliconas representou 2,5% das isoflavonas totais e foi 2,9 vezes maior do que na soja não germinada.

O cozimento dos brotos de soja germinados por 3, 5 e 7 dias, causou perdas significativas na maioria dos ácidos fenólicos individuais, grupo hidroxibenzoico, grupo hidroxicinâmico, CFT, isoflavonas individuais e isoflavonas totais. Entretanto, nos brotos germinados por 1 e 2 dias ocasionou alterações mínimas na composição de ácidos fenólicos. O tempo de cozimento por 20 min, 7 min ou 5 min dos brotos de soja germinados por 1 e 2 dias, 7 dias e 3 e 5 dias, respectivamente, para manter a textura e palatabilidade, exerceu maior impacto no CFT e atividade antioxidante do que o tempo de germinação. Enquanto que o tratamento térmico dos brotos de soja interconverteu e degradou as isoflavonas (KUMARI; CHANG, 2016).

A fermentação em estado sólido (FES) da soja tem sido utilizada para produzir alimentos ou como uma estratégia de enriquecimento de produtos à base de soja que contenham compostos fenólicos livres, isoflavonas agliconas e com atividade antioxidante. A FES das sojas amarela e preta, cozida, descascada e fermentada em estado sólido por 24 h com diferentes cepas de *Bacillus subtilis* (MTCC 1747 e MTCC 5480) causou drástica mudança no CFT e aumentou o poder antioxidante das sojas fermentadas (SANJUKTA et al., 2015). O CFT das sojas amarelas e pretas não fermentadas foi de 1,93 e 1,64 mg GAE/g de soja, respectivamente, e aumentou para 7,9-8,4 e 6,9-7,5 mg GAE/g de soja fermentada, respectivamente. Foi observado que o aumento do poder antioxidante das sojas fermentadas foi dependente da variedade da soja e do micro-organismo utilizado sendo que a soja amarela e o *Bacillus subtilis* 5480 apresentaram maior potencial antioxidante.

3.4 Processo de Fermentação

O processo de fermentação da soja pode influenciar substancialmente no conteúdo, perfil e composição de fenólicos e peptídeos, com consequente biodisponibilidade desses compostos bioativos. Em geral, a fermentação dependendo do micro-organismo utilizado aumenta os níveis de ácidos fenólicos e flavonoides, principalmente as isoflavonas agliconas (DUEÑAS et al., 2012). A biodisponibilidade das isoflavonas ocorre por meio da ação da flora intestinal durante a degradação dos compostos e, é influenciada pela dose de isoflavonas ingerida na dieta e pela ação de enzimas bacterianas, especialmente a enzima β-glucosidase (SETCHELL et al., 2001). Estas enzimas hidrolisam as isoflavonas glicosiladas, que não podem ser absorvidas, produzindo as suas formas agliconas. Desta forma, pela ação das enzimas β-glucosidases produzidas durante a fermentação, há formação de diferentes compostos bioativos, possibilitando que independentemente da sua microbiota intestinal, todos os consumidores possam obter benefícios à saúde desses compostos (LANDETE et al., 2015).

No processo de fermentação, os compostos fenólicos podem ser produzidos por meio de via metabólica secundária ou liberados a partir do substrato pela ação de enzimas produzidas por micro-organismos, melhorando o teor de fenólicos e atividade antioxidante (BEI et al., 2017; VILLARES et al., 2011; WANG et al., 2017). A fermentação da soja envolve transformações químicas que podem resultar em perda de isoflavonas, conversão de isoflavonas glicosiladas em agliconas e, em alguns casos, ocorre à formação de derivados de isoflavonas pela adição ou substituição de grupos funcionais na estrutura química (DEY et al., 2016; DULF; VODNAR; SOCACIU, 2016). Ainda, pode reduzir os RFOs pela atuação de carboidrases produzidas durante a fermentação (WANG et al., 2014).

A FES é um processo antigo e utilizado para produzir enzimas e metabólitos secundários. Assim, sua aplicação na indústria é importante, pois além de enzimas pode produzir também biomoléculas como ácidos orgânicos, pigmentos, compostos fenólicos e de sabor (SOCCOL et al., 2017). A FES tem sido aplicada na soja ou coprodutos do seu processamento para produzir isoflavonas (YAAKOB et al., 2011), enzimas, como β-glucosidase (HANDA et al., 2014) e corantes naturais (MHALASKAR; THORAT, 2016), além de melhorar a capacidade antioxidante (XIAO

et al., 2015). Tem sido aplicada também para aumentar o teor de aminoácidos e para diminuir o conteúdo de ácido fítico, inibidores de tripsina e oligossacarídeos como rafinose, estaquiose e verbascose (CHEN; MADL; VADLANI, 2013; HASSAAN; SOLTAN; ABDEL-MOEZ, 2015).

McCue e Shetty (2003) avaliaram o papel de enzimas carboidrases na liberação de compostos fenólicos com capacidade antioxidante durante a FES da soja integral com *Rhizopus oligosporus*. Verificaram que o CFT aumentou 120,0-135,0% nos extratos, a atividade antioxidante aumentou 61,0%, este aumento foi limitado ao período inicial de fermentação de até dois dias e diminuiu nos tempos subsequentes. A maior atividade antioxidante foi associada ao aumento das atividades de (α e β) glucosidase e β-glucuronidase, enquanto o alto CFT foi parcialmente ligado ao aumento da atividade da α-amilase.

A fermentação da farinha de soja por *Aspergillus oryzae* por 36 h reduziu o teor dos oligossacarídeos rafinose, estaquiose e verbascose e do inibidor de tripsina, cujo valor inicial foi de 9,48 mmol/ 100 g para um nível não detectável. Os polissacarídeos estruturais diminuíram 59,0% (p/p) e após 36 h de fermentação, também modificou os fatores nutricionais, como por exemplo, o teor de proteína que aumentou de 50,5 para 58,9% (p/p) (CHEN; MADL; VADLANI, 2013).

A FES da FDS converteu as isoflavonas glicosiladas em agliconas e foi dependente do fungo utilizado. O teor de agliconas na FDS fermentada com *Monascus purpureus* aumentou em 5 vezes, enquanto que na FDS fermentada com *Aspergillus oryzae*, o aumento foi de apenas 1,8 vezes, no entanto, este fungo produziu a enzima β-glucosidase com atividade de 10,7 vezes superior a enzima produzida pelo *Monascus purpureus* (HANDA et al., 2014). Observaram também que a etapa de esterilização da farinha desengordurada de soja por autoclavagem reduziu o teor de isoflavonas malonil-β-glicosídeo em 74,0% que foram convertidas em outras formas de isoflavonas.

A eficiência de FES para a obtenção do produto desejado depende do micro-organismo, bem como das condições operacionais e do meio ambiente, tais como temperatura, pH, teor de umidade, aeração, concentração de nutrientes e natureza do substrato (FARINAS, 2015). Os fungos são preferidos para serem utilizados no processo de FES, uma vez que as condições de cultivo são semelhantes

às condições exigidas pelos micro-organismos no ambiente natural (RAIMBAULT, 1998).

Dentre os vários fungos utilizados na FES, destacam-se o *Aspergillus* e *Monascus* que são utilizados no continente asiático desde longa data em alimentos fermentados tradicionais. O *Aspergillus oryzae* é um dos fungos mais utilizados na FES, sendo parte da lista do FDA (Food and Drug Administration-EUA) como GRAS "Geralmente Reconhecido Como Seguro (do inglês General Recognized As Safe). O *Aspergillus oryzae* tem sido amplamente utilizado na indústria de alimentos devido às suas altas atividades proteolíticas e amilolíticas, sendo utilizados na produção alimentos tradicionais fermentados como molho de soja, saquê, temperos e vinagre (KAWAUCHI; IWASHITA, 2014; LI et al., 2016; MACHIDA; YAMADA; GOMI, 2008). Este fungo é conhecido também por apresentar grande potencial de produção de várias enzimas, sendo, portanto, utilizado na produção de enzimas industriais na área da biotecnologia moderna (MACHIDA; YAMADA; GOMI, 2008).

O *Monascus purpureus* é um fungo comestível e versátil, que produz metabólitos secundários, principalmente pigmentos e há séculos tem sido usado na FES na Ásia (SRIANTA et al., 2016). Os *Monascus* spp. são fungos filamentosos e amplamente conhecidos por seus produtos fermentados, especialmente o arroz vermelho, cujo alimento fermentado é tradicional no Leste Asiático. Este gênero de fungo é capaz de produzir vários metabólitos secundários que apresentam efeitos benéficos, como monacolin (relacionado com a redução da síntese de colesterol), ácido γ-amino butírico (anti-hipertensivo), ácido dimerumico (antioxidante) e pigmentos (corantes de qualidade alimentar). No entanto, algumas cepas podem também secretar a citrinina, um metabólito nefrotóxico (CHEN et al., 2015b).

Embora a FES tenha sido definida como um bioprocesso realizado com limitada quantidade de água, a umidade é um fator chave, uma vez que o substrato deve possuir suficiente quantidade de água para sustentar o crescimento e atividade metabólica do micro-organismo (THOMAS; LARROCHE; PANDEY, 2013). Entre os parâmetros da FES, o pH do substrato é um dos mais difíceis de controlar e as mudanças no pH podem afetar o crescimento dos micro-organismos e a produção de metabólitos. Assim, o ajuste do pH inicial do substrato tem sido explorado para avaliar a variação do micro-organismo e a produção de enzimas durante o processo da FES (HANDA et al., 2014; ZOU et al., 2016). A temperatura também é considerada

como um dos parâmetros mais críticos para FES, influenciando no crescimento de micro-organismos, atividade enzimática e produção de metabólitos (PANDEY, 2003; THOMAS; LARROCHE; PANDEY, 2013).

Na FES com *Monascus purpureus*, dependendo do substrato utilizado e do produto desejado, o teor de umidade do substrato ou a quantidade de água adicionada ao substrato tem variado de 30,0 a 60,0% ou de 1:4 a 1,2:1 mL água/g substrato (v/m); enquanto que o pH inicial do substrato varia de 3 a 7 e a temperatura de incubação varia de 20 a 50 °C (ABD RAZAK et al., 2015; BABITHA; SOCCOL; PANDEY, 2006; HANDA et al., 2014; HUANG; ZHANG; XUE, 2017; SRIANTA et al., 2016; VELMURUGAN et al., 2011; WANG; LEE; PAN, 2003). Na FES com *Aspergillus oryzae* a variação da quantidade de água adicionada ao substrato é de 40,0 a 76,0% de umidade ou 2:1 a 6:1 água/g substrato (v/m); enquanto que o pH inicial do substrato varia de 3 a 7 e a temperatura de incubação varia de 20 a 40 °C (CHEN; MADL; VADLANI, 2013; CHUTMANOP et al., 2008; DE CASTRO; SATO, 2013; HANDA et al., 2014; PURI; ARORA; SARAO, 2013; SANGEETHA; RAMESH; PRAPULLA, 2004; SZABO et al., 2015).

Além da seleção dos parâmetros mais apropriados da FES para obtenção do produto desejado, é importante também otimizar a extração dos compostos desejados. O produto obtido por FES pode ser recuperado da massa fermentada sólida por extração com solventes (misturas aquosas ou outros solventes). O tipo de solvente e sua concentração, bem como a proporção de solvente para o sólido e o pH são variáveis importantes que influenciam na extração do produto. Além disso, uma vez que os metabólitos difundem-se ao longo da massa sólida durante a fermentação, podem ser necessários tempo de extração prolongado para a recuperação completa do produto (MARTINS et al., 2011).

3.5 Extração de Compostos Fenólicos

A extração dos compostos bioativos de produtos naturais é uma etapa crítica, pois a sua eficiência depende de vários parâmetros, como o tipo de amostra, tipos de compostos a serem extraídos, localização na amostra (MUSTAFA; TURNER, 2011), tipo de solvente extrator (XYNOS et al., 2012), método de extração e temperatura de extração (GALANAKIS; TORNBERGB; GEKASC, 2010). Os fenólicos

simples e flavonoides são compostos relativamente solúveis e de baixa massa molar, dependendo da polaridade e estrutura química (grau de hidroxilação, acetilação, glicosilação etc). Porém, alguns destes podem estar ligados aos componentes da parede celular. Dependendo da natureza dessas ligações, estes compostos podem ser solubilizados em condições alcalinas ou ácidas, ou permanecerem retidos na matriz (BRAVO, 1998).

Para extração de amostras sólidas é necessário realizar a transferência destes compostos de interesse para a fase líquida e utilizar um solvente extrator adequado (LUQUE DE CASTRO; PRIEGO-CAPOTE, 2010). Esse processo é um dos mais antigos empregados no preparo de amostras e é conhecido como extração sólido-líquido ou lixiviação (LUQUE DE CASTRO; GARCÍA-AYUSO, 1998; MILIĆ et al., 2014).

A extração sólido-líquido ideal envolve o contato íntimo entre um material sólido, geralmente finamente moído e um solvente que tem uma solubilidade máxima para o composto de interesse. Utiliza-se uma solubilidade mínima para a matriz, com forças externas adicionais para acelerar o processo de extração. Amostras sólidas de soja, tais como, grãos de soja ou proteínas de soja, necessitam apenas de moagem antes da extração, porém, muitas vezes são liofilizadas para fornecer um pó homogêneo. Os métodos mais comuns para a extração das isoflavonas a partir de amostras sólidas incluem a extração com solvente orgânico puro ou solução aquosa de metanol, etanol, acetonitrila ou acetona com e sem a adição de ácidos, utilizando imersão simples, mistura, agitação, extração soxhlet ou técnicas de ultrassom (ROSTAGNO et al., 2009; ROSTAGNO; ARAÚJO; SANDI, 2002).

Há diversos solventes que são utilizados para extração de compostos fenólicos e isoflavonas. A composição do solvente extrator varia conforme o estudo e depende muito da matriz em que se encontra. O fato dos compostos fenólicos estarem localizados em estrutura subcelular, principalmente, nos vacúolos e, ainda, podendo ser encontrados na forma solúvel ou suspensos em combinação com os componentes da parede celular ou outras moléculas, pode resultar em um importante impacto para sua extração (WACH; PYRZYŃSKA; BIESAGA, 2007).

O etanol, metanol e água têm boa polaridade e, portanto, são utilizados para extrair os compostos polares, tais como, os compostos fenólicos como

os flavonoides. O etanol é um solvente orgânico e não tóxico e portanto, é frequentemente utilizado para extração. Quando se utiliza a água há necessidade de uma etapa diferente de liofilização para removê-la a partir do extrato após a extração. A toxicidade do metanol limita sua utilização na extração e em algumas análises subsequentes. Os solventes não polares tais como éter e de baixa polaridade, como clorofórmio e éster, são usados em casos específicos, além do fato que a disponibilidade também limita o uso desses solventes no processo de extração (ALAM; BRISTI; RAFIQUZZAMAN, 2013).

Os solventes polares têm sido recomendados na extração de compostos bioativos. O etanol aquoso a 77,0% foi utilizado como solvente em extrações de isoflavonas de soja e produtos derivados, nos quais estes compostos estão presentes, na sua maioria (>95,0%), nas formas glicosiladas e ésteres malonil (KUDOU et al., 1991).

Genovese e Lajolo (2001) verificaram que o metanol aquoso a 80,0% foi o melhor meio extrator de isoflavonas de derivados protéicos de soja e alimentos industrializados, em termos de rendimento e perfil obtido, e a temperatura de 40°C de rota-evaporação foi a mais adequada para a concentração dos extratos.

Yoshiara et al. (2012) otimizaram a extração das diferentes formas de isoflavonas (β-glicosídeo, malonil-β-glicosídeo e aglicona) de FDS utilizando o delineamento experimental simplex-centroide com quatro solventes de polaridade variada (água, acetona, etanol e acetonitrila). A maior quantidade de isoflavonas totais foi extraída quando utilizou a mistura ternária de água, acetona e etanol (1:1:1; v:v:v).

Xu e Chang (2007) observaram que solventes com diferentes polaridades têm efeitos significativos sobre o CFT, teor de flavonoides totais, taninos condensados e atividade antioxidante de diferentes leguminosas. Os extratos de acetona a 50,0% exibiram maior CFT total para ervilha amarela, ervilha verde, grão de bico e soja amarela. Os extratos ácidos de acetona a 70,0% (0,5% de ácido acético) exibiram maior CFT, teor flavonoide total e maior atividade redutora do Fe^{3+} (FRAP) para feijão preto, lentilha, soja preta e feijão vermelho. Os extratos de acetona 80,0% exibiram a maior atividade de eliminação de radicais livres de DPPH e conteúdo de flavonoide total e taninos condensados para ervilha amarela, ervilha verde, grão de bico e soja amarela. Os extratos de etanol a 70,0% exibiram o maior valor ORAC para todas as leguminosas avaliadas.

Os solventes que apresentaram melhor eficiência nas extrações dos compostos fenólicos foram as misturas de água com metanol, etanol ou acetona, sendo empregados em concentrações que variaram de 50,0 a 80,0% (v/v). No entanto, o etanol tem sido preferido ao metanol e à acetona por ser considerado um solvente GRAS e por apresentar baixa toxicidade (RODRÍGUEZ-ROJO et al., 2012).

Neste contexto, há evidências de que a extração de compostos fenólicos bem como a atividade antioxidante estes extratos podem ser influenciados pelo sistema de solvente extrator. Além disso, a fermentação em estado sólido da FDS pode contribuir para a obtenção de uma FDS fermentada com maior quantidade de isoflavonas agliconas, atividade antioxidante e com a redução no conteúdo de oligossacarídeos. Contudo, ressaltam-se a importância de investigar os efeitos dos parâmetros (pH inicial da FDS, quantidade de água adicionada e temperatura de incubação) e do tempo de fermentação sobre os compostos bioativos e atividade antioxidante. Ressalta-se ainda, a necessidade de avaliar a atividade anti-hipertensiva de alimentos de soja após a digestão gastrointestinal *in vitro*.

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4 MATERIAL E MÉTODOS

Este item **4 MATERIAL E METODOS** foi contemplado com as publicações dos quatro artigos científicos abaixo relacionados e serão apresentados nesta Tese no item **5 RESULTADOS E DISCUSSÃO.**

5.1 ARTIGO CIENTÍFICO 1

HANDA, C. L.; DE LIMA, F. S.; GUELFI, M. F. G.; GEORGETTI, S. R.; IDA, E. I. Multi-response optimisation of the extraction solvent system for phenolics and antioxidant activities from fermented soy flour using a simplex-centroid design. **Food Chemistry**, v. 197, p. 175-184, 2016.

5.2 ARTIGO CIENTÍFICO 2

HANDA, C. L.; DE LIMA, F. S.; GUELFI, M. F. G.; FERNANDES, M. S.; GEORGETTI, S. R.; IDA, E. I. Parameters of the fermentation of soybean flour by *Monascus purpureus* or *Aspergillus oryzae* on the production of bioactive compounds and antioxidant activity. Será submetido para publicação - **Food Chemistry.**

5.3 ARTIGO CIENTÍFICO 3

HANDA, C. L.; CHANG, S. K. C.; VERRI, JR., W, A.; DA CUNHA, P. H. C.; GEORGETTI, S., R.; IDA, E. I. Fermentation time of soybean flour by *Monascus purpureus* or *Aspergillus oryzae* on the content of bioactive compounds with antioxidant potential and oligosaccharides. Será submetido para publicação – **Journal of Functional Foods.**

5.4 ARTIGO CIENTÍFICO 4

HANDA, C. L.; ZHANG, Y.; KUMARI, S.; XU, J.; IDA, E. I.; CHANG, S. K. C. Potential anti-hypertensive of several digested soy foods as a source of angiotensin I converting enzyme (ACE) inhibitors. Será submetido para publicação – **Journal of Functional Foods.**

5 RESULTADOS E DISCUSSÃO

Este item **5 RESULTADOS E DISCUSSÃO** foi contemplado com as publicações dos quatro artigos científicos supracitados na ordem que foi descrita

5.1 ARTIGO CIENTÍFICO 1

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Multi-response optimisation of the extraction solvent system for phenolics and antioxidant activities from fermented soy flour using a simplex-centroid design

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ABSTRACT

A simplex-centroid design comprising three solvents (water, ethanol and methanol) was used to optimise the extraction mixture for phenolics and antioxidant activities from defatted soy flour fermented with *Monascus purpureus* or *Aspergillus oryzae*. Total phenolics were more efficiently extracted using only water for both samples. The highest antioxidant activities by the DPPH and ABTS methods were obtained using extraction mixtures containing at least 75 wt% water. Specific water:ethanol:methanol ratios promoted the joint optimisation of the total phenolic and isoflavone contents as well as antioxidant activities: 0.5:0.375:0.125 (wt/wt/wt) and 0.5:0.3:0.2 (wt/wt/wt) from defatted soy flour fermented with *M. purpureus* or *A. oryzae*, respectively. However, a water:ethanol ratio of 0.5:0.5 (wt/wt) was deemed optimal because it is comprised of green solvents and yielded results that were greater than 90% of the multi-response maximum values. Both the solvents and the sample matrix strongly influenced the extractability of total phenolics and isoflavones.

1. Introduction

Among the secondary metabolites of various plant species, phenolic compounds have been extensively investigated because of their antioxidant, anticarcinogenic, anti-inflammatory and antihyperglycaemic effects in humans (Crozier, Jaganath, & Clifford, 2009).

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Flavonoids, specifically isoflavones, are the major polyphenols found in soybeans and their derivatives (Chung, Seo, Ahn, & Kim, 2011). Soy isoflavones are divided into four different classes by chemical structure: aglycones, β-glucosides, 6″-*O*-acetylglucosides and 6″-*O*-malonylglucosides (Supplementary Material: Fig. S1). The phenolic contents and antioxidant activities of soy-based products can be altered by certain types of processing, such as soaking, thermal treatment and microbial fermentation (Handa, Couto, Vicensoti, Georgetti, & Ida, 2014; Lima, Kurozawa, & Ida, 2014; Xu & Chang, 2008).

Solid-state fermentation is a promising process to obtain enzymes, bioactive compounds (Thomas, Larroche, & Pandey, 2013) and functional soybean flours (Fernandez-Orozco et al., 2007). Among the microorganisms used in this fermentation process, the *Aspergillus oryzae* and *Monascus purpureus* fungi have been two of the most investigated. These fungi have different characteristics from each other in the solid-state fermentation process (Handa et al., 2014), and therefore, the physicochemical alterations promoted by them in the same substrate may differ drastically. The use of defatted soy flour (byproduct of soybean oil extraction) as substrate is particularly advantageous in solid-state fermentation because it is one of the simplest soybean protein ingredients. More research related to the production and extraction of soybean-derived phenolics is needed because the phenolic extracts may have potential applications in different foods, such as tofu (Hong, Lee, Kim, & Imm, 2012), soymilk and bakery products. Moreover, these natural antioxidants could replace synthetic antioxidants in food formulations.

During microbial fermentation, many biochemical reactions occur, including the synthesis, conversion and degradation of substances. Thus, polar and apolar phenolics may be formed, converted and/or degraded during the fermentation process and the physicochemical properties of these compounds can be altered depending on their interaction with proteins, carbohydrates and lipids present in the system (Jakobek, 2015). In this context, variability in the literature data on phenolics and antioxidant activities for the same sample type cannot be explained exclusively by differences in processing or a lack of standard extraction and quantification methods. Furthermore, inefficient extraction of these compounds due to a lack of optimisation of the solvent system for each particular sample should also be considered.

Aqueous mixtures of methanol, ethanol and acetonitrile have showed similar extraction efficiency of phenolics from soybeans (Rostagno, Palma, & Barroso, 2003), and therefore, these solvents have been the most commonly used to extract phenolics from soy-based products (Rostagno, Villares, Guillamón, García-Lafuente, & Martínez, 2009). The use of acidified solvents can cause the degradation of malonylglycoside isoflavones and the hydrolysis of β-glucosides during the extraction process, thus reducing the recovery of the total isoflavones from the sample (Lin & Giusti, 2005). Therefore, the use of acidified solvents has been reported as unnecessary to improve the extraction of isoflavones.

The recovery level of total phenolics from a given sample depends on the sample-solvent interactions and the efficiency of the solvent system to extract the predominant forms of phenolics present in this sample matrix. Although the maximum extraction yield of phenolics is needed to meet the requirements of the functional food and pharmaceutical industries, the health and environmental risks should also be considered when selecting an extraction solvent. In this context, green extraction solvents, such as water and ethanol (Chemat, Vian, & Cravotto, 2012), have been more preferred than methanol, acetone and acetonitrile.

Solvent type, solvent-sample ratio, solvent–solvent ratio, extraction time, temperature and pH have all been investigated to optimise the extraction of phenolics in different samples (Lin & Giusti, 2005; Murphy et al., 1999; Xu & Chang, 2007). However, it is not possible to state that such studies were able to optimise the solvent:solvent ratio in extraction process since all solvent proportions (0–100%) were not investigated. The investigation of all solvent proportions on the extraction yield of phenolics is experimentally infeasible. Nevertheless, this problem can be overcome by the use of experimental designs.

Statistical methods, particularly the response surface design, have been used in food science to explore the effects of process variables on the specific responses of a system. Mixture design, such as simplex-centroid, allows for the investigation of the synergistic or antagonistic effects of the mixture components on response variables because the proportion of the components is interdependent on the mixture (Cornell, 2002, chap. 2). This is an important statistical tool that can help clarify the relationship between the solvent composition and sample matrix on the extraction yield of phenolics.

The extraction of phenolics and antioxidant activity values from samples should not be evaluated separately, as several studies have made, because the optimal solvent system to extract total phenolics may be different of that for other antioxidants. In this context, the multi-response optimisation approach is an important tool for simultaneous evaluation of these data and could lead to interesting findings. Despite some studies (Achouri, Boye, & Belanger, 2005; Murphy et al., 1999; Rostagno et al., 2009; Xu & Chang, 2007) have mentioned about the importance of the solvent system in maximising both the extraction of phenolics and other antioxidants in different soy food matrices, a comprehensive discussion on this finding has not been reported.

The aim of this study was to optimise the extraction solvent system for phenolics and antioxidant activities (DPPH, FRAP and ABTS) from defatted soy flour fermented with *M. purpureus* or *A. oryzae* using a simplex-centroid design.

2. Materials and methods

2.1. Samples and standards

Defatted soy flour was purchased from BRF Brasil Foods S.A. (Curitiba, PR, Brazil). The chemical composition of this flour was as follows: 8.95% moisture, 1.07% fat, 48.96% protein (N x 6.25), 5.98% ash and 35.04% carbohydrates (by difference). The *A. oryzae* IOC 3999/1998 and *M. purpureus* NRRL 1992 (GenBank: JQ614061.1) fungi used in fermentation were obtained from the Oswaldo Cruz Foundation (Fiocruz, Rio de Janeiro, RJ, Brazil) and the Laboratory of Biochemistry and Microbiology at the Institute of Applied Science and Food Technology at the Federal University of Rio Grande do Sul (Porto Alegre, RS, Brazil), respectively. Isoflavone standard solutions were prepared from 6″-*O*-acetylglucosides and 6″-*O*-malonylglucosides (Wako Pure Chemical Industries Ltd., Osaka, Japan) and from β-glucosides and aglycones (Sigma Aldrich Co., St. Louis, MO, USA). 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH·), 2,4,6-tri(2-pyridyl)-*S*-triazine (TPTZ), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Folin–Ciocalteu reagent, gallic acid and 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox) were acquired from Sigma Aldrich Co. and used to assess the antioxidant activities and quantify the total phenolic content of the sample extracts. All reagents used were of analytical or liquid chromatography grade.

2.2. Reactivation of microorganisms and obtaining spores suspensions

The *A. oryzae* IOC 3999/1998 and *M. purpureus* NRRL 1992 fungi were activated separately before inoculation. Prior to the spore counting, 5 mL of 2% Tween 20 (v/v) was added to the inoculant and then the spores from the surface of the culture medium were

suspended with the aid of a glass rod. The suspension was diluted by 1:10 (v/v) and the spore counting was performed in a Neubauer chamber (Handa et al., 2014).

2.3. Solid-state fermentation of defatted soy flour and experimental design

A mixture of 10 g of defatted soy flour in 10 mL of distilled water was used in solid-state fermentation after adjusting the pH to 6. The mixture was then autoclaved at 121 °C for 15 min, inoculated with a suspension containing 10^7 spores, and finally incubated at 30 °C for 48 h (Handa et al., 2014). Each fungus was used separately in the fermentation process. After incubation, the samples were immediately frozen, lyophilised (Christ Alpha 2–4 LD Plus, Osterode am Harz, Germany), ground (electric grinder, model MDR301, Cadence Eletrodomésticos SA, Navegantes, SC, Brazil) and stored at −22 °C until use in further analysis.

The phenolics were extracted from defatted soy flour fermented with *M. purpureus* or *A. oryzae* using a simplex-centroid design comprising three pure solvents (x_1 = ultra-pure water, x_2 = ethanol and x_3 = methanol), three binary mixtures and one ternary mixture with two replicates (Table 1). The response functions were expressed as follows: TPC (total phenolic content), M.GLU (malonylglucoside isoflavones), A.GLU (acetylglucoside isoflavones), GLU (β-glucoside isoflavones), AGLY (aglycone isoflavones), DPPH (antioxidant activity determined by the DPPH$^{\cdot}$ assay), ABTS (antioxidant activity determined by the ABTS$^{\cdot+}$ assay) and FRAP (ferric reducing antioxidant power).

2.4. Extraction of total phenolics

Prior to the extraction of total phenolics, the defatted soy flour fermented with *M. purpureus* or *A. oryzae* was lyophilised and then it was ground in an electric grinder to pass through a standard 40-mesh sieve (particle size < 0.42 mm). Approximately 0.6 g of each sample was used in 6 g of extraction solution according to the experimental design (Table 1). The mixtures were then shaken vigorously by vortexing (Ika®lab dancer, Ika Works, Inc., Wilmington, NC, USA) every 15 min for 1 h at 25 °C. Thereafter, the mixtures were placed in an ultrasonic bath at 25 °C for 15 min, centrifuged (794×g at 4 °C for 15 min; Centrifuge 5804R, Eppendorf, Hamburg, Germany) and filtered (Millex-GV, PVDF hydrophilic membrane, 0.22 µm, Millipore, Billerica, MA, USA). The extracts obtained were analysed separately for total phenolic content, isoflavone groups and antioxidant activities.

2.5. Determination of different isoflavone groups

Separation and quantification of isoflavones were performed using ultra-high-pressure liquid chromatography (UHPLC; Acquity UPLC® System, Waters, Milford, MA, USA) according to Handa et al. (2014). The external calibrations were calculated from standard solutions (0.1, 0.05, 0.01, 0.005, 0.001 and 0.0005 mg/mL) for each isoflavone. The isoflavone content of the different groups was expressed on a molar basis (micromoles of malonylglucosides, acetylglucosides, β-glucosides or aglycones per gram of sample). The total concentration of isoflavones was expressed as mg of aglycone equivalents per 100 g of sample aiming a proper comparison with previously reported literature values. Thus, the mass of each isoflavone form was multiplied by the ratio of the molar mass of its aglycone to the molar mass of the individual isoflavone form before summing (Lima et al., 2014).

2.6. Determination of total phenolic content

Total phenolic content was measured by the Folin–Ciocalteu colorimetric method (Kumazawa et al., 2002; Singleton, Orthofer, & Lamuela-Raventos, 1999) using standard gallic acid. Total phenolic content was expressed as mg of gallic acid equivalents per gram of sample using the calibration curve of gallic acid (0.002–0.012 mg).

2.7. Antioxidant activity analyses

2.7.1. DPPH$^{\cdot}$ radical scavenging activity

The antioxidant activity of the extracts was measured based on the ability of their phenolic components to donate hydrogen to the stable free radical DPPH$^{\cdot}$ (Blois, 1958). Radical scavenging

Table 1
Simplex-centroid design and response functions expressed in the defatted and fermented samples.

Sample[a]	Mixture[b] (x_1,x_2,x_3)	Response functions[c]							
		M.GLU (µmol/g)	A.GLU (µmol/g)	GLU (µmol/g)	AGLY (µmol/g)	TPC (mg GAE/g)	DPPH (µmol TE/g)	FRAP (µmol TE/g)	ABTS (µmol TE/g)
DSSF-Mp	(1,0,0)	0	0	0	0	2.23	7.78	6.78	50.21
	(0,1,0)	0	0	0	0.13	0.04	4.00	0.48	0
	(0,0,1)	0.20	0	0.77	2.66	0.52	5.66	5.52	28.97
	(1/2,1/2,0)	0.88	0.12	1.26	3.07	2.13	7.57	17.13	68.21
	(1/2,0,1/2)	0.86	0.09	1.13	1.86	1.92	7.99	15.82	53.54
	(0,1/2,1/2)	0.00	0.00	0.00	0.81	0.12	4.25	1.37	4.11
	(1/3,1/3,1/3)	0.89	0.13	1.48	3.21	1.47	7.67	16.08	51.44
	(1/3,1/3,1/3)	0.80	0.11	1.39	2.92	1.52	7.69	15.49	55.0
	(1/3,1/3,1/3)	0.87	0.13	1.48	3.19	1.53	7.94	15.98	61.29
DSSF-Ao	(1,0,0)	0	0	0	0	6.54	9.61	12.75	186.95
	(0,1,0)	0	0	0	0	0.08	3.81	2.96	0.000
	(0,0,1)	0	0.07	1.07	1.32	0.57	5.88	8.77	28.67
	(1/2,1/2,0)	0.56	0.06	2.17	2.31	3.82	10.08	14.95	134.18
	(1/2,0,1/2)	0.27	0.19	1.00	2.53	4.38	9.25	17.16	122.55
	(0,1/2,1/2)	0	0	0	0.41	0.20	4.21	3.98	4.94
	(1/3,1/3,1/3)	0.28	0.24	2.88	2.05	2.67	8.60	10.39	76.65
	(1/3,1/3,1/3)	0.29	0.24	2.90	2.08	2.51	9.56	9.74	91.55
	(1/3,1/3,1/3)	0.30	0.25	3.02	2.10	2.60	9.18	10.10	91.03

GAE = gallic acid equivalents, TE = Trolox equivalents.
TPC = total phenolic content, DPPH = antioxidant activity determined by the DPPH$^{\cdot}$ assay, FRAP = ferric reducing antioxidant power, ABTS = antioxidant activity determined by the ABTS$^{\cdot+}$ assay.
[a] DSSP-Mp = defatted soy flour fermented with *Monascus purpureus*, DSSP-Ao = defatted soy flour fermented with *Aspergillus oryzae*.
[b] Proportion of water:ethanol:methanol (x_1:x_2:x_3; g:g:g), respectively.
[c] M.GLU = malonylglucoside isoflavones, A.GLU = acetylglucoside isoflavones, GLU = β-glucoside isoflavones, AGLY = aglycone isoflavones,

measurements were performed according to Dinis, Madeira, and Almeida (1994). The free radical scavenging activity of the extracts was expressed as micromoles of Trolox equivalents per gram of sample using the calibration curve of Trolox (0.01–0.04 μmol).

2.7.2. FRAP assay

The ferric reducing antioxidant power of the extracts was estimated as reported by Benzie and Strain (1996) with slight modifications. Briefly, 900 μL of FRAP reagent, freshly prepared and warmed to 37 °C, was mixed with 70 μL of distilled water and 30 μL of either the test sample, the standard or the appropriate reagent blank. The FRAP reagent consisted of 2.5 mL of a 10 mmol/L TPTZ solution in 40 mmol/L HCl, 2.5 mL of 20 mmol/L $FeCl_3{\cdot}6H_2O$ and 25 mL 0.3 mmol/L acetate buffer at pH 3.6. The temperature was maintained at 37 °C. The absorbance at 595 nm was measured after 30 min (Biochrom Libra S22, Cambridge, England), and ethanolic solutions of known Trolox concentrations were used for calibration. The ferric reducing ability of the extracts was expressed as micromoles of Trolox equivalents per gram of sample using the calibration curve of Trolox (0.003–0.018 μmol).

