UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Fração enriquecida em isoflavonas agliconas da soja (*Glycine max*): Estudos farmacotécnicos, físico-químicos, analíticos e de permeação cutânea

MARINA CARDOSO NEMITZ

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Fração enriquecida em isoflavonas agliconas da soja (*Glycine max*): Estudos farmacotécnicos, físico-químicos, analíticos e de permeação cutânea

Tese apresentada por **MARINA CARDOSO NEMITZ** para obtenção do GRAU DE DOUTOR em Ciências Farmacêuticas.

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Coorientador: Profa. Dra. Gilsane von Poser

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“A educação é a arma mais poderosa que você pode usar para mudar o mundo.”

Nelson Mandela

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RESUMO

Isoflavonas são substâncias fenólicas conhecidas por promoverem diversos efeitos benéficos à pele. Na soja, esses compostos são essencialmente encontrados nas formas glicosiladas. No entanto, as atividades biológicas são principalmente relacionadas com as suas formas agliconas. A presente tese teve como principal objetivo a obtenção de uma fração enriquecida em isoflavonas agliconas e a sua incorporação em formulações tópicas visando o aumento da retenção cutânea de tais compostos. Para tanto, foi realizada primeiramente a extração de soja em meio hidroetanólico seguida de processo clássico hidrolítico (hidrólise ácida) para obtenção das formas livres das isoflavonas. O extrato obtido foi avaliado por um método cromatográfico de ultraeficiência, o qual foi desenvolvido e validado para a determinação quantitativa das isoflavonas agliconas (IA), bem como para avaliação das principais impurezas furânicas formadas. O método mostrou-se específico, linear, preciso, robusto e exato para a quantificação das IA daidzeína, gliciteína e genisteína, e para a quantificação das impurezas hidroximetilfurfural (HMF) e etoximetilfurfural (EMF). Além disso, o método foi concomitantemente validado para a quantificação das IA em diferentes matrizes analíticas e bioanalíticas visando estudos de desenvolvimento de produtos tópicos e estudos de permeação/retenção cutânea. Devido à toxicidade relatada na literatura para o HMF, o presente estudo investigou *in vitro* o potencial genotóxico e mutagênico do EMF, principal impureza formada após degradação ácida dos açúcares presentes no extrato hidroetanólico de soja. O EMF apresentou potencial genotóxico em linhagem celular do tipo HepG2 nas concentrações próximas ao teor encontrado no extrato. Devido a isso, foi realizada uma investigação de métodos alternativos ao da hidrólise ácida visando à obtenção das IA da soja sem a presença de HMF e EMF, e facilitando, desta forma, os processos de purificação até uma fração enriquecida em IA. Para tanto, foram realizadas otimizações de processos hidrolíticos mais seletivos, tais como hidrólise enzimática com β-glicosidase e fermentação com *Saccharomyces cerevisiae*. Os processos foram primeiramente avaliados por desenho experimental de Plackett-Burman para determinação dos principais fatores que podem influenciar durante os bioprocessos hidrolíticos. Posteriormente, os métodos foram otimizados por meio de desenho experimental de Box-Behnken. Destaca-se que os processos foram

realizados com um extrato equivalente ao extraído de 1,0 g de soja desengordurada. Para o método enzimático, as condições ótimas de processo compreenderam 838 unidades de β-glicosidase, em pH 6.0, durante 4,5 h à 37 ºC. Para o método fermentativo, as condições ótimas de processo hidrolítico compreenderam 1500 mg de fermento comercial contendo *Saccharomyces cerevisiae,* em pH 7.6, durante 24 h à 33 ºC. Os extratos foram avaliados quanto ao teor de IA, sempre realizando a sua comparação com um extrato não hidrolisado (controle negativo) e com um extrato hidrolisado pela via ácida (controle positivo). O teor de IA após hidrólise enzimática foi estatisticamente semelhante ao controle positivo. No entanto, o processo de fermentação não permitiu completa hidrólise das isoflavonas, sendo necessária a realização de um processo adicional de hidrólise ácida ao final do processo fermentativo. Todos os extratos hidrolisados, ou seja, os obtidos pelas vias ácida, enzimática, fermentativa, fermentativa+ácida, foram avaliados quanto à presença ou ausência de açúcares, compostos furânicos, triterpenos e saponinas. Além disso, todos passaram por processo de partição líquido-líquido com acetato de etila para obtenção de diferentes frações. A fração que apresentou maior pureza e que foi considerada a mais promissora para escalonamento de produção foi obtida após partição do extrato de soja hidrolisado pela via enzimática. Essa fração foi avaliada quanto ao seu potencial *in vitro* de proliferação de queratinócitos (HaCaT). Após 48 h de tratamento celular, os resultados demonstraram que a fração na concentração de 0,1 µg de IA/mL foi considerada não citotóxica para as células e apresentou promissora atividade proliferativa de queratinócitos. Desta forma, tal fração foi escolhida para ser incorporada em nanoemulsões visando uma futura aplicação tópica durante processos regenerativos de tecidos cutâneos. As formulações foram espessadas, ou não, até hidrogéis de ácido hialurônico. Por fim, foi realizada a avaliação da capacidade de permeação/retenção cutânea das IA a partir das formulações utilizando modelo experimental *in vitro* com pele de orelha suína. Tanto as nanoemulsões quanto os hidrogéis demonstraram ser promissores sistemas de liberação das IA na pele, aumentando sua retenção em camadas cutâneas consideradas importantes para exercerem seus efeitos benéficos.

Palavras-chave: Fração enriquecida em isoflavonas agliconas; Impurezas furânicas; Nanoemulsões; Permeação/retenção cutânea; Processos hidrolíticos.

ABSTRACT

Isoflavone aglycones-rich fraction from soybeans (*Glycine max*): Technological, physicochemical, analytical and skin permeation studies

Isoflavones are phenolic compounds that promote several benefic effects on the skin. In soybeans, these substances are present in different chemical conformations, mostly as glycoside conjugates. However, the beneficial activities are mainly credited to their aglycone forms. The main goal of this study was the production of an isoflavone aglycone-rich fraction to be incorportated into topical products intended to increase the skin retention of isoflavone aglycones (IA). For that, firstly it was performed a soybean extraction in a hydroethanolic media followed by a classical hydrolysis procedure (acid hydrolysis) to breakdown the conjugated forms until their respective aglycones. To analyze the obtained extract, an ultra-fast liquid chromatography method was developed and validated. The method showed to be specific, linear, precise, robust and accurate for quantification of daidzein, glycitein and genistein, as well as for hydroxymethylfurfural (HMF) and ethoxymethylfurfural (EMF), the main impurities present in the acid hydrolyzed extract. Besides, the method was concomitantly validated for determination of IA in different analytical and bioanalytical matrices during development of topical products and permeation/retention assays. Due to the well-documented toxicity of HMF, this study also investigated *in vitro* the genotoxic and mutagenic potential of EMF, a sugar degradation product formed after acid hydrolysis of soybean extract. EMF showed to be genotoxic for HepG2 cells at concentrations very close to those present in the extract. So, alternative hydrolysis methods were carried out aiming to obtain the IA from soybeans without the presence of HMF and EMF, and thus facilitating the processes of purification until new IA-rich fractions. For that, procedures with more selectivity properties were accomplished, such as enzymatic hydrolysis with β-glucosidase and fermentation with *Saccharomyces cerevisiae*. These methods were firstly evaluated by a Plackett-Burman design, to screen the main factors that could be influencing during the hydrolysis of isoflavones present in soybean extract. Further, the methods were optimized by Box-Behnken design. It is important to highpoint that the processes were always carried out with a sample corresponding to

the extracted from 1.0 g of defatted soybeans. The highest IA content was achieved under the optimal conditions: enzymatic hydrolysis with 838 units of β-glucosidase, during 4.5 h at pH 6.0 and 37 ºC, or fermentative process with 1500 mg of commercial bakery yeast (*Saccharomyces cerevisiae)* during 24 h at pH 7.6 and 33 ºC. The extracts were investigated to evaluate the IA content, and the results were always compared with a non-hydrolyzed extract (negative control) and with an acid hydrolyzed extract (positive control). The IA amount after enzymatic hydrolysis was statistically similar than positive control. However, the fermentation did not hydrolyze completely the isoflavones, being necessary an additional acid hydrolysis at end of fermentative process. The extracts obtained by acid, enzymatic, fermentative and fermentative+acid processes were investigated to analyze the presence or absence of sugars, furanic compounds, triterpenes and saponins. Besides, all of them were partitioned with ethyl acetate to obtain IA-rich fractions. The most pure and easily achieved fraction was the one obtained from enzymatic hydrolyzed extract. This fraction was examined to explore its *in vitro* proliferation ability in keratinocytes cells (HaCaT). After 48 h of cellular treatment, the results showed that the fraction at 0,1 µg de IA/mL was considered non-cytotoxic for cells, and showed a promising proliferative activity. Therefore, this fraction was chosen to be incorporated in nanoemulsions viewing a future topic application during regenerative processes of skin. Moreover, the formulations were thickened, or not, until hyaluronic acid hydrogels. Lastly, it was performed the skin permeation/retention evaluation for IA from formulations using an *in vitro* experimental model with porcine ear skin. Both nanoemulsions and hydrogels were considered as promisor delivery systems for IA, increasing their retention in skin layers (epidermis and dermis).

Palavras-chave: Furanic impurities; Hydrolysis methods; Isoflavone aglycones-rich fraction; Soybean extract; Topical formulations.

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LISTA DE ABREVIATURAS

ANOVA: análise de variância

BBD: *Box Behnken Design*

CD: *cyclodextrin* – ciclodextrina

CL: Cromatografia Líquida

CLAE: Cromatografia Líquida de Alta Eficiência

CLN: carreador lipídico nanoestruturado

CLUE: Cromatografia Líquida de Ultraeficiência

CO_2: dióxido de carbono

DAD: detector de arranjo de diodos

DAID: *daidzein* – daidzeína

DAI: *daidzein* – daidzeína

DF: *damage frequency* – frequência de dano

DI: *damage index* – índice de dano

DMEM: *Dulbecco's Modified Eagle's medium*

DMSO: *dimethyl sulfoxide*

DNA: ácido desoxirribonucleico

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

DS*: defatted soybeans* –soja desengordurada

DSS*: defatted soybean seeds* – sementes de soja desengordurada

EDTA: *ethylenediaminetetraacetic acid*

EH: *enzymatic hydrolysis* – hidrólise enzimática

EMBRAPA: Empresa Brasileira de Pesquisa Agropecuária

EMEA: *European Medicines Agency*

EMF: 5-etoximetil-2-furfural

EPO: *European Patent Office*

ER: *estrogen receptor* – receptor estrr

ESI: electrospray ionization

FBS: *Fetal bovine serum*

FCO: Fosfatidilcolina de Ovo

FDA: *Food and Drug Administration*

FIA: fração de isoflavonas agliconas da soja

FP: *fermentative process* – processo de fermentação

FPG: *Formamido pyrimidine glycosylase protein*

F-SE_{AH}: *fraction from soybean acid hydrolyzed extract*

F-SE_{EH}: *fraction from soybean extract after enzymatic hydrolysis*

F-SE_{FP}: *fraction from soybean fermented extract*

F-SFE_{AH}: *fraction from soybean fermented extract after acid hydrolysis process*

GEN: *genistein* - genisteína

GLY: *glycitein* - gliciteína

HA: *hyaluronic acid* – ácido hialurônico

HaCaT: *spontaneously transformed aneuploid immortal keratinocyte cell line from adult human skin*

HCl: ácido clorídrico

HMF: 5-hidroximetilfurfural

H-NE: nanoemulsão espessada em hidrogel

HPLC: *high pressure liquid chromatography*

HRT: *hormone replacement therapies* – terapias de reposição hormonal

HSE: hydroethanolic soybean extract – extrato de soja hidroetanólico

IA: *isoflavone aglycones* – isoflavonas agliconas

IAF: *isoflavone aglycones fraction* – fração enriquecida em isoflavonas agliconas

ICH: *International Conference on Harmonisation*

INPI: Instituto Nacional de Propriedade Industrial

IP: *intellectual property* – propriedade intelectual

JPO: *Japan Patent Office*

LO: lecitina de gema de ovo

LogP: partition coeficiente – coeficiente de partição

MCT: *medium chain triglycerides* – triglicerídeos de cadeia média

MEG: monoestearato de glicerila

MI: mutagenic index – índice de mutagenicidade

MSR: Metodologia de superfície de resposta

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt

NaOH: hidróxido de sódio

NaCl: cloreto de sódio

NC: *negative control* – controle negativo

NaN_3: azida de sódio

NE: *nanoemulsions* – nanoemulsões

NE_B: *blank nanoemulsions* – nanoemulsões brancas

NE_{FIA}: *nanoemulsions containing fraction enriched in isoflavone aglicones*– nanoemulsões contendo fração enriquecida em isoflavonas agliconas

NLS: nanopartícula lipídica sólida

NMR: *Nuclear Magnetic Resonance* – ressonância nuclear magnética

o/w: *oil in water* – óleo em água

OECD: *Organisation for Economic Co-operation and Development*

PBS: *phosphate-buffered saline* – tampão fosfato salina

P&D: Pesquisa e Desenvolvimento

P.E/D: *porcine epidermis/dermis* – epiderme+derme de pele de orelha suína

P.EM: *porcine esophageal mucosa* – mucosa de esófago suíno

PAR-2: *protease type 2* – protease tipo *?*

PC: *positive control* – controle positivo

PCT: *Patente Cooperation Treaty* – Tratado de Cooperação de Patentes

PDA: *photodiode array detection* - detector de arranjo de diodos

PDI: *polydispersity index* – índice de polidispersão

PI: propriedade intelectual

PG: propilenoglicol

PSC: *porcine stratum corneum layer after tape stripping method*

R&D: *Research and Development* - Pesquisa e Desenvolvimento

r^2: *coefficient of determination* – coeficiente de determinação

r: *coefficient of determination* – coeficiente de correlação

RE: receptor estrogênico

RF: *receptor fluid* – fluído receptor

ROS: *reactive oxygen species* – espécies reativas de oxigênio

RSD: relative *standard deviation* – desvio padrão relativo

RSM: *response surface methodology* – metodologia de superfície de resposta

S9 mix: external *metabolic activation* – ativação metabólica externa

SAE: *soybean acid extract* – extrato de soja hidrolisado pela via ácida

SD: *standard deviation* – desvio padrão

SE_{AH}: *soybean acid hydrolyzed extract*

SE_{EH}: *soybean extract after enzymatic hydrolysis*

SE_{FP}: *soybean fermented extract*

SFE_{AH}: *soybean fermented extract after acid hydrolysis process*

SIPO: *State Intellectual Property Office of the People's Republic of China*

SLN: *solid lipid nanoparticles* – nanopartículas lipídicas sólidas

TCM: triglicerídeos de cadeia média

TIA: *total isoflavone aglycones* – isoflavonas agliconas totais

TGF-β1: *transforming growth factor β1*

UFLC: *Ultra-Fast Liquid Chromatography*

USPTO: *U.S. Patent and Trademark Office*

UV: *ultraviolet* - ultravioleta

UV-Vis: *ultraviolet - visible* ; ultravioleta - visível

w/o*: water in oil* – água em óleo

WIPO: *World Intellectual Property Organization*

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INTRODUÇÃO

A soja (*Glycine max*) é uma leguminosa amplamente cultivada e consumida (CHEN *et al*., 2012). O Brasil é atualmente o segundo maior produtor mundial e está entre os maiores exportadores de seus grãos. Esse cenário postivo se deve, dentre muitos fatores, ao importante papel exercido pela Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) no desenvolvimento de cultivares altamente produtivas, enriquecidas em compostos específicos e adaptadas a diferentes regiões climáticas do país (EMBRAPA, 2016a).

Pertencente à família Fabaceae, a soja é caracterizada por ser um alimento funcional rico em proteínas e fitoestrógenos, apresentando diversos efeitos benéficos ao organismo (MESSINA, 1999; MESSINA, 2010; MESSINA, 2014). Nos grãos de soja, assim como em seus derivados, as características biológicas de interesse são comumente atribuídas às isoflavonas. Essas substâncias são estruturalmente semelhantes ao 17-β-estradiol, possuindo atividade estrogênica comprovada e por isso denominadas de fitoestrógenos (KUIPER *et al*., 1998; SONG, 1999; MUELLER, 2002; HWANG *et al*., 2006). As isoflavonas desempenham um papel importante na prevenção de diversos eventos crônicos como doenças cardíacas, osteoporose, câncer e diabetes, além de atuarem na redução de sintomas climatéricos na mulher em menopausa e pós-menopausa (SETCHELL, 1998; BARNES, 1998, BARNES *et al.*, 2000; ALBERTAZZI & PURDIE, 2002; MORTENSEN *et al*., 2009; FRITZ *et al*., 2013).

Além dos efeitos supracitados, uma vasta literatura descreve os efeitos benéficos que as isoflavonas apresentam sobre a pele, estando estes geralmente relacionados com as suas formas agliconas. Diversos estudos *in vitro* e *in vivo* sugerem que as isoflavonas da soja apresentam atividade antioxidante (WEI *et al*., 1995; WEI *et al*., 2002; GEORGETTI *et al*., 2006; GEORGETTI *et al*., 2009; GEORGETTI *et al*., 2013), anti-melanoma (PARK *et al*., 2011a; DANCIU *et al*., 2013), anti-inflamatória (GEORGETTI *et al*., 2008; KHAN *et al*., 2012), anti-herpética (ARGENTA *et al*., 2015), anti-envelhecimento, fotoprotetora (SUDEL *et al*., 2005; HUANG *et al*., 2008; LIN *et al*., 2008; HUANG *et al*., 2010; IOVINE *et al*., 2012; IOVINE *et al*., 2014) e cicatrizante (HWANG *et al*., 2001; EMMERSON *et al*., 2010; PARK *et al*., 2011b; TIE *et al*., 2013).

Com base nas diversas atividades dermatológicas apresentadas pelas isoflavonas, observa-se um interesse crescente no desenvolvimento de produtos/formulações contendo extratos de soja, frações e/ou isoflavonas isoladas visando aplicação em diferentes áreas das indústrias farmacêutica e cosmética (SHIDHAYE *et al*., 2008; CHEN *et al*., 2012; WAQAS *et al*., 2015). No entanto, apesar de ser promissora a sua administração tópica, a incorporação das isoflavonas agliconas em formulações livres de solventes orgânicos é desafiadora, especialmente devido à reduzida hidrossolubilidade que essas substâncias apresentam (SCHMID *et al*., 2003; ALBULESCU & POPOVICI, 2007).

Nesse contexto, o uso da nanotecnologia apresenta-se como uma alternativa promissora para a veiculação de isoflavonas agliconas em formulações de uso tópico de caráter hidrofílico. Na literatura são encontrados estudos de incorporação dessas substâncias, principalmente genisteína, em sistemas nanoestruturados, tais como lipossomas, nanopartículas e nanocápsulas (SCHMID *et al*., 2003; DWIECKI *et al*., 2009; ZAMPIERI *et al*., 2013; DESHMUKH & AMIN, 2013). Além desses sistemas, nosso grupo de pesquisa demonstrou as potencialidades da veiculação da genisteína em nanoemulsões, relatando um aumento da permeação/retenção cutânea desta isoflavona isolada quando aplicada sobre a pele (SILVA *et al*., 2007, SILVA *et al*., 2009, ARGENTA *et al.,* 2014).

Embora a grande maioria dos estudos de desenvolvimento de formulações tópicas descreva produtos contendo apenas genisteína, alguns pesquisadores têm destacado que o uso de extratos ou misturas de isoflavonas pode potencializar especialmente as ações estrogênicas e fotoprotetoras quando comparado com as das isoflavonas isoladas (KAO & CHEN, 2006; RANDO *et al.*, 2009; HUANG *et al.,* 2010; IOVINE *et al.,* 2011). Dessa forma, extratos de soja contendo simultaneamente mais de uma isoflavona, principalmente as agliconas, podem ser considerados insumos promissores em relação a substâncias isoladas durante a fabricação de produtos tópicos.

Para obter um extrato enriquecido nas isoflavonas agliconas é recomendada a realização da extração das sementes moídas e desengorduradas de soja com posterior etapa de hidrólise mediada por catalisadores químicos ou biológicos (ROSTAGNO *et al*., 2009). Isso se deve, basicamente, ao fato de que naturalmente

na soja são encontradas as formas conjugadas das isoflavonas. No entanto, é válido destacar que a escolha do método hidrolítico a ser empregado é fundamental na qualidade do produto obtido, uma vez que produtos de degradação indesejáveis oriundos de outros componentes da soja podem ser formados quando métodos não seletivos são utilizados (NEMITZ *et al.,* 2015). Dessa forma, para evitar processos longos de purificação para retirada de impurezas, métodos biotecnológicos de alto índice de seletividade podem ser utilizados a fim de hidrolisar apenas compostos β-glicosilados, tais como isoflavonas, evitando-se produtos de degradação proveniente de outros compostos presentes na soja.

Face ao exposto, a presente tese visa obter uma fração enriquecida em isoflavonas agliconas (FIA) e realizar a sua incorporação em formulações para administração tópica cutânea. Destaca-se que para alcançar tal objetivo, diversos estudos se tornam necessários, e para efetuar uma melhor organização dos experimentos a serem realizados, estes foram divididos em etapas, tais como (1) o desenvolvimento e a validação de um método cromatográfico para avaliação quantitativa das isoflavonas agliconas nos produtos, bem como nos ensaios bioanalíticos de retenção/permeação cutânea; (2) a avaliação das impurezas que podem estar presentes nos extratos de soja obtidos por diferentes vias hidrolíticas; (3) a obtenção de uma FIA altamente pura por meio de um processo simples e rápido; (4) a incorporação da FIA em nanoemulsões espessadas, ou não, até hidrogéis e a avaliação da retenção/permeação cutânea das isoflavonas a partir das formulações. Nesse sentido, a presente tese foi dividida em capítulos, com encarte de publicações, onde primeiramente estão descritas as revisões de literatura científica e tecnológica, e na sequência estão apresentados os capítulos da parte experimental. Os capítulos foram organizados da seguinte forma:

- Capítulo I – Artigo de revisão científica: *Bioactive soy isoflavones - Extraction and purification procedures, potential dermal use and nanotechnology-based delivery systems*;

- Capítulo II – Artigo de revisão tecnológica: *The international scenario of patents concerning isoflavones*;

- Capítulo III – Artigo científico: *A versatile, stability-indicating and high-throughput ultra-fast liquid chromatography method for the determination of isoflavone aglycones in soybeans, topical formulations and permeation assays*;

- Capítulo IV – Artigo científico: *Determination of furanic compounds in soybean acid hydrolyzed extracts and the in vitro mutagenicity and genotoxicity studies of 5-ethoxymethyl-2-furfural*;

- Capítulo V - Artigo científico: *Optimization of soybean extracts by different biocatalysis systems and purification processes aiming to obtain an isoflavone aglycone-rich fraction with promising wound healing property*;

- Capítulo VI - Artigo científico: *In vitro skin permeation/retention of daidzein, genistein and glycitein from a soybean isoflavone rich fraction-loaded nanoemulsions and derived hydrogels*.

OBJETIVOS

Objetivo geral

A presente tese de doutorado tem por objetivo otimizar e caracterizar uma fração enriquecida em isoflavonas agliconas da soja (*Glycine max*) visando o desenvolvimento de nanoemulsões espessadas com ácido hialurônico para aplicação cutânea.

Objetivos específicos

- Realizar a extração de sementes desengorduradas de soja seguida de hidrólise por diferentes métodos, bem como realizar a purificação dos extratos para obtenção das isoflavonas agliconas;

- Desenvolver e validar um método cromatográfico para determinação quantitativa de isoflavonas agliconas (daidzeína, gliciteína e genisteína) em extratos hidrolisados de soja, formulações tópicas e ensaios de permeação/retenção cutânea;

- Avaliar a adequabilidade e validar o método cromatográfico para determinação quantitativa de impurezas furânicas (hidroximetilfurfural e etoximetilfurfural) que porventura tenham sido formadas durante os processos hidroliticos de extratos de soja;

- Avaliar o potencial mutagênico e genotóxico do etoximetilfurfural, uma das principais impurezas furânicas formada após hidrólise ácida do extrato de soja;

- Avaliar diferentes bioprocessos hidrolíticos das isoflavonas da soja por meio de métodos enzimático (β-glicosidase) e fermentativo (*Saccharomyces cerevisiae*), bem como otimizar as condições desses processos por meio de ferramentas estatísticas;

- Obter diferentes frações a partir dos extratos da soja hidrolisados pelas vias ácida, enzimática e fermentativa, e determinar a fração mais promissora em termos de pureza de isoflavonas agliconas e facilidade de obtenção;

- Avaliar a capacidade proliferativa *in vitro* da fração escolhida em cultura celular de queratinócitos;

- Preparar e caracterizar nanoemulsões e hidrogéis derivados contendo a fração de isoflavonas agliconas da soja e avaliar a retenção/permeação cutânea destes compostos a partir das formulações desenvolvidas.

CAPÍTULO I

Revisão da literatura científica

1.1. INTRODUÇÃO

A obtenção de compostos ativos a partir de matérias-primas vegetais pode ser realizada através de difentes tipos de processos extrativos e o rendimento final dos compostos de interesse dependerá das condições escolhidas a serem empregadas durante tais processos (SEIDEL, 2006).

No caso de processos envolvendo a soja, muitos estudos são encontrados na literatura descrevendo diferentes condições extrativas para realizar a obtenção das isoflavonas. Essas substâncias apresentam diversas atividades biológicas, e, devido a isso, pesquisadores das áreas farmacêutica, alimentar e cosmética vêm constantemente aperfeiçoando as técnicas de extração e etapas adicionais de processos, tais como desengorduramento e hidrólise, a partir dos grãos e derivados de soja (LUTHRIA & NATARAJAN, 2009; ROSTAGNO *et al*., 2009).

Dentre as diversas atividades biológicas apresentadas pelas isoflavonas, destacam-se as atividades estrogênica e antioxidante (SCHMID & ZULLI, 2002; SUDEL *et al*., 2005; HUANG *et al*., 2008; BROWNLOW *et al*., 2015). Devido a isso, ampla literatura é encontrada a fim de avaliar as atividades que as isoflavonas isoladas, ou contidas em derivados de soja, podem apresentar na pele (WAQAS *et al*., 2015).

