Universidade Estadual de Londrina

FERNANDO SANCHES DE LIMA

EFEITOS DO TEMPO E TEMPERATURA DE HIDRATAÇÃO DA SOJA SOBRE AS PROPRIEDADES DO GRÃO, CONTEÚDO DE ISOFLAVONAS E AÇÚCARES E ATIVIDADES DE β-GLICOSIDASES E α-GALACTOSIDASES

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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 Doutor em Ciência de Alimentos.

Orientadora: Dra. Elza Iouko Ida.
Coorientadora: Dra. Louise Emy Kurozawa.

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Londrina, 30 de maio de 2017.

Dedico

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RESUMO

A hidratação da soja é uma etapa prévia à elaboração de vários produtos de soja que visa reduzir a dureza dos grãos e pode influenciar a solubilidade das proteínas, a lixiviação, a conversão e/ou a degradação de isoflavonas e/ou açúcares. O objetivo deste trabalho foi investigar os efeitos do tempo e temperatura de hidratação da soja sobre as propriedades do grão (umidade, dureza e teor de proteínas solúveis), conteúdo de diferentes formas de isoflavonas e açúcares e atividades de β-glicosidases e α-galactosidases. Os grãos de soja foram hidratados na proporção de 1 g de soja: 1,5 g de água deionizada por 0, 1, 2, 3, 4, 5, 6 e 7 h a 25, 40, 55 e 70 °C. O conteúdo de isoflavonas foi quantificado por cromatografia líquida de ultra eficiência (CLUE) e de açúcares por cromatografia de troca iônica de alta performance (HPAEC). Os efeitos do tempo e temperatura de hidratação da soja sobre o conteúdo de isoflavonas e açúcares foram avaliados por meio de análises de regressão linear e não linear. O tempo e temperatura de hidratação da soja foram fatores importantes e significativos na absorção de água, dureza, conteúdos de proteínas solúveis, isoflavonas e açúcares e na ação de β-glicosidases e α-galactosidases dos grãos hidratados. A soja hidratada por 3 h a 55 ou 2 h a 70 °C atingiu o teor de umidade de 120% em base seca. A partir de 5 h de hidratação da soja e temperatura acima de 40 °C, a dureza dos grãos hidratados foi mantida e a extração das proteínas solúveis aumentou. O tempo de hidratação da soja a 25 °C não influenciou o conteúdo e perfil das isoflavonas das séries de daidzeína (daidzeína, daidzina e malonil daidzina), genisteína (genisteína, genistina e malonil genistina) e gliciteína (glicitina e malonil glicitina). Entretanto, o tempo de hidratação da soja a 40, 55 ou 70 °C influenciou o fenômeno de lixiviação, conversão e/ou degradação de isoflavonas. A partir do modelo de regressão foi estimado um conteúdo máximo de daidzeína em grãos hidratados por 5,43 h a 40 °C. Os grãos hidratados por 6 h a 55 °C apresentaram um conteúdo de daidzeína e genisteína, 6 e 7 vezes maior do que nos grãos não hidratados, respectivamente. A hidratação da soja a partir de 1 h a 70 °C promoveu a lixiviação e degradação de isoflavonas malonil daidzina, malonil genistina e malonil glicitina. O tempo e temperatura de hidratação da soja a 25 ou 40 °C não promoveu alterações significativas no conteúdo de rafinose e estaquiose. A hidratação da soja por até 3 h a 55 ou 70 °C favoreceu a ação da α-galactosidase hidrolisando os oligossacarídeos rafinose e estaquiose em galactose e sacarose. Após 3 h de hidratação da soja a 55 °C houve uma redução no conteúdo de rafinose de 45% e estaquiose de 25%. Contudo, o tempo de hidratação dos grãos de soja a 25, 40, 55 e 70 °C não influenciou significativamente o conteúdo de sacarose. Portanto, independentemente do tempo de hidratação dos grãos de soja a 40, 55 ou 70 °C, ocorreram fenômenos de lixiviação, conversão e/ou degradação de isoflavonas e açúcares.

Palavras-chave: Isoflavonas. Oligossacarídeos. β-glicosidase. α-galactosidase.

LIMA, Fernando Sanches de. **The effects of the time and temperature of soybean soaking on the grain properties, isoflavone and oligosaccharide contents, and activities of β-glucosidases and α-galactosidases.** 2017. 86 p. Tese (Doutorado em Ciência de Alimentos) – Universidade Estadual de Londrina, Londrina, 2017.

ABSTRACT

Soybean soaking is a prior step to preparation of various soy products and aims to reduce the hardness of the grains and can influence the solubility of the proteins, leaching, conversion and/or degradation of isoflavones and/or sugars. The objective of this work was to investigate the effects of the time and temperature of soybean soaking on the grain properties (moisture, hardness and soluble proteins content), contents of different forms of isoflavones and sugars, and activities of β-glucosidases e α-galactosidases. Soybean soaking was performed in at a 1:1.5 (g:g, soybean: deionised water) ratio for 0, 1, 2, 3, 4, 5, 6 and 7 h at 25, 40, 55 and 70 °C. The content of isoflavones was determined by ultra high-performance liquid (UHPLC) and for sugars was by chromatography high performance anion exchange chromatography (HPAEC). The effects of the time and temperature of soybean soaking on the isoflavone and sugar contents were submitted to linear and non-linear regression analyzes. The time and temperature of soybean soaking were significant and important factors in the water absorption, hardness, soluble protein, isoflavones and sugar contents, and action of β-glucosidases e α-galactosidases of soaked soybeans. A moisture content of 120% (dry basis) was achieved by soaking the soybeans at 55 °C and 70 °C for 3 and 2 h, respectively. From 5 h of soybean soaking and temperature above 40 °C, the hardness of the soaked grains was maintained and the extraction of the soluble proteins improved. The duration of soybean soaking at 25 °C did not influence the contents and profiles of isoflavones of the daidzein (daidzein, daidzin and malonyl daidzin), genistein (genistein, genistin and malonyl genistin) and glycitein (glycitin and malonyl glycitin) series. However, the soybean soaking at 40, 55 or 70 °C influenced the isoflavones leaching, conversion and/or degradation. The maximum content of daidzein was estimated in soybeans soaked for 5.43 h at 40 °C. Soybeans soaked for approximately 6 h at 55 °C showed 6- and 7-fold higher daidzein and genistein contents than did unsoaked soybeans, respectively. Soybean soaking from 1 h at 70 °C favoured the leaching and degradation of malonyl daidzin, malonyl genistin and malonyl glycitin. Soybean soaking at 25 or 40 °C did not show significant changes in the raffinose and stachyose contents. The α-galactosidases are catalyse the hydrolysis of raffinose and stachyose oligosaccharides to galactose in soybeans soaked at 55 or 70 °C for 3 h. After 3 h of soybean soaking at 55 °C, there were reductions of 45% for raffinose and 25% for stachyose. However, at temperatures of 25, 40, 55 and 70 °C, there were no significant changes of the sucrose content. Therefore, regardless of soybean soaking time at 40, 55 or 70 °C, leaching, conversion and/or degradation of isoflavones and sugars occurred.

Keywords: Isoflavones. Oligosaccharides. β-glucosidase. α-galactosidase.

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LISTA DE ABREVIATURAS E SIGLAS

ANVISA	Agência Nacional de Vigilância Sanitária
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária
FDA	*Food and Drug Administration*
USDA	*United States Department of Agriculture*

SUMÁRIO

1 INTRODUÇÃO

A soja [*Glycine max* (L.) Merrill] é uma leguminosa de grande importância na economia mundial. Em 2016/2017, o Brasil ainda deve permanecer como segundo maior produtor mundial de soja em grãos e estima-se uma produção de 104 milhões de toneladas (USDA, 2017).

Os efeitos fisiológicos de vários compostos bioativos da soja têm sido amplamente investigados. Embora os ácidos fenólicos e as saponinas da soja tenham sido estudados por um certo tempo, as isoflavonas e os oligossacarídeos têm sido os mais explorados. A relação entre a ingestão de isoflavonas da soja e os efeitos associados à redução do risco de doenças cardiovasculares, da incidência de diferentes tipos de câncer e ao combate do diabetes *mellitus* tem sido reportada (YUN, 2010; FEI; LING; HUA, REN, 2014; MESSINA; BADGER, 2017). Embora a fermentação dos oligossacarídeos rafinose e estaquiose produza gases que resultam em desconforto abdominal e flatulência nos indivíduos, a ação prebiótica destes açúcares tem sido destacada (CHEN et al., 2012; SAAD et al., 2013).

As isoflavonas da soja estão divididas em quatro grupos distintos conforme a sua estrutura química e são denominados de agliconas, β-glicosídeos, malonilglicosídeos e acetilglicosídeos. Uma vez que as isoflavonas são derivadas das formas agliconas, estas podem ser agrupadas como séries de daidzeína (daidzeína, daidzina, acetil daidzina e malonil daidzina), genisteína (genisteína, genistina, acetil genistina e malonil genistina) e gliciteína (gliciteína, glicitina, acetil glicitina e malonil glicitina). As isoflavonas da soja estão associadas à redução do risco de doenças cardiovasculares, controle do diabetes *mellitus*, diminuição da incidência de alguns tipos de câncer, alívio nos sintomas da menopausa e outros potenciais efeitos, os quais são frequentemente atribuídos às isoflavonas agliconas (VITALE et al., 2013; MESSINA; BADGER, 2017).

Além da sacarose, os oligossacarídeos rafinose e estaquiose são os principais açúcares solúveis da soja. Estes oligossacarídeos são considerados prebióticos, cuja fermentação pelas bactérias do cólon produz ácidos graxos de cadeia curta. Estes ácidos graxos podem estar associados à redução de ocorrências de constipação, câncer de cólon, doenças cardiovasculares e inflamações sistêmicas associadas à obesidade (MEIJER; VOS; PRIEBE, 2010; MACFARLANE; MACFARLANE, 2011; LI; LU; YANG, 2013).

O conteúdo e o perfil de isoflavonas e oligossacarídeos na soja são dependentes de fatores, tais como a genética da cultivar e a região de cultivo da mesma (BRITZ; SCHOMBURG; KENWORTHY, 2011; SALDIVAR; WANG; CHEN; HOU, 2011; HAGELY; PALMQUIST; BILYEU, 2013). As isoflavonas glicosiladas e os oligossacarídeos estão sujeitos às conversões em estruturas menores por meio da ação das enzimas β-glicosidase e α-galactosidase, respectivamente, as quais podem ser encontradas naturalmente na soja.

A hidratação da soja é uma operação unitária de pré-processamento utilizada para o preparo de vários produtos à base de soja, tais como extrato de soja, *tofu* e *tempeh*. O tempo e temperatura de hidratação são fatores importantes que devem ser monitorados neste processo, pois influenciam na textura, trituração e cozimento dos grãos. Além disto, tais fatores influenciam na lixiviação e degradação de substâncias presentes na soja e ativam as enzimas endógenas (PAN; TANGRATANAVALEE, 2003; LIMA; KUROZAWA; IDA, 2014; FABBRI; CROSBY, 2016). Neste contexto, as variáveis tempo e temperatura podem ser estabelecidas de modo a promover a formação de isoflavonas agliconas e preservar o conteúdo total de isoflavonas e proteínas solúveis, sem afetar a absorção de água e dureza dos grãos hidratados. Assim sendo, produtos de soja com maior conteúdo de isoflavonas agliconas e reduzido teor de oligossacarídeos podem ser obtidos a partir de grãos de soja tratados hidrotermicamente sob condições pré-estabelecidas.

As alterações no conteúdo e perfil de isoflavonas durante a hidratação da soja foram descritas por Toda et al. (2000), Sutil et al. (2008), Góes-Favoni, Carrão-Panizzi e Beleia (2010). Contudo, os autores utilizaram apenas uma condição de temperatura e não avaliaram outros fatores de interesse e de forma conjunta.

Portanto, o objetivo deste trabalho foi investigar os efeitos do tempo e temperatura de hidratação da soja sobre as propriedades do grão, conteúdo de isoflavonas e oligossacarídeos e atividades de β-glicosidases e α-galactosidases endógenas.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar os efeitos dos tempos e temperaturas de hidratação da soja sobre as propriedades do grão, conteúdo de isoflavonas e oligossacarídeos e atividades de β-glicosidases e α-galactosidases endógenas.

2.2 OBJETIVOS ESPECÍFICOS

Investigar os efeitos dos tempos e temperaturas de hidratação da soja sobre:

- Os conteúdos de umidade, proteínas solúveis e diferentes formas de isoflavonas; dureza e atividade de β-glicosidades dos grãos hidratados e/ou solução residual.
- As transformações no conteúdo das diferentes formas de isoflavonas dos grãos hidratados.
- As alterações no conteúdo de açúcares e atividade de α-galactosidase dos grãos hidratados.

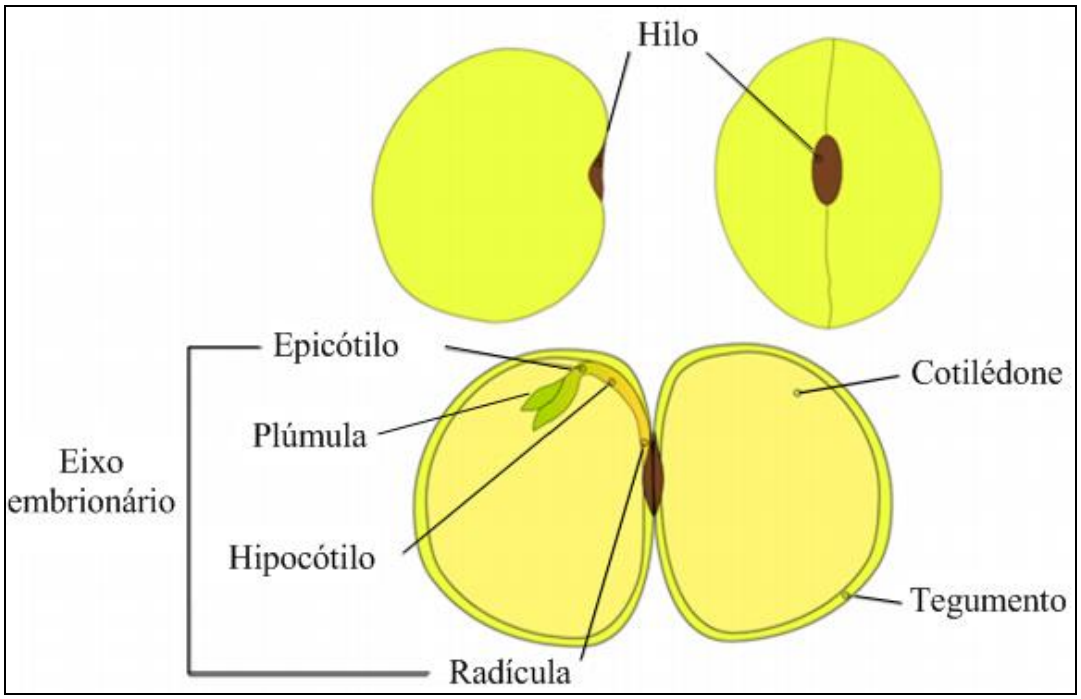
3 REVISÃO BIBLIOGRÁFICA

3.1 COMPOSIÇÃO QUÍMICA, VALOR NUTRITIVO E ASPECTOS TECNOLÓGICOS DE PROCESSAMENTO DA SOJA

A soja é uma leguminosa muito importante na economia brasileira e de outros países, tais como Estados Unidos, Argentina e China. Na previsão de 2016/2017, o Brasil deverá permanecer como segundo maior produtor mundial de soja em grãos, com uma produção estimada em 104 milhões de toneladas (USDA, 2017).

O grão de soja se destaca pelo elevado teor de proteínas (40-41 g/100 g) e lipídeos (18-22 g/100 g) e contém quantidades significativas de carboidratos (30-35 g/100 g) e cinzas (5-7 g/100 g) em base seca. A composição química da soja pode variar em função da cultivar, estádio de maturação e localização geográfica de cultivo (MEDIC; ATKINSON; HURBURGH, 2014). Ainda, estes constituintes da soja estão distribuídos (Tabela 1) de forma distinta nas diferentes partes componentes do grão (Figura 1).

Figura 1 – Partes do grão de soja.



Fonte: Medic, Atkinson e Hurburgh (2014) – Adaptado.

Tabela 1 – Composição química do grão e das partes componentes da soja.

Partes componentes	% em massa do grão	Composição química*			
		Proteínas (N x 6,25)	Lipídeos	Carboidratos**	Cinzas
Cotilédone	90	43	23	29	5
Tegumento	8	9	1	86	4
Hipocótilo	2	41	11	43	5
Grão	100	40	20	35	5

*O conteúdo da composição química foi calculado em relação a cada componente (100 %) do grão em base seca. **Por diferença.
Fonte: Berk (1992) – Adaptado e corrigido.

Os cotilédones, tegumento e hipocótilo (Figura 1) são os principais componentes da soja e representam 90%, 8% e 2% da massa do grão (Tabela 1). Assim sendo, as proteínas, lipídeos, carboidratos e cinzas correspondem a 43%, 23%, 29% e 5% da massa total de cotilédone, que corresponde a 90% da massa total do grão (Tabela 1). Estes conteúdos elevados de nutrientes no cotilédone estão relacionados com a sua maior proporção em massa do grão. Observa-se que estes mesmos constituintes apresentam valor absoluto baixo no tegumento e hipocótilo. Contudo, quando são expressos em relação à massa total do tegumento ou hipocótilo as concentrações de proteínas, lipídeos, carboidratos e/ou cinzas aumentam (Tabela 1).

Reconhecendo a importância das proteínas de soja do ponto de vista nutricional e de saúde, o Food and Drug Administration (FDA), órgão americano regulador de alimentos e medicamentos, aprovou desde 1999 a alegação que "*o consumo diário de 25 g de proteínas de soja, como parte de uma dieta pobre em gordura saturada e colesterol, pode reduzir o risco de doença cardíaca*". Somente os produtos de soja contendo no mínimo 6,25 g de proteínas de soja por porção podem ter a referida alegação no rótulo (FDA, 1999). A Agência Nacional de Vigilância Sanitária (ANVISA) também autorizou descrições similares na rotulagem de alimentos derivados de soja (BRASIL, 2014). Contudo, ainda não está bem elucidada a relação entre a ingestão de proteínas de soja e a redução do nível de colesterol LDL no sangue. Assim sendo, a categoria "A", que indica o nível máximo

de evidência científica sobre as alegações de saúde de um composto, tem sido questionada para as proteínas de soja (GIRGIH et al., 2013).

Os principais ácidos graxos constituintes da soja são o oleico [C18:1 (9)], linoleico [(C18:2 (9,12)] e α-linolênico [C18:3 (9,12, 15)], sendo este último conhecido como ω-3 e que está associado à redução do risco de doenças cardiovasculares (DECKELBAUM; TORREJON, 2012). As insaturações na cadeia do ácido α-linolênico o tornam muito suscetível à rancidez oxidativa e oxidação enzimática (MEDIC; ATKINSON; HURBURGH, 2014) promovida pelas lipoxigenases (EC 1.13.11.12). Estas reações podem ser intensificadas nos grãos durante a sua estocagem e até mesmo durante o processamento de produtos de soja (IASSONOVA et al., 2009). Como consequência, são formados compostos responsáveis pelos sabores de “feijão” cru e ranço, os quais reduzem significativamente a aceitação dos alimentos de soja pelos consumidores (SHIN; KIM; KIM, 2013).

A Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) tem desenvolvido cultivares de soja, tais como a BRS 257 (genealogia BR93-32109 X BR94-23396), isentas das isoenzimas lipoxigenases L1, L2 e L3, cujas características têm sido importantes para a elaboração do extrato de soja com sabor mais aceitável pelos ocidentais (MORAES et al., 2007). Embora as cultivares de soja isentas dessas três isoenzimas apresentem um menor conteúdo de aldeídos e cetonas, responsáveis, em parte, pelo sabor e odor característicos dos grãos de soja, as mesmas ainda podem apresentar outras substâncias que também contribuem com essas características sensoriais. Estas substâncias parecem ser provenientes da ação de outras enzimas sobre os fosfolipídeos (IASSONOVA et al., 2009).

Além da sacarose, os oligossacarídeos da família rafinose (rafinose, estaquiose e verbascose) são os principais açúcares solúveis da soja. Estes oligossacarídeos, algumas vezes referidos como α-galactosídeos ou α-galacto-oligossacarídeos, fazem parte do grupo das fibras alimentares ou dietéticas (CHEN et al., 2012). Os carboidratos insolúveis da soja consistem de polissacarídeos complexos constituídos essencialmente por celulose, hemicelulose e pectina e são classificados como fibra alimentar ou dietética, cujo teor nos grãos de soja varia entre 15,1 a 24,4 g de fibra alimentar total por 100 g de soja madura em base seca (REDONDO-CUENCA; VILLANUEVA-SUÁREZ; MATEOS-APARICIO, 2008). As

fibras da soja podem auxiliar na redução dos níveis de colesterol no sangue e exercer efeitos anticarcinogênicos no sistema digestório humano (ANDERSON et al., 2009; TRINIDAD et al., 2010; CHEN et al., 2012). Cultivares de soja com elevado teor de sacarose são importantes para conferir doçura aos produtos de soja e, consequentemente, melhorar a aceitação destes pela população ocidental (Kumar et al., 2010).

Os grãos de soja contêm aproximadamente 5 % de cinzas em base seca. Os constituintes deste resíduo mineral fixo, dependendo da sua concentração, podem ser classificados como micronutrientes ou macronutrientes. Os conteúdos de potássio, fósforo, magnésio, enxofre, cálcio, cloro e sódio variam de 0,2 a 2,1 g por 100 g de soja seca e são os principais macronutrientes minerais da soja. Os micronutrientes incluem o silício, ferro, zinco, manganês, cobre e outros que apresentam um conteúdo de 0,01 a 140 mg por kg de soja seca (LIU, 2004). Os minerais são importantes para o crescimento e reparação de tecidos, ativação de enzimas, condução de impulsos nervosos e contração muscular, entre outras funções.

Portanto, observa-se que o valor nutritivo da soja está relacionado com os seus constituintes primários (proteínas, lipídeos e carboidratos) e minerais e, por isso, tem sido investigada principalmente quanto a sua composição química. No entanto, há carência de informações sobre outros constituintes, tais como compostos bioativos que são benéficos à saúde humana, bem como as alterações ou transformações que estes podem sofrer ao longo do processamento da soja.