2.7.3. ABTS radical cation

The ABTS assay was carried out as reported by Re et al. (1999) with slight modifications. The generation of free radical $ABTS^{\cdot+}$ was performed by reacting 7 mmol/L ABTS stock solution with 2.45 mmol/L potassium persulphate and allowing the mixture to rest in the dark at 25 °C for 12–16 h before use. The $ABTS^{\cdot+}$ solution was diluted with 20 mmol/L phosphate buffered saline (pH 7.4) to an absorbance of 0.70 ± 0.02 at 730 nm. Next, 10 μL of sample or Trolox standard was added to 4 mL of diluted $ABTS^{\cdot+}$ solution, and the absorbance was measured after 6 min at 730 nm (Biochrom Libra S22, Cambridge, England). Ethanolic solutions containing known amounts of Trolox were used for calibration. The free radical scavenging activity of the extracts was expressed as micromoles of Trolox equivalents per gram of sample using the calibration curve of Trolox (0.015–0.100 μmol).

2.8. Statistical analysis

All results in triplicate were expressed on a dry weight basis in relation to the fermented or control samples. A Scheffé special cubic model was expressed for each response function from the simplex-centroid design:

$$y = \sum_{i=1}^{q} b_i^* x_i + \sum_{i<j}^{q}\sum_{j}^{q} b_{ij}^* x_i x_j + \sum_{i<j}^{q}\sum_{j<k}^{q}\sum_{k}^{q} b_{ijk}^* x_i x_j x_k + \cdots + b_{12\ldots q}^* x_1 x_2 \cdots x_q$$

where y = estimated response, b^* = coefficient estimated by the least squares method, and x_i = independent variable, with $1 > x_i > 0$ and $\sum x_i = 1$ (i.e., 100 wt%). The b_i^* parameter is the linear coefficient related to the pure component i; b_{ij}^* is the quadratic coefficient of binary interaction for components i and j; and b_{ijk}^* is the cubic coefficient of ternary interaction for components i, j and k (Cornell, 2002, chap. 2).

The mathematical models were subjected to analysis of variance (ANOVA) and regression analysis using Statistica 10.0 software (StatSoft, Tulsa, OK, USA). Contour plots of the responses were generated from adjusted models. The simultaneous optimisation of the response variables was based on the overall desirability function (Derringer & Suich, 1980). Models were validated using Student's t-test ($\alpha = 0.05$), where the average ($n = 3$) of the experimental values was compared with the estimated responses ($n = 3$) of the models. In addition, a one-sample t-test ($\alpha = 0.05$) was used to compare the optimised results from each response function studied herein with previously reported literature values, whose population standard deviation is unknown.

3. Results

3.1. Effects of the solvent system and sample matrix on isoflavone extraction

The response functions were expressed separately for defatted soy flour fermented with *M. purpureus* or *A. oryzae* (Table 2). The models fitted to the experimental data had determination coefficients (R^2) of 99% and adjusted R^2 values between 96% and 99% (Table 3).

Regarding the isoflavone extraction from defatted soy flour fermented with *M. purpureus*, the binary interaction coefficients (water/ethanol and water/methanol) were significant ($p < 0.05$) for the response functions malonylglucosides and acetylglucosides (Table 2). The maximum responses (malonylglucosides = 0.93 μmol/g and acetylglucosides = 0.13 μmol/g) were estimated when the same solvent combination (0.5:0.25:0.25, wt/wt/wt, water:ethanol:methanol; Supplementary Material: Fig. S1a,b) was used. Both binary and ternary interactions were significant for the response function β-glucosides, and the binary mixture ethanol/methanol negatively influenced this response. Thus, the maximum β-glucoside concentration (1.50 μmol/g) was estimated using the following solvent proportions: 0.5:0.25:0.25 (wt/wt/wt, water:ethanol:methanol) (Supplementary Material: Fig. S2c). The β-glucosides and aglycones were the only response functions related to the isoflavones that showed one significant linear term (x_3 = methanol), and the ternary mixture (water/ethanol/methanol) strongly and positively affected these response functions, as evidenced by the high value of the regression coefficient (Table 2). A synergistic effect of the solvents on the extraction of aglycone isoflavones was observed in the extraction mixture with 0.5:0.375:0.125 (wt/wt/wt, water:ethanol:methanol) (Supplementary Material: Fig. S2d), which was estimated to extract the largest amount of aglycones (3.21 μmol/g).

On the basis of the mathematical models (Table 2) related to the isoflavones extracted from defatted soy flour fermented with *A. oryzae*, the coefficients of the interactions water/ethanol and water/methanol as well as water/ethanol/methanol were significant for the response function malonylglucosides, in which the highest estimated response was 0.56 μmol/g when a ratio of 0.5:0.5:0 (wt/wt/wt, water:ethanol:methanol) (Supplementary Material: Fig. S3a) was used. The dependent variables acetylglucosides, β-glucosides and aglycones had the same significant terms (Table 2) despite exhibiting different effects. The maximum responses (acetylglucosides = 0.25 μmol/g, β-glucosides = 2.90 μmol/g and aglycones = 2.55 μmol/g) were estimated from the following combinations: 0.33:0.25:0.42 (wt/wt/wt, water:ethanol:methanol) (Supplementary Fig. S3b); 0.5:0.3:0.2 (wt/wt/wt, water:ethanol:methanol) (Supplementary Fig. S3c); and 0.33:0:0.67 (wt/wt/wt, water:ethanol:methanol) (Supplementary Fig. S3d), respectively (Supplementary Material).

3.2. Impacts of the solvent system and sample matrix on total phenolic content and antioxidant activities

Analysing the mathematical models related to total phenolic content (TPC) and antioxidant activities from defatted soy flour fermented with *M. purpureus*, the linear terms (x_1 = water and x_3 = methanol) and binary interaction (water/ethanol) were significant ($p < 0.05$) for the response functions TPC_{Mp}, $DPPH_{Mp}$, $FRAP_{Mp}$ and $ABTS_{Mp}$. Furthermore, the coefficient from the ternary mixture was significant for $DPPH_{Mp}$ and $FRAP_{Mp}$. These terms had a positive

Table 2
Regression coefficients of the special cubic models.

Sample[a]	Response functions[b]	Regression coefficients						
		x_1	x_2	x_3	x_1x_2	x_1x_3	x_2x_3	$x_1x_2x_3$
DSSF-Mp	$M.GLU_{Mp}$	0	0	−0.20	3.54*	3.05*	−0.40	2.56
	$A.GLU_{Mp}$	0	0	0	0.49*	0.34*	0	0.84
	GLU_{Mp}	0	0	0.77*	5.04*	2.98*	−1.53*	12.75*
	$AGLY_{Mp}$	0	0.13	2.66*	12.03*	2.13	−2.32	23.34*
	TPC_{Mp}	2.23*	0.04	0.52*	3.99*	2.18*	−0.63	−1.05
	$DPPH_{Mp}$	7.78*	4.00*	5.66*	6.71*	5.07*	−2.34	24.45*
	$FRAP_{Mp}$	6.78*	0.48	5.52*	54.00*	38.68*	−6.52	54.50*
	$ABTS_{Mp}$	50.21*	0	28.97*	172.43*	55.79	−41.51	236.75
DSSF-Ao	$M.GLU_{Ao}$	0	0	0	2.24*	1.06*	0	−2.09*
	$A.GLU_{Ao}$	0	0	0.07*	0.25*	0.61*	−0.14*	3.80*
	GLU_{Ao}	0	0	1.07*	8.69*	1.85*	−2.14*	44.34*
	$AGLY_{Ao}$	0	0	1.32*	9.25*	7.50*	−0.98*	−3.10*
	TPC_{Ao}	6.53*	0.08	0.57*	2.07*	3.32*	−0.47	−9.33
	$DPPH_{Ao}$	9.61*	3.81*	5.88*	13.46*	6.01	−2.53	21.59
	$FRAP_{Ao}$	12.75*	2.96*	8.77*	28.37*	25.60*	−7.54*	−87.57*
	$ABTS_{Ao}$	186.95*	0	28.67	162.82	58.95	−37.59	−160.07

x_1 = water, x_2 = ethanol and x_3 = methanol.
TPC = total phenolic content, DPPH = antioxidant activity determined by the DPPH· assay, ABTS = antioxidant activity determined by the $ABTS^{+\bullet}$ assay, FRAP = ferric reducing antioxidant power.
[a] DSSP-Mp = defatted soy flour fermented with *Monascus purpureus*, DSSP-Ao = defatted soy flour fermented with *Aspergillus oryzae*.
[b] M.GLU = malonylglucoside isoflavones, A.GLU = acetylglucoside isoflavones, GLU = β-glucoside isoflavones, AGLY = aglycone isoflavones,
* Significant ($p < 0.05$).

effect on the extraction of phenolic compounds and their antioxidant activities. The combinations of solvents used to obtain the highest values for each response function were estimated as follows: 1:0:0 (wt/wt/wt, water:ethanol:methanol; TPC_{Mp} = 2.23 mg of gallic acid equivalents/g); 0.75:0.125:0.125 (wt/wt/wt, water:ethanol:methanol; $DPPH_{Mp}$ = 8.39 μmol of Trolox equivalents/g); 0.5:0.25:0.25 (wt/wt/wt, water:ethanol:methanol; $FRAP_{Mp}$ = 17.77 μmol of Trolox equivalents/g); and 0.75:0.2:0.05 (wt/wt/wt, water:ethanol:methanol; $ABTS_{Mp}$ = 68.42 μmol of Trolox equivalents/g) (Supplementary Material: Fig. S4a–d).

Regarding the total phenolic content and antioxidant activities from defatted soy flour fermented with *A. oryzae*, the coefficients related to pure solvents, primarily water (x_1), were important for increasing the response functions TPC_{Ao}, $DPPH_{Ao}$, $FRAP_{Ao}$ and $ABTS_{Ao}$. All of the terms in the $FRAP_{Ao}$ response function were significant ($p < 0.05$), whereas $ABTS_{Ao}$ had only one significant term (x_1 = water). The solvent mixtures that yielded maximum values for each response function include the following: 1:0:0 (wt/wt/wt, water:ethanol:methanol; TPC_{Ao} = 6.53 mg of gallic acid equivalents/g); 0.75:0.125:0.125 (wt/wt/wt, water:ethanol:methanol; $DPPH_{Ao}$ = 10.46 μmol of Trolox equivalents/g); 0.5:0:0.5 (wt/wt/wt, water:ethanol:methanol; $FRAP_{Ao}$ = 17.16 μmol of Trolox equivalents/g); and 1:0:0 (wt/wt/wt, water:ethanol:methanol; $ABTS_{Ao}$ = 186.95 μmol of Trolox equivalents/g) (Supplementary Material: Fig. S5a–d).

3.3. Multi-response optimisation and model validation

According to the joint optimisation of the total phenolic and isoflavones contents as well as antioxidant activities from defatted soy flour fermented with *M. purpureus* or *A. oryzae* (Figs. 1 and 2), the maximum responses were estimated at solvent ratios of 0.5:0.375:0.125 and 0.5:0.3:0.2 (wt/wt/wt, water:ethanol:methanol), respectively. However, a water:ethanol ratio of 0.5:0.5 (wt/wt) was chosen as optimal because it consists of green extraction solvents and yielded results that were greater than 90% of the multi-response maximum values. Thus, this solvent system was used to validate all of the models for both the defatted soy flour fermented with *M. purpureus* or *A. oryzae*. A sample of non-fermented defatted soy flour (control) was also extracted with the optimal solvent mixture and characterised using the same response functions expressed in the fermented samples. The models were verified to be significant (Table 3) and appropriate for estimative finalities because the experimentally observed values were not significantly different ($p > 0.05$) from the estimated values of the models (Table 4). Extraction yields of total phenolics and isoflavones as well as antioxidant activity values that were obtained using the optimal solvent mixture (0.5:0.5, wt/wt, water:ethanol) were compared to another previously reported literature values for defatted soy flour and soybeans (Table 5).

The fermentation process caused significant changes in the isoflavone profiles and antioxidant activities and increased the total extractable phenolics from fermented defatted soy flour. The highest values of total phenolic content and antioxidant activities for DPPH and ABTS were found in defatted soy flour fermented with *A. oryzae*, whereas the aglycone isoflavone content was higher in defatted soy flour fermented with *M. purpureus*. The antioxidant activities determined by the ABTS and FRAP methods were not significantly different between the defatted soy flour fermented with *M. purpureus* and the control (Table 4).

4. Discussion

All of the groups of isoflavones (aglycones, acetylglucosides, malonylglucosides and β-glucosides) present in the fermented samples were more efficiently extracted using solvent mixtures containing water as the primary solvent. Furthermore, the water:ethanol:methanol solvent mixture substantially increased the isoflavone content of the extracts. Likewise, Yoshiara, Madeira, Delaroza, Silva, and Ida (2012) reported that the extraction of different isoflavone forms from defatted cotyledon soy flour was affected by the solvent system and the solvent:solvent ratio, and a water:acetone:ethanol solvent mixture extracted these compounds most effectively. These observations may be attributed to differences in the hydrophobicity (aglycones > acetylglucosides > malonylglucosides > β-glucosides) among the isoflavones (Murphy et al., 1999). The solubility of flavonoids in aqueous media is primarily associated with hydroxyl groups (Supplementar Material: Fig. S1), whereas methyl and acetyl radicals decrease the polarity of the flavonoids. Thus, the presence of glucose in the

Table 3
ANOVA of the special cubic models adjusted to the experimental data.

Sample[a]	Source	df	Response functions[b]													
			M.GLU		A.GLU		GLU		AGLY		TPC		DPPH		FRAP		ABTS	
			SS	*F*-Value	SS	*F*-Value	SS	*F*-Value	SS	*F*-Value	SS	*F*-Value	SS	*F*-Value	SS	*F*-Value	SS	*F*-Value
DSSF-Mp	Model	6	1.49	112.69*	0.03	65.49*	3.50	198.31*	14.20	89.55*	5.64	829.25*	21.36	159.44*	380.59	635.34*	4834.63	32.41*
	Total error	2	0.004		0.0002		0.006		0.05		0.002		0.04		0.20		49.73	
	Pure error	2	0.004		0.0002		0.006		0.05		0.002		0.04		0.20		49.73	
	Total adjusted	8	1.49		0.03		3.50		14.25		5.64		21.41		380.79		4884.36	
	R^2			0.99		0.99		0.99		0.99		0.99		0.99		0.99		0.99
	Adjusted R^2			0.99		0.98		0.99		0.98		0.99		0.99		0.99		0.96
DSSF-Ao	Model	6	0.32	613.28*	0.10	394.79*	13.78	401.44*	8.38	2225.43*	36.40	896.55*	48.24	34.34*	170.49	267.52*	30947.14	72.11*
	Total error	2	0.0002		0.00008		0.01		0.001		0.01		0.47		0.21		143.05	
	Pure error	2	0.0002		0.00008		0.01		0.001		0.01		0.47		0.21		143.05	
	Total adjusted	8	0.32		0.10		13.79		8.39		36.42		48.70		170.70		31090.19	
	R^2		0.99			0.99		0.99		0.99		0.99		0.99		0.99		0.99
	Adjusted R^2		0.99			0.99		0.99		0.99		0.99		0.96		0.99		0.98

[a] DSSF-Mp = defatted soy flour fermented with *Monascus purpureus*, DSSF-Ao = defatted soy flour fermented with *Aspergillus oryzae*.
[b] M.GLU = malonylglucoside isoflavones, A.GLU = acetylglucoside isoflavones, GLU = β-glucoside isoflavones, AGLY = aglycone isoflavones, TPC = total phenolic content, DPPH = antioxidant activity determined by the DPPH• assay, FRAP = ferric reducing antioxidant power, ABTS = antioxidant activity determined by the ABTS•+ assay.
* Significant ($p < 0.05$).

chemical structure of acetylglucosides, malonylglucosides and β-glucosides increases the water solubility of these isoflavones.

The recovery level of total isoflavones from a given sample depends on the sample-solvent interactions and the efficiency of the solvent system to extract the predominant forms of isoflavones that are present in this sample matrix, and therefore, is almost impossible to suggest a single solvent composition that ensures maximum extraction yields for all isoflavones from any soy sample (Rostagno et al., 2009). Because the β-glucosides and aglycones were the major forms of isoflavone found in the samples fermented with *M. purpureus* or *A. oryzae* (Table 4), these compounds strongly influenced the multi-response optimisation of the extraction solvent composition. With the exception of the extraction of aglycones and acetylglucosides from the sample fermented with *A. oryzae*, the best solvent mixture to extract isoflavones was comprised of 50 wt% water (Supplementary Material: Figs. S2 and S3). On the basis of the overall desirability function, the optimal solvent mixtures had this same proportion of water (Figs. 1 and 2). Rostagno et al. (2003) also found that extraction mixtures containing from 40 to 60 v% water is needed for efficient extraction of soybean isoflavones. Similarly, Murphy et al. (1999) reported that extraction mixtures containing at least 29 v% water substantially improved the recovery of isoflavones from soy protein isolate, soy flour, tofu and miso. This observed increase in extractability of isoflavones into media with high water content can be due to their interactions with proteins and carbohydrates, which have high water solubility. Even though the pure methanol showed good affinity towards aglycones because of its intermediate polarity, pure methanol and ethanol or a mixture of both were not good enough for extraction of the different isoflavone groups (Table 1).

The solubility order of isoflavone standards is not completely consistent with the polarity order of solvents. Fan, Xu, Shen, and Zhang (2015) verified that the solubility order of genistin standard (β-glucoside) in pure solvents was acetone > tetrahydrofuran > methanol > ethanol > isopropanol > n-butyl alcohol > acetonitrile ≈ ethyl acetate > cyclohexane > n-hexane > chloroform, despite the polarity order of these solvents is methanol > ethanol > n-butyl alcohol > isopropanol > acetonitrile > acetone > ethyl acetate > chloroform > tetrahydrofuran > n-hexane > cyclohexane. Methanol and ethanol can form strong hydrogen bonds between solvent molecules because of their hydrogen bond donation and acceptance abilities, which would explain the lower solubility of genistin in methanol and ethanol than that in tetrahydrofuran and acetone. Nan et al. (2014) reported that the solubility of daidzein standard (aglycone) in six pure solvents was acetone > methanol > ethyl ethanoate > hexane > trichloromethane > water, whereas the solubility order of genistein standard (aglycone) was acetone > ethyl ethanoate > methanol > hexane > trichloromethane > water. The differences in the solubility of these isoflavones were mainly attributed to the 5-hydroxyl group of genistein (Supplementary Material: Fig. S1). However, the solubility characteristics of the isoflavones from a food matrix (real system) may be different from the isoflavone standards, since the physicochemical properties of these phenolics can be altered depending on their interaction with proteins, carbohydrates and lipids present in the matrix (Achouri et al., 2005; Jakobek, 2015; Speroni, Milesi, & Añón, 2010). For instance, although the isoflavone standards exhibit poor water solubility, this solvent is essential to extract isoflavones from soy food matrices by the solid–liquid extraction process.

The solvent viscosity can also affect the extractability of bioactive compounds from plant materials. A low solvent viscosity enables greater diffusion into the pores of the plant matrices, improving the extraction of substances (Wijekoon, Bhat, & Karim, 2011). Among the response functions, only TPC and DPPH had the same optimal extraction solvent compositions (1:0:0, wt/wt/wt, water:ethanol:methanol for TPC and 0.75:0.125:0.125, wt/wt/

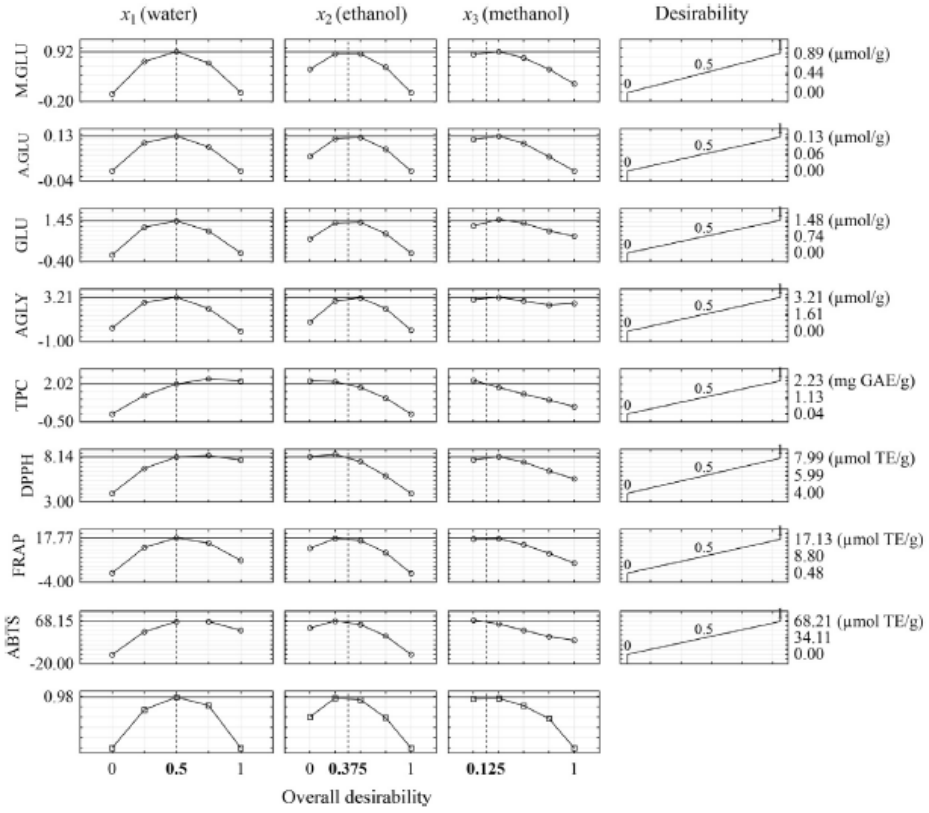

Fig. 1. Profiles for the predicted values and overall desirability as a function of the solvent system for defatted soy flour fermented with *Monascus purpureus*. M.GLU = malonylglucoside isoflavones, A.GLU = acetylglucoside isoflavones, GLU = β-glucoside isoflavones, AGLY = aglycone isoflavones, TPC = total phenolic content, DPPH = antioxidant activity determined by the DPPH· assay, FRAP = ferric reducing antioxidant power, ABTS = antioxidant activity determined by the ABTS·⁺ assay. GAE = gallic acid equivalents, TE = Trolox equivalents.

wt, water:ethanol:methanol for DPPH) for both fermented samples, indicating that both the solvent system and solvent:solvent ratio are influenced by the target compounds and characteristics of each fermented sample. For instance, the high proteolytic and amylolytic activities of *A. oryzae* (Castro & Sato, 2014) likely affected protein–phenolic and carbohydrate–phenolic interactions, such as hydrogen binding and hydrophobic interactions, which can influence the solubility and extractability of isoflavones and phenolic acids from defatted soy flour fermented with *M. purpureus* (Jakobek, 2015; Malaypally & Ismail, 2010). This partially explains the differences observed in the solvent system and solvent:solvent ratio when comparing these parameters to a similar response function expressed for both the defatted soy flour fermented with *M. purpureus* or *A. oryzae*.

Phenolic compounds occur as soluble conjugates and insoluble forms. The sugars are very common in the soluble forms, whereas the insoluble forms are covalently bound to cellulose, hemicellulose, lignin, pectin and structural proteins. In plants, phenolic acids are found in insoluble or bound forms, whereas flavonoids typically occur as glucosides (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014). Enzymes such as pectinases, cellulases, amylases, hemicellulases and glucanases are also typically applied to disintegrate plant cell wall matrices and facilitate the extraction of phenolics (Stalikas, 2007). Differences between *M. purpureus* and *A. oryzae* with respect to their ability to synthesise these enzymes may have also contributed to the observed differences in the solvent systems, solvent:solvent ratios and compound profiles for the same response function expressed in the fermented samples. Kim et al. (2013) observed a reduction in the total flavonoid content of samples fermented with *A. oryzae* accompanied by a significant increase in antioxidant activity. This phenomenon was partially attributed to the increased levels of unidentified metabolites formed during the fermentation process. Handa et al. (2014) verified that the fermentation of defatted soy flour contributed to the conversion of β-glucoside isoflavones into aglycones by means of β-glucosidase synthesised by *M. purpureus* or *A. oryzae*. However, the β-glucosides were more efficiently hydrolysed by the enzyme from *M. purpureus* compared with that from *A. oryzae*.

With the exception of the FRAP assay, all assays used in this study showed that the antioxidant activities were influenced by the profile of compounds extracted from fermented defatted soy flour. According to Xu, Yuan, and Chang (2007), the antioxidant capacities of legumes are strongly correlated with the total phenolic content. Kim et al. (2006) found that syringic, chlorogenic, gallic and ferulic acids are the major phenolic compounds in whole soybean, and DPPH activity had a strong positive correlation with all phenolic compounds except for myricetin, naringenin, hesperetin and biochanin A. Tyug, Prasad, and Ismail (2010) found a strong positive correlation between total phenolic content and FRAP and between total phenolic content and ABTS in soybean by-products. Moreover, the vanillic, ferulic and chlorogenic acids; daidzein and genistein may possess the capacity to donate a single

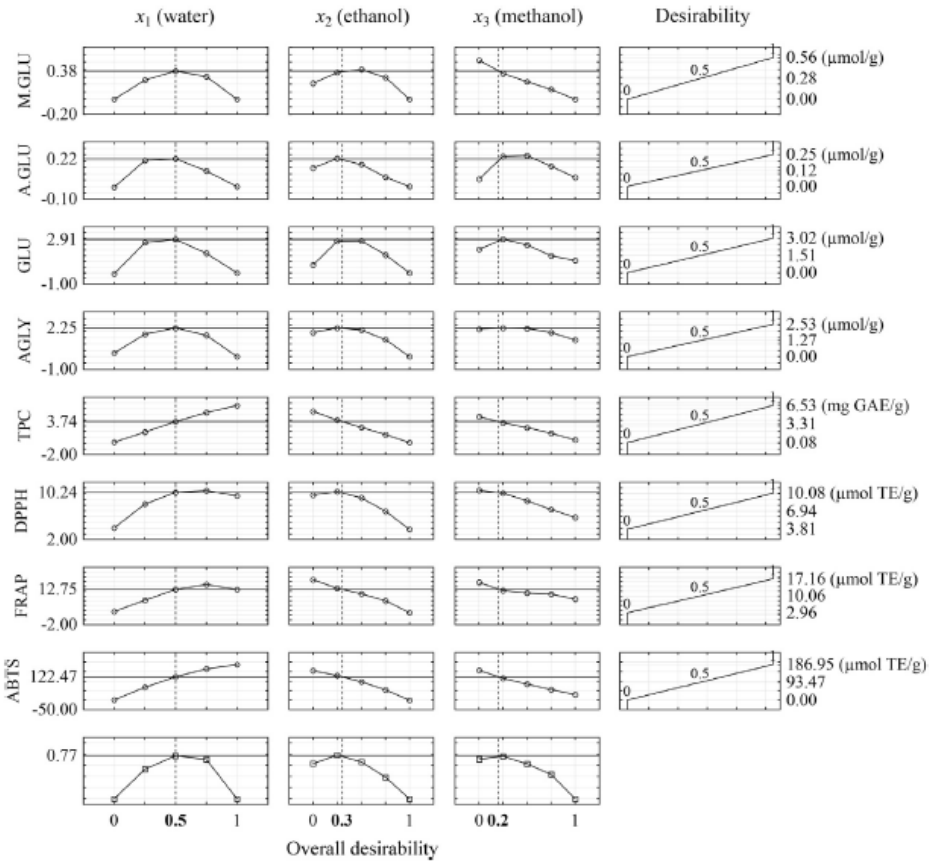

Fig. 2. Profiles for the predicted values and overall desirability as a function of the solvent system for defatted soy flour fermented with *Aspergillus oryzae*. M.GLU = malonylglucoside isoflavones, A.GLU = acetylglucoside isoflavones, GLU = β-glucoside isoflavones, AGLY = aglycone isoflavones, TPC = total phenolic content, DPPH = antioxidant activity as determined by the DPPH· assay, FRAP = ferric reducing antioxidant power, ABTS = antioxidant activity as determined by the $ABTS^{\cdot+}$ assay. GAE = gallic acid equivalents, TE = Trolox equivalents.

Table 4
Total phenolic content (mg of gallic acid equivalents/g), isoflavones (μmol/g) and antioxidant activities (μmol of Trolox equivalents/g) that were obtained using the optimal solvent mixture (50 wt% aqueous ethanol).

Response functions[a]	DSFF-Mp		DSFF-Ao		DSF-control
	Predicted values	Observed values	Predicted values	Observed values	
M.GLU	0.88^{A}	0.84 ± 0.01^{Ab}	0.56^{A}	0.50 ± 0.00^{Ac}	0.89 ± 0.00^{a}
A.GLU	0.12^{A}	0.11 ± 0.00^{Ab}	0.06^{A}	0.09 ± 0.00^{Ac}	0.55 ± 0.00^{a}
GLU	1.26^{A}	1.13 ± 0.06^{Ac}	2.17^{A}	1.81 ± 0.02^{Ab}	3.77 ± 0.01^{a}
AGLY	3.07^{A}	2.83 ± 0.02^{Aa}	2.31^{A}	2.22 ± 0.01^{Ab}	0.78 ± 0.00^{c}
TPC	2.13^{A}	2.24 ± 0.03^{Ab}	3.82^{A}	4.28 ± 0.2^{Aa}	1.60 ± 0.03^{c}
DPPH	7.57^{A}	6.77 ± 0.80^{Ab}	10.08^{A}	8.25 ± 0.24^{Aa}	2.10 ± 0.15^{c}
FRAP	17.13^{A}	16.98 ± 0.36^{Aa}	14.95^{A}	16.54 ± 0.65^{Aa}	17.09 ± 0.84^{a}
ABTS	68.21^{A}	67.19 ± 1.39^{Ab}	134.18^{A}	144.06 ± 5.48^{Aa}	61.31 ± 3.12^{b}

Results are expressed as the mean ($n = 3$) ± standard deviation.
Means with identical capital letters in the same line were not significantly different ($p > 0.05$).
Means followed by different superscript lowercase letters in the same line were significantly different ($p < 0.05$).
DSSP-Mp = defatted soy flour fermented with *Monascus purpureus*, DSSP-Ao = defatted soy flour fermented with *Aspergillus oryzae* and DSF-control = non-fermented defatted soy flour.
[a] M.GLU = malonylglucoside isoflavones, A.GLU = acetylglucoside isoflavones, GLU = β-glucoside isoflavones, AGLY = aglycone isoflavones, TPC = total phenolic content, DPPH = antioxidant activity determined by the DPPH· assay, FRAP = ferric reducing antioxidant power, ABTS = antioxidant activity determined by the $ABTS^{\cdot+}$ assay.

electron to the FRAP reagent and $ABTS^{\cdot+}$. These compounds also had the greatest contribution to total phenolic content.

Hydroxyl substitutions on the aromatic B-ring particularly affect the antioxidant activities of flavonoids. Although genistein and daidzein have the same B-ring structure, genistein might have higher free radical scavenging capacity because it has an additional hydroxyl group on the AC-ring (Supplementary Material: Fig. S1). One way that polyphenolic compounds act as antioxidants is by

Table 5
One-sample *t*-test: comparison of optimised[a] results for the total phenolic content, total isoflavones and antioxidant activity with previously reported literature values for defatted soy flour or soybeans.

Function response[b]	Mean (n = 3) ± SD	Comparison value[c]	t	Reference
TISO (control)	90.6 ± 1.13	101	−11.7*	Pinto, Lajolo, and Genovese (2005)
$TISO_{Mp}$	142.19 ± 3.81	101	18.3*	Sample: defatted soy flour
$TISO_{Ao}$	134.53 ± 3.3	101	17*	Technique: magnetic stirring
				Solvent: 80 v% aqueous methanol
				Sample-solvent ratio: 1:20 (wt/v)
				Temperature/time: 4 °C for 2 h
TISO (control)	90.6 ± 1.13	112.4	−24.6*	Wang and Murphy (1994)
$TISO_{Mp}$	142.19 ± 3.81	112.4	12.6*	Sample: defatted soy flour
$TISO_{Ao}$	134.53 ± 3.3	112.4	11.3*	Technique: magnetic stirring
				Solvent: acetonitrile:0.1 N HCl (5:1, v/v).
				Sample-solvent ratio: 1:6 (wt/v)
				Temperature/time: room temperature for 2 h
TPC (control)	1.60 ± 0.03	2.62	−88.3*	Xu and Chang (2007)
TPC_{Mp}	2.24 ± 0.03	2.62	−23.7*	Sample: yellow soybean (full-fat)
TPC_{Ao}	4.28 ± 0.2	2.62	14.4*	Technique: shaking
				Solvent: 50 v% aqueous acetone
				Sample-solvent ratio: 1:10 (wt/v)
				Temperature/time: 25 °C for 3 h followed by 12 h in the dark overnight
DPPH (control)	2.10 ± 0.15	1.83	3.4	Xu and Chang (2007)
$DPPH_{Mp}$	6.77 ± 0.8	1.83	10.1*	Sample: yellow soybean (full-fat)
$DPPH_{Ao}$	8.25 ± 0.24	1.83	53.6*	Technique: shaking
				Solvent: 80 v% aqueous acetone
				Sample-solvent ratio: 1:10 (wt/v)
				Temperature/time: 25 °C for 3 h followed by 12 h in the dark overnight

Control = non-fermented defatted soy flour.
The subscripts Mp and Ao refer to the defatted soy flour fermented with *Monascus purpureus* or *Aspergillus oryzae*, respectively.
[a] Optimal solvent mixture = 0.5:0.5 (wt/wt, water:ethanol).
[b] TISO = total isoflavones expressed as aglycone equivalents (mg/100 g), TPC = total phenolic content (mg of gallic acid equivalents/g), DPPH = antioxidant activity determined by the DPPH· assay (μmol of Trolox equivalents/g).
[c] Experimental values reported in the corresponding reference.
* Significant parameters ($p < 0.05$).

transferring a hydrogen atom. The reaction pathway involving the 4'-OH site of the daidzein isoflavone is energetically more favourable than the reaction pathway involving the 7-OH site of daidzein (Chakraborty & Biswas, 2012).

The fermentation process caused an increase in the content of aglycone isoflavones, antioxidant activities and total extractable phenolics from fermented defatted soy flour (Table 4). The defatted soy flour fermented with *M. purpureus* or *A. oryzae* had total phenolic contents that were 1.4- and 2.7-fold higher, respectively, than that of the control. The bioconversion of isoflavones from defatted soy flour led to 3.7- and 2.9-fold increases in aglycones compared to the control after fermentation with *M. purpureus* and *A. oryzae*, respectively. Nevertheless, the increase in antioxidant activity was more pronounced in the sample fermented with *A. oryzae*, which agrees with the results reported by Lin, Wei, and Chou (2006) for soybeans fermented with different filamentous fungi. Similarly, Juan and Chou (2010) observed that the total phenolic and flavonoid contents and antioxidant activity increased in black soybeans after solid-state fermentation with *Bacillus subtilis*. The observed increase in antioxidant activity after fermentation can be partially attributed to the individual phenolic compounds potentially released from the sample matrix and bioconverted during fermentation. Moreover, this increase in antioxidant activity may also be due to antioxidant peptides formed from hydrolysis of proteins throughout fermentation (Qin, Jin, & Heui, 2010). Yokomizo, Takenaka, and Takenaka (2002) verified that the antioxidant activity of hydrolysates obtained from okara protein using proteases from *A. oryzae* depended on the characteristics of the peptide amino acid sequences.