Em relação à aplicação dérmica, diversos estudos descrevem o desenvolvimento de formulações tópicas contendo isoflavonas, principalmente genisteína. Destaca-se que diversas são as formulações que estão sendo propostas, apresentando descrições desde sua incorporação a sistemas tradicionais, como emulsões óleo em água, até estudos mais diferenciados utilizando nanotecnologia (GEORGETTI *et al*., 2006; DWIECKI *et al*., 2009; VARGAS *et al*., 2012; ZAMPIERI *et al.,* 2013). Por outro lado, poucos relatos são encontrados visando o desenvolvimento de formulações tópicas contendo extratos de soja.

Para melhor relatar a vasta gama de literatura acerca do tema exposto, este capítulo descreve uma revisão bibliográfica abordando as diferentes formas de obtenção das isoflavonas da soja, suas atividades sobre a pele e tipos de formulações contendo tais substâncias. O conteúdo está apresentado na forma de artigo de revisão, redigido nas normas do periódico em que foi publicado.

1.2. ARTIGO

Phytochemistry Reviews 14 (2015) 849–869

BIOACTIVE SOY ISOFLAVONES: EXTRACTION AND PURIFICATION PROCEDURES, POTENTIAL DERMAL USE AND NANOTECHNOLOGY-BASED DELIVERY SYSTEMS

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ABSTRACT

Isoflavones are polyphenol compounds found mainly in legumes such as soybeans (*Glycine max* (L.) Merrill). These compounds can be found in different chemical forms; however, the beneficial effects for skin care have been mainly credited to their free forms. This manuscript claims to review the main effects of isoflavone aglycones on the skin, the different techniques for obtaining bioactive forms from soybeans, and the interest in incorporating them into topical systems. The benefits of dermatological application of isoflavones, as anti-aging action, estrogenic activity, wound healing properties, and antiphotocarcinogenic effects are highlighted. Moreover, the advantages and drawbacks of the extraction techniques of soybeans, methods for converting glucosides into aglycones, and purification procedures are described. Different strategies to incorporate these poorly soluble compounds in conventional or nanostructured delivery systems are also discussed. Illustrative examples especially for genistein-loaded liposomes, nanoemulsions, nanocapsules and cyclodextrin complexation are reported.

Keywords: dermatological application; extraction techniques; nanostructured delivery systems; skin care; soybean isoflavones

INTRODUCTION

Soy (*Glycine max* (L.) Merrill) is a legume originating from Asia, widely distributed throughout the world and of great economic importance (Zuanazzi and Mayorga 2010; Chen et al. 2012). At first, it represented a rich source of protein and vegetable oil, widely used for preparing food products (Chen et al. 2012). More recently, however, its pharmacological importance has been recorded, as its consumption is linked to the reduction of menopausal symptoms and prevention of chronic diseases such as certain cancers, cardiovascular disease, osteoporosis and diabetes (Barnes 1998; Setchell 1998).

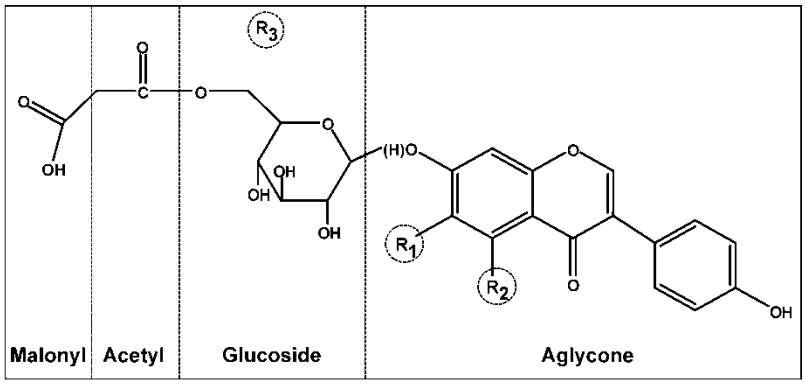
Among the various compounds present in this plant, isoflavones have attracted the most attention in the pharmaceutical field. Isoflavones are structurally similar to 17-β-estradiol, having proven estrogenic activity and, therefore, are called phytoestrogens (Barnes 2010). Studies have shown that they play an important role in preventing several chronic diseases, in addition to reducing climacteric symptoms in menopausal and postmenopausal women (Barnes 1998; Setchell 1998; Albertazzi and Purdie 2002) and having beneficial effects on the skin (Wei et al. 1995a; Song et al. 1999; Miyazaki et al. 2002; Huang et al. 2008).

Isoflavones are phenolic compounds belonging to the flavonoids class and are widely distributed in the plant kingdom, mainly in the Leguminosae family (Barnes 2010). There are twelve main isoflavones in soybeans, namely genistein, daidzein, glycitein and their respective acetyl-glucoside, malonyl-glucoside, and glucoside forms. As shown in Figure 1.1, the chemical structure of these substances is based on the presence of a flavone nucleus, which is composed of two aromatic rings attached to a heterocyclic ring, with some characteristic substituents (Rostagno et al. 2009; Shao et al. 2011).

Isoflavones are found in soybeans and their products (Barnes 2010), such as flour, fermented foods (tofu, miso, and tempeh), milk, and concentrated soy proteins, among others (Chen et al. 2012). To obtain extracts of these compounds, a number of studies have proposed increasingly elaborate, fast, and reliable extraction and quantification technologies (Rostagno et al. 2009; Luthria and Natarajen 2009). As

such, the industrial sector has become increasingly interested in extracts enriched in isoflavones for obtaining herbal and phytocosmetic products (Chen et al. 2012).

In view of the activities attributed to isoflavones, there are topical products in the market containing soy extracts. The Brazilian (Adcos®, Natura®, and Payot®), American (Aveeno®, SkinCeuticals®), and European (Vichy®) brands are examples of companies investing in products containing soy extracts mainly recommended for anti-aging action on the skin.



Isoflavone	R_1	R_2	R_3
Genistein	H	OH	H
Daidzein	H	H	H
Glycitein	OCH_3	H	H
Genistin	H	OH	$C_6O_5H_{11}$
Daidzin	H	H	$C_6O_5H_{11}$
Glycitin	OCH_3	H	$C_6O_5H_{11}$
Acetyl-genistin	H	OH	$C_6O_5H_{11} + COCH_3$
Acetyl-daidzin	H	H	$C_6O_5H_{11} + COCH_3$
Acetyl-glycitin	OCH_3	H	$C_6O_5H_{11} + COCH_3$
Malonyl-genistin	H	OH	$C_6O_5H_{11} + COCH_2COOH$
Malonyl-daidzin	H	H	$C_6O_5H_{11} + COCH_2COOH$
Malonyl-glycitin	OCH_3	H	$C_6O_5H_{11} + COCH_2COOH$

Figure 1.1. Chemical structures of soybean isoflavones (adapted from Rostagno et al. 2009).

Nevertheless, the extracts incorporated into cosmetic formulations very often have isoflavones in conjugated forms that may, in some cases, limit their biological action due to a lower possibility of penetrating the skin. To get the desired effects of

isoflavones, their free forms should preferably be used. However, they are less soluble than the conjugated forms, which may hinder their incorporation into traditional topical delivery systems (Schmid et al. 2003).

To allow better use of the aglycones in formulations and enhance their penetration, some alternatives to the traditional systems such as liposomes, micro and nanosystems, or complexes with dendrimers and cyclodextrins, are being reported (Schmid et al. 2003; Silva et al. 2009; Kitagawa et al. 2010; Xavier et al. 2010; Zhao et al. 2011).

In this context, this article aims to report the main effects of isoflavone aglycones on the skin, as well as the different techniques of obtaining bioactive forms from soybeans, and the interest in incorporating them into topical systems, including nanotechnology-based systems.

THE ACTION OF ISOFLAVONES ON THE SKIN

The skin is composed of three distinct layers: the epidermis, dermis, and hypodermis (Förster et al. 2009). Its aging is associated with the loss of elasticity, an increase of skin roughness and development of wrinkles, whose causes may be of intrinsic or extrinsic origin.

When intrinsic, the phenomenon occurs due to aging, leading to a depletion of the extracellular matrix by a decrease in collagen and elastin synthesis. When extrinsic, it takes place through exposure to ultraviolet (UV) radiation, leading to the formation of reactive oxygen species (ROS) and causing extensive tissue damage (Sudel et al. 2005; Masaki 2010).

After menopause, women experience a significant reduction in hormone levels, which is one of the intrinsic skin aging factors. Hormone replacement therapies (HRT), both oral and topical, are alternatives to maintaining the skin's thickness and elasticity. Due to their similarity to the 17-β-estradiol (**Figure 1.2**), isoflavones are strong candidates for this type of therapy since they can bind to estrogen receptors (ERs), with beneficial effects on the skin (Sator et al. 2004).

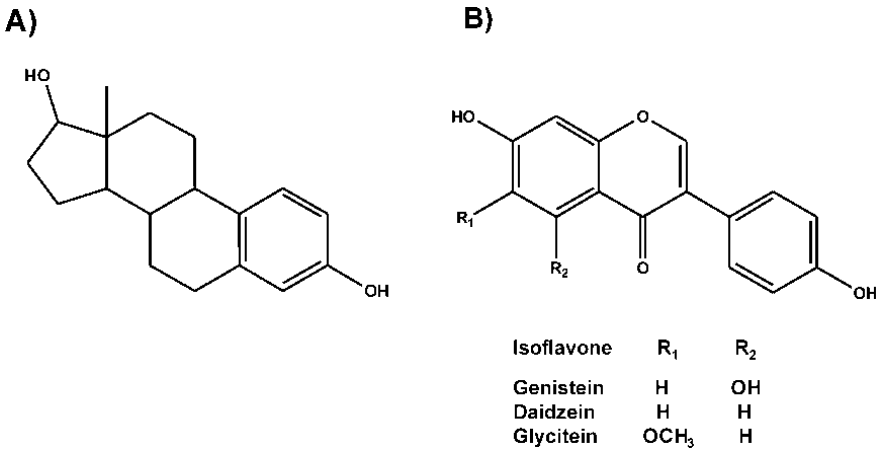


Figure 1.2. Structural similarity of: A) 17-β-estradiol and B) isoflavone aglycones, as well as the chemical orientation proposed by Barnes (2010).

There are two subtypes of estrogen receptors, ER-α and ER-β, the latter widely found in the skin, present in the epidermis, fibroblasts, and keratinocytes (Kuiper et al. 1998). Estrogens couple with ERs in the cell's nucleus, switching linked genes. This may lead to proliferative or differentiation responses (Pinnel 2003). Once bound by estrogen, the ER undergoes a conformational change allowing the receptor to interact with chromatin and modulate transcription of target genes (Kuiper et al. 1998). The presence of ER-β in the skin cells suggests that estrogen may directly modulate the synthesis of collagen, hyaluronic acid, and elastin (Sator et al. 2004).

Among the different varieties of phytoestrogens, genistein and daidzein stimulate the transcriptional activity of both ER subtypes at concentrations of 1 - 10 nM, with an affinity about 20 times greater for ER-β receptors (Kuiper et al. 1998). Even though genistein and daidzein have showed relative ER affinities 1000-fold lower than [^{3}H]-17β-estradiol (Kuiper et al. 1998), the isoflavones have shown beneficial estrogenic effects on the skin. In a clinical study by Schmid and Zulli (2002), the authors showed the occurrence of an increase in skin thickness in 20 volunteers who applied a cream containing genistein at a concentration of 90 mg/g twice a day, during three-months' treatment. Bayer and Keil (2002) showed the skin effects in a controlled multi-center study with 234 post-menopausal volunteers using a cosmetic cream preparation including isoflavone (Neovadiol®). The isoflavone cream was applied twice daily (in the morning at a concentration of 0.0075%

isoflavone and in the evening at a concentration of 0.015% isoflavone) on the face, neck and one upper arm, during 12 weeks. Skin dryness and roughness were significantly improved on the treated areas and facial wrinkles were significantly reduced. Moraes et al. (2009) conducted a randomized, double-blind study of topical administration of estradiol (0.01%) and 40% isoflavones (4% of which was genistein) on volunteers for 24 weeks. The facial skin was evaluated by biopsy, before and after treatment, to determine epidermal thickness parameters and the number of dermal papillae, fibroblasts, and blood vessels. The results showed that women treated with estradiol experienced more effective results than those treated with isoflavones, but the authors reported that additional studies need to be carried out to confirm the behavior of collagen, elastic fibers, and glycosaminoglycans present in the skin after topical treatment. In this context, another clinical study was conducted recently by Patriarca et al. (2013) in which 30 post-menopausal volunteers applied a gel with estradiol (0.01%) or genistein (4%) for 24 weeks. Afterwards, skin biopsies showed an increased concentration of hyaluronic acid in the patients in both treatments.

Moreover, several works have examined the molecular role of estrogen on the cells and metabolic processes involved in wound repair (Ashcroft et al. 1999; Brincat et al. 2005). Age related delays in wound healing have been partially attributed to low levels of transforming growth factor-β1 (TGF-β1), decreased collagen synthesis, and increased presence of proteases, specifically elastase (Brincat et al. 2005). Systemic HRT is able to accelerate healing of acute cutaneous wounds in elderly females, linked to its potent anti-inflammatory activity. However, the beneficial effects on skin wound healing are primarily mediated by epidermal ER-β, promoting restoration of the epidermis (Campbell et al. 2010). In this context, genistein has been considered a good candidate for the treatment of wounds, since different administration routes have shown wound repairs. Hwang et al. (2001) reported that topical genistein treatment in rats (1 mg / 200 g / day and 4 mg / 200 g / day) increased the production of collagen in wounds after 14 days of treatment. Emmerson et al. (2010) demonstrated that the subcutaneous administration of genistein (50 mg / kg / day) in ovariectomised female mice increased the capacity of wound healing by different pathways. The mechanisms reported were the reduction of inflammation along with an increase in fibroblast and macrophage migration, which occurred through non-ER dependence, and an increase in keratinocyte migration, which occurred through the

ER mechanism. Park et al. (2011) reported the effect of oral genistein treatment (0.1%) in mice during two weeks in the initial stages of wound healing. They showed the antioxidant actions of genistein in the tissue repair process, observing a decrease in Cu, Zn-SOD and Mn-SOD expression and a modulation in ROS production during the early stage of wound healing.

Conversely, isoflavones have shown potent anticarcinogenic effects that are largely independent of their estrogenic activities (Pinnel 2003). Alternative mechanisms that promote chemopreventive effects have been suggested, including induction of cancer cell differentiation, inhibition of protein kinases, suppression of angiogenesis, and direct antioxidant effects (Wei et al. 2003). These alternative pathways may occur at isoflavone concentrations much higher (>5 μM) than the concentrations at which estrogenic effects are detected (<100 nM), and show a different structure-activity relationship (Kuiper et al. 1998).

Many *in vitro* and *in vivo* studies, as well as a clinical study with volunteers, show the topical effect of genistein on cancer caused by UV-B radiation (Wei et al. 2003). UV-B from sunlight is closely associated with skin diseases such as melanoma and non-melanoma skin cancer. It produces inflammation and proliferation in skin, both mediated in part by activation of protein kinase C and metabolites of arachidonic acids (Wei et al. 2002). Evidences from studies of the effect of isoflavones, especially genistein, in cultured tumor cells have demonstrated antiproliferative activity due to the inhibition of certain enzymes, such as tyrosine kinase and topoisomerase II (Akiyama et al. 1987; Okura et al. 1988).

The *in vitro* antiphotocarcinogenic effect of genistein, including the increase of tyrosinase activity and dendrite-like structure formation, was reported by Kiguchi et al. (1990) in five human melanoma cell linesafter six days of treatment at a concentration of 45 μM. Wang et al. (2002) reported that genistein and daidzein exerted multiple suppressive effects in murine and human melanoma cells, including growth inhibition, cell cycle arrest, and induction of cell differentiation effects. The genistein treatment resulted in arrest of melanoma cells in the G2 phase of the cell cycle, whereas daidzein, which lacks a hydroxyl group, induced cell accumulation in the G1 phase. Genistein also induced melanoma cells to acquire dendrite-like structures and increased the production of melanin. In contrast, daidzein only

retarded the growth of these cells and failed to induce differentiation, suggesting that these isoflavones can inhibit certain malignant phenotypes of melanoma via different mechanisms (Wang et al. 2002).

Studies in animals have shown that genistein inhibits the UVB-induced skin carcinogenesis and photodamage (Wei et al. 1993; Wei et al. 1995b; Wei et al. 1998; Wei et al. 2002). The possible mechanisms of its action include the inhibition of tyrosine protein kinase, the anti-inflammatory pathways, scavenging of ROS, blocking of oxidative and photodynamic damage to DNA, downregulation of epidermal growth factor receptor phosphorylation, and suppression of oncoprotein expression.

A clinical study indicates that genistein (5 μM genistein/cm^2 human skin) effectively protects human skin against UVB-induced skin photodamage (Wei et al. 2003). Analyses of data from six subjects showed that pre-UVB application of genistein significantly inhibited both cutaneous erythema and discomfort whereas post-UVB application improved the discomfort score with a minimal effect on erythema (Wei et al. 2003). A phase II clinical trial is underway to determine the effect of genistein together with interleukin-2 in patients with metastatic melanoma or renal clear cell carcinoma. This trial is based on the assumption that genistein may stop the growth of tumor cells through its antiangiogenic effect, whereas interleukin-2 may stimulate the immune system to kill tumor cells (http://www.clinicaltrials.gov).

Table 1.1 shows a number of pre-clinical studies that summarize diverse anti-aging and antiphotocarcinogenic actions of isoflavones. It is noteworthy that most of the studies have reported biological activity for the aglycone forms of isoflavones. Once in the skin, enzymes that hydrolyze the glucosylated molecules are not found (Schmid and Zulli 2002), contrary to what happens when isoflavones are taken orally, in which their transformation takes place while passing through the intestinal tract (Sánchez-Calvo et al. 2013). Therefore, when extracting isoflavones intended for topical use, it is important to carry out procedures that allow the increase of aglycone forms, such as soybean extraction followed by hydrolysis and purification.

Table 1.1. Pre-clinical studies concerning the action of isoflavones on the skin

Samples	Evaluation	Method	Conclusions	References
Genistein solution	Antioxidant activity after acute and cronic UV-B exposure	*in vivo:* hairless mice	↓ H_2O_2 production ↓MDA production ↓ 8-OHdG production	Wei et al. (2002)
Isoflavones aglycone solutions or soy extract	Inhibition of retinoid-induced epidermal hyperplasia	*in vitro:* Keratinocyte culture cells (HDK) Fibroblast culture cells (HDF)	↓ hyperplasia ↓ keratinocyte proliferation ↑ fibroblasts ↑ synthesis of type I procollagen	Varani et al. (2004)
o/w emulsion containing 2% of genistein, daidzein or soy extract	Effects on parameters of skin aging after exposure to UV-A radiation	*in vitro:* Fibroblast culture cells (HDF)	↑ collagen ↑ hyaluronic acid ↑ glycosaminoglycan	Sudel et al. (2005)
Isoflavones aglycone solutions	Keratinocytes damage after exposure to UV-B radiation: Production of intracellular H_2O_2 and cell viability	*in vitro:* Keratinocyte culture cells (HaCaT) *in vivo:* hairless mice	↓ H_2O_2 production Keratinocyte protection	Huang et al. (2008)
Isoflavone aglycone solutions	Photoprotection after UV-B exposure	*in vivo:* Pig skin	Decrease of sunburn cell formation and/or erythema	Lin et al. (2008)
Isoflavones aglycone solutions or Soy extract rich in aglycones and acetyl-glucosides	Photoprotection after UV-B exposure	*in vitro:* Keratinocyte culture cells (HaCaT) *in vivo:* hairless mice	↓ oxidative stress ↓ inflamation Keratinocyte protection Decrease of erythema	Huang et al. (2010)

H_2O_2: hydrogen peroxide; MDA: malondialdehyde; 8-hydroxy-2'-deoxyguanosine; o/w: oil in water; ↑: increase; ↓: decrease.

OBTAINING BIOACTIVE ISOFLAVONES FROM SOYBEANS

General considerations

Morphologically, soybeans are generally composed of a seed coat (integument), cotyledon, radicle, hypocotyl, and hilum (Thorne 1981), and isoflavones are diversely distributed in these parts depending on the degree of the soybeans' maturation or variety (Eldridge and Kwolek 1983; Cho et al. 2009; Yuan et al. 2009; Britz et al. 2011) as shown in **Table 1.2**.

Table 1.2. Anatomical distribution of isoflavones in different samples of soybeans

Soybean variety	Soybean part	Total isoflavone Content (mg/ 100g)	References
USA - Tiger soybeans (TS-280)	Hull Hypocotyl Cotyledon	20.0 1405.2 319.2	Eldridge and Kwolek (1983)
Green soybean (AGA3)	Radicle Cotyledon Seed coat	203.04 102.03 61.7	Phommalth et al. (2008)
Korea - Sprouts	Whole sprout Hypocotyl Cotyledon Root	144.0 53.0 218.0 202.0	Cho et al. (2009)
China - Yellow soybean	Seed coat Hypocotyl Cotyledon	1.09 159.4 1239.2	Yuan et al. (2009)

However, when viewed as a whole, the soybean typically has isoflavone concentration between 1-2 mg/g, predominantly in the malonyl-glucoside and glucoside forms. The aglycone forms are rarely seen in the fresh plant; however, some by-products may exhibit higher levels of these free forms due to a chemical change that takes place in the compounds during production, which involves heat and/or a fermentation process (Barnes 2010).

The presence of heat during soybean extractions or food processing may trigger chemical changes, with the decarboxylation of malonyl-glucosides to acetyl-glucosides and breakdown of the ester bond being frequently observed, the latter leading to the formation of glucosides. However, sugar hydrolysis for conversion to the aglycone forms only takes place under conditions of extreme pH or in the presence of certain enzymes (Albulescu and Popovici 2007; Sontag and Schwartz 2009; Barnes 2010).

Among the sample's physicochemical properties, polarity is a key extraction factor. When working with isoflavones, it is important to note that the glucosylated forms are soluble in water, whereas the aglycone forms are hydrophobic, although they are highly soluble in polar organic solvents, such as ethanol and methanol (Albulescu and Popovici 2007).

The pretreatments of soybeans are very important, because they can actively influence the removal of the chemical compounds of interest. The most frequently used processes reported steps for soybeans and their products are drying, grinding, sieving, and degreasing (Becker 1978; Rostagno et al. 2009).

Techniques for extracting isoflavones from soybeans

Among the most commonly used solid-liquid extraction techniques for soybean extraction, the traditional ones such as reflux, Soxhlet, static and dynamic maceration, and such unconventional ones as ultrasound, microwave, supercritical fluid, and extraction with pressurized liquid, are the most noteworthy (Vacek et al. 2008; Rostagno et al. 2009; Luthria and Natarajan 2009).

The earliest studies of extraction of soy isoflavones reported extensive use of Soxhlet and reflux (Walter 1941; Naim et al. 1974). Reflux is a widely described technique for laboratory use, facilitating routine analysis because it is an easy and quick process that allows high yields during extraction. Despite having some similar features to reflux, Soxhlet has the advantage to exhaust the drug extraction when subjected to a prolonged period of time with a suitable solvent (Seidel 2006). However, both techniques have the disadvantage of using heat with a refrigeration system and, therefore, rarely used in obtaining extracts on a large scale.

Publications began appearing in the 1980s reporting conditions for optimizing the extraction of isoflavones aimed at ensuring better performance, reliability, and making it easier to enhance the production scale. Thus, the use of static and dynamic maceration began to be explored, because it enables greater large-scale use (Murphy 1981; Carrara et al. 2009). Murphy (1981) published one of the earliest studies of soy extraction by dynamic maceration at room temperature and showed that the two-hour-long extraction process was similar for isoflavone recovery when compared with the Soxhlet extraction process, regardless of the solvent used. According with Carrão-Panizzi et al. (2002), soybean flour extraction with or without constant agitation at room temperature, both with ethanol 70% (v/v), had similar results for isoflavone concentration after 1 hour or 24 hours of extraction; however, comparing the average total isoflavone recovery, the authors reported the highest isoflavone concentration when extraction was performed with agitation. A technique involving shaking and heating is often described for soybeans (Rostagno et al. 2009) and, more recently, was optimized by Zhang et al. (2007). The use of high temperatures during dynamic maceration usually modifies the prevalent isoflavone forms extracted, but it is not normally used to increase their total yield of isoflavones at the end of process (Coward et al. 1998).

Another conventional method widely described for soybeans extraction is the ultrasound process, which allows high yields of isoflavones (Rostagno et al. 2009). Ultrasound extraction, in general, is made through the use of sound wave energy generated at specific frequencies (20-100 kHz), which causes cavitation in the sample, generating ruptures that allow the chemical constituents to be extracted from the matrix. Its advantages over other techniques are the speed of extraction and low solvent consumption, which makes this technique specifically applicable to analyzing the raw material in laboratories, but the drawback is the difficulties concerning scaling up (Seidel 2006). Rostagno et al. (2003) optimized the ultrasound extraction (200 W and 24 kHz) of soybeans and evaluated variables such as the type of solvent, with ethanol, methanol, or acetonitrile at concentrations of 30-70% in water, temperature of 10 ºC or 60 ºC, a drug ratio of 0.5 g per 25 mL of solvent, and time of 10, 20, or 30 minutes. For quantification purposes, the best conditions for extracting all proposed isoflavones were a temperature of 60°C and 50% ethanol for 20 minutes.

Other techniques aimed at increasing extraction yields, process speed, and duplication of results have been evaluated for the extraction of soy isoflavones. Extractions with supercritical fluid, microwaves, or pressurized solvents are examples of these techniques, and for this reason, they were recently optimized by Rostagno and co-workers (Rostagno et al. 2002; Rostagno et al. 2004; Rostagno et al. 2007).

Supercritical fluid extraction employs a fluid in a supercritical state that has intermediate properties between gas and liquid, with carbon dioxide (CO_2) being the most common solvent used. Despite the high cost of purchasing the equipment, this technique is being increasingly used as it offers such benefits as high precision and sensitivity, good selectivity, low solvent consumption, speed, and safety (Rostagno et al. 2002; Sovová and Stateva 2011). Rostagno et al. (2002) evaluated this procedure for extracting soy isoflavones on a laboratory scale, using soybean seeds, static extraction for 10 minutes, followed by dynamic extraction for 20 minutes at different temperatures (40-70 °C), pressures (200-360 bar), and percentage of the modifier (0.5 and 10% ethanol 70%). The best condition for all compounds was 1 g of the drug and 55.2 g of CO_2, with 10% of ethanol: water modifier (7:3, v/v).