3.2 ISOFLAVONAS DA SOJA COMO COMPOSTOS BIOATIVOS E BENEFÍCIOS À SAÚDE

As substâncias bioativas ou compostos bioativos são nutrientes ou não nutrientes que possuem ação metabólica ou fisiológica específica em humanos (BRASIL, 2002). Os compostos bioativos geralmente são metabólitos secundários de plantas e, por isto, muitas vezes são denominados de fitoquímicos. Os alimentos funcionais são justificados pela presença de compostos bioativos, entretanto estes alimentos não podem ter finalidade medicamentosa ou terapêutica, qualquer que seja a forma de apresentação ou o modo como são administrados. Além disso, esta classe de produtos deve ser segura para o consumo humano, sem necessidade de

orientação e/ou acompanhamento médico, a não ser que sejam destinados a grupos populacionais específicos (BRASIL, 2002).

A soja possui diversos compostos bioativos, tais como proteínas, peptídeos, fibras, oligossacarídeos, ácidos fenólicos, flavonoides, saponinas, fitosteróis, ácido fítico e outros (VILLARES et al., 2011). Embora somente as proteínas da soja possuam alegação de saúde autorizada pela ANVISA, estudos clínicos e epidemiológicos têm demonstrado um efeito potencial e benéfico destes outros constituintes sobre a saúde (XU et al., 2010; CHEN et al., 2012; MESSINA; BADGER, 2017). Os estudos epidemiológicos, de farmacocinética e o uso de biomarcadores têm contribuído para definir os possíveis efeitos dos compostos bioativos à saúde humana, além de estabelecer a relação dose-resposta e segurança destas substâncias em células e tecidos específicos (BIESALSKI et al., 2009).

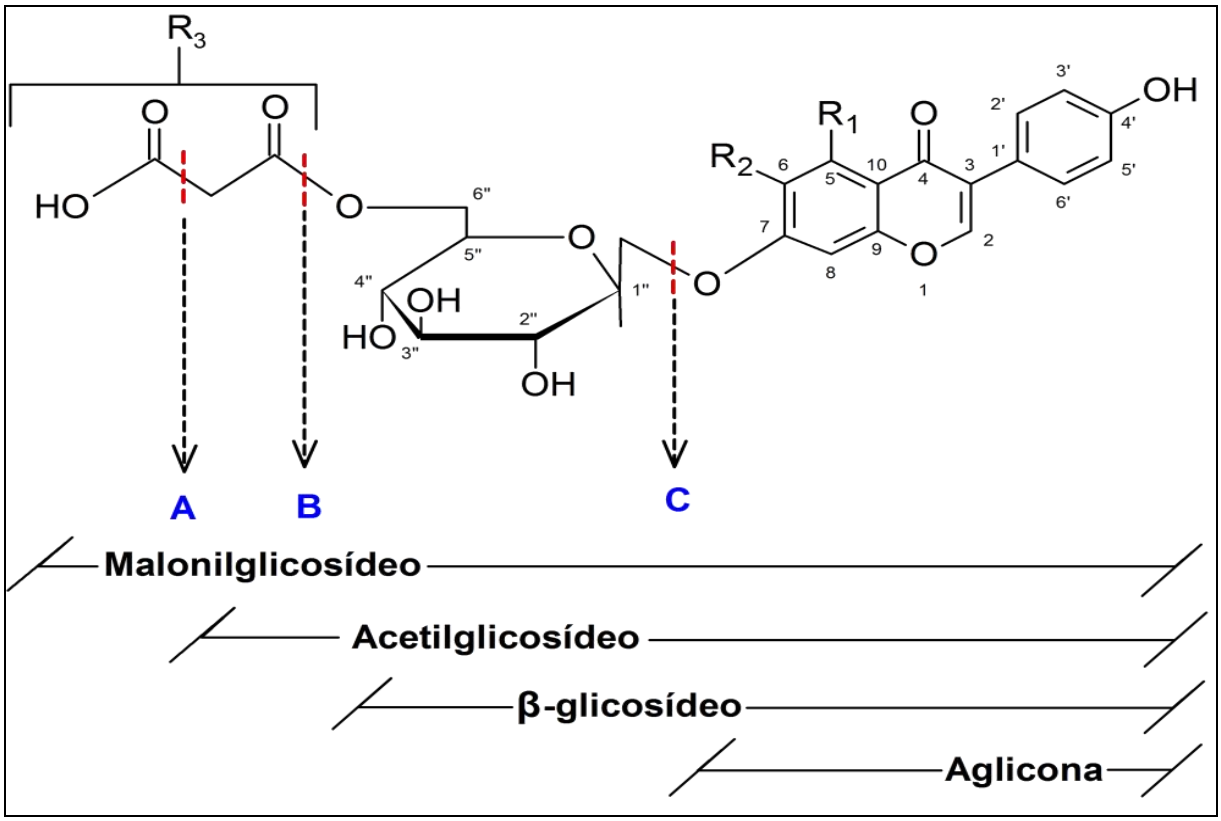
As isoflavonas são compostos polifenólicos e correspondem a uma subclasse, denominada de isoflavonoides, do grupo dos flavonoides (Figura 3). Ao todo, há doze formas principais de isoflavonas que podem ser encontradas na soja e seus derivados. Estas são divididas em quatro grupos conforme a sua estrutura química e denominadas de agliconas (daidzeína, genisteína e gliciteína), β-glicosídeos (daidzina, genistina e glicitina), acetilglicosídeos (6''-*O*-acetildaidzina, 6''-*O*-acetilgenistina e 6''-*O*-acetilglicitina) e malonilglicosídeos (6''-*O*-malonildaidzina, 6''-*O*-malonilgenistina e 6''-*O*-malonilglicitina). A Tabela 2 apresenta os grupos de isoflavonas da soja e seus principais radicais químicos (R1, R2 e R3). A Figura 3, além das estruturas químicas, apresenta também as possíveis transformações por clivagem das ligações por meio de descarboxilação, desesterificação ou hidrólise. Considerando que as isoflavonas são derivadas das formas agliconas, as mesmas podem ser agrupadas nas séries de daidzeína (daidzeína, daidzina, acetil daidzina e malonil daidzina), genisteína (genisteína, genistina, acetil genistina e malonil genistina) e gliciteína (gliciteína, glicitina, acetil glicitina e malonil glicitina), conforme descrito por Wang e Murphy (1996) e Yuan et al. (2009).

Tabela 2 - Grupos de isoflavonas da soja e seus principais radicais químicos.

Grupos	Isoflavonas	R_1	R_2	R_3
Agliconas	Daidzeína	-H	-H	-
	Genisteína	-OH	-H	-
	Gliciteína	-H	$-OCH_3$	-
β-glicosídeos	Daidzina	-H	-H	-H
	Genistina	-OH	-H	-H
	Glicitina	-H	$-OCH_3$	-H
Acetilglicosídeos	6''-*O*-acetildaidzina	-H	-H	$-COCH_3$
	6''-*O*-acetilgenistina	-OH	-H	$-COCH_3$
	6''-*O*-acetilglicitina	-H	$-OCH_3$	$-COCH_3$
Malonilglicosídeos	6''-*O*-malonildaidzina	-H	-H	$-COCH_2COOH$
	6''-*O*-malonilgenistina	-OH	-H	$-COCH_2COOH$
	6''-*O*-malonilglicitina	-H	$-OCH_3$	$-COCH_2COOH$

Fonte: Liu (2004) – Adaptado.

Figura 2 - Estrutura química das isoflavonas da soja e possíveis transformações por clivagem de ligações.



A = descarboxilação de malonilglicosídeos com a formação de acetilglicosídeos.
B = desesterificação de malonil e acetilglicosídeos com a formação de β-glicosídeos.
C = hidrólise de malonil, acetil e β-glicosídeos com a formação de agliconas.

No metabolismo secundário dos vegetais compostos específicos são formados conforme as necessidades de cada planta e estes compostos geralmente estão envolvidos no mecanismo de defesa do vegetal. Dentre os metabólitos secundários da soja, as isoflavonas são as mais investigadas. Segundo Yoo et al. (2013), as isoflavonas são importantes na relação simbiótica rizóbio-leguminosa e podem atuar contra os patógenos microbianos.

O conteúdo e perfil de isoflavonas nos grãos de soja são influenciados por vários fatores, entre os quais se destacam a genética da cultivar, grupo de maturidade, safra, clima da região de cultivo, condições de estocagem e outros (WANG; MURPHY, 1994; BRITZ; SCHOMBURG; KENWORTHY, 2011).

O conteúdo de flavonoides e isoflavonoides em diversos alimentos tem sido armazenado em um banco de dados que foi elaborado pelo *Nutrient Data Laboratory* (NDL) do USDA com base em dados de pesquisas e de indústrias de alimentos (USDA, 2008). Neste banco de dados é informado que os grãos de soja maduros apresentam em média 12,86 mg, 18,77 mg e 2,88 mg de massas equivalentes das séries de daidzeína, genisteína e gliciteína, respectivamente, por 100 g de soja em base seca.

Uma grande parte de produtos desenvolvidos com soja apresentam teor e perfil de isoflavonas distintos (VILLARES et al., 2011; ALEZANDRO et al., 2011) devido ao grande número de variáveis que afetam o conteúdo destas substâncias desde a matéria prima até o produto final. Além disto, há dificuldade de se estabelecer um processo de obtenção de produtos de soja que seja capaz de promover um padrão com conteúdo adequado de isoflavonas.

Além de estudos epidemiológicos com humanos ou testes com animais, as isoflavonas da soja têm sido amplamente investigadas devido a sua relevante atividade biológica. A bioatividade das isoflavonas da soja tem sido associada à redução de incidência de doenças cardiovasculares em mulheres no período pré-menopausa, alguns tipos de câncer, hiperglicemia, Alzheimer, sintomas da menopausa e outras doenças (DONG et al., 2013; FILIBERTO et al., 2013; PARK et al., 2013; REED et al., 2013; WADA et al., 2013; MESSINA; BADGER, 2017).

Segundo a ANVISA (2017), as isoflavonas são consideradas medicamentos fitoterápicos e necessitam, obrigatoriamente, de registro para sua comercialização. Além disto, no rótulo destes medicamentos contendo isoflavonas pode ser alegado apenas o auxílio na redução do colesterol e alívio nos sintomas da

menopausa. Todavia, a alegação de conteúdo ("contém isoflavonas", "fonte de isoflavonas", "rico em isoflavonas", dentre outras), de função ou de saúde em rótulo de alimentos que naturalmente contenham isoflavonas não está autorizada.

A ingestão de isoflavonas da soja pelas diferentes populações e países varia consideravelmente. A população asiática ingere de 20 a 100 mg de isoflavonas por dia, enquanto que a população ocidental consome de 1 a 10 mg de isoflavonas por dia por ingerirem menos do que 4 g de soja e seus derivados por dia (NAKAMURA; TSUJI; TONOGAI, 2000; MESSINA; NAGATA; WU, 2006; SONG et al., 2007; GIRGIH et al., 2013). As diferenças observadas na ingestão de isoflavonas da soja por estas populações também podem ser responsáveis pela variabilidade encontrada na significância e intensidade dos seus efeitos benéficos descritos em estudos epidemiológicos.

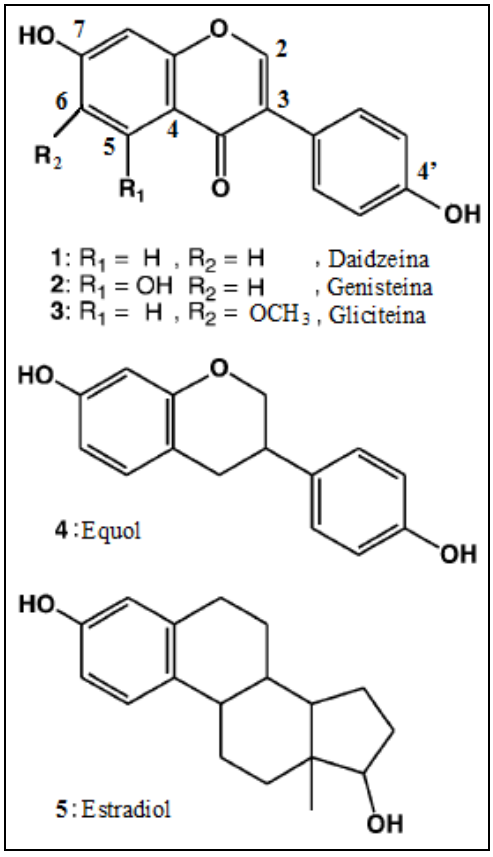
Hendrich et al. (1994) estimaram que a ingestão de 1,5 a 2 mg de isoflavonas da soja por kg de massa corpórea humana seria suficiente para promover um potencial efeito anticarcinogênico. Contudo, na área médica e farmacológica não há um consenso sobre a dose-resposta efetiva das isoflavonas para exercer potenciais efeitos benéficos à saúde humana (MESSINA; BADGER, 2017). Em 2006, a Comissão de Segurança de Alimentos do gabinete do governo do Japão, a partir de recomendações do comitê que revisa a segurança de alimentos para fins específicos à saúde ou "*Foods for Specified Health Use*"- FOSHU, definiu o limite máximo da ingestão diária de isoflavonas como sendo 30 mg de isoflavonas, em equivalente de agliconas, para um consumo adicional à dieta normal (YANAKA et al., 2010).

Na biodisponibilidade de polifenóis, a microbiota intestinal desempenha um papel chave e pode afetar a bioatividade destes compostos (CARDONA et al., 2013). É possível que a população chinesa apresente um perfil genético diferente das populações cujo consumo de soja é relativamente recente. Assim sendo, a população chinesa provavelmente tenha desenvolvido uma maior capacidade de extrair e digerir os nutrientes da soja, uma vez que consomem soja há mais de 5000 anos (HE; CHEN, 2013). Ainda neste contexto, alguns estudos sugerem que as crianças têm uma maior exposição sistêmica aos isoflavonoides do que os adultos, quando considerada a mesma ingestão de soja ajustada à massa corporal. Isto poderia resultar em crianças com maiores benefícios da soja à saúde em relação aos adultos e, possivelmente, explicaria o risco reduzido de câncer de

mama em mulheres adultas que ingeriram soja desde criança, especialmente no período antes da puberdade (KORDE et al., 2009; FRANKE; LAI; HALM, 2014).

As isoflavonas agliconas são denominadas de fitoestrógenos, pois apresentam estrutura química e funcionalidade similar ao estrógeno 17 β-estradiol (Figura 3) (UZZAN; LABUZA, 2004). A daidzeína pode ser metabolizada em equol pela microbiota intestinal e esse metabólito também pode exercer ação estrogênica e antiestrogênica (DECROOS et al., 2005). Contudo, estima-se que apenas 30 a 50 % da população mundial possui uma microbiota intestinal capaz de promover a metabolização da daidzeína em equol (LAMPE, 2009; TSENG et al., 2013).

Figura 3 - Estrutura química das agliconas (1, 2, 3), equol (4) e estradiol (5).



As isoflavonas agliconas têm se destacado como potenciais inibidores das enzimas digestivas de carboidratos e podem apresentar um papel importante no combate ao diabetes *mellitus* tipos 1 e 2. Choi et al. (2008) verificaram que tanto a genisteína quanto a daidzeína foram importantes na homeostase de glicose em ratos que apresentaram diabetes tipo 1. Desta forma, estas isoflavonas também podem ser úteis para prevenir o aparecimento desta doença autoimune.

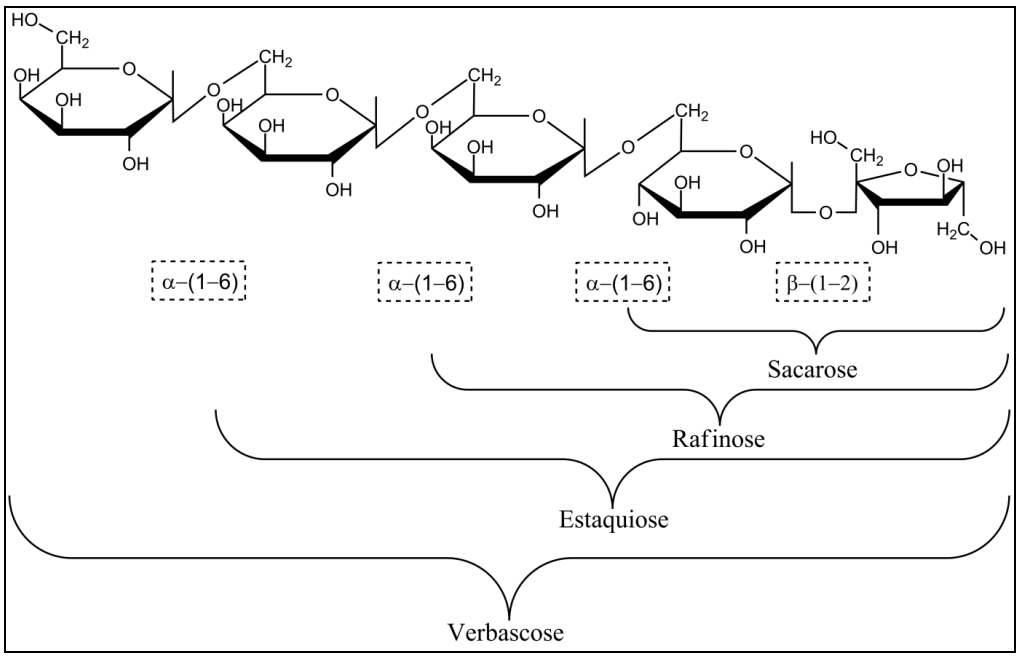
Park et al. (2013), em um estudo controlado em ratos, reportaram que a daidzeína foi mais efetiva na inibição da α-glicosidase e α-amilase do que o fármaco acarbose. Portanto, a inserção de produtos de soja com maior quantidade de isoflavonas agliconas na alimentação humana pode ser potencialmente benéfica.

3.3 OLIGOSSACARÍDEOS DA SOJA E BENEFÍCIOS À SAÚDE

Na soja, além da sacarose, os oligossacarídeos rafinose, estaquiose e verbascose são os principais açúcares solúveis. Estes oligossacarídeos algumas vezes são referidos como α-galactosídeos ou α-galacto-oligossacarídeos e fazem parte do grupo das fibras alimentares ou dietéticas. A rafinose é um trissacarídeo que contém em sua estrutura química uma galactose ligada por α-(1→6) a uma molécula de sacarose. A estaquiose é um tetrassacarídeo constituído por uma molécula de galactose ligada por α-(1→6) à galactose terminal da rafinose. A verbascose é um pentassacarídeo cuja estrutura química apresenta uma galactose ligada por α-(1→6) à galactose terminal da estaquiose (Figura 4).

Os carboidratos solúveis da soja desempenham um importante papel na tolerância de sementes à dessecação (OBENDORF et al., 1998) e estresse ao frio (OBENDORF et al., 2008).

Figura 4 - Estrutura química dos oligossacarídeos da soja.



Os oligoassacarídeos representam de 8 a 13 % do conteúdo total de carboidratos presente nas sementes de soja (HOLLUNG et al., 2005; OLIVEIRA et al., 2010). Este conteúdo pode variar conforme a cultivar, estádio de maturação, região de cultivo, dentre outros fatores (KUMAR et al., 2010; SALDIVAR et al., 2011). Segundo Kumar et al. (2010), os conteúdos de açúcares de 148 genótipos de soja cultivados em diferentes regiões da Índia foram de 1,70-4,07 g de sacarose; 0,33-1,28 g de rafinose e 1,39-4,73 g de estaquiose em 100 g de amostra em base seca. A sacarose e estaquiose são os carboidratos solúveis predominantes em grãos maduros de cultivares de soja convencional ou tipo alimento e representam 60 e 36 % do conteúdo total de carboidratos solúveis, respectivamente (OLIVEIRA et al., 2010).

Diversos fatores, incluindo os genéticos e ambientais, podem causar considerável variabilidade no conteúdo de oligossacarídeos na soja. Portanto, os produtos de soja, sem considerar as formas de processamento, poderão apresentar um perfil e conteúdo distinto destes açúcares. Na hidratação da soja ocorre a difusão dos açúcares e é a primeira etapa de pré-processamento da soja comumente utilizada para a elaboração de produtos de soja e é um importante passo para assegurar ou manter parcialmente estes açúcares na elaboração do extrato de soja, tofu e outros derivados.

Os humanos não possuem a enzima α-galactosidase (EC 3.2.1.22), responsável pela clivagem das ligações α-(1–6) galactosídicas dos oligossacarídeos da soja e, portanto, estes açúcares não podem ser digeridos. Estes açúcares, quando atingem o cólon, permanecem intactos e são preferencialmente fermentados pelas bifidobactérias que sintetizam esta enzima. Na fermentação destes açúcares são produzidos gases, tais como o dióxido de carbono, hidrogênio, metano e outros que podem resultar em um desconforto abdominal e flatulência nos indivíduos (SUAREZ et al., 1999). O principal efeito adverso e reconhecido até o momento dos diferentes galacto-oligossacarídeos, incluindo os anômeros alfa (α) e beta (β), é a “diarreia osmótica” transitória que ocorre quando estes açúcares são consumidos em excesso. Em humanos, estima-se que a administração diária entre 0,3 a 0,4 g de β-galacto-oligossacarídeos por kg de massa corporal ou até 20 g destas substâncias por pessoa não induza à diarreia (SAKO; MATSUMOTO; TANAKA, 1999; TORRES et al., 2010).

Os oligossacarídeos têm sido reconhecidos por exercerem funções promotoras à saúde, uma vez que podem ser utilizados como prebióticos para estimular o crescimento de bifidobactérias (CHEN et al., 2012; SAAD et al., 2013). A ingestão de 0,83 g de α-galacto-oligossacarídeos por kg de massa corporal de ratos mostrou ter um efeito prebiótico relevante nestes animais (LI; LU; YANG, 2013).