Regarding the fermented samples, the extraction yields of total phenolics and isoflavones as well as antioxidant activity values that were obtained using the optimal solvent mixture (0.5:0.5, wt/wt, water:ethanol) indicate that is possible achieve similar or even higher extraction yields of antioxidant compounds than those reported in the literature for defatted soy flour or soybeans (Table 5). These results can be attributed to the higher aglycone content from the fermented samples than that non-fermented and this is highly desirable because aglycones have high bioactivity, including antioxidant and anticarcinogenic activities (Crozier et al., 2009). Therefore, the solid-state fermentation should be encouraged for obtaining functional defatted soy flour and ethanolic extracts rich in bioactive compounds and with high antioxidant activity.

The results of this study help to clarify the relationship between the solvent composition and sample matrix on the extraction yield of phenolics. This work may contribute to the preparation of various functional foods. For instance, the defatted soy flour fermented with *M. purpureus* or *A. oryzae* can be added to bakery products (biscuits and breads) during their production process. After the extraction process of phenolics, the ethanolic extracts may be concentrated in a rotary evaporator to remove ethanol and then used in different food formulations, such as cookies, tortilla, pancake mixes and soymilk.

5. Conclusion

A simplex-centroid design proved to be an efficient tool for the optimisation of the extraction of phenolics from defatted soy flour fermented with *M. purpureus* or *A. oryzae* and their antioxidant activities. Because the sample matrix can strongly influence the extraction of total phenolics and isoflavones, the solvent system should be optimised for each sample type, especially in fermented samples. The use of green solvents for extraction of phenolics should be highly encouraged when their efficiency is similar to that of organic solvents. The extraction mixture comprised of water: ethanol (0.5:0.5, wt/wt) can be used to obtain extracts with high

phenolics content and antioxidant activity from defatted soy flour fermented with *M. purpureus* or *A. oryzae*. This environmentally friendly extraction condition can have significant contribution in the development of food and nutraceutical products.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.10.124.

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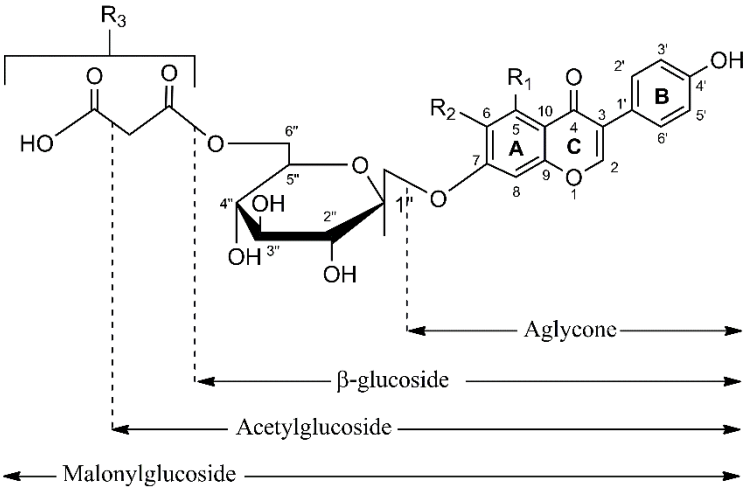
Supplementary Material for the Manuscript Entitled:

Multi-response optimisation of the extraction solvent system for phenolics and antioxidant activities from fermented soy flour using a simplex-centroid design

(Handa et al., 2015)



Group	Isoflavone	R_1	R_2	R_3
Aglycone	Daidzein	-H	-H	-
	Genistein	-OH	-H	-
	Glycitein	-H	$-OCH_3$	-
β-glucoside	Daidzin	-H	-H	-H
	Genistin	-OH	-H	-H
	Glycitin	-H	$-OCH_3$	-H
Acetylglucoside	6″-*O*-acetyldaidzin	-H	-H	$-COCH_3$
	6″-*O*-acetylgenistin	-OH	-H	$-COCH_3$
	6″-*O*-acetylglycitin	-H	$-OCH_3$	$-COCH_3$
Malonylglucoside	6″-*O*-malonyldaidzin	-H	-H	$-COCH_2COOH$
	6″-*O*-malonylgenistin	-OH	-H	$-COCH_2COOH$
	6″-*O*-malonylglycitin	-H	$-OCH_3$	$-COCH_2COOH$

Fig. S1. Chemical structure of soy isoflavones and their classification into groups.

Information related to the estimated responses of the mathematical models fitted to the experimental data for each response variable is provided below.

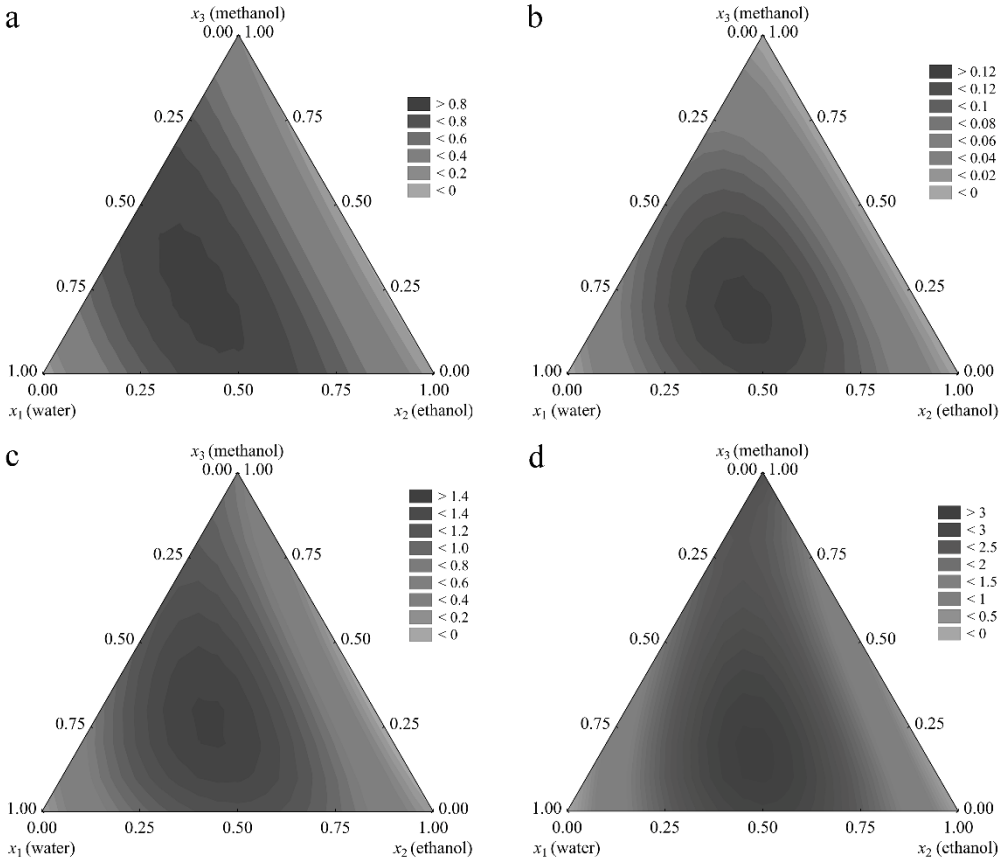


Fig. S2. Contour plots for isoflavones (µmol/g) from defatted soy flour fermented with *Monascus purpureus* as a function of the solvent system used in the extraction process. (a) malonylglucosides, (b) acetylglucosides, (c) β-glucosides, (d) aglycones.

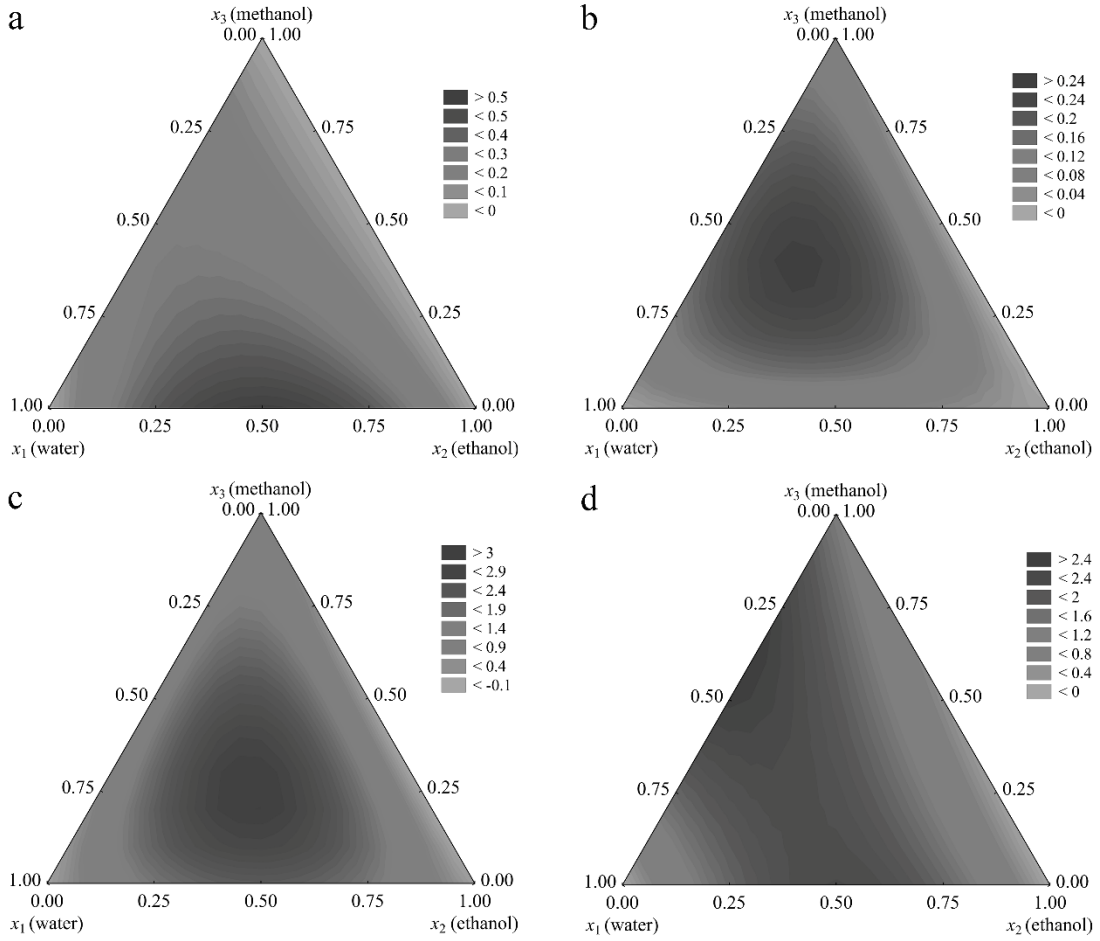


Fig. S3. Contour plots for isoflavones (µmol/g) from defatted soy flour fermented with *Aspergillus oryzae* as a function of the solvent system used in the extraction process. (a) malonylglucosides, (b) acetylglucosides, (c) β-glucosides, (d) aglycones.

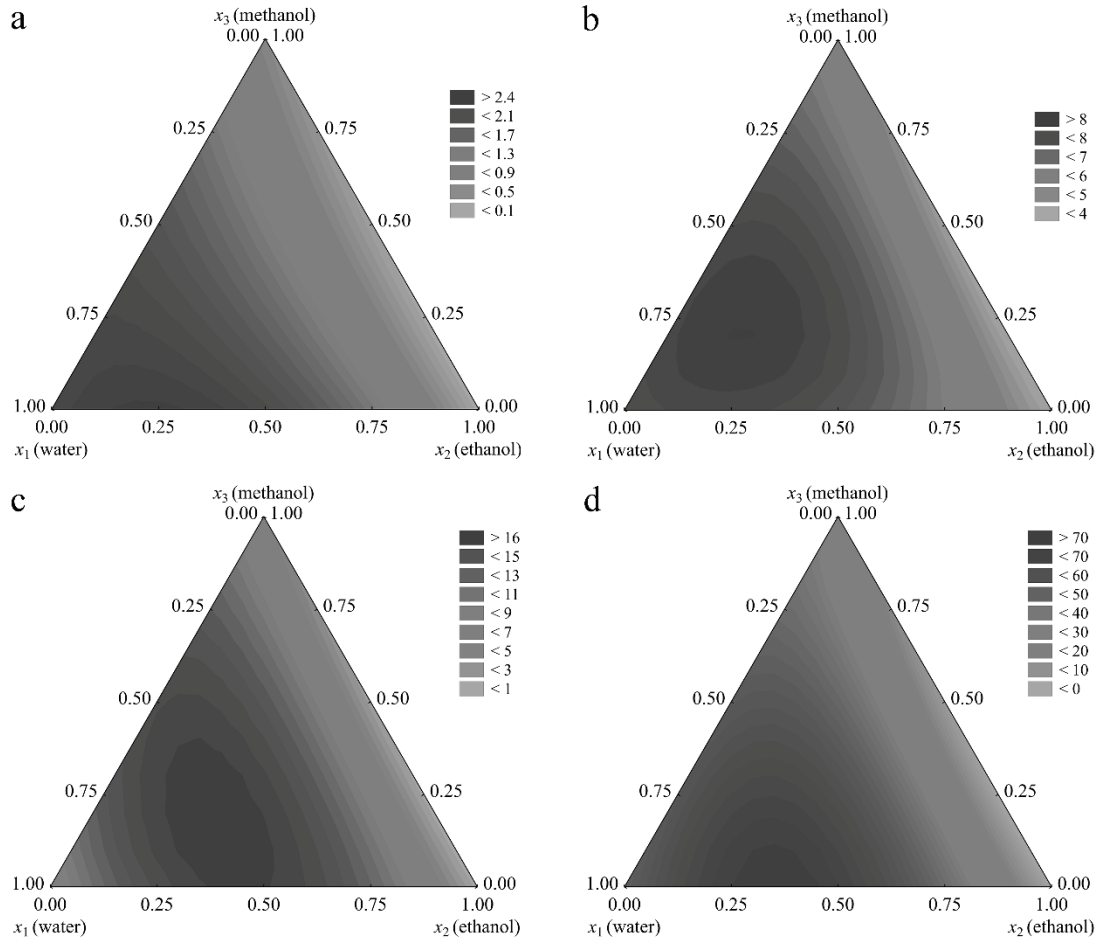


Fig. S4. Contour plots for total phenolics (mg of gallic acid equivalents/g) and antioxidant activities (µmol of Trolox equivalents/g) from defatted soy flour fermented with *Monascus purpureus* as a function of the solvent system used in the extraction process. (a) Total phenolic content, (b) antioxidant activity determined by the DPPH$^{\bullet}$ assay, (c) ferric reducing antioxidant power, (d) antioxidant activity determined by the ABTS$^{\bullet+}$ assay.

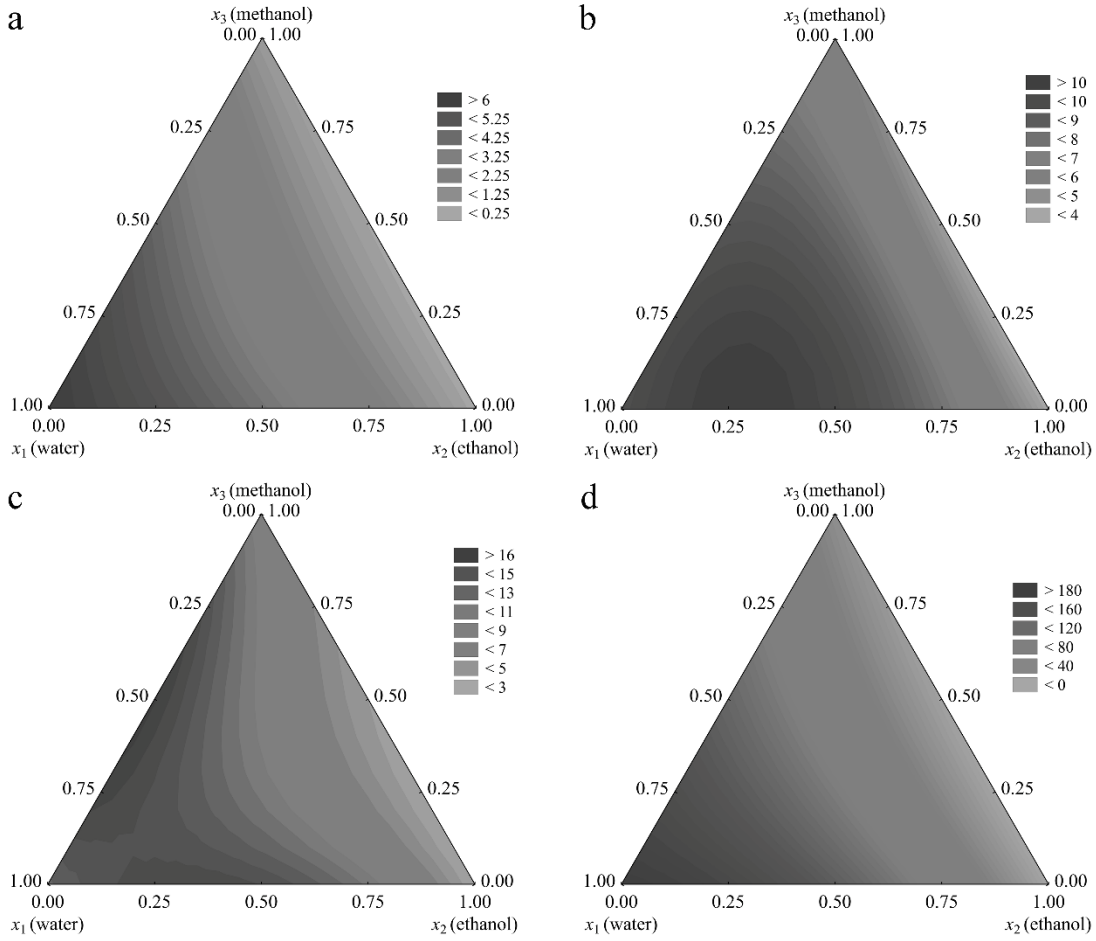


Fig. S5. Contour plots for total phenolics (mg of gallic acid equivalents/g) and antioxidant activities (μmol of Trolox equivalents/g) from defatted soy flour fermented with *Aspergillus oryzae* as a function of the solvent system used in the extraction process. (a) Total phenolic content, (b) antioxidant activity determined by the $DPPH^{\bullet}$ assay, (c) ferric reducing antioxidant power, (d) antioxidant activity determined by the $ABTS^{\bullet+}$ assay.

5.2 ARTIGO CIENTÍFICO 2

Parameters of the fermentation of soybean flour by *Monascus purpureus* or *Aspergillus oryzae* on the production of bioactive compounds and antioxidant activity

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Abstract

The objective of this work was to evaluate the effects that the solid-state fermentation parameters (initial pH, water added, and incubation temperature) of defatted soy flour by *Monascus purpureus* (DSFF-Mp) or *Aspergillus oryzae* (DSFF-Ao) have on the isoflavone and total phenolic content (TPC) and antioxidant activity (DPPH, ABTS, and FRAP). Central composite rotatable design, multi-response optimization, and Pearson's correlation were used. For DSFF-Mp, all fermentation parameters affected the content of acetyl-β-glucoside, β-glucosides, and aglycone

isoflavones but did not affect the TPC or antioxidant activity. The DSFF-Mp should be performed at an initial pH of 5.2-6.4, in 10-18 mL of water, and at 30-47 °C. For DSFF-Ao, the water and incubation temperature affected all of the isoflavone forms, the TPC, and the antioxidant activities. For DSFF-Ao, the initial pH was independent and the water should be 10-14 mL, at 22-38 °C. The correlation test indicated that fermentation favoured the formation of bioactive compounds in both DSFF-Mp and DSFF-Ao.

Keywords: Solid-state fermentation, Initial pH of substrate, Water added to substrate, Incubation temperature, Antioxidant activity.

Chemical compounds studied in this article: Daidzein (PubChem CID: 5281708), Daidzin (PubChem CID: 107971), 6''-O-Acetyldaidzin (PubChem CID: 156155), 6''-O-Malonyldaidzin (PubChem CID: 9913968), Genistein (PubChem CID: 5280961), Genistin (PubChem CID: 5281377), 6"-O-Acetylgenistin (PubChem CID: 5315831), 6''-O-Malonylgenistin (PubChem CID: 53398685), Glycitein (PubChem CID: 5317750), Glycitin (PubChem CID: 187808), 6''-O-Acetylglycitin (PubChem CID: 10228095), 6''-O-Malonylglycitin (PubChem CID: 23724657).

1. Introduction

Soybeans are the most important oilseed in the world. A large quantity of defatted soybean flour (DSF) is a product obtained from the oil extraction process. DSF has a high protein content and typically consists of 50% proteins, 40% carbohydrates, 7.5% water, and other minor components such as saponins, phenolic compounds, isoflavones, and essential amino acids (Chen, Lin, Rao, & Zeng, 2013; Hassaan, Soltan, & Abdel-Moez, 2015; Jong, 2007; Muttakin, Kim, & Lee, 2015).

Soybean fermentation has been used to develop specific foods and as a strategy for enriching soy-based products with phenolic antioxidants, which are associated with good health and well-being (McCue & Shetty, 2003). In the fermentation process by microorganisms, antioxidant phenolic compounds are either produced via secondary metabolic pathways or released from the substrate by enzymes produced by the microorganisms, thus improving the phenolic content and antioxidant activity (Dey, Chakraborty, Jain, Sharma, & Kuhad, 2016; Dulf, Vodnar, & Socaciu, 2016).

Solid-state fermentation (SSF) is an ancient culture method that is still used to produce enzymes and secondary metabolites; therefore, its application in the food industry is important for producing biomolecules such as organic acids, pigments, phenolic compounds, and flavour (Soccol, Scopel, Alberto, Letti, Karp, & Woiciechowski, 2017). SSF has been applied to soybean products to produce isoflavones (Yaakob, Malek, Misson, Jalil, Sarmidi, & Aziz, 2011), enzymes (Handa, Couto, Vicensoti, Georgetti, & Ida, 2014; Li, Loman, Coffman, & Ju, 2017), and food bio-colour (Mhalaskar & Thorat, 2016); to increase proteins and hydrolysed amino acids content; to decrease the levels of phytic acid, trypsin inhibitors, raffinose, stachyose, and verbascose (Chen, Madl, & Vadlani, 2013; Hassaan et al., 2015); and to improve the antioxidant activity (Xiao, Zhang, Miao, Rui, Li, & Dong, 2015).

The efficiency of SSF depends on the microorganism as well as the operational and environmental conditions, such as temperature, pH, moisture content, aeration, nutrient concentrations, and nature of the substrate (Farinas, 2015). Fungi are preferred for use in the SSF process because the culture conditions are similar to those that the microorganisms require in the natural environment (Raimbault, 1998). Among the fungi, *Aspergillus oryzae* has been used in SSF and is listed as “Generally

Recognized as Safe (GRAS)". *Aspergillus oryzae* has a long history of use in the food industry due to its high proteolytic and amylolytic activities and is producing traditional fermented foods (Kawauchi & Iwashita, 2014; Li et al., 2016). *Monascus purpureus* is an edible and versatile fungus, which produces secondary metabolites, mainly pigments, and has been used in SSF for centuries (Srianta, Zubaidah, Estiasih, Yamada, & Harijono, 2016). The evaluation of different strains, carbon sources and process parameters to optimize the production process has been previously investigated (Thomas, Larroche, & Pandey, 2013).

Temperature, moisture content, and pH are considered the main parameters that should be evaluated in SSF, as are the interactions among parameters (Farinas, 2015). These parameters can be optimized based on factorial design experiments and response surface methodology to identify the critical parameters and their interactions (Thomas et al., 2013). Handa et al. (2014) investigated the effects that the initial pH of the DSF, the volume of water added and the temperature of incubation during SSF by *Monascus purpureus* or *Aspergillus oryzae* have on the production of β-glucosidase; however, the effect of these parameters on the production of bioactive compounds has not been investigated. Therefore, the objective of this study was to evaluate the effects that the fermentation parameters of defatted soy flour (DSF) by *Monascus purpureus* or *Aspergillus oryzae* have on the content of bioactive compounds and antioxidant activity using a central composite rotatable design (CCRD), multi-response optimization, and Pearson's correlation.

2. Material and methods

2.1. Material

Commercial DSF purchased from a local store in Londrina, PR, Brazil, (8.95% moisture, 1.07% lipids, 48.96% protein (N x 6.25), 5.98% ash, and 35.04% carbohydrates) was used as a substrate for solid-state fermentation (SSF). *Aspergillus oryzae* IOC 3999/1998 (Oswaldo Cruz Foundation, Fiocruz, RJ, Brazil) and *Monascus purpureus* NRRL 1992 (GenBank: JQ614061.1, Laboratory of Biochemistry and Applied Microbiology of the Institute of Food Science and Technology of the Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil) were used in this study. Acetyl-β-glucosides (6”-O-acetyldaidzin, 6”-O-acetylgenistin and 6”-O-acetylglycitin) and malonyl-β-glucosides (6”-O-malonyldaidzin, 6”-O-malonylgenistin, and 6”-O-malonylglycitin) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). β-glucosides (daidzin, genistin, and glycitin) and aglycones (daidzein, genistein and glycitein) were purchased from Sigma Aldrich Co. (St. Louis, MO, EUA). 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH•), 2,4,6-Tri(2-pyridyl)-S-triazine (TPTZ), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Folin-Ciocalteu reagent, gallic acid, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Aldrich Co. All reagents used in the analysis were of analytical grade or liquid chromatography grade.

2.2. Procedures

2.2.1. Evaluating the fermentation parameters of defatted soy flour by Monascus purpureus (DSFF-Mp) or Aspergillus oryzae (DSFF-Ao) for the production of fermented flour containing bioactive compounds

The solid-state fermentation of DSF by *Monascus purpureus* or *Aspergillus oryzae* was performed in two blocks according to the CCRD (Table 1), as described by Handa et al. (2014). The first block was exploratory and consisted of 10 experimental trials, eight factorial points and two central points. In the second block, the axial points were added to optimize the fermentation parameters for DSFF-Mp or DSFF-Ao containing a higher content of bioactive compounds (aglycone isoflavones, TPC, and higher antioxidant activity). In the CCRD, the coded independent variables, x_1, x_2 and x_3 and uncoded variables, X_1 (initial pH of DSF), X_2 (mL - water added in 10 g DSF) and X_3 (°C - incubation temperature), were studied (Table 1). The SSF employed 10 g of DSF and the initial pH (X_1) was adjusted according to the levels presented in Table 1. The amount of water added to DSF (X_2) (Table 1) was previously prepared by using distilled water with its pH adjusted by the addition of aliquots of 1M hydrochloric acid or 1M sodium carbonate until the pH (X_1) of the DSF reached the correct levels (Table 1). The DSF was homogenized and the material was uniformly distributed into 250 mL Erlenmeyer flasks and sterilized by autoclaving at 121 °C for 15 min. A suspension of 10^7 spores was spread evenly over the surface of each sample and incubated (Fanem, mod. 347F, Sao Paulo, Brazil) for 48 h at incubation temperature (X_3) according to pre-determined levels (Table 1).

The DSFF-Mp and DSFF-Ao were immediately frozen, lyophilized (Christ Alpha 2–4 LD Plus, Osterode am Harz, Germany), ground (30 mesh) (electric grinder, model MDR301, Cadence Eletrodomésticos SA, Navegantes, SC, Brazil) and stored at -22 °C until used in further analyses. The range of initial pH of the DSF, the amount of water added to DSF and the incubation temperature were selected from a search of the literature and based on the maximum production of several products via the SSF of many substrates by *Monascus purpureus* and *Aspergillus oryzae*.

The experimental results of CCRD were fitted using the response surface regression procedure with the following second-order polynomial equation: $\mathcal{Y} = \beta_0 + \beta_j + \beta_1 x_1 + \beta_{11}\beta x_1^2 + \beta_2 x_2 + \beta_{22}\beta x_2^2 + \beta_3 x_3 + \beta_{33}\beta x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 3 + \beta_{23} x_2 x_3 + e$ (Eq. 1), where $\mathcal{Y}$ is the predicted response; x_1, x_2 and x_3 are coded variables; β is the model coefficient that represents the regression coefficients of variables for linear, quadratic, and interactive regression terms; β_j is the estimated coefficient of the block; and e is the error. The response variables evaluated were as follows: MGLU (µmol malonyl-β-glucoside isoflavones/g sample), AGLU (µmol acetyl-β-glucoside isoflavones/g sample), GLU (µmol β-glucoside isoflavones/g sample), AGLY (µmol aglycone isoflavones/g sample), TPC (mg gallic acid equivalent (GAE)/g sample), DPPH (µmol Trolox equivalents (TE)/g sample), ABTS (µmol TE/g sample) and FRAP (µmol TE/g sample). The mathematical models were subjected to an analysis of variance (ANOVA) and regression analysis using the Statistica 10.0 software (StatSoft, Tulsa, OK, USA). The response surfaces were generated from the adjusted models using Statistica 10.0 software.

2.2.2. Multi-response optimization to obtain DSFF-Mp or DSFF-Ao with higher content of bioactive compounds and antioxidant activity

The multi-response optimization to obtain DSFF-Mp or DSFF-Ao with a high content of AGLY, TPC and high antioxidant activity (DPPH, ABTS, and FRAP) was performed using the overall desirability function. According to Yolmeh and Jafari (2017), the overall desirability function, d_i (Y_i), assigns numbers between 0 and 1 to the possible values of Y_i; d_i (Y_i) = 0 represents a completely undesirable value of Y_i; and di (Y_i) = 1 represents a completely desirable or ideal response value. The individual desirability values were combined using the geometric mean, which gives the overall

desirability. The overall desirability was calculated using the Statistica 10.0 software (StatSoft, Tulsa, OK, USA). Models were validated using Student's t-test (α = 0.05), where the average (*n* = 3) of the experimental values was compared with the estimated responses (*n* = 3) of the models. In addition, correlations among experimental values from the response variables (MGLU, AGLU, GLU, AGLY, TPC, DPPH, FRAP and ABTS) of DSFF-Mp and DSFF-Ao were calculated using Pearson´s correlation (*α* = 0.05 e *α* = 0.01).

2.3. Analytical Methods

2.3.1. Quantification of isoflavone forms by UPLC

The DSFF-Mp and DSFF-Ao were previously degreased using hexane in a 1:10 ratio (g: mL, sample: hexane) under continuous stirring at 25 °C for 1 h, followed by vacuum filtration. The isoflavones were extracted in triplicate according to the methodology described by Yoshiara, Madeira, Delaroza, da Silva, and Ida (2012) and quantified by Handa et al. (2014), using ultra-performance liquid chromatography (UPLC®) Waters (Acquity UPLC System, Waters, USA). The identification of the different forms of isoflavones was done by comparing the retention time and the UV spectra of the respective standards. The results of the sum of different forms of isoflavones were expressed as μmol of isoflavone/g of sample (dry weight basis).

2.3.2. Extraction and determination of total phenolic content

The extraction of TPC from the DSFF-Mp and DSFF-Ao was performed as described by Handa, De Lima, Guelfi, Georgetti, and Ida (2016). The extracts were used to determine the TPC and the antioxidant activity of the fermented products, as

measured by the DPPH, FRAP, and ABTS methods. The TPC of the DSFF-Mp and DSFF-Ao extracts was determined by the Folin-Ciocalteu colourimetric method (Kumazawa, Taniguchi, Suzuki, Shimura, Kwon, &, 2002; Singleton, Orthofer, & Lamuela-Raventós, 1999) using gallic acid (GA) as a standard. The TPC was expressed as mg of GA equivalents/g of sample on a dry weight basis (mg of GAE/g).

2.3.3. Determination of antioxidant activity

DPPH$^{\bullet}$ scavenging ability - The antioxidant activity of the phenolic extracts using the DPPH$^{\bullet}$ radical was measured following Blois (1958) and Dinis, Madeira, and Almeida (1994). Trolox was used for the calibration curve. The hydrogen-donating ability of extracts was expressed as µmol of Trolox equivalents (TE)/g of sample (dry weight basis).

FRAP assay - The ferric reduction antioxidant power of extracts was estimated using the method reported by Benzie and Strain (1996) with modifications (Handa et al., 2016). The ability of the extract to reduce Fe^{3+} was expressed as µmol TE/g of sample (dry weight basis).

Cation ABTS radical assay - The ABTS assay was performed as reported (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans (1999). The ability to scavenge the ABTS free radical of the extracts was expressed as µmol of TE/g of sample (dry weight basis).

4. Results and Discussion

4.1. Effects of solid-state fermentation parameters of DSF by Monascus purpureus or Aspergillus oryzae on isoflavones content

The response variables, $MGLU_{Mp}$, $AGLU_{Mp}$, GLU_{Mp}, and $AGLY_{Mp}$, from the DSFF-Mp experimental trials (Table 1) varied in the maximum and minimum contents (based on the ratio between the maximum and minimum values) of these isoflavones by 3.0-, 2.2-, 4.8- and 6.3-fold, respectively. For the experimental trials of the DSFF-Ao response variables, $MGLU_{Ao}$, $AGLU_{Ao}$, GLU_{Ao}, and $AGLY_{Ao}$, varied in the maximum and minimum contents of these isoflavones by 3.0-, 2.0-, 1.9- and 2.8-fold, respectively. For the TPC, DPPH, FRAP, and ABTS of DSFF-Ao and DSFF-Mp, the variation between the maximum and minimum values of these measures was 2.2, >3.0-, 2.6-, and 1.9-fold and 1.3-, 1.2-, 1.2-, and 1.4-fold, respectively. It was also observed that the DSFF-Mp had a higher content of the different forms of isoflavones, whereas DSFF-Ao had higher TPC and antioxidant activity. *Aspergillus oryzae* has been described as the key organism in the production of traditionally fermented soy foods such as miso and shoyu and is responsible for the high productivity of hydrolases (Machida, Yamada, & Gomi, 2008). Thus, the higher variation in TPC of DSFF-Ao may be due to the potent enzyme systems of *Aspergillus oryzae*, which include the amylases, proteases, and lipases that release phenolic compounds bound to macromolecules. The lower TPC mobilized by *Monascus purpureus* can be explained by a selective action of its β-glucosidase, resulting in polyphenol aglycones with a lower capacity to reduce the Folin–Ciocalteu reagent or by synthesis of β-glucosidases with low activity on other soybean flour phenolic glucosides. Handa et al. (2014) and Handa et al. (2016) confirmed that the fermentation of soybean flour by *Aspergillus oryzae* produced β-glucosidase with higher activity and higher TPC than did fermentation by *Monascus purpureus*, which was more effective at converting the glycosylated isoflavones into aglycones.

The model's adequacy was evaluated by the coefficient of determination (R^2) and the lack of fit for the model from the ANOVA (Table 2). The independent variables, X_1 (initial pH of the DSF), X_2 (mL - water added to 10 g of DSF) and X_3 (°C - incubation temperature) in the solid state fermentation of DSF by the fungi *Monascus purpureus* and *Aspergillus oryzae* presented different effects on the evaluated response variables.

Thus, for the $MGLU_{Mp}$ from DSFF-Mp, the linear (x_1, x_2 and x_3), quadratic (x_1^2), and interaction (x_1x_2) effects were significant. However, the model was not used for predictive purposes due to the low R^2 value (0.53) and the significant lack of fit (Table 2). For an appropriate proposed model, it is desirable that the value of R^2 should be close to 1 because this value indicates a better explanation of the variability of the experimental data by the proposed model; in other words, a better correlation exists between observed and predicted values (Gangadharan, Sivaramakrishnan, Nampoothiri, Sukumaran, & Pandey, 2008; Yolmeh, & Jafari, 2017). This inadequacy of the model may be associated with the lack of β-glucosidase specificity produced by the fungus *Monascus purpureus* to malonyl-β-glucoside isoflavones, as previously observed (Handa et al. 2014).