A highly efficient extraction may be carried out using the pressurized solvent extraction technique, which employs high temperatures and pressures ranging from 50-200 °C and 40-200 bar, respectively. These conditions allow a high extraction of compounds, given that this technique facilitates the diffusion of the solvent from the pores of the matrix, increasing mass transfer to the liquid extractant (Rostagno et al. 2004; Wijngaard et al. 2012). Rostagno et al. (2004) conducted a study to optimize isoflavone extraction using the pressurized solvent technique on a laboratory scale. Parameters evaluated were the type of solvent (ethanol or methanol at concentrations of 30% to 80%), temperature (60-200 °C), pressures (100-200 atm), amount of sample (0.5 to 0:05 g), and cycle time (5-10 minutes). The best results were obtained using 100 atm, 100 °C, three static cycles at seven minutes each, and ethanol 70% in water.

When extraction is done using microwaves, substances are extracted from the matrix through changes in their cellular structures due to the action of electromagnetic waves (0.3 to 300 GHz), with heat generation. This allows the solvent molecules to be exposed, resulting in high yields at the end of the process

(Rostagno et al. 2007; Wijngaard et al. 2012). Rostagno et al. (2007) evaluated the extraction of isoflavones on a laboratory scale using microwaves at 500 W. Different situations were assessed in that study, such as the use of methanol or ethanol at different concentrations (30-70%), temperature between 50-150 °C, amount of sample and solvent, and extraction time (5-30 minutes) in samples of ground, lyophilized soybeans. The best extraction of all forms of isoflavones was: 0.5 g of the drug and 25 ml of 50% ethanol at 50 ºC for 20 minutes.

In summary, there is a vigorous motivation to find different conditions for extracting isoflavones from soy. Conventional techniques are generally slower, whereas modern ones are considerably faster. However, isoflavone yield needs to be taken into account to assess the real advantage between the different techniques, along with the possibility of scale up. **Table 1.3** shows several comparative studies on different methods of isoflavone extraction from soybean seeds. Thus, it may be suggested that extraction by microwave, ultrasound, and pressurized solvent are the most predisposed candidates for extracting isoflavones from soybeans.

Hydrolysis of isoflavones

Hydrolysis of isoflavones is a procedure that aims at transforming conjugated compounds into their free forms (Lee et al. 2008) and is interesting for two reasons: obtaining their active forms in the extract when the goal is the incorporation into products (medicines and cosmetics) or reducing the time of quantitative analysis in routine laboratory quality control by simplifying the number of substances to be analyzed (Lee et al. 2008; Schwartz and Sontag 2009).

For analytical purposes or the industrial production of isoflavones from soybean, the literature describes three types of hydrolysis: acidic, basic, and enzymatic (Sontag and Schwartz 2009). Acidic and enzymatic hydrolysis is responsible for the breakdown of the isoflavone between the glucoside and aglycone, resulting in a higher yield of the latter. Conversely, in basic hydrolysis, breakdown takes place in the ester parts of the compound, resulting in greater conversion for the glucosylated forms (Sontag and Schwartz 2009; Lee et al. 2008).

Table 1.3. Comparative studies on different methods of isoflavone extraction from soybean

Techniques	Experimental conditions	Best conditions based on isoflavone yield extraction	References
SE US-B SFE	SE: 80% MeOH, 9 h US-B: 22 kHz, 80% MeOH (3 cycles, 1h each one) SFE: 40-70 ºC; 200-360 bar CO_2, with 10% modifier agent	US-B	Rostagno et al. (2002)
US-B US-P HSE	Different combination of solvents, time and temperature	US-B 50% EtOH, 60 ºC, 20 min	Rostagno et al. (2003)
PLE US SE	PLE: hexane followed of MeOH during 2 cycles of 5 min, 145 ºC, 140 bar US-P: 38 kHz, 90% MeOH, 5 min SE: 90% MeOH, 70 min	PLE + US	Kledjus et al. (2005)
US-P MW	US-P: kHz, 50% EtOH, 60 ºC, 20 min MW: 500 W, 50% EtOH, 50 ºC, 20 min	Comparable yields	Rostagno et al. (2007)
VE SHE US-B SE PLE	Different solvents by: VE: 1 min SHE: 60 min US-B: 15 min SE: 3 h PLE 1000 psi, 100 ºC (3 static cycles, 7 min each one)	PLE DMSO:ethanol: water (5:75:25, v/v/v)	Luthria et al. (2007)
SHE SE US	Different solvents by: SHE: 15 h SE: 4 h, 80 ºC US: 40 W, 3 min	US: 50% acetone	Chung et al. (2010)

DMSO: dimethylsulfoxide; EtOH: ethanol; HSE: hot and shaking extraction; MeOH: methanol; MW: microwave extraction; PLE: pressurized liquid extraction; SHE: shaker extraction; SE: Soxhlet extraction; SFE: supercritical fluid extraction; US: ultrasound extraction; US-B: ultrasonic bath extraction; US-P: ultrasonic probe extraction; VE: vortex extraction

Studies attempting to compare the best conditions to obtain the isoflavone aglycones (IA) after acidic or enzymatic hydrolysis can be found in the literature as shown in **Table 1.4.** However, the choice of technique is always in accordance with the intended purpose. For analytical purposes or quality control in laboratories, studies can be found with acidic hydrolysis, in which the concentration of acid, time, and temperature of the procedure are optimized; and enzymatic hydrolysis, in which the choice of the enzyme varied considerably in an attempt to find the best yields and percentage of isoflavone recovery (Rostagno et al. 2009).

The most frequently used acid for the hydrolysis of isoflavones is hydrochloric acid (HCl); while for enzymatic hydrolysis, the most frequently used enzymes are β-glucosidase (from almonds or *Escherichia coli*) and β-glucuronidase (from *Helix pomatia*). However, in recent years, there has been an increase in studies for the discovery of new enzymes involved in the process of transforming isoflavones into their active forms. Some examples are β-glucosidase from *Aspergillus oryzae*, *Paecilomyces thermophila*, *Thermotoga maritime, Pyrococcus furiosus*, and *Sulfolobus solfataricus* (Aguiar and Park 2004; Yang et al. 2009; Sun et al. 2010; Song et al. 2011; Yeom et al. 2012; Kim et al. 2012; Kuo et al. 2012).

Some studies have reported the optimization of the hydrolysis process to successfully convert the isoflavone glucosides into their free forms. Chiang et al. (2001) optimized the acid hydrolysis conditions of soybean hypocotyls by response surface methodology (RSM). Optimum hydrolysis conditions were obtained using 3.42 M HCl at 44.6 ºC for 205.5 min in a water bath, resulting in an isoflavone recovery close to 100%. Cesar et al. (2006) optimized the acid hydrolysis efficiency of soybean flour at five different concentrations of HCl (1.0, 1.5, 2.0; 2.5, and 3.0 M) at different hydrolysis times. After sonication for 5 min, the highest IA content was achieved after 40 min of steam bath (temperature not indicated), using a 3.0 M HCl in ethanolic solution.

Using the Plackett-Burman design and RSM, Tipkanon et al. (2010) optimized the enzymatic hydrolysis conditions of soybean flour using the β-glucosidase enzyme. The optimized process was obtained with a proportion of soy: deionized water (1:5, w/v), β-glucosidase at 1 unit/ g of soy flour, pH 5, and incubation temperature/time of 45 ºC/ 5 h. Lee and Choung (2011) studied the optimal acid

hydrolysis condition using drying oven and microwave assisted methods. All isoflavone glucosides were completely converted into their aglycones at 120 min with drying oven and 50 min with microwave using 1 M HCl at 100 ºC. Shao et al. (2011) studied the acid and enzymatic hydrolysis processes to compare the conversion of isoflavone glucosides into aglycones. The optimized acid method (1.2 M HCl in ethanolic media, 80 °C, 2 h) converted only 92% of isoflavones, while the enzymatic method with 1 mg/mL of β-glucuronidase (from *H. pomatia*) effectively converted 100% of isoflavone into their aglycone forms at 37°C, pH 5.0 for 16 h.

Moreover, a few studies have reported the hydrolysis processes of soybeans as a way to obtain a rich IA fraction to be used in the pharmaceutical field. Utkina et al. (2004) optimized the hydrolysis conditions to obtain a rich IA fraction from a soybean supplement (NovaSoy®). The authors studied the enzymatic hydrolysis using *Aspergillus heteromorphous* 3010, or the acidic hydrolysis of the glycosides in ethanol solution using 1 M HCl for 2 h at 100 °C, and the acid hydrolysis of glycoside powder using 6 M HCl for 5 h at 100 °C. The best IA yield was achieved with the latter process. After purification, the antioxidant activity was studied within the range of 0.1–10 mM, demonstrating that at no less than 1 mM concentrations, the rich IA fraction inhibits phospholipid peroxidation registered through the peroxide formation and malonic dialdehyde accumulation.

Zhang et al. (2007) optimized the extraction of isoflavones from soybean flour, followed by the optimization of acid hydrolysis. The best condition to obtain the IA was 0.13 M HCl in ethanolic media at 80 ºC for 6 h. After purification, the authors not only obtained a rich fraction with high level of IA but also identified its estrogenic activity at 0.4 µg of total isoflavones/mL (0.84 µM of genistein and 0.64 µM of daidzein) by the MTT assay using MCF-7 cells. Deshmukh and Amin (2013) obtained a rich IA fraction from a commercial soy extract by refluxing the extract with 4 M HCl in an ethanolic media for 2 h followed by purification. The antioxidant activity was studied within the range of 50 – 500 µg/mL, demonstrating that at a concentration of 125 µg/mL the fraction was able to inhibit 50 % of DPPH reaction.

Table 1.4. Comparative studies for different methods of isoflavone hydrolysis

Samples	Type of hydrolysis	Best conditions to obtain IA	References
Soybeans seeds and tofu	Acid: Reflux of 1.0 g using 50 mL of 2 M HCl in 77% ethanol during 1- 4 h Enzimatic: Reflux of 1.0 g using 50 mL of 77% ethanol during 3 h, solvent evaporation and addition of pH 5.0 acetate buffer + 2 mg β-glucosidase from almonds + 40 µL β-glucoronidase from *Helix pomatia* during 24 h at 37 ºC	Acid hydrolysis, during 1 h for genistein and 2 h for daidzein	Franke et al. (1994)
Soybeans flour	Acid: 2.5 g in 5 mL of 80% methanol, sonication during 10 min followed by addition of 3 M HCl and reaction during 2 h at 80 ºC Enzimatic: 5000 Units of β-glucuronidase from *H. pomatia* in 5 mL of pH 5.0 acetate buffer Enzimatic: 100 Units of cellulase from *Aspergllus niger* in 5 mL of pH 5.0 acetate buffer Enzimatic: 100 Units of β-glucosidase from almonds in 5 mL of pH 6.8 phosphate buffer Note: for the enzimatic procedures, the reaction was conducted *overnight* in a concentration of 0.5 mg/mL	Enzimatic with cellulase from *A. niger*	Liggins et al. (1998)
Standards of isoflavones	Enzimatic: 3, 6 or 30 Units of β-glycosidase from *E. coli* in pH 7.0 phosphate buffer at 37 ºC Enzimatic: 3, 6 or 30 Units of β-glycosidase from almonds in pH 7.0 phosphate buffer at 37 ºC Note: Reactions using standards solutions at 500 ppm	30 U. of β-glycosidase from *E. coli* during 4 h	Ismail et al. (2005)
Soybeans seeds, tofu, soybean milk or soybeans cereals	Alkaline: 400 µL of extract + 30 µL of 2M NaOH during 10 min at room temperature Acid: 100 µL of extract + 900 µL 1.3 M HCl at 80 ºC during 2 h Enzimatic: 1 mL of dry extract followed by addition of 1 mL of enzimatic solution (1mg/mL de β-glucuronidase, type H-5 from *H. pomatia* in pH 5.0 phosphate buffer) during 16 h at 37 ºC Note: extracts were obtained by shaking 1.0 of sample with 10 mL of 80% methanol during 12 h	All treatments showed an equivalent quantification for the isoflavones evaluated, however only the acid and enzimatic hydrolysis converted all them until the free forms.	Shao et al. (2011)

Extract purification

To obtain isoflavone-rich extracts, subsequent extraction steps may be carried out to purify the sample by removing impurities and making it highly concentrated in isoflavones. Such steps include centrifugation, precipitation, ultrafiltration, passage through columns containing specific resins, or purification by polarity selectivity (Murphy 1981; Xu et al. 2004; Cho et al. 2009).

Among these processes, the solid phase extraction technique has been well-described for the purification of soy extracts on a laboratory scale. This is based on separating compounds by the interaction between a stationary and a mobile phase through different physical-chemical phenomena. To purify soy isoflavones, the use of different types of adsorbents, such as silica gel or C18 polymeric materials, such as Diaion HP-20 and Amberlite XAD16, has been reported in the literature (Thorne 1981; Hirota et al. 2004; Li-Hsun et al. 2004).

The liquid-liquid partition in conjunction with solid phase extraction is a complementary alternative for purifying soybean extract on a laboratory scale, as demonstrated in the study by Cho et al. (2009). The authors used three purification steps: passing the hydroethanolic extract through a column of Diaion HP-20, followed by acid hydrolysis, and liquid-liquid partition with an appropriate solvent polarity. A good recovery of genistein and daidzein was obtained by enriching the extract by about 20 times when using solvent partition with ethyl ether. The final amount of IA obtained was 229 mg/ g of fraction.

Purification of aglycone isoflavones may also occur with other separation techniques, including those that would allow scale-up production, as reported by Zhang et al. (2007). The authors used a high polar solvent, such as water, to turn the aglycone forms insoluble in the medium, thereby causing their precipitation, which, with subsequent filtration and drying of the residue, resulted in obtaining an enriched fraction of these substances. This study was carried out to optimize the amount of antisolvent, and it was concluded that, by using a 1:4 ratio (hydrolyzed extract:water), it is possible to get a high recovery of genistein and daidzein, thereby showing the process to be efficient in obtaining a fraction with isoflavone content twice that of the extract prior to the process. The final amount of IA obtained was 0.87 mg of total isoflavones (aglycone equivalency)/ g of soybeans.

Ultrafiltration is an easily scale-up purification process, in which the choice of filters may play a role in selecting compounds with regard to the separation of interesting substances. The study performed by Xu et al. (2004) reveals the efficacy of this process concentrating isoflavones from soybean milk. The process consisted of centrifuging the aqueous extract of soybean seeds, passing it through a 30,000 Da cellulose membrane, diafiltratering, followed by passing it through a 1,000 Da cellulose membrane, and finally passing it through a reverse osmosis membrane; in the latter step, only substances that were not of commercial interest were able to permeate, with only isoflavones being retained in the filter. This process allowed for increased isoflavone concentration, resulting in a final IA amount of 10.9 mg/ g of fraction.

Recently, Wang et al. (2013) developed a green strategy to obtain IA from soybean. First an ethanol-alkaline extraction method was designed and optimized. The high extraction yield of isoflavones was achieved by the optimal extraction conditions of pH 9.0 at 70 °C for 60 min, using ethanol 65% (v/v). Next, enzymatic hydrolysis with cellulase (GC-220) gave an excellent conversion of 95%. Lastly, the crude isoflavone aglycones were purified by a procedure consisting of ethanol precipitation, ethyl acetate liquid-liquid extraction, and water rinse. The final amount of aglycone isoflavones obtained was 1.279 mg/ g of defatted soybean.

Obtaining isolated isoflavones

As previously mentioned, the aglycone forms of isoflavones are of great industrial interest (Setchell 1998). Isoflavones may be isolated by purifying the soybean extract or they may also be purchased commercially, since their synthesis routes have been reported for these compounds (Baker and Robinson 1928; Baker et al. 1933; Antus et al. 1975).

Genistein was first isolated from a plant called Dyer's Broom (*Genista tinctoria*) by Perkin and Newbury (1899); three decades later, it was synthesized by Baker and Robinson (1928). In soybeans, genistein was first isolated by Walz (1931), and its physical and chemical characteristics were reported by Walter (1941), who studied and further described this compound. Daidzein was also isolated from soybeans by

Walz (1931), and its synthesis was successfully accomplished by Baker, Robinson, and Simpson (1933). Glycitein was isolated years later by Naim et al. (1973). This compound is a minor isoflavone in soybean; therefore, it is the most difficult to obtain. Glycitein was successfully synthesized few years later (Antus et al. 1975).

Even though soy isoflavone aglycones are individually available for purchase, studies have been described their isolation from soybeans. Farmakalidis and Murphy (1984) isolated genistein and daidzein from soybean by a semi-preparative high-performance liquid chromatographic method. First, the flakes were extracted with acetone 0.1 M HCl, passed through silica gel column eluted with chloroform-methanol with increasing polarity. Further, isoflavones have been isolated by the semi-preparative chromatography method using a reversed-phase 250 x 9.4 mm Partisil ODS-3 column, a non-linear methanol-water gradient, and flow-rate at 5 mL/min. Yang et al. (2001) reported the isolation of daidzin, glycitin, genistin, acetyldaidzin, glycitein, acetylgenistin, and daidzein from soybeans using high-speed counter-current chromatography. Three solvent systems were used: chloroform-methanol-water (4:3:2, v/v); chloroform-methanol-*n*-butanol-water (4:3:0.5:2, v/v); and methyl tert-butyl ether-tetrahydrofuran-0.5% aqueous trifluoroacetic acid (2:2:0.15:4, v/v). The isolated compounds presented 98-99% purity. More recently, Wang et al. (2013) reported a chromatographic method for isolating and purifying isoflavones from soybean extracts by using 12% cross-linked agarose gel as the separation media, and methanol at concentrations of 30% or 65% as gradient elution. Soybean extracts were separated by the proposed method and six soy isoflavones, including glycitin, daidzin, genistin, glycitein, daidzein, and genistein, were obtained with purity higher than 97%.

INCORPORATION OF ISOFLAVONES INTO TOPICAL SYSTEMS

As shown in **Figure 1.3**, both commercially obtained pure substances and the extract obtained without the hydrolysis step (preferably containing glucosylated forms), or hydrolyzed and purified extracts (primarily containing aglycone forms), can be incorporated into different types of topical formulations.

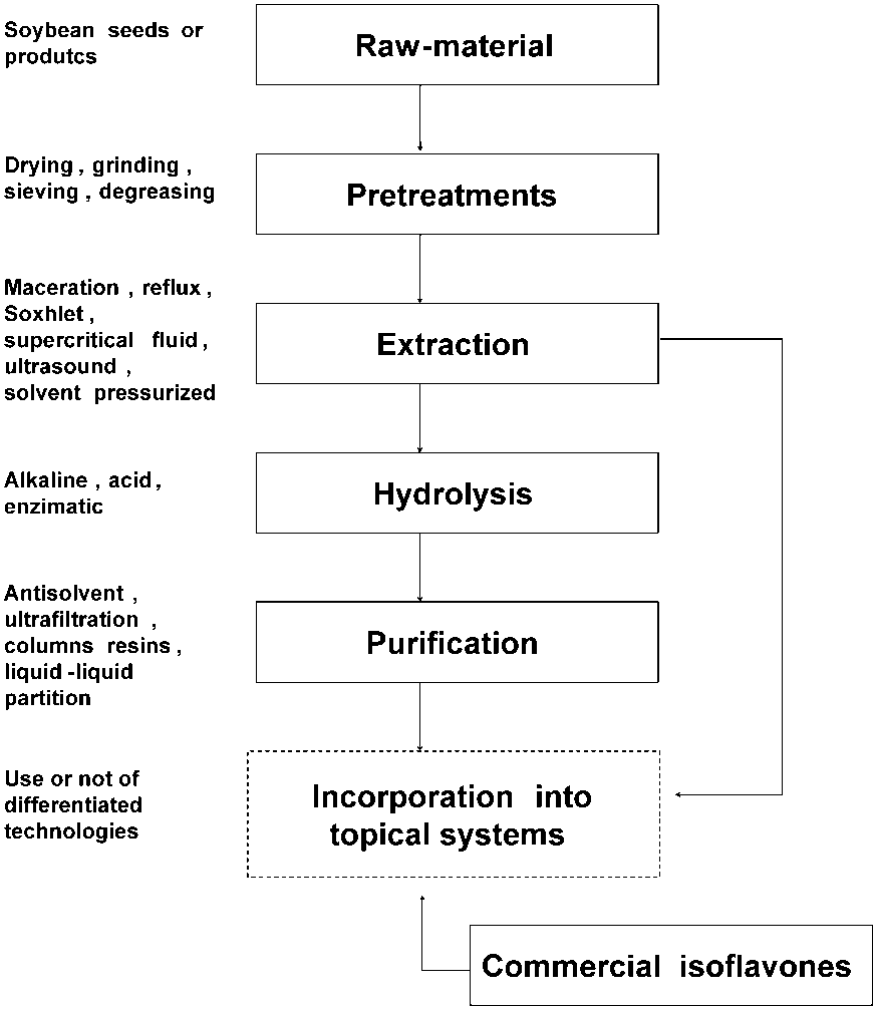


Figure 1.3. Steps described in this review for the development of topical products containing isoflavones from soybeans or isolated commercial compounds

When developing products (medicines and cosmetics) for topical use, parameters such as skin characteristics, type of formulation, and solubility of substances, are of great importance to maintaining product stability and allowing the active ingredients to penetrate into the skin (Förster et al. 2009; Weiss 2011).

The epidermis is the first layer of the skin, whose function is to protect the skin from the external environment. The stratum corneum is found on the surface and consists of dead cells that function as a protective lipophilic barrier. When a topical formulation is administered, it is precisely this barrier that active substances must penetrate to effect action on the skin (Förster et al. 2009).

In this context, it is important to point out that skin penetration depends on the physicochemical properties of each substance, their behavior when added to a formulation, and, last but not least, the conditions of the user's skin (Förster et al. 2009). The physical and chemical characteristics of the active substances are relevant for predicting behavior after cutaneous application. Those with a high hydrophilicity will have difficulty penetrating the skin. Conversely, if they have high lipophilicity, they will tend to be retained in the formulation when it is also lipophilic in nature. For this reason, it is important that the substance has characteristics that allow its partition, with favorable partition coefficient values (log P) ranging between 1 and 3 (Förster et al. 2009).

Different vehicles used in topical formulations may also influence the amount and extent to which a substance is able to permeate the skin. In the case of highly permeable substances, the rate of penetration can be controlled by using a suitable carrier. In these conditions, the diffusion of the active ingredient in the formulation is the limiting step. Therefore, the partition coefficient of a substance of low solubility in water between a hydrophilic matrix and the stratum corneum promotes its migration to the latter, characterizing a good "transfer" to the skin (Förster et al. 2009).

It has been postulated that the highly hydrophilic isoflavone glucosides will have difficulty penetrating the stratum corneum, whereas genistein, daidzein, and glycitein have a tendency to more easily penetrate this layer because they have values near the partition coefficients of 2.98, 2 78 and 2.57, respectively (these values are determined through the ACD/I-Lab online service). However, even with a suitable log P, the type of formulation will influence their penetration, a parameter that should always be taken into account when developing a product.

Conventional topical systems

In traditional cosmetics systems, cream type formulations are widely used, and the choice of system is determined by the solubility of the active substance to be incorporated. Thus, when the compound is hydrophilic, it will be more readily incorporated into oil-in-water (o/w) emulsions and, when hydrophobic, in water-in-oil (w/o) emulsions. However, the latter is characterized as a more oily formulation and, therefore, less accepted by users (Weiss 2011).

In the case of incorporating isoflavones into these types of emulsions, two situations can be observed: (1) when the purpose is to incorporate glucosylated forms, o/w emulsions may be used, although penetration could be somewhat difficult, and (2) when the goal is to use aglycone forms, the use of w/o emulsions is more described, despite lower user acceptance (Schmid et al. 2003; Georgetti et al. 2006; Kitagawa et al. 2010).

In a study by Georgetti et al. (2006), four o/w emulsion formulations were prepared with the aim of incorporating 2% commercial extract, containing both glucosylated and aglycone isoflavones (Isoflavin Beta ®). These formulations were evaluated using the physical-chemical parameters of the products and *in vitro* antioxidant activity. The systems comprised distilled water, methylparaben, propylparaben, macadamia oil, propylene glycol, imidazolidinyl urea, and a self-emulsifying base (Polawax® or Crodabase®) with or without carboxypolymethylene as a stabilizer. Among these formulations, all showed a similar antioxidant potential when in the presence of isoflavones, but the formulation consisting of Crodabase® without the added stabilizer was found to be unstable (Georgetti et al. 2006).

In a subsequent study, the same authors evaluated the stability of formulations containing the seemingly more promising Polawax® for six months, and also studied the *in vitro* dermal absorption and *in vivo* antioxidant activity of formulations containing the isoflavones. The formulations were stable in the evaluated conditions (4 ºC and 40 ºC and 70% RH, 30 ºC and 70% RH) for six months. The evaluation of cutaneous antioxidant activity in mice was shown by the inhibition of lipid peroxidation and the cutaneous retention in porcine skin was found to be low, as quantification was not possible, neither on the skin nor in the Franz cell receptor (Georgetti et al. 2008).

It is worth mentioning that the glucosylated forms may have unfavorable penetration, due to their high water solubility, in addition to not being metabolized into active forms due to the lack of specific enzymes in the skin. This situation was reported by Schmid et al. (2003) through a clinical study with ten volunteers, showing no transformation of genistin into genistein in the skin’s outer layers after topical application of the glucosylated form.

Kitagawa et al. (2010) developed two types of microemulsions with the intent of incorporating genistein, daidzein, and biochanin A. The formulations were composed of the following components: isopropyl myristate, aqueous solution containing sodium chloride (NaCl) 150 mM, Tween 80 and ethanol, which, when in a ratio of 8:25:20:47, constituted an o/w emulsion, and when in a ratio of 33:7:30:30, formed a w/o emulsion. Based on these results, the authors concluded that the best formulation for isoflavones was the one that used the second ratio, given that the substance solubility was higher, providing better system stability and greater cutaneous retention of isoflavone aglycone in porcine skin.

Topical systems involving different technologies

Due to the poor water solubility of the active forms of isoflavones, several alternative technologies have been studied to improve this feature and make it possible to incorporate them into hydrophilic pharmaceutical forms. Some of them are listed in **Table 1.5**, which describes the technologies and type of sample used.