O conceito de prebiótico, segundo Gibson e Roberfroid (1995), é “um ingrediente alimentar, não digerível, que afeta beneficamente o hospedeiro ao estimular seletivamente o crescimento e/ou atividade de uma ou de um número limitado de bactérias no cólon e, portanto, melhora a saúde do hospedeiro”. Os efeitos bifidogênicos dos oligossacarídeos da família rafinose são decorrentes principalmente dos ácidos graxos de cadeia curta, particularmente o ácido acético, propiônico e butírico, que são produtos de fermentação destes açúcares pelas bactérias do cólon. Diversos efeitos benéficos à saúde têm sido relacionados à formação destes ácidos, podendo ser destacada a associação na redução de ocorrências de constipação, câncer de cólon, doenças cardiovasculares e inflamações sistêmicas e associadas à obesidade (SAKO; MATSUMOTO; TANAKA, 1999; WONG et al., 2006; MEIJER; VOS; PRIEBE, 2010; MACFARLANE; MACFARLANE, 2011; LI; LU; YANG, 2013).

Os oligossacarídeos da soja são considerados seguros pela FDA e possuem a classificação de *Generally Recognized as Safe* (GRAS) (FEI et al., 2014). No Japão, estes açúcares também são reconhecidos como ingredientes funcionais pelo sistema FOSHU e no rótulo de produtos contendo estes componentes pode-se afirmar, por exemplo, que estes promovem o aumento de bifidobactérias intestinais e, assim, ajudam a manter as condições gastrointestinais saudáveis (YAMADA et al., 2008). Contudo, a ANVISA não autoriza qualquer alegação de propriedade funcional e/ou de saúde no rótulo dos alimentos que contêm oligossacarídeos da soja.

Os efeitos bifidogênicos dos oligossacarídeos da família rafinose têm sido observados em alguns estudos (HAYAKAWA et al., 1990; WADA et al., 1992; HARA et al., 1997; NAGURA et al., 2002) não controlados com humanos provavelmente devido aos efeitos indesejáveis destes oligossacarídeos, tais como flatulência, desconforto abdominal e diarréia, que foram observados em alguns indivíduos e animais que consumiram alimentos à base de soja. Assim sendo, as pesquisas relacionadas aos oligossacarídeos da soja têm sido conduzidas apenas com o objetivo de reduzir ou remover estes açúcares nos grãos ou produtos de soja

(MULIMANI; THIPPESWAMY; RAMALINGAM, 1997; GUIMARÃES et al., 2001; VIANA et al., 2005; BRASIL et al., 2010).

Os efeitos dos oligossacarídeos da soja sobre a atividade de enzimas antioxidantes (superóxido dismutase, glutationa peroxidase e catalase) e resistência à insulina em mulheres grávidas com diabetes mellitus gestacional foram avaliados por Fei, Ling, Hua e Ren (2014). Os resultados indicaram que os oligossacarídeos da soja foram capazes de reduzir o estresse oxidativo e atenuar a resistência à insulina nas pacientes, indicando que estes açúcares podem desempenhar um papel importante no controle de complicações do diabetes *mellitus* gestacional.

Portanto, produtos de soja com maior conteúdo de isoflavonas agliconas e a presença de oligossacarídeos podem ser benéficos à saúde humana. Desta forma, estudos sobre os impactos do processamento da soja, especialmente a hidratação, sobre o conteúdo e perfil destes açúcares devem ser conduzidos, uma vez que vários produtos de soja podem ser obtidos a partir da hidratação da soja como um pré-tratamento.

3.4 HIDRATAÇÃO DOS GRÃOS DE SOJA

Antes do preparo de vários produtos de soja, tais como extrato de soja, *tofu*, *tempeh* e outros, geralmente os grãos de soja são hidratados (ou macerados) em temperatura ambiente por um determinado tempo e proporção adequada de grão:solvente de hidratação. Esta etapa visa alterar principalmente as características de textura do grão e pode afetar os processos posteriores, tais como a extração de proteínas e outros constituintes hidrossolúveis, a trituração e cocção. No método de Illinois para obtenção do extrato de soja, os grãos são hidratados até 12 h a 25 °C (NELSON; STEINBERG; WEI, 1976). Contudo, informações sobre a dureza de grãos de soja hidratados em função do tempo e temperatura de hidratação não têm sido reportadas na literatura.

Pan e Tangratanavalee (2003) recomendaram que a hidratação da soja deveria ser realizada até atingir um conteúdo mínimo de umidade de 120 g de água por 100 g de matéria seca, antes da realização dos processos de trituração dos grãos. Temperaturas de hidratação acima de 30 °C reduziu significativamente o tempo de hidratação do grão requerido para atingir esse conteúdo de umidade de referência (GOWEN et al., 2007). Por outro lado, temperaturas de hidratação de soja

acima de 40 °C aumentaram a transferência de massa por difusão e contribuíram com uma considerável perda de sólidos (PAN; TANGRATANAVALEE, 2003), que incluem as proteínas, açúcares, isoflavonas e outros constituintes, para o meio de hidratação. Além disto, a hidratação da soja em temperaturas mais elevadas por um tempo prolongado pode promover a intensa desnaturação das proteínas da soja, cujas temperaturas iniciais de desnaturação da β-conglicinina (7 S) e glicinina (11 S) ocorreram na faixa de 65 a 75 °C e 85 a 95 °C, respectivamente (KITABATAKE; TAHARA; DOI, 1990). Desta forma, o tempo e temperatura de hidratação dos grãos de soja são relevantes para minimizar a insolubilização das proteínas, cujas propriedades tecnológicas são dependentes da sua solubilidade (SHIN; KIM; KIM, 2013).

O tratamento hidrotérmico de grãos de soja influencia também o perfil de isoflavonas, favorecendo a atividade das β-glicosidases endógenas na conversão dos β-glicosídeos em agliconas (MATSUURA; OBATA; FUKUSHIMA, 1989; GÓES-FAVONI; CARRÃO-PANIZZI; BELEIA, 2010; LIMA; IDA, 2014). As β-glicosidases (β-D-glicosídeo-*O*-glicohidrolases, EC 3.2.1.21) são enzimas que hidrolisam as ligações β-glicosídicas dos dissacarídeos, oligossacarídeos e outros glicosídeos conjugados. Estas enzimas constituem um grupo heterogêneo de enzimas que são amplamente distribuídas na natureza e possuem diferentes aplicações em processos biológicos, tais como na hidrólise dos glicolipídeos, modificação de metabólitos secundários (isoflavonas e glicosídeos terpênicos) e produção de bioetanol (ESEN, 1993; HSIEH; GRAHAM, 2001; GONZÁLES-POMBO et al., 2011; SINGHANIA et al., 2013). No tecido vegetal, as β-glicosidases estão compartimentalizadas e isoladas dos seus substratos. Quando o tecido vegetal sofre alguma injúria, as β-glicosidases agem sobre os seus substratos e, em determinadas condições de hidratação dos grãos, estas enzimas também podem ser ativadas (MORANT et al., 2008).

Grãos de soja hidratados (1:3, g:g – soja: água destilada) por 1, 2, 5, 10 e 15 h a 20 °C foram investigados por Toda et al. (2000) quanto ao teor e perfil de isoflavonas; foi observado que, com o aumento do tempo de hidratação, ocorreu um pequeno aumento no conteúdo de isoflavonas agliconas e uma diminuição das isoflavonas β-glicosídeos. O aumento no teor de agliconas foi atribuído à temperatura da água de hidratação, que não foi adequada para proporcionar uma boa atividade das β-glicosidases.

O efeito do tratamento hidrotérmico dos grãos e farinha de soja a diferentes pH na formação de agliconas foi investigado por Sutil et al. (2008). A hidratação dos grãos foi realizada por 15 h a 50 °C em soluções tampão citrato-fosfato de pH variando entre 3,5 a 7,5. Após os tratamentos foi observado que em todos os valores de pH ocorreu maior formação de agliconas na farinha de soja hidratada em relação aos grãos hidratados, devido à trituração dos grãos, que favoreceu a interação entre as β-glicosidases e os β-glicosídeos. Em geral, com o aumento do pH de 6,0 para 6,5 houve uma maior conversão de isoflavonas glicosiladas em agliconas, tanto nos grãos hidratados quanto na farinha de soja hidratada. A faixa de pH com maior formação de agliconas foi semelhante à observada por Matsuura, Obata e Fukushima (1989), os quais relataram que a máxima formação destas isoflavonas em grãos de soja submetidos à hidratação (1:5, g:g – soja: água destilada) ocorreu em pH 6,0 a 50 °C.

As alterações no perfil de isoflavonas dos cotilédones de soja hidratados em dois volumes de água foram avaliados por Góes-Favoni, Carrão-Panizzi e Beleia (2010). Os grãos da cultivar de soja BRS 213 (safras de 2004 e 2005) foram descascados, com remoção do tegumento e hipocótilo e obtenção dos cotilédones. Os cotilédones foram hidratados em água deionizada a 50 °C por 12 h nas proporções 1:1,2 (g:g, cotilédone:água) e 1:3 (g:g, cotilédone:água), respectivamente. Os cotilédones não hidratados apresentaram 0,08 μmol e 0,23 μmol de agliconas por g em base seca, respectivamente. Após a hidratação dos cotilédones, o teor de agliconas foi de 0,49 μmol e 0,76 μmol de agliconas por g de amostra em base seca, respectivamente. Ainda, observaram que a relação entre o teor de agliconas nos cotilédones hidratados e teor de agliconas nos cotilédones antes do tratamento hidrotérmico foi maior quando empregou-se o menor volume de água. Parte das agliconas presentes nos cotilédones da safra de 2005 foram lixiviadas para a água de hidratação. Além disso, segundo os autores, no ensaio em que foi utilizada a menor proporção de água não foi possível quantificar as isoflavonas na água residual de hidratação, pois quase toda a água foi absorvida pelos cotilédones.

O efeito da hidratação dos grãos de soja na proporção de 1:1,5 (g:g, soja:solução tampão citrato-fosfato 0,05 mol L^{-1} a pH 6) em diferentes combinações de tempo (3, 4, 6, 8 e 9 h) e temperatura (41, 45, 55, 65 e 69 °C ± 1 °C) sobre a

máxima conversão de isoflavonas β-glicosídeos em agliconas na cultivar de soja BRS 257 foi investigado por Lima e Ida (2014) utilizando a metodologia de superfície de resposta. Verificaram que a máxima conversão em agliconas ocorreu a 55 °C ± 1 °C por 6 h de hidratação. Nesta condição, os grãos de soja tratados hidrotermicamente apresentaram um conteúdo mínimo de β-glicosídeos (0,18 μmol de β-glicosídeos por g de matéria seca e integral), máximo teor de agliconas (1,22 μmol de agliconas por g de matéria seca e integral) e a atividade de β-glicosidase a 55 °C foi praticamente nula. Ressalta-se que os grãos de soja utilizados para a hidratação continham, inicialmente, 0,11 μmol de agliconas por g de matéria seca e integral, o que indicou um aumento no conteúdo de agliconas de aproximadamente 11 vezes nos grãos hidratados na condição ótima em relação aos grãos iniciais.

Há poucos estudos relatando o efeito de diferentes tempos e temperaturas de hidratação da soja sobre a lixiviação e degradação dos oligossacarídeos. Bianchi, Silva e Campos (1983) hidrataram a cultivar de soja Santa Rosa em água na proporção de 1:3 e 1:10 (g:mL, soja:água) por 3, 6, 12, 18 e 24 h a 25 °C. Observaram que o tempo e proporção de água de hidratação não influenciaram significativamente o conteúdo dos açúcares sacarose (4,35 g por 100 g de matéria seca), rafinose (0,74 g por 100 g de matéria seca) e estaquiose (3,53 g por 100 g de matéria seca) dos grãos hidratados em relação ao grão bruto inicial. A hidratação da soja na proporção de 1:10 (g:mL, soja:água destilada) por 4, 8, 12 e 16 h a 25 °C foi investigada por Mulimani, Thippeswamy e Ramalingam (1997) e verificaram que o aumento do tempo de hidratação diminuiu o conteúdo de rafinose e estaquiose da soja hidratada. A hidratação da soja por 16 h reduziu o conteúdo de estaquiose e rafinose de 44,8 e 80,3 %, respectivamente, em relação ao grão inicial. Essa redução não foi discutida ou justificada pelos autores, mas foi provavelmente devida à lixiviação destes oligossarídeos ou hidrólise por meio da ação das α-galactosidases endógenas da soja ou devido ambos os fatores.

Neste contexto, há evidências de que determinadas condições de hidratação dos grãos de soja podem contribuir para o preparo de produtos de soja com maior quantidade de isoflavonas agliconas e com a redução no conteúdo de oligossacarídeos. Contudo, ressaltam-se a importância de investigar os efeitos do tempo e temperatura de hidratação da soja sobre as propriedades dos grãos, tais como absorção de água e textura, bem como sobre a lixiviação de proteínas

solúveis, conversão ou degradação de isoflavonas e oligossacarídeos, uma vez que diversos produtos de soja são elaborados a partir de uma hidratação prévia dos grãos.

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4 MATERIAL E MÉTODOS

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

ARTIGO CIENTÍFICO 1:

LIMA, F. S.; KUROZAWA, L. E.; IDA, E. I. The effects of soybean soaking on grain properties and isoflavones loss. **LWT- Food Science and Technology**, v. 59, p. 1274-1282, 2014.

ARTIGO CIENTÍFICO 2:

LIMA, F. S.; HANDA, C. L.; FERNANDES, M. S.; RODRIGUES, D.; KUROZAWA, L. E.; IDA, E. I. Soybean soaking changes the contents of different forms of isoflavones. Submetido para publicação - **Food Chemistry**.

ARTIGO CIENTÍFICO 3:

LIMA, F. S.; HANDA, C. L.; FERNANDES, M. S.; RODRIGUES, D.; KUROZAWA, L. E.; IDA, E. I. Soybean soaking changes the contents of sugars and activity of α-galactosidases. Submetido para publicação - **LWT-Food Science and Technology**.

5 RESULTADOS E DISCUSSÃO

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5.1 ARTIGO CIENTÍFICO 1

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5.1 ARTIGO CIENTÍFICO 1

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The effects of soybean soaking on grain properties and isoflavones loss

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ABSTRACT

The aim of this study was to investigate the effects of soybean soaking on grain properties and isoflavone loss in order to find suitable conditions for this step of soybean processing. A moisture content of 120% (dry basis) was achieved by soaking the soybeans at 55 °C and 70 °C for 3 and 2 h, respectively. Soybeans soaked at temperatures above 25 °C showed no significant difference ($p > 0.05$) in hardness after 1 h of soaking. The contents of total isoflavones and soluble proteins were better preserved at soaking temperatures of 25 °C and 40 °C. The β-glucosidase activity and contents of aglycone and β-glucoside isoflavones were closely related. Soybeans soaked at 55 °C for 5 h had a 6-fold higher aglycone isoflavone content than did whole soybeans (81.4 μg aglycones g^{-1}) without considerable impairment of the aforementioned characteristics; soaking under this condition is therefore recommended before subsequent processing. This work helps to estimate the loss in soluble protein content and the loss and degradation of isoflavones during soybean soaking at different temperatures.

1. Introduction

Soybean soaking is a traditional process that is mainly used to soften the grains and to facilitate their subsequent cooking. In the Illinois process that is used in the production of soymilk, the soybean grains are soaked for 12 h at room temperature (Nelson, Steinberg, & Wei, 1976). Texture changes in the grains result from water absorption, which is affected by the soaking time and temperature (Pan & Tangratanavalee, 2003), and continue throughout the cooking step, with the added effect of the cooking temperature. Lengthy soaking time until reaching equilibrium moisture content in the soaked soybeans is not necessary to promote additional softening of the grains because it does not produce further improvements in cooking rate or cooking quality of the soybeans (Wang, Swain, Hesseltine, & Heath, 1979).

Processes using high temperatures and reduced soaking times can be applied in the preparation of various soy products, such as soymilk, tofu, and tempeh. Soaking temperatures within the range of 40–60 °C can decrease the lipoxygenase activity and improve the digestibility of soybean proteins (Shin, Kim, & Kim, 2013). However, high soaking temperatures increase the mass transfer rate by diffusion, resulting in a significant loss of solids, such as proteins and isoflavones, into the aqueous medium (Pan & Tangratanavalee, 2003).

High levels of protein denaturation may impair the solubility of the soy protein, whose denaturation onset temperatures for β-conglycinin (7S) and glycinin (11S) were observed to be 65–75 °C and 85–95 °C, respectively (Kitabatake, Tahara, & Doi, 1990). The technological properties of soy proteins are dependent on their solubility (Shin et al., 2013). Thus, proper times and temperatures for soybean soaking should be considered to minimise protein insolubilisation.

Soybean isoflavones are bioactive compounds that may benefit human health and can be affected during processing. Isoflavones are divided into four groups: β-glucosides (daidzin, genistin, and glycitin), 6″-*O*-acetylglucosides, 6″-*O*-malonylglucosides and aglycones (daidzein, genistein, and glycitein). Soybean isoflavones have been widely investigated, particularly aglycones, because of their ability to reduce the incidence of breast cancer (Wada et al., 2013) and other diseases. Thus, there is a growing interest in preparing soy products with higher amounts of aglycones, and soybean hydrothermal treatment has been used to favour endogenous β-glucosidase activity in the conversion of β-glucoside isoflavones to aglycones (Lima & Ida, 2014). Therefore, the formation of aglycone isoflavones must be stimulated during soybean soaking without impairing the other aforementioned properties. In this context, the aim of this study was to investigate the effects of soybean soaking time and temperature on the moisture, hardness, soluble proteins, β-glucosidase activity, and isoflavones of the soaked grains and/or

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of the residual solution in order to find a suitable condition for this step of soybean processing.

2. Materials and methods

2.1. Materials and standards

Isoflavone standard solutions were prepared from 6″-*O*-acetylglucosides and 6″-*O*-malonylglucosides (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as well as from β-glucosides and aglycones (Sigma–Aldrich Co., St. Louis, MO, USA). For the soluble protein assay, bovine serum albumin (fraction V, Sigma–Aldrich Co., St. Louis, MO, USA) was used as the protein standard. The β-glucosidase activity was measured using *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) (Sigma–Aldrich Co. St. Louis, MO, USA) as the substrate, and the chromogenic reagent *p*-nitrophenol (*p*-NP) (Sigma–Aldrich Co., St. Louis, MO, USA) was used to construct a calibration curve. All the reagents used in the analysis were of analytical grade or liquid chromatography grade.

2.2. Hydrothermal treatment

Approximately 50 g of cleaned and unbroken soybeans [*Glycine max* (L.) Merr.], lipoxygenase-null cultivar BRS 257 (Empresa Brasileira de Pesquisa Agropecuária, Londrina/Paraná, Brazil) from the crop year 2013 was used in a 1:1.5 (g:g, soybean:deionised water) ratio for each soaking assay. This soybean:deionised water ratio was used on the basis of a preliminary study and literature data (Góes-Favoni, Carrão-Panizzi, & Beléia, 2010; Gulati, Chakrabarti, Singh, Duvuuri, & Banerjee, 2010). Glass bottles containing water to soak the whole soybeans were pre-incubated in a thermostatically controlled water bath (Marconi, MA 159, Piracicaba, Brazil) at the required soaking temperature (25, 40, 55, and 70 °C) until reaching thermal equilibrium before the addition of the grains. In addition, the soybeans were also soaked in a 0.1 mol L^{-1} phosphate-citrate buffer solution at pH 6 at a soaking temperature that promoted the highest content of aglycones in the soaked soybean to provide optimal conditions for the β-glucosidase enzyme (Matsuura, Obata, & Fukushima, 1989; Sutil et al., 2008). After soaking for regular time intervals (0, 0.5, 1, 2, 3, 4, 5, 6, and 7 h) at each temperature, the flasks were successively withdrawn from the water baths, and then the soaked soybeans and drained solution were immediately cooled in an ice bath until they reached 25 °C. The assays were performed in closed systems to avoid water evaporation into the environment. The temperature of the soaking medium was continuously monitored with a high accuracy (±0.2 °C) mercury-in-glass thermometer (Incoterm®, Porto Alegre, Brazil) and was maintained at the required level (±1 °C) throughout the soaking period. At the end of each assay, the soaked soybeans were superficially dried at 30 °C for 10 min in a vacuum oven before being weighed. The mass of the residual solution (water not absorbed by the soybeans and compounds leached) was obtained by calculating the difference between the total mass of the system (the sum of the whole soybeans and the mass of the soaking medium) and the mass of the grains superficially dried. A portion of each sample was reserved for the analysis of soluble proteins (residual solution), hardness (soaked soybeans), and moisture content (soaked soybeans), and the remaining samples were frozen, lyophilised (Christ Alpha 2-4 LD plus, Osterode am Harz, Germany), milled (Ika A11 basic, St. Louis, MO, USA) and stored at −22 °C until further analysis.

2.3. Moisture content determination

The moisture content was determined using 2 g of soaked soybeans for each soaking assay. The samples were dried in an oven at 105 °C until reaching a constant weight (AOAC, 2002). The results were expressed as g of H_2O/100 g dry matter (%, dry basis).

2.4. Hardness measurement

The hardness of the soaked soybeans was measured by a penetration test using a TA-XT2i Texture Analyser (Stable Micro Systems, Surrey, UK) equipped with an HDP/MTP multiple pea rig. In this test, 18 soaked soybeans were simultaneously tested by applying a force in the direction perpendicular to the hilum. The settings used were as follows: pre-test speed (2 mm s^{-1}), test speed (2 mm s^{-1}), penetration distance (3.5 mm), and force (0.05 N). The maximum force (N) necessary to penetrate the soaked soybeans (with tegument) was determined as an indicator of hardness.

2.5. Soluble protein content

The soluble protein contents in the soaking residual solution and the soaked soybeans were determined according to the Bradford method (Bradford, 1976) using bovine serum albumin as the protein standard (fraction V; 25–250 μg mL^{-1}). The residual solution from each soaking assay was filtered (nylon membrane, 0.45 μm; Millipore, Billerica, MA, USA) before the protein assay, and when necessary, the samples were diluted with deionised water. Soluble protein extraction from the soaked soybeans was carried out using 0.2 g of lyophilised sample and 10 mL of deionised water, which was kept under continuous rotary agitation (305 rpm) for 1 h at 25 °C. Then, the mixture was centrifuged (4 °C, 8200 × *g*; Centrifuge 5804R e Eppendorf, Hamburg, Germany), and the supernatant was diluted with deionised water and filtered before analysis. The results were expressed as mg of soluble protein leached into the soaking medium or g of soluble protein retained in the soaked soybeans.