The $MGLU_{Ao}$ from DSFF-Ao presented significant linear (x_3), quadratic (x_2 and x_3), and interaction (x_1x_2; x_1x_3; x_2x_3) effects. The model ($MGLU_{Ao} = 0.15 - 0.02^* + 0.03x_2^{2*} - 0.02x_3{}^* + 0.05x_3^{2*} + 0.02x_1x_2^* - 0.02x_1x_3^* + 0.04x_2x_3^*$) (Eq. 2) had an R^2 value of 0.79, and the lack of fit was not significant. The response surface (Supplementary Fig. S2A) and the desirability parameters showed an optimal region with a low $MGLU_{Ao}$ (0.112 µmol malonyl-β-glucoside/g of DSFF-Ao) when $0<x_1<1.68$ or X_1 (initial pH) was between 6 and 6.8; $-1.68<x_2<0$ or X_2 (water added) was between 2 and 10 mL; and $0<x_3<1.68$ or X_3 (incubation temperature) was between 30 and 47 °C. During

fermentation with *Aspergillus oryzae,* there may have been a transformation of malonyl-β-glucoside isoflavones by decarboxylation, de-esterification or deglycosylation into acetyl-β-glucosides, β-glucosides or aglycones, respectively. Chen et al. (2015) suggest that the interconversion of the 12 isoflavones in soybeans and soy products may occur during the different processing methods, such as fermentation, soaking, germination, and heat treatments. According to Chen Erh, Su, Liu, Chou, and Cheng (2012) the deglycosylation of isoflavones can be achieved during the fermentation process by using β-glucosidase produced by several strains of microorganisms, such as lactic acid bacteria, basidiomycetes, filamentous fungi, and *Bacillus subtilis*. This transformation is important because humans absorb aglycone isoflavones faster and in greater amounts than their glucoside forms.

The $AGLU_{Mp}$ from DSFF-Mp presented significant linear (x_2), quadratic (x_1^2, x_2^2, x_3^2), and interaction (x_1x_2) effects. The model ($AGLU_{MP} = 0.41^* + 0.02 + 0.03x_1^{2*} - 0.08x_2^* + 0.06x_2^{2*} + 0.04x_3^{2*} + 0.04x_1x_2$) (Eq. 3) may be used for predictive purposes since it presented a good fit to the experimental data ($R^2 = 0.89$) and the lack of fit was not significant. The response surface (Supplementary Fig. S1A) and desirability parameters indicated an optimal region with low $AGLU_{Mp}$ (0.402 µmol acetyl-β-glucoside/g of DSFF-Mp) when $-1.68<x_1<0.84$ or X_1 was between 5.2 and 6.4; $0<x_2<1.68$ or X_2 was between 10 and 18 mL; and $-0.84<x_3<0.84$ or X_3 was between 22 and 38 °C. The $AGLU_{Ao}$ response variable from the DSFF-Ao showed significant linear (x_2) and quadratic (x_2^2) effects. However, the linear (x_1 and x_3) and quadratic (x_3^2) terms were kept in the model because of their contributions to the R^2 and $R_{adjusted}$ values (Table 2). Thus, the model equation, $AGLU_{Ao} = 0.50^* + 0.03 - 0.04x_1 - 0.05x_2{}^* + 0.05x_2^{2*} - 0.03x_3 + 0.04x_3^2$ (Eq. 4), was used for predictive purposes since it presented a good fit to the experimental data ($R^2 = 0.70$) and the lack of fit was not significant.

The response surface (Supplementary Fig. S2B) and the desirability parameters showed an optimal region with a low $AGLU_{Ao}$ (0.458 μmol acetyl-β-glucoside/g of DSFF-Ao) when $084<x_1<1.68$ or X_1 was between 5.6 and 6.8; $-0.84<x_2<1.68$ or X_2 was between 6 and 18 mL; and $-0.84<x_3<1.68$ or X_3 was between 22 and 47 °C.

The GLU_{Mp} response variable from the DSSF-Mp presented significant linear (x_2, x_3), quadratic (x_1^2, x_2^2, x_3^2), and interactive (x_2x_3) effects. The model ($GLU_{Ao} = 2.64^* - 0.12 + 0.13x_1 - 0.16x_2 - 0.22x_3{}^* + 0.37x_3^{2*} - 0.21x_2x_3$) (Eq. 5) may be used for predictive purposes since it presented a good fit to the experimental data (R^2 = 0.85) and the lack of fit was not significant. The response surface (Supplementary Fig. S1B) and the desirability parameters indicated an optimal region with low GLU_{Mp} (0.902 μmol β-glucoside isoflavones/g of DSFF-Mp) when $-0.84<x_1<0.84$ or X_1 was between 5.6 and 6.4; $0<x_2<1.68$, or X_2 was between 10 and 18 mL; and $0 <x_3<1.68$, or X_3 was between 30 and 47 °C. The GLU_{Ao} from DSFF-Ao showed significant linear (x_3) and quadratic (x_3^2) effects. Nevertheless, the linear (x_1 and x_2) and interactive (x_2x_3) terms were kept in the model because of their contribution to the R^2 and $R_{adjusted}$ values. The model equation $GLU_{Ao} = 2.64^* - 0.12 + 0.13x_1 - 0.16x_2 - 0.22x_3{}^* + 0.37x_3^{2*} - 0.21x_2x_3$ (Eq. 6), was used for predictive purposes since it presented a good fit to the experimental data (R^2 = 0.76) and the lack of fit was not significant. The response surface (Supplementary Fig. S2C) and the desirability parameters presented an optimal region with lower GLU_{Ao} (1.929 μmol β-glucoside/g of DSFF-Ao) when $-1.68<x_1<1.68$, or X_1 was between 5.2 and 6.8; $0<x_2<1.68$, or X_2 was between 10 and 18 mL; and $0<x_3<1.68$, or X_3 was between 30 and 47 °C.

The $AGLY_{Mp}$ response variable for the DSSF-Mp had significant linear (x_2, x_3), quadratic (x_1^2, x_2^2, x_3^2), and interactive (x_2x_3) effects. The model ($AGLY_{Mp} = 3.48^* - 0.18^* - 0.46x_1^{2*} + 0.68x_2{}^* - 0.43x_2^{2*} + 0.71x_3{}^* - 0.69x_3^{2*} + 0.39x_2x_3{}^*$) (Eq. 7) may be

used for predictive purposes since it presented a great fit to the experimental data (R^2 de 0.90) and the lack of fit was not significant. The response surface (Supplementary Fig. S1C) and the desirability parameters indicated an optimal region with high $AGLY_{Mp}$ (3.947 µmol aglycones/g of DSFF-Mp) when $-0.84<x_1<0.84$ or X_1 was between 6.6 and 6.4; $0.84<x_2<1.68$ or X_2 was between 14 and 18 mL; and $0<x_3<0.84$ or X_3 was between 30 and 38 °C. The $AGLY_{Ao}$ response variable for the DSFF-Ao presented significant linear (x_2, x_3), quadratic (x_2^2, x_3^2), and interactive(x_2x_3) effects. The ($AGLY_{Ao} = 1.70^* + 0.14^* + 0.29{x_2}^* - 0.19x_2^{2*} + 0.16{x_3}^* - 0.30x_3^{2*} + 0.16x_2{x_3}^*$) (Eq. 8) may be used for predictive purposes since it presented a great fit to the experimental data (R^2 de 0.92) and the lack of fit was not significant. The response surface (Supplementary Fig. S2D) and the desirability parameters showed an optimal region with high $AGLY_{Ao}$ (1.984 µmol aglycones/g of DSFF-Ao) when $1.68<x_1<1.68$ or X_1 was between 5.2 and 6.8; $0<x_2<1.68$, or X_2 was between 10 and 18 mL; and $0<x_3<0.84$, or X_3 was between 30 and 38 °C.

4.2. Effects of solid-state fermentation parameters on the total phenolic content and antioxidant activity of defatted soy flour fermented by Monascus purpureus or Aspergillus oryzae

The adequacy of the models was evaluated using the coefficient of determination (R^2) and the lack of fit for the model from the ANOVA table (Table 2). The response surface, TPC_{Mp}, $DPPH_{Mp}$, and $ABTS_{Mp}$ for the DSFF-Mp did not present any significant term (linear, quadratic, or interactive) (Table 2), which indicated that these response variables were not influenced by the independent variables X_1 (initial pH of DSF), X_2 (mL - water added to 10 g of DSF) and X_3 (°C - incubation temperature)

348 (Table 1), possibly due to the low variation between the experimental trials; therefore,
349 they were not evaluated. For the $FRAP_{Mp}$ from DSFF-Mp, it was observed that only the
350 quadratic term (x_3^2) was significant. However, the linear (x_2 and x_3), quadratic (x_2^2),
351 and interactive (x_1x_2) terms were kept in the model because of the contribution to the
352 R^2 and $R_{adjusted}$ values. The model ($FRAP_{Mp} = 14.57^* - 0.02 + 0.24x_2 + 0.26x_2^2 -$
353 $0.17x_3 - 0.55x_3^{2*} - 0.24x_1x_2$) (Eq. 9) may be used for predictive purposes since it
354 presented a good fit to the experimental data (R^2 = 0.82) and the lack of fit was not
355 significant. The response surface (Supplementary Fig. S1D) and the desirability
356 parameters presented an optimal region with a high $FRAP_{Mp}$ (15.645 µmol TE/g of
357 DSFF-Mp) when $-1.68<x_1<0.84$ or X_1 was between 5.2 and 6.4; $0<x_2<1.68$ or X_2 was
358 between 10 and 18 mL; and $-0.84<x_3<0.84$ or X_3 was between 22 and 38 °C. Therefore,
359 it was verified that the independent variables of the fermentation process of DSFF-Mp
360 influenced the potential for reducing the Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ.

361 The fermentation parameters in the solid-state fermentation of DSF by
362 *Aspergillus oryzae* influenced the TPC and antioxidant activity of the product. The
363 TPC_{Ao} from DSFF-Ao had significant linear (x_2) and quadratic (x_2^2, x_3^2) effects. The
364 model ($TPC_{Ao} = 3.62^* + 0.33^* + 0.34x_2{}^* - 0.27x_2^{2*} - 0.66x_3^{2*}$) (Eq. 10) may be used for
365 predictive purposes since it presented a good fit to the experimental data (R^2 = 0.87)
366 and the lack of fit was not significant. The response surface (Supplementary Fig. S2E)
367 and the desirability parameters showed an optimal region with high TPC_{Ao} (4.033 mg
368 GAE / g DSFF-Ao) when $-1.68<x_1<1.68$ or X_1 was between 5.2 and 6.8; $0 <x_2<1.68$ or
369 X_2 was between 10 and 18 mL; and $x_3 = 0.84$ or X_3 was 30 °C.

370 The $DPPH_{Ao}$ from DSFF-Ao had significant linear (x_2) and quadratic (x_1^2, x_3^2)
371 effects. However, the interaction (x_2x_3) term was kept in the model due to its
372 contribution to the R^2 and $R_{adjusted}$ values. The model ($DPPH_{Ao} = 0.25^* - 0.03^* +$

373 $0.04x_1^{2*} + 0.03x_2{}^* - 0.09x_3^{2*} + 0.03x_2x_3$) (Eq. 11) may be used for predictive purposes
374 since it presented a good fit to the experimental data (R^2 = 0.87) and the lack of fit was
375 not significant. The $FRAP_{Ao}$ from DSFF-Ao showed significant linear (x_2) and quadratic
376 (x_2^2, x_3^2) effects. However, the interaction terms (x_1x_2 and x_2x_3) were kept in the model
377 due to their contribution to the R2 and $R_{adjusted}$ values. The model ($FRAP_{A0} = 25.34^* +$
378 $1.36 + 2.55x_2{}^* - 2.53x_2^{2*} - 4.53x_3^{2*} - 0.98x_1x_2 + 1.25x_2x_3$) (Eq. 12) may be used for
379 predictive purposes since it presented a good fit to the experimental data (R^2 = 0.86)
380 and the lack of fit was not significant. The $ABTS_{Ao}$ from DSFF-Ao had significant linear
381 (x_2) and quadratic (x_3^2) terms. However, the quadratic term (x_1^2) was kept in the model
382 because of the contribution to the R^2 and $R_{adjusted}$ values. The model ($ABTS_{Ao} =$
383 $79.62^* + 5.99^* + 7.17x_2{}^* - 3.63x_2^2 - 9.48x_3^{2*}$, Eq. 13) may be used for predictive
384 purposes since it presented a good fit to the experimental data (R_2 = 0.80) and the lack
385 of fit was not significant (Table 2). The $DPPH_{Ao}$ (Supplementary Fig. S2F), $FRAP_{Ao}$
386 (Supplementary Fig. S2G), and $ABTS_{Ao}$ (Supplementary Fig. S2H) response surface
387 and desirability parameters had an optimal region with a high $DPPH_{Ao}$ (0.355 µmol
388 TE/g of DSFF-Ao), $FRAP_{Ao}$ (28.440 µmol TE/g of DSFF-Ao), and $ABTS_{Ao}$ (89.750 µmol
389 TE/g of DSFF-Ao) when: $1.68<x_1<1.68$ or X_1 was between 5.2 and 6.8; $0<x_2<1.68$ or
390 X_2 was between 10 and 18 mL; and $-0.84<x_3<0.84$, or X_3 was between 22 and 38 °C.
391
392 *4.3. Multi-response optimization of the solid-state fermentation parameters of DSF by*
393 *Monascus purpureus or Aspergillus oryzae*
394
395 Multi-response function optimizations ($AGLU_{Mp}$, GLU_{Mp}, $AGLY_{Mp}$, $FRAP_{Mp}$ and
396 $AGLU_{Ao}$, GLU_{Ao}, $AGLY_{Ao}$, TPC_{Ao}, $DPPH_{Ao}$, $FRAP_{Ao}$, $ABTS_{Ao}$) of the fermentation
397 parameters of DSFF-Mp and DSFF-Ao, were performed from the overall desirability

parameters of the respective, fitted models. The desired fermentation conditions for DSFF-Mp, with low $AGLU_{Mp}$ and GLU_{Mp} values and high $AGLY_{Mp}$ and $FRAP_{Mp}$ values occurred when $-1.68<x_1<0.84$ or X_1 (initial pH) was between 5.2 and 6.4; $0<x_2<1.68$ or X_2 (water added) was between 10 and 18 mL; and $0<x_3<1.68$ or X_3 (incubation temperature) was between 30 and 47 °C (Fig 1). These results are in agreement with those reported by Mhalaskar and Thorat (2016), who found that the best optimized conditions for producing food bio-colour by *Monascus purpureus* (MTCC 410) using soybean meal were 65% (w/v), a temperature of 30 °C and a pH of 6. According to Haque, Kachrimanidou, Koutinas, and Lin (2016), the use of a low initial moisture content in the SSF by *Monascus purpureus* results in insufficient fungal growth; this species can produce multi-enzyme at temperature of 30, 35 and 37 °C. For DSFF-Ao (Fig. 2), the desirable fermentation conditions occurred when $-1.68<x_1<1.68$ or X_1 was between 5.2 and 6.8; $0<x_2<0.84$ or X_2 was between 10 and 14 mL; and $-0.84<x_3<0.84$ or X_3 was between 22 and 38 °C. Similar results were reported by Muñiz-márquez, Contreras, Rodríguez, Mussatto, Teixeira, and Aguilar (2016) who observed that the initial pH had no significant influence on the production of fructosyltransferase by SSF when using aguamiel (agave sap) as culture medium and *Aspergillus oryzae* and that the ideal incubation temperature was 32 °C.

A comparison of the parameters of overall desirability for DSFF-Mp and DSFF-Ao (Fig. 1 and 2) shows that the initial pH values (X_1) of DSFF-Mp were low while for DSFF-Ao, the initial pH values were independent in the range investigated. The pH level is more difficult to control in a SSF and the changes can affect the growth of the microorganism and metabolite production. Thus, the adjustment of the initial pH of the substrate has been explored to evaluate the variation of microorganisms during the process (Zou, Ding, Zhang, Yao, Jiang, & Liang, 2016). Velmurugan et al. (2011)

423 reported that the initial pH of corn influenced the yield of yellow and red pigments;
424 yellow pigment production was maximal at pH 6 and red pigment production was
425 maximal at pH 5, whereas the overall pigment production was reduced at a pH above
426 6.

427 Volumes of water added (X_2) to DSFF-Mp at levels above 10 mL provided
428 desirable values of for $AGLU_{Mp}$, GLU_{Mp}, $AGLY_{Mp}$, and $FRAP_{Mp}$. While in the DSFF-Ao,
429 the water added (X_2) to DSFF-Ao was restricted to a range of 10-14 mL. Although SSF
430 has been defined as a bioprocess that occurs in the absence or near-absence of free
431 water, moisture is a key factor in SFF since the substrate must possess sufficient
432 moisture to support the growth and metabolic activity of the microorganism (Thomas,
433 et al., 2013). However, high moisture fermentation requires vast amounts of energy to
434 remove the water (Zhao, Guo, & Zhu, 2017). Therefore, a volume of 10 mL of water
435 added to the DSF was the most appropriate value for producing DSFF-Mp and DSFF-
436 Ao because this was the minimum volume observed within the optimal range for
437 fermentation by both fungi.

438 In addition, the desirable variable X_3 (incubation temperature) was higher for
439 DSFF-Mp (30-47 °C) than for DSFF-Ao (22-38 °C). In previous studies of fermentation
440 by *Aspergillus oryzae*, it was very important to keep the temperature below 40 °C to
441 prevent the inhibition of the fungal production of several hydrolases (Machida et al.,
442 2008). The microbial growth under aerobic conditions results in considerable heat
443 production, causing a fast increase of temperature (Soccol et al., 2017). Temperature
444 is one of the most important parameters for SSF and influences the growth of the
445 microorganisms, enzyme activity, and metabolite production (Pandey, 2003; Thomas
446 et al., 2013).

447

448 *4.4. Validation of the models*

449

450 The predictive models were validated under the conditions in which the
451 independent variables were of low financial cost with respect to the amount of water
452 added and the incubation temperature. To obtain DSFF-Mp and DSFF-Ao, the
453 conditions used were $x_1 = 0$, or X_1 with an initial pH of 6.0; $x_2 = 0$, or X_2 of 10 mL of
454 water added to DSF; and $x_3 = 0$, or X_3 at 30 °C. Thus, Table 3 shows that the estimated
455 values of the response variables ($AGLU_{Mp}$, GLU_{Mp}, $AGLY_{Mp}$, and $FRAP_{Mp}$) did not differ
456 ($p>0.05$) of the respective values observed. Similarly, it is also observed in Table 3 that
457 for DSFF-Ao, the values of the estimated response variables ($AGLU_{Ao}$, GLU_{Ao},
458 $AGLY_{Ao}$, TPC_{Ao}, $DPPH_{Ao}$, $FRAP_{Ao}$, and $ABTS_{Ao}$) did not differ ($p>0.05$) from the
459 observed values. Therefore, these models can be used for predictive purposes.
460 However, when the model validation of the $MGLU_{Ao}$ from DSFF-Ao was performed,
461 the observed value (0.13 µmol malonyl-β-glucosides/g of DSFF-Ao) differed
462 significantly from the estimated value (0.31 µmol malonyl-β-glucosides/g of DSFF-Ao).
463 Therefore, this proposed model was not adequate for the experimental data and
464 cannot be used for predictive purposes. This inadequacy of the model can be attributed
465 to the thermal instability of malonyl-β-glucoside isoflavones in the sterilization
466 treatment (autoclaving) of DSF and the low specificity of β-glucosidase produced by
467 *Aspergillus oryzae* for its substrate, malonyl-β-glucoside isoflavones, as observed by
468 Handa et al. (2014), who evaluated the production of β-glucosidase during solid-state
469 fermentation of FDS by *Monascus purpureus* or *Aspergillus oryzae.*

470

471 *4.5. Correlations between the different isoflavone forms, total phenolic content and*
472 *antioxidant activity of DSFF-Mp and DSFF-Ao*

From the validation of the models, the Pearson's correlation test was applied to the observed values of the response variables from DSFF-Mp and DSFF-Ao (Table 4). MGLU did not correlate with the other response variables ($p>0.05$). AGLU and GLU presented a strong negative correlation with AGLY and DPPH and a strong positive correlation with TPC, FRAP, and ABTS. These results indicated that the isoflavones AGLU and GLU were converted into AGLY. AGLY presented a strong negative correlation with TPC, FRAP and ABTS, but had a strong positive correlation with DPPH. Thus, these results confirm that the aglycone isoflavones contribute largely to antioxidant activity by the mechanism of hydrogen donation (DPPH) (Blois, 1958; as Alam, Bristi, & Rafiquzzaman, 2013). The TPC had a strong positive correlation with FRAP and ABTS and a strong negative correlation with DPPH. TPC presents a more pronounced antioxidant activity by the mechanism of electron donation (FRAP and ABTS) (Alam et al., 2013). Although the aglycone isoflavones showed a positive correlation with DPPH and TPC showed a positive correlation with ABTS and FRAP, it was difficult to establish a correlation between structure and antioxidant properties due to the diversity of the compounds present in the extracts of both DSFF-Mp and DSFF-Ao. In addition, according to Mishra, Ojha, and Chaudhury (2012) the number of phenolic hydroxyl groups is not always the only factor determining the antioxidant activity of an antioxidant. As reported by Dulf et al. (2016) during microbial fermentation, the bond between phenolic compounds and other substituents in conjugated molecules are broken down based on the enzymes produced by the microorganisms, thereby improving the antioxidant capacity of the final products.

5. Conclusion

The initial pH, volume of added water and incubation temperature had a great effect on the AGLU, GLU, and AGLY isoflavone content of DSFF-Mp. No effect of these parameters was observed on the TPC and antioxidant activity measured by DPPH and ABTS. The incubation temperature influenced the antioxidant activity, as measured by FRAP.

The volume of water added and the incubation temperature had significant effects on the content of all of the isoflavone forms (MGLU, AGLU, GLU, and AGLY), the TPC, and antioxidant activity of the DSFF-Ao, as measured by DPPH, FRAP, and ABTS. In contrast, the initial pH exerted an effect only on the antioxidant activity, as measured by DDPH.

The solid-state fermentation of DSF by *Monascus purpureus* should be carried out using an initial pH between 5.2 and 6.4, between 10 and 18 mL of water, and an incubation temperature between 30 and 47 °C. In the fermentation by *Aspergillus oryzae,* the initial pH was an independent variable, the optimal volume of water added was between 10 and 14 mL, and the optimal incubation temperature was between 22 and 38 °C.

The strong negative correlation of AGLU and GLU with AGLY indicates that, during the fermentation of DSF by *Monascus purpureus* or *Aspergillus oryzae,* there was a conversion of these glycosylated isoflavones into aglycones. The positive correlation of aglycone isoflavones with antioxidant activity, as measured by DPPH, and of TPC with antioxidant activity as measured by FRAP and ABTS, confirmed that the fermentation of DSF favoured the formation of bioactive compounds in DSFF-Mp and DSFF-Ao.

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Table1 - Central composite rotatable design and response variables expressed in the defatted soy flour fermented by *Monascus purpureus* (DSFF-Mp) and defatted soy flour fermented by *Aspergillus oryzae* (DSFF-Ao), X_1 (initial pH of DSF); X_2 (mL water added to 10 g of DSF); X_3 (°C incubation).

Independent variables coded and uncoded				Response-functions of DSFF- Mp								Response-functions of DSFF- Ao							
Run	x_1 (X_1)	x_2 (X_2)	x_3 (X_3)	MGLU Mp	AGLU Mp	GLU Mp	AGLY Mp	TPC Mp	DPPH Mp	FRAP Mp	ABTS Mp	MGLU Ao	AGLU Ao	GLU Ao	AGLY Ao	TPC Ao	DPPH Ao	FRAP Ao	ABTS Ao
1	-1(5.5)	-1(5)	-1(20)	0.25	0.59	3.13	0.76	2.15	0.69	13.85	47.4	0.24	0.67	3.34	0.78	2.09	0.22	16.04	59.5
2	-1(5.5)	-1(5)	+1(40)	0.23	0.60	2.47	2.04	2.02	0.72	13.58	58.7	0.26	0.59	2.95	0.81	2.02	0.12	11.37	56.58
3	-1(5.5)	+1(15)	-1(20)	0.43	0.46	3.15	1.28	2.42	0.7	14.96	55.53	0.2	0.63	3.65	0.93	2.42	0.21	17.11	59.43
4	-1(5.5)	+1(15)	+1(40)	0.28	0.36	0.72	4.57	2.45	0.69	14.87	54.95	0.22	0.54	2.39	1.87	2.45	0.24	23.62	62.73
5	+1(6.5)	-1(5)	-1(20)	0.35	0.59	3.44	0.94	2.32	0.63	14.66	60.03	0.36	0.51	3.21	0.72	2.42	0.25	12.5	54.98
6	+1(6.5)	-1(5)	+1(40)	0.24	0.52	2.19	2.12	2.06	0.69	13.57	50.88	0.14	0.5	3.15	0.9	2.07	0.17	15.99	51.95
7	+1(6.5)	+1(15)	-1(20)	0.32	0.48	3.45	1.19	2.43	0.65	14.42	65.28	0.26	0.6	3.77	0.9	2.43	0.28	15.85	64.97
8	+1(6.5)	+1(15)	+1(40)	0.32	0.55	1.19	3.48	2.4	0.64	14.29	53.59	0.32	0.45	2.95	1.44	2.5	0.28	18.14	59.36
9	0(6.0)	0(10)	0(30)	0.18	0.36	1.24	3.86	2.29	0.72	15.03	64.82	0.17	0.44	2.66	1.67	3.4	0.36	25.31	78.01
10	0(6.0)	0(10)	0(30)	0.16	0.41	1.55	3.59	2.32	0.69	14.38	56.92	0.16	0.49	2.39	1.71	3.63	0.31	27.46	83.98
11	-1.68(5.2)	0(10)	0(30)	0.48	0.53	2.14	2.18	2.25	0.73	14.57	59.67	0.13	0.56	2.05	2.01	3.8	0.36	23.46	89.28
12	+1.68(6.8)	0(10)	0(30)	0.29	0.50	2.73	1.86	2.14	0.72	14.81	57.76	0.12	0.49	2.67	1.68	4.47	0.33	29.55	79.5
13	0(6.0)	-1.68(2)	0(30)	0.31	0.78	3.27	0.73	2.12	0.73	15.22	58.97	0.18	0.87	3.30	0.75	2.23	0.19	15.65	55.82
14	0(6.0)	+1.68(18)	0(30)	0.29	0.42	1.40	3.46	2.25	0.75	15.44	61.66	0.28	0.47	1.97	1.95	4.26	0.32	25.16	100.08
15	0(6.0)	0(10)	-1.68(13)	0.48	0.55	3.20	0.85	2.03	0.74	13.25	63.23	0.33	0.66	3.63	0.88	2.23	0.00	15.1	64.3
16	0(6.0)	0(10)	+1.68(47)	0.18	0.53	2.70	1.87	1.91	0.72	12.85	59.09	0.22	0.6	3.32	1.18	2.08	0.00	14.4	58.51
17	0(6.0)	0(10)	0(30)	0.48	0.44	1.32	3.2	1.95	0.72	14.16	58.38	0.13	0.51	2.55	1.85	3.73	0.14	26.11	79.29
18	0(6.0)	0(10)	0(30)	0.47	0.43	1.04	3.25	2.15	0.76	14.49	61.47	0.16	0.55	2.72	1.65	3.45	0.16	24.29	83.97

MGLU = malonyl-β-glucoside isoflavones (µmol/g); AGLU = acetyl-β-glucoside isoflavones (µmol/g); GLU = β-glucoside isoflavones (µmol/g); AGLY = aglycone isoflavones (µmol/g); TPC = total phenolic content (mg GAE/g); DPPH = antioxidant activity determined by DPPH (µmol TE/g); ABTS = antioxidant activity determined by ABTS (µmol TE/g); FRAP = ferric reducing antioxidant power (µmol TE/g).
Mp = DSFF-Mp; Ao = DSFF-Ao.

Table 2 - ANOVA of the predictive models of the defatted soy flour fermented by *Monascus purpureus* (DSFF-Mp) and defatted soy flour fermented by *Aspergillus oryzae* (DSFF-Ao).

Source	$MGLU_{MP}$		$AGLU_{MP}$		GLU_{MP}		$AGLY_{MP}$		TPC_{MP}		$DPPH_{MP}$		$FRAP_{MP}$		$ABTS_{MP}$	
	SS	*p**	SS	*p**	SS	*p**	SS	*p**	SS	*p**	SS	*p**	SS	*p**	SS	*p**
Blocks	0.041	0.00	0.008	0.07	0.035	-	0.556	0.03	0.153	0.06	0.011	0.05	0.005	0.86	43.000	0.26
x_1	0.005	0.03	-	-	-	-	-	-	-	-	0.003	0.15	0.775	0.14	-	-
x_1^2	0.005	0.03	0.012	0.04	1.706	0.02	2.686	0.01	0.015	0.34	0.001	0.32	-	-	17.776	0.43
x_2	0.005	0.03	0.085	0.01	2.515	0.02	6.256	0.00	0.136	0.07	-	-	-	-	20.850	0.39
x_2^2	-	-	-	-	1.406	0.03	2.370	0.01	0.012	0.39	0.001	0.40	0.900	0.12	-	-
x_3	0.045	0.00	0.049	0.01	4.050	0.01	6.973	0.00	0.026	0.25	-	-	-	-	21.384	0.39
x_3^2	-	-	0.020	0.03	3.839	0.01	6.046	0.00	0.027	0.24	-	-	3.907	0.03	-	-
x_1x_2	0.004	0.04	0.010	0.05	-	-	1.213	0.02	-	-	-	-	0.459	0.20	-	-
x_1x_3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	124.451	0.12
x_2x_3	-	-	-	-	0.965	0.04	-	-	0.020	0.30	0.001	0.29	-	-	26.022	0.35
Lack of Fit	0.093	0.01	0.020	0.22	2.202	0.14	2.488	0.06	0.065	0.66	0.003	0.78	1.239	0.62	67.555	0.85
Pure Error	0.000		0.001		0.085		0.039		0.020		0.001		0.264		36.007	
Total SS	0.196		0.180		14.884		25.225		0.492		0.022		8.382		360.157	
R^2	0.53		0.89		0.85		0.90		0.83		0.79		0.82		0.71	
R adj.	0.27		0.82		0.74		0.83		0.71		0.70		0.75		0.56	
Source	**$MGLU_{Ao}$**		**$AGLU_{Ao}$**		**GLU_{Ao}**		**$AGLY_{Ao}$**		**TPC_{Ao}**		**$DPPH_{Ao}$**		**$FRAP_{Ao}$**		**$ABTS_{Ao}$**	
	SS	***p****	**SS**	***p****	**SS**	***p****	**SS**	***p****	**SS**	***p****	**SS**	***p****	**SS**	***p****	**SS**	***p****
Blocks	0.005	0.03	0.013	0.08	0.240	0.09	0.334	0.03	1.879	0.02	0.016	0.04	32.585	0.06	633.359	0.02
x_1	-	-	0.018	0.06	0.233	0.10	-	-	-	-	-	-	-	-	-	-
x_1^2	-	-	-	-	-	-	-	-	-	-	0.016	0.04	-	-	-	-
x_2	-	-	0.038	0.03	0.330	0.07	1.155	0.01	1.559	0.02	0.015	0.04	88.698	0.02	701.888	0.02
x_2^2	0.014	0.01	0.033	0.03	-	-	0.472	0.02	0.991	0.03	-	-	84.074	0.02	173.516	0.07
x_3	0.006	0.03	0.014	0.08	0.683	0.04	0.353	0.03	-	-	-	-	-	-	-	-
x_3^2	0.032	0.00	0.019	0.06	1.844	0.01	1.211	0.01	5.742	0.01	0.096	0.01	270.063	0.01	1183.826	0.01
x_1x_2	0.003	0.05	-	-	-	-	-	-	-	-	-	-	7.656	0.19	-	-
x_1x_3	0.005	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	-
x_2x_3	0.010	0.02	-	-	0.340	0.07	0.198	0.05	-	-	0.005	0.10	12.462	0.13	-	-
Lack of Fit	0.019	0.06	0.052	0.18	1.120	0.18	0.309	0.26	1.501	0.20	0.028	0.20	73.875	0.21	660.187	0.21
Pure Error	0.000		0.002		0.052		0.022		0.062		0.001		3.964		28.784	
Total SS	0.090		0.179		4.917		3.956		11.631		0.195		547.823		3400.655	
R^2	0.79		0.70		0.76		0.92		0.87		0.85		0.86		0.80	
R adj.	0.64		0.53		0.63		0.87		0.82		0.79		0.78		0.74	

x_1 (X_1 = initial pH of DSF); x_2 (X_2 = mL water added to 10 g DSF); x_3 (X_3 = °C incubation temperature). MGLU = malonyl-β-glucoside isoflavones (µmol/g); AGLU = acetyl-β-glucoside isoflavones (µmol/g); GLU = β-glucoside isoflavones (µmol/g); AGLY = aglycone isoflavones (µmol/g); TPC = total phenolic content (mg GAE/g); DPPH = antioxidant activity determined by DPPH (µmol TE/g); ABTS = antioxidant activity determined by ABTS (µmol TE/g); FRAP = ferric reducing antioxidant power (µmol TE/g).
Mp = DSFF-Mp; Ao = DSFF-Ao.
* Significant ($p < 0.05$).

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Table 3 - Isoflavone concentrations (µmol/g), total phenolic content (mg of gallic acid equivalents/g), and antioxidant activity (µmol of Trolox equivalents/g) in the defatted soy flour fermented by *Monascus purpureus* (DSFF-Mp) and defatted soy flour fermented by *Aspergillus oryzae* (DSFF-Ao), obtained using optimal fermentation conditions (initial pH= 6.0; 10 mL water added to 10 g of DSF; incubated at 30 °C).

Response variables	DSFF-Mp		Response variables	DSFF-Ao	
	Predicted values	Observed values		Predicted values	Observed values
$MGLU_{Mp}$	-	0.31±0.03	$MGLU_{Ao}$	$0.13±0.04^{B}$	$0.31±0.03^{A}$
$AGLU_{Mp}$	$0.49±0.11^{A}$	$0.41±0.02^{A}$	$AGLU_{Ao}$	$0.53±0.16^{A}$	$0.52±0.02^{A}$
GRU_{Mp}	$1.34±1.04^{A}$	$1.23±0.13^{A}$	GRU_{Ao}	$2.52±0.75^{A}$	$2.23±0.13^{A}$
$AGLY_{Mp}$	$3.30±0.69^{A}$	$3.53±0.02^{A}$	$AGLY_{Ao}$	$1.84±0.49^{A}$	$1.76±0.10^{A}$
TPC_{Mp}	-	2.20±0.03	TPC_{Ao}	$3.94±0.83^{A}$	$3.68±0.19^{A}$
$DPPH_{Mp}$	-	0.74±0.02	$DPPH_{Ao}$	$0.22±0.12^{A}$	$0.38±0.01^{A}$
$FRAP_{Mp}$	$14.55±1.72^{A}$	$14.26±0.44^{A}$	$FRAP_{Ao}$	$26.70±6.68^{A}$	$24.57±0.42^{A}$
$ABTS_{Mp}$	-	59.61±6.68	$ABTS_{Ao}$	$85.61±17.99^{A}$	$81.35±3.41^{A}$

Results are expressed as the mean (n = 3) ± standard deviation. Means with identical capital letters in the same line were not significantly different ($p > 0.05$).
MGLU = malonyl-β-glucoside isoflavones (µmol/g); AGLU = acetyl-β-glucoside isoflavones (µmol/g); GLU = β-glucoside isoflavones (µmol/g); AGLY = aglycone isoflavones (µmol/g); TPC = total phenolic content (mg GAE/g); DPPH = antioxidant activity determined by DPPH (µmol TE/g); ABTS = antioxidant activity determined by ABTS (µmol TE/g); FRAP = ferric reducing antioxidant power (µmol TE/g).
Mp = DSFF-Mp; Ao = DSFF-Ao.