Delivery systems containing active substances can be formed by (1) encapsulation in micelles and vesicles formed by polymers, surfactants, or phospholipids, and (2) complexation techniques to host macromolecules. In both cases, it is possible to increase the compounds' water solubility, making them very attractive in terms of the technological development of topical formulations (Forster et al. 2009). Among the main types of such systems, the literature reports the use of liposomes, nanoemulsions, nanocapsules, nanospheres, or cyclodextrin and dendrimer complexes. These structures offer a number of advantages when compared with the hydrophobic active substance, for, in addition to improving solubility, they also increase stability and control delivery (Förster et al. 2009).

Liposomes are formed by a lipid bilayer primarily consisting of phospholipids and cholesterol, which form a colloidal vesicular system. These structures have the ability to incorporate hydrophilic substances in their core, or even hydrophobic ones in the phospholipid bilayer. The main benefit of this system is its biocompatibility with the skin's outer layer, which increases the likelihood that the active compounds will be able to penetrate it (Förster et al. 2009).

Table 1.5. Technological strategies to enhance the release characteristics of isoflavone aglycones

Sample	System	Other constituents	References
Daidzein	Liposome	Phosphatidilcoline	Dwiecki et al. (2009)
Genistein	Liposome	Phospholipids, polysorbate 80	Schmid et al. (2003)
Genistein	Nanoemulsion	MCT, egg lecithin	Silva et al. (2009)
Genistein	Nanocapsule	PLA	Zampieri et al. (2013)
Soybean extract	Microcapsule	NaCMC	Sansone et al. (2013)
Rich fraction of IA	Nanoparticle Lipid Solid	Softsan 601, polysorbate 20	Deshmukh et al. (2013)
Genistein	CD complex	β-CD; hydroxypropyl-β-CD or methyl-β-CD β-CD or γ-CD β-CD amphiphilic CD	Crupi et al. (2007) Daruhazi et al. (2008) Xavier et al. (2010) Cannavá et al. (2010)
Genistein+daidzein	CD complex	Hydroxypropyl-β-CD	Stancanelli et al. (2007)
Rich fraction of IA	CD complex	β-CD or hydroxypropyl-β-CD	Yatsu et al. (2013)
Daidzein	CD complex	β-CD; methyl-β-CD or hydroxypropyl-β-CD	Borghetti et al. (2011)
Daidzein	Dendrimers	PAMAM or PPI	Zhao et al. (2011)

CD: cyclodextrin; IA: isoflavone aglycones; MCT: Medium chain triglycerides; NaCMC: sodium-carboxymethylcellulose; PAMAM: poly(amidoamine); PLA: poly (acid lactic); PPI: poly(propylene imine); Softsan 601: glyceryl cocoate (and) hydrogenated coconut Oil (and) ceteareth – 25

The use of liposomes to encapsulate daidzein was evaluated by Dwiecki et al. (2009), who proposed a weak interaction of the molecule with a lipid bilayer composed of egg phosphatidylcholine and a greater interaction with the polar portion of this membrane. The *in vitro* antioxidant activity of this system was evaluated and it was found that there was an increase in such activity when compared to the free substance. Schmid et al. (2003) conducted a study of the ability of genistein to penetrate skin under different formulations in ten volunteers. The objective was to compare the accumulation of genistein on the skin, whether or not the compound had

been incorporated into liposomes. The results showed that the best penetration of this isoflavone into the stratum corneum took place when the volunteers applied the formulation containing genistein associated with the liposomes, thereby demonstrating one of the strong points of this alternative technology.

Like liposomes, nanoemulsions have been considered as a potential colloidal system for topical administration of hydrophobic molecules. These systems are defined as a nano-dispersion of oily droplets in an external aqueous phase, stabilized by a suitable surfactant system (Förster et al. 2009; Vargas et al. 2012).

A study describing the incorporation of genistein in nanoemulsions was reported by Silva et al. (2009), who developed a topical nanoemulsion containing 1 mg/mL of this substance by using the spontaneous emulsification technique. The best formulation was composed of water, medium chain triglycerides (MCT), stabilized with egg lecithin, and presented an average particle size ranging between 200-300 nm, with association efficiency very close to 100%. In the same study, the cutaneous permeation in porcine skin was also studied, and the results indicated that the use of such nanoemulsions led to a delay in the cutaneous flow of genistein and an increase in its retention in the skin layers. In a continuation of the study, Vargas et al. (2012) incorporated genistein nanoemulsions in Carbopol 940® hydrogels dispersed in water at a 5% concentration. An evaluation of genistein permeation in porcine skin once again showed better retention when using MCT as an oily core, and the main benefit of using hydrogel is based on its better viscosity and product appearance, while maintaining the nanostructures' physical-chemical characteristics.

Solid lipid nanoparticles (SLNs) are composed of physiological and biodegradable solid lipids. SLNs have the advantage of protection against chemical degradation when compared with liposomes and nanoemulsions (which are also lipid carriers but without a solid structure). However, the main drawback of SLNs is that during storage the bioactive component can be expulsed due to a change in lipid conformation to a lower energy crystal state (Förster et al. 2009). Deshmukh and Amin (2013) reported the production of an isoflavone aglycone-rich fraction from commercial soy extract intended to be incorporated into a SLN-based gel at a final concentration of 1 mg/mL. To prepare the SLN, the fraction was added in a warm microemulsion followed by dilution in cold water (2–3 °C) under mechanical stirring.

The selected compounds for microemulsion formation were glyceryl cocoate (and) hydrogenated coconut oil (and) ceteareth – 25 (Softsan 601) as solid lipid. The microemulsions were stabilized by the surfactant tween 20. Carbopol (971 P) was selected as the gelling agent based on compatibility with the nanoparticulate dispersion, ease of preparation, and aesthetic appeal. The SLN-based gel was characterized by pH, spreadability, rheology, bioactive content, and *in vitro* permeation using porcine ear skin model; its safety was assessed using primary skin irritation studies. The developed SLN-based gel showed about 60% deposition of soy isoflavones in dermal matrix and showed no skin irritation on intact rabbit skin.

Nanocapsules, or nanospheres, are another type of nanometric system that can be formed from suitable techniques when in the presence of polymers. Zampieri et al. (2013) encapsulated genistein in nanostructures formed with the poly (lactic acid) biodegradable polymer by the nanoprecipitation technique and evaluated their physical and chemical characteristics, stability, and *in vitro* cutaneous permeation of porcine skin. Permeation studies showed that genistein reached the deepest layers, characterizing a penetration enhancement when incorporated into gel-type formulations.

Sansone et al. (2013) described a sodium carboxymethylcellulose matrix obtained by spray-drying to microencapsulate soy extract rich in isoflavones, thereby increasing the solubility of these substances, and evaluating the permeation profile in nitrocellulose membranes and the compounds' dissolution profile. The authors suggested that microparticles of isoflavones could be potentially used for topical and oral use.

Cyclodextrins (CD) are structures formed by cyclic oligosaccharides composed of 6, 7, or 8 toroidal-shaped dextrose molecules whose interior is characterized as a hydrophobic cavity whereas the exterior is hydrophilic. These characteristics make complexation possible with apolar substances, thus increasing their water solubility (Förster et al. 2009; Xavier et al. 2010). Several studies have demonstrated the efficiency of complexation of genistein with cyclodextrins, thus increasing the solubility of that particular isoflavone in water (Crupi et al. 2007; Stancanelli et al. 2007; Daruházi et al. 2008; Cannavá et al 2010; Xavier et al. 2010). Among them, the efficiency of complexation and characterization of complexes are well described;

nevertheless, their incorporation into final topical formulation still needs to be evaluated as well as their release behavior and activities.

Crupi et al. (2007) obtained genistein complexes by using the co-precipitation technique with β-cyclodextrin (β-CD), hydroxypropyl-β-CD, and methyl-β-CD, characterizing the associations by infrared and UV-Vis spectrophotometry. Daruházi et al. (2008) achieved complexation with β-CD and γ-CD, with the technique having enabled an association with 9.9% genistein, using a molar ratio of 2:1 CD-genistein, and the complexes were characterized by nuclear magnetic resonance (^{1}H NMR). Inclusion complexes were prepared by Xavier et al. (2010) in an aqueous solution with β-CD in 1:1 molar ratio, dried by lyophilization to obtain a powder with 19.22% genistein concentration. Molecular modeling studies carried out in the latter work suggest that it is the isoflavone's A ring that is included in the cyclodextrin's hydrophobic cavity.

Cannavà et al. (2010) also evaluated the association of genistein to amphiphilic CD by the emulsification-diffusion technique, with evaluation by UV-Vis, circular dichroism, and infrared analysis. The authors found that the complexes organized themselves to form nano-aggregates due to the presence of polyethylene structures, thus increasing the solubility of isoflavones by about ten times. Stancanelli et al. (2007) complexed genistein and daidzein into hydroxypropyl-β-CD in an aqueous phase, and the associations were later evaluated by UV-Vis and circular dichroism. Both substances successfully complexed with CD, thereby, increasing the water solubility of isoflavones.

Multiple complexation of a fraction enriched in isoflavone aglycones (genistein, daidzein, and glycitein) with β-CD or hydroxypropyl-β-CD was recently proposed by Yatsu et al. (2013). Different techniques for obtaining complexes were studied, which were characterized by infrared spectroscopy, x-ray diffraction, scanning electron microscopy, differential scanning calorimetry, and NMR. The results showed that the complexes obtained with hydroxypropyl-β-CD allowed a greater increase in solubility for isoflavones, and complexation may occur by inserting the isoflavone's A or B ring into the hydrophobic cavity of CD.

Among the studies found in the literature concerning cyclodextrin complexation with isoflavones, only the study by Borguetti et al. (2011) reported

complexation of daidzein aimed at its incorporation into hydrogels for topical application. The study was conducted by using different gels, with hydroxypropylmethylcellulose or polyvinylpyrrolidone presenting results of being viable for use. In this case, molecular modeling assumes that the B and C rings enter into the hydrophobic cavity of CD, contrary to what was found for genistein in earlier studies.

Another organized system known as dendrimers is a class of macromolecules of nanometer size with a certain degree of repetition of a known grouping, forming branches around a core substance. Dendrimers have a part that allows complexation with apolar substances, while the remainder is considered polar and, therefore, a macromolecule with hydrophilic characteristics (Zhao et al. 2011).

Zhao et al. (2011) evaluated daidzein complexation with dendrimers formed by poly-amidoamine (PAMAM) or poly-propyleneimine (PPI). The results showed that both complexes effectively enhanced the solubility of isoflavone, improved the pharmaceuticals' delivery profile, prolonged their action, and were stable for 30 days. However, the complex formed with PAMAM dendrimer was the best candidate to be used for antioxidant action, as it presented less cytotoxicity against two cellular lines.

CONCLUSIONS

Soy is a legume that is widely exploited economically and pharmacologically. The chemical constituents present in this plant with benefits to the skin are mainly genistein and daidzein, but they are more easily extracted in conjugated forms with sugars. In this sense, hydrolysis of glucosides present in the extracts, with subsequent purification to enrich the aglycone forms or the use of these synthetic compounds is essential to developing skin permeable delivery systems.

However, the active forms of isoflavones are poorly water soluble, often requiring the use of alternative technologies, such as nanotechnology, to facilitate their ability to incorporate into topical formulations of hydrophilic character and promote their retention within the skin.

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CAPÍTULO II

Revisão da literatura tecnológica

2.1. INTRODUÇÃO

As inovações tecnológicas são um dos principais elementos de desenvolvimento econômico dos países (IDRIS, 2003; FRIETSCH *et al*., 2010). Uma importante maneira de instigar a inovação é a proteção da propriedade intelectual (PI), visto que os direitos de PI protegem inventores e empresas de ter suas criações e inovações exploradas por terceiros de forma não autorizada por um determinado período (IDRIS, 2003). Dentre as formas de garantir os direitos de PI está a escolha de patentear um produto ou um processo (IDRIS, 2003; FRIETSCH *et al*., 2010).

Um dos parâmetros de avaliação do perfil de inovação de uma região ou segmento específico são os estudos de prospecção através de mapeamentos tecnológicos (ERNST, 2003; LEE *et al*., 2015). Para isso, são normalmente utilizados dados e análises com base em indicadores tecnológicos, sendo o número de depósitos de patentes um importante indicador de desenvolvimento industrial (BASBERG, 1987; YOON *et al*., 2002). Este indicador normalmente é utilizado para espelhar o presente perfil tecnológico das inovações, bem como auxiliar nas atividades futuras em termos de pesquisa e desenvolvimento (P&D) (FRIETSCH *et al.*, 2010). Além disto, o conjunto das informações obtidas nos levantamentos de patentes pode auxiliar no planejamento estratégico de instituições, políticas públicas e diretrizes industriais (ERNST, 2003; SPEZIALI *et al*., 2012; LEE *et al.,* 2015).

Dessa forma, devido ao grande interesse no uso farmacológico e cosmético de isoflavonas, bem como a diversidade de aplicações desses compostos na área alimentícia, o mapeamento tecnológico de patentes envolvendo isoflavonas é um atrativo objeto de trabalho para pesquisadores que visam avaliar a prospecção da inovação de temáticas com grande impacto mundial em termos econômicos e científicos.

Contemplando a ideia de auxiliar em novas decisões a cerca de novos investimentos em tecnologias envolvendo isoflavonas, esta etapa do trabalho objetivou realizar uma busca sobre o estado da arte de patentes (intervalo de tempo de 20 anos) envolvendo as principais isoflavonas e suas formas isoladas agliconas: genisteína, daidzeína e gliciteína. Assim, para realizar a pesquisa foi efetuado um mapeamento tecnológico sobre os assuntos acima descritos entre os anos de 1994

– 2014, destacando-se o número de patentes por país, tipos de depositantes, e os objetivos das patentes, sendo eles divididos em três *clusters*: (a) obtenção de alimentos, extratos de plantas e derivados contendo isoflavonas; (b) uso de isoflavonas em produtos cosméticos, e (c) sistemas diferenciados contendo isoflavonas, a citar a complexação com ciclodextrinas e a encapsulação em micro ou nanoestruturas.

Para abranger o tema em questão, este capítulo está apresentado na forma de artigo de revisão, redigido nas normas do periódico em que foi publicado.

2.2. ARTIGO

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THE INTERNATIONAL SCENARIO OF PATENTS CONCERNING ISOFLAVONES

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ABSTRACT

Isoflavones are a class of phytoestrogens that has been considered important raw material for healthcare products, mainly as dietary supplements for hormone replacement and active ingredients in skin care cosmetics. Because of this, many scientific reviews are found regarding the extraction, analysis methods and biological activities of these compounds. However, it is emphasized that, to date, no studies have been found concerning technological mapping of patents involving isoflavones. The present study aimed to map patents covering the last 20 years of technology innovation comprising isoflavones. To that, a patent survey was conducted between the years of 1994 – 2014 in an international patent database (*Espacenet*) using the title's keywords: isoflavones, genistein, daidzein, glycitein, biochanin A and formononetin. The resulting data allowed the identification of the major countries, universities and companies that invest in products containing these compounds. In addition, patents were separated into interest groups. The three clusters discussed in this study were: processes for obtaining enriched isoflavone fractions from different plant materials intended to healthcare products, such as dietary supplements; the use of isoflavones in cosmetic products; and the incorporation of isoflavones in micro and nanostructured systems to mask unpleasant tastes or overcome the low water solubility of bioactive forms. In conclusion, this study, along with other literature reviews, could assist in designing strategic research and worldwide development of new products containing isoflavones.

Keywords: cosmetics; enriched fractions; isoflavones; micro and nanostructured systems; technological indicators.

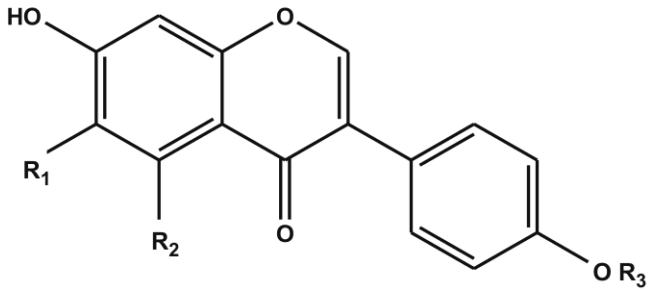
INTRODUCTION

Isoflavones are a group of phytoestrogens of widespread interest in nutritional, medicinal and cosmetic fields. These compounds are chemically similar to the hormone 17-β-estradiol, conferring several beneficial effects on the organism. Among several human foods containing isoflavones, soybeans are the most abundant, presenting predominantly the aglycones genistein, daidzein, glycitein and their respective acetyl, malonyl and glucoside conjugated forms (Chen *et al*., 2012; Nemitz *et al*., 2015). Red clover is another example of enriched natural source of isoflavones, which contain the same ones of soybeans, but predominantly present the free and conjugated forms of formononetin and biochanin A (Vacek, Klejdus, Lojkova & Kuban*,* 2008).

Aglycones (**Fig. 2.1**) are forms of isoflavones with a great capacity to be absorbed both in the gastrointestinal tract and skin, and have a substantial therapeutic potential when compared to the conjugated forms (Izumi *et al*., 2000; Nemitz *et al*., 2015). These compounds can be obtained by different ways, such as purchase of the isolated and synthetic forms or by extraction from plant materials. Since soybeans predominantly present the conjugated forms of isoflavones, extraction followed by hydrolysis and purification processes has been the method most often described for obtaining isoflavone aglycones from defatted soybeans or soyfoods (Rostagno, Villares, Guillamón, Gárcia-Lafuente & Martinéz, 2009; Nemitz et al. 2015). On the other hand, red clover predominantly presents isoflavones in their aglycone forms (Tsao, Papadopoulos, Yang, Young, & Mcrae, 2006), so their obtainment is basically the extraction from the leaves followed by purification processes (Vacek, Klejdus, Lojkova & Kuban*,* 2008).

Many patents and scientific studies can be found in the literature because of the clinical importance of isoflavones. Consequently, to facilitate the insight of the state of the art involving the use of isoflavones, several researchers have constantly written review articles. Some examples are: review on isoflavones regarding their biological activity (Barnes, 1998; Setchell, 1998; Albertazzi & Purdie, 2002; Wuttke, Jarry & Seidlova-Wuttke, 2007; Andres, Donovan & Kuhlenschmidt, 2009; Mortensen *et al*., 2009; Chen *et al*., 2012; Kolodziejczyk-Czepas, 2012; Fritz *et al*., 2013),

metabolism (Setchell, 1998; Heinonen, Wähälä & Adlercreutz, 2002; Yuan, Wang & Liu, 2007; Aura, 2008; Barnes, 2010), byossintesis (Tian, Pang & Dixon, 2008), meta-analysis of preclinical and clinical studies (Anderson, Johnstone & Cook-Newell, 1995; Zhan & Ho, 2005; Taku, *et al*., 2010; Yang *et al*., 2011; Gartoulla & Han 2014), description of the different ways of obtaining them (Rostagno, Villares, Guillamón, Gárcia-Lafuente & Martinéz, 2009; Chen *et al*., 2012; Nemitz *et al*., 2015), analytic methods for their quantitative measurement (Wang, Prasain & Barnes, 2002; Vacek, Klejdus, Lojkova & Kuban, 2008; Mortensen *et al*., 2009; Luthria & Nataranjan 2009; Raju, Kadian, Taneja & Wahajuddin, 2015), their topical use (Wei *et al*., 2003; Leyden & Wallo, 2011; Nemitz *et al*., 2015), the unpleasant taste that these compounds cause in foods (Drewnowski & Gomez-Carneros, 2011), or a description of technological alternatives to circumvent solubility challenges during the production of topical products (Nemitz *et al*., 2015). However, to date, none of the review studies has presented a technological mapping of patents involving isoflavones.



Isoflavone	R_1	R_2	R_3
Genistein	H	OH	H
Daidzein	H	H	H
Glycitein	OCH_3	H	H
Formononetin	H	H	CH_3
Biochanin A	H	OH	CH_3

Figure. 2.1 . Chemical structure of soybean isoflavone aglycones

It is noteworthy that to assess the state of the art of a particular subject, not only the scientific literature should be evaluated, but also the technologies protected by patents (Okubo, 1997). The reviews of patents are important tools for measuring the rates of production and dissemination of knowledge, and assist in making

decisions about new research projects to be undertaken either by universities or private sectors. Bibliometric indicators related to the patent also serve as a tool for assessing the degree of a country's technological development, in addition to identifying potential partners or market competitors (Frietsch *et al*., 2010).

In this context, considering both the importance and originality of the subject, this study aimed to conduct a search of the state of the art on patents within a 20-year timeframe involving the main isoflavones and their isolated forms: genistein, daidzein, glycitein, formononetin and biochanin A. The justification for undertaking the study was to help make new decisions concerning studies involving isoflavones, especially when it comes to risk management and technological innovations, improving the technological competitiveness, reducing investment uncertainty, and supporting new decision-making in research and development (R&D) of new products containing isoflavones.

THE IMPORTANCE OF PATENTS

Technological innovations are recognized as strategic elements of growth and development for all types of industries (Idris, 2003; Frietsch *et al*., 2010). An important way to induce innovation is the intellectual property (IP), since IP rights protect inventors and companies for a determined period from having their creations and innovations exploited in an unauthorized manner by third parties (Idris, 2003).

One way to ensure IP rights is by patenting a product or process (Idris, 2003; Frietsch *et al*., 2010). A patent is a legal document in the public domain aimed at ensuring the inventor of the right to economically exploit his invention or utility model, either individually or in the form of a license, for a determinate period of time, generally 20 years from the filing date of the application. In return, the inventor is obliged to provide technical information about the invention so as to allow for technological diffusion of innovations covered by patents (Auerbach, 2006). Furthermore, patents are territorial rights. In general, the exclusive rights are only applicable in the country or region in which a patent has been filed and granted. However, to ensure the exploitation in different countries, the applicant can also

submit an international patent application under the Patent Cooperation Treaty (PCT) which is administered by the World Intellectual Property Organization (WIPO).

Some assessment ways for summarizing the innovation profile of a specific region or segment are the prospecting studies through technological mapping (Ernst, 2003; Lee, Kang & Shin, 2015). That way, data and analyses based on technology indicators are typically used, and the number of patent applications is an important industrial development indicator (Basberg, 1987; Yoon, Yoon & Park, 2002). This indicator is normally used to mirror the profile of technological innovations as well as assists in future research and development activities (Frietsch *et al*., 2010). In addition, all of the information derived from patent surveys can assist in strategic planning for institutions, developing public policies, and industrial management (Ernst, 2003; Speziali, Guimarães & Sinisterra, 2012; Lee, Kang & Shin, 2015).

During the technological mapping, the correct searching of information is essential. While exhaustive searches can be difficult to conduct, the correct choice of research variables, such as the selection of keywords and database, is critical to determining the scenario to be evaluated (Noh, Jo & Lee, 2015).

The technological monitoring of patents in different countries can be made by means of national office databases or commercial databases. National or regional offices strive to maintain free access to documents. Some examples are the Brazilian National Institute of Intellectual Property (INPI), the U.S. Patent and Trademark Office (USPTO), the Japan Patent Office (JPO), and the State Intellectual Property Office of the People's Republic of China (SIPO).

The Espacenet database is another free website, part of the European Patent Office (EPO), characterized by the fact that it can locate patents filed in over 70 countries. Nonetheless, it is interesting to note that there are widely-specialized patent files, such as the Derwent Innovations Index (IBD), Questel Orbit, and Micropatent, but they are commercial databases and, therefore, access to their files is done by prepayment (Carvalho, Winter, Mothé & Carestiato, 2011; WIPO, 2015).

CLINICAL IMPORTANCE OF ISOFLAVONES AND TECHNOLOGICAL CHALLENGES

Isoflavones are plant compounds belonging to the group of polyphenols, known for their considerable estrogenic activity. In general, these compounds are found in the Leguminosae family, being predominantly present in soybeans, alfalfa sprouts, and red clover leaves (Vacek, Klejdus, Lojkova & Kuban, 2008). Phytoestrogens, and more specifically, the isoflavone aglycone genistein, are characterized by having various beneficial health effects, such as preventing heart disease, osteoporosis, cancer, diabetes and climacteric symptoms, besides offering beneficial effects when applied on the skin (Barnes, 1998; Albertazzi & Purdie 2002; Chen *et al*., 2012; Rodrigues *et al*., 2013; Nemitz *et al*., 2015; Rodrigues, Almeida, Sarmento, Amaral & Oliveira, 2014).

Because of these beneficial effects, both isolated isoflavones and plant extracts containing isoflavones are found in several food, dietary supplements, pharmaceutical, and cosmetic products. However, studies report that extracts containing isoflavones have higher clinical effects when compared to the individual phytoestrogens (Kim, Jeong & Kim, 2008; Rando, Ramachandran, Rebecchi, Ciana & Maggi, 2009; Hsu, Bray, Helferich, Doerge & Ho, 2010; Iovine, Iannella, Gasparri, Monfrecola & Bevilacqua, 2011). Moreover, it is important to highlight that to allow the gastrointestinal and skin absorption, the products should preferably contain the aglycone forms of isoflavones (Izumi *et al*., 2000; Nemitz *et al*., 2015).

On the other hand, isoflavone aglycones are less soluble than the conjugated forms, which often hinder the product development, especially those related to hydrophilic systems (Nemitz *et al*., 2015). Besides, the aglycone forms have bitterness and astringency, producing an unpleasant taste in food products and beverages (Drewnowski & Gomez-Carneros, 2011). To overcome these limitations, some technological alternatives are being proposed, such as: the development of liposome carriers, micro/nanostructures, and the formation of complexes with cyclodextrins to incorporate aglycone into hydrophilic systems or mask their disagreeable taste (Nemitz *et al*., 2015).

PRODUCTS CONTAINING ISOFLAVONES - FOODS, DIETARY SUPPLEMENTS, MEDICINES AND COSMETICS

Foods that contain high amounts of isoflavones include soy, peanuts, chick peas, alfalfa, fava beans, and kudzu. Between these foods, soybeans are the most abundant source of isoflavones. Soybean products (soyfoods), reported as potential functional foods, are often associated with the reduction of menopausal symptoms (Chen *et al*., 2012). These products are classified as non-fermented and fermented soyfoods, and the most common are soymilk, tofu, soy sauce, miso, infant formula, natto, tempeh, among others. The soy flour is a very attractive raw-material to obtaining soy protein concentrate and bioactive fractions, such as fractions enriched in isoflavones, which normally are marked as dietary supplements.