2.6. β-Glucosidase activity

The extraction of the β-D-glucoside glucohydrolase (EC 3.2.1.21) enzyme was carried out according to Carrão-Panizzi and Bordignon (2000) using 0.4 g of lyophilised sample and 5 mL of extraction solution. The enzyme activity was assessed using the method of Matsuura and Obata (1993), with minor modifications. The substrate *p*-NPG was diluted with a 0.1 mol L^{-1} phosphate-citrate buffer solution at pH 6. The calibration curve was prepared by varying the concentration of *p*-NP from 0.04 to 0.32 μmol in a 5 mL total reaction volume. One activity unit (AU) was defined as the quantity of enzyme necessary to release 1 μmol of *p*-NP min^{-1} under the experimental conditions.

2.7. Determination of isoflavones

Prior to the analysis of the isoflavones, the samples were defatted with hexane in a 1:10 (g:mL, sample:hexane) ratio by continuous rotary agitation for 1 h at 25 °C, followed by vacuum filtration. The residual solution was only lyophilised. For the isoflavone extraction from the soaked soybeans, 0.3 g of lyophilised sample was used in 6 mL of extraction solution containing ultrapure water, acetone, and ethanol in a 1:1:1 (mL:mL:mL) ratio, as described by Yoshiara, Madeira, Delaroza, Silva, and Ida (2012). The isoflavone extraction from the soaking residual solutions was performed separately with 0.2 g of lyophilised sample in 1.5–2 mL of extraction solution. The separation and quantification of the isoflavones were carried out according to Handa, Couto, Vicensoti, Georgetti, and Ida (2014). External calibrations were prepared from standard solutions (0.1, 0.05, 0.01, 0.005, 0.001, and 0.0005 mg mL^{-1}) of each isoflavone form for quantification. The

isoflavone contents in the different groups were reported after normalising (Eq. (1)) the molecular weight differences between the glucosylated forms and the corresponding aglycones.

$$W_N = \frac{W_{NN} \times MW_{aglycone}}{MW_{glucosylated}} \quad (1)$$

where W_N, W_{NN}, $MW_{aglycone}$, and $MW_{glucosylated}$ are the normalised mass (mg) of the glucosylated isoflavone, the non-normalised mass (mg) of the glucosylated isoflavone, the molecular weight (g mol^{-1}) of the corresponding aglycone isoflavones, and the molecular weight (g mol^{-1}) of the glucosylated isoflavone, respectively. The molecular weights (g mol^{-1}) were as follows: malonyldaidzin = 502.42, malonylglycitin = 532.45, malonylgenistin = 518.42, daidzin = 416.38, glycitin = 446.40, genistin = 432.38, daidzein = 254.24, glycitein = 284.26, genistein = 270.24.

The content of each isoflavone group was expressed in mass units to eliminate the effects of the concentration or dilution of these components due to differences in the mass transfer rate between the soybeans and the soaking medium throughout the soaking process at different temperatures. For the soaked soybeans, the results were expressed as the isoflavone masses retained in the soaked soybeans (dry basis and full fat) in mg of β-glucosides, mg of malonylglucosides, and mg of aglycones. For the residual solutions, the results were expressed as the isoflavone masses leached into the soaking medium in mg of β-glucosides, mg of malonylglucosides, and mg of aglycones. The mass balance of isoflavones in the systems was expressed as the mg of total isoflavones (the sum of the mass of total isoflavones retained in the soaked soybeans and the mass of total isoflavones leached into the soaking medium). In addition, the % mass retention of total isoflavones in the soaked soybeans (RI_{SS}), the % mass loss of total isoflavones into the soaking medium (LI_{RS}), and the % mass degradation of total isoflavones in the systems (DI_{SY}) were estimated on the basis of the mass balance of total isoflavones after normalisation (Eq. (1)).

2.8. Statistical analysis

The soaking assays and analyses were performed in duplicate. Thus, the data are presented as the mean ($n = 4$) ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test ($\alpha = 0.05$) was carried out using the software program Statistic 10.0 (StatSoft, Tulsa, OK, USA). Values of $p < 0.05$ were considered statistically significant. The data were previously tested for homogeneity of variance (Hartley's test) and distribution of within-cell residuals.

3. Results and discussion

3.1. Effects of soybean soaking on moisture content, hardness and soluble proteins

During soybean soaking at different temperatures (25, 40, 55, and 70°C ± 1 °C), it was observed that the water absorption rate by the grains was faster ($p < 0.05$) during the first 3 h of soaking, particularly at 55 °C and 70 °C, followed by asymptotic behaviour after 3 h (Fig. 1a). Likewise, Gowen, Abu-Ghannam, Frias, and Oliveira (2007) found that the maximum water absorption by the soaked soybeans in a 1:50 (g:g soybean:distilled water) ratio at 60 °C was achieved after 2 h of soaking with a moisture content of 122% (dry basis). High soaking temperatures (>40 °C) increase the tegument permeability, water diffusion, and mass transfer. According to Pan and Tangratanavalee (2003), the equilibrium moisture content of soaked soybeans in a 1:10 (g:g, soybean:distilled water) ratio at 40 °C was reached after 7 h of soaking, which was a consequence of the low temperature used. In the present study, the leaching of water soluble compounds (proteins, carbohydrates, and others) from the soaked soybeans at 70 °C was most likely responsible for the decreasing moisture content of the grains after 2 h of soaking (Fig. 1a), in agreement with previous studies (Gowen et al., 2007; Pan & Tangratanavalee, 2003). The diffusion process throughout soaking is influenced by the concentration gradient between the soaked grains and the soaking medium.

When the soybeans were soaked at 25 °C, there was no significant difference ($p > 0.05$) in hardness after 2 h of soaking. Soybeans soaked at temperatures above 25 °C showed no significant difference ($p > 0.05$) in hardness after 1 h of soaking at a constant temperature (Fig. 1b). Soybean soaking is a traditional process that is mainly used to promote the softening of the grains and to facilitate their posterior cooking, as texture changes in the grains result from water absorption during soaking (Pan & Tangratanavalee, 2003). Data on the hardness of soaked and cooked soybeans as a function of the processing time and temperature have not been described in the literature. During the initial stages of soaking, texture changes are a function of the soaking time. Once the soaking process reaches equilibrium, the changes in texture become minimal and do not depend on the soaking time or temperature (Abu-Ghannam, 1998). However, the minimum value recommended for the moisture content of the soybeans before grinding processes has been described as 120% in dry basis (Pan & Tangratanavalee, 2003). Thus, soybeans soaked at 25 °C would require a soaking time of over 7 h (Fig. 1a); this observation is consistent with previous studies (Pan & Tangratanavalee, 2003; Wardhani, Vázquez, & Pandiella, 2008). On the other hand, an adequate moisture content in the soaked soybeans at 55 °C and 70 °C was achieved after 3 and 2 h of soaking, respectively. Thus, soaking temperatures above 40 °C may be used to reduce the time needed for soybean soaking and cooking steps, in addition to improving the digestibility of the soybean proteins (Shin et al., 2013). However, high soaking temperatures may result in a significant loss of solids into the soaking medium (Pan & Tangratanavalee, 2003). Therefore, traditional processes of soybean soaking, which involve lengthy times beyond what is necessary to achieve this moisture, are not needed to promote additional softening of the grains because no further improvements in cooking rate or cooking quality of the beans will be achieved, as described by Wang et al. (1979). The soaking of the soybeans in buffer solution at pH 6 negatively affected the hardness of the soaked grains (Fig. 1b), which had a lower water absorption rate (Fig. 1a) than did the beans soaked at 55 °C with deionised water, possibly due to excessive migration of soluble solids into the aqueous medium and the acidic buffer. Soybean soaking in alkaline medium (pH > 8) has been shown to improve the dissolution of pectic substances in the middle lamella of the cells and promote protein destabilisation, with a reduction in the hard-to-cook phenomenon (del Valle, Cottrell, Jackman, & Stanley, 1992). However, for an industrial application, such as the preparation of soymilk, where the desired pH is near neutral, a neutralisation step would be required before packaging, increasing processing time and costs.

The soluble protein content retained in the soaked soybeans at 55 °C was significantly reduced ($p < 0.05$) after 2 h of hydration, followed by an increase after 5 h of soaking, and finally remained stable ($p > 0.05$) until the end of the process (Fig. 1c). Similar behaviour was observed in the grains soaked at 70 °C, although the soluble protein content retained in the soaked soybeans had already been reduced during the first hour of soaking. These observations indicate that increased leaching of soluble proteins into the soaking medium occurred at higher soaking temperatures (Fig. 1d). In contrast, the excessive diffusion of solids into the soaking medium must have been impeded, in part, due to the use of

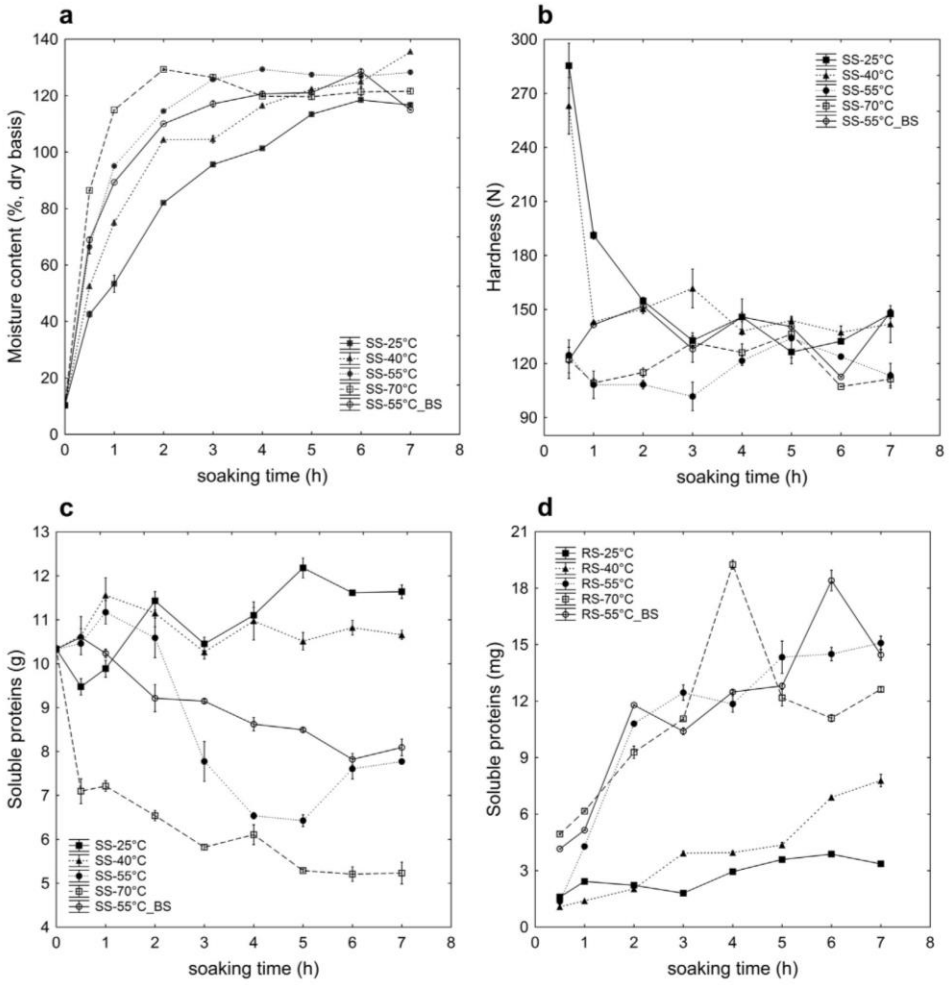

Fig. 1. Changes in moisture, hardness, and soluble proteins throughout the soybean soaking period at different temperatures. BS = buffer solution. (a) Moisture content of the soaked soybeans, (b) Hardness of the soaked soybeans, (c) Soluble protein content retained by the soaked soybeans, (d) Soluble protein content leached into the soaking medium. The error bars are the standard deviation ($\alpha = 0.05$; $n = 4$). The solid lines are provided as a visual guide only.

a low ratio of soybeans to aqueous medium (1:1.5, g:g), where the concentration gradient between the soaked grains and the soaking medium was being established throughout the processing time. Nevertheless, the total content of soluble proteins (retained and leached) in the systems at 55 °C and 70 °C was lower ($p < 0.05$) after 1 h and 0.5 h of soaking, respectively, compared to the whole soybeans. High levels of protein denaturation may impair the solubility of the soy protein, whose denaturation onset temperatures for β-conglycinin (7S) and glycinin (11S) were observed to be 65–75 °C and 85–95 °C, respectively (Kitabatake et al., 1990; Malaypally & Ismail, 2010). The level of protein denaturation may be related to the processing temperature and time. Moreover, the stability of proteins is different in the soaking medium than it is in the protein bodies within the soybeans. In this study, there was an increase from 11 to 19% in the total content of soluble proteins in the systems at 25 °C after 4 h of soaking. The soaking of the grains at this temperature most likely favoured the subsequent protein extraction (Vishwanathan, Singh, & Subramanian, 2011). The soybeans soaked at 25 °C and 40 °C had the lowest rates of protein leaching, but more time would be required for the soaking of the grains at 25 °C to prepare for subsequent grinding processes (Pan & Tangratanavalee, 2003). Lengthy soaking times may lead to excessive loss of solids into the soaking medium, which is usually discarded after this process, affecting the preparation and nutritional value of soy products, such as soy-bulgur (Bayram, Kaya, & Öner, 2004).

3.2. *Effects of soybean soaking on the different isoflavone groups and β-glucosidase activity*

The whole soybeans (starting material for the soaking assays) showed the following contents of different isoflavone forms: 524.7 ± 2.8 μg of β-glucosides g^{-1} (223.1 ± 0.9 μg of daidzin g^{-1}, 34.3 ± 2.6 μg of glycitin g^{-1}, and 267.3 ± 0.6 μg of genistin g^{-1}), 1640.7 ± 5.0 μg of malonylglucosides g^{-1} (575.4 ± 1.8 μg of malonyldaidzin g^{-1}, 175.5 ± 4.4 μg of malonylglycitin g^{-1}, and 889.8 ± 1.4 μg of malonylgenistin), and 81.4 ± 3.4 μg of aglycones g^{-1} (43.5 ± 3.3 μg of daidzein g^{-1} and 37.9 ± 1.1 μg of genistein g^{-1}).

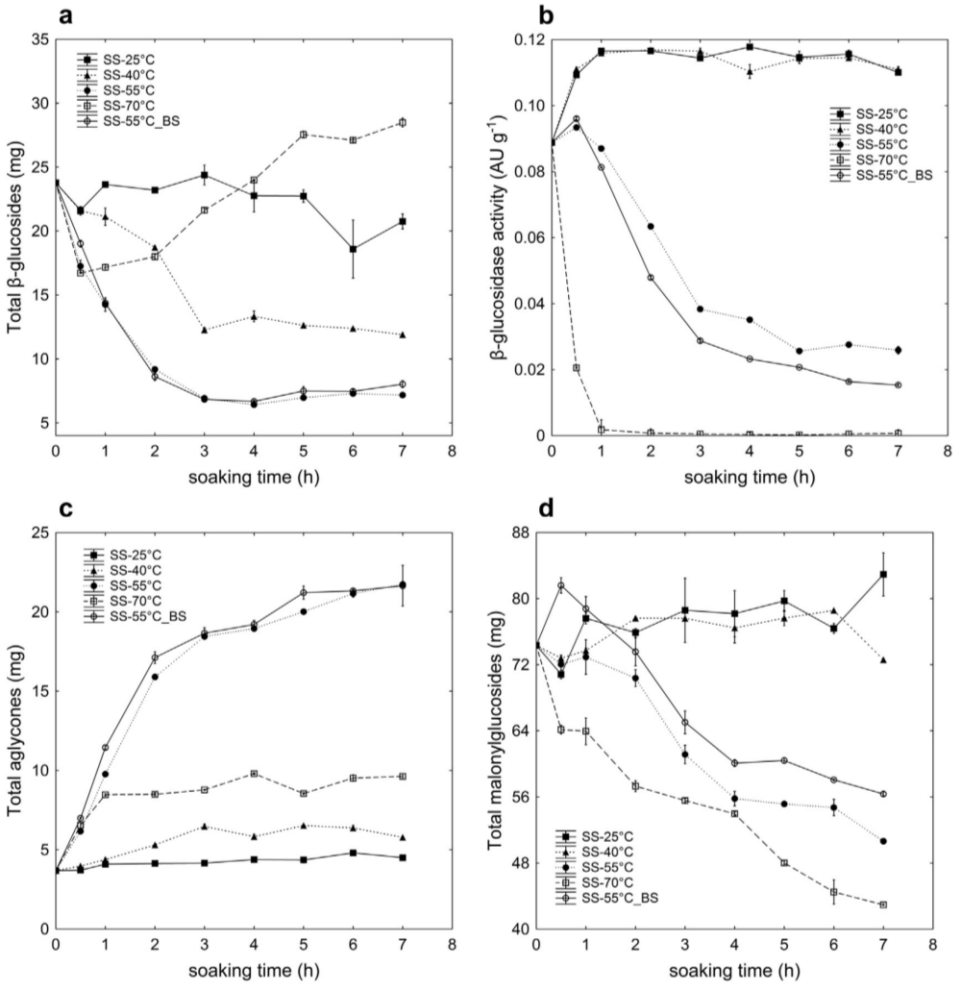

Fig. 2. Contents of β-glucoside, aglycone, and malonylglucoside isoflavones and β-glucosidase activity in the soaked soybeans at different temperatures. BS = buffer solution. (a) Total β-glucosides (sum of the daidzin, glycitin, and genistin), (b) β-glucosidase activity (AU = activity unit), (c) Total aglycones (sum of the daidzein and genistein), (d) Total malonylglucosides (sum of malonyldaidzin, malonylglycitin, and malonylgenistin). The error bars are the standard deviation ($\alpha = 0.05$; $n = 4$). The solid lines are provided as a visual guide only.

Altogether, 2246.8 ± 6.7 μg of total isoflavones g^{-1} was quantified, comprising 23.4% of β-glucosides, 3.6% of aglycones, and 73% of malonylglucosides. However, acetylglucoside isoflavones and glycitein were not detected by UPLC®, as reported for most soybean cultivars (Britz, Schomburg, & Kenworthy, 2011).

Compared with the β-glucoside content in the soybeans soaked at 25° C, there was a significant difference ($p < 0.05$) only in the grains soaked for 6 h. On the other hand, the soybeans soaked at 40 °C displayed reduced β-glucoside content during the first 3 h of soaking, while after this period there was no significant difference ($p > 0.05$). Regarding the grains soaked at 55 °C (with deionised water or buffer solution), the same behaviour was observed throughout the soaking time (Fig. 2a). At this temperature, it was also observed that after 5 h of soaking, there was no significant difference in the aglycone content of the soaked soybeans; this is in agreement with the study conducted by Lima and Ida (2014) optimising soybean hydrothermal treatment for the formation of aglycone isoflavones. In contrast, there was no substantial increase in the aglycone contents of the soybeans soaked at 25, 40 or 70 °C. Only the soybeans soaked at 70 °C showed an increase in β-glucoside content. These observations were closely related to the β-glucosidase activity (Fig. 2b) and the aglycone (Fig. 2c) and malonylglucoside contents (Fig. 2d). When the soybeans were soaked at 55 °C, there was a reduction in the β-glucoside content and β-glucosidase activity, whereas the aglycone content increased. This indicates that the β-glucosidase in the soybeans was responsible for the conversion of the β-glucosides to aglycones during the soaking of the grains. The soybeans were also soaked in a 0.1 mol L^{-1} phosphate-citrate buffer solution (pH 6) at 55 °C to evaluate the possible increase in aglycones in the soaked soybeans because the optimal pH range for β-glucosidase is between 5 and 7 (Matsuura et al., 1989; Sutil et al., 2008). Lima and Ida (2014) utilised a buffer solution at pH 6 as a medium for soybean soaking for the conversion of β-glucoside isoflavones to aglycones, but this condition was unnecessary for this purpose throughout the time period evaluated herein (Fig. 2c). The aglycone content in the soybeans

soaked in deionised water at 55 °C for 5 h was 20 mg (or 470.1 μg of aglycones g^{-1} of dry hydrothermally treated soybean), which comprised 50.3% daidzein and 49.7% genistein. Glycitein was not detectable, confirming the complete absence described (Britz et al., 2011) in most soybean cultivars and the apparently lower affinity of β-glucosidase toward glycitin (Ismail & Hayes, 2005). Wardhani, Vαzquez, and Pandiella (2008) verified that the maximum β-glucosidase activity in soaked soybeans occurred after 1 h of soaking at 50 °C, which was followed by a reduction in the enzymatic activity of the soaked grains, as likewise observed in this study. The β-glucosidase enzyme can be inhibited by its reaction products, such as glucose and aglycones (Esen, 2003), which justifies its low activity in the soybeans soaked at 55 °C (with deionised water or buffer solution) with increasing aglycone content (Fig. 2b, c). In addition, under the same soaking conditions (55 °C for 5 h, deionised water), the sum of the contents of each group of isoflavones (Fig. 2a, c, d) corresponded to a total isoflavone content of 82.1 mg (or 1928.8 μg of total isoflavones g^{-1} of dry hydrothermally treated soybean), which comprised 8.5% β-glucosides, 24.4% aglycones, and 67.1% malonylglucosides. Among the isoflavones, aglycones have been highlighted for their high bioactivity, including their high antioxidant and anticarcinogenic activities (Arora, Nair, & Strasburg, 1998; Wada et al., 2013). Thus, soaking soybeans at 55 °C in deionised water may be advantageous for developing soy products with greater amounts of aglycones, as these soybeans were found to possess a 6-fold higher aglycone isoflavone content than that of whole soybeans (81.4 μg aglycones g^{-1}). The hardness and soluble protein content in the soybeans soaked under this condition were not adversely affected, as previously discussed. In particular, the soybeans soaked at 70 °C showed an increase in β-glucoside content up to 5 h of soaking (Fig. 2a) and the inactivation of the β-glucosidase enzyme after the first hour of soaking (Fig. 2b), whereas the content of aglycone isoflavones was nearly constant after 1 h of soaking (Fig. 2c) and the content of malonylglucoside isoflavones decreased considerably (Fig. 2d). The thermal instability of the malonylglucosides suggests that these isoflavones were decarboxylated to their corresponding β-glucoside forms (Kudou et al., 1991). The increase in the content of aglycones in the first hour of soaking was most likely due to the β-glucosidase activity converting the β-glucoside isoflavones to aglycones, as malonylglucosides are poor substrates for this enzyme (Ismail & Hayes, 2005). It also seems that once formed, the aglycones exhibited good thermal stability (Fig. 2c). This is interesting to consider for subsequent processes under more drastic conditions, such as the cooking of soaked soybeans.