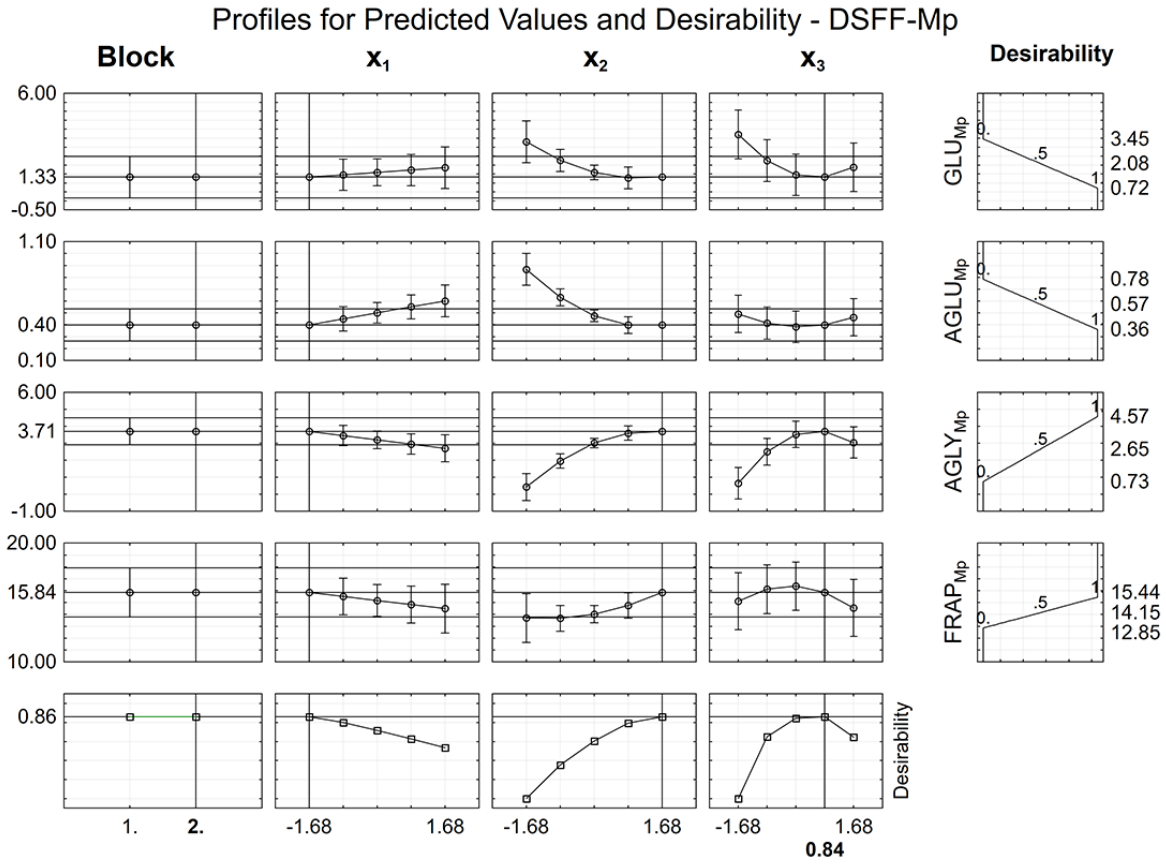
Table 4 - Pearson's correlation coefficients (r) between different forms of isoflavones, TPC and antioxidant activity for defatted soy flour fermented by *Monascus purpureus* (DSFF-Mp) and defatted soy flour fermented by *Aspergillus oryzae* (DSFF-Ao).

	GLU	AGLU	MGLU	AGLY	TPC	DPPH	FRAP
AGLU	0.98*	-	-	-	-	-	-
MGLU	0.15	0.19	-	-	-	-	-
AGLY	-0.98*	-0.95*	-0.09	-	-	-	-
TPC	0.95*	0.96*	0.10	-0.98*	-	-	-
DPPH	-0.96*	-0.94*	-0.01	0.99*	-0.99*	-	-
FRAP	0.98*	0.97*	0.08	-0.99*	0.99*	-0.99*	
ABTS	0.95*	0.91**	0.12	-0.93*	0.91**	-0.91**	0.94*

MGLU = malonyl-β-glucoside isoflavones (µmol/g); AGLU = acetyl-β-glucoside isoflavones (µmol/g); GLU = β-glucoside isoflavones (µmol/g); AGLY = aglycone isoflavones (µmol/g); TPC = total phenolic content (mg GAE/g); DPPH = antioxidant activity determined by DPPH (µmol TE/g); ABTS = antioxidant activity determined by ABTS (µmol TE/g); FRAP = ferric reducing antioxidant power (µmol TE/g).
*Significant correlation test, $p < 0.01$
**Significant correlation test, $p < 0.05$

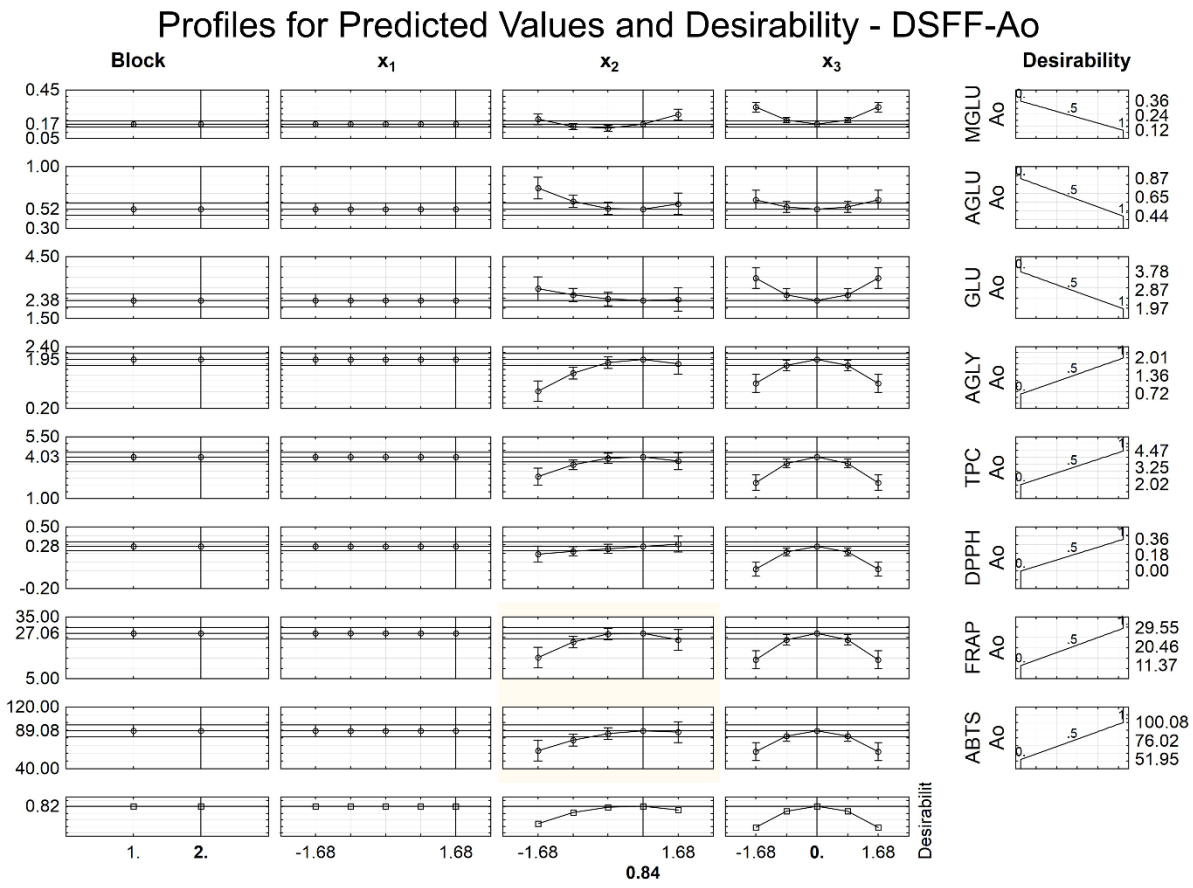
Figures:

Figure 1 - Profiles for the predicted values and overall desirability for defatted soy flour fermented by *Monascus purpureus* (DSFF-Mp).



x_1 (initial pH of DSF); x_2 (mL water added to10 g DSF); x_3 (°C incubation); AGLU = acetyl-β-glucoside isoflavones; GLU = β-glucoside isoflavones; AGLY = aglycone isoflavones; FRAP = ferric reducing antioxidant power.

Figure 2 - Profiles for the predicted values and overall desirability for defatted soy flour fermented by *Aspergillus oryzae* (DSFF-Ao).



x_1 (initial pH of DSF); x_2 (mL water added to 10 g DSF); x_3 (°C incubation); MGLU = malonyl-β-glucoside isoflavones; AGLU = acetyl-β-glucoside isoflavones; GLU = β-glucoside isoflavones; AGLY = aglycone isoflavones; TPC = total phenolic content; DPPH = antioxidant activity determined by the DPPH; ABTS = antioxidant activity determined by the ABTS; FRAP = ferric reducing antioxidant power.

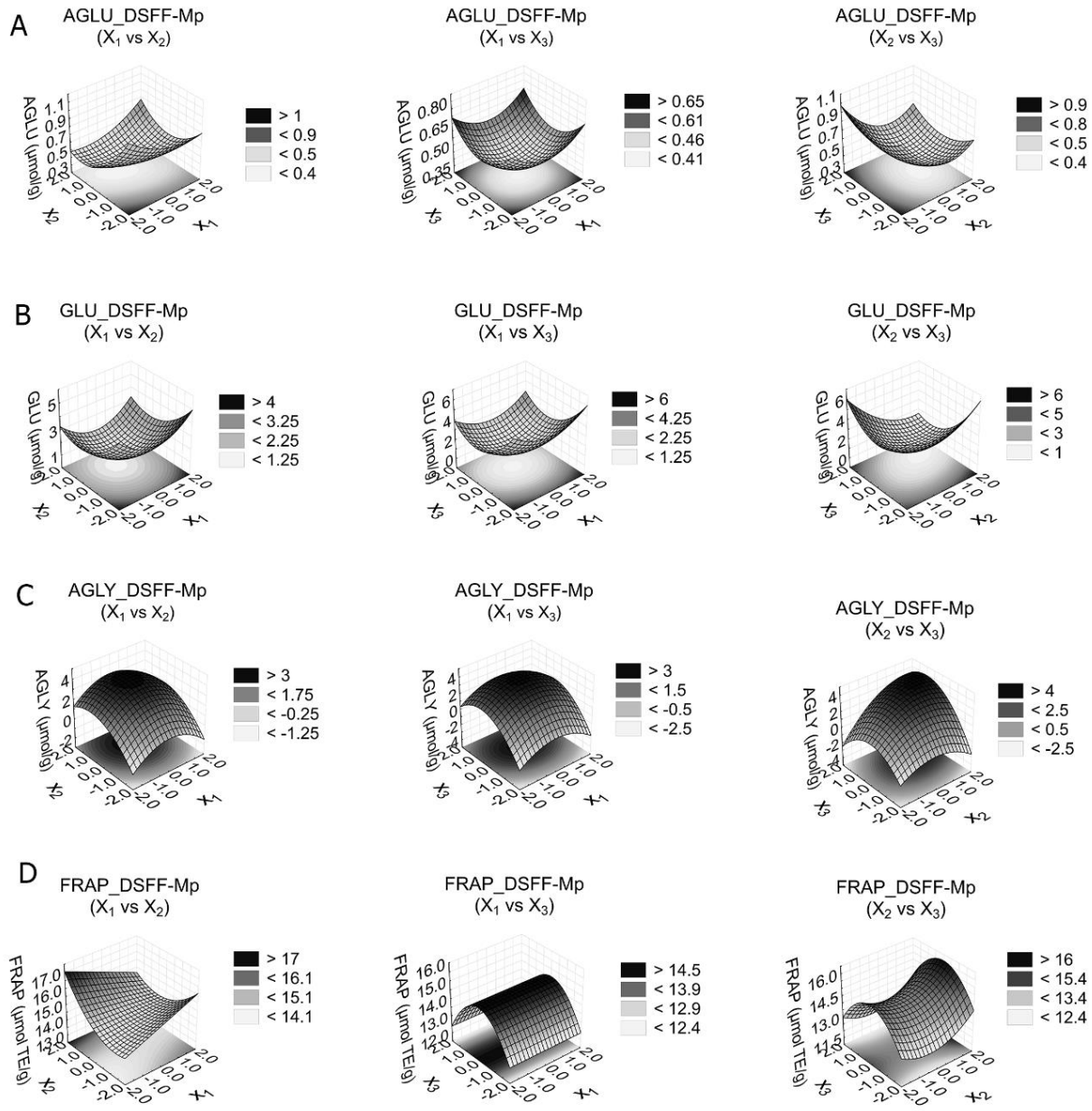
Supplementary Material for the Manuscript Entitled:

Parameters of the fermentation of soybean flour by *Monascus purpureus* or *Aspergillus oryzae* on the production of bioactive compounds and antioxidant activity

Cintia Ladeira Handa, Fernando Sanches de Lima, Marcela Fernanda Geton Guelfi, Meg da Silva Fernandes, Sandra Regina Georgetti, Elza Iouko Ida

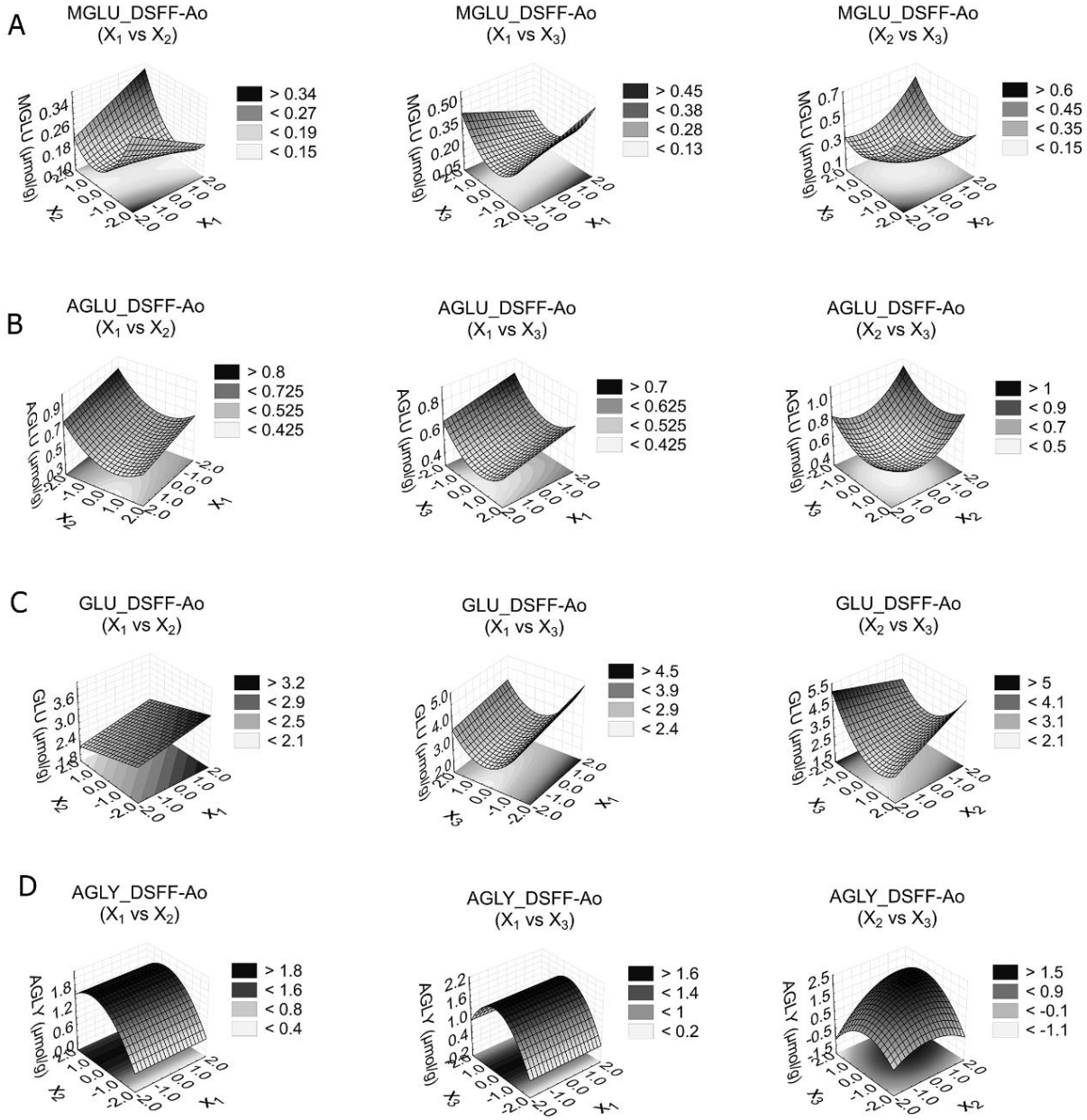
Supplementary Material

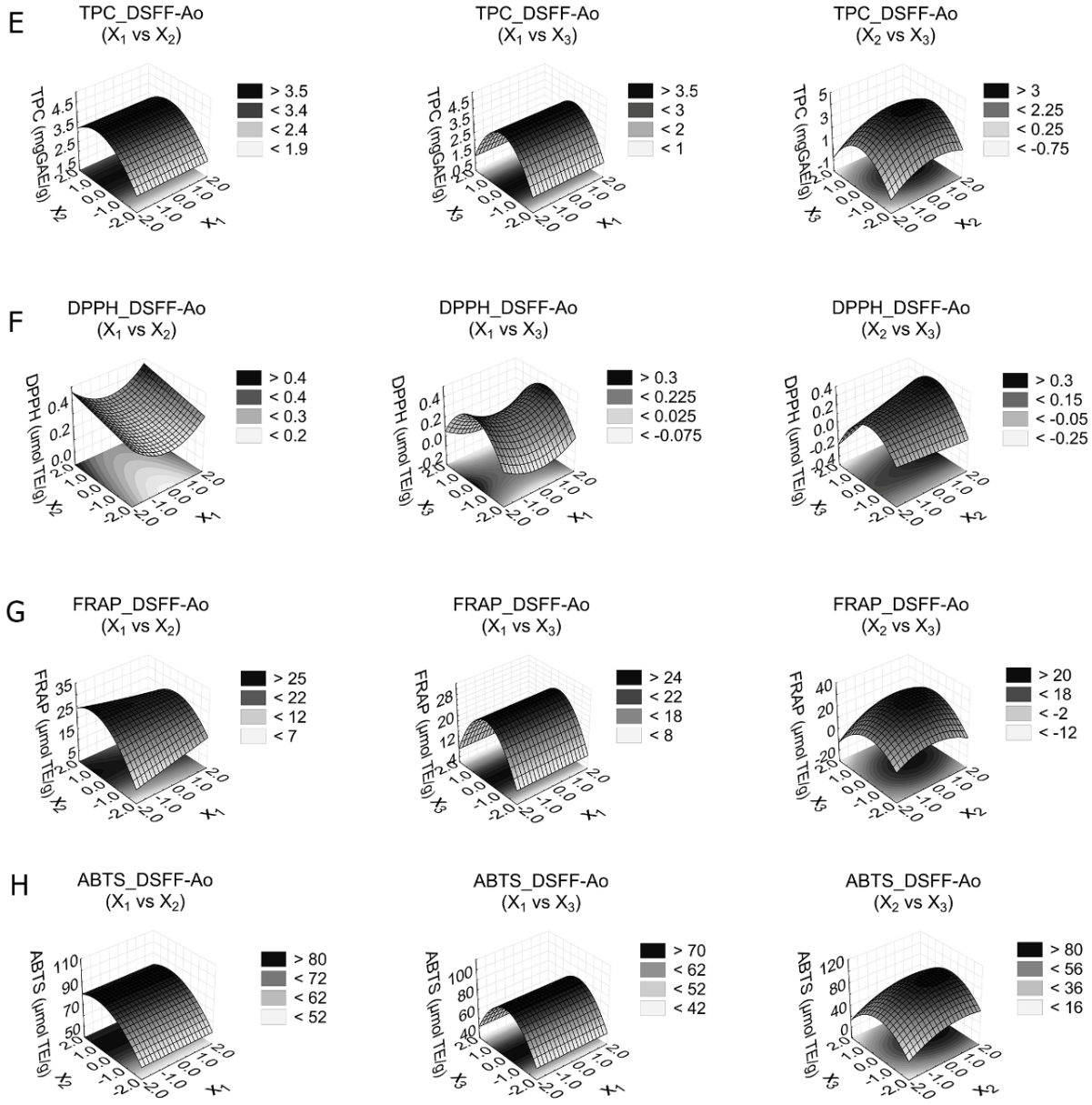
Supplementary Figure S1- Response Surface defatted soy flour fermented by *Monascus purpureus* (DSFF-Mp).



x_1 (initial pH of DSF); x_2 (mL water added to 10 g DSF); x_3 (°C incubation).
A) AGLU = acetyl-β-glucoside isoflavones;
B) GLU = β-glucoside isoflavones;
C) AGLY = aglycone isoflavones;
D) FRAP = ferric reducing antioxidant power.

Supplementary Figure S2- Response Surface for defatted soy flour fermented by *Aspergillus oryzae* (DSFF-Ao).





x_1 (initial pH of DSF); x_2 (mL water added to 10 g DSF); x_3 (°C incubation).

A) MGLU = malonyl-β-glucoside isoflavones
B) AGLU = acetyl-β-glucoside isoflavones
C) GLU = β-glucoside isoflavones
D) AGLY = aglycone isoflavones
E) TPC = total phenolic content
F) DPPH = antioxidant activity determined by the DPPH
G) ABTS = antioxidant activity determined by the ABTS
H) FRAP = ferric reducing antioxidant power.

5.3 ARTIGO CIENTÍFICO 3

Fermentation time of soybean flour by *Monascus purpureus* or *Aspergillus oryzae* on the content of bioactive compounds with antioxidant potential and oligosaccharides

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Abstract

The objective was to evaluate the effect of solid-state fermentation (SSF) time of the defatted soybean flour (DSF) by *Monascus purpureus* (DSFF-Mp) or *Aspergillus*

oryzae (DSFF-Ao) on the β-glucosidase activity, isoflavones content, total phenolic content (TPC), antioxidant capacity, total proteins, and soluble sugars content. The fermentation time (1-7 days) of DSFF-Ao increased the β-glucosidase activity, TPC, protein and antioxidant activity; reduced the content of isoflavones and soluble sugars. For DSFF-Mp, it increased the β-glucosidase activity, TPC, aglycone isoflavones and sucrose; did not alter the proteins content and antioxidant activity; reduced the glycosylated isoflavones and oligosaccharides. For DPPH and ABTS, the IC_{50} of DSF>DSFF-Mp>DSFF-Ao. For iron reducing ability DSFF-Ao>DSFF-Mp>DSF. For Fe^{2+} chelating activity, the IC_{50} of DSF=DSFFAo>DSFF-Mp. Therefore, it is recommended to ferment the DSF: (i) by *Aspergillus oryzae* for 4-7 days to obtain a DSFF-Ao with higher TPC and antioxidant and (ii) by *Monascus purpureus* for 3-7 days to obtain a DSFF-Mp with higher aglycone isoflavones and sucrose.

Keywords: β-glucosidase; Isoflavones; Soluble sugars; Solid-state fermentation.

Chemical compounds studied in this article: Daidzein (PubChem CID: 5281708), Daidzin (PubChem CID: 107971), 6''-O-Acetyldaidzin (PubChem CID: 156155), 6''-O-Malonyldaidzin (PubChem CID: 9913968), Genistein (PubChem CID: 5280961), Genistin (PubChem CID: 5281377), 6"-O-Acetylgenistin (PubChem CID: 5315831), 6''-O-Malonylgenistin (PubChem CID: 53398685), Glycitein (PubChem CID: 5317750), Glycitin (PubChem CID: 187808), 6''-O-Acetylglycitin (PubChem CID: 10228095), 6''-O-Malonylglycitin (PubChem CID: 23724657).

1. Introduction

Soybean is an important food source to humans and animals providing proteins, oil, carbohydrates, isoflavones, and other nutrients (Porfiri, Cabezas, & Wagner, 2016).

The major sugars present in soybean include sucrose, raffinose, and stachyose, which represent in average 55.16%, 33.97%, and 8.50% of total soluble sugars, respectively (Hou, Chen, Shi, Zhang, & Wang, 2009). Both raffinose and stachyose belong to the raffinose family of oligosaccharides (RFOs) (Hagely, Palmquist, & Bilyeu, 2013). RFOs are considered potential prebiotics that may be used to improve immune function (Ma, Wu, Giovanni, & Meng, 2016). However, RFOs have been described to cause flatulence after the ingestion of soybean and other legumes, which restrict their acceptance (De Fátima Viana et al., 2005; Hagely et al., 2013). Isoflavones are the main secondary metabolites in soybean and derivative products. There are three main basic molecular forms namely genistein, daidzein and glycitein (aglycone forms) with three derivatives per form denominated of β-glucosides, 6-O″-acetyl-β-glucosides and 6-O″-malonyl-β-glucosides, giving a totality 12 isoflavones (Benjamin, Stéphane, Gérard, & Pierre-Yves, 2017). In soybeans, glycosylated forms are present in greater amounts, although aglycones are more bioactive (Xie, Hettiarachchy, Cai, Tsuruhami, & Koikeda, 2003). In addition to isoflavones, soybean contains other phenolic compounds that have antioxidant potential (Handa, De Lima, Guelfi, Georgetti, & Ida, 2016). Most phenolic compounds occur mainly in conjugated forms, bound to sugar or other compounds, which reduce their ability to act as antioxidants, since the resonance stabilization of free radicals depends on the availability of the free hydroxyl groups in the phenolic rings (Dulf, Vodnar, & Socaciu, 2016).

Defatted soybean flour (DSF) is a product obtained from the oil extraction process and has been used for animal feed or as a source of proteins (Villalobos et al., 2016). The DSF contains 52.00% proteins and 36.00% carbohydrates (Bainy, Tosh, Corredig, Poysa, & Woodrow, 2008; Li, Loman, Coffman, & Ju, 2017). The mainly carbohydrates in DSF are non-starch polysaccharides and oligosaccharides

(stachyose and raffinose) (Dersjant-Li & Peisker, 2010). DSF has higher concentration of isoflavones than the whole soybean flour and has a similar profile of isoflavones (Genovese, Barbosa, Pinto, & Lajolo, 2007).

There are many methods for improving the digestibility of legumes, such as cooking, soaking, steaming, extrusion, bleaching or biotechnology processes, such as fermentation. Fermentation has been used to produce fermented soybean foods or microbial fermentation broth feeding with soybean flour. Fermentation involves chemical transformations that may result in loss of isoflavones, conversion of glucosides to aglycones, and, in some cases the formation of isoflavone derivatives by the addition or substitution of functional groups in the chemical structure (Chang, 2014; Villares, Rostagno, García-Lafuente, Guillamón, & Martínez, 2011). Fermentation has a great potential for utilization of by-products by enhancing their antioxidant ability by cleaving the bond between phenolics and other substituents in conjugated molecule, based on the enzymes produced by microorganisms (Zhang et al., 2017). The fermentation process may also reduce the RFOs in legumes by carbohydrase produced during fermentation (Wang et al., 2014).

Monascus and *Aspergillus* have been used for fermentation process to improve antioxidant activity, to immobilize bioactive phenolic compounds, to convert the glycosylated isoflavones to aglycones and to hydrolyze the RFOs present in many substrates (Huang, Zhang, & Xue, 2017; Zhang et al., 2017; Chen, Madl, & Vadlani, 2013). Handa et al. (2014) studied the production of β-glucosidase by solid-state fermentation (SSF) of DSF by *Monascus purpureus* or *Aspergillus oryzae*. They observed that the highest production of β-glucosidase and conversion from glycosylated to aglycones isoflavones occurred when using *Monascus purpureus* or *Aspergillus oryzae* with an initial pH of 6.0, addition of 10 mL of water into DSF, and

incubation temperature at 30 °C. However, the effect of fermentation time on the immobilization of bioactive phenolic compounds, antioxidant activity, conversion of isoflavones to aglycones, and hydrolysis of RFOs in DSF has been little explored.

Therefore, the objective of this study was to evaluate the effect of SSF time of DSF by *Monascus purpureus* or *Aspergillus oryzae* on the β-glucosidase activity, isoflavones content, TPC, antioxidant capacity, total proteins, and soluble sugars content.

2. Material and methods

2.1. Material

DSF from commercial source was used as a substrate for SSF. *Monascus purpureus* NRRL 1992 (GenBank: JQ614061.1) from the Laboratory of Biochemistry and Applied Microbiology of the Institute of Food Science and Technology of the Federal University of Rio Grande do Sul (Porto Alegre, RS, Brazil). *Aspergillus oryzae* IOC 3999/1998 was donated by the Oswaldo Cruz Foundation (Fiocruz, Rio de Janeiro, Brazil) were used in this study. 6”-O-acetylglucoside and 6”-O-malonylglucoside (Wako Pure Chemical Industries Ltd., Osaka, Japan), β-glucoside and aglycone isoflavones (Sigma Aldrich Co., St. Louis, MO, U.S.A.) were used as individual standards in the quantification by UPLC® (Waters, Milford, MA, U.S.A.). *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) and *p*-nitrophenol (*p*-NP) (Sigma Aldrich Co.) were used for determination of β-glucosidase activity. 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH•), 2,4,6-tri(2-pyridyl)-S-triazine (TPTZ), 2,2`-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Folin–Ciocalteu reagent, gallic acid

and 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox) were acquired from Sigma Aldrich Co. and used to assess the antioxidant activity and quantify the TPC of the samples. All reagents used were of analytical or liquid chromatography grade.

2.2. Effect of the solid-state fermentation time of the defatted soybean flour by Monascus purpureus or Aspergillus oryzae on β-glucosidase activity, bioactive compounds, antioxidant activity, and total protein and soluble sugars content

The effect of the SSF time of DSF fermented (DSFF) by *Aspergillus oryzae* (DSFF-Ao) or *Monascus purpureus* (DSFF-Mp) on the β-glucosidase activity, bioactive compounds, antioxidant activity, total proteins, and soluble sugars content was evaluated in fermented products. The fermentation with the two fungi was carried out separately and as described by Handa, Couto, Vicensoti, Georgetti, and Ida (2014). In the fermentation, each substrate was prepared from 10 g of DSF and 10 mL of solution acidified with 0.5 mol/L of HCl and initial pH adjusted to 6.0. The homogenized substrate was transferred to a 250 mL Erlenmeyer flask, autoclaved at 121 °C for 15 min. After cooling, it was inoculated with a suspension of 10^7 spores and incubated at 30 °C for 0, 1, 2, 3, 4, 5, 6 e 7 days. After each incubation period, non-fermented DSF (control without fungi), DSFF-Ao, and DSFF-Mp were immediately frozen, lyophilized (Christ Alpha 2-4 LD plus, Osterode am Harz, Germany), milled (Ika A11 basic, St. Louis, MO, U.S.A.) and stored at -20 °C until the analysis. To evaluate the effect of fermentation time for up to 7 days, the following analyzes were performed: β-glucosidase specific activity, quantification of different isoflavone forms, TPC,

antioxidant activity measured by DPPH, ABTS and FRAP, total proteins, and soluble sugars content (galactose, glucose, fructose, sucrose, raffinose, and stachyose).

Principal component analysis (PCA) and cluster analysis were performed to cluster and classify the DSFF-Ao and DSFF-Mp and to establish an adequate fermentation time. The active variables were β-glucosidase activity, the content of the different isoflavone forms, TPC, soluble sugars content (sucrose, raffinose, and stachyose), and antioxidant activity by DPPH, ABTS, and FRAP. Supplementary variables were galactose, glucose and fructose. The projections of the variables on the principal components were designated as quadrants (Q), QI being the 1st quadrant and so on up to the 4th quadrant (QI, QII, QIII and QIV). The PCA was performed using the software program Statistic 10 (StatSoft, Tulsa, OK, U.S.A.).

After establishing the appropriate fermentation time of the DSFF-Ao and DSFF-Mp the IC_{50} values were calculated for fermented samples and DSF non-fermented by the concentration that caused 50.00 % inhibition to DPPH, ABTS and iron-chelating activity assays. In addition, the ferric-reducing antioxidant power was expressed in micromoles/liter of Trolox equivalent (TE) / micrograms/milliliters of extract (µmol/L of TE/µg/mL).

2.3. Analytical procedures

2.3.1 Determination of β-glucosidase activity

The β-glucosidase enzyme was extracted from DSFF-Ao and DSFF-Mp as described by Carrão-Panizzi and Bordingnon (2000). In the obtained extract, the β-glucosidase activity was determined as described by Matsuura and Obata (1993). The standard curve of *p*-NP (0.016–0.32 µmol) was constructed to determine the activity of

the enzyme. One activity unit (AU) was defined as the quantity of enzyme necessary to release 1 µmol of p-NP / min, under the experimental conditions. The specific activity (SA) was expressed as activity units/mg protein (AU/mg).

2.3.2. Determination of total and soluble proteins content

The total proteins content of DSFF-Ao and DSFF-Mp were determined using the Kjeldahl method with a conversion factor of 6.25 (AOAC, 1995) and expressed as percentage (%) of total proteins on the dry weight basis. The soluble proteins content was determined according to the method described by Bradford (1976) using bovine serum albumin (BSA). The results were expressed as mg of soluble proteins/g of sample (dry weight basis).

2.3.3. Determination of isoflavones by UPLC

The samples were previously degreased using hexane in the ratio 1:10 (g: mL, sample: hexane) under continuous stirring at 25 °C for 1 h, followed by vacuum filtration. The isoflavones were extracted in triplicate according to the methodology described by Yoshiara, Madeira, Delaroza, da Silva, and Ida (2012) and quantified as Handa et al. (2014). The quantification of the individual isoflavones was performed by external standardization from standard solutions (0.1; 0.05; 0.01; 0.005; 0.001 and 0.0005 mg/mL) of each isoflavone form. The identification of the isoflavones was done by comparing the retention time and the UV spectrum of the respective standards. The results were expressed as µmol of isoflavones/g of sample (dry weight basis).

2.3.4. Determination of total phenolic content

The TPC was extracted in duplicate from the previously degreased samples as described by Handa, De Lima, Guelfi, Georgetti, and Ida (2016). In the extracts obtained were determined the TPC by the colorimetric method of Folin-Ciocalteu (Kumazawa et al., 2002) using a standard curve of gallic acid (GA). The results of TPC were expressed as mg of GA equivalents/g of sample (dry weight basis).

2.3.5. Determination of antioxidant activity

DPPH$^{\bullet}$ scavenging ability - The antioxidant activity of the phenolic extracts of the samples using the DPPH$^{\bullet}$ radical was measured as Blois (1958). Solutions with known concentrations of Trolox were used for the calibration curve. The free radical scavenging activity was expressed as µmol TE/g of samples (dry weight basis). For calculation of IC_{50}, The DPPH scavenging ability of extracts was determined by the decrease in absorbance at 517 nm and the following equation was applied: % of activity = (1-sample absorbance/control absorbance) x 100 (Equation 1) (Georgetti, Casagrande, Moura-de-Carvalho Vicentini, Verri, & Fonseca, 2006).