Dietary supplements are products intended for ingestion that contains a dietary ingredient purposed to add further nutritional value to supplement the diet. According to the Food and Drug Administration (FDA) conceptions, a dietary ingredient may be one, or any combination, of the following substances: vitamins, minerals, herb and botanicals, amino acid, metabolite, constituent, or an extract. The dietary supplements may be found in many forms such as tablets, capsules, softgels, gelcaps, liquids, or powders. Some dietary supplements can help ensure an adequate dietary intake of essential nutrients; others may help to reduce risk of diseases. Whatever the form, dietary supplements are considered a special category of food and are not considered drugs (Melethil, 2006).

The differences between medicinal products (drugs) and dietary supplements are based on their regulation routes, composition, claims and presentation (Coppens, da Silva & Pettman, 2006). The most commercialized products containing isoflavones, in USA and Europe, are the dietary supplements obtained from soybeans and red clover. These supplements are promoted worldwide for the treatment of menopausal symptoms and the maintenance of health and welfare after the menopause (Coppens et al, 2006; Melethil, 2006; Eisenbrand, 2007).

Because of the large demand for products containing isoflavones, especially by climacteric women, many studies and patents concerning the production of enriched-isoflavones products from soybeans and red clover are found in literature,

as well as, many commercial products are found in the market portfolio of industries. Soy extracts are marketed as food supplements and “dietary foods for special medical purposes”, whereas in most cases red clover extracts have been marketed as food supplements. These preparations are frequently market without medical prescriptions, and usually are available in pharmacies, supermarkets, and on the internet. The dosage recommended varies greatly according to the manufacture; however, it is generally between 20 and 80 mg isoflavone/day (Eisenbrand, 2007).

Cosmetic formulations containing isoflavones are market available with the appeal of anti-aging products. These formulations are supposed to reduce wrinkles and dryness, and increase skin elasticity (Kapuscinska & Nowak, 2015). According to literature and several clinical trials (Nemitz *et al*., 2015), the isoflavones have shown beneficial estrogenic effects on the skin, being largely attributed to the aglycone forms (Schmid & Zulli, 2002).

PATENT SURVEY CONCERNING ISOFLAVONES

Owing to the diverse applications of isoflavones in food and health fields, technological mapping of patents involving this particular topic is an attractive way to assess innovation prospecting in this sector. Thus, this study conducted a data compilation of patents involving isoflavones. The method used was based on a patent search in the international databases Espacenet and Espacenet-LATIPAT, bellowing to EPO, over a period of 20 years (1994 - 2014). The selected title’s keywords were "isoflavona", "genisteina", "daidzeina", "gliciteina", “formononetina” and “biochanina” with truncation symbol (*) used for searches in the Latin databases (lp.espacenet.com), and the words "isoflavone", "genistein”, “daidzein’, “glycitein”, “formononetin” and “biochanin” with the truncation symbol (*) for the international database (ep.espacenet.com).

The first set of data was carried out by sorting patents by year using the keywords "isoflavona*” and "isoflavone*" (**Fig. 2.2a**). As can be seen, the number of patent filings, as found on both Espacenet-LATIPAT and Espacenet, underwent significant growth between 1994 and 2002, signaling a potential increase in product and process innovation in a global context. Then, between 2004 and 2005, there was

a steady peak in terms of innovation, with a slight decrease and constancy in the following years. Besides, **Fig. 2.2b** shows the number of patents filed using the title's keywords "genistein", "daidzein", "glycitein" "formononetin" and "biochanin" during the 20 years assessed. It is noteworthy that the greatest innovative interest occurred with the isoflavone genistein. This scenario may have arisen as a result of scientific and clinical demonstrations that this is the most bioactive compound when compared to other isoflavones (Polkowski & Mazureki, 2000; Chen *et al*., 2012).

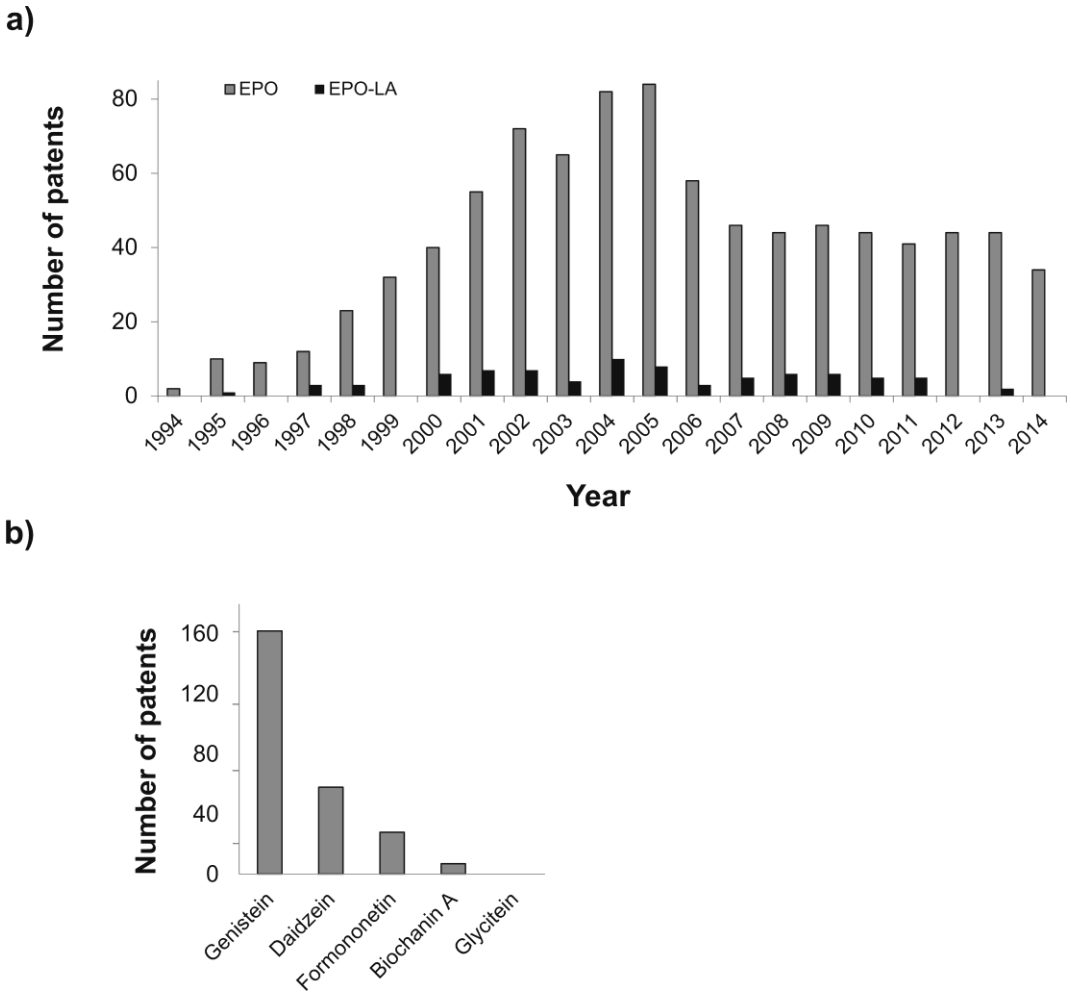


Figure. 2.2. Relationship of patent applications between 1994 and 2014, where: (a) annual distribution of patents with the title's keywords "isoflavona*" found in the Espacenet-LATIPAT (EPO-LA) database, and with the title's keywords "isoflavone*" found in the Espacente (EPO) database; (b) number of patents with the title's keywords "genistein*", "daidzein*", "glycitein*", "formononetin*" and "biochanin*" found in the Espacenet (EPO) database

In general, technologies have evolution cycles with slow onset of entering the market, followed by an ascension phase, and finally a decline when a new alternative is introduced. Cycle duration is variable because there are more stable industries in which improvements are essentially complementary or incremental, and there are more dynamic industries in which more radical technological changes are noted (Galembeck, dos Santos, Schumacher, Rippel & Rosseto, 2007). **Fig. 2.2a** shows that current technologies involving isoflavones are in decline, meaning that product innovations and improvements in processes are needed for a new ascension phase in this industrial area.

In view of the potential interest that food companies can display in the production of isoflavones from natural sources for their use as dietary supplements or cosmetics, as well as taking into account a recent scientific review published by Nemitz *et al*. (2015), the present manuscript selected and divided the patents described in **Fig. 2.2** into three major clusters: (1) processes for obtaining fractions enriched in isoflavones from plant materials; (2) the use of isoflavones in cosmetic products; and (3) incorporation of isoflavones into micro- and nanostructured systems designed to mask unpleasant flavors or circumvent the poor water solubility of their bioactive forms. The results of the divide-by-cluster patents from **Fig. 2.2a** and **Fig. 2.2b** are shown in **Table 2.1** and **Table 2.2**, respectively.

It is important to point out some peculiarities in the interpretation of the **Tables 2.1** and **2.2**, especially with regard to data compilation of the first cluster. Thus, it is highlighted that only the patents that describe methods of obtaining extracts, fractions, or isolated forms from plant materials and food products, resulting in a powder of isoflavones were compiled. Therefore, patents that describe obtaining food products in different forms than solid powder products, such as tofu, milk, cheese, cereal, noodles, sauce were not taken into consideration, even when the technology described ways to increase the content of isoflavones in these products. In addition, while preparing the tables, it was not taken into account, patents that describe different isoflavone synthesis routes, their by-products, analytical methods for quantification, metabolization, incorporation into classic pharmaceutical or supplement systems (e.g., capsules, tablets, and solutions), action mechanisms, types of pharmaceutical use, and changes in the culture process modifications.

Table 2.1. Patents separated by clusters after examination of documents obtained in Espacenet and Espacenet-LATIPAT databases, between the years of 1994 – 2014, with the keywords “isoflavone*” and “isoflavona*”

Cluster	Patent number					
Obtaining fraction containing isoflavones	US2002004458 (A)	WO2004002469 (A)	JP2000262244 (A)	CN101066960 (A)	CN101063158 (A)	EP0837139 (A)
	US2003104084 (A)	WO2004037020 (A)	JP2000281673 (A)	CN101084978 (A)	CN102319284 (A)	EP1038531 (A)
	US2003119759 (A)	WO2004043945 (A)	JP2001204486 (A)	CN101104866 (A)	CN102351827 (A)	EP1135142 (A)
	US2004047927 (A)	WO2004057983 (A)	JP2001302689 (A)	CN101129447 (A)	CN102406693 (A)	EP1135142 (A)
	US2004121059 (A)	WO2005042757 (A)	JP2002003487 (A)	CN101168759 (A)	CN103265614 (A)	EP1174144 (A)
	US2004126443 (A)	WO2005094604 (A)	JP2004147532 (A)	CN101255150 (A)	CN103342691 (A)	EP1197154 (A)
	US2004170713 (A)	WO2005094607 (A)	JP2005080655 (A)	CN101278729 (A)	CN103585254 (A)	EP1254132 (A)
	US2004215003 (A)	WO2008021699 (A)	JP2005224162 (A)	CN101407507 (A)	CN103652346 (A)	EP1303187 (A)
	US2004215003 (A)	WO2008070940 (A)	JP2005281251 (A)	CN101423521 (A)	CN103751260 (A)	EP1489918 (A)
	US2005085632 (A)	WO2012007978 (A)	JP2005318880 (A)	CN101532039 (A)	CN1111946 (A)	EP1491192 (A)
	US2005123633 (A)	WO2014030832 (A)	JP2005333973 (A)	CN101928273 (A)	CN1226805 (A)	EP1501919 (A)
	US2005202139 (A)	WO03093456 (A)	JP2005531616 (A)	CN101947251 (A)	CN1321640 (A)	EP1576893 (A)
	US2006116510 (A)	WO03077904 (A)	JP2007217367 (A)	CN101974577 (A)	CN1390946 (A)	EP1659118 (A)
	US2007179099 (A)	WO03000674 (A)	JP2007223915 (A)	CN102167688 (A)	CN1422855 (A)	EP1659119 (A)
	US2008044861 (A)	WO03084340 (A)	JP2007520192 (A)	CN102174621 (A)	CN1448394 (A)	EP1993579 (A)
	US2010048689 (A)	WO03082888 (A)	JP2008022834 (A)	CN102702161 (A)	CN1456557 (A)	EP2593570 (A)
	US2010190844 (A)	WO03088907 (A)	JP2011190245 (A)	CN102747116 (A)	CN1456558 (A)	EP1166643 (A)
	US2011305782 (A)	WO02056700 (A)	JP2013538187 (A)	CN102783647 (A)	CN1456559 (A)	EP0647408 (B)
	US2012003337 (A)	WO02080697 (A)	JP2000095792 (A)	CN102885263 (A)	CN1493695 (A)	EP0656786 (B)
	US2013231291 (A)	WO9726269 (A)	JP2004520066 (A)	CN102961436 (A)	CN1515523 (A)	EP0812837 (B)
	US5352384 (A)	WO9510512 (A)	JPH11155592 (A)	CN102964327 (A)	CN1515524 (A)	EP1233747 (B)
	US5670632 (A)	WO9510529 (A)	JPH11255792 (A)	CN102965184 (A)	CN1537865 (A)	EP1359215 (B)
	US5679806 (A)	WO9510530 (A)	JPH11263786 (A)	CN102994583 (A)	CN1570125 (A)	EP1391208 (B)
	US5702752 (A)	WO9737547 (A)	JPH08283283(A)	CN102977066 (A)	CN1584038 (A)	EP0795553 (B)
	US5726034 (A)	WO9935138 (A)	JPH11221048 (A)	CN103140588 (A)	CN1594307 (A)	EP1466609 (B)
	US5827682 (A)	WO9849153 (A)	JPH1067770 (A)	CN103145675 (A)	CN1757642 (A)	EP1450786 (B)
	US5851792 (A)	WO0032204 (A)	JPH1189589 (A)	CN103146774 (A)	CN1861592 (A)	EP0980363 (B)
	US5994508 (A)	WO0017217 (A)	JPH1099089 (A)	CN103773820 (A)	CN1940081 (A)	EP0906029 (B)
	US6013771 (A)	WO0158262 (A)	JP4354611 (B)	CN103788156 (A)	CN1944634 (A)	EP0827698 (B)
	US6033714 (A)	WO0234229 (A)	JP4279982 (B)	CN103848804 (A)	CN1986556 (A)	EP0804462 (B)
	US5637561 (A)	WO0139738 (A)	JP3609273 (B)	CN103755674 (A)	CN100345505 (C)	EP0794960 (B)
	US5763389 (A)	WO0017217 (A)	JP4031703 (B)	CN103739583 (A)	CN100345837 (C)	EP0647408 (B)

Table 2.1 continued

US6132795 (A)	KR100200182 (B)	JP3787844 (B)	CN1966705 (B)	CN100351388 (C)	EP0896795 (B)
US5990291 (A)	KR100361356 (B)	JP3118451 (B)	CN1911924 (B)	CN100356914 (C)	EP1377176 (B)
US5919921 (A)	KR100375981 (B)	JP4685202 (B)	CN1813711 (B)	CN100371341 (C)	EP1377273 (B)
US5637562 (A)	KR100379642 (B)	JP3777390 (B)	CN101029320 (B)	CN100383136 (C)	EP0723536 (B)
US6140469 (A)	KR100379642 (B)	JP3777389 (B)	CN101085768 (B)	CN100393710 (C)	EP0656786 (B)
US5789581 (A)	KR100380827 (B)	JP4343828 (B)	CN101086002 (B)	CN100405058 (C)	EP1054008 (B)
US5792503 (A)	KR100409054 (B)	JP3157168 (B)	CN101200744 (B)	CN100410267 (C)	EP0943245 (B)
US5821361 (A)	KR100411647 (B)	JP4090438 (B)	CN101239961 (B)	CN100415732 (C)	PT9800305 (A)
US6228993 (B)	KR100412116 (B)	JP4098385 (B)	CN101348811 (B)	CN100441555 (C)	PT827698 (E)
US6245536 (B)	KR100412117 (B)	JP4403800 (B)	CN101353340 (B)	CN100454003 (C)	PT906029 (E)
US6261565 (B)	KR100427679 (B)	JP4442422 (B)	CN101357933 (B)	CN100465281 (C)	CA2443383 (C)
US6323018 (B)	KR100442205 (B)	JP3118451 (B)	CN101386613 (B)	CN100484931 (C)	CA2218236 (C)
US6391310 (B)	KR100460697 (B)	JP4397525 (B)	CN101422504 (B)	CN100523210 (C)	CA2214665 (C)
US6410699 (B)	KR100475129 (B)	JP3919871 (B)	CN101503469 (B)	CN100577656 (C)	CA2207360 (C)
US6444239 (B)	KR100491186 (B)	JP3534844 (B)	CN101559094 (B)	CN100591225 (C)	CA2643973 (A)
US6495141 (B)	KR100525875 (B)	JP3239121 (B)	CN101701232 (B)	CN1095468 (C)	CA2249366 (C)
US6497906 (B)	KR100557006 (B)	AT192284 (T)	CN101736052 (B)	CN1107065 (C)	CA2249501 (C)
US6500965 (B)	KR100613764 (B)	AT201128 (T)	CN101766673 (B)	CN1109682 (C)	CA2307064 (C)
US6518319 (B)	KR100620568 (B)	AT222064 (T)	CN101775023 (B)	CN1114351 (C)	CA2290004 (C)
US6521282 (B)	KR100683182 (B)	AT241282 (T)	CN101781278 (B)	CN1069903 (C)	CA2484258 (A)
US6562380 (B)	KR100823896 (B)	AT282032 (T)	CN101792779 (B)	CN1120163 (C)	CA2173743 (A)
US6579561 (B)	KR100847225 (B)	AT302767 (T)	CN101824019 (B)	CN1132830 (C)	CA2448513 (A)
US6664382 (B)	KR100858280 (B)	AT312605 (T)	CN101921300 (B)	CN1137629 (C)	CA2133382 (C)
US6703051 (B)	KR100882280 (B)	AT361741 (T)	CN102002029 (B)	CN1158273 (C)	CA2173999 (C)
US6703051 (B)	KR100891165 (B)	AT415169 (T)	CN102206209 (B)	CN1163150 (C)	CA2307061 (C)
US6706292 (B)	KR100996952 (B)	AT437575 (T)	CN102302539 (B)	CN1176084 (C)	CA2403584 (C)
US6818246 (B)	KR101198426 (B)	AT460474 (T)	CN102424674 (B)	CN1193683 (C)	CA2288321 (C)
US6987098 (B)	KR101233117 (B)	AT460497 (T)	CN102558192 (B)	CN1216151 (C)	CA2174120 (A)
US7045155 (B)	KR101302376 (B)	RU2124896 (C)	CN102702158 (B)	CN1233639 (C)	CA2803322 (A)
US7060470 (B)	KR101393240 (B)	RU2130073 (C)	CN102702160 (B)	CN1237896 (C)	CA2240795 (C)
US7208594 (B)	KR20000005290 (A)	RU2142957 (C)	CN102838575 (B)	CN1257899 (C)	CA2217649 (C)
US7524526 (B)	KR20000055133 (A)	RU2151775 (C)	CN102838576 (B)	CN1261586 (C)	CN2666177 (Y)
US7553505 (B)	KR20010016220 (A)	RU2152434 (C)	CN103223001 (B)	CN1300135 (C)	NO20045212 (A)
US7618671 (B)	KR20010027341 (A)	RU2180662 (C)	CN103262941 (B)	CN1314678 (C)	NO325127 (B)
US6395279 (B)	KR20010039376 (A	RU2197095 (C)	CN1321992 (C)	CN1241922 (C)	AR016141 (A)
US5858449 (B)	KR20010073600 (A)	RU2197095 (C)	CN1321993 (C)	CN1305869 (C)	SG43879 (A)
US6320028 (B)	KR20010089863 (A)	RU2207006 (C)	CN1065864 (C)	CN1328274 (C)	US6146668 (A)
US6369200 (B)	KR20010091098 (A)	RU2216991 (C)	CN1055931 (C)	CN1282650 (C)	US7033621 (B)

Table 2.1 continued

	US6391308 (B)	KR20030092338 (A)	RU2219785 (C)	CN1055932 (C)	CN1273609 (C)
	US6391309 (B)	KR20030095669 (A)	RU2273147 (C)	CN1214020 (C)	CN1210407 (C)
	US6399072 (B)	KR20040038481 (A)	RU2309603 (C)	CN103965154 (A)	CN1305392 (C)
	US6479054 (B)	KR20040009971 (A)	HK1024918 (A)	CN103623048 (A)	CN1309716 (C)
	US6517840 (B)	KR20050065486 (A)	HK1071512 (A)	CN103598579 (A)	CN1094044 (C)
	US6818246 (B)	KR20040048225 (A)	US7595080 (B)	ITRM20100378 (A)	CN1127495 (C)
	US7083819 (B)	KR20040098612 (A)	TWI260205 (B)	RU2013105731(A)	CN1195750 (C)
	US7084263 (B)	KR20070106255 (A)	TWI377254 (B)	KR101451298 (B)	DK0827698 (T)
	US7112573 (B)	KR20070111655 (A)	TW574225 (B)	ZA200307700 (A)	DK0906029 (T)
	US7306821 (B)	KR20100035786 (A)	TWI241893 (B)	DE69430314 (T)	DK0943245 (T)
	US7354765 (B)	KR20100035840 (A)	TWI245603 (B)	DE69429931 (T)	DK1450786 (T)
	US7378114 (B)	KR20120059198 (A)	AU4099897 (A)	DE69429673 (T)	HU0002624 (A)
	US7560131 (B)	KR20140026725 (A)	AU5956699 (A)	DE69422124 (T)	NZ500715 (A)
	BRPI0502309 (A)	BR9805069 (A)	AU3690601 (A)	DE69709472 (T)	ZA9702978 (A)
	BRPI0903222 (A)	BR9809316 (A)	AU1847097 (A)	DE69908219 (T)	NZ331691 (A)
	BR0002609 (A)	BR9809316 (A)	AU1243402 (A)	DE69921778 (T)	NZ506701 (A)
	BR0001876 (A)	BR9815832 (B)	AU1704601 (A)	DE69714734 (T)	IL120409 (A)
	BR0004237 (A)	BR9815832 (B)	AU2475600 (A)	DE69704861 (T)	IL140953 (A)
	BR0208798 (A)	BR9915896 (A)	AU777632 (B)	DE69701842 (T)	NO984695 (A)
	BR1101092 (A)	BR9915896 (A)	AU674437 (B)	DE69734042 (T)	NO325456 (B)
	BR1101108 (A)	AU718810 (B)	AU2003285804 (A)	ZA9808962 (A)	ES2147660 (T)
	BR9404054 (A)	AU748832 (B)	AU2002210886 (A)	TW474930 (B)	ES2160895 (T)
	BR9407792 (A)	AU717144 (B)	AU2003231985 (A)	TW491688 (B)	ES2160895 (T)
	BR9407820 (A)	AU720462 (B)	AU2003231977 (A)	TW491851 (B)	ES2180979 (T)
	BR9407822 (A)	AU742569 (B)	AU2003223644 (A)	TW491852 (B)	ES2180979 (T)
	BR9703526 (A)	AU764493 (B)	AU2003284189 (A)	TW491895 (B)	ES2199523 (T)
	BR9704589 (A)	AU720838 (B)	AU2002328824 (A)	TW493002 (B)	ES2232107 (T)
	BR9705019 (A)	AU680554 (B)	AU2003291446 (A)	TW526081 (B)	ES2247617 (T)
	BR9705428 (A)	AU732423 (B)	AU2002254573 (B)	GB2339429 (B)	ES2254725 (T)
	BR9705428 (A)	AU696553 (B)	AU2003227617 (B)	FR2817866 (B)	ES2315332 (T)
	BR9708545 (A)	AU696574 (B)	AU2003296185 (B)	FR2815539 (B)	ES2333304 (T)
	BR9708545 (A)	AU738774 (B)	CL31972002 (A)	IL158569 (A)	IL158263 (A)
Use of isoflavones in cosmetics	US2002106388 (A)	WO2004002435 (A)	DE202005012206 (U)	MXPA05000073 (A)	EP1158975 (A)
	US2002107282 (A)	WO2005030157 (A)	DE102004020712 (A)	AU2003264673 (A)	EP1205179 (A)
	US2002160064 (A)	WO2007000192 (A)	DE102004006829 (A)	ITMI20060392 (A)	EP1259221 (A)
	US2004170655 (A)	WO02074278 (A)	KR20040091178 (A)	TW201021843 (A)	EP1377254 (A)
	US2005256061 (A)	WO02076409 (A)	CN101212965 (A)	KR100500641 (B)	EP1667641 (A)

Table 2.1 continued

	US6017893 (A)	WO02089757 (A)	CN101732180 (A)	KR100530199 (B)	EP1896009 (A)	
	US8685456 (B)	WO0234229 (A)	CN102113977 (A)	KR100868904 (B)	EP1233747 (B)	
	AU1243402 (A)	WO0139738 (A)	CN102525850 (A)	KR101195970 (B)	EP1234572 (B)	
	AU1704601 (A)	WO0152840 (A)	CN102631362 (B)	KR101229511 (B)	FR2841470 (B)	
	AU3555101 (A)	WO0164177 (A)	DE10009424 (A)	KR101314689 (B)	HK1074404 (A)	
	FR2803747 (B)	EP1361854 (B)	DE10114305 (A)	BRPI0612705 (A)	ES2311730 (T)	
	FR2815539 (B)	EP1515696 (B)	DE10122342 (A)	JP2002193726 (A)	AT493109 (T)	
	FR2816502 (B)	EP1834628 (B)	DE10344531 (A)	CN1460465 (A)	JP5129130 (B)	
	FR2820974 (B)	DE4432947 (C)	DE60219432 (T)	CN1671354 (A)	CA2491150 (A)	
	FR2822068 (B)	JP3271840 (B)	AU4647101 (A)	CN1205915 (C)		
Micro and nanotechnology systems containing isoflavones	US2002160064 (A)	BRPI0612705 (A)	KR20000030509 (A)	JP2003183166 (A)	CN101757640 (B)	EP2640188 (A)
	US2005220949 (A)	BRPI0612848 (A)	KR20000030512 (A)	JP2013545752 (A)	CN102652736 (B)	EP1234572 (B)
	US2009035336 (A)	CN103961716(A)	KR100504379 (B)	JP2002155072 (A)	CN201657748 (U)	EP1904053 (B)
	US2012121654 (A)	WO0113890 (A)	KR101242851 (B)	JPH09309902 (A)	CN101212964 (A)	
	US2013189320 (A)	US5847108 (A)	KR101314689 (B)	JPH10298175 (A)	CN101212965 (A)	
	US2013190392 (A)	US8551530 (B)	CN1861058 (A)	JP5129130 (B)	CN101947251 (A)	
	WO2007000192 (A)	US8685456 (B)	AU6591400 (A)	JP5134535 (B)	CN103099798 (A)	
	WO2007000193 (A)	US6890561 (B)	ES2369928 (T)	EP1210066 (A)	CN103211750 (A)	
	WO2012068140 (A)	CA2382218 (A)	AR044000 (A)	EP1896009 (A)	CN103211769 (A)	
	KR100482355 (B)	CA2852410 (A)	AT520396 (T)	IL131508 (A)	CN1660075 (A)	

Considering the total number of patents described in the three clusters in **Table 2.1**, it is important to note that the 705 patents represent a total of 346 technologies. This difference is due to the fact that some technologies were filed in more than one country. Among the protected technologies, 46 patents were filed via international application. The technology that has the highest number of patents filed via PCT is internationally entitled "*Novel isoflavone-enriched soy protein product and method for its manufacture*", first filed by the American company DuPont in 1995 under the number WO9737547 (A). This technology has been patented in over 17 countries, resulting in a total of 18 patents. The technology internationally entitled "*Recovery of isoflavones from soy molasses*", filed by Protein Technologies International (PTI) first and foremost in the European patent office under the number EP0812837 (A) in 1997 and granted in 2005 under the number EP0812837 (B), is the one that has the largest number of patents in different countries. This patent was not filed via PCT; but the company filed 20 separate patents in different countries.