The isoflavone content leached into the soaking medium was also analysed, and it was demonstrated that a higher leaching of isoflavones, mainly β-glucosides and malonylglucosides, occurred with an increase in the soaking temperature (Fig. 3 a–c). The soybeans soaked at 25 °C and 40 °C showed no substantial leaching of isoflavones throughout the soaking period. The chemical structural differences in soybean isoflavones affect their solubility in aqueous media, which is attributed mainly to hydroxyl groups. According to Jankowiak, Trifunovic, Boom, and Goot (2014), the aglycone isoflavones present in okara were less soluble in water than in slightly less polar solvents, and therefore, aglycone loss by leaching was more difficult. Góes-Favoni et al. (2010) described that soybean hydrothermal treatment at 50 °C for 12 h resulted in a reduction in the content of malonylglucosides due to their conversion to β-glucosides and to leaching into the soaking medium. These authors also verified that the soybean cotyledons that were soaked in a reduced volume of water (1:1.2, g:g; cotyledons to water deionised) resulted in less migration of isoflavones into the soaking medium. In this study, the residual solutions resulting from soaking at 55 °C showed higher contents of aglycones than did the solutions at 25, 40, and 70 °C. This clearly demonstrates that higher amounts of aglycones were formed in the soybeans soaked at 55 °C than at any other condition studied herein because both the soaked soybeans and the residual solution showed the highest amounts of aglycones.

3.3. Mass balance of isoflavones throughout soybean soaking

The percentages of mass retention of total isoflavones by the soaked soybean (RI_{SS}, Eq. (2)), the mass loss of total isoflavones into the soaking medium (LI_{RS}, Eq. (3)), and the mass degradation of total isoflavones in the systems (DI_{SY}, Eq. (4)) were expressed (Table 1) on the basis of the mass balance of total isoflavones (Eq. (5)) of the systems in relation to the mass of isoflavones from the whole soybeans before soaking. It is noteworthy that DI_{SY} was considered only when there was a significant difference ($p < 0.05$) between the contents of total isoflavones in the system compared to that in the whole soybeans. Likewise, RI_{SS} was considered as 100% when the content of total isoflavones in the soaked grains was not significantly different ($p > 0.05$) in relation to the raw soybean. In addition, the masses of the soaked soybeans throughout the soaking were also reported (Table 1).

$$RI_{SS} = \frac{\dot{W}_{SS} \times 100}{\dot{W}_{WS}} \tag{2}$$

$$LI_{RS} = \frac{\dot{W}_{RS} \times 100}{\dot{W}_{WS}} \tag{3}$$

$$DI_{SY} = 100 - RI_{SS} - LI_{RS} \tag{4}$$

$$\dot{W}_{WS} = \dot{W}_{SS} + \dot{W}_{RS} \tag{5}$$

where $\dot{W}_{WS}$, $\dot{W}_{SS}$, and $\dot{W}_{RS}$ express the mass (mg) of total isoflavones in the whole soybeans, the soaked soybeans and the residual solution, respectively.

When the soybeans were soaked at 25 °C, there was no significant difference ($p > 0.05$) in the content of total isoflavones of the systems in relation to that in the whole soybeans, indicating that there was no degradation of isoflavones (Table 1). Moreover, almost no leaching of isoflavones ($LI_{RS} < 1\%$) was observed at this temperature. The soybeans soaked at 40 °C had low leaching of total isoflavones ($LI_{RS} < 1.5\%$), and the DI_{SY} was estimated from 3 h of soaking. However, Wang and Murphy (1996), investigating the effects of processing techniques on the distribution of isoflavones, verified that the tempeh made from soybean grains soaked in a 1:3.3 (g:g, soybean:tap water) ratio at 24 °C for 12 h showed a 12% mass loss of total isoflavones into the soaking medium. The low leaching of isoflavones at 25 °C and 40 °C found herein must have been impeded in part because of the low ratio of soybeans to soaking solution (1:1.5, soybean:soaking medium). Both the soaking processes at 55 °C and 70 °C resulted in higher degradation (DI_{SY} up to 15%) and leaching (LI_{RS} up to 12%) of isoflavones. In contrast, Wang and Murphy (1996) prepared tofu from soybean grains that were soaked in tap water at 25 °C for 13 h, followed by heating at 95 °C for 7 min. They found that the total isoflavone recovery in tofu processing was not different from the total amount of isoflavones in the whole soybean (starting material). These results indicate that the isoflavones were not degraded but were instead fractionated into the okara and whey. Nevertheless, the results of this study demonstrated that the degradation of isoflavones may occur at temperatures from 40 °C (Table 1). Various phenomena occur simultaneously during soybean hydrothermal treatment, such as the leaching of isoflavones into the soaking medium (Lima & Ida, 2014), the thermal decarboxylation of

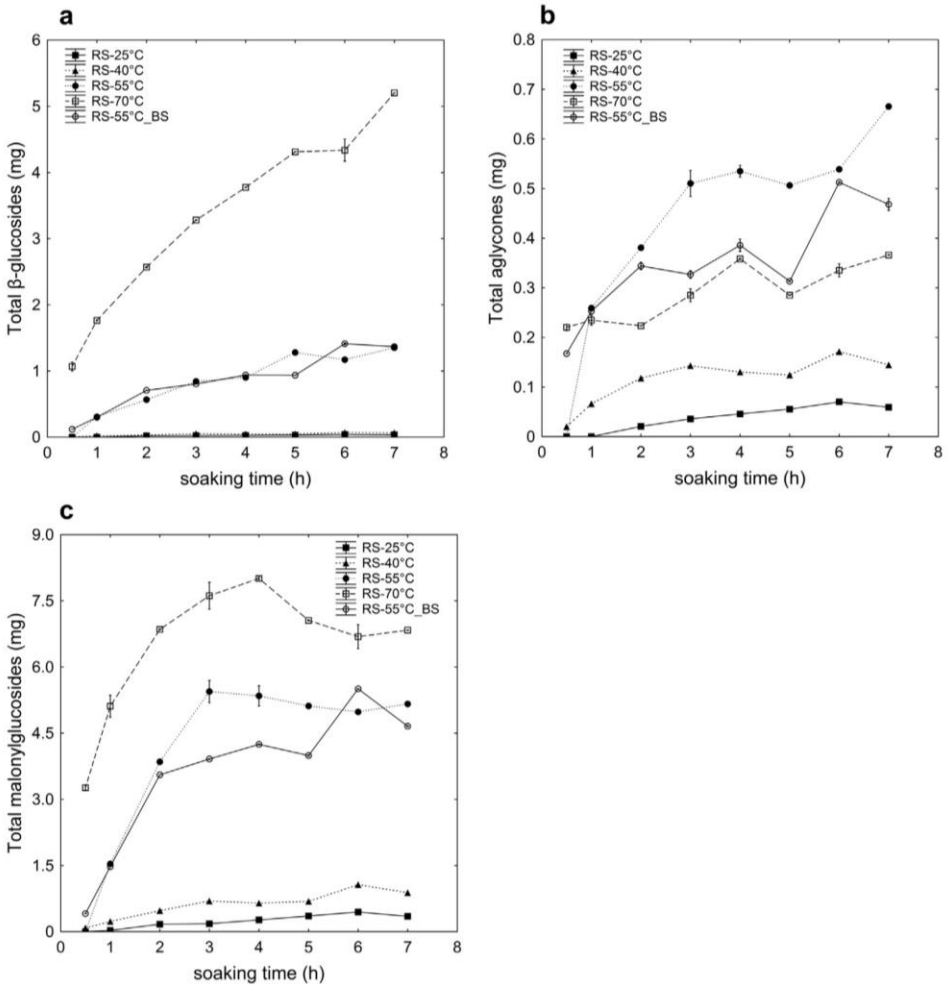

Fig. 3. Content of β-glucoside, aglycone, and malonylglucoside isoflavones leached into the soaking medium from soybeans soaked at different temperatures. BS = buffer solution. (a) Total β-glucosides (sum of daidzin, glycitin, and genistin), (b) Total aglycones (sum of daidzein and genistein), (c) Total malonylglucosides (sum of malonyldaidzin, malonylglycitin, and malonylgenistin). The error bars are the standard deviation ($\alpha = 0.05$; $n = 4$). The solid lines are provided as a visual guide only.

malonylglucoside isoflavones to β-glucosides (Kudou et al., 1991), the conversion of β-glucoside isoflavones to aglycones (Góes-Favoni et al., 2010; Lima & Ida, 2014), and the degradation of these bioactive compounds (Wardhani et al., 2008). Niamnuy, Nachaisin, Poomsa-ad, and Devahastin (2012) evaluated the rate constants of degradation and conversion of all isoflavones and verified that isoflavones were more susceptible to inter-conversion than they were to thermal and oxidative degradation during the infrared drying of soybean (50, 70, 130, and 150 °C). Interestingly, a higher degradation of isoflavones occurred at 55 °C (with deionised water) than at the other temperatures used herein. However, an increase in the content of total isoflavones was observed after soaking at 55° C (with buffer solution) for 1 h (Table 1) because of the formation of malonylglycosides (Fig. 2d). It is probable that isoflavones were not formed at 55 °C, but their interactions with soybean proteins may have led to an underestimation of the isoflavone contents of the samples that underwent mild heating (<55 °C) (Malaypally & Ismail, 2010; Nufer, Ismail, & Hayes, 2009). Nevertheless, the water imbibition by soybean seeds corresponds to the initial phase of the germination process, and metabolic pathways can be induced for the conversion of the precursors (naringenin and isoliquiritigenin) of isoflavonoids to isoflavones (Paucar-Menacho, Berhow, Mandarino, Chang, & Mejia, 2010). Thus, the increase that was observed in the content of malonylglucosides in the soaked grains at 25 °C (Fig. 2d) would not be justified only by the improved extraction of isoflavones in the soybean samples that were subjected to the soaking process.

4. Conclusion

The changes in the properties of soybeans and the loss of isoflavones during grain soaking were monitored in this study, providing important information of this step of soybean processing. Soaking times can be reduced by using high soaking temperatures. Lengthy soaking times are not necessary to promote additional softening of the grains. Temperatures that considerably impair the solubility of soy proteins should be avoided, or an appropriate processing time should be used. The leaching and degradation of

Table 1
Mass balance of total isoflavones during soybean soaking at different temperatures.

		Soaking time								
		0 h	0.5 h	1 h	2 h	3 h	4 h	5 h	6 h	7 h
SS (g)	25 °C	50 ± 0.02^{hA}	62.9 ± 0.8^{gD}	70.1 ± 0.7^{fD}	81.4 ± 0.8^{eC}	86.9 ± 1.0^{dC}	91.8 ± 0.9^{cC}	96.3 ± 1.2^{bA}	98.5 ± 0.9^{abA}	100.3 ± 1.0^{aA}
	40 °C	50 ± 0.03^{eA}	70.2 ± 0.9^{dC}	81.1 ± 0.7^{cC}	92.5 ± 1.0^{bB}	91.2 ± 1.1^{bB}	96.9 ± 0.7^{aAB}	98.7 ± 0.9^{aA}	99.1 ± 1.2^{aA}	99.5 ± 1.0^{aAB}
	55 °C	50 ± 0.02^{dA}	77.4 ± 0.7^{cB}	88.4 ± 0.9^{bB}	95.2 ± 1.1^{aAB}	96.5 ± 0.8^{aA}	97.8 ± 0.9^{aA}	96.8 ± 0.8^{aA}	97.1 ± 1.1^{aA}	96.5 ± 0.7^{aBC}
	70 °C	50 ± 0.04^{dA}	83.0 ± 0.7^{cA}	92.5 ± 0.8^{bA}	96.5 ± 0.7^{aA}	95.9 ± 0.9^{aA}	94.1 ± 0.8^{abBC}	96.3 ± 0.7^{aA}	95.6 ± 0.9^{aA}	92.3 ± 0.9^{bD}
	55 °C_BS	50 ± 0.03^{eA}	76.1 ± 0.8^{dB}	86.1 ± 0.7^{cB}	92.3 ± 0.9^{bB}	94.6 ± 0.7^{abAB}	95.3 ± 0.7^{aAB}	95.7 ± 0.6^{aA}	95.7 ± 0.7^{aA}	94.5 ± 0.8^{abCD}
TI_{SY} (mg)	25 °C	101.8 ± 0.1^{abA}	96.2 ± 0.5^{bBC}	105.4 ± 0.7^{abAB}	103.4 ± 0.4^{abA}	107.4 ± 3.3^{aA}	105.6 ± 4.2^{aA}	107.3 ± 1.6^{aA}	100.3 ± 2.9^{abA}	108.6 ± 3.2^{aA}
	40 °C	101.8 ± 0.1^{abA}	98.4 ± 0.7^{bcB}	99.5 ± 0.5^{abcBC}	102.3 ± 0.3^{aA}	97.2 ± 0.5^{cB}	96.4 ± 2.4^{cBC}	97.7 ± 1.0^{cB}	98.6 ± 0.1^{abcAB}	91.4 ± 0.3^{dBC}
	55 °C	101.8 ± 0.1^{aA}	95.5 ± 1.8^{bcBC}	99.1 ± 2.8^{abBC}	100.2 ± 1.2^{abA}	93.3 ± 1.2^{cdB}	88.0 ± 1.4^{deC}	89.0 ± 0.1^{deC}	89.9 ± 1.4^{deC}	86.7 ± 0.2^{eC}
	70 °C	101.8 ± 0.1^{aA}	92.0 ± 0.7^{eC}	96.7 ± 1.7^{bcdC}	93.4 ± 0.9^{deB}	97.1 ± 0.7^{bcB}	99.9 ± 0.1^{abAB}	95.8 ± 0.5^{cdB}	92.5 ± 1.1^{eBC}	93.5 ± 0.2^{deB}
	55 °C_BS	101.8 ± 0.1^{bA}	108.3 ± 1.2^{aA}	106.6 ± 1.8^{abA}	103.9 ± 2.4^{abA}	95.5 ± 1.9^{cB}	91.6 ± 0.3^{cBC}	94.4 ± 0.4^{cB}	94.3 ± 0.2^{cABC}	92.5 ± 1.7^{cBC}
RI_{SS} (%)	25 °C	–	100	100	100	100	100	100	100	100
	40 °C	–	96.6	97.5	100	94.6	93.9	95.1	95.6	88.7
	55 °C	–	93.8	95.2	93.7	85.0	79.7	80.7	81.7	78.1
	70 °C	–	85.8	88.0	82.3	84.4	86.2	82.6	79.7	79.6
	55 °C_BS	–	100	100	97.5	88.9	84.5	87.5	85.3	84.5
LI_{RS} (%)	25 °C	–	–	–	–	–	–	–	–	–
	40 °C	–	0.1	0.3	–	0.9	0.8	0.8	1.3	1.1
	55 °C	–	0.0	2.1	4.7	6.7	6.7	6.8	6.6	7.1
	70 °C	–	4.5	7.0	9.5	11.0	11.9	11.4	11.2	12.2
	55 °C_BS	–	–	–	4.5	5.0	5.5	5.1	7.3	6.4
DI_{SY} (%)	25 °C	–	–	–	–	–	–	–	–	–
	40 °C	–	–	–	–	4.5	5.3	4.1	–	10.2
	55 °C	–	6.2	–	–	8.4	13.6	12.6	11.7	14.8
	70 °C	–	9.7	5.0	8.2	4.6	–	5.9	9.2	8.2
	55 °C_BS	–	–	–	–	6.2	10.1	7.3	7.4	9.1

Results are expressed as the mean ($n = 4$) ± standard deviation. The isoflavone content was normalised to the corresponding aglycones.
Means with identical lowercase letters in the same line (a–h) and capital letters (A–D) in the same column do not differ significantly ($\alpha = 0.05$).
SS = soaked soybeans, TI_{SY} = total isoflavones of the systems, RI_{SS} = retention of total isoflavones by the soaked soybean, LI_{RS} = loss of total isoflavones into the soaking medium, DI_{SY} = degradation of total isoflavones of the systems.
Missing DI_{SY} values indicate that there was no significant difference ($p > 0.05$) in relation to the TI_{SY} value from the whole soybeans (time zero).
Missing LI_{RS} values indicate that the loss of isoflavones was not significant ($p > 0.05$).
BS = buffer solution.

isoflavones can occur at soaking temperatures above 25 °C. Process conditions that allow increased formation of aglycone isoflavones and preserve the total isoflavone content without substantially impairing the water absorption, hardness, or soluble protein content of soybeans may be interesting for the preparation of soy products. In this context, soybean soaking at 55 °C for 5 h before subsequent processes is recommended.

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5.2 ARTIGO CIENTÍFICO 2

SOYBEAN SOAKING CHANGES THE CONTENTS OF DIFFERENT FORMS OF ISOFLAVONES

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Abstract

The effects of the time and temperature of soybean soaking on changes in the contents of different forms of isoflavones were evaluated. Soybean soaking (1:1.5 g:g soybean: deionised water ratio) was performed at 25, 40, 55 and 70 °C for 0, 1, 2, 3, 4, 5, 6 and 7 h, and the contents and profiles of different forms of isoflavones were determined by ultra-high-performance liquid chromatography (UHPLC). The duration of soybean soaking at 25 °C did not influence the contents of isoflavones of the daidzein, glycitein and genistein series. The isoflavones of the daidzein and glycitein series were the most unstable in soybeans soaked at 55 and 70 °C. Soybeans soaked for 6 h at 55 °C showed 6- and 7-fold higher daidzein and genistein contents than did unsoaked soybeans, respectively. Therefore, soybeans soaked for 5 h at 55 °C can be used to produce soy products rich in aglycones.

Keywords: soybean soaking, isoflavones, bioactive compounds, β-glucosidases.

Chemical compounds:

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), malonylglycitin (PubChem CID: 23724657).

1. Introduction

Soybean [*Glycine max* (L.) Merrill] is a legume that is being widely investigated due to its contents of isoflavones, which are associated with beneficial effects on human health (Chen, Erh, Su, Liu, Chou, & Cheng, 2012; Zhang, Chang, & Liu, 2015). Isoflavonoids comprise one of the major classes of phenolic compounds found in soybeans and their derivatives. These compounds are divided into four groups and twelve distinct forms called aglycones (daidzein, genistein and glycitein); 7-*O*-β-glucosides (daidzin, genistin and glycitin); 7-*O*-(6''-*O*-acetyl-β-glucosides) (acetyl daidzin, acetyl genistin and acetyl glycitin); and 7-*O*-(6''-*O*-malonyl-β-glucosides) (malonyl daidzin, malonyl genistin and malonyl glycitin). Since these isoflavones are derived from the aglycone forms, they can be grouped into the daidzein (daidzein, daidzin, acetyl daidzin and malonyl daidzin), genistein (genistein, genistin, acetyl genistin and malonyl genistin) and glycitein (glycitein, glycitin, acetyl glycitin and malonyl glycitin) series. These compounds, particularly the aglycones daidzein and genistein, have been further investigated because of their anticarcinogenic, antioxidant, cardioprotective, antihyperglycemic and antiobesity effects (Crozier, Jaganath, & Clifford, 2009; Messina & Badger, 2017).

Soybean soaking is a preliminary step used for the preparation of several products, such as soymilk, tofu and tempeh. The soaking time and temperature are important factors that can substantially influence the textural characteristics (Pan & Tangratanavalee, 2003) and cooking of the grains (Fabbri & Crosby, 2016), as well as the leaching of hydrosoluble substances (Lima, Kurozawa, & Ida, 2014). In a food matrix, the solubility of isoflavones depends not only on their chemical structure but also on their interactions with carbohydrates, proteins and lipids, which can alter the physicochemical characteristics of the isoflavones (Jakobek, 2015). The solubility of the isoflavones in aqueous media is related to their hydroxyl groups, which interact with the water by means of hydrogen bonds.

Endogenous β-glucosidase enzymes can be activated during soybean soaking to convert glucosylated isoflavones, particularly β-glucosides, into aglycones (Chiou, Lin, Su, & Lee, 2010). This conversion is desirable because the aglycones daidzein and genistein have been associated with reduced incidence of diabetes (Choi, Jung, Yeo, Kim, & Lee, 2008), cancer (Dong, Xu, Sikes, & Wu, 2013; Russo et al., 2016) and other diseases.

Changes in different forms of isoflavones during soybean soaking were investigated at only one temperature. Toda et al. (2000) investigated the profiles of isoflavones in soybeans soaked (1:3 g:g soybean: distilled water) for 1, 2, 5, 10 and 15 h at 20 °C and observed that with increasing soaking time, the contents of aglycones increased slightly, while the β-glucoside content decreased. Sutil et al. (2008) reported that soybeans soaked at 50 ° C for 15 h showed higher formation of aglycones. The molar concentrations of malonyl and β-glucosides decreased from 33% to 56.5% in soybeans soaked at 50 °C for 12 h, while the aglycones daidzein and genistein increased from 0 to 0.5-0.8 μmol g^{-1} (Góes-Favoni, Carrão-Panizzi, & Beleia, 2010). However, to define the best conditions of soybean soaking for the formation of aglycone isoflavones, it is necessary to investigate joint changes in the contents and profiles of these isoflavones as a function of soaking temperature and time. Consequently, the results of this investigation may contribute to preparation of aglycone-rich soy products from hydrothermally treated soybeans.

Therefore, the objective of this study was to investigate the effects of the time and temperature of soybean soaking on the changes in the contents of different forms of isoflavones.

2. Materials and methods

2.1. Sample and reagents

Soybean [*Glycine max* (L.) Merrill] lipoxygenase-null cultivar BRS 257 (Empresa Brasileira de Pesquisa Agropecuária, Londrina/Paraná, Brazil) was used in this study.