FRAP assay - The reducing power of the Fe^{+3} was estimated as reported by Benzie and Strain (1996) with modifications (Handa et al., 2016). The ability of the extracts obtained to reduce Fe^{3+} as expressed as µmol of TE/g of sample (dry weight basis).

Cation ABTS Radical - The ABTS assay was performed as reported by Re et al. (1999). The free radical scavenging activity of the extracts obtained was expressed as µmol of TE/g of samples (dry weight basis). For calculation of IC_{50} the ABTS scavenging ability of extracts was determined by the decrease in absorbance at 730 nm and the equation 1 was applied.

Determination of iron-chelating activity using the bathophenanthroline (BPS) assay – BPS is a strong chelator of ferrous ion and this reaction results in a colored complex. Samples extracts chelating activity of iron was monitored through the suppression of the $Fe_2(BPS)_3$ complex formation. Measurements were performed at 530 and 700 nm and the IC_{50} value was calculated by equation 1 (Casagrande et al., 2006).

2.3.6. Calculation of inhibitory concentration (IC_{50}) for determination of antioxidant capacity

The antioxidant capacity of DSFF-Mp and DSFF-Ao (fermented for 5 days) and non-fermented DSF was expressed by the measure of IC_{50}. The IC_{50} which is the necessary concentration of the antioxidant to reduce the radical by 50.00 %; IC_{50}, the antioxidant activity of the extract or its anti-radical power (Negri, Possamai, & Nakashima, 2009). In order to calculate the IC_{50} the phenolic extracts were obtained from the DSFF-Mp and DSFF-Ao (fermented for 5 days) and from the non-fermented DSF (as described in item 2.3.4). The extracts were then concentrated using the 40 °C, lyophilized, vacuum-packed and the dried extract was stored at -20 °C until the of IC_{50} analysis. For determination of IC_{50}, the lyophilized extracts were solubilized at different concentrations using ethanol to obtain at least five concentrations. The IC_{50} value was obtained using the software GraphPad Prism®, version 5.00, 2007, using a hyperbolic curve (one site binding hyperbola). Results were expressed as mean ± standard error. For the iron-reducing power test, the results were expressed as µmol/L of TE/µg/mL of the sample using the standard curve prepared with the Trolox.

2.3.7. Determination of soluble sugar content by HPAEC

The DSFF-Ao and DSFF-Mp were previously degreased and the extraction of sugars was performed with 0.2 g of sample and 8 mL of ethanol solution (80 mL: 20 mL, absolute ethanol: ultra-pure water) was added, followed by continuous stirring (orbital shaker, 305 rpm) for 1 h at 25 °C. The mixture was then centrifuged (2070 × g at 25 °C for 15 min; Centrifuge 5804R and Eppendorf, Hamburg, German) and 0.5 mL of the supernatant was transferred into a micro-centrifuge tube and submitted to centrifugal vacuum concentration at 927 × g at 25 °C (Jouan®, model RC 10.22, Jouan, Inc., Winchester, VA, USA) until evaporation of the solvent. The concentrated material was solubilized in 10 mL of ultra-pure water and filtered (Millex-GV, PVDF hydrophilic membrane, 0.22 µm, Millipore, Billerica, MA, USA) prior to injection into the high-performance anion exchange chromatography (HPAEC) instrument. Aliquots of 10 µL of filtered extract were automatically injected into an ICS 5000 (Dionex Canada Ltd., Oakville, Canada) chromatograph equipped with a CarboPac® PA1 column (250 mm × 4 mm, 10 µm; Dionex/Thermo Fisher Scientific), preceded by a CarboPac® PA1 guard column (50 mm × 4 mm, 10 µm), and a pulsed amperometric detector (PAD; Dionex/Thermo Fisher Scientific). Sugars were separated using 20 mmol of NaOH/L of ultra-pure water, which was comprised of 90.00% solvent A (ultra-pure water) and 10.00% solvent B (200 mmol of NaOH/L of ultra-pure water) with isocratic elution for 52 min at 1 mL/min at 25 °C. At the end chromatographic run, a column washing step was performed with 200 mmol of NaOH/L of ultra-pure water for 10 min at 25 ºC followed by column stabilization with 20 mmol of NaOH/L of ultra-pure water for 15 min. For the detection of sugars, a working gold electrode connected to a pH-Ag/AgCl reference electrode (Dionex/Thermo Scientific) was used to promote the oxidation of the sugars by means of a waveform (E = potential, t = duration): E1 = +0.1 V, t1 = 400 ms; E2 = -2.0 V, t2 = 20 ms; E3 = +0.6, t3 = 10 ms; and E4 = -0.10, t4 = 70 ms. For the

quantification of individual sugars, external calibration curves were constructed from standard aqueous solutions with ultra-pure water using 0.5-25 µg of galactose/mL, 0.5-25 µg of glucose/mL, 0.5-30 µg of fructose/mL, 0.5-60 µg of sucrose/mL, 0.5-60 µg of raffinose/mL and 0.5-60 µg of stachyose/mL. Chromeleon software 6.8 (Dionex/Thermo Scientific) was used for data acquisition. The sugar content was expressed as mg sugar/g of sample (dry weight basis).

2.4. Statistical analysis

To evaluate the effect of the fermentation time in obtaining of the DSFF-Ao and DSFF-Mp on the β-glucosidase activity, isoflavones content, TPC, antioxidant capacity, total protein, and soluble sugars content, the fermentations were performed in duplicate and the analytical procedures in triplicate (n = 2 x 3 = 6). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test (α = 0.05) was carried out using the software program Statistic 10 (StatSoft, Tulsa, OK, U.S.A.).

3. RESULTS AND DISCUSSION

3.1. Effect of the solid-state fermentation time of the defatted soybean flour by Aspergillus oryzae or Monascus purpureus on the activity of β-glucosidase and isoflavone content

The fermentation time of up to 4 days of DSFF-Ao and by 5 days for DSFF-Mp showed a significant effect on the β-glucosidase activity with increase 2.71 and 7.66-fold in comparison with the 1st day of fermentation and remained constant until the 7th

day, respectively (Tables 1 and 2). The β-glucosidase activity of DSFF-Ao was 5.29-fold higher than DSFF-Mp (Tables 1 and 2) and indicated that *Aspergillus oryzae* had a greater ability to produce the enzyme. Therefore, the fermentation of DSF by *Aspergillus oryzae* may be useful to obtain fermented DSF with high β-glucosidase activity. This enzyme of high activity and produced by this fungus, may be extracted, purified, characterized and applied in different sectors, such as in the pharmaceutical or food industries.

The fermentation time of DSFF-Ao influenced significantly the content of glycosylated isoflavones (β-glucoside, malonyl, and acetyl isoflavones) (Table 1). Regarding the zero fermentation time, the daidzin content reduced 69.00% by the 3rd day and was not detected from the 4th to 7th day. However, the content of glycitin reduced by 19.00% on the 1st day and was not detected from the 2nd to 7th day. Furthermore, the genistin content decreased by 92.00% until the 6th day of fermentation and was not detected on the 7th day. Therefore, the fermentation time influenced in the reduction of 100.00% in the isoflavone content of β-glucosides (glycitin, daidzin, and genistin), which occurred in distinct fermentation times: 2nd, 4th and 7th day, respectively. The reduction of these isoflavones may be associated with the action of β-glucosidase, whose activity increased until the 4th day of fermentation and remained constant until the 7th day (Table 1). The fermentation time of DSFF-Ao did not influence the content of malonyl daidzin, malonyl glycitin, acetyl daidzin, and acetyl glycitin, whose content was not detected until 7 days of fermentation. However, the fermentation time of DSFF-Ao influenced the content of malonyl genistin and acetyl genistin with reduction of 51.00% and 44.00% until the 3rd day of fermentation, respectively, and were not detected from the 4th to 7th day, respectively (Table 1). In relation to the aglycone isoflavones, the fermentation time of DSFF-Ao influenced in a

different manner, whereas from the time zero to 7th day of fermentation, the glycitein was not detected. The content of daidzein and genistein increased by twice until the 3rd day of fermentation and reduced by 60.00% and 44.00% until the 7th day of fermentation, respectively (Table 1).

As observed in DSFF-Ao, the fermentation time of DSFF-Mp significantly influenced the content of glycosylated isoflavones (β-glucoside, malonyl, and acetyl isoflavones) (Table 2). The daidzin content in the DSSF-Mp decreased by 97.00% until the 3rd day, remained unchanged until the 6th day, and it was not detected on the 7th day. The glycitin content decreased by 11.00% on the 1st day of fermentation and was not detected from the 2nd to 7th day. The genistin content reduced by 87.00% until the 5th and 6th day of fermentation and was not detected on the 7th day. Regarding the content of the isoflavones malonyl and acetyl glucosides, the fermentation time of DSFF-Mp did not influence the content of malonyl daidzin, malonyl glycitin, acetyl daidzin, and acetyl glycitin, whose content was not detected until 7 days of fermentation. Regarding zero fermentation time, the malonyl genistin content decreased by 69.00% until the 7th day of fermentation and the acetyl genistin content decreased by 43.00% until the 2nd day of fermentation and was not detected from the 3rd to 7th day. In relation to the aglycone isoflavones content of the DSFF-Mp, the fermentation time had a different influence, and the glycitein was not detected until the 7th day of fermentation. However, regarding the zero fermentation time, the content of daidzein and genistein increased 4.3 and 5.3-fold until the 7th day, respectively. The results indicated that glycosylated isoflavones were converted to their aglycone forms with the fermentation time by *Monascus purpureus*. The interconversion of the 12 isoflavones in soybeans and soy products may occur during the different processing,

such as fermentation, soaking, germination, and heat treatment, by reaction of decarboxylation, de-esterification or deglycosylation (Chen et al., 2015).

Comparing the effect of fermentation time between DSFF-Ao and DSFF-Mp on the conversion of the glycosylated isoflavones to aglycones (Tables 1 and 2), the aglycones content (daidzein and genistein) was maximal on the 3rd and 7th days of fermentation for DSFF-Ao and DSFF-Mp, respectively. After the 3rd day of fermentation, DSFF-Ao the aglycone isoflavones content reduced until the 7th day, indicating that aglycones were metabolized in the fermentation process by *Aspergillus oryzae*. However, until the 7th day of fermentation in the DSFF-Mp, there was an increase in the content of aglycone isoflavones, indicating that aglycones were not metabolized in the fermentation process by *Monascus purpureus*. These results are in agreement with the observed by Chang, Ding, Tai, and Wu (2007) who found that *Aspergillus oryzae* metabolized daidzein and genistein to 8-hydroxydaidzein and 8-hydroxygenistein after 2 and 3 days of submerged fermentation of medium, respectively. On the other hand, no metabolite was observed when *Monascus purpureus* was used. It has further been observed (Tables 1 and 2) that the maximum level of daidzein and genistein in the DSFF-Mp was 2.15 and 2.84-fold higher than in the DSFF-Ao, respectively. Although the β-glucosidase activity was higher in DSFF-Ao than in DSFF-Mp (Tables 1 and 2), these results may be due to the greater specificity of the β-glucosidase produced by *Monascus purpureus* than by *Aspergillus oryzae* in the SSF of the DSF for the conversion of the glycosylated isoflavones to their aglycone forms as observed by Handa et al. (2014). Therefore, the fermentation of DSF by *Monascus purpureus* may be useful for obtaining a DSFF-Mp containing β-glucosidase with high specificity for conversion of glycosylated isoflavones to aglycones. This enzyme produced by *Monascus purpureus* may be extracted, purified,

characterized and applied in different foods containing glycosylated isoflavones, such as soybean products, in order to obtain a product with higher aglycones content.

3.2. Effect of the solid-state fermentation time of the defatted soybean flour by Aspergillus oryzae or Monascus purpureus on TPC, antioxidant activity, and protein content

The fermentation time of DSFF-Ao and DSFF-Mp had a significant effect on TPC (Tables 1 and 2). Regarding the zero fermentation time, in the DSFF-Ao fermented for 5 days, the TPC presented an increase of 3.96-fold and remained constant until the 7th day (Table 1). While the fermentation by only 1 or 2 days of the DSFF-Mp (Table 2) increased 1.43-fold, reduced 9.70% on the 3rd day and did not change until the 6th fermentation. After 7 days of fermentation, the TPC increased by 5.94% and did not differ of the DSFF-Mp for 2 days. Thus, it was observed that fermentation using the two fungi increased TPC; this may be associated with the release of phenolic compounds from macromolecules such as lignin, proteins, and carbohydrates of the cell wall of the DSF. McCue and Shetty (2003) found that carbohydrate-conjugated phenolics can be biochemically rearranged for full mobilization by fungal glucosidases, and possibly by lignocellulolytic enzymes.

The fermentation time of the DSFF-Ao and DSFF-Mp influenced the antioxidant activity (Tables 1 and 2). Thus, it was observed that, in relation to zero time, the antioxidant activity of DSFF-Ao (Table 1) measured by FRAP and ABTS increased 4.67 and 2.98-fold, respectively, until the 4th day of fermentation and remained constant until the 7th day. Likewise, the antioxidant activity measured by DPPH increased from 5.73-fold until the 7th day. However, the fermentation time of the DSFF-

Mp did not influence the antioxidant activity measured by FRAP and ABTS (Table 2). Meanwhile, after two days fermentation of DSFF-Mp, the antioxidant activity measured by DPPH increased by 1.27-fold and then reduced by 10.80% until the 7th, when did not differ from zero and 1 day of fermentation. In another study, Lee, Yang, and Mau (2008) described that non-fermented soybeans were more effective in antioxidant activity and scavenging ability on DPPH radicals whereas fermented soybean by *Monascus purpureus* or *Monascus pilosus* were more effective in reducing power and scavenging ability on hydroxyl radicals. Therefore, fermentation of DSF by *Aspergillus oryzae* was more effective for increasing antioxidant activity than by *Monascus purpureus*.

The fermentation time of DSF influenced the total protein content of DSFF-Ao (Table 1), whereas for DSFF-Mp the fermentation time had no significant effect (Table 2). By the 5th day of fermentation, the total protein content of DSFF-Ao increased 1.25-fold and remained constant up to 7 days (Table 1). Chen, Madl, and Vadlani (2013) also observed the increase in total protein content in soybean flour fermented by *Aspergillus oryzae*. It was reported that during SSF of soybean flour using *Aspergillus oryzae,* the protein content kept increasing from the original 50.00% to approximately 63.00% after 7 days of fermentation. This increase was possibly due to the utilization of carbohydrates that contributed to the dry matter loss. In addition, it was observed that *Aspergillus oryzae* single cell protein and dry matter losses were the reasons for protein content increase and the fermentation was a manner to enhance protein content of soybean products.

Comparing the fermentation times of DSFF-Ao and DSFF-Mp (Tables 1 and 2), it was observed that the maximum TPC, antioxidant activity, and total protein occurred at different times. The maximums of TPC, FRAP, DPPH, ABTS and total protein of

DSFF-Ao were 2.76, 4.48, 4.49, 2.50, and 1.22-fold higher than DSSF-Mp, respectively. These results indicated that *Aspergillus oryzae* showed a greater capacity to release phenolic compounds from macromolecules or other compounds with antioxidant activity than *Monascus purpureus*.

3.3. Effect of the solid-state fermentation time of the defatted soybean flour by Aspergillus oryzae or Monascus purpureus on soluble sugars

The fermentation time of DSF by fungi *Aspergillus oryzae* or *Monascus purpureus* had a distinct effect on the soluble sugars content of DSFF-Ao and DSFF-Mp (Figure 1). In relation to zero time and after the 1st and the 2nd day of fermentation of DSFF-Ao, the content of galactose and glucose increased 9.2 and 21-fold, respectively, followed by reduction until the 4th and 5th day, respectively, when did not differ from zero and remained constant until the 7th day (Figure 1a). After the 1st day of fermentation of DSFF-Ao, the fructose content increased 8-fold followed by a 95.66% reduction by the 3rd day, increased from 3.7-fold until the 4th day and decreased by 48.64% until the 5th day, which did not differ from zero and remained constant until the 7th day. Regarding DSFF-Mp (Figure 1c), the fermentation time influenced the content of galactose, glucose, and fructose. The lowest galactose content occurred on the 3rd day of fermentation and presented a reduction of 80.00% in comparison with zero time. On the 6th day of fermentation, the galactose content was maximum and 1.2-fold higher than on zero time. The glucose content reduced by 90.00% from the zero to 2nd day of fermentation, on the 3rd day did not change, then increased 5.94-fold until the 6th day and did not differ from the 7th day of fermentation, when the content was 42.00% lower than on the zero time. The fructose content of

DSFF-Mp reduced 39.00% after the 1st day of fermentation and until the 2nd day remained constant, it increased in the 3rd day, whose content did not differ from zero time and was unchanged until the 7th day of fermentation (Figure 1c). The effect of the fermentation time of DSFF-Ao and DSFF-Mp on the content of monosaccharides was distinct and dependent on the fungus used (Figures 1a and 1c). Since the maximum galactose, glucose and fructose content in the DSFF-Ao was 17, 21 and 9-fold higher than in the DSFF-Mp, respectively, and it occurred in different fermentation time. Therefore, *Aspergillus oryzae* produced enzymes with higher specificity for the production of these sugars than *Monascus purpureus*.

The fermentation time influenced the sucrose, raffinose and stachyose content of DSFF-Ao. From the zero to 3rd day of fermentation of DSFF-Ao a 98.00% reduction in sucrose content was observed and it was not detected from the 4th day to 7th. However, after the 1st day of fermentation, the raffinose content increased 1.4-fold, followed by 99.00% reduction by 4th day and remained constant until the 7th day. After four days of fermentation, the stachyose content decreased 99.00% and did not differ until the 7th day. Therefore, from the 4th day of fermentation of DSF, the *Aspergillus oryzae* consumed all the major sugars of DSF, such as sucrose, raffinose and stachyose. Nevertheless, Chen, Madl, and Vadlani (2013) observed up to 20 h soybean flour SSF by *Aspergillus oryzae*, the sucrose was completely consumed and oligosaccharides started to be used and after 28 h of fermentation were completely reduced. The sucrose content of DSFF-Mp increased 1.4-fold after the 1st day of fermentation and remained constant until the 7th day. The raffinose content increased 1.3-fold after the 2nd day of fermentation and reduced 96.00% by the 6th day, which did not differ from the 7th day. However, after the 1st day of fermentation, the stachyose content was reduced by 83.00% and reduced completely (99.00%) on the

5th day. Therefore, sucrose was not hydrolyzed in the fermentation of DSF by *Monascus purpureus*, whereas the content of oligosaccharides (raffinose and stachyose) reduced almost completely after the 3rd day of fermentation, indicated that *Monascus purpureus* was unable to utilize sucrose. It may be the reason of the low content of monosaccharides found the DSFF-Mp. Babitha, Soccol, and Pandey (2006) reported that the addition of sucrose as a source of carbon reduced the growth of *Monascus purpureus* under SSF of jackfruit seeds (Babitha, Soccol, & Pandey, 2006). In the fermentation in submerged cultures, *Monascus sp*. assimilated and grow up well on media containing glucose or raffinose in comparison with the other tested carbon sources; they weakly utilize maltose and sucrose and practically cannot assimilate lactose (Pisareva & Kujumdzieva, 2006).

Comparing fermented DSF by both fungi, it was observed that *Monascus purpureus* did not degrade sucrose, whereas *Aspergillus oryzae* consumed 50.00% sucrose on the first 24 h of fermentation. The raffinose content increased after one or two days of fermentation of DSF by *Aspergillus oryzae* or *Monascus purpureus*, respectively, and reduced completely at subsequent times. This increase in the raffinose content on the early fermentation times can be attributed to the fact that raffinose is an intermediary product of stachyose hydrolysis. Therefore, it is confirmed that in the fermentation of DSF by the two fungi, raffinose and stachyose were completely hydrolyzed. The reduction of raffinose and stachyose may be due to α-galactosidase produced by the both fungi, which catalyzes the disruption of the α-D-galactosidic linkages of both simple and complex oligo and polysaccharides. *Aspergillus oryzae* has been reported to secrete α-galactosidase, which acts on gal-gal bonds in the tetrasaccharide stachyose, releasing galactose and raffinose; it also acts on gal-glu bonds with the release of sucrose (Chen, Madl, & Vadlani, 2013).

According to Wong et al. (1986) sugars with α-galactoside bonds (melibiose, raffinose, and stachyose) and galactose all showed good enzyme-inducing ability for producing of Monascus α-galactosidase. Raffinose was hydrolyzed to galactose and sucrose, while stachyose was hydrolyzed to galactose and sucrose, with raffinose as the intermediate compound.

3.4. Principal component analysis of the active and supplementary variables of defatted soy flour fermented by Monascus purpureus and defatted soy flour fermented by Aspergillus oryzae

The results of TPC, FRAP, DPPH, ABTS, malonyl, acetyl, β-glucoside, and aglycone isoflavones, sucrose, raffinose and stachyose from DSFF-Ao and DSFF-Mp were considered as active variables. As supplementary variables, the content of galactose, glucose and fructose was considered. Two principal components (PC1 and PC2) were obtained, and these explained 93.57% of the total variance of the data (Figure 2a). The total variance of 64.20% was explained by PC1, which was characterized mainly by sucrose (Q I), raffinose and malonyl isoflavone (Q IV), TPC, β-glucosidase and antioxidant activity (DPPH, ABTS and FRAP) (Q III). At the figure 2b, is noted that the DSFF-Ao in the intermediate and advanced fermentation times were characterized by greater TPC, highest β-glucosidase and antioxidant activity. While the DSFF-Mp and DSFF-Ao in the initial fermentation times were characterized by the higher content of malonyl isoflavones, sucrose, and raffinose.

The total variance of 29.37% was explained by PC2, which was characterized mainly by aglycone isoflavones (Q I), acetyl and β-glucoside isoflavones, and stachyose (Q IV). The DSFF-Mp in the intermediate and advanced fermentation times

were characterized by a higher content of aglycone isoflavones (Figure 2b). It is noteworthy that in the initial times of fermentation (Figures 2a and b), the DSFF-Ao and DSFF-Mp were characterized by higher content of acetyl and β-glucoside isoflavones and stachyose. The similarities among the DSFF-Mp and DSFF-Ao with different fermentation times were evaluated using cluster analysis (linkage distance = 3.04878, Ward`s method, Euclidean distances) which resulted in seven clusters (Figure 2c). Clusters 1, 2 and 3 were formed by non-fermented DSF (C0), DSFF-Ao fermented for one day (Ao1) and two days (Ao2), respectively. They are located in Q III (Ao2) and Q IV (C0 and Ao1) and were characterized as containing higher content of malonyl, acetyl and β-glucoside isoflavones and oligosaccharides (raffinose and stachyose) (Figure 2b). The cluster 4 was composed of DSFF-Ao fermented for three days (Ao3) and is located in the Q III. It was characterized by the higher content of monosaccharides galactose, glucose, and fructose. The cluster 5 was composed of DSFF-Ao fermented for 4 (Ao4), 5 (Ao5), 6 (Ao6), and 7 (Ao7) days and is located in Q III. It was characterized by higher TPC, higher β-glucosidase and antioxidant activity (DPPH, ABTS and FRAP). Cluster 6 was composed of DSFF-Mp fermented for one (Mp1) and two days (Mp2) and is located at the interface between the Qs I and IV (Figure 2b). It was characterized by the higher content of malonyl isoflavones and the oligosaccharide raffinose. The cluster 7 was composed of DSFF-Mp fermented for 3 (Mp3), 4 (Mp4), 5 (Mp5), 6 (Mp6), and 7 (Mp7) days and is located in the Q I. It was characterized by the higher content of aglycone isoflavones and sucrose. Therefore, by the PCA, the DSFF-Ao fermented for 4, 5, 6 and 7 days presented higher TPC, β-glucosidase and antioxidant activity (DPPH, ABTS and FRAP), and low sucrose and oligosaccharide content (raffinose and stachyose). Nevertheless, the DSFF-Mp fermented for 3, 4, 5, 6 and 7 days presented higher content of aglycone isoflavones

and sucrose and low content of oligosaccharides (raffinose and stachyose). Therefore, depending on the purpose of the soybean flour, as functional use or as an ingredient. It is recommended to ferment the DSF: (i) by *Aspergillus oryzae* for 4 to 7 days to obtain DSFF-Ao with higher TPC and antioxidant activity and (ii) by *Monascus purpureus* for 3 to 7 days to obtain a DSFF-Mp with higher content of aglycone isoflavones and sucrose. Finally, 5 day of fermentation time was selected for antioxidant capacity studies for both DSSF-Ao and DSFF-Mp.

3.5. Antioxidant capacity of phenolic extracts from non-fermented defatted soybean flour, defatted soy flour fermented by Monascus purpureus, and defatted soy flour fermented by Aspergillus oryzae

The antioxidant capacity of the extracts obtained from DSFF-Ao and DSFF-Mp with 5 days of fermentation and non-fermented DSF was evaluated regarding IC_{50} values measured by the ABTS, DPPH, and Fe^{+2} ion chelator activity, besides the iron reducing capacity by FRAP (Figure 3).

The results showed that the $DPPH^{\bullet}$ scavenging ability of extracts was concentration-dependent for the extracts analyzed. The maximal activity found for the non-fermented DSF, DSFF-Ao, and DSFF-Mp extracts were 69.00% at the concentration of 2.500 mg/mL of the reaction medium, 72.00% at the concentration of 1.250 mg/mL and 70.00% at the concentration of 2.500 mg/mL, reaching IC_{50} values of 1.150 mg/mL, 0.580 mg/mL and 0.980 mg/mL, respectively (Figures 3 a-c). Thus, these results indicated that the fermentative process of DSF by *Aspergillus oryzae* or *Monascus purpureus* provided extracts with greater hydrogen-donor capacity than the non-fermented DSF, being this activity superior when using *Aspergillus oryzae*.

For the ABTS radical scavenging test, the maximum activity achieved for non-fermented DSF, DSFF-Ao, and DSFF-Mp, using the concentration of 0.160 mg/mL in the reaction medium were similar, with values of 94.00%, 99.40% and 98.70%. Although the maximum antioxidant activity percentages of the extracts presented close values, the IC_{50} values were 0.061 mg/mL, 0.023 mg/mL and 0.052 mg/mL, respectively (Figures 3 d-f), demonstrating that DSFF-Ao presented better electron donating activity.

Therefore, the results indicated that the DSFF-Ao presents a higher free radical scavenging potential. In addition, by comparing between the two *in vitro* methodologies applied, the electron donor activity to the radical cation ($ABTS^{\bullet+}$) was more expressive than the hydrogen donor activity at the radical anion ($DPPH^{\bullet}$).

The Fe^{2+} chelating activity was concentration-dependent for all extracts, and the maximum chelator activity was found at 600 µg/mL in the reaction medium reaching a plateau. IC_{50} values were 124 µg/mL for control, 125 µg/mL for DSFF-Ao and 88.5 µg/mL for DSFF-Mp (Figures 3 g-i). These results suggest that the fermentation of DSF with *Monascus purpureus* provided an extract with compounds having a higher iron chelating capacity.

In the FRAP method, the result was expressed as Trolox equivalent (Figure 3 j-l), the higher the value, the greater the compound's ability to reduce Fe^{3+}, since it will be equivalent to the Trolox standard (water-soluble vitamin E analog) that has a high reducing power. The results were calculated using the Trolox analytical curve, which presented a linear correlation coefficient (R^2) greater than 0.99. The reducing power of the non-defatted DSF, DSFF-Ao, and DSFF-Mp was of 0.046 µmol/L; 0.064 µmol/L and 0.054 µmol/L of TE/µg of dried extract/mL, respectively.

By the results of the different antioxidant methods used, it was observed that the solid-state fermentation process of DSF by *Monascus purpureus* or *Aspergillus oryzae* improved the antioxidant capacity. The extract of DSFF-Ao presented a greater capacity of free radical sequestration and iron reducing capacity, while the extract of DSFF-Mp presented higher Fe^{2+} ion chelating capacity. The DSFF-Mp results may be due its high content of aglycone isoflavones. According to studies performed by Toscano and Russo (2016), the daidzein, genistein and glycitein anions form very stable 2:1 stoichiometry complexes with iron (II) cation and may act as powerful chelating agent of iron (II) metal cation. Chelating agents may inhibit the formation of radicals by stabilizing the transition metals, consequently reducing free radical damage (Xiao et al., 2015). Free radical scavenging can disrupt the chain of reaction or retard oxidation and may occur mainly through three mechanisms: i) the hydrogen atom transfer (HAT), ii) the electron transfer followed by a proton transfer (SET-PT), and iii) the sequential proton loss electron transfer (SPLET) (Toscano & Russo, 2016).

4. Conclusion

The SSF time of DSF by *Monascus purpureus* or *Aspergillus oryzae* influenced the β-glucosidase activity, content of the different forms of isoflavones and TPC, antioxidant activity and total proteins and soluble sugars content. The DSFF-Ao fermented for 4, 5, 6 or 7 days showed similarities and were characterized with higher TPC, higher activity of β-glucosidase and antioxidant (DPPH, ABTS and FRAP) and lower content of sucrose, raffinose and stachyose. Consequently, due to the antioxidant potential, DSFF-Ao can be useful as an ingredient in foods by adding in several foods such as meat and bakery products. While the DSFF-Mp fermented for 3,

619 4, 5, 6 or 7 days presented similarity and were characterized with higher content of
620 aglycone isoflavones and sucrose and lower content of raffinose and stachyose.
621 Therefore, DSFF-Mp can be useful as a raw material for the preparation of foods
622 containing aglycone isoflavones and sucrose.

623 The solid-state fermentation for 5 days of DSF by *Monascus purpureus* or
624 *Aspergillus oryzae* improved the antioxidant capacity assessed by the IC_{50} value. The
625 DSFF-Ao extract presented a higher capacity of free radical scavenging (DPPH and
626 ABTS) and reductive iron, whereas DSFF-Mp presented higher Fe^{2+} ion chelation
627 capacity. Therefore, both DSFF-Ao and DSFF-Mp presented great antioxidant ability
628 and have potential to be used in such a way as to prevent the oxidative stress due the
629 presence of free radicals in the human body.

630

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Tables:

Table 1 - Effect of fermentation time of defatted soy flour fermented by *Aspergillus oryzae* (DSFF-Ao) on the β-glucosidase, isoflavone content, TPC, and antioxidant activity.

Time (Day)	β-glucosidase (UA/mg)	Daidzin (μmol/g)	Glycitin (μmol/g)	Genistin (μmol/g)	M. Genistin (μmol/g)	A. genistin (μmol/g)	Dadzein (μmol/g)	Genistein (μmol/g)	TPC (mg GAE/g)	FRAP (μmol. TE/g)	DPPH (μmol TE /g)	ABTS (μmol TE /g)	Total Proteins (%)
0	-	1.593± 0.032 ^{a}	0.279± 0.005 ^{a}	2.243± 0.045 ^{a}	0.667± 0.020 ^{a}	0.273 ± 0.006 ^{b}	0.496± 0.007 ^{e}	0.469± 0.005 ^{d}	1.584± 0.043 ^{g}	17.002± 0.997 ^{d}	2.186± 0.198 ^{e}	58.911± 4.982^{d}	52±1^{e}
1	0.598± 0.258 ^{d}	1.553± 0.036 ^{a}	0.226± 0.005 ^{b}	2.197± 0.053 ^{a}	0.630± 0.026 ^{b}	0.286± 0.003 ^{a}	0.556± 0.004 ^{e}	0.503± 0.004^{d}	2.904± 0.018 ^{f}	18.783± 0.775 ^{d}	2.734± 0.085 ^{e}	70.225± 4.829 ^{d}	51±2 ^{d,e}
2	1.221± 0.233 ^{c}	0.972± 0.040 ^{b}	ND	1.601± 0.056 ^{b}	0.445± 0.039 ^{c}	0.221± 0.006 ^{c}	0.905± 0.024 ^{b}	0.764± 0.015 ^{b}	4.353± 0.025 ^{e}	41.533± 2.441 ^{c}	5.244± 0.107 ^{d}	117.890 ± 6.136 ^{c}	55±2 ^{c,d}
3	1.304± 0.091 $^{b\,c}$	0.494± 0.048^{c}	ND	0.873± 0.084 ^{c}	0.327± 0.022 ^{d}	0.154± 0.002 ^{d}	0.992± 0.040 ^{a}	0.844± 0.029 ^{a}	5.204± 0.083 ^{d}	53.760± 2.196 ^{b}	7.401± 0.526 ^{c}	136.417 ± 6.162 ^{b}	57±2 ^{c}
4	1.520± 0.049 ^{a,b}	ND	ND	0.407± 0.100 ^{d}	ND	ND	0.797± 0.064 ^{c}	0.760± 0.032 ^{b}	6.141± 0.054 ^{b,c}	76.674± 2.779 ^{a}	9.741± 0.744 ^{b}	177.201 ± 5.493 ^{a}	61±1^{b}
5	1.512± 0.115 ^{a,b}	ND	ND	0.333± 0.088 ^{d}	ND	ND	0.693± 0.072 ^{d}	0.696± 0.048 ^{c}	6.269± 0.068 ^{a}	80.567± 4.845 ^{a}	10.634± 0.932 ^{b}	177.446 ± 7.714 ^{a}	64±1 ^{a}
6	1.592± 0.098 ^{a,b}	ND	ND	0.187± 0.110 ^{e}	ND	ND	0.552± 0.039 ^{e}	0.631± 0.007 ^{c}	6.097± 0.024 ^{c}	78.058± 5.403 ^{a}	9.945± 0.979 ^{b}	170.630 ± 8.786 ^{a}	65±1 ^{a}
7	1.874± 0.178 ^{a}	ND	ND	ND	ND	ND	0.404± 0.029 ^{e,f}	0.476± 0.031^{d}	6.262± 0.080^{a,b}	82.203± 2.786 ^{a}	12.524± 0.661 ^{a}	177.118 ± 6.124 ^{a}	66±2 ^{a}

Results are expressed as the mean ± standard deviation (dry weight basis). Values followed by different superscript lowercase letters in the same column differ significantly ($p < 0.05$).