The patents that were reviewed and presented in **Table 2.1** refer to obtaining isoflavones by means of extraction and purification from various plants. The different raw materials include a description of isoflavone extraction from *Glycine max* (soybeans), and their by-products or from other plant species such as *Trifolium pretense* (red clover), *Iris florentine, Astralagus membranaceous, Pueraria montana, Butea monosperma, Sophorae japonica, Pueraria thunbergiana, Belamcanda chinensis* and *Dalbergia odoriferous,* among others.

To illustrate obtaining isoflavone fractions from plant materials for use as dietary supplement, it can be cited US6146668 (A) filed in the United States in 1997 by the American company Novogen, that posterior assistance of in the creation of the patent US6599536 (B), filed by the same company. These technologies refers to obtaining a fraction enriched in isoflavones from plant material (preferably red clover, *Trifolium pratense*), and the oral administration of tablets containing the fraction, respectively. These patents have given rise to Promensil®, Rimostil® and Trinovin®, supplements recommended for the treatment of climacteric symptoms in menopausal women, bone health, and to maintain prostate health and urinary function in men, respectively (Booth *et al*., 2006). The dietary supplement protected by the second technology was also protected in several countries or regions, such as

South Africa (ZA200005070 A), Turquia (TR200002770 T), Portugal (PT1063990 E), Norway (NO325456 B), Japan (JP2002507568 A), Israel (IL138129 A), Spain (ES2281169 T), Europe (EP 1063990 B), Denmark (DK1063990 T), Germany (DE69934877 T), Canada (CA2325631 A), and Brazil (BR9909105 A).

Another example of successful marketing of a food product from protected technology, comprising an innovative way to obtain fractions containing isoflavones, is Novasoy®, a supplement belonging to the American company ADM (Ray, 2004). This product was launched in 1998 and consists of concentrated isoflavones, commercially sold as a raw material for nutritional supplements, medicine, or cosmetics with a composition of 40% genistin, daidzin, and glycitin and their aglycone forms in a ratio of 1.3: 1.0: 0.3. As an example of its use in the medicinal products, it is cited the technology filed in the United States under number US2003175345 (A), in Europe (EP1463515 A), Canada (CA2484528 A), and Australia (AU20023666380 A), which describes the use of this supplement in the production of controlled-release tablets for oral use. In the cosmetics field, an example of using Novasoy® technology is a patent filed by L'oreal under number FR2818148 (A) in 2002, granted in 2005, and also filed in Europe (EP1343459 B), Japan (JP2004515516 A), United States (US6852326 B), Spain (ES2278680 T), Germany (DE60125823 T), Australia (AU2080302 A) and Austria (AT350111 T).

With regard to patents shown in the cosmetic cluster, those filed by German company Biersdorf, represented by the Nivea brand, are of particular interest since this brand has a strong global presence in the cosmetics sector (Chen & Liang, 2013). Technologies filed by the company include WO02076409 (A), WO02089757 (A) filed in 2002 and WO2005030157 (A), DE102004020712 (A), DE102004006829 (A) filed in 2005, and describe formulations containing isoflavones among the products' active ingredients, which are suitable for different cosmetic actions, such as a reduction of sebum, antiperspirant and anti-aging actions, and a decrease in sensitive skin irritation. In the cosmetics cluster, it is also interesting to mention the patent WO0234229 (A) filed by the French company Silab in 2002. This technology describes obtaining a fraction enriched in isoflavone aglycones from rhizomes of plants belonging to the genus *Florentine* with subsequent incorporation into gels and creams suitable for anti-aging skin action.

In the nanotechnology cluster, some patents describe product innovations containing isoflavones for dietary supplements or cosmetic use. The patent WO2007000193 (A) describes the production of genistein powder with nano-sized particles resulting from the substance's self-aggregation, but without being incorporated into a nanocarrier. Subsequently, the patent WO2007000192 (A) describes the use of this nano-sized particle in cosmetic products. The patents JPH09309902 (A), US5847108 (A), JPH10298175 (A), JP2002155072 (A) describe the combination of cyclodextrins with isoflavone aglycones, particularly aimed at reducing their bitterness and astringency or increasing their water solubility. The patent CN1660075 (A) uses the combination of isoflavones and cyclodextrin to manufacture various kinds of medicinal products. The patents KR20000030509 (A) and KR20000030512 (A) describe the production of microcapsules with sugars and emulsifiers to increase the isoflavone's water solubility. The patent WO0113890 (A) describes the production of modified release microcapsules for oral administration, and patent KR100482355 (B) describes the production of liposomes and nanoemulsions containing soy isoflavones and dipalmitoyl hydroxyproline for incorporation into cosmetics. Finally, the United State patent US2002160064 (A) describes the production of liposomes, preferably containing genistein and daidzein, for incorporation into cosmetics. This patent was subsequently licensed to Mibelle for the production of a marketable product known as Lipobelle Soyaglycone®.

To better visualize the most prominent countries in technological innovation involving isoflavones, the technologies shown in **Table 2.1** were organized by countries in which patents were filed. Thus, the most prominent countries for the cluster related to obtaining isoflavone fractions are China, USA, Korea and Japan, with 132, 63, 44 and 28 protected technologies, respectively. The most prominent countries in the cosmetic cluster are France, Germany, China, and Korea with 08, 07, 05 and 05 protected technologies, respectively. The most prominent countries in the nanotechnology cluster are China, Japan, and Korea with 10, 04, and 04 protected technologies, respectively. The total number of patented technologies is a valuable indicator of the degree of innovative development of the countries.

However, it is noteworthy that this indicator is considered controversial when the objective is the comparison of aggregated value, for example, estimates of

economic, technological, social, and strategic value in different countries, since some companies may file patents without subsequently launching marketable products involving the patented technology, or there are institutions such as universities filing patents that end up not being licensed afterwards (Frietsch *et al*., 2010).

To better visualize the types of patent applicants involving isoflavones, the technologies presented in **Table 2.1** were organized by type of holder, which was broken down into three categories: independent, company, or university. The proportion of patents filed independently, by companies, and by universities within each cluster can be seen in **Fig. 2.3**.

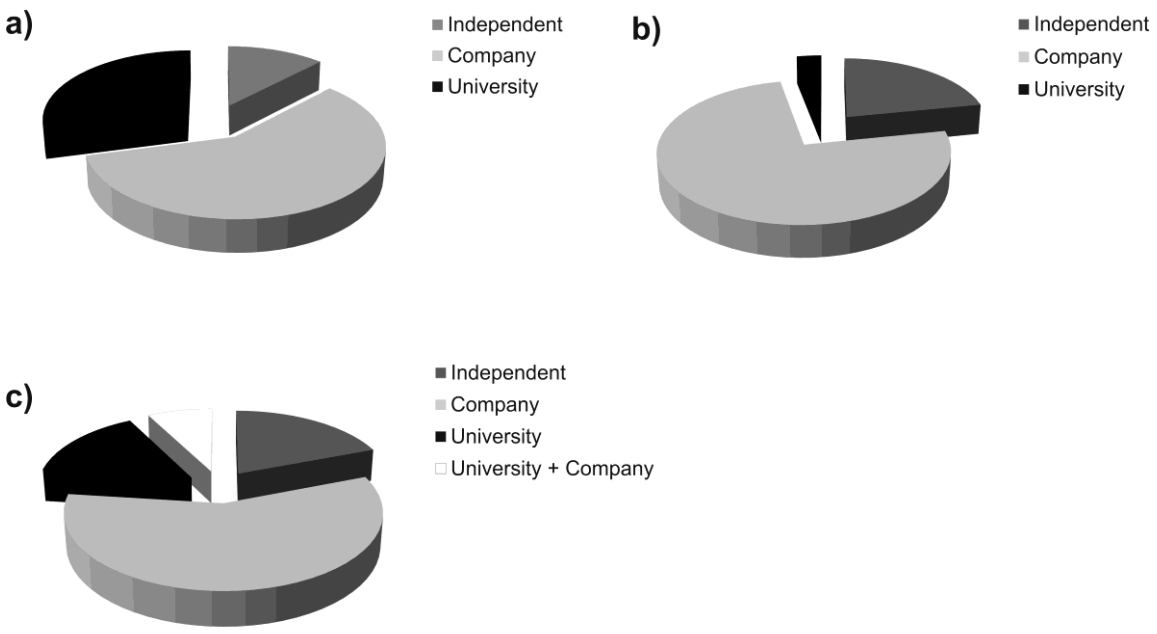


Figure. 2.3. Types of patent applicants according to technologies filed for each cluster, where: (a) obtaining fraction containing isoflavones; (b) use of isoflavones in cosmetics; (c) micro and nanotechnology systems containing isoflavones

For the cluster related to obtaining fractions (**Fig. 2.3a**), 168 technologies represent filings made by companies, 83 technologies by universities, and 35 independent filers. With regard to the cosmetic cluster (**Fig. 2.3b**), 24 technologies represent filings made by companies, 01 technology by a university, and 07 by independent filers. For the cluster related to nanotechnology systems (**Fig. 2.3c**), 15

technologies represent filings made by companies, 04 by universities, 02 by partnerships between universities and companies, and 05 by independent filers. Among the patents filed by universities, China is the country that most protects its technologies, and when it comes to obtaining fractions, the educational institutions with more patent applications are Northeast Agricultural University, Zhejiang University, and the Institute of Jilin of Chemical Technology, with 06, 05, and 03 protected technologies, respectively.

To better visualize the most outstanding companies in products containing isoflavones, the technologies in **Table 2.1** were organized by filing companies. These data prove to be important, especially when the interest is in seeking potential partners, competitors, or markets for future innovations and business strategies. Thus, in the area of obtaining isoflavone fractions, the most prominent companies are the American's DuPont (34 technologies), ADM (07 technologies), Novogen (06 technologies), the Japan's Kokkoman (04 technologies), Fuji Oil (04 technologies), and the South Korea's Eugenbio (05 technologies) and Rexgene (04 technologies). In the cosmetics area, the most prominent companies that invest in products containing isoflavones are the German Biersdorf (05 technologies), the French Pharmascience (03 technologies), Korean Amorepacific (02 technologies), and Kolmar (02 technologies). In the area involving isoflavones and the complexation of cyclodextrins or incorporation into nanostructures, the leading companies in terms of protected technologies are the Japanese Fuji Tsuko (03 technologies), the Chinese Nano and Advanced Materials Institute Ltd (02 technologies), and the Netherlands' DSM (02 technologies).

To get a better understanding of the history of companies that innovate in products containing isoflavones, especially those of the first cluster, a search was made of the literature and websites of the most prominent companies in the field. Protein Technologies International (PTI), founded in 1958 in the United States, was bought in 1997 by DuPont and Bunge. Up until 2002, PTI was the leader in the protection of new technologies involving obtaining isoflavones from plant materials, especially soybeans. In 2003, PTI changed its name to Solae LLC and, in 2012, DuPont became its majority shareholder. According to data reported by Shurtleff and Aoyagi (2013), the biggest competitor of DuPont is the American company Archer

Daniels Midland (ADM). This company, founded in 1902, has a consolidated trade in the food market. However, according to the results here expressed, DuPont, through its subsidiaries PTI and Solae LLC, is the one that has the most protected technologies involving obtaining fractions that contain isoflavones.

Taking account all the patents described in **Table 2.2**, the 122 patents represent, in real, a total of 56 technologies. In the cluster of obtaining fractions, the sum of technologies that describe obtaining individuals or mixtures of genistein, daidzein, formononetin and biochanin A is 37, in which: 19 filed by companies, 15 by universities, and 03 by independent filers. Six technologies were found in the cosmetic cluster involving genistein and/or daidzein: 03 filed by companies, 02 by universities, and 01 by independent filer. Thirteen technologies were found in the nanotechnology cluster involving genistein and/or daidzein: 10 filed by universities, 02 independent, and 01 by a company.

A literature review was recently conducted by Nemitz *et al*. (2015), which took into account papers published in scientific journals addressing the same clusters of interest described herein. Thus, the set of previously published scientific data and the technological data described in this paper may help researchers from universities and the private sector look for ideas and input for new product innovations containing isoflavone aglycones, especially with regard to the use of these substances in dietary supplements and skin care products.

CONCLUSIONS

Owing to the importance of technological prospecting for the development of new projects and research, this paper has presented a technological mapping related to isoflavones, specifically their use in cosmetics, their incorporation into different delivery systems, and the processes for obtaining enriched fractions from plant materials. The results presented herein refer to patents filed over a period of 20 years (1994 - 2014), showing the current scenario by country and type of filers. With the present data, it is possible to see the state of the art of technologies applied to food (extracts for dietary supplements) and cosmetic fields. However, the results should be interpreted with caution when considering society's social, economic, and political aspects or the field of knowledge involved.

Table 2.2. Patents separated by clusters after examination of documents obtained in Espacenet and Espacenet-LATIPAT databases, between the years of 1994 – 2014 with interested keywords

Keywords	Patent number			
	Cluster 1		**Cluster 2**	**Cluster 3**
Genistein Genisteína	KR20040005113 (A) KR100858280 (B) KR100849145 (B) KR100412116 (B) CN103773820 (A) CN101760488 (A) CN101619338 (A) CN102351828 (A) CN101544626 (B) CN101497594 (B) CN101709057 (B) CN100523210 (C) CN101781278 (B) CN102491965 (B) CN101210058 (B) MX9706806 (A) DE69701842 (T) DE69704861 (T) CN1210407 (C) CN1269810 (C) CN1352896 (A) CN1216878 (C) RU2152434 (C) RU2309603 (C) NZ252051 (A)	WO9706273 (A) US5554519 (A) US5726034 (A) US5851792 (A) CA2218236 (C) CA2214665 (C) BR0304014 (A) BR9705428 (A) BR9704589 (A) DK0827698 (T) AU4099897 (A) AU6716196 (A) AU718810 (B) EP0827698 (B) ES2147660 (T) ES2160895 (T) PT827698 (E) AT201128 (T) AT192284 (T) TW574225 (B) JPH11155592 A JPH1099089 A JP3078694 (B) JP3482558 (B)	JP2000511907 (A) US2012083524 (A) US2009286872 (A) US5824702 (A) CA2257579 (C) DK0918504 (T) DE69719994 (T) WO9746208 (A) PT918504 (E) AT234599 (T) AU716131 (B) KR20070014672 (A) WO2007116052 (A) KR100500641 (B) CN102488210 (B) ES2188963 (T) EP0918504 (B) EP2004161 (B)	CN102319438 (A) CN103271892 (A) CN103301090 (A) CN103446055 (A)
Daidzein Daidzeína	AU718810 (B) AT192284 (T) AT201128 (T) TW574225 (B) CN1210407 (C) JPH1099089 (A) DE69704861 (T) DE69701842 (T) JPH11155592 (A) CN100523210 (C) JP2007223915 (A) KR100412116 (B) KR100855741 (B) CN102911978 (A) CN103059008 (A) CN101210058 (B) KR20040009971 (A)	US5851792 (A) US5726034 (A) EP0827698 (B) ES2160895 (T) ES2147660 (T) CA2214665 (C) CA2218236 (C) BR0304014 (A) BR9705428 (A) BR9704589 (A) DK0827698 (T) RU2309603 (C) RU2152434 (C) AU4099897 (A) MX9706806 (A) PT827698 (E) NZ252051 (A)	KR20070014672 (A)	CN101204392 (A) CN102008454 (B) CN102058528 (B) CN102060870 (B) CN102258475 (B) CN102274202 (B) CN102552208 (B) CN102727482 (B) CN100487032 (C)
Glycitein Gliciteína	No patents			
Formononetin Formononetina	CN102532083 KR2008007534	NZ252051 (A) CN101775418		

Table 2.2 continued

Biochanina A	CN103773820 (A)
Biochanin A	CN102373248 (A)
	NZ252051 (A)

Cluster 1: Obtaining fraction containing isoflavones; Cluster 2: Use of isoflavones in cosmetics; Cluster 3: Micro and nanotechnology systems containing isoflavones

Finally, this study contributes to the food sciences, once it helps to visualize opportunities for future decision-making and expansion in research concerning new routes of obtaining isoflavones from natural sources, and impulses the innovation for development of new products intended mainly for the area of dietary supplements and cosmetic products.

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CAPÍTULO III

Desenvolvimento e validação de método cromatográfico para avaliação quantitativa das isoflavonas agliconas em amostras analíticas e bioanalíticas

3.1. INTRODUÇÃO

Durante o desenvolvimento de produtos farmacêuticos é necessário realizar a quantificação dos compostos ativos continuamente em todas as fases dos estudos, tais como etapas de formulação, estabilidade e testes bioanalíticos. Para isso, diferentes técnicas podem ser utilizadas, bem como diferentes métodos podem ser desenvolvidos.

Dentre diversas técnicas existentes, a cromatografia líquida (CL) é uma das mais utilizadas na pesquisa e rotina de laboratórios (LANÇAS, 2008). Recentente, o uso de métodos denominados de ultraeficiência vem a aprimorar as técnicas de CL, pois suportam altas pressões, possibilitam uso de colunas cromatográficas com diâmetro interno reduzido (<3 µm), que aumentam a resolução dos picos e diminuem tanto a corrida cromatográfica como o consumo dos reagentes químicos (GAIKWAD *et al*., 2010).

Para garantir que um novo método de quantificação de compostos em produtos da saúde gere informações confiáveis e interpretáveis sobre a amostra, é recomendado pelos guias e compêndios mundiais a execução de uma etapa denominada de validação de método analítico e/ou bioanalítico. A validação de um método é um processo contínuo que começa no planejamento da estratégia analítica e continua ao longo de todo o seu desenvolvimento, confirmando que o mesmo é apropriado para as aplicações pretendidas, e dessa forma, assegurando a confiabilidade dos resultados (BRASIL, 2003).

Nesse contexto, tendo em vista que o objetivo geral desta tese é a obtenção de uma fração enriquecida em isoflavonas agliconas da soja para incorporação em um produto tópico dérmico, é de extrema importância realizar a determinação quantitativa dos compostos de interesse durante os experimentos por meio de um método analítico sensível, preciso, exato, robusto e indicativo de estabilidade.

O presente capítulo descreve, portanto, o desenvolvimento e a validação de um método analítico e bioanalítico por cromatografia líquida de ultraeficiência para a quantificação de genisteína, daidzeína e gliciteína em amostras do extrato hidrolisado de soja e seu derivado (fração), bem como nos produtos obtidos de nanoemulsões e hidrogéis contendo a fração, e ensaios bioanalíticos de permeação das isoflavonas agliconas na pele de orelha ou mucosa de esôfago suíno.

Os dados experimentais deste capítulo estão apresentados na forma de artigo científico, redigido nas normas do periódico em que foi publicado.

3.2. ARTIGO

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A VERSATILE, STABILITY-INDICATING AND HIGH-THROUGHPUT ULTRA-FAST LIQUID CHROMATOGRAPHY METHOD FOR THE DETERMINATION OF ISOFLAVONE AGLYCONES IN SOYBEANS, TOPICAL FORMULATIONS, AND PERMEATION ASSAYS

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ABSTRACT

There is a growing interest in the pharmaceutical field concerning isoflavones topical delivery systems, especially with regard to their skin care properties and antiherpetic activity. In this context, the present work describes an ultra-fast liquid chromatography method (UFLC) for determining daidzein, glycitein, and genistein in different matrices during the development of topical systems containing isoflavone aglycones (IA) obtained from soybeans. The method showed to be specific, precise, accurate, and linear (0.1 to 5 µg mL^{-1}) for IA determination in soybean acid extract, IA-rich fraction obtained after the purification process, IA loaded-nanoemulsions, and topical hydrogel, as well as for permeation/retention assays in porcine skin and porcine esophageal mucosa. The matrix effect was determined for all complex matrices, demonstrating low effect during the analysis. The stability indicating UFLC method was verified by submitting IA to acidic, alkaline, oxidative, and thermal stress conditions, and no interference of degradation products was detected during analysis. Mass spectrometry was performed to show the main compounds produced after acid hydrolysis of soybeans, as well as suggest the main degradation products formed after stress conditions. Besides the IA, hydroxymethylfurfural and ethoxymethylfurfural were produced and identified after acid hydrolysis of the soybean extract and well separated by the UFLC method. The method's robustness was confirmed using the Plackett-Burman experimental design. Therefore, the new method affords fast IA analysis during routine processes, extract purification, products development, and bioanalytical assays.

Keywords: Isoflavone aglycones; Matrix effect; Permeation/retention assays; Soybean acid extract; Stability indicating UFLC method; Topical delivery systems.

INTRODUCTION

The use of soybeans is of interest from scientific and economic points of view, basically due to the presence of isoflavones [1]. These compounds have demonstrated beneficial health effects, especially for preventing heart disease, osteoporosis, cancer, and diabetes [2], plus reducing climacteric symptoms in menopausal and postmenopausal women [3]. Moreover, isoflavones act against acute virus infections [4], and have many beneficial effects on the skin [5,6], demonstrating a growing interest on their topical use in the pharmaceutical and cosmetics field.

In general, isoflavones are incorporated into topical products in their glycoside forms [7]. In some cases, this may limit the biological action of these conjugated forms due to the low possibility of penetration through different skin layers or mucosa tissues [7]. Therefore, to get the isoflavones' desired effects, their aglycone forms such as daidzein, glycitein, and genistein, should preferably be used [7, 8]. However, it is important to highlight that the isoflavone aglycones (IA) are less hydrosoluble than the conjugated forms, and this may limit their incorporation into traditional topical delivery systems [7]. To allow better use of IA in formulations and enhance their penetration, some alternative technologies have been studied, such as nanoemulsions [9-12].

During products development, routine analysis, or biological assays, it is essential to use an adequate method for quantification of the compounds of interest. In this way, a number of analytical methods have been reported for isoflavones analysis [13,14]. Nevertheless, high-pressure liquid chromatography (HPLC) with ultraviolet-visible (UV-Vis) detector is, undoubtedly, the method of choice applied in this field [13,14]. Although the HPLC methods have some advantages when it comes to analyzing isoflavones in terms of specificity, sensitivity, and straightforward operation, they require a relatively long period of time, normally from 20 min [15] to 65 min [16]. To overcome this lengthy amount of time, the use of high-throughput liquid chromatography technologies has been reported in the last decade for isoflavone analysis, reducing the chromatographic time to less than 10 min [17-21]. These techniques allow the use of short columns, packed with <3 μm particles,

supporting elevated pressures, thus reducing the analysis time, solvent consumption, and, consequently, the environmental impact [22].

Among the high-throughput methods reported in the literature for isoflavones analysis, ultra-performance liquid chromatography (UPLC) and ultra-fast liquid chromatography (UFLC) are cited for their determination in soybeans cultivars [21], phytoestrogen-rich plants [17,23,24], soy bits [17], soymilk [25], texturized soy protein [20], and soy-based nutritional supplements [26]. However, none of them have reported the use of UFLC or UPLC for pharmaceutical or cosmetic formulation containing isoflavones in permeation/retention studies of these compounds on skin or mucosa tissues.

Recently, our research group reported a stability-indicating HPLC method for routine analysis of IA present in soybeans after acid hydrolysis, as well as in nanoemulsions containing the extract [27]. The method has been reported as a new approach for isoflavones analysis, since it can be used to indicate the stability of these compounds in different stress conditions, but it has one big drawback: the total analysis time is quite long (30 min) as compared with the new scenario of chromatographic methods [17, 20-26]. Therefore, the development of a fast and reliable method to analyze IA in complex biological matrices, even in the presence of their degradation products, becomes a new challenge in the present context of chromatographic systems for isoflavones analysis.

In this context, the aim of this work was the development and validation of a stability-indicating UFLC method for the determination of daidzein (DAID), glycitein (GLY), and genistein (GEN) applied to different assays, such as routine analysis of soybeans, monitoring the purification process, development of nanoemulsions and hydrogels containing IA, and their permeation/retention assay in porcine esophageal mucosa and porcine skin. The matrix effect for all of these applications was assessed to demonstrate the versatility and reliability of the UFLC method for IA analysis. Mass spectrometry analysis was performed to show the main products formed after acid hydrolysis of the soybeans, demonstrates the method specificity, and suggests the main degradation products formed after stress conditions. In addition, the most complex matrix used in this study (soybean acid extract) was also analyzed by a

previously validated HPLC method to show the advantages in time and resolution of the new method being proposed.

MATERIAL AND METHODS

Chemicals and Materials

Isoflavone standards, daidzein, glycitein and genistein (≥95%, ≥98% and ≥98% of purity, respectively) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Methanol and acetonitrile liquid chromatography grade were obtained from Merck (Darmstadt, Germany). Egg-lecithin (Lipoid E-80®) and medium chain triglycerides (MCT) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Ultrapure water was obtained from a Milli-Q apparatus (Millipore, Billerica, USA). The soybeans (cultivar BRS 262) were obtained from *Empresa Brasileira de Pesquisa Agropecuária* (EMBRAPA), kindly donated by SEMEL seeds (São Paulo, Brazil). Porcine ears and porcine esophageal mucosa were obtained from a local slaughterhouse.