Calibration curves for isoflavones were constructed from standard solutions of 7-*O*-(6''-*O*-acetyl-β-glucosides) and 7-*O*-(6''-*O*-malonyl-β-glucosides) (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 7-*O*-β-glucosides and aglycones

(Sigma-Aldrich Co., St. Louis, MO, USA). All the reagents used in the analyses were of analytical grade or liquid chromatography grade.

2.2. Effects of time and temperature of soybean soaking on contents of isoflavones

Soybean soaking was investigated to evaluate the effects of time (0, 1, 2, 3, 4, 5, 6 and 7 h) and temperatures (25, 40, 55 and 70 °C) on the contents and profiles of different forms of isoflavones. Soybean soaking was performed as described by Lima, Kurozawa and Ida (2014). In this step, 50 g of soybean grains in a 1:1.5 (g:g, soybean: deionised water) ratio was used for each soaking condition. After soaking, the soaked soybeans were drained, frozen, lyophilised (Christ Alpha 2-4 LD plus, Osterode am Harz, Germany) and ground (Ika A11 basic, St. Louis, MO, USA). These soybeans and flours of soaked soybeans were stored at -22 °C until use for analysis of different forms of isoflavones. The residual soaking solution was discarded.

2.3. Determination of different forms of isoflavones

The isoflavones were extracted after defatting of the soybeans (time zero or control) and of the flours of soaked soybeans, as described by Lima, Kurozawa and Ida (2014). Separation and quantification of isoflavones were performed as described by Yoshiara, Madeira, Delaroza, Silva and Ida (2012), with a modified extraction ratio to 0.2 g of defatted flour: 8 mL of solvent. This material was centrifuged at 8200 × *g* for 15 min at 4 °C (Centrifuge 5804R-Eppendorf, Hamburg, Germany) and filtered (Millex-GV, PVDF hydrophilic membrane, 0.22 µm, Millipore, Billerica, MA, USA). Aliquots of 1.4 µL of filtered extract were automatically injected into an ultra-high-performance liquid chromatography (UHPLC) system (Acquity UPLC® System, Waters, Milford, MA, USA) equipped with a reverse-phase BEH C18 column (50 mm x 2.1 mm, 1.7 µm, Waters) and photodiode array (PDA) detector (Waters) set to monitor the absorption of the eluate at 260 nm. The separation of isoflavones was carried out by means of a mobile phase in a non-linear gradient (Table 1) at 0.3 mL/min and 27 °C. Equipment control and acquisition and treatment of data were performed using Empower® 3 software (Waters).

For the quantification of the different forms of isoflavones, external calibration curves were constructed using standard solutions (0.2, 7.2, 10.7, 14.2, 17.7, 21.2 and 28.2 μg/mL) for each isoflavone form. The isoflavone contents were expressed as μmol per g of sample on a dry and full fat basis. Since these isoflavones are derived from the aglycone forms, the isoflavone profiles were grouped into daidzein (daidzein, daidzin, acetyl daidzin and malonyl daidzin), genistein (genistein, genistin, acetyl genistin and malonyl genistin) and glycitein (glycitein, glycitin, acetyl glycitin and malonyl glycitin) series.

2.4. Statistical analysis

For evaluation of effects of time and temperature of soybean soaking (n = 2) on isoflavone contents (n = 4), the results were subjected to analysis of variance (ANOVA) followed by least-square regression analysis and mathematical modelling when the results were properly adjusted to the models. Relative minima and maxima of quadratic models were estimated from the first derivative test (dy/dx = 0). The results not adjusted to the mathematical models were subjected to one-way ANOVA followed by Tukey's multiple comparisons test (α = 0.05). All data were treated using Statistica 10.0 software (StatSoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Profiles of the different forms of isoflavones in soybeans

The soybean cultivar BRS 257 showed 5.58 μmol of total isoflavones/g of sample on a dry and full fat basis (Table 2), consisting of a higher content of malonyl glucosides (63.08%), followed by β-glucosides (31.18%) and lower contents of aglycones (5.55%) and acetyl glucosides (0.18%). The content of β-glucosides was 1.74 μmol/g of sample on a dry and full fat basis, which consisted of 40.23% daidzin, 41.38% genistin and 18.39% glycitin (Table 2). Of the total content of malonyl glucosides (3.52 μmol/g of sample on a dry and full fat basis), the malonyl daidzin, malonyl genistin and malonyl glycitin contents represented 37.22%, 51.14% and 11.65%, respectively. The Brazilian soybean cultivars studied by Quinhone Júnior and Ida (2015) and Ribeiro et al., (2007) also exhibited predominance in the content

of malonyl glucosides, which comprised 72.86% of the total content of isoflavones. Acetyl daidzin and acetyl glycitin were not detected in soybeans, whereas the acetyl genistin content was low (Table 2). A similar profile of isoflavones in different soybean genotypes has been described by Britz, Schomburg and Kenworthy (2011). Soybean isoflavones participate in plant defence mechanisms, and their profile is related to a complex biosynthesis involving glycosylation, esterification and/or malonylation from aglycone isoflavones. The conjugation step promotes stability and solubility of aglycone isoflavones *in vivo*, enabling their compartmentalisation in vacuoles or their transport to different parts of the plant (Dhaubhadel, Farhangkhoee, & Chapman, 2008). The contents of the aglycones daidzein and genistein were similar, whereas glycitein was not detected (Table 2). The absence of glycitein has also been observed in several soybean cultivars (Britz, Schomburg, & Kenworthy, 2011). It is noteworthy that the contents and profiles of soybean isoflavones may be affected by several factors, such as cultivar, maturity group, crop year, growing region, climate, storage conditions, and others (Britz, Schomburg, & Kenworthy, 2011; Kim et al., 2014; Wang & Murphy, 1994).

3.2. Effect of time and temperature of soybean soaking on the contents and profiles of isoflavones of the daidzein series

According to the regression analysis, it was observed that the duration of soybean soaking at 25 °C did not influence the contents of daidzin, daidzein and malonyl daidzin. It was also observed that the contents of these isoflavones were equal or similar to those of unsoaked soybeans (Table 2), and therefore, the data were not adequately adjusted to the proposed mathematical model (Fig. 1a). Lima and Ida (2014) verified that in soaked soybeans, the hydrolysis of β-glucoside isoflavones to aglycones was dependent on the temperature of the soaking water, but the changes in isoflavone contents were not investigated at different times during soaking. Moreover, the content of total isoflavones was best preserved in soaked soybeans at 25 °C (Lima, Kurozawa, & Ida, 2014). Similar results were observed in soybeans soaked at 20 °C, and the total loss of isoflavones was negligible in relation to the total isoflavone content of unsoaked soybeans (Zhang, Chang, & Liu, 2015). The soaking process also promotes the softening of the grains, and this facilitates the grinding, cooking, and extraction of components into the soaking medium (Palavalli,

Natarajan, Wang, & Krishnan, 2012). Furthermore, soybean soaking alters textural characteristics of the grains and affects subsequent processes in the production of several products, such as soymilk, tofu and tempeh (Nelson, Steinberg, & Wei, 1976; Pan & Tangratanavalee, 2003).

Regression analysis indicated that soybean soaking time at 40 °C produced models with linear and quadratic effects on changes in contents of daidzin (R^2 = 0.93, p < 0.05) and daidzein (R^2 = 0.86, p < 0.05), respectively (Fig. 1b). However, malonyl daidzin content (Fig. 1b) was not significantly altered (p > 0.05), and therefore, the data were not properly adjusted to the proposed models. It is noteworthy that the maximum content of daidzein (0.27 μmol/g of sample on a dry and full fat basis) was estimated at 5.43 h for the first derivative of the proposed model. A significant conversion of β-glucoside isoflavones to aglycones was also observed by Lima and Ida (2014) after soaking soybeans for 5 to 6 h at 40 to 65 °C. Changes in the daidzin content were adjusted to a linear model, and the negative slope indicated a reduction in the content of this isoflavone during soybean soaking (Fig. 1b). This reduction can be explained by the leaching of this isoflavone to the immersion medium of soybeans soaked for 3 h at 40 °C, as was also observed by Lima, Kurozawa and Ida (2014). In addition, the malonyl glucosides have been described as thermally unstable (Coward, Smith, Kirk, & Barnes, 1998; Mathias, Ismail, Corvalan, & Hayes, 2006) and were partially degraded.

Regression analysis for soybean soaking time at 55 °C showed models with quadratic effects on changes in the contents of daidzin (R^2 = 0.95, p < 0.05) and daidzein (R^2 = 0.96, p < 0.05) and a linear effect for malonyl daidzin (R^2 = 0.85, p < 0.05) (Fig. 1c). For daidzin, the quadratic coefficient of the model was positive (0.0180), and for daidzein it was negative (-0.0185). In addition, the absolute values of these coefficients were almost identical, and it can therefore be inferred that at 55 °C, there was a stoichiometric (1 mol → 1 mol) conversion of daidzin to daidzein. Therefore, from the first derivative of the model (Fig. 1c), it was possible to estimate the minimum content of daidzin (0.23 μmol/g of sample on a dry and full fat basis) and the maximum daidzein content (0.95 μmol/g of sample on a dry and full fat basis) in soybeans soaked at 55 °C for 4.64 and 6.44 h, respectively. It is noteworthy that this maximum daidzein content was 6-fold higher than that in the unsoaked soybeans (Table 2). These results confirmed the conversion of β-glucoside isoflavones to aglycones in soybeans soaked for 6 h at 55 °C, due the ideal conditions for the action

of endogenous β-glucosidases, as was also observed by Lima and Ida (2014). Greater leaching of these isoflavones occurred when the soybean soaking time was increased from 4.54 h to 6.44 h. Furthermore, the solubility of the isoflavones in aqueous media is associated with their hydroxyl groups, whereas methyl and acetyl radicals decrease the polarity of these substances (Handa, Lima, Guelfi, Georgetti, & Ida, 2016). In addition, the extractability of isoflavones into aqueous media can be attributed to their interactions with proteins and carbohydrates, which have high water solubility (Jakobek, 2015; Speroni, Milesi, & Añón, 2010). The linear model for malonyl daidzin showed a negative slope (Fig. 1c) and therefore indicates that as the soybean soaking time increased, the content of this isoflavone decreased. This reduction can be explained by its partial leaching and degradation, as was also observed by Lima, Kurozawa and Ida (2014), and by the low specificity of soybean β-glucosidases towards the malonyl glucosides (Chiou, Lin, Su, & Lee, 2010; Ismail, & Hayes, 2005). Thus, during soybean soaking, the conversion of malonyl daidzin to daidzein may not have occurred.

Regression analysis for soybean soaking time at 70 °C showed models with linear effects and with good fitting of data for changes in daidzin (R^2 = 0.91, p < 0.05) and malonyl daidzin (R^2 = 0.90, p < 0.05) contents (Fig. 1d). However, the daidzein content was not significantly altered (p > 0.05) during soybean soaking at 70 °C (Fig. 1d), due to the thermal stability of this isoflavone and the inactivation of endogenous soybean β-glucosidases, as reported by Lima, Kurozawa and Ida (2014). Therefore, it was not possible to estimate a model (Fig. 1d). In models with linear effects for the contents of daidzin and malonyl daidzin, the slopes were positive (0.0433) and negative (-0.0688), respectively, indicating that malonyl daidzin was partially converted to daidzin by decarboxylation and deesterification reactions, as reported by Andrade, Mandarino, Kurozawa, and Ida (2016); Coward, Smith, Kirk and Barnes (1998); and Mathias, Ismail, Corvalan and Hayes (2006). In addition, malonyl daidzin may have been transformed into degradation products.

3.3. Effect of soybean soaking time and temperature on the contents and profiles of isoflavones of the genistein series

According to the regression analysis, it was found that the duration of soybean soaking at 25 °C did not influence the contents of genistin, genistein and malonyl genistin, since the data were not adequately adjusted to the proposed mathematical

model (Fig. 2a). It was also observed that the contents of these isoflavones were equal or similar to those of the unsoaked soybeans (Table 2). This observation was similar for isoflavones of the daidzein series in soybeans soaked at 25 °C (Fig. 1a). In addition, the conversion of genistin to genistein was not significant at 25 °C, because the temperature was not ideal for the action of endogenous soybean β-glucosidases, as was also observed by Lima and Ida (2014).

Regression analysis for soybean soaking time at 40 °C indicated models with quadratic effects and with good fitting of data for changes in genistin (R^2 = 0.98, p < 0.05) and genistein (R^2 = 0.93, p < 0.05) contents, while for the content of malonyl genistin, the model was linear and adequately fitted (R^2 = 0.95, p < 0.05) to the experimental data (Fig. 2b). The quadratic model for genistin (Fig. 2b) showed a relative minimum when x = 6.11 h, and therefore, the estimated value of y ($\hat{y}$) was 0.41 μmol genistin/g of sample on a dry and full fat basis. In contrast, the model for genistein (Fig. 2b) showed a relative maximum when x = 5.18 h. Thus, the value of $\hat{y}$ was 0.30 μmol of genistein/g of sample on a dry and full fat basis and was 2-fold higher than that in unsoaked soybeans (Table 2). These results agree with those described by Lima, Kurozawa, and Ida (2014) and Lima and Ida (2014), who observed that 40 °C was not an optimum temperature for soybean endogenous β-glucosidase activity but was responsible for the hydrolysis of β-glucoside isoflavones in aglycones. The slope of the linear model for changes in malonyl genistin was positive (Fig. 2b), indicating that during soybean soaking, an increase in the content of this isoflavone occurred. This fact may be explained by changes in the tissue of the soybean grains leading to increases in extraction.

Regression analysis for soybean soaking time at 55 °C indicated models with quadratic effects on changes in contents of genistin (R^2 = 0.92, p < 0.05) and genistein (R^2 = 0.97, p < 0.05) and a linear effect for malonyl genistin (R^2 = 0.74, p < 0.05) (Fig. 2c). The coefficients of the quadratic model for genistin and genistein were positive (0.0254) and negative (-0.0185), respectively. Therefore, it can be inferred that at 55 °C, there was a stoichiometric (1 mol → 1 mol) conversion of genistin to genistein, since the coefficients had similar absolute values. These changes also occurred in daidzein content at same soaking temperature (Fig. 2c). The model for genistin (Fig. 2c) had a relative minimum when x = 4.77 h, and therefore, the estimated minimum content for genistin was 0.20 μmol of genistin/g sample on a dry and full fat basis. However, the model for genistein (Fig. 2c) had a relative maximum

content of 1.03 μmol genistein/g of sample on a dry and full fat basis when x = 6.13 h. It was observed that even with the leaching of genistein during soaking, the maximum content was 7-fold higher than that of untreated soybeans (Table 2). This fact indicates that during the soaking of soybeans at the optimum temperature for β-glucosidase activity, 55 °C, more conversion of the β-glucoside isoflavones to their corresponding aglycones occurred, as described by Góes-Favoni, Carrão-Panizzi and Beleia (2010); Lima, Kurozawa, and Ida (2014); and Lima and Ida (2014). The model for malonyl genistin showed a negative slope, indicating that as soybean soaking time increased, the content of this isoflavone decreased. This reduction was due to partial leaching and degradation, as was also observed by Lima, Kurozawa, and Ida (2014). It is noteworthy that the use of residual water from soaked soybeans may be an alternative for obtaining bioactive compounds, such as the different forms of isoflavones, for which approaches need to be better investigated. For instance, soybeans soaked at 50 °C for 8 h release large amounts of enzymatically active Bowman-Birk protease inhibitor into the soaking media; when concentrated, this inhibitor can be applied as a cancer chemoprotective agent (Palavalli, Natarajan, Wang, & Krishnan, 2012).

Regression analysis for soybean soaking time at 70 °C indicated properly adjusted models with linear effects for changes in contents of genistin (R^2 = 0.85, p < 0.05), malonyl genistin (R^2 = 0.84, p < 0.05) and acetyl genistin (R^2 = 0.94, p < 0.05). In soaked soybeans, the content of genistein was not significantly altered (p > 0.05), and therefore, the proposed model did not fit the experimental data (Fig. 2d). The formation of acetyl genistin may occur due to the deesterification of malonyl genistin (Coward, Smith, Kirk, & Barnes, 1998; Jackson et al., 2002; Mathias, Ismail, Corvalan, & Hayes, 2006). Wet heat favours the conversion of malonylglucosides to acetylglucosides and β-glucosides better than dry heat does, as verified by Chien, Hsieh, Kao, & Chen (2005) for isoflavone standards treated at 100, 150 and 200 °C from 0 to 200 min. Therefore, the wet heat involved in soybean hydrothermal treatment probably followed this phenomenon. In addition, it was verified that soaked soybeans had a smaller reduction in malonyl genistin content (Fig. 2d) than in malonyl daidzin content (Fig. 1d). This observation is accordance with the study of Mathias, Ismail, Corvalan and Hayes (2006), who reported that the loss in contents of daidzein-series isoflavones was significantly greater than that found for genistein-series isoflavones.

3.4. Effect of soybean soaking time and temperature on the contents and profiles of isoflavones of the glycitein series

According to the regression analysis, the soybean soaking time at 25 °C and 40 °C did not influence the contents of glycitin and malonyl glycitin, and consequently, the data did not adequately fit the proposed models (Fig. 3a-b). The same result was observed for the isoflavones of the daidzein and genistein series (Fig. 1a and Fig. 2a).

Regression analysis for soybean soaking time at 55 °C indicated models with quadratic effects on changes in contents of glycitin (R^2 = 0.88, p < 0.05) and glycitein (R^2 = 0.96, p < 0.05) and a linear effect for malonyl glycitin (R^2 = 0.79, p < 0.05) (Fig. 3c). In the quadratic model for glycitin (Fig. 3c), there was a relative minimum (0.17 µmol of glycitin/g of sample on a dry and full fat basis) when x = 4.89 h. However, in the quadratic model for glycitein (Fig. 3c), there was a relative maximum (0.20 µmol of glycitein/g of sample on a dry and full fat basis) when x = 5.58 h, although in unsoaked soybeans, this compound was not detected (Table 2). In soybeans soaked at 55 °C, thermal hydrolysis of glycitin and formation of glycitein probably occurred, since according to Ismail and Hayes (2005), the specificity of β-glucosidases for glycitin is low. Zhang and Chang (2016) verified that ultra-high temperature processing of soy milk also increased the glycitein content, which could be partially attributed to the thermal conversion of glycitin to glycitein.

Regression analysis for soybean soaking time at 70 °C produced models with negative linear or quadratic effects that were properly adjusted to the changes in malonyl glycitin (R^2 = 0.86, p < 0.05) and glycitein (R^2 = 0.72, p < 0.05) contents, respectively. Therefore, the malonyl glycitin content decreased with increasing soybean soaking time (Fig. 3d), which may be attributed to leaching and degradation rather than to conversion to glycitein, since soybean β-glucosidases are inactive at temperatures above 70 °C (Lima, Kurozawa, & Ida, 2014). The changes in glycitein contents in soybeans soaked at 70 °C did not exceed 0.09 µmol of glycitein/g of sample on a dry and full fat basis, whereas high conversion of glycitin into glycitein was observed in soybeans soaked at 55 °C for 5.58 h (Fig. 2c). At 70 °C, the glycitin contents in soaked soybeans did not vary significantly (p > 0.05), and therefore, the data were not fitted to the proposed model (Fig. 3d), indicating that leaching,

degradation and partial thermal conversion of malonyl glycitin to glycitin may have occurred simultaneously.

Therefore, comparing the changes in contents of isoflavones of the daidzein, genistein and glycitein series, the soaking of soybeans at 55 °C may be advantageous for the preparation of soy products such as soy milk, tofu, soybeans, tempeh, miso and others, since these products require soaking prior to processing. The main advantage of soaking would be to obtain soy products rich in the aglycones daidzein, genistein and glycitein, which have been widely investigated because of their beneficial effects on human health (Choi, Jung, Yeo, Kim, & Lee, 2008; Crozier, Jaganath, & Clifford, 2009; Dong, Xu, Sikes, & Wu, 2013; Messina & Badger, 2017; Russo et al., 2016). It is noteworthy that soybean soaking, especially at 55 °C, promoted the formation of daidzein, genistein and glycitein in significant quantities, and therefore, these substances should be preserved in subsequent thermal processing, such as grinding, and cooking at high temperatures, which are usually used in the elaboration of soy products.

4. Conclusion

The duration of soybean soaking at 25 °C did not influence the contents and profiles of isoflavones of the daidzein (daidzein, daidzin and malonyl daidzin), genistein (genistein, genistin and malonyl genistin) and glycitein (glycitin and malonyl glycitin) series.

Regardless of the duration of soybean soaking at 40, 55 and 70 °C, leaching, conversion and/or degradation of isoflavones occur. Soybeans soaked for 5.43 h at 40 °C had the maximum content of daidzein, while maximum conversion of genistin to genistein was observed from 5 to 6 h. Soybeans soaked for approximately 6 h at 55 °C showed significant increases in the contents of the aglycones daidzein and genistein. Soybean soaked from 1 h at 70 °C favoured the leaching and degradation of malonyl daidzin, malonyl genistin and malonyl glycitin.

Stoichiometric conversion (1 mol: 1 mol) of genistin to genistein, daidzin to daidzein and glycitin to glycitein were verified during soybean soaking at 55 °C. Therefore, soybean soaked for 5 h at 55 °C could be used to produce soy products rich in the aglycones daidzein, genistein and glycitein.

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Graphical abstract

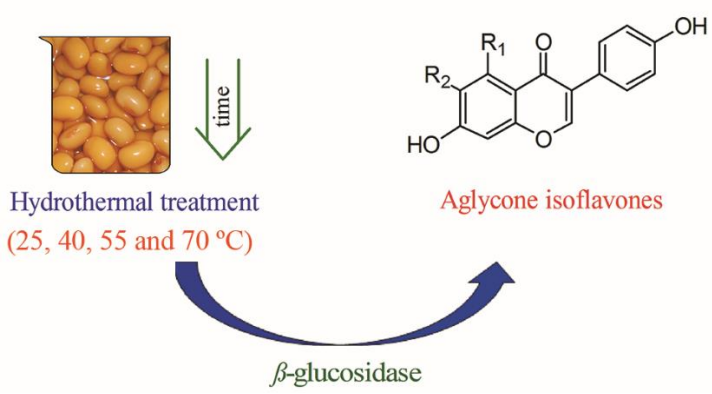

Tabela 1 - Conditions of separation of different forms of isoflavones using UHPLC*.