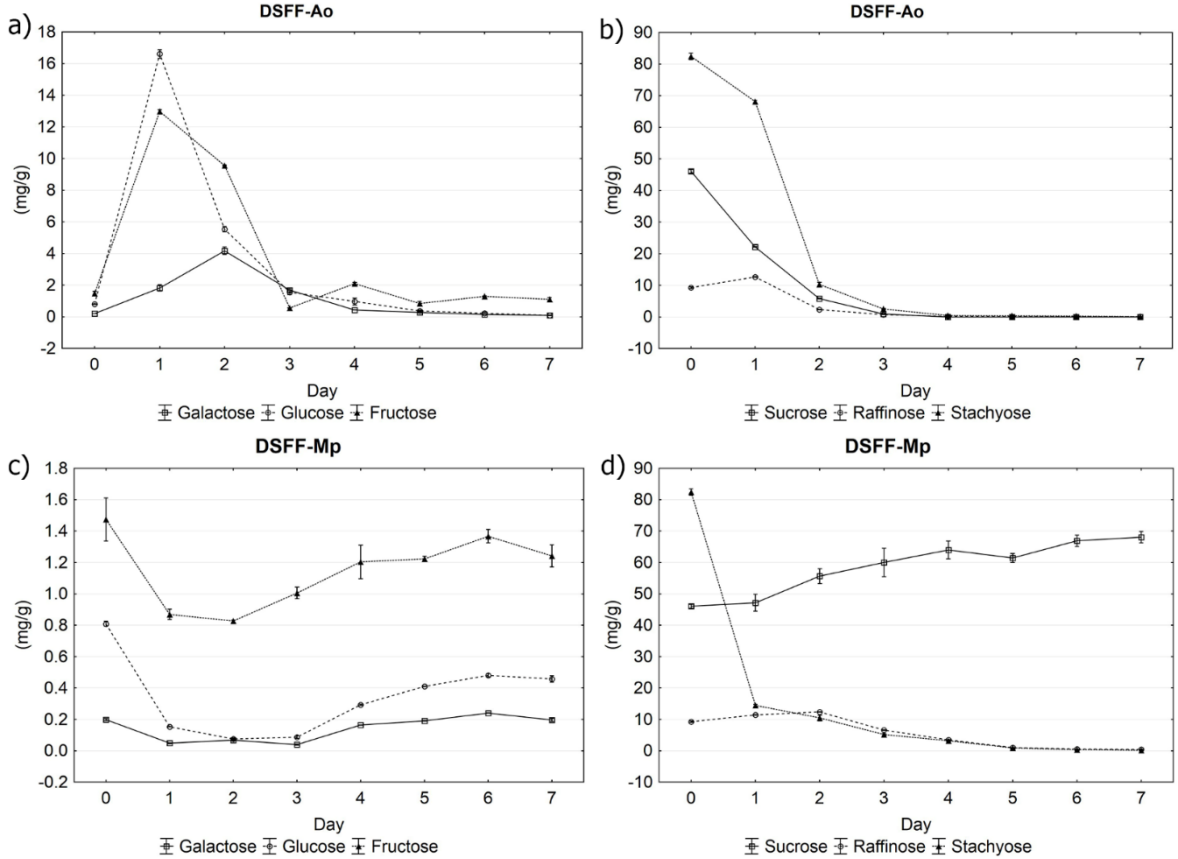
Table 2 - Effect of fermentation time of defatted soy flour fermented by *Monascus purpureus* (DSFF-Mp) on the β-glucosidase, isoflavone content, TPC, and antioxidant activity.

Time (Day)	β-glucosidase (UA/mg)	Daidzin (μmol/g)	Glycitin (μmol/g)	Genistin (μmol/g)	M. genistin (μmol/g)	A. genistin (μmol/g)	Dadzein (μmol/g)	Genistein (μmol/g)	TPC (mg GAE/g)	FRAP (μmol TE/g)	DPPH (μmol TE/g)	ABTS (μmol TE/g)	Total Proteins (%)
0	-	1.593± 0.032 ^{a}	0.279± 0.005 ^{a}	2.243± 0.045 ^{a}	0.667± 0.020 ^{a}	0.279± 0.005 ^{a}	0.496± 0.007 ^{g}	0.471± 0.003 ^{g}	1.584± 0.043 ^{d}	17.002± 0.997^{a}	2.186± 0.198 ^{d}	58.911± 4.982 ^{b}	52±1 ^{b}
1	0.040± 0.003^{e}	1.132± 0.028^{b}	0.248± 0.006 ^{b}	1.805± 0.039^{b}	0.477± 0.023^{c}	0.265± 0.006^{b}	0.900± 0.022^{f}	0.821± 0.030^{f}	2.271± 0.025^{a}	18.713± 1.114^{a}	2.385± 0.060^{b,c,d}	61.466± 3.345^{a,b}	52±1^{a,b}
2	0.112± 0.019^{d}	0.491± 0.016^{c}	ND	0.950± 0.094^{c}	0.545± 0.054 ^{b}	0.158± 0.020^{c}	1.446± 0.030^{e}	1.451± 0.095^{e}	2.240± 0.033^{a,b}	18.021± 0.667^{a}	2.787± 0.091^{a}	56.466± 5.224 ^{b}	52±2^{a,b}
3	0.179± 0.012^{c}	0.054± 0.000^{d}	ND	0.562± 0.053^{d}	0.412± 0.026^{d,e}	ND	1.796± 0.036^{d}	1.947± 0.049^{d}	2.054± 0.075^{c}	18.265± 0.847^{a}	2.594± 0.191^{a,b}	57.764± 0.968 ^{b}	53±1^{a,b}
4	0.230± 0.032^{b}	0.055± 0.003^{d}	ND	0.436± 0.044^{e}	0.390± 0.017^{e}	ND	1.877± 0.036^{c}	2.076± 0.043^{c}	2.070± 0.026^{c}	18.036± 1.043^{a}	2.511± 0.117^{a,b,c}	58.786± 5.1798^{b}	53±1^{a,b}
5	0.301± 0.022^{a}	0.054± 0.000^{d}	ND	0.290± 0.00^{f}	0.447± 0.050^{c,d}	ND	1.971± 0.024^{b}	2.224± 0.047^{b}	2.053± 0.045^{c}	17.805± 1.002^{a}	2.509± 0.125^{a,b,c}	60.358± 3.124^{b}	54±1^{a}
6	0.302± 0.029^{a}	0.053± 0.001^{d}	ND	0.222± 0.016^{f}	0.333± 0.023^{f}	ND	1.971± 0.026^{b}	2.241± 0.037^{b}	2.035± 0.080^{c}	18.339± 1.227^{a}	2.391± 0.123^{b,c,d}	61.521± 1.978^{a,b}	53±1^{a,b}
7	0.317± 0.025^{a}	ND	ND	ND	0.210± 0.008^{g}	ND	2.135± 0.055^{a}	2.515± 0.075^{a}	2.175± 0.027^{b}	18.337± 1.076^{a}	2.246± 0.031^{c,d}	70.507± 3.663^{a}	54±1^{a}

Results are expressed as the mean ± standard deviation (dry weight basis). Values followed by different superscript lowercase letters in the same column differ significantly ($p < 0.05$).

Figures:

Figure 1 – Effect of the solid-state fermentation time of the defatted soybean flour by *Aspergillus oryzae* (DSSF-Ao) or *Monascus purpureus* (DSFF-Mp) on soluble sugars content.



a) Galactose, fructose and glucose content from defatted soybean flour by *Aspergillus oryzae;* b) sucrose, raffinose and stachyose content from defatted soybean flour by *Aspergillus oryzae;* c) galactose, fructose and glucose content from defatted soybean flour by *Monascus purpureus;* d) sucrose, raffinose and stachyose content from defatted soybean flour by *Monascus purpureus.*

Figure 2 – Scatterplot of the Principal Components Analysis (a) representing 11 active and 3 supplementary variables, (b) representing defatted soybean flour fermented by *Aspergillus oryzae* (DSSF-Ao) and *Monascus purpureus* (DSFF-Mp) at different fermentation, and (c) cluster analysis.

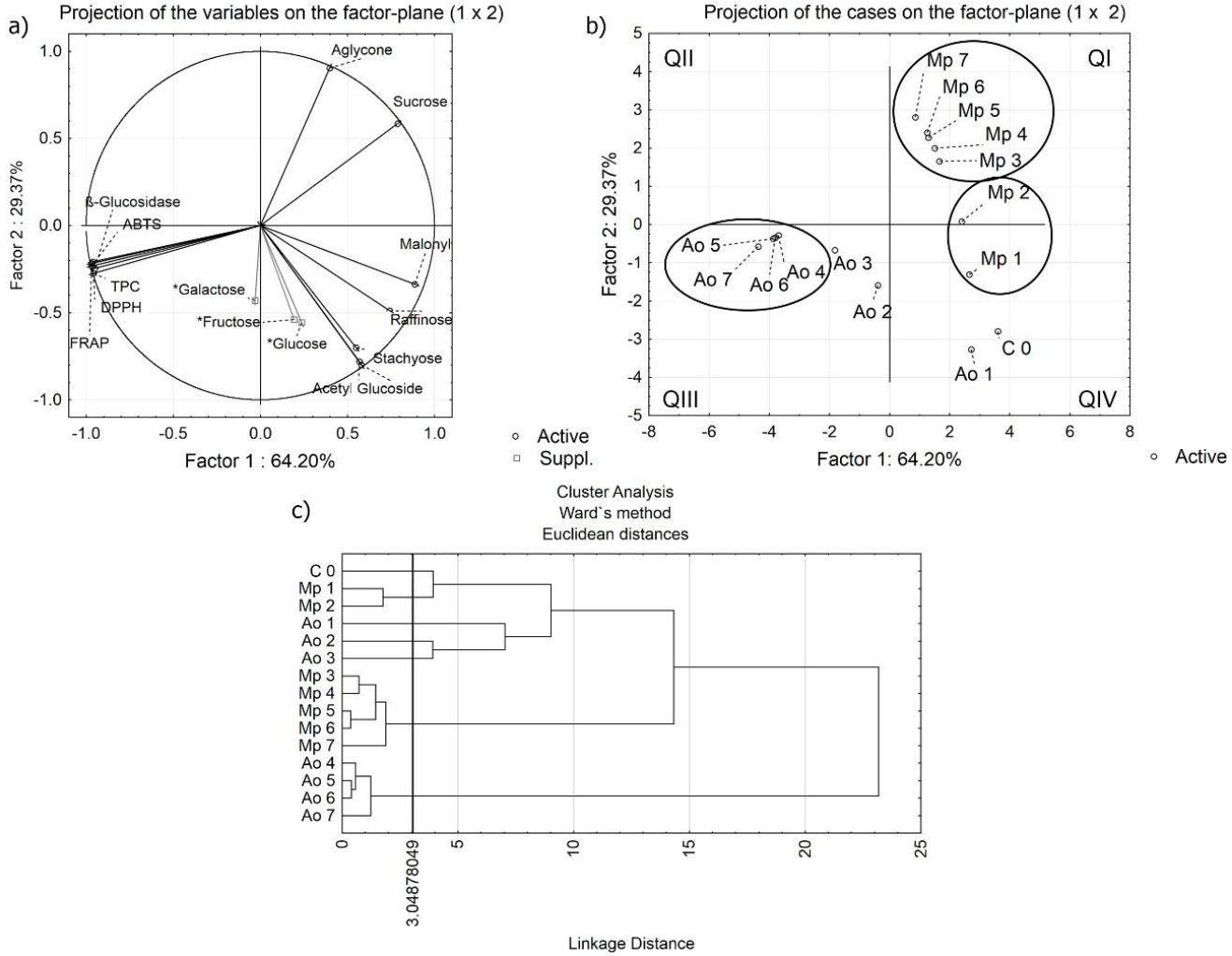
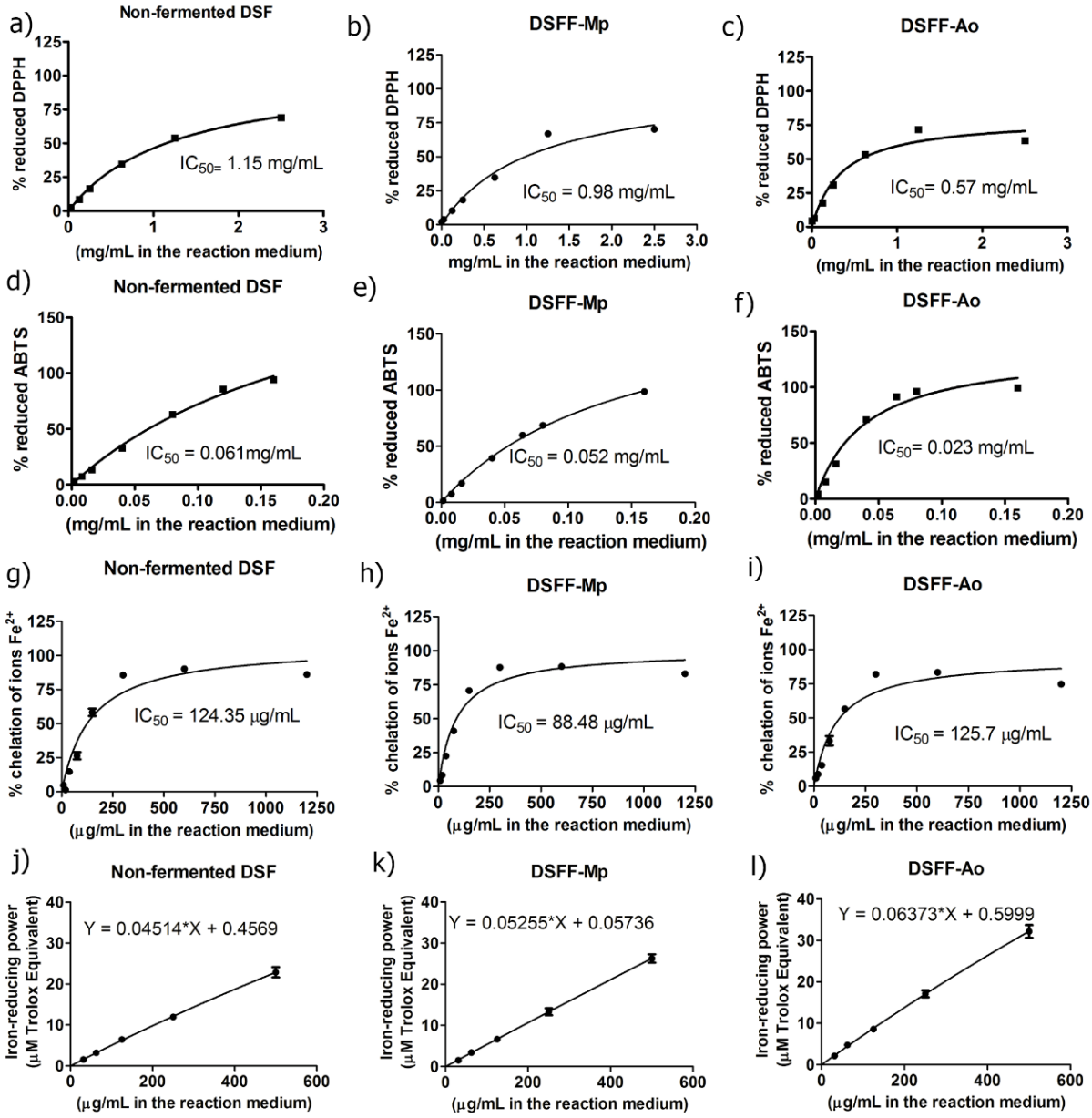


Figure 3 - Antioxidant capacity of the phenolic extracts from the non-fermented defatted soybean flour (DSF), defatted soybean flour fermented by *Aspergillus oryzae* (DSSF-Ao) or *Monascus purpureus* (DSFF-Mp) (fermented for 5 days).



IC_{50}= necessary concentration of the antioxidant to reduce the radical by 50.00 %

5.4 ARTIGO CIENTÍFICO 4

Potential anti-hypertensive of several digested soy foods as a source of angiotensin I converting enzyme (ACE) inhibitors

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Abstract

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The objective of this work was to evaluate ACE inhibitory potential of digested fermented and non-fermented soy foods and hydrolyzed 7S and 11S soy protein fractions. Soy foods were *in vitro* digested for the determination of ACE inhibitory activity, peptide molecular weight (MW) distribution, total phenolic content (TPC), individual phenolics, and soluble proteins. 7S and 11S fractions were hydrolyzed using pepsin, trypsin, and α-chymotrypsin and determined the ACE inhibitory capacity and peptide MW distribution. Raw and cooked soymilk showed the highest ACE inhibitory potential. Bacterial fermented soy foods had higher ACE inhibitory activity than fungal, and sprouts germinated for 3 days had higher than for 5 and 7 days. 11S hydrolysates showed higher ACE inhibitory capacity than 7S. Peptides of 1-4.5 kDa showed a higher contribution to reducing IC_{50}. Soy foods may be used as functional food, and 7S and 11S hydrolysates as ingredients to prevent or control hypertension.

Keywords: Peptides, enzymatic protein hydrolysates, hypertension, phenolic compounds, *in vitro* digestion.

Chemical compounds studied in this article

Hippuric acid (PubChem CID: 464); Hippuryl-L-histidyl-L-leucine (PubChem CID: 94418).

1. Introduction

High blood pressure is a major risk factor for heart attacks and strokes (Merai et al., 2016). ACE (peptidyl-dipeptidase, E.C. 3.4.15.1) plays a vital role in the regulation of blood pressure in the renin-angiotensin system. Inhibition of ACE is the dominant therapeutical approach to the treatment of high blood pressure due to the inhibited angiotensin II generation and preserved bradykinin. In reason of side effects of synthetic anti-hypertensive drugs (Atkinson & Robertson, 1979), food-derived ACE

inhibitors are drawing more attention due to safety concerns (Fitzgerald & Murray, 2006). Different types of natural food-derived compounds have been investigated for their ACE inhibitory activity. Various peptides with ACE inhibitory activity derived from animals, plants, and microorganisms have been reported. Phenolic extracts from various plant sources and pure phenolic compounds have also been proven to be potent ACE inhibitors (Al Shukor et al., 2013). ACE inhibitory activity of phenolic extracts from soybean has been studied (Ademiluyi & Oboh, 2013). ACE inhibitory peptides have been characterized from soy protein hydrolysates (Gibbs, Zougman, Masse, & Mulligan, 2004), fermented soy foods such as soybean paste (Li, Ohnishi-Kameyama, Takahashi, & Yamaki, 2013), tempeh and natto (Gibbs et al., 2004), and from non-fermented soy foods such as soybean seeds, soybean based infant formulas and soymilk (Alauddin et al., 2015; Capriotti, Caruso, Cavaliere, & Samperi, 2015; Puchalska, Marina, & García, 2014; Tomatsu, Shimakage, Shinbo, Yamada, & Takahashi, 2013). Many food-derived peptides have been verified to be potent ACE inhibitors *in vitro*, however establishing a direct relationship between ACE inhibitory activity and antihypertensive activity remains to be difficult. Since ACE, inhibitory peptides must remain active during gastrointestinal digestion and absorption and reach the cardiovascular system (Gu & Wu, 2013).

Soybeans is a source of oil, high-quality proteins, isoflavones, and a considerable number of phenolic acids and flavonoids (Messina, 2014). Glycinin (11S) and β-conglycinin (7S) are the most important soy proteins and represent more than 70.00% of the total storage proteins (Fukushima, 2001). Many technologies, such as physical, chemical, biological or a combination of these, are used in making various fermented (natto, tempeh and soy yogurt) and non-fermented soy foods (soymilk, tofu, and soy sprouts). The processing practices employed not only affect sensory quality,

but also the nutritional and physiological properties of resultant soy foods. The peptides and phenolic compounds may be released during food processing or *in vitro* digestion and usually showed a multifunctional nature including antioxidant, immunomodulatory, antimicrobial, antithrombotic, hypocholesterolemic and anti-hypertensive potential (Erdmann, Cheung, & Schröder, 2008; Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014). Peptides from soybean has been identified and characterized for their potential ACE inhibition (Capriotti et al., 2015; Gu & Wu, 2013). However, to the best of our knowledge, the ACE inhibitory activity of several digested fermented and non-fermented soy foods from the same soybean seeds has not been clarified. The objectives of this study were: i) to investigate the ACE inhibitory activity of *in vitro* digested soy foods, such as soymilk, tofu, sprout, natto, tempeh and soy yogurt; ii) to evaluate the effect of hydrolysis of 7S and 11S fractions on ACE inhibitory capacity, and iii) to evaluate the relationship between ACE inhibitory capacity and peptides MW.

2. Material and methods

2.1. Plant material and chemicals

The soybean [*Glycine max* (L.) Merrill] cultivar Prosoy harvested in 2012 was obtained from Sinner Brothers and Bresnahan Co. grown in Casselton, North Dakota, U.S.A. Soybean seeds were denominated as raw soybean (RSoy) and stored in a cool and dry air-conditioned room (5°C) prior to use.

Pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, trypsin from porcine pancreas, α-chymotrypsin from bovine pancreas, captopril, hippuric acid

(HA), ACE, N-α-hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma-Aldrich. (St. Louis, Mo., U.S.A.). Gallic, protocatechuic, 2,3,4-trihydroxybenzoic, p-hydroxybenzoic, gentistic, vanillic, caffeic, chlorogenic, syringic, p-coumaric, m-coumaric, o-coumaric, ferullic, salicylic, sinapic, trans-cinnamic acid, vanillin, syringaldehyde, protocatechualdehyde, (+)-catechin, (+)-epicatechin, epigallo-catechin, epicatechin-gallate, epigallatecatechin-gallate,myricetin, luteolin, quercetin, apigenin, kaempferol, quercetin-3-rutinoside, Folin–Ciocalteu reagent, and HPLC-grade trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside, and quercetin-3-O-glucoside were purchased from Extrasynthèse (Genay, France).

2.2. Preparation of soy foods

2.2.1. Soymilk

Raw and cooked traditional soymilk (RTSoyM and CTSoyM), raw and cooked soymilk slurry (RSoyMS and CSoyMSF) were prepared. RTSoyM was prepared according to Zhang, Guo, Liu, and Chang (2012). For CSoyMSF the soy slurry was subjected to similar cooking method followed by filtration through muslin cloth to separate the okara from the soymilk. Both types of soymilk were cooled in an ice bath and freeze-dried.

2.2.2. Tofu

Pressed tofu (PT) and Filled tofu (FT) were prepared according to reported by Meng, Chang, Gillen, and Zhang (2016). PT and FT were freeze-dried.

2.2.3. Natto

Natto was prepared as reported by our previous study (Wei, Wolf‐Hall, & Chang, 2001). *Bacillus natto* culture was prepared from a commercial natto purchased from Asian grocery store in Starkville, Mississippi, U.S.A. Natto (N) samples were stored at 4 ºC for 2 (Nd2), 4 (Nd4) and 6 days (Nd6) before freeze drying.

2.2.4. Tempeh

Tempeh was prepared using starter culture, *Rhizopus oryzae* obtained from the Cultures for Health (Morrisville, NC, U.S.A.). Manufacturer's recommended protocol was followed with minor modifications. After that, soybeans were soaked in the water at 30 ºC until pH reached 6.5, they were then washed, steamed for 15 min and dried on a mesh screen with an electric fan to evaporate the water off the beans. Soybeans were inoculated with culture and packaged in zip lock bags with holes. The sample was incubated at 31 °C for 48 h. Tempeh (T) samples were kept at 4 °C for 2 (Td2), 4 (Td4) and 6 days (Td6) before freeze drying.

2.2.5. Soy yogurt

Soy yogurt was made according to Zhang et al. (2012) with minor modifications. The bean-to-water ratio used was 1:7 (w/w). Soymilk was sterilized at 121 °C for 5 min in glass bottles. When soymilk reached 40 °C, aseptic inoculation was conducted according to manufacturer's recommended rate with a yogurt culture (YC-087; Chr. Hansen Laboratory, Inc., Milwaukee, WI) that contained strains of *Streptococcus thermophilus* and *L. delbrueckii ssp. bulgaricus.* The inoculated soymilk was poured into 500 mL sterile transparent plastic cups with lids (300 mL per cup) and incubated at 40 ºC until the pH decreased to between 4.2 and 4.5. The soy yogurt (Y) samples were stored at 4 °C for 0 (Yd0), 2(Yd2), 6 (Yd6) and 8 days (Yd8) before freeze drying.

147

148 *2.2.6. Soybean sprout*

149 Soybean sprouts were prepared as reported by Kumari and Chang (2016) using
150 Freshlife automatic sprouter (Tribest Corporation, Cerritos, CA, U.S.A.). Soybean
151 sprouts germinated for 1, 2, 3, 5 and 7 days were designated as RSd1, RSd2, RSd3,
152 RSd5, and RSd7, respectively. Meanwhile, a portion of each type of sprout was cooked
153 using common household cooking practice according to Kumari and Chang (2016).
154 The cooked soybean sprouts were named as CSd1, CSd2, CSd3, CSd5, and CSd7,
155 respectively. All sprout samples were freeze dried for further analysis.

156

157 *2.3. In vitro digestion of soy foods*

158

159 The freeze dried soy foods were subjected to simulated *in vitro* gastrointestinal
160 digestion using pepsin and pancreatin in sequence (done in duplicate). Freeze dried
161 soy foods (3 g) were put in a 50 mL conical flask containing 30 mL of distilled water
162 and mixed for 30 s. For each sample, 0.3 mL of pepsin (10000 units/mL) was added,
163 mixed, and the pH was adjusted to 2.0 using 5 N HCl, before the flasks were incubated
164 at 37 °C in a shaking water bath (220 rpm) for 2 h. The pH of the pepsin digests was
165 adjusted to 8.0 using 5 N NaOH and 3 mL of pancreatin (40 mg/mL) was added to
166 each flask. The pH was adjusted again to 8.0 using 5 N NaOH. Incubation was
167 continued for 2 h. The digested sample was held in the boiling water for 15 min to
168 inactivate the digestive enzymes, and then it was cooled and centrifuged (3000 *g*, 15
169 min). The digested sample was filtered, freeze-dried and stored at -20 °C until use.
170 The freeze-dried digested samples were suspended in water (8.6 mg freeze dried
171 sample/mL), diluted in water to 0.25 mg/mL (on dried basis) and were evaluated for

ACE inhibitory activity and, when applicable, as to study the holding of the ACE inhibitory activity during storage (natto, tempeh and soy yogurt).

2.4. Characterization of digested soy foods

From the results of ACE inhibitory activity of all digested soy foods, the representative digested soy foods of each type of processing, which presented a higher percentage of ACE inhibition, were selected for characterization as to ACE inhibitory capacity (IC_{50} = concentration of extracts required to inhibit 50.00% of the enzyme activity), TPC, individual phenolic acids and flavonoids, soluble proteins, and peptide MW distribution.

2.5. Hydrolysis of 7S and 11S fractions

The 7S and 11S fractions were isolated from low-temperature defatted soybean meal according to the method of Liu et al. (2007) and were freeze dried and stored at -20 °C. Before hydrolysis, in order to remove residues of phenolic compounds, the freeze-dried 7S and 11S fractions were suspended into 80.00% acetone, shaken overnight at 25 °C and centrifuged at 12000 rpm for 30 min. The precipitates were dried in oven at 40 °C for 48 h. The hydrolysis in duplicate was carried out in 5 steps: (A) 7S and 11S fractions were suspended in deionized water (10 g into 100 mL); (B) 1 mL of pepsin (10000 units/mL) was added, the pH value was adjusted to 2 with 5 N HCl and the suspension was incubated at 37 °C in a shaking water bath (220 rpm) for 2 h; (C) 0.7 mL of trypsin (520000 – 800000 units/mL) was added, the pH was adjusted to 8 using 5 N NaOH and incubation was continued for 2 h; (D) 0.4 mL of α-chymotrypsin (1600 units/mL) was added, the pH was adjusted to 8 using 5 N NaOH,

198 and incubated for more 2 h; and (E) 7S and 11S fractions were suspended in deionized
199 water (3 g into 30 mL), 0.3 mL of pepsin (10000 units/mL) was added, the pH value
200 was adjusted to 2.0 with 5 N HCl and the flasks incubated at 37 °C in a shaking water
201 bath (220 rpm) for 2 h, 0.3 mL of trypsin (520000 – 800000 units/mL) and 0.3 mL of α-
202 chymotrypsin (1600 units/mL) were added, the pH was adjusted to 8 using 5 N NaOH
203 and incubation was continued for 2 h. Aliquots of 30 mL were collected at (B), (C), (D),
204 and (E) steps from reaction mixtures and heated at 100 °C for 10 min for enzyme
205 inactivation and were labeled as follows: A- no hydrolyzed; B- hydrolyzed by pepsin for
206 2h; C- hydrolyzed by pepsin for 2h + trypsin for 2h; D- hydrolyzed by pepsin for 2h +
207 trypsin for 2h + α-chymotrypsin for 2h; and E- hydrolyzed by pepsin for 2h + trypsin
208 and α-chymotrypsin for 2h. The hydrolysates were cooled, freeze-dried and stored at
209 −20 °C until use. These hydrolysates were suspended in water (10 mg/mL, on dried
210 basis) and characterized as to ACE inhibitory capacity (IC_{50}) and peptide MW
211 distribution.
212
213 *2.6. Determination of ACE inhibitory activity*
214
215 ACE inhibitory activity of the samples was measured *in vitro* according to
216 Cushman and Cheung (1971) with modifications. N-α-hippuryl-L-histidyl-L-leucine
217 (HHL) and ACE were dissolved in 100 mM borate buffer (pH = 8.3, 300 mM NaCl), at
218 concentrations of 2.5 mM and 10 mU/mL, respectively. The reaction mixture containing
219 75 μL HHL, 35 μL sample, and 75 μL ACE was incubated at 37 °C in a shaking water
220 bath (200 rpm) for 30 min. The reaction was stopped by heating at 85 °C for 15 min
221 and 185 μL of water was added before injecting into the ultra-high performance liquid
222 chromatography (UHPLC) sample loop. UHPLC analyses were performed to quantify
223 the hippuric acid (HA) produced by the enzymatic hydrolysis of the HHL using a

Thermo Scientific Dionex UltiMate 3000 RSLC UHPLC focused System (Thermo Scientific Dionex, Fürstenfeldbruck, GE). Data acquisition was performed with Chromeleon 7 software. Separations were accomplished on a Waters UPLC column (CORTECS® UPLC®, C18 1.6 µm, 2.1 × 50 mm) and column temperature was maintained at 37 °C. The analytical procedure for quantification of HA by UHPLC was previously validated. The injection volume was 100µL and the detection wavelength was set at 228 nm. The mobile phase consisted of a gradient of 0.10% TFA in water (A) and 0.10% TFA in acetonitrile (B). The flow rate was set at 0.176 mL/min, and the gradient profile was as follows: $t_{0–0.96min}$: linear gradient from 5 to 30.00% of B; $t_{0.96–1.60min}$: linear gradient from 30.00 to 60.00% of B; $t_{1.60–1.90min}$: curve 7 gradient from 60 to 70.00% of B; $t_{1.90–2.20min}$: isocratic elution with 70.00% of B; $t_{2.20–2.60min}$: linear gradient from 70.00 to 5.00% of B; total time of 4 min. The HA control contained water instead of sample solution and the blank sample contained buffer instead of ACE solution. The ACE inhibitory activity was calculated according to the following equation: ACE inhibitory activity (%)=[(A-B)/(A)]x100. Where *A* is HA from control containing water instead of sample solution and *B* is HA from sample reaction with the subtraction of sample blank. The inhibitory concentration to inhibit ACE by 50 % (IC_{50}) (mg/mL on dried basis) was calculated using GraphPad Prism software.

2.7. Determination of peptide molecular weight distribution

MW distribution of peptides from samples was analyzed by size exclusion chromatography (You, Udenigwe, Aluko, & Wu, 2010) with modifications. Each sample was dissolved in water, at a concentration of 10 mg/mL (on dried basis) for 7 S and 11S hydrolysates, and 8.6 mg/mL (on dried basis) for digested soy foods. A 40 µL aliquot was injected onto the Superdex™ peptide 10/300 GL column (GE Healthcare,

Piscataway, NJ) coupled with an Agilent Technologies Chromatography 1200 series system (Agilent Technologies, Santa Clara, Calif., U.S.A.), and eluted with 30.00% acetonitrile at a flow rate of 0.380 mL/min. The eluate was monitored at 214 nm. A calibration curve of MW was obtained from the following standards: Cytochrome C (12,384 Da), Aprotinin (6500 Da), Vitamin B12 (1855 Da), and L-Reduced Glutathione (307 Da).

2.8. Determination of total phenolic content and soluble proteins

The TPC was measured by the Folin-Ciocalteu colorimetric method (Singleton, Orthofer, & Lamuela-Raventós, 1999) using standard gallic acid (GA) and expressed as micrograms of gallic acid equivalents/milligram of sample (μg GAE/mg) on dried basis. Soluble proteins was measured by bicinchoninic acid (BCA) method (Smith, et al., 1985) and expressed as micrograms/milligram of sample (μg/mg) on dried basis with BSA as reference.

2.9. HPLC analysis of phenolic acids and flavonoids

HPLC analysis was performed according to the method of Xu and Chang (2009) using an Agilent 1260 Infinity LC system (Agilent Technologies, Santa Clara, Calif., U.S.A.), equipped with a quaternary pump, a DAD detector, an online degasser, an auto plate-sampler and a thermostatically controlled column compartment. Chromatographic separation was carried out at 40 °C on Zorbax Stablebond Analytical SB-C18 column (4.6 mm × 250 mm, 5 μm, Agilent technologies, Rising Sun, MD).

2.10. Statistical analysis

The soy foods, *in vitro* gastrointestinal digestion and analyses were carried out in duplicate, duplicate and triplicate, respectively. One-way ANOVA followed by Tukey's multiple comparisons test ($\alpha = 0.05$) was carried out using the software program Statistic 10 (StatSoft, Tulsa, OK, U.S.A.).

3. Results and discussion

3.1. ACE inhibitory activity of digested fermented and non-fermented soy food

Processing methods employed to manufacture soy foods, such as cooking, grinding, soaking, coagulation, germination, dilution, and fermentation, influenced the ACE inhibitory activity of the digested soy foods (Figure 1). Soymilk, tofu, raw sprout germinated for 1, 2 and 3 days, and fermented soy foods presented increases in the ACE inhibition percentage as compared to RSoy. However, RSd7, cooked sprouts and Td6 presented ACE inhibition percentage smaller than RSoy. The influence of the thermal treatment on ACE inhibitory activity was dependent on the type of soy food, as may be observed in the RTSoyM and CTSoyM, whose ACE inhibition percentage did not distinguish from each other, while the CSoyMSF showed a 14.00% reduction in the ACE inhibitory activity as compared to RSoyMS. The greatest impact of heat treatment on ACE inhibitory activity of sprouts was observed in the CSd3, which showed a 68.90% reduction in comparison with RSd3.

The thermal treatments applied in the preparation of the different soy foods, possibly, altered the protein structure in different ways, affecting the enzyme activity during *in vitro* digestion. Consequently, different peptides may be formed (da Costa, Rocha Gontijo, & Netto, 2007). In addition, Kumari and Chang (2016) observed the

cooking of soy sprouts caused significant losses in most individual phenolic acids, benzoic group, cinnamic group, total phenolic composition, individual isoflavones, and total isoflavones. All these factors may have affected the results.

The germination time also significantly affected the ACE inhibition percentage of the sprouts with a 1.5-fold increase after 24 h of germination. The ACE inhibition percentage remained constant until the third day and then decreased until the seventh day, which presented 41.80% smaller inhibition than RSoy. The increase in the ACE inhibition percentage followed by reduction can be attributed to the excessive hydrolysis of the proteins or to the breakdown of the complex phenolic compounds caused by long germination time followed by *in vitro* digestion. According to Zakharov, Carchilan, Stepurina, Rotari, Wilson, and Vaintraub (2004), at the beginning of germination, the proteins start to be hydrolyzed by endopeptidases to oligopeptides, and then are hydrolyzed by exopeptidases to free amino acids, which may be used to synthesize new proteins and tissues. Yang and Li (2010) found by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) that the α' and α subunits of β-conglycinin (7S) and the acidic chains of glycinin (11S) were gradually degraded with germination time right after the soybean imbibition. Simultaneously, increase in the activity of polyphenol oxidase and other catabolic enzymes modified the phenolic compounds profile (Kumari & Chang, 2016). During gastrointestinal digestion, the additional presence of exopeptidases and endopeptidases would further degrade these compounds (Capriotti et al., 2015).

The ACE inhibitory activity was dependent on the microorganism used in the making of fermented soy foods. Bacteria and fungi were used in the solid-state fermentation to obtain natto and tempeh, respectively. Whereas, soy yogurt was fermented with bacteria by submersion fermentation. As observed in Figure 1, Nd2

showed an increase of 40.00% in the ACE inhibitory activity as compared to RSoy and remained constant until sixth day of storage. Td2 increased just 17.00% in the ACE inhibitory activity compared to RSoy, and the activity decreased with the storage time. However, the ACE inhibitory activity of soy yogurt was 36.00% higher than RSoy and 11.46% smaller than CTSoyM and remained unchanged during the storage.