Apparatus and analytical conditions

UFLC analysis

The UFLC analysis was performed on a Shimadzu Prominence system device coupled to a photodiode array (PDA) detection and an automatic injector controlled by LC-Solution Multi-PDA software (Kyoto, Japan). The stationary phase was a Shim-pack XR ODS column (Shimadzu, 100 x 2.0 mm i.d.; particle size, 2.2 µm) guarded by an in-line pre-column filter Ultra KrudKatcher (Phenomenex, USA). The mobile phase consisted of (A) trifluoroacetic acid 0.1% (v/v) and (B) acetonitrile. The gradient elution was 20 - 25% B (0 - 2 min), 25 - 30% B (2 - 3 min), 30 - 35% B (3 - 4 min), and 35 - 20% B (4 - 6 min). The column was re-equilibrated with 20% B for 2 min before the next analysis. The flow rate was a gradient of 0.35 mL min^{-1} (0 - 1.5 min), 0.35 - 0.5 mL min^{-1} (1.5 - 2 min), 0.5 mL min^{-1} (2 - 3 min), 0.5 - 0.35 mL min^{-1} (3 – 4 min), and 0.35 mL min^{-1} up to 8 minutes. The wavelength was adjusted to 260 nm, injection volume of 3 µL, and the analysis was carried out at 55 °C.

HPLC analysis

The HPLC analysis was carried out as described by Yatsu et al. [27], and was performed on a Shimadzu LC-20AT system (Kyoto, Japan), coupled to PDA detector controlled by LC-Solution Multi-PDA software. The stationary phase was a Synergi-Fusion-RP column (Phenomenex, 150 x 4.6 mm i.d.; particle size, 4.0 µm), protected with a C-18 guard column. The mobile phase consisted of (A) acidified Milli-Q water (0.01% trifluoroacetic acid) and (B) acidified acetonitrile (0.1% trifluoroacetic acid). The gradient elution was: 20-25 % B (0 - 10 min), 25-30 % B (10 - 15 min), 30-35 % B (15 - 23 min), 35% B (23 - 26 min), and 35-20 % B (26 - 30 min), maintained at a flow rate of 1.0 mL min^{-1}. The wavelength was adjusted to 260 nm, injection volume of 10 µL, and the analysis was carried out at 40 ºC.

Mass spectrometry (MS)

The MS analysis was performed on a Micromass-LCT Premier Time of Flight (TOF) mass spectrometer (Waters, MA, USA) with an electrospray interface and coupled with an Acquity UPLC system (Waters, MA, USA). The chromatography parameters were similar to those of the UFLC analysis; nevertheless TFA was changed for acid formic. The ESI conditions were: capillary voltage 3000 V, sample cone 30 V, source temperature 120 °C, desolvation temperature of 300 °C, cone gas flow 70 $L.h^{-1}$ and desolvation gas flow of 350 L/h. Detection was performed in positive ion mode (ESI+) in the m/z range 50–300. Software used to control spectrometer, data acquisition and data processing was MassLynx (v 4.1). The molecular ions $[M+H]^+$ monitored during the analysis were m/z 255, 285 and 271, correspondingly of DAID, GLY and GEN, respectively. The criteria for fragments selection were intensity and specificity being selected 3 fragments for compound in accordance with Wu et al. [28].

Solutions

Stock and reference solutions

A stock solution (30 µg mL^{-1}) of DAID, GLY and GEN was prepared in methanol by weighing approximately 3.0 mg of the compounds into a 100 mL calibrated

volumetric flask and diluting to volume. The reference solutions were prepared by the stock solution dilution with acetonitrile 50% (v/v).

Matrices solutions

Soybean acid extract (SAE)

Soybeans were previously grinded and defatted by exhaustive extraction with *n*-hexane in a Soxhlet apparatus. The extraction of isoflavones from defatted soybean material (5,0 g) was performed in a Soxhlet using 200 mL of ethanol 80% at the temperature of 70-80 °C for 4 h. After, 1.3 M hydrochloric acid (HCl) was added and the mixture was heated at temperature of 80 °C for 2 h under reflux. An adequate aliquot was diluted in acetonitrile 50% (v/v), filtered by membrane of 0.22 µm and analyzed.

Isoflavone aglycones-rich fraction

To obtain the isoflavone aglycones-rich fraction (IAF), the soybean acid extract was evaporated under reduced pressure to remove ethanol, leaving only an aqueous phase. Liquid-liquid partition with salting-out procedure was performed adding 20% (w/v) potassium chloride into the aqueous phase. The mixture was partitioned with ethyl acetate and washed with water three times to neutralize the partitioned fraction, that was subsequently evaporated under reduced pressure, and subjected to silica gel column. The ethyl acetate fraction was eluted successively with a gradient system with *n*-hexane: methylene chloride: ethyl acetate of increasing polarity. Six new fractions were collected, chloroform was added on fraction 4 and the isoflavone aglycones precipitated. The residue was filtrated, dried and resuspended in methanol. An adequate aliquot was diluted in acetonitrile 50% (v/v), filtered by membrane of 0.22 µm and analyzed.

Nanoemulsions and hydrogels

Blank nanoemulsions (NE_B) or IAF loaded-nanoemulsions (NE_{IAF}) were composed with absence or presence of 0.1% (w/w) of IAF. The other compounds of formulations were medium chain triglycerides 8.0% (w/w), egg lecithin 2.0% (w/w),

polysorbate 80 1.0% (w/w) and water up to 100%. The formulations were obtained by means of spontaneous emulsification procedure as firstly described by Yatsu et al. [27]. An adequate aliquot was diluted in acetonitrile 50% (v/v), filtered by membrane of 0.22 µm and analyzed.

The hydrogels containing NE_B or NE_{IAF} were prepared incorporating the nanoemulsions into carbomer-hydrogel at a final concentration of 0.5% [11]. For this, Carbopol 940® was dispersed in the nanoemulsion and the mixture was stirred at room temperature and neutralized with triethanolamine to give a hydrogel with a pH of approximately 7.0. An adequate aliquot was diluted in acetonitrile 50% (v/v), filtered by membrane of 0.22 µm and analyzed.

Porcine skin layers and esophageal mucosa

Full thickness skin was excised from the outer region of the porcine ear. After the removal of subcutaneous fat, the skin was cut into circle pieces, and the separation of stratum corneum was made by the tape stripping process. The first stripped tape was discarded, while the following 14 tapes were placed in test tubes and used for the stratum corneum analysis. The remaining layer (epidermis/dermis) was reduced to tiny pieces and placed in different test tubes. To extract the IA from skin layers, 5 mL of methanol were added and the samples were maintained in an ultrasound bath for 30 minutes, as previously reported by Vargas et al. [12].

The porcine esophageal mucosa was separated from the muscular layer by cutting the loose connective fibers with a scalpel. The remaining mucosa was then perforated into tiny pieces, and placed in test tubes. To extraction procedure, 5 mL of methanol were added on the tubes and the samples were maintained in an ultrasound bath for 30 minutes. An adequate aliquot was filtered by membrane of 0.22 µm and analyzed.

Receptor fluid for permeation studies

The chosen solution to be the receptor fluid for the permeation/retentions studies was 30% ethanol in phosphate buffer pH 7.4, as reported by Vargas et al. [12]. An adequate aliquot was filtered by membrane of 0.22 µm and analyzed.

Validation of UFLC method

The developed method was validated according to the official guidelines. For pharmaceutical products (nanoemulsions and hydrogel) it was used the ICH specifications [29], while for biotechnological products (derivative from soybeans) and biological matrices (skin layers and mucosa tissue) it was used the FDA and EMEA recommendations [30,31]. The results were analyzed by Student's t test and analysis of variance (ANOVA) using a significance level of α=0.05.

Assessment of the matrix effect

The analyses of matrix effects (ME) were performed as reported by Watanabe et al. [32] and Yatsu et al. [27]. The slopes obtained in standard curves of IA standards diluted in the mobile phase were compared with the slopes obtained in standard curves of IA-spiked in each matrix. The studied matrices were SAE, IAF, NE_B, H-NE_B, porcine epidermis/dermis skin layers (P.E/D), porcine stratum corneum layer after *tape stripping* method (P.SC), porcine esophageal mucosa (P.EM) and receptor fluid (RF). Three standard curves were obtained, in three consecutive days, by plotting the peak area *versus* the concentration of the isoflavone aglycone standards (0.1, 1.0, 2.0, 4.0, 5.0 µg mL^{-1}) in acetonitrile 50% (v/v) and in the matrices solutions. Five replicates were analyzed for each concentration level.

The matrix effect was calculated based on the following equation: ME% = 100 x [1 – (S_m/S_s)], where S_m = slopes of the standard curves of the isoflavone standards in the mobile phase and S_s = slopes of the standard curves of the isoflavone standards in the matrix.

Specificity

The interference of the matrix composition was determined by the injection of samples containing only the matrices, and samples containing matrices spiked with DAID, GLY and GEN at the concentrations of 2 µg mL^{-1} each one. The IA present in the different matrices were identified based on their UV spectra between 200 and 400 nm, their electrospray ionization (ESI+) mass spectra, and their retention times, with respect to the reference materials. The stock solutions of standards were also

submitted to a forced degradation as reported by Yatsu et al. [27]. Hydrochloric acid (HCl) was added to the standard solutions to achieve the final concentration of 0.5 M to verify the acid hydrolysis. Sodium hydroxide (NaOH) was added to the standard solutions to achieve the final concentration of 0.5 M to verify the basic hydrolysis. After a pre-determined period of time, both stress solutions were neutralized with base and acid, respectively. The oxidative degradation was induced by storing the sample solutions in 30% hydrogen peroxide (H_2O_2). All these solutions were kept in 25 ºC and 60 ºC and protected from light to prevent the interference of photolytic degradation. After pre-determined times, the samples were diluted in mobile phase and analyzed using a PDA detector determining the peak purity of isoflavones and MS analysis was also performed for the degradation peaks obtained.

Linearity, precision and accuracy

The linearity of the method for each matrix was evaluated by regression analysis using the least square method. Three standard curves were obtained, in three consecutive days, by plotting the peak area *versus* the concentration of the isoflavone aglycones standards (0.1, 2.0, 4.0, 6.0, 8.0 and 10.0 μg mL^{-1}) in acetonitrile 50% (v/v) and (0.1, 1.0, 2.0, 3.0, 4.0 and 5.0 μg mL^{-1}) in matrices. Each concentration level was analyzed in six replicates. The limit of detection (LOD) and the limit of quantitation (LOQ) of the method were calculated based on the standard deviation of the intercept and on the slope of the standard curves.

The intra-day precision (repeatability) was determined by analyzing IA at three levels (0.1, 2.0, 4.0 μg mL^{-1}) in the presence of matrices, and with six determinations per concentration, during the same day under the same experimental conditions. Inter-day precision (intermediate precision) values were obtained by assaying IA samples of the same concentration levels on three different days. The standard deviation and the relative standard deviation (RSD) were calculated for each level.

The accuracy was evaluated by adding known amounts of IA standards at three different concentration levels (0.1, 2.0, 4.0 μg mL^{-1}) to the post extraction matrices. At each level, samples were prepared in six replicates and analyzed. The accuracy was evaluated as the standardized correlation between the measured value and the

theoretical value, as follows: RE% = [(mean calculated concentration – theoretical value)/theoretical value].

Robustness

The robustness in each matrix was investigated by the Plackett-Burman design. The factors, analyzed in low levels (-1) and high levels (+1), were: column oven temperature (53; 57 ºC), initial organic composition (19; 21 %), initial flow rate (0.34; 0.36 µg mL^{-1}) and TFA concentration (0.08; 0.12 %). The four factors selected were tested with 8 experiments designed in accordance with Heyden et al. [33]. The responses evaluated were the percentages of IA in the matrices obtained in relation to the standard solutions. After the calculation of the effects for each parameter (by the sums of the responses of the positive and negative levels), the statistical interpretation (*t*-test) allowed determination of the similarity or difference of the results. The DAID, GLY and GEN standards and the samples were analyzed under identical experimental conditions, and for this reason no additional experiments were necessary. A half-normal probability plot for the effects in combination with the dummy factors was used to estimate the error and identify significant effects.

Recovery of IA after extraction from porcine skin and mucosa

Before the extraction procedure of porcine skin layers or mucosa, the matrices were spiked with isoflavone aglycones leading a theoretical concentration of 0.1, 2 and 4 µg mL^{-1}. Methanol was added in each matrix test tube, and the samples were maintained in an ultrasound bath for 30 minutes, filtered through a 0.22 µm membrane and analyzed by UFLC.

IA stability in matrices

The stability of IA-spiked matrices was determined after 48 h of storage at ambient temperature. The stability of these solutions was studied by performing the analysis and observing any change in the chromatographic pattern, compared with freshly prepared solutions.

System suitability

The system suitability test was also carried out to evaluate the adequacy of the system for the analysis. The parameters measured were peak area, retention time, theoretical plates, tailing factor, and resolution between DAID, GLY and GEN.

Method application

The determination of DAID, GLY and GEN in SAE and IAF were performed after dilution of samples as described in sections of matrices solutions "*Soybean acid extract*" and "*Isoflavone aglycone-rich fraction*", and the IA determination in NE_{IAF} and H-NE_{IAF} was carried out as described in section "*Nanoemulsions and hydrogels*".

The permeation/retention studies for IA were evaluated using Franz type diffusion cells, which presented a surface area for diffusion of 1.77 cm^2 and a receptor volume of 10.0 mL. The excised circular porcine ear skin and esophageal mucosa were previously dipped in PBS pH 7.4 solution during 30 minutes. Then, they were mounted in a Franz-type diffusion cell between the donor and receptor compartments, with the inner part facing the upper inside portion of the cell. The bathing solution was kept under a controlled temperature (32 ± 1.0 ºC) and stirred at 650 rpm. About 400 µL of NE_{IAF} were placed in the donor compartment, maintaining the sink conditions for the assay. At the end of experiment (8 h), an aliquot of RF was withdrawn for analysis and the skin or mucosa was removed from the cell and cleaned using a cotton swab. Next, the skin layers and mucosa were treated as described in section of "*Matrices solutions – Porcine skin layers and esophageal mucosa*" to extract the IA, and analyzed by the UFLC method.

RESULTS AND DISCUSSION

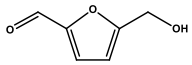
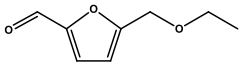
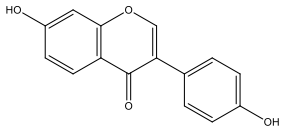
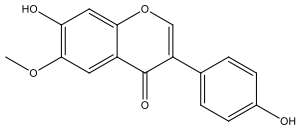
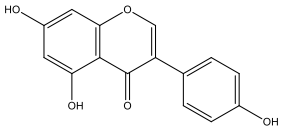
UFLC method development and advantages

For optimization studies of pharmaceutical formulations and permeation assays through skin or mucosa, numerous samples must be analyzed during the tests. Consequently, it is often necessary to develop an easy, fast, and reliable method to quantify the compounds of interest.

The UFLC was chosen in this study to achieve IA separation with good resolution in a short time. The choice of an appropriate column and other factors were evaluated considering the main chromatographic parameters obtained for the major compounds present in the SAE, since this sample was the most complex matrix used in the present work (**Table 3.1**). The greatest IA separation, with high resolution between all peaks, was achieved with a C18 column packed with 2.2 µm particle size. To allow a rapid and satisfactory separation of the main products, the use of solvent gradient, high temperature, and gradient of flow rates was needed. As previously reported by our research group, higher temperatures is a useful tool for reducing analysis time since mobile phase viscosity is significantly reduced and this, in turn, decreases the pressure and peak width [27]. The most effective separation occurred with a gradient of 0.1% (v/v) trifluoroacetic acid in water and acetonitrile. UV detection was at 260 nm, since all IA have good absorption at this wavelength.

The soybean extract was submitted to acid hydrolysis because isoflavone glycosides are the predominant forms in soybeans, but the activities and higher skin permeation are mainly credited to the aglycone forms [34,7]. However, when soy derivative products such as foods or extracts are submitted to acid conditions, other compounds could be obtained. For instance, the presence of sugars under extreme pH conditions and high temperatures usually induce the Maillard reaction, or caramelization process. Both phenomena can result in the production of furanic aldehydes, such as hydroxymethylfurfural (HMF) and furfural [35]. Recently, our research group had isolated HMF and ethoxymethylfurfural (EMF) from soybeans after acid hydrolysis in an ethanolic media [36]. These compounds are reported as toxic [37, 38], and for this reason, the purification of acid extracts becomes essential before they can be used in pharmaceutical products. Consequently, the development of an analytical method that enables IA quantification and the detection of HMF and EMF come to be necessary to make sure these toxic compounds are removed from the extract. In this way, the UFLC method proved to be capable of separating the toxic compounds from the IA and is, therefore, suitable for the quality control of the purification processes.

Table 3.1. Chemical structure for the main products obtained after acid hydrolysis of soybeans, as well as their UFLC retention times, UFLC peak parameters, maximum UV absorption (λ max), and molecular ion $[M+H]^+$ acquired by MS analysis

Compound	Chemical structure	Retention time	Theoretical plates	Tailing factor	Resolution	λ (max)	$[M+1]^+$
HMF		0.92	10046.42	2.03	-	229/282	127
EMF		2.87	14104.95	1.38	17.86	229/281	155
Daidzein		3.63	21105.32	1.27	8.93	248/301	255
Glycitein		4.07	32900.98	1.14	4.98	256/319	285
Genistein		5.05	63837.17	1.13	11.5	260	271

HMF: hydroxymethylfurfural; EMF: ethoxymethylfurfural

In addition, to demonstrate the advantage of time-related analysis and reduced solvent consumption, the SAE was analyzed by the UFLC method and an HPLC method recently reported by our research group [27]. The representative chromatographic separations for both methods are shown in **Fig 3.1**. As can be observed, the UFLC method greatly shortens the analysis time by up to three times that of the HPLC method, while maintaining the resolution between all peaks. Moreover, it is important to highlight that the environmental impact and cost are minimized, since the UFLC flow rate was 0.5 mL min^{-1}, half that of the HPLC value.

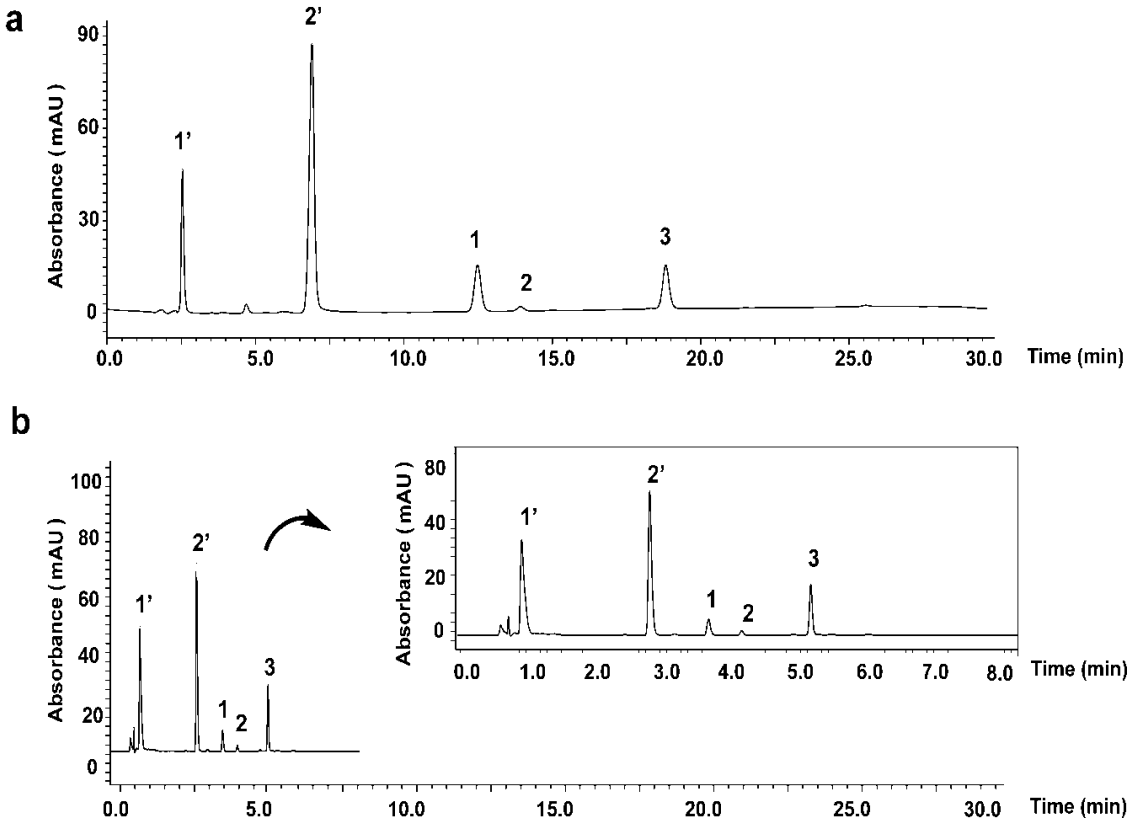


Figure 3.1. Representative chromatographic profiles of soybean acid extract obtained by (a) HPLC and (b) UFLC methods at 260 nm. Where (1') HMF, (2') EMF, (1) DAID, (2) GLY and (3) GEN

Comparing the UFLC method described herein with other ultra-fast methods reported in the literature, it is possible to observe that the total run time is a little longer than the methods reported by Kiss et al. [24] and Fiechter et al. [21], which

showed the IA analysis in soybeans and phytoestrogen-rich plants with chromatographic time of under 5 min. However, it is important to note that the separation of HMF, EMF, DAID, GLY, and GEN with high resolution was herein achieved, even with the difference in polarity between the furfural compounds and isoflavones.

UFLC method validation

Assessment of the matrix effect

Method validation is the process by which the compounds of interest are reliably quantified independently of the matrix [31]. For this reason, during the development of an analytical and bioanalytical method, it is essential to consider the effect of all matrices involved, which can be easily detected by comparing the response obtained from a standard solution with that from a spiked pre-treated sample [27].

When the response range is between -20% < ME% <20%, the matrix effects is deemed low; when it is between -50% < ME% <-20% or 20%> ME% > 50%, it is considered medium, and when it is between ME% < -50% or ME% > 50%, it is considered high [39].

The matrix effects for each IA present in the SAE, IAF, NE_{IAF}, H- NE_{IAF}, P. E/D skin layer, P. SC layer after tape stripping process, RF and P. EM, were expressed by ME% and are presented in **Table 3.2**. The data indicate that samples exhibited low matrix effects (ME% < 12.42) for isoflavones determination, when analyzed according to Niessen et al. [39].

Furthermore, when IA spiked-matrices were analyzed by UPLC-MS/MS, no interfering signals (m/z) were found in the same retention time of DAID, GLY, and GEN. Therefore, the proposed method dispenses with the need for clean-up pre-treatment of the samples and is classified as a fast and simple method for analyzing IA in complex matrices.

Table 3.2. Linearity data of the isoflavone aglycone standards and the matrix effect for each matrix studied

Matrix	IA	Equation	R^2	LOD	LOQ	ME (%)
				μg mL^{-1}		
Standards	DAID	y=19866x-337.73	0.999	0.10	0.35	-
	GLY	y= 21752x+142.0	0.999	0.09	0.32	-
	GEN	y=36619x+355.32	0.999	0.10	0.32	-
SAE	DAID	y= 20071x-325.95	0.998	0.08	0.28	1.02
	GLY	y= 22006x-624.98	0.998	0.09	0.32	1.15
	GEN	y= 37002x-298.39	0.998	0.08	0.28	1.04
IAF	DAID	y= 19486x+2760.6	0.993	0.17	0.57	-1.95
	GLY	y= 21953x+104.11	0.988	0.21	0.71	0.92
	GEN	y= 36988x+3127.8	0.996	0.12	0.38	1.00
NE_B	DAID	y= 19389x-195.21	0.993	0.17	0.58	2.40
	GLY	y= 21567x-119.18	0.995	0.13	0.45	0.85
	GEN	y= 35753x+1182.1	0.994	0.15	0.50	2.36
H-NE_B	DAID	y= 19722x-106.42	0.998	0.08	0.27	-0.73
	GLY	y= 21986x-177.26	0.998	0.08	0.26	1.06
	GEN	y= 36700x+181.32	0.998	0.08	0.28	0.22
P. E/D	DAID	y= 20003x-312.91	0.998	0.09	0.31	0.68
	GLY	y= 22079x+54.060	0.998	0.08	0.26	1.48
	GEN	y= 36669x+576.41	0.998	0.10	0.32	0.14
P. SC	DAID	y= 19709x-1007.9	0.995	0.14	0.48	-0.80
	GLY	y= 21605-640.460	0.995	0.13	0.44	-0.68
	GEN	y= 37172x-2245.4	0.998	0.09	0.32	1.49
P. EM	DAID	y= 19722x-24.812	0.998	0.10	0.33	0.72
	GLY	y= 21886x+144.67	0.997	0.10	0.33	-0.62
	GEN	y= 36757+939.10	0.997	0.10	0.33	-0.38
RF	DAID	y= 22682x-489.67	0.998	0.08	0.28	12.42
	GLY	y= 23718x+44.349	0.998	0.09	0.30	8.29
	GEN	y= 39345x-988.07	0.999	0.09	0.39	6.93

IA : isoflavone aglycone; SAE: soybean acid extract; IAF: isoflavone aglycone-rich fraction; NE_B: blank nanoemulsion; H-NE_B: hydrogel containing NE_B; P. E/D: porcine epidermis/dermis; P. SC: porcine stratum corneum after *tape stripping* process; P. EM: porcine esophageal mucosa; RF : receptor fluid; R^2 = determination coefficient; LOD = limit of detection; LOQ = limit of quantitation; ME : matrix effect

Specificity

The chromatographic separation of IA standards at 260 nm is shown in **Fig. 3.2A**. The retention times for DAID, GLY, and GEN peaks were 3.60, 4.07, and 5.05 min, with characteristic λ_{max} (maximum UV absorption) at 248/301, 256/319 and 260 nm (**Fig. 3.2B**), and MS/MS spectra with characteristic molecular $[M+H]^+$ ions *m*/*z* 255 for DAI, *m*/*z* 285 for GLY, and *m*/*z* 271 for GEN (**Fig. 3.2C**). The main fragmentation ions were in accordance with those reported by Wu et al. [28], in which the characteristic retro-Diels-Alder fragments m/z 137 for DAID, m/z 167 for GLY, and m/z 153 for GEN were significantly present.