Time (min)	Flow-rate (mL/min)	% A (0.4 g of formic acid /100 mL of ultra-pure water)	%B (Acetonitrile)	Curve
Initial	0.3	95	5	Initial
1	0.3	85	15	7
4	0.3	82	18	7
5	0.3	75	25	7
6	0.3	75	25	7
8.5	0.3	20	80	7
9.5	0.3	95	5	6
12	0.3	95	5	6

*Ultra-high-performance liquid chromatography.

Tabela 2. Contents and profiles of isoflavones* in unsoaked soybeans.

	Daidzein series	Genistein series	Glycitein series	Total in row
β-glucoside	0.70 ± 0.04	0.72 ± 0.01	0.32 ± 0.07	1.74 ± 0.08
Malonyl glucoside	1.31 ± 0.07	1.80 ± 0.04	0.41 ± 0.09	3.52 ± 0.12
Acetylglucoside	ND	0.01 ± 0.0002	ND	0.01 ± 0.0002
Aglycone	0.16 ± 0.004	0.15 ± 0.005	ND	0.31 ± 0.006
Total in column	2.17 ± 0.08	2.68 ± 0.04	0.73 ± 0.11	5.58 ± 0.15

*In µmol/g on a dry weight and full fat basis.

β-glucoside (daidzin, genistin or glycitin), malonyl glucoside (malonyl daidzin, malonyl genistin or malonyl glycitin)

Acetylglucoside (acetyl daidzin, acetyl genistin or acetyl glycitin), aglycone (daidzein, genistein or glycitein)

ND = non detected.

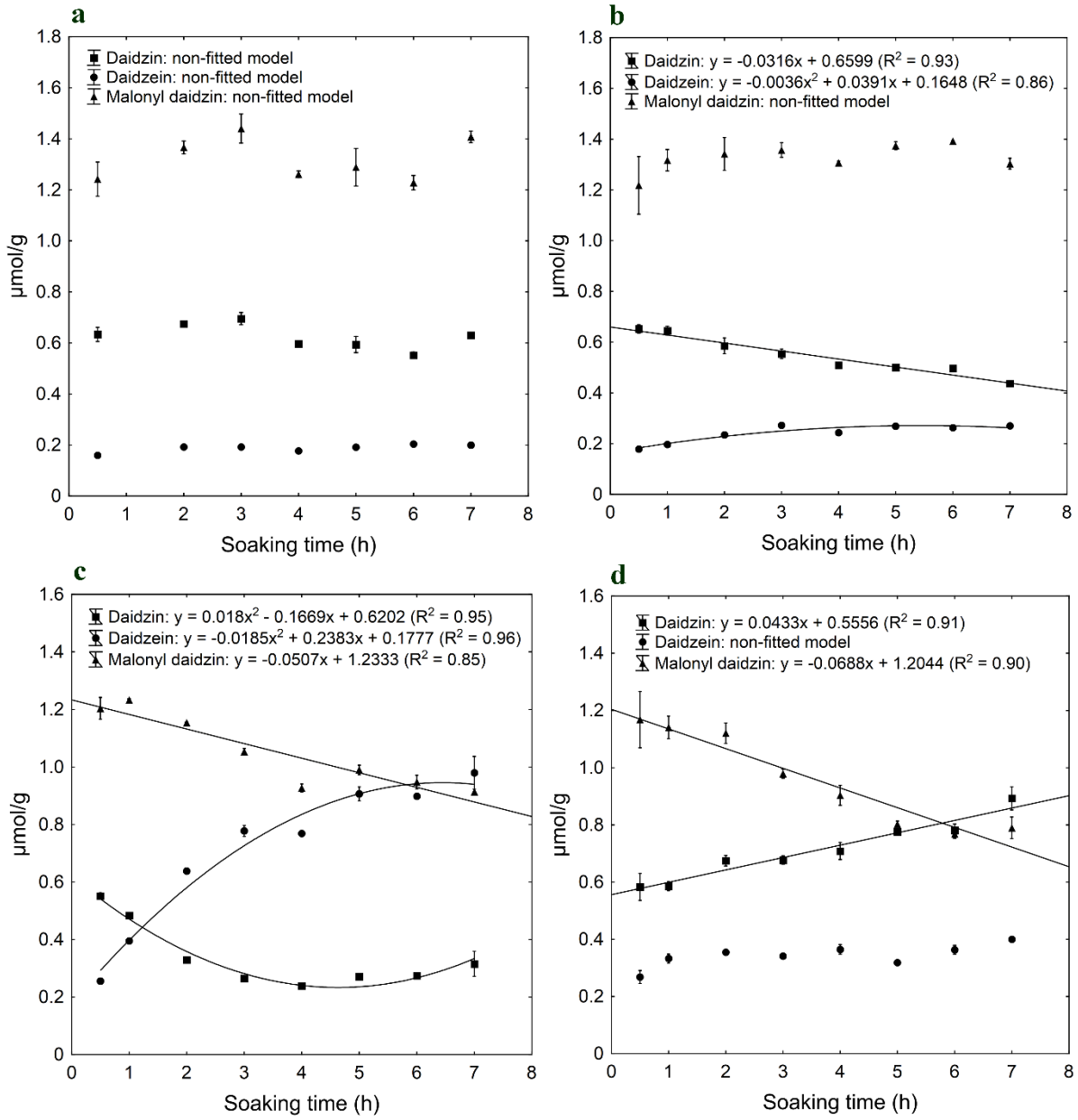

Figura 1 - Effect of soybean soaking (n = 2) time at (a) 25 °C, (b) 40 °C, (c) 55 °C and 70 °C on the contents (n = 4) and profiles of the isoflavones of the daidzein series. The solid lines without regression lines are provided as a visual guide only.

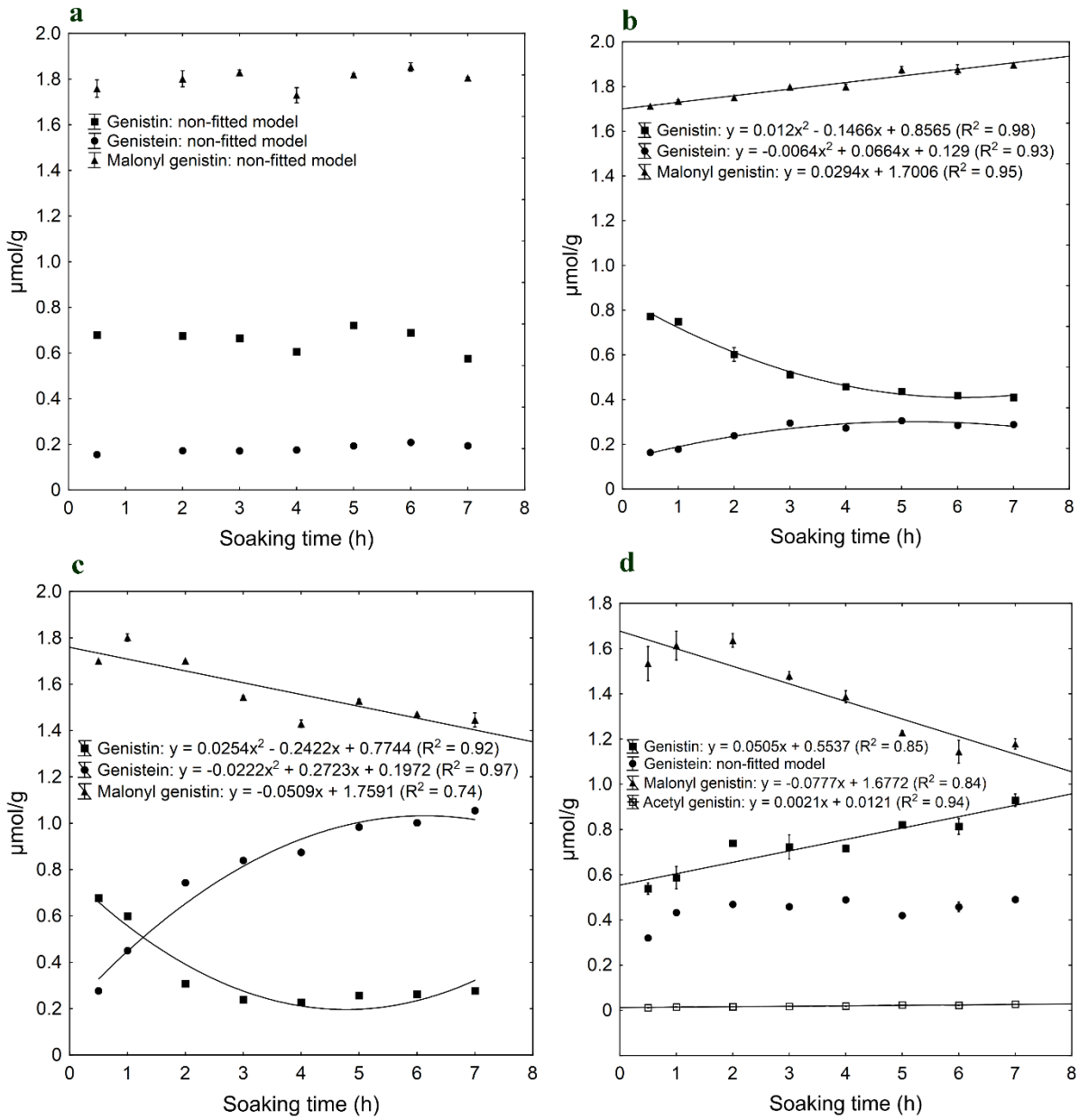

Figura 2 - Effect of soybean soaking (n = 2) time at (a) 25 °C, (b) 40 °C, (c) 55 °C and 70 °C on the contents (n = 4) and profiles of the isoflavones of the genistein series. The solid lines without regression lines are provided as a visual guide only.

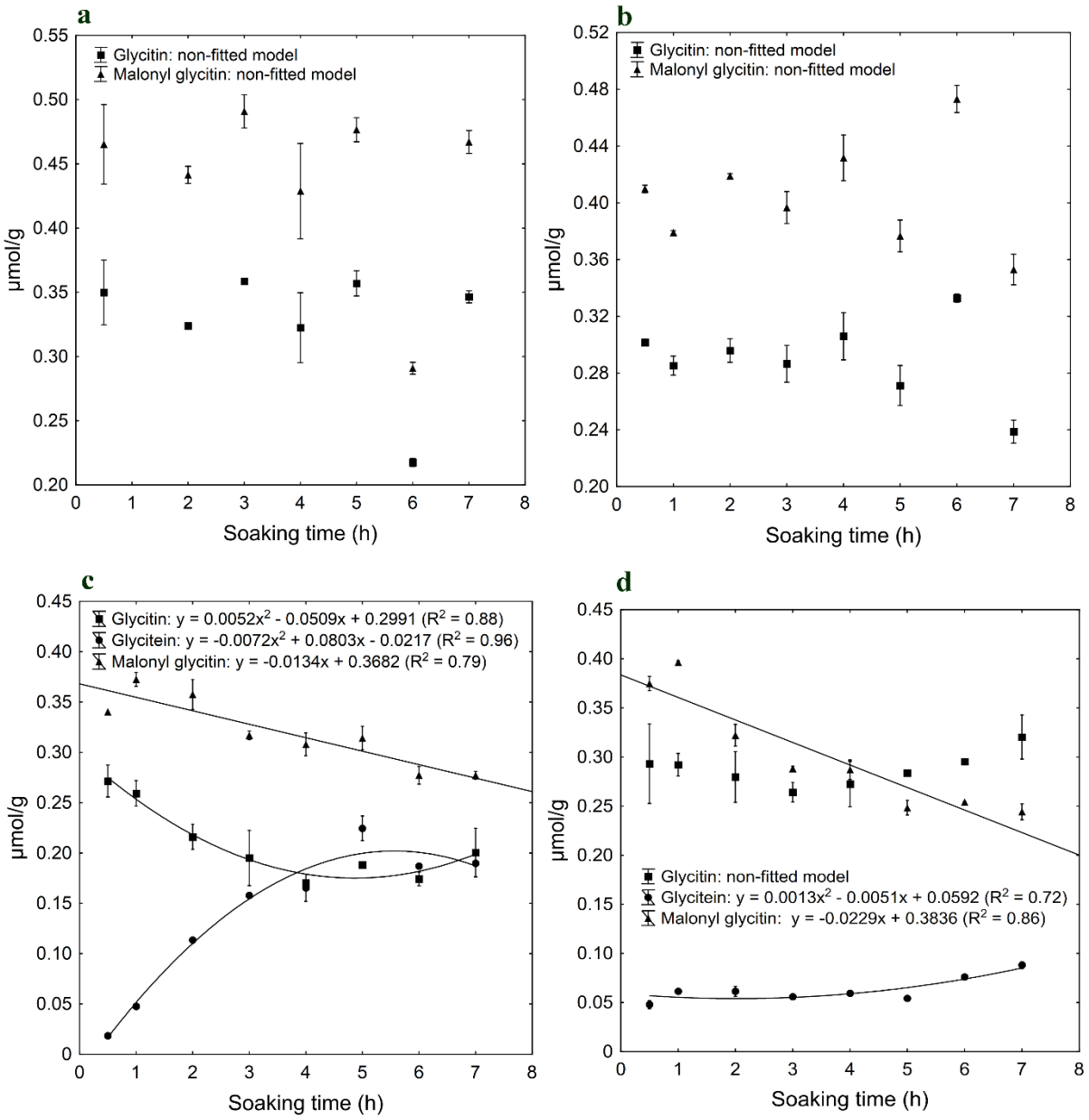

Figura 3 - Effect of soybean soaking (n = 2) time at (a) 25 °C, (b) 40 °C, (c) 55 °C and 70 °C on the contents (n = 4) and profiles of the isoflavones of the glycitein series. The solid lines without regression lines are provided as a visual guide only.

5.3 ARTIGO CIENTÍFICO 3

SOYBEAN SOAKING CHANGES THE CONTENTS OF SUGARS AND ACTIVITY of α-GALACTOSIDASES

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Abstract

The effects of the time and temperature of soybean soaking on the changes in sugar content and α-galactosidases activity were evaluated. Soybean soaking was performed at a 1:1.5 (g:g, soybean: deionised water) ratio at 25, 40, 55 and 70 °C for 0, 1, 2, 3, 4, 5, 6 and 7 h, and the content and profile of sugars and α-galactosidases activity were determined by high performance anion exchange chromatography (HPAEC) and spectrophotometry, respectively. Soybean soaked at 25 or 40 °C did not show significant changes in raffinose and stachyose contents. Soybean soaking for 3 h at 25, 40, 55 or 70 ° C influenced the activity of α-galactosidases, which hydrolyse oligosaccharides in soaked soybeans at 55 or 70 °C. Soybean soaking at 55 °C for longer than 3 h showed mean reductions of 45% for raffinose and 25% for stachyose. However, soybean soaking at temperatures of 25, 40, 55 or 70 °C showed no significant changes ($p > 0.05$) in the sucrose content. Therefore, soybean soaking at 40 or 55 °C can be used to promote the partial hydrolysis of raffinose and stachyose to galactose and sucrose.

Keywords: soaking, raffinose, stachyose, sucrose, α-galactosidases.

Chemical compounds:

D-(-)-fructose (PubChem CID: 5984); D-galactose (PubChem CID: 6036); D-sucrose (PubChem CID: 5988); D-(+)-raffinose: (PubChem CID: 439242); D-stachyose hydrate (PubChem CID: 439531).

1. Introduction

The soybean [*Glycine max* (L.) Merrill] is a legume widely investigated because of its bioactive compounds, particularly isoflavones and oligosaccharides, which are associated with beneficial effects on human health (Chen, Erh, Su, Liu, Chou, & Cheng, 2012; Zhang, Chang, & Liu, 2015).

Sucrose and the oligosaccharides raffinose and stachyose are the major soluble sugars found in soybeans. These two oligosaccharides contain one and two molecules of galactose bound to sucrose by means of α-(1→6) linkages, respectively, and, they are not digested in the gastrointestinal tract, causing abdominal discomfort and flatulence due to the formation of gases during their fermentation in the colon (Suarez, Springfield, Furne, Lohrmann, Kerr, & Levitt, 1999). However, these oligosaccharides have been considered as prebiotics because they selectively stimulate the growth of and/or activity of microorganisms that promote health benefits to the host (Chen, Erh, Su, Liu, Chou, & Cheng, 2012; Gibson & Roberfroid, 1995; Li, Lu, & Yang, 2013; Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013). Fei, Ling, Hua and Ren (2014) reported that soy oligosaccharides were able to reduce oxidative stress and attenuate insulin resistance in patients, indicating that these sugars may play an important role in the management of complications of gestational diabetes mellitus. Moreover, an intake of 0.83 g of α-galactooligosaccharides per kg of body weight was shown to have a relevant prebiotic effect in mice (Li, Lu, & Yang, 2013).

Soybean soaking is a pre-processing operation used for the preparation of various products, such as soymilk, tofu, tempeh and others. The time and temperature are important factors that should be monitored in this process because they influence the grinding and cooking of the grains, the leaching of some substances into the soaking medium and the activation of endogenous enzymes of the soybean (Fabbri & Crosby, 2016; Lima, Kurozawa, & Ida, 2014; Pan & Tangratanavalee, 2003).

The α-galactosidase enzymes convert the oligosaccharides raffinose and stachyose to galactose and sucrose (Viana et al., 2005). However, the relationship between changes in the oligosaccharide content and activity of the α-galactosidases during soybean soaking at different temperatures has not yet been investigated. Soybean seeds with high sucrose content are desired because sucrose is a sweetness-imparting component and thus helps in the wider acceptance of soy-derived food products (Kumar et al., 2010).

The impact of the processing of soy products on the oligosaccharide contents have been described by Egounlety and Aworh (2003) and Mulimani, Thippeswamy, and Ramalingam (1997). However, to define the best conditions of this step for the hydrolysis and partial leaching of raffinose and stachyose, the conditions should be investigated based on joint changes in the profiles of these compounds throughout the soybean soaking as a function of the temperature and time. This approach may contribute to the preparation of soy products with proper oligosaccharide and sucrose contents from soaked soybeans.

Therefore, the objective of this study was to investigate the effects of the time and temperature of soybean soaking on the changes in the contents of sugars and the activity of α-galactosidases.

2. Materials and methods

2.1. Sample and reagents

Soybeans [*Glycine max* (L.) Merril], lipoxygenase-null cultivar BRS 257 (Empresa Brasileira de Pesquisa Agropecuária, Londrina/Paraná, Brazil), were used in this study.

Calibration curves for the sugars were constructed from standard solutions of glucose, fructose, galactose, sucrose, raffinose and stachyose (Sigma-Aldrich Co., St. Louis, MO, USA). The substrate *p*-nitrophenyl-α-D-galactopyranoside (Sigma-Aldrich Co., St. Louis, MO, USA) was used to determine the α-galactosidase activity (EC 3.2.1.21) and *p*-nitrophenol (Sigma-Aldrich Co., St. Louis, MO, USA) was used to construct the calibration curve. All of the reagents used in the analyses were of analytical grade or liquid chromatography grade.

2.2. Effects of the time and temperature of the soybean soaking on the sugar contents

Soybean soaking was investigated to evaluate the effects of time (0, 1, 2, 3, 4, 5, 6 and 7 h) and temperature (25, 40, 55 and 70 °C) on the contents and profiles of different sugars as well as the activity of α-galactosidases. Soybean soaking was performed as described by Lima, Kurozawa and Ida (2014). In this step, 50 g of soybean grains in a 1:1.5 (g:g, soybean:deionised water) ratio was used for each soaking condition. Afterwards, the soaked soybeans were drained, frozen, lyophilised (Christ Alpha 2-4 LD plus, Osterode am Harz, Germany) and ground (Ika A11 basic, St. Louis, MO, USA). These soaked soybean flours were stored at -22 °C until use for the determination of different sugars and α-galactosidase activity. The residual soaking solution was discarded.

2.3. Determination of the sugars by HPAEC-PAD

Prior to the extraction of the sugars from the raw soybeans and soaked soybean flours, these sample were defatted as described by Lima, Kurozawa and Ida (2014). Approximately 0.2 g of the defatted sample was transferred into a 50 mL flask, 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 centrifuged at 2070 × *g* for 15 min at 25 °C (Centrifuge 5804R-Eppendorf, Hamburg, Germany), and 0.5 mL of the extract was transferred into a microcentrifuge tube and submitted to centrifugal vacuum concentration at 927 × *g* at 30 °C (Jouan®, model RC 10.22, Jouan, Inc., Winchester, VA, USA) until evaporation of the solvent. The concentrated material was solubilised 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% solvent A (ultra-pure water) and 10% 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 °C followed by column stabilisation 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): E_1 = +0.1 V, t_1 = 400 ms; E_2 = -2.0 V, t_2 = 20 ms; E_3 = +0.6, t_3 = 10 ms; and E_4 = -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 g of an individual sugar per 100 g of raw soybeans or soaked soybean flour on a dry and full fat basis.

2.5. Determination of the α-galactosidase activity

The α-galactosidases enzymes were extracted from 0.2 g of soybeans and soaked soybean flour with 6 mL of 0.05 mol/L citrate buffer solution (pH = 4.5) containing 0.1 mol of NaCl/L. This mixture was vortexed at 15 min intervals for 1 h at 25 °C and centrifuged at 8200 × *g* for 15 min at 4 °C (Centrifuge 5804R-Eppendorf, Hamburg, Germany). These extracts were diluted 20- to 40-fold with the aforementioned buffer. Test tubes containing 0.8 mL of 1 mmol of *p*-nitrophenyl-α-D-galactopyranoside substrate/L in 0.1 mol/L acetate buffer (pH = 4.7) were pre-incubated for 10 min at 30 °C. Aliquots of 0.2 mL of the enzyme extracts were added to these test tubes, vortexed and kept in water bath for 30 min at 30 °C. The reaction was quenched with 1 mL of 0.5 mol of Na_2CO_3/L, and the absorbances were measured in a spectrophotometer (Biochrom Libra S22, Cambridge, England) at 400 nm. The quantity of *p*-nitrophenol (*p*-NP) released by the enzymatic reaction was determined from the calibration curve plotted for the *p*-NP solutions from 0.04 to 0.32 µmol in a total volume of 2 mL of the reaction mixture. One activity unit (AU) was defined as the quantity of enzyme necessary to release 1 µmol of *p*-NP per min

under the assay conditions. The activity of α-galactosidases was expressed as AU per g of raw soybeans or soaked soybean flour on a dry and full fat basis.