The difference in the ACE inhibitory activity between natto and tempeh could be due to presence of different enzymes, since fungi usually produce a wider range of extracellular enzymes than bacteria (Kirk, Gifford, Drive, & Farrell, 1987). The proteases from Bacillus and Rhizopus strains may hydrolyze the main soy proteins into large peptides (Gibbs et al., 2004), and the subsequent hydrolysis of these peptides by *in vitro* digestion could lead to formation of different peptides. Therefore, high ACE inhibition percentage and storage stability of natto and soy yogurt compared to tempeh indicated that bacteria could be promising for production of fermented soy foods with ACE inhibitory activity.

3.2. Characterization of digested soy foods

The representative digested soy foods of each type of processing which presented a higher percentage of ACE inhibition were selected for characterization as to ACE inhibitory capacity (IC_{50}), TPC, individual phenolic acids and flavonoids, soluble proteins, and peptide MW distribution. (Table 1 and Figure 2). As shown in Table 1, soymilk exhibited the highest ACE inhibition with RTSoyM and CTSoyM representing 47.37% and 36.84% IC_{50} decreases in comparison with RSoy.

However, when the soymilk was fermented for making soy yogurt or coagulated for making tofu, the IC_{50} values were not significantly different from that of Rsoy ($p >$

352 0.05). The effect of solid-state fermentation on ACE inhibitory capacity was dependent
353 on microorganism used in the making of soy foods. IC_{50} value of the Nd2 was not
354 significantly different from that of RSoy, whereas Td2 showed a 1.36-fold increase in
355 the IC_{50} value as compared with RSoy. The germination of soybean for 3 days (RSd3)
356 reduced 21.05% the IC_{50} value in comparison with RSoy, but the cooking of sprout
357 (CSd3) increased IC_{50} value, which was equivalent to that of RSoy.

358 Comparing the IC_{50} values from different digested fermented and non-
359 fermented soy foods from the same soybean seeds had not been reported in the
360 literature. In addition, the IC_{50} values depend on the experimental protocol, protein
361 extraction procedure, and differences in the peptide mixture composition after
362 digestion process (Barbana & Boye, 2010).

363 Thus, IC_{50} value of the synthetic inhibitor, captopril, was used to verify ACE
364 inhibition efficiency of the digested soy foods. Results showed captopril was a very
365 strong inhibitor as compared with digested soy foods. The IC_{50} value of captopril was
366 1.05 nM, which was comparable to that reported by Lahogue, Réhel, Taupin, Haras,
367 and Allaume (2010). According to Hayes and Tiwari (2015), bioactive peptides derived
368 from natural sources need higher concentrations than synthetic drugs to be effective.

369 The fermented soy foods Nd2 and Td2 presented TPC increases of 30.11% and
370 13.85% ($p < 0.05$), respectively, while all of other soy foods were not significantly
371 different from RSoy. The TPC was affected by thermal treatment of sprout and CSd3
372 exhibited 12.37% lower in TPC than RSd3. TPC remained unchanged with cooking of
373 soymilk.

374 A total of 16 phenolic acids, 5 flavan-3-ols and 6 flavonols or flavones were
375 evaluated in the digested soy foods. However, under the experimental conditions
376 studied, they were not detected. These phenolic compounds may have been degraded

completely or undergone transformation during *in vitro* gastrointestinal digestion, since, according to Bermúdez-Soto, Tomás-Barberán, and García-Conesa (2007) the phenolic compounds are susceptible to the mild alkaline conditions in the duodenum phase of the digestion, where they may be transformed into some unknown or undetected structural forms. Furthermore, the low concentration of these compounds after *in vitro* digestion may have made impossible to quantify these phenolic compounds. Although the soy foods had high TPC values, the color measured may be due to other non-phenolic substances (such as aromatic amino acids) from food, which also are oxidized by the Folin–Ciocalteu reagent and could contribute to the estimation of TPC (Prior, Wu, & Schaich, 2005).

Nd2, Td2, RTSoyM, CTSoyM and RSd3 showed relatively higher soluble proteins, while RSoy gave the lowest soluble proteins (Table 1). The soluble proteins content was influenced by heat treatment the same way as observed in the ACE inhibitory capacity and TPC. The size exclusion chromatography was carried out to estimate the peptide MW distribution in digested soy foods. The peptides were grouped in six MW ranges: 4.5-10, 2-4.5, 1-2, 0.5-1, 0.1-0.5, and <0.1 kDa. As shown in Figure 2a, the processing had little effect on peptide MW distribution. Except Nd2, which showed about 47.00% and 43.00% lower than Rsoy in the 4.5-10 and 2-4.5 kDa ranges, respectively, all other soy foods exhibited similar MW distribution.

The relative content of peptides ranging from 1-2 and 0.5-1 kDa were similar among digested soy foods ($p > 0.05$). The highest percentages between 0.1 and 0.5 kDa were observed in the Nd2 and PT, which were 1.43 and 1.27-fold higher than RSoy, respectively, while no differences in the relative content of these peptides were found between other soy foods and RSoy. CTSoyM and Td2 showed higher percentage of compounds < 0.1 kDa than RSoy. The compounds less the 0.5 kDa

could be amino acids released by *in vitro* digestion. In terms of total area in the chromatogram, which are corresponding to peptides, Td2, Yd2, and CTSoyM were 1.61, 1.46 and 1.27-fold higher than RSoy, respectively (Table 1). Besides the processing, the differences in the peptide MW distribution and total area of peptides among the digested soy foods also could be attributed to the lack of specificity of digestive enzymes that, with the exception of trypsin and chymotrypsin, randomly hydrolyze peptide bonds within protein molecules (Capriotti et al., 2015).

3.3. ACE inhibitory capacity and molecular weight distribution of 7S and 11S hydrolysates

As shown in Table 2, each enzyme sequentially involved changed the ACE inhibitory capacity and the two protein fractions also exhibited significant ($p < 0.05$) differences. The IC_{50} value of non-hydrolyzed 7S-A fraction was 2.16-fold higher than non-hydrolyzed 11S-A. Pepsin hydrolysis (B) for 2 h of 7S and 11S fractions reduced the IC_{50} value by 31.73% and 23.65%, respectively. The IC_{50} values of 7S and 11S hydrolyzed by pepsin for 2 h + trypsin for 2 h (C) were 80.96% and 83.40% smaller than 7S-A and 11S-A, respectively, and remained constant with addition of α-chymotrypsin for 2 h (D). No significant differences were observed between D and E hydrolysates of both 7S and 11S fractions, indicating sequential and simultaneous actions of trypsin and α-chymotrypsin had no influence on ACE inhibitory capacity. By comparing lowest IC_{50} values of each protein fraction, 7S-C and 11S-E, the IC_{50} value of 11S-E was 84.85% smaller than that of 7S-C. The difference in the IC_{50} values observed among 7S and 11S hydrolysates can be due to the difference in their structures, amino acids composition, and processing properties (Nik, Tosh, Poysa, Woodrow, & Corredig, 2008). Furthermore, Gibbs et al. (2004) found the biologically

428 active peptides were mostly derived from glycinin (11S). The peptide YVVFK that is
429 part of the glycinin protein (positions 465–469) was found after soy protein hydrolysis
430 and has been reported as strong ACE inhibitory activity (Capriotti et al., 2015).

431 Hydrolysis of 7S and 11S fractions by different proteases resulted in distinct
432 peptide MW distribution for each fraction (Figure 2: b and c). In the non-hydrolyzed
433 fractions (A) peptides of 4.5-10 kDa made up 77.00 and 55.00% of 7S-A and 11S-A,
434 respectively. With pepsin these peptides reduced to less than 10.00% and unchanged
435 with addition of other enzymes for both fractions ($p > 0.05$). The percentage of peptides
436 between 2 and 4.5 kDa was constant in all 7S and 11S hydrolysates (B, C, D and E).

437 Peptides between 1 and 2 kDa were not found in the non-hydrolyzed 7S-A, but
438 higher percentages were observed in the 7S-B and 7S-C, and they decreased in the
439 7S-D and 7S-E. While in the 11S fraction, the relative content of theses peptides
440 increased 2.9-fold in the 11S-B and unchanged in the 11S-C and 11S-D. The
441 percentage of peptides of MW ranging from 0.5-1 kDa increased until 7S-C and
442 remained unaltered in the 7S-D and 7S-E hydrolysates. However, the relative content
443 of these peptides remained unchanged with hydrolysis of 11S fraction ($p > 0.05$). No
444 difference was observed in the percentage of peptides ranging from 0.1 to 0.5 kDa
445 between 7S-A and 7S-B, but it increased in the 7S-C and reduced in the 7S-D and 7S-
446 E. In the 11S-A, the relative content of these peptides was 21.00%, decreasing to
447 12.00% in the 11S-B and increasing in the 11S-D and 11S-E. The amount of substance
448 with MW < 0.1 kDa was about 10.00% in the 7S-A, and was above 35.00% in the 7S-
449 B, D and E. The highest percentages of compounds <0.1 kDa in the 11S hydrolysates
450 were found in the 11S-B and 11S-C, 31 and 28.00%, respectively, and 11S-E had
451 lower percentage than 11S-D.

In the chromatograms of peptides, the peptide total area of 7S-D was 8.62-fold higher than 7S-A and 1.34-fold lower than 7S-E (Table 2). The total area of peptides of 11S fraction increased 23.69-fold in the 11S-D compared to 11S-A, and 11S-D was 1.14-fold higher than 11S-E. Comparing the peptide total area of 7S-C and 11S-D, which showed the best ACE inhibitory capacity, it is observed the total area of 11S-D was 3-fold bigger than 7S-E (Table 2). The difference in the peptide MW distribution among 7S and 11S hydrolysates probably may be due to diversity of generated peptides from each fraction. Glycitin is a hexamer with a molecular mass of 320-380 kDa, and each monomer subunit involves one basic and one acidic polypeptide, linked via disulfide bond (Fukushima, 2001). Whereas, β-conglycinin is a trimeric glycoprotein containing approximately 4.00% of carbohydrate, with a molecular mass of 180 kDa consisting of three subunit associated by hydrophobic and hydrogen bonding (Thanh & Shibasaki, 1978). Gibbs et al. (2004) found glycinin was the precursor for 95.00% of the peptides isolated in theirs experiments, while the β-conglycinin was found to be more resistant to proteolytic attack.

3.4. Effect of molecular weight of peptides on ACE inhibitory activity

PCA analysis was performed between peptide MW and IC_{50} values from digested soy foods and 7S and 11S hydrolysates (Figure 3a, b). The projection on the factorial plane (CPI x CPII) of peptide MW and IC_{50} values is shown in the Figure 3a, and the projection of the soy foods and 7S and 11S hydrolysates is shown in the Figure 3b. The percentage of variance among samples was 83.12 and 9.06% for PCA axes 1 and 2, respectively. The vectors of CP1 that are close indicate that the variables are positively correlated with each other and therefore, a positive correlation was observed between the peptides of different MW, except the substances smaller than 0.1 kDa

presented a lower correlation with others. The vectors that form an angle close to 180° indicate a negative correlation, and thus peptides between 1 and 2 kDa were those that most contributed to the reduction of the IC_{50} values, followed by the peptides between 2 and 4.5 kDa. Substances smaller than 0.1 kDa showed a weak negative correlation, indicating a smaller contribution to reducing the IC_{50} value (Figure 3a). This result was consistent with reports by Puchalska, García, and Marina (2014), who found the most potent ACE inhibitory activity were observed in peptides with MW below 3 kDa. Although has been studied intensively, the structure−function relationship between the ACE inhibitory activity and peptides remain unclear. Moreover, the number of amino acids in the composition of potentially antihypertensive peptides is not well specified, it may vary from two to several amino acids. Wu, Aluko, and Nakai, (2006a, 2006b) proposed models for ACE-inhibitory peptides through computational analysis, they indicated that for dipeptides, amino acid residues with a large bulk chain as well as hydrophobic side chains are preferred, such as phenylalanine, tyrosine, and tryptophan. The structure of the carboxyl terminal of a dipeptide is more relevant to the potency of ACE inhibitory activity than N-terminal. For tripeptides, the most favourable structure is to include an aromatic amino acid residue in the C-terminal, and the hydrophobic amino acids such as leucine, tryptophan in the N-terminal. Besides, the tetrapeptide residue from the C-terminal end mainly decides the potency of peptides that contain 4 – 10 amino acid residues.

Thus, additional studies on identification of the active peptides of each soy food should be performed. In addition, is necessary to clarify how the processing used to obtain soy foods affect the formation/degradation of bioactive peptides during the digestion.

PCA was used to group the samples according to the degree of similarity (Figure 3b). Thus, it is observed 7S-A, 7S-B and 11S-A are located in the fourth quadrant and therefore are characterized by the higher IC_{50} values, and the 11S-A fraction was similar to 7S-B. The 11S-B is located in the first quadrant and grouped with 7S-C, D, and E. 11S-C, D and E are grouped in the second quadrant, indicating that they are similar and with a lower IC_{50} values. However, 11S-C, D and E with low IC_{50} values did not show enough similarities to be clustered with the digested soy foods. The digested soy foods Yd2, Td2 and CTSoyM are grouped in the third quadrant, whereas RTSoyM, RSd3, CSd3, PT, Nd2 and RSoy are clustered between quadrant 2 and 3. Therefore, it was confirmed the processing exerted an effect on protein hydrolysis during *in vitro* digestion and ACE inhibitory activity.

Considering the differences between digested soy foods and 7S and 11S hydrolysates, it was observed peptides of MW between 1 and 4.5 kDa had a higher antihypertensive capacity. However, it was not possible to confirm that peptides in general were the only ones responsible for ACE inhibition activity, since it was indicated by the lower IC_{50} value (less 53.00%) of RTSoyM (a complex matrix) compared to 11S-E (protein hydrolysate). Therefore, it is recommended to investigate which other compounds have ACE inhibitory activity and if synergistic effect occurs with other bioactive compounds. In addition, according to Capriotti et al. (2015) the extensive hydrolysis of soybean proteins undergo during simulated gastrointestinal digestion generated a large number of peptides some of which with established biological activity. However, one should take into account that the *in vivo* digestion is a process far more complicated and the enzymes produced by the gut bacteria should also be considered.

4. Conclusion

The ACE inhibitory properties of digested fermented and non-fermented soy and of hydrolysates of 7S and 11S fractions were reported for the first time in this study. Processing to manufacture soymilk, tofu, natto, tempeh and soy yogurt improved the ACE inhibition after *in vitro* simulated gastrointestinal digestion. Raw and cooked traditional soymilk showed the highest anti-hypertensive activity. Among fermented soy foods, natto and soy yogurt had higher ACE inhibitory activity than tempeh. The germination time for 3 days was the most optimal to obtain sprouts with better anti-hypertensive activity. The highest TPC was found in the natto and tempeh. *In vitro* digestion of soy foods probably changed the individual phenolic compounds into undetectable or unknown compounds. Hydrolysis of 7S and 11S fractions using pepsin followed by trypsin was enough for the released peptides with good ACE inhibitory capacity, and 11S hydrolysates were more powerful than 7S hydrolysates in ACE inhibition. Peptides between 1 and 4.5 kDa showed the highest ACE inhibitory activity. Further analysis is required for identification of peptides from digested soy foods with anti-hypertensive potential.

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Tables

Table 1 - Characteristics of digested soy foods.

Digested Soy Foods	IC_{50} (mg/mL)	TPC (µg GAE/mg)	Soluble protein (µg/mg)	Total area of peptides
RSoy	0.19±0.01 ^{d}	16.17±0.30 ^{c,d}	368.64±4.25 ^{d}	2.29E+6±1.32E+5^{d}
RTSoyM	0.10±0.01 ^{a}	17.16±0.42 ^{b,c}	546.36±2.04 ^{a,b}	2.67E+6±7.29E+4^{c,d}
CTSoyM	0.12±0.01^{a,b}	17.00±0.35 ^{b,c}	482.38±18.82 ^{a,b,c,d}	2.91E+6±1.22E+5^{b,c}
PT	0.20±0.00 ^{d}	17.10±0.46 ^{b,c}	432.00±15.31 ^{b,c,d}	2.71E+6±2.18E+4^{c,d}
RSd3	0.15±0.00 ^{b,c}	16.73±0.33 ^{c}	562.46±10.91 ^{a}	2.56E+6±1.20E+5^{c,d}
CSd3	0.20±0.02 ^{d}	14.66±0.28 ^{d}	381.32±15.81 ^{c,d}	2.54E+6±5.24E+4^{c,d}
Nd2	0.16±0.01 ^{c,d}	21.04±0.41 ^{a}	569.60±8.98 ^{a}	2.78E+6±2.92E+5^{c,d}
Td2	0.26±0.00 ^{e}	18.41±0.31 ^{b}	487.44±2.57 ^{a,b,c}	3.70E+6±5.48E+4^{a}
Yd2	0.17±0.01 ^{c,d}	16.56±0.16 ^{c}	418.19±8.07 ^{c,d}	3.34E+6±3.84E+4^{a,b}

Results are expressed as the mean ± standard deviation (on dried basis). Values followed by different superscript lowercase letters in the same column differ significantly ($p < 0.05$).
IC_{50}: inhibitory concentration to inhibit ACE by 50 %; TPC: total phenolic content; RSoy: raw soybean; RTSoyM: Raw traditional soymilk; CTSoyM: Cooked traditional soymilk; RSoyMS; PT: Pressed tofu; RSd3: Raw sprout germinated for 3 days; CSd3: Cooked sprout germinated for 3 days; Nd2: Natto stored for 2 days; Td2: tempeh stored for 2 days; and Yd2: Yogurt stored for 2 days.

Table 2 – Angiotensin convert enzyme (ACE) inhibitory capacity of the 7S and 11S hydrolysates.

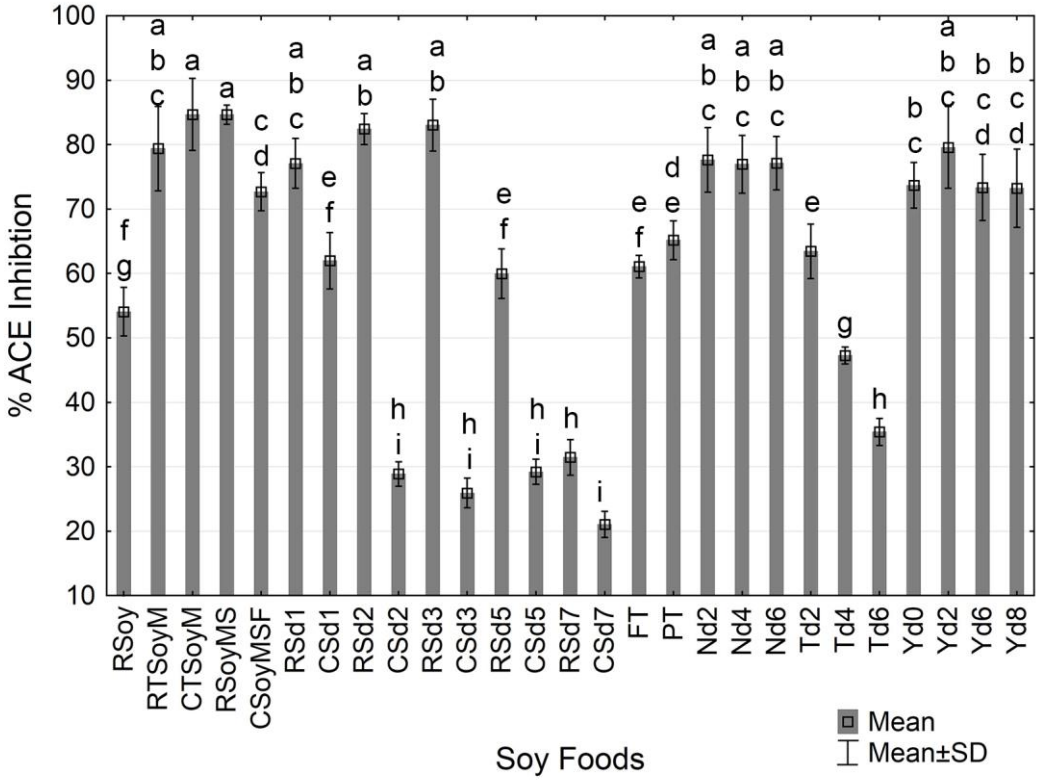
Samples	IC_{50} (mg/mL)	Total area of peptides
7S-A	5.20±0.13 ^{a,A}	1.00E+4±1.39E+3^{d}
7S-B	3.55±0.03 ^{b,A}	2.19E+5±3.18E+4 ^{c}
7S-C	0.99±0.01 ^{c,A}	3.71E+5±3.68E+4^{c}
7S-D	1.20±0.01 ^{c,A}	8.64E+5±3.60E+4^{b}
7S-E	1.04±0.01 ^{c,A}	1.16E+6±1.27E+5^{a}
11S-A	2.41±0.38 ^{a,B}	1.45E+4±5.87E+3^{d}
11S-B	1.84±0.06 ^{b,B}	7.34E+5±1.23E+5^{c}
11S-C	0.40±0.04 ^{c,B}	3.30E+6±9.70E+4^{a,b}
11S-D	0.21±0.02 ^{c,d,B}	3.43E+6±3.80E+4^{a}
11S-E	0.15±0.07 ^{d,B}	3.01E+6±1.40E+5^{b}

Results are expressed as the mean ± standard deviation (on dried basis). Values followed by different superscript lowercase letters in the same column differ significantly ($p < 0.05$) among the hydrolysates of same fraction (7S or 11S).
Values followed by different superscript uppercase letters in the same column differ significantly ($p < 0.05$) between hydrolysates from the different fraction (7S or 11S) and the same treatment (A, B, C, D or E).
IC_{50}: inhibitory concentration to inhibit ACE by 50 %.

7S and 11S: A- no hydrolyzed; B- hydrolyzed by pepsin for 2h; C- hydrolyzed by pepsin for 2h + trypsin for 2h; D- hydrolyzed by pepsin for 2h + trypsin for 2h + α-chymotrypsin for 2h; and E- hydrolyzed by pepsin for 2h + trypsin + α-chymotrypsin for 2h.

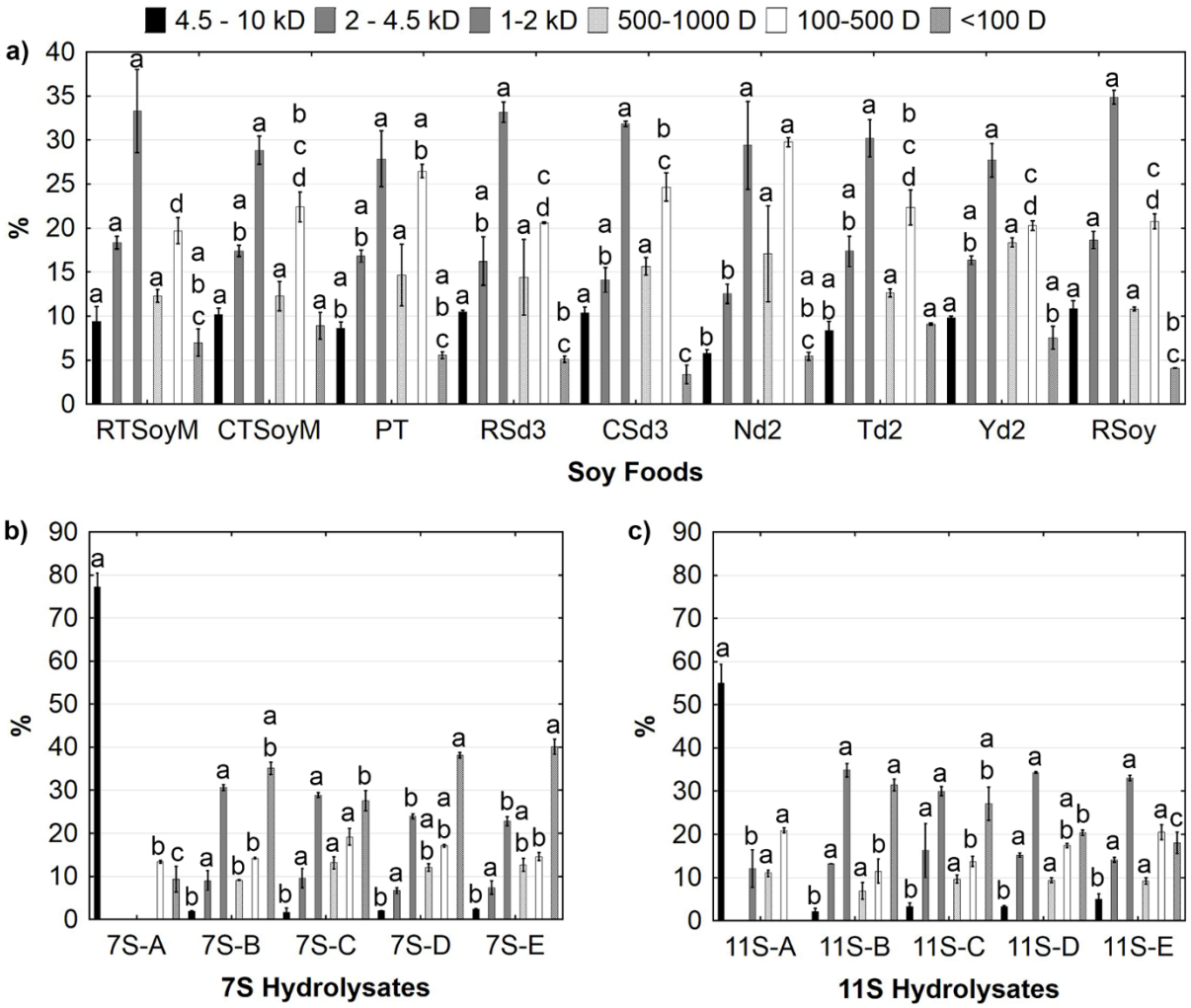
Figures

Figure 1 - Percentage inhibition of ACE of digested fermented and non-fermented soy foods.



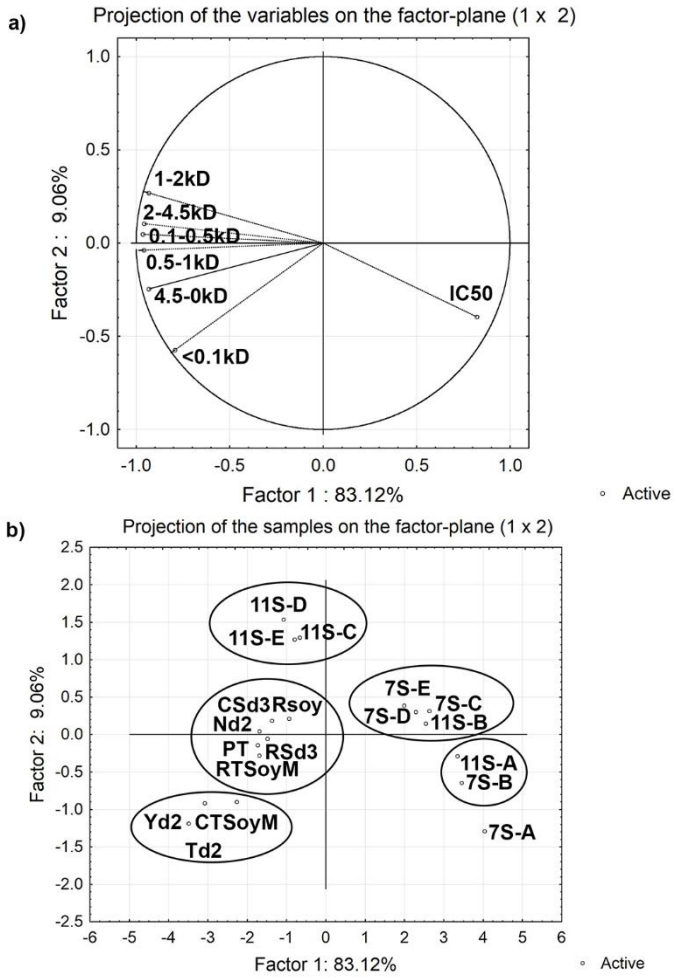
Different lowercase letters over the bar are significantly ($p < 0.05$) different among samples. Results are expressed as the mean ± standard deviation. Sample concentration = 0.25 mg/mL in water (on dried basis).
RSoy: raw soybean; RTSoyM: Raw traditional soymilk; CTSoyM: Cooked traditional soymilk; SoyMS: Raw soymilk slurry; CSoyMSF: Cooked soymilk with okara and filtered; PT: Pressed tofu; FT: Filled tofu; RSd (1, 2, 3, 5, and 7): Raw sprout germinated for 1, 2, 3, 5, and 7 days; CSd (1, 2, 3, 5, and 7): Cooked sprout germinated for 1, 2, 3, 5, and 7 days; Nd (2,4, and 6): Natto stored for 2, 4, and 6 days; Td (2, 4, and 6): tempeh stored for 2, 4, and 6 days; and Yd (2, 4, 6, and 8): Yogurt stored for 2, 4, and 6 days.

Figure 2 - Molecular weight distribution of peptides from digested soy foods and 7S and 11S hydrolysates.



Different lowercase letters over the bar are significantly different among samples ($p < 0.05$) on the same range of peptide molecular weight.
RSoy: raw soybean; RTSoyM: Raw traditional soymilk; CTSoyM: Cooked traditional soymilk; RSoyMS; PT: Pressed tofu; RSd3: Raw sprout germinated for 3 days; CSd3: Cooked sprout germinated for 3 days; Nd2: Natto stored for 2 days; Td2: tempeh stored for 2 days; and Yd2: Yogurt stored for 2 days.
7S and 11S: A- no hydrolyzed; B- hydrolyzed by pepsin for 2h; C- hydrolyzed by pepsin for 2h + trypsin for 2h; D- hydrolyzed by pepsin for 2h + trypsin for 2h + α-chymotrypsin for 2h; and E- hydrolyzed by pepsin for 2h + trypsin and α-chymotrypsin for 2h.

Figure 3 - Scatterplot of the PCA (a) representing 8 variables and (b) representing 9 soybean foods, 5 hydrolysates of 7S and 5 hydrolysates of 11S.



RSoy: raw soybean; RTSoyM: Raw traditional soymilk; CTSoyM: Cooked traditional soymilk; RSoyMS; PT: Pressed tofu; RSd3: Raw sprout germinated for 3 days; CSd3: Cooked sprout germinated for 3 days; Nd2: Natto stored for 2 days; Td2: tempeh stored for 2 days; and Yd2: Yogurt stored for 2 days.
7S and 11S: A- no hydrolyzed; B- hydrolyzed by pepsin for 2h; C- hydrolyzed by pepsin for 2h + trypsin for 2h; D- hydrolyzed by pepsin for 2h + trypsin for 2h + α-chymotrypsin for 2h; and E- hydrolyzed by pepsin for 2h + trypsin and α-chymotrypsin for 2h.

6 CONCLUSÕES

- O delineamento simplex centroide foi uma ferramenta eficiente para otimizar a extração de isoflavonas e compostos fenólicos com atividade antioxidante das FDSF-Mp e FDSF-Ao.
- Os solventes de extração e a amostra matriz influenciaram na capacidade de extração de isoflavonas e fenólicos totais.
- A composição ótima da mistura de solventes para extração de compostos bioativos da FDSF-Mp e FDSF-Ao foi água, etanol e metanol nas proporções em peso de 0,500: 0,375: 0,125 e 0,500: 0,300: 0,200, respectivamente.
- Extração de compostos bioativos com rendimento acima de 90% foi obtido utilizando o solvente extrator não tóxico constituído por água e etanol na proporção em peso de 0,500: 0,500.
- Os parâmetros de fermentação (pH inicial, água adicionada e temperatura de incubação) da FDSF-Mp influenciaram no teor das isoflavonas acetil-β-glicosídeos, β-glicosídeos e agliconas e não apresentaram efeitos sobre o CFT e atividade antioxidante (DPPH e ABTS). A fermentação da FDSF-Mp para obtenção de maior teor de compostos bioativos e atividade antioxidante, deve ser realizada em pH inicial entre 5,2 e 6,4, com adição de água entre 10 e 18 mL e temperatura de incubação entre 30 e 47 °C.
- Os parâmetros de fermentação (água adicionada e temperatura de incubação) da FDSF-Ao influenciaram no teor de todas as formas de isoflavonas, CFT e atividade antioxidante (DPPH, FRAP e ABTS). A fermentação da FDSF-Ao para obtenção de maior teor de compostos bioativos e atividade antioxidante, deve ser realizada com adição de água entre 10 e 14 mL, temperatura de incubação entre 22 e 38 °C e independente do pH inicial na faixa investigada.
- A correlação negativa do teor de isoflavonas acetil-β-glicosídeos e β-glicosídeos com o teor de agliconas indicou que a fermentação da DSF com *Monascus purpureus* ou *Aspergillus oryzae* promoveu a conversão das isoflavonas glicosiladas em agliconas.
- A correlação positiva do teor de isoflavonas agliconas com atividade antioxidante (DPPH) e do teor de compostos fenólicos totais com atividade antioxidante (FRAP e ABTS) confirmou que a fermentação da FDS com

Monascus purpureus ou *Aspergillus oryzae* favoreceu a formação de compostos bioativos.

- O tempo de fermentação da FDS com *Monascus purpureus* ou *Aspergillus oryzae* influenciou a atividade de β-glucosidase, conteúdo de diferentes formas de isoflavonas, CFT, atividade antioxidante, e teor de proteínas totais e açucares solúveis.
- O tempo de fermentação por 3, 4, 5, 6 e 7 dias da FDSF-Mp apresentou similaridades e foi caracterizado com maior teor de isoflavonas agliconas e sacarose e baixo teor de rafinose e estaquiose. Portanto, esta pode ser útil como matéria prima para elaboração de alimentos contendo isoflavonas agliconas e sacarose.
- O tempo de fermentação por 4, 5, 6 e 7 dias da FDSF-Ao apresentou similaridades e foi caracterizado com maior CFT, atividade de β-glucosidase e antioxidante (DPPH, ABTS e FRAP) e menor teor de sacarose, rafinose e estaquiose. Consequentemente, devido ao potencial antioxidante a FDSF-Ao pode ser útil como ingrediente de alimentos adicionando em diversos produtos alimentícios como em produtos cárneos e de panificação.
- A capacidade antioxidante mensurada pelo valor de IC_{50} indicou que o extrato da FDSF-Ao apresentou maior capacidade de eliminação dos radicais livres (DPPH e ABTS) e atividade redutora do Fe^{3+}, enquanto que o extrato da FDSF-Mp apresentou maior capacidade quelante do Fe^{2+}. Ambos extratos das farinhas fermentadas apresentaram elevada capacidade antioxidante e potencial de uso na prevenção do estresse oxidativo.
- A avaliação do potencial anti-hipertensivo de diversos alimentos de soja tradicionais digeridos *in vitro* indicou que entre todos os produtos, o extrato de soja apresentou maior potencial anti-hipertensivo.
- Produtos digeridos de soja fermentados com bactéria (natto e iogurte de soja) apresentaram maior inibição da ECA do que o digerido fermentado de soja com fungo (tempeh).
- Brotos germinados por 3 dias e digerido apresentaram maior inibição da ECA do que os brotos germinados por 5 ou 7 dias e digeridos.
- A fração hidrolisada de proteica de soja 11S exibiu maior inibição da ECA do que a 7S. Os peptídeos de massa molar entre 1 e 4,5 kDa foram os que mais

contribuíram para a inibição da ECA. Os alimentos de soja ou frações proteicas de soja hidrolisadas apresentam potencial para serem utilizados na prevenção e controle da hipertensão.