2.6. Statistical analysis

For evaluation of the effects of the time and temperature of the soybean soaking (n = 2) on the oligosaccharide contents (n = 4) and the activity (n = 4) of α-galactosidases, the results were subjected to analysis of variance (ANOVA) followed by least-square regression analysis and mathematical models with the results properly adjusted to the models. Relative minima and maxima of quadratic models were estimated from the first derivative test. Results not adjusted to the mathematical models were subjected to one-way analysis of variance followed by Tukey's multiple comparisons test (α = 0.05). All data were treated using the Statistica 10.0 software (StatSoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Profile of sugars in raw soybeans

Soybean BRS 257 presents, on a dry basis, 5.22 g of total sugars/100 g of sample comprised of 10.34% raffinose, 30.27% stachyose and 59.39% sucrose. It is noteworthy that the sugars galactose, glucose and fructose were not detected. Moreover, it exhibited an α-galactosidase activity of 1.36 AU/g dry sample. Sucrose and stachyose are soluble sugars predominant in mature seeds of conventional or food-type soybeans and represent 58-60% and 31-36% of the total soluble carbohydrate content, respectively (Fan, Zang, & Xing, 2015; Oliveira, Carrão-Panizzi, & Mandarino, 2010). The different contents of sucrose (1.70-4.07 g/100 g), raffinose (0.33-1.28 g/100 g) and stachyose (1.39-4.73 g/ 100 g) were described in cultivated soybeans in India by Kumar et al. (2010), which described that the contents of these sugars are influenced by genotypes and the planting location. The profiles and contents of soybean sugars can also be affected by other factors, such as the cultivar, maturity group, crop year, growing region, climate, storage conditions and others (Hagely, Palmquist, & Bilyeu, 2013; Saldivar, Wang, Chen, & Hou, 2011).

3.2. Effects of the soybean soaking time and temperature on the sugar profile and activity of α-galactosidases

Soybeans soaked at 25 or 40 °C did not show significant changes ($p > 0.05$) in their raffinose and stachyose contents (Fig. 1 and Fig. 2). In contrast, the activity of α-galactosidases was adjusted to the quadratic model ($R^2 = 0.92$ for 25 °C and $R^2 = 0.81$ for 40 °C, $p < 0.05$) and had a significant increase for up to 5 h of soaking, followed by a reduction for up to 7 h of soaking (Fig. 1 and Fig. 2). It is noteworthy that in this study, the soybean:deionised water ratio (1:1.5, g:g) in the soaking was low, and therefore, there was low leaching. However, Mulimani, Thippeswamy, and Ramalingam (1997) reported that when the soybeans were soaked for 16 h at 25 °C in a 1:10 (g:g, soybean:distilled water) ratio, mean decreases of 44.8% for stachyose and 80.3% for raffinose in relation to the raw soybeans occurred. Soaking the beans for 12–14 h reduced the oligosaccharide and sucrose contents by 20–35% (Egounlety & Aworh, 2003). In the soybean soaking process, the diffusion of water and their compounds occurs, which are dependent mainly on the temperature and concentration gradient between the soaked soybeans and the soaking medium (Lima, Kurozawa, & Ida, 2014). The activity of α-galactosidases (Fig. 1 and Fig. 2) of soybeans soaked at 25 or 40 °C increased possibly due to the efficiency of the extraction resulting from the increase of the permeability of the grain tissue during soaking.

Regression analysis for the soaking time of the soybeans at 55 °C indicated a model with quadratic effects on changes in raffinose ($R^2 = 0.81$, $p < 0.05$) and galactose ($R^2 = 0.98$, $p < 0.05$), whose data were adequately fitted to the proposed model. However, the stachyose content of the soybeans soaked at 55 °C for 7 h was not significantly altered ($R^2 = 0.63$, $p > 0.05$) (Fig. 3) by the soaking time, and consequently, only 63% of the data were fitted to the proposed model. The changes in raffinose, galactose and stachyose contents can be explained by the higher specificity of α-galactosidases toward raffinose than stachyose (Gao & Schaffer, 1999). Since the stachyose hydrolysis yields raffinose, endogenous soybean α-galactosidases simultaneously hydrolyse these two substrates. According to Porter, Herrmann and Ladisch (1990), stachyose hydrolysis gives a nearly constant level of raffinose shortly after hydrolysis begins. Soybeans soaked at 55 °C after 3 h showed mean reductions of 45% for raffinose and 25% for stachyose (Fig. 3). However, at 55

°C and for up to 7 h of soybean soaking (Fig. 3), the activity of α-galactosidases decreased, and in these conditions, the raffinose and stachyose contents decreased simultaneously while the galactose content increased. These results indicate that endogenous α-galactosidases hydrolysed these oligosaccharides since these compounds contain one and two molecules of galactose bound to sucrose by means of α-(1→6) linkages. The soybean soaking possibly activated the activity of endogenous α-galactosidases hydrolysing the oligosaccharides to galactose and sucrose, which according to Herman and Shannon (1985) are used as energy sources in the germination process. According to Viana et al. (2005), the optimum temperature for the activity of α-galactosidases is 50 °C. However, the reduction in the activity of α-galactosidases (Fig. 3) can be attributed mainly to inhibition by the reaction products galactose and sucrose (Porter, Herrmann, & Ladisch, 1990) or the loss of their stability, as reported by Lima, Kurozawa and Ida (2014) for β-glucosidases from soybeans soaked at 55 °C.

In the soybeans soaked at 70 °C, the raffinose and stachyose contents did not significantly vary ($p > 0.05$) throughout soaking, and therefore, the data of both sugars did not fit properly to the proposed models (Fig. 4). In relation to the changes in the galactose content of soybeans soaked at 70 °C, it was observed that the model (Fig. 4) showed a quadratic effect and good fit ($R^2 = 0.88$, $p < 0.05$) to the experimental data. It is noteworthy that the maximum content of galactose (0.21 g/100 g of dry soybean) was estimated at 6.8 h for the first derivative of the proposed model. This increase in galactose content during the soybean soaking at 70 °C was possibly due to activity of α-galactosidases at the start of soaking and the improved permeability of the soaked grains, which favoured the subsequent extraction of galactose. In relation to the activity of α-galactosidases of soybeans soaked at 70 °C (Fig. 4), it was observed that the model showed a quadratic effect for the soaking time of the soybeans with a good fit ($R^2 = 0.85$, $p < 0.05$) to the experimental data. It is verified that the minimum activity of α-galactosidases (0.05 AU/g) was estimated at 4.88 h from the first derivative of the proposed model. This model indicated that the activity of α-galactosidases was highest at the start of soybean soaking at 70 °C with a significant reduction until 5 h. Porter, Sarikaya, Herrmann and Ladisch (1992) described that the activity of α-galactosidases showed a substantial loss starting at 60 °C.

Soybeans soaked at 25, 40, 55 or 70 °C did not show significant changes ($p > 0.05$) in their sucrose content (Fig. 5). Although partial leaching of sucrose has occurred throughout the soybean soaking, its content was close to that of the unsoaked soybeans, possibly due to the hydrolysis of raffinose and stachyose to sucrose. Moreover, the higher permeability of the soaked grains should have improved the subsequent extraction of sucrose. Soybeans with a high sucrose content are desired because it is a sweetness-imparting component and thus helps in wider acceptance of soy-derived food products (Kumar et al., 2010).

4. Conclusion

Soybean soaking at 25 or 40 °C did not show significant changes ($p > 0.05$) in the raffinose and stachyose contents. However, the α-galactosidases activity in soybeans soaked at 25, 40, 55 or 70 °C was influenced by the soaking time. These enzymes catalysed the hydrolysis of raffinose and stachyose oligosaccharides to galactose in soybeans soaked at 55 or 70 °C for 3 h. After 3 h of soybean soaking at 55 °C, there were reductions of 45% for raffinose and 25% for stachyose. However, at temperatures of 25, 40, 55 or 70 °C, there were no significant ($p > 0.05$) changes of the sucrose content. Therefore, soybean soaking at 40 or 55 °C can be used to promote the partial hydrolysis of raffinose and stachyose to galactose and sucrose.

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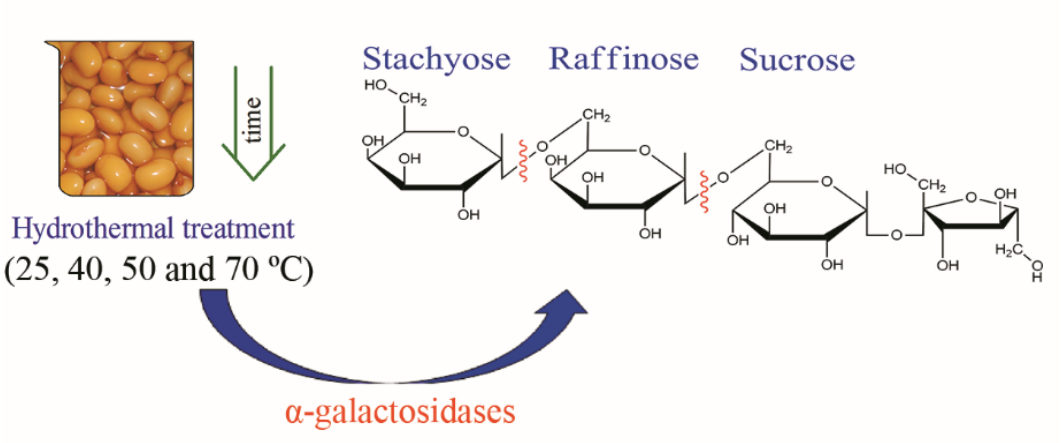
Graphical abstract



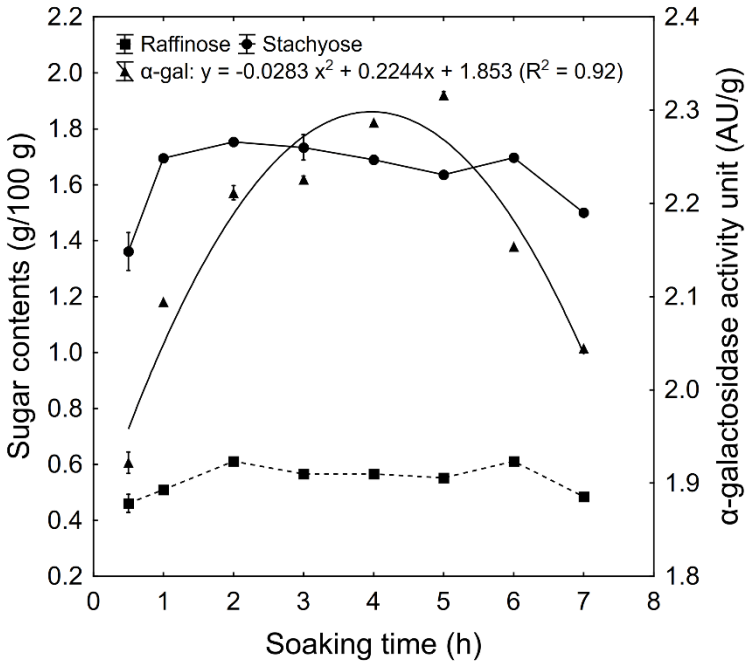


Figura 1 - Changes in the contents (n = 4) of oligosaccharides and activity (n = 4) of α-galactosidases throughout the soybean soaking (n = 2) period at 25 °C.
The solid lines without regression lines are provided as a visual guide only.

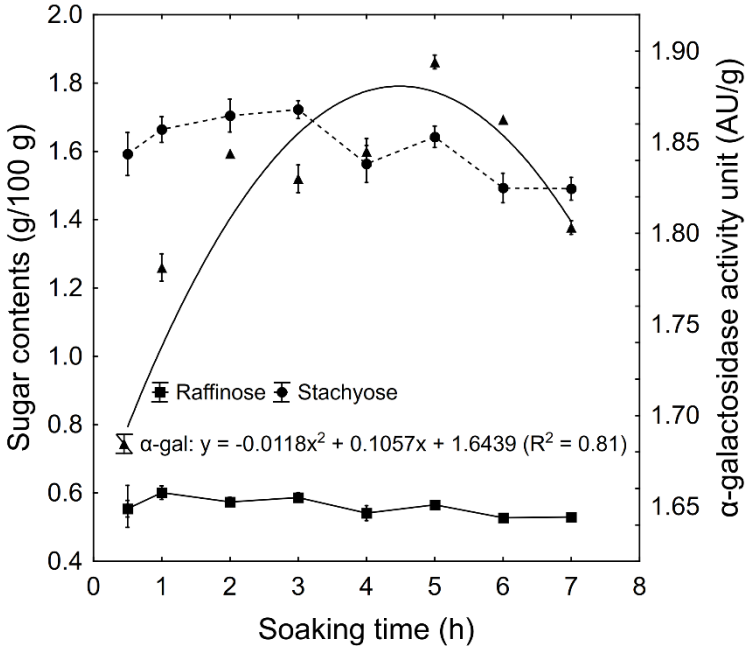


Figura 2 - Changes in the contents (n = 4) of oligosaccharides and activity (n = 4) of α-galactosidases throughout the soybean soaking (n = 2) period at 40 °C.
The solid lines without regression lines are provided as a visual guide only.

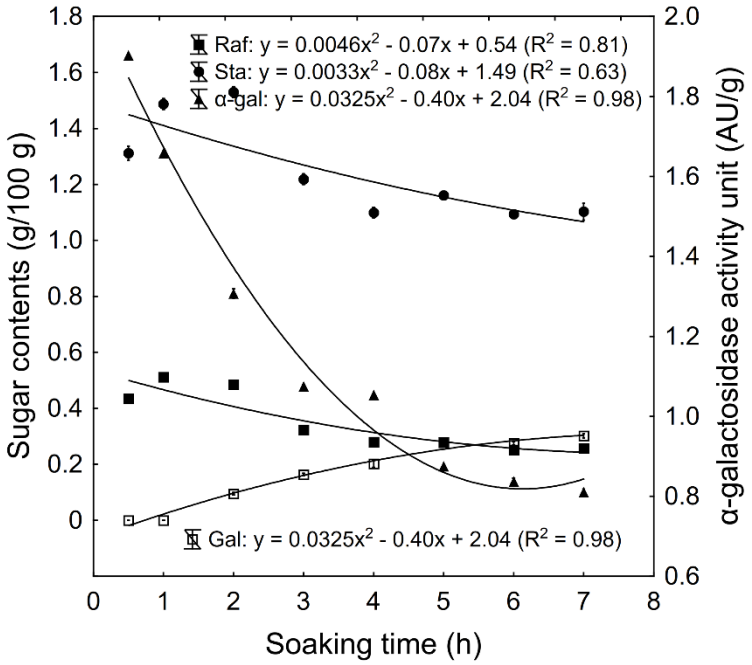


Figura 3 - Changes in the contents (n = 4) of oligosaccharides and galactose as well as the activity (n = 4) of α-galactosidases throughout the soybean soaking (n = 2) period at 55 °C. Raf = raffinose, Sta = stachyose and Gal = galactose.
The solid lines without regression lines are provided as a visual guide only.

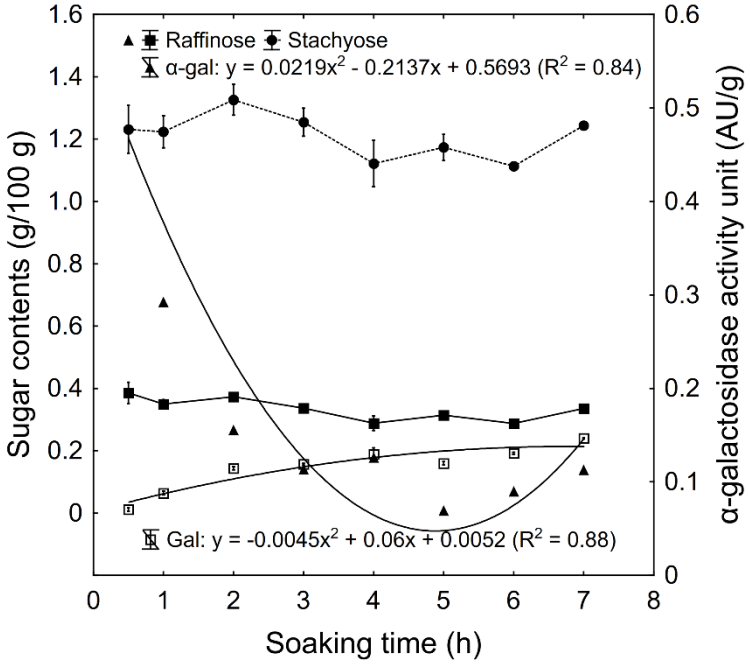


Figura 4 - Changes in the contents (n = 4) of oligosaccharides and galactose as well as the activity (n = 4) of α-galactosidases throughout the soybean soaking (n = 2) period at 70 °C. The solid lines without regression lines are provided as a visual guide only.

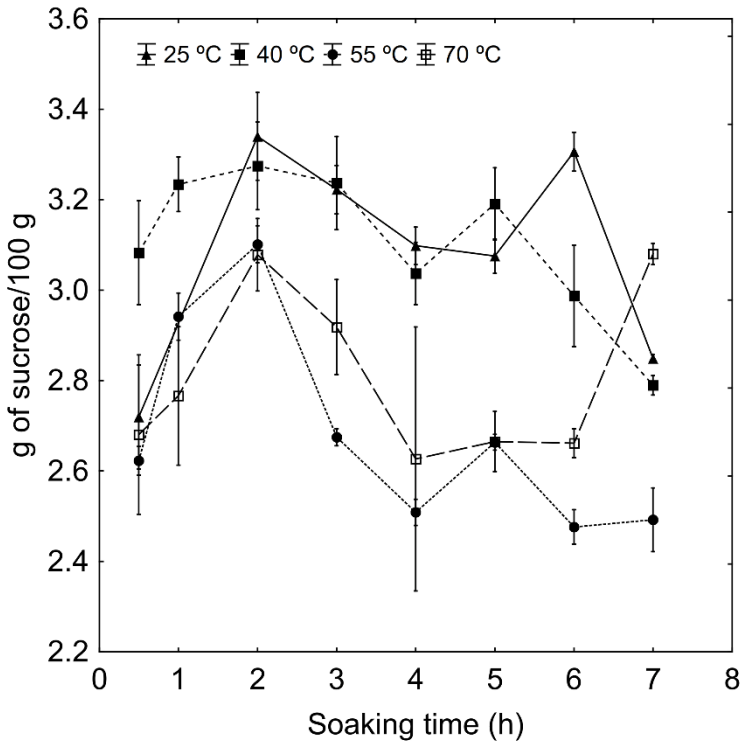


Figura 5 - Changes in sucrose content (n = 4) throughout the soybean soaking (n = 2) period at 25, 40, 55 and 70 °C. The solid lines without regression lines are provided as a visual guide only.

6 CONCLUSÕES

O tempo e temperatura de hidratação da soja foram fatores importantes e significativos na absorção de água, dureza, conteúdos de proteínas solúveis, isoflavonas e açúcares e na ação de β-glicosidases e α-galactosidases dos grãos hidratados.

A partir de 5 h de hidratação da soja e temperatura acima de 40 °C manteve-se a dureza dos grãos hidratados e melhorou a extração das proteínas solúveis.

O tempo de hidratação da soja a 25 °C não influenciou o conteúdo e perfil das isoflavonas das séries de daidzeína (daidzeína, daidzina e malonil daidzina), genisteína (genisteína, genistina e malonil genistina) e gliciteína (glicitina e malonil glicitina). Entretanto, o tempo de hidratação da soja a 40, 55 ou 70 °C influenciou o fenômeno de lixiviação, conversão e/ou degradação de isoflavonas. A partir do modelo de regressão foi estimado que no tempo de 5,43 h de hidratação dos grãos a 40 °C o conteúdo de daidzeína foi máximo e no tempo de 5 a 6 h a conversão de genistina em genisteína foi máxima. Os grãos hidratados por 6 h a 55 °C apresentaram um conteúdo de daidzeína e genisteína de respectivamente, 6 e 7 vezes maior do que nos grãos não hidratados. A hidratação da soja a partir de 1 h a 70 °C promoveu a lixiviação e degradação de isoflavonas malonil daidzina, malonil genistina e malonil glicitina.

O tempo e temperatura de hidratação da soja a 25 ou 40 °C não promoveu alterações significativas no conteúdo de rafinose e estaquiose. Contudo, o tempo de hidratação da soja a 25, 40, 55 e 70 °C influenciou a ação da α-galactosidase endógena. A hidratação da soja por até 3 h a 55 ou 70 °C favoreceu a ação da α-galactosidase para hidrolisar os oligossacarídeos rafinose e estaquiose em galactose e sacarose. Após 3 h de hidratação da soja a 55 °C houve uma redução de 45% no conteúdo de rafinose e 25% no conteúdo de estaquiose. Contudo, o tempo de hidratação dos grãos a 25, 40, 55 e 70 °C não influenciou significativamente o conteúdo de sacarose.

Portanto, independentemente do tempo de hidratação dos grãos de soja a 40, 55 ou 70 °C, ocorreram fenômenos de lixiviação, conversão e/ou degradação de isoflavonas e açúcares.

7 CONSIDERAÇÕES FINAIS

As condições de processo de hidratação da soja são relevantes para o preparo de diferentes produtos de soja e as variáveis tempo e temperatura devem ser estabelecidas adequadamente para promover a formação de isoflavonas agliconas, preservar o conteúdo total de isoflavonas e proteínas solúveis, sem afetar a absorção de água e dureza dos grãos hidratados. Além disto, a hidratação da soja entre 40 e 55 °C pode ser utilizada para hidrólise parcial de rafinose e estaquiose e aumento no teor de galactose e sacarose.

Neste contexto, considerando os efeitos das variáveis tempo e temperatura de hidratação da soja sobre as alterações das propriedades dos grãos e teor de isoflavonas e açúcares, recomenda-se que a hidratação da soja seja realizada por 5 h a 55 °C como uma etapa prévia à elaboração de produtos de soja para promover maior presença de isoflavonas agliconas e redução parcial de oligossacarídeos.