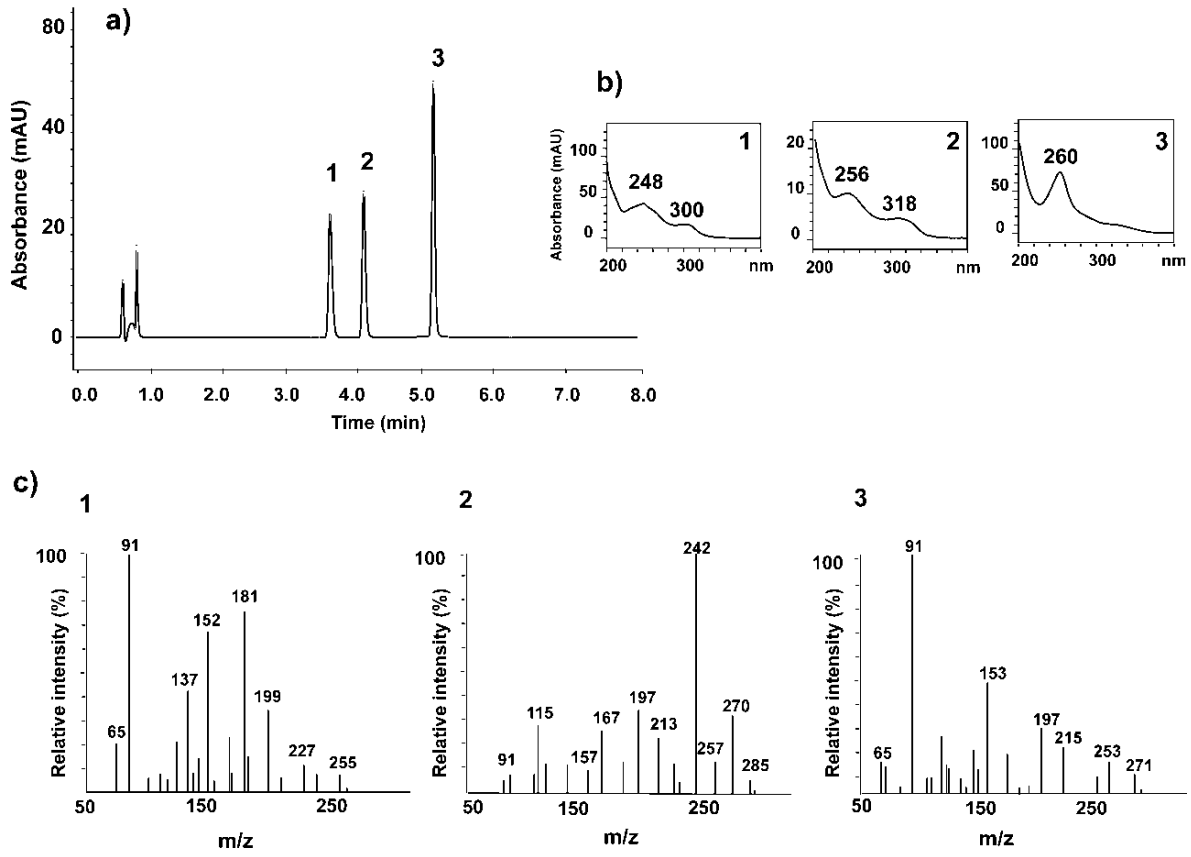


Figure 3.2. Analysis of isoflavones (1) DAID, (2) GLY and (3) GEN by (a) UFLC method, (b) UV-VIS absorption spectra; (c) MS/MS spectra measured in positive mode

The UFLC method specificity was assessed by injecting blank matrices and evaluating the peak purity of each IA spiked-matrix. No matrix-related interference was found, showing that the IA peaks were free from any co-eluting substance and demonstrating that the proposed method is specific for the simultaneous analysis of IA in all of the assessed matrices (**Fig. 3.3A**).

Moreover, forced degradations were done to provide stability-indicating properties. A stability-indicating method is defined as an analytical method that accurately quantifies the compounds of interest without interference from degradation products. In the present work, the IA standards were submitted to acidic, alkaline, neutral, oxidative, and thermal stress conditions. No significant changes were observed in the isoflavones after stress exposure in acid, oxidative or neutral conditions at 25 ºC and 60 ºC during 6 h of exposure. On the other hand, when the IA were submitted to the alkaline condition at 60 ºC for 6 h (**Fig. 3.3B**), DAID degraded more than 50%, GEN more than 20%, and GLY more than 15%.

However, only the degradation products of DAID and GEN could be observed in the developed UFLC method. To enhance the knowledge about the possible degraded products and confirm the purity of the isoflavones, the peaks were analyzed by PDA and MS detector following the stress process. The degradation products D1, D2, D3, D4, D5, and D6 had retention times of 1.80, 4.33, 4.63, 4.75, 5.29, and 5.75 min, respectively. In addition, they were well separated from the other ones in the chromatographic system, resulting in a good resolution (>1.4 between all peaks). Evaluation of DAID, GLY, and GEN purity after the stress condition demonstrated that these peaks were free from any co-eluting substance. The maximum UV spectra of the main degraded peaks showed λmax: 255 nm for D1, λmax: 236/289 nm for D2, λmax: 230/277 nm for D3, λmax: 240/283 nm for D4, λmax: 260/290/322 nm for D5, and λmax: 260/318 nm for D6. Furthermore, the MS spectra of three out of six degradation products were compared with the literature data, and the results showed that D1, D2, and D3 have the same precursor ions $[M+H]^+$, m/z 255, 259 and 273, than the degradation products reported by Yatsu et al. [27]. However, more studies are needed on the other three degradation products to propose their identities.

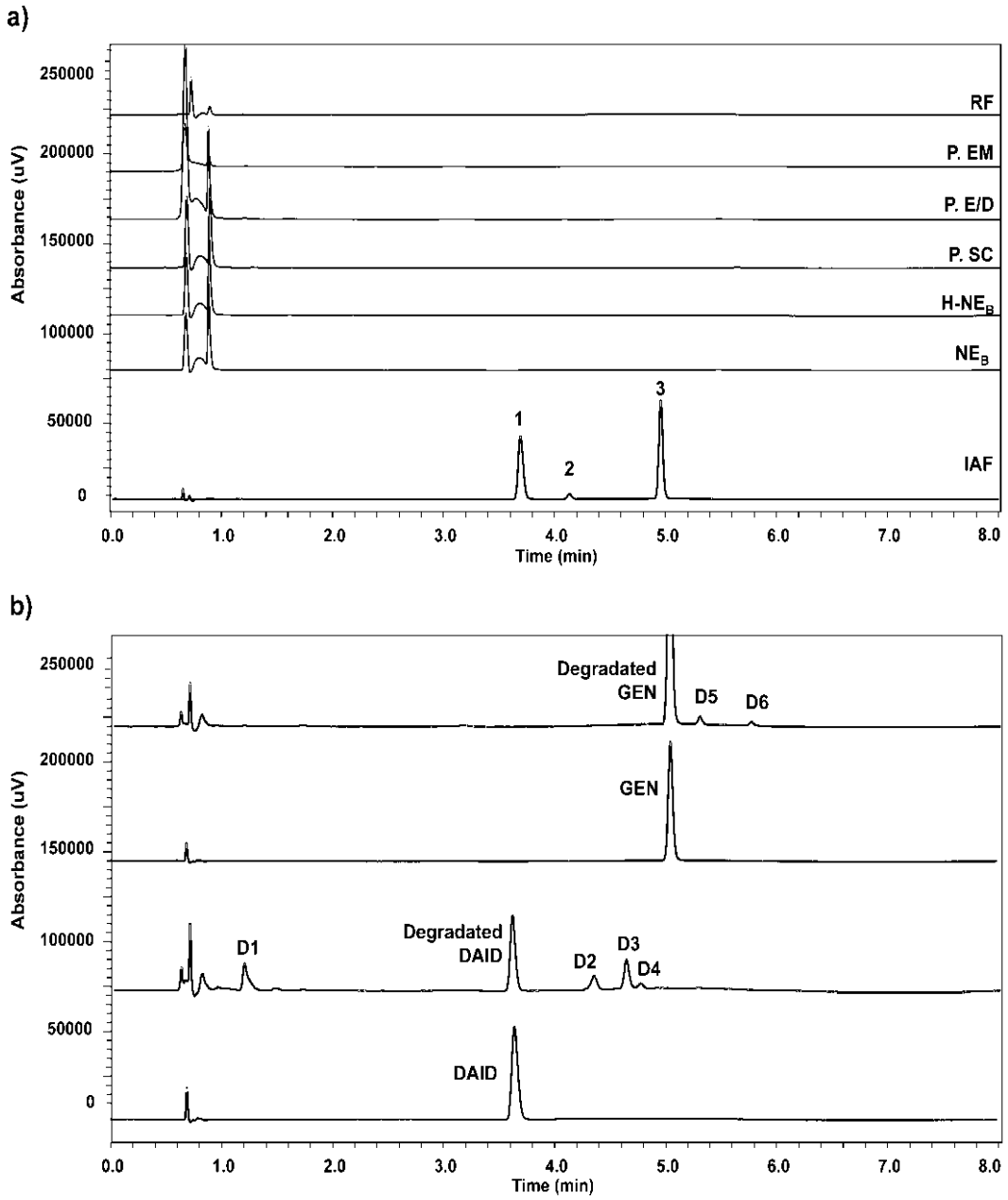


Figure 3.3. Representative chromatographic profiles obtained in the specificity assay for the (a) different matrices, where, IAF: isoflavone aglycone-rich fraction, 1: DAID, 2: GLY, 3: GEN, NE_B: blank nanoemulsions, H-NE_B: topical hydrogel containing nanoemulsions, P. SC: porcine stratum corneum after tape stripping method, P. E/D: porcine epidermis/dermis skin layer, P. EM: porcine esophageal mucosa, RF: receptor fluid; and for the (b) degradation products of DAID (D1, D2, D3, D4) and GEN (D5, D6) obtained after alkaline hydrolysis of separated standard solutions of DAID and GEN

Linearity, precision and accuracy

The results for linearity are shown in **Table 3.2.** Analysis of the determination coefficients demonstrated that the method is linear for all of the standard compounds within the tested range. The confidence interval observed in the *t*-test on the intercepts and the graphic examination of the residuals also demonstrated the absence of constant systematic errors (data not shown).

The LOD and LOQ calculated by standard curves are also presented in **Table 3.2**. The lower LOQ (LLOQ) measurement is a great concern in the validation methods, which are used to quantify low concentrations of drugs in biological matrices. The LLOQ was fixed at 0.1 µg mL^{-1} for all isoflavones in all assessed matrices, since it was the lowest IA concentration that could be determined with acceptable precision and accuracy (RSD < 15%).

The precision and accuracy of post-extraction spiked-matrices were evaluated by analyzing the IA at concentrations of 0.1 µg mL^{-1} (lowest concentration), 2.0 µg mL^{-1} (medium concentration), and 4.0 µg mL^{-1} (highest concentration) for each sample. The intra-day precision data for IA showed a relative standard deviation (RSD) value between 0.04 - 3.96 for the analytical assays (NE, H-NE, RF), between 0.02 - 8.40 for the bioanalytical assays (P.SC, P. E/D, P. EM), and between 0.09 - 6.21 for the biotechnological products (SAE, IAF). The inter-day precision data for IA showed a RSD value between 0.17 - 5.12 for the analytical assays, between 0.66 - 8.00 for the bioanalytical assays, and between 0.96 - 10.70 for the biotechnological products. The accuracy results for IA in all matrices were within the 90.37% to 104.81% range. Despite the complexity of the different matrices, the UFLC method can be considered precise and accurate according to official guidelines.

Robustness

A model's robustness refers to its ability to remain unaffected by small, deliberate variations in the analysis conditions [33]. A multivariate approach using design of experiments is often recommended in robustness testing since a number of different factors can be analyzed concurrently with a reduced number of experiments [40].

The responses after assessing the Plackett-Burman design are the percentage of DAID, GLY, and GEN in the samples in relation to the standard solutions in each

experiment. As shown in **Fig. 3.4**, no significant factors were revealed for all analyses as the calculated *t*-values were lower than the *t*-critical values (α = 0.05). Thus, there were no significant changes in the assay results in terms of the percentage of IA contents with the changes made in the experimental conditions, thereby demonstrating the proposed method's robustness.

IA stability in matrices

The stability evaluation results showed that the concentration of isoflavones in the different matrices remained constant after 48 h of storage at room temperature. The DAID, GLY, and GEN amounts in all matrices after this period of time were found to be between 99.45% and 101.99%.

IA extraction from skin and mucosa

The recovery data for IA quantification after extraction of previously spiked-matrices are shown in **Table 3.3**, and was within FDA recommendations for bioanalytical method validation [30]. Taken together, the recovery yields are highly satisfactory and demonstrated that 30 min was sufficient for the complete IA extraction from matrices, and no matrix components interfered during the procedure. Furthermore, IA recovery showed adequate precision in all assessed matrices (RSD < 8.3%).

System suitability

Routine analyses of the standard substances were performed under the developed experimental conditions. Parameter values and their variability (RSD, %) for each compound were: (i) DAID analysis: 3.6 (0.13) min for migration time, 20166 (2.11) for theoretical plates, and 1.28 (0.40) for tailing factor; (ii) GLY analysis: 4.07 (0.09) min for migration time, theoretical plates 32122 (1.95), and tailing factor 1.23 (0.81); (iii) GEN analysis: 5.05 (0.09) min for migration time, 60526 (2.06) for theoretical plates, and 1.16 (0.74) for tailing factor. The resolution between DAID and GLY peaks was 4.9 (0.99) and 11.39 (0.85) between GLY and GEN. The parameters indicate that the system is suitable for the analysis.

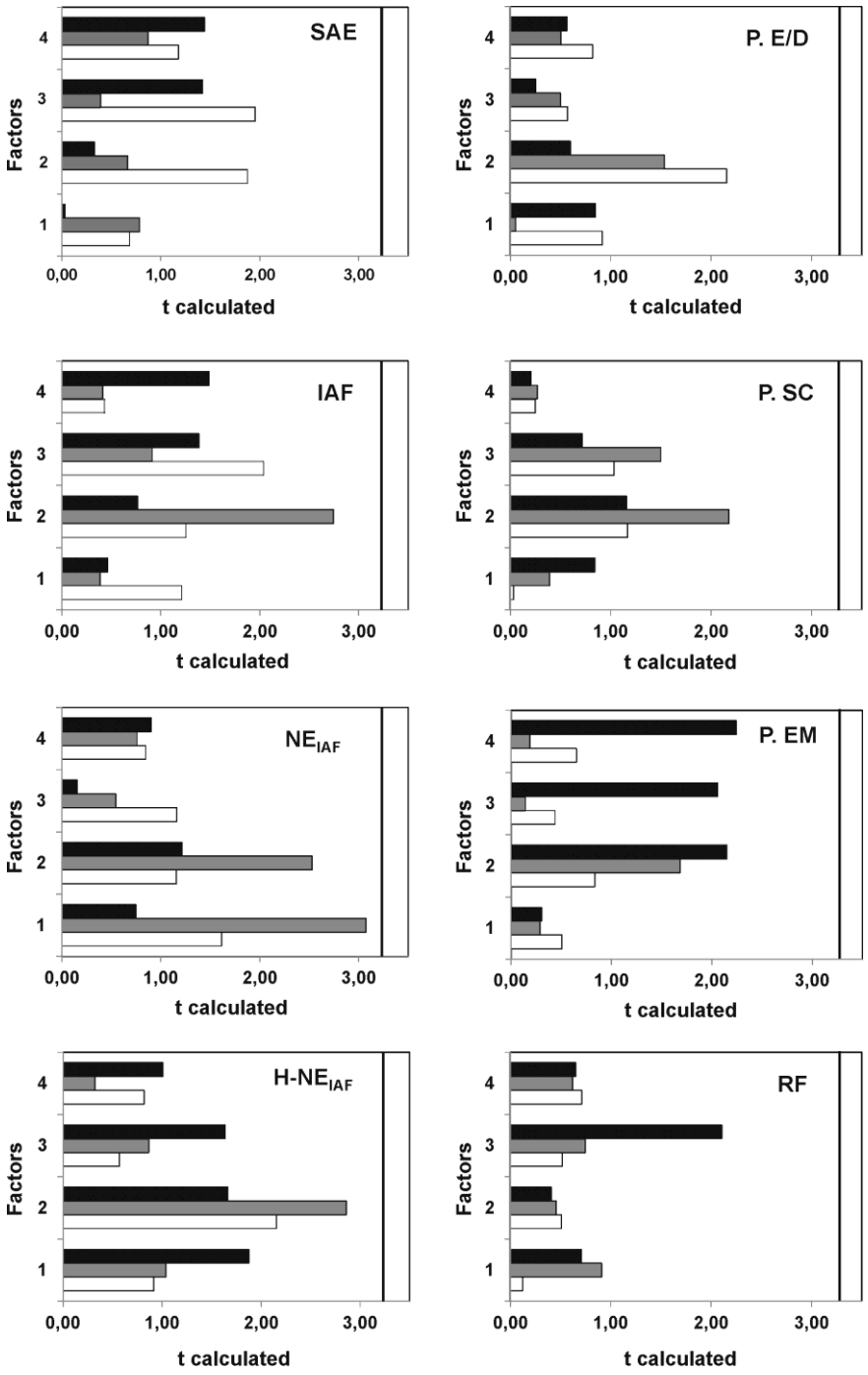


Figure 3.4. Bar charts representing the *t*-calculated for quantitative determination (assay) of the investigated factors (1,2,3 and 4) in Plackett-Burman experimental design and their *t*-critical, represented by the vertical line, for each isoflavone in the matrices. Where, 1: column oven temperature, 2: initial flow rate, 3: TFA concentration, 4: initial organic composition, SAE: soybean acid extract, IAF: isoflavone aglycone-rich fraction, NE_{IAF} isoflavone aglycone rich-fraction loaded nanoemulsion, H-NE_{IAF} topical hydrogel containing nanoemulsions, P. SC: porcine stratum corneum layer after tape stripping method, P. E/D: porcine epidermis/dermis skin layer, P. EM: porcine esophageal mucosa, RF: receptor fluid and the bar charts: DAID (black columns), GLY (gray columns), and GEN (white columns).

Table 3.3. Recovery data of the IA added in biological matrices

Matrix	Nominal (μg mL^{-1})	Recovery (%) (RSD)		
		DAID	GLY	GEN
P. E/D skin layer	0.1	98.09 (8.3)	95.12 (3.7)	95.43 (5.2)
	2	97.94 (1.4)	99.45 (2.0)	99.93 (1.1)
	4	95.15 (2.8)	94.56 (2.3)	100.8 (8.1)
P. SC skin layer	0.1	101.8 (2.6)	99.60 (5.9)	99.80 (4.2)
	2	101.1 (1.6)	99.10 (1.8)	100.9 (2.6)
	4	101.8 (1.5)	100.0 (1.3)	102.0 (5.7)
P. E mucosa	0.1	98.81 (1.6)	97.92 (3.2)	99.13 (1.5)
	2	99.16 (2.3)	93.49 (3.1)	93.24 (0.3)
	4	98.24 (2.2)	95.49 (2.3)	96.32 (1.4)

P. E/D: porcine epidermis/dermis; P. SC: porcine stratum corneum after tape stripping process; P. E: porcine esophageal mucosa ; RSD = relative standard deviation

Method application

As a last objective of this work, the UFLC method was applied to determine the IA amount in the real samples. The IA content in SAE, IAF, NE_{IAF}, H-NE_{IAF}, receptor fluid, and in skin or mucosa layers are shown in **Table 3.4**, and the results indicate the precision of the method in all samples, in which the RSD was lower than 3.14% in the analytical assays and lower than 18.52% in the bioanalytical assays.

As previously reported by Yatsu et al. [27], the soybean cultivar used in this work had a total isoflavone aglycones content greater that had been reported for 14 different soybeans cultivars [21], demonstrating the importance of this sample for industrial applications. To obtain the IA in a pure fraction, we described an appropriate method of purification and acquisition of DAID, GLY, and GEN from SAE, which allowed obtaining a fraction with 0.89 mg of IA per mg of powder (**Table 4**) and free from HMF and EMF (**Fig 3A - IAF**).

When this fraction was incorporated into nanoemulsions, the quantification of all IA showed adequate repeatability. This also occurred during the analysis of

hydrogels containing the NE_{IAF}. Both formulations had successfully incorporated the IA, since their amount was 0.86 mg mL^{-1}, resulting in more than 96% of IA when compared with the 0.89 mg mL^{-1} initially added.

Regarding the *in vitro* skin permeation/retention study, no IA could be detected in the receptor fluid after 8 h of assay. Conversely, DAID and GEN could be quantified with adequate precision in the stratum corneum, as well as in the epidermis and dermis layers. Yet, the same was not observed with GLY, which was detected in all skin layers, but could not be quantified, because its peak areas were always lower than the LLOQ.

Table 3.4. Determination of the isoflavone aglycones in real samples

Application of UFLC method	**Mean of six replicates (RSD)**			
	DAID	**GLY**	**GEN**	**IA Total**
Samples				
SAE (mg 100 g^{-1} of DSS)	75.14 (2.49)	16.54 (2.66)	112.16 (0.40)	203.85 (1.16)
IAF (mg mg^{-1})	0.45 (1.18)	0.043 (1.02)	0.39 (2.64)	0.89 (1.14)
NE_{IAF} (mg mL^{-1})	0.44 (1.81)	0.037 (1.68)	0.37 (3.14)	0.86 (2.39)
H-NE_{IAF} (mg g^{-1})	0.45 (1.40)	0.038 (1.18)	0.37 (1.47)	0.86 (1.42)
Skin retention				
P. SC (µg $cm^{2\ -1}$)	0.47 (13.50)	LLQ	0.16 (16.25)	0.63 (14.87)
P.E/D (µg $cm^{2\ -1}$)	0.24 (18.52)	LLQ	0.39 (14.50)	0.63 (16.50)
RF (µg $cm^{2\ -1}$)	LLQ	LLQ	LLQ	-
Mucosa permeation				
P.E mucosa (µg $cm^{2\ -1}$)	2.70 (10.16)	0.29 (11.44)	3.43 (10.78)	6.42 (10.80)
RF (µg $cm^{2\ -1}$)	6.65 (9.87)	0.79 (18.02)	4.25 (18.10)	11.69 (15.30)

SAE: soybean acid extract; DSS: defatted soybean seeds; IAF: isoflavone aglycone-rich fraction; NE_{IAF}: isoflavone aglycone rich-fraction loaded nanoemulsion; H-NE_{IAF}: hydrogel containing isoflavone aglycone rich-fraction loaded nanoemulsion; P. E/D: porcine epidermis/dermis; P. SC: porcine stratum corneum after tape stripping process; RF: receptor fluid; LLQ: lower than limit of quantification; P. E: porcine esophageal mucosa; RSD = relative standard deviation

In the literature, no report was found concerning the validation of a method for simultaneous quantification of DAID, GLY, and GEN after skin permeation/retention assays. Among some studies comprising such matters, Vargas et al. [11] showed GEN quantification in the skin layers and receptor fluid after the cutaneous permeation/retention assay using a previously validated HPLC method [12]. On the other hand, for simultaneous IA quantification, Huang et al. [6] have reported *in vitro* and *in vivo* skin retention/permeation of DAID and GEN, without demonstrating the validation of the method used for the assessment. That being said, the present study reports, for the first time, a fast and validated method for skin studies involving all soybean isoflavone aglycones.

In addition, the method was able to quantify IA in a mucosa permeation assay in both receptor fluid and tissue with satisfactory precision. This is an important finding, considering that isoflavone aglycones have shown anti-herpetic activities [4], requiring application on some mucosas to carry out their functions. The choice of porcine esophageal mucosa to investigate the permeation study was done in accordance with Consuelo et al. [41], since the authors showed that this membrane is a useful and practical substitute for buccal mucosa for *in vitro* permeability studies. Lastly, it is important to emphasize that this was the first time this application had been reported for isoflavone aglycones.

CONCLUSIONS

The overall results showed that the developed UFLC method is an excellent tool for determing isoflavone aglycones present in soybean acid extract, isoflavone aglycone-rich fractions, isoflavone aglycones loaded-nanoemulsions, topical hydrogel containing isoflavone aglycones loaded-nanoemulsions, porcine skin layers, porcine esophageal mucosa, and in a 30% ethanol solution in phosphate buffer pH 7.4 (receptor fluid for permeation studies). The method had a low matrix effect, without any interference from the matrices and degradation products, besides being simple, quick, and able to quantify isoflavones with precision, robustness, and accuracy in different complex matrices.

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CAPÍTULO IV

Determinação quantitativa das principais impurezas formadas após hidrólise ácida do extrato de soja e avaliação in vitro da genotoxicidade e mutagenicidade da impureza majoritária

4.1. INTRODUÇÃO

No desenvolvimento de novos medicamentos é necessário garantir as características de qualidade, segurança e eficácia dos produtos (VIEIRA *et al.*, 2013). Um dos testes que deve ser realizado é a avaliação da presença de impurezas nas matérias-primas e produtos finais (AHUJA, 2007). As impurezas são classificadas como orgânicas, inorgânicas ou resídos de solvente (ICH, 2006a). As impurezas orgânicas podem ser provenientes de produtos resultantes dos processos de sínteses de fármacos e excipientes, compostos gerados durante processamento do medicamento ou da degradação dos fármacos e excipientes durante estabilidade do produto final (BRASIL, 2013). Caso elas estejam presentes, devem ser realizados diversos testes de identificação, quantificação e avaliação toxicológica para garantir a segurança dos medicamentos (MCGOVERN & JACOBSON-KRAM, 2006; AHUJA, 2007; BASAK *et al*., 2007; JACOBSON-KRAM & MCGOVERN, 2007).

Muito se discute sobre a importância da avaliação qualitativa, quantitativa e toxicológica dos produtos farmacêuticos e das impurezas que podem estar presente nos produtos sintéticos (ICH, 1999a; ICH, 2006a; BRASIL, 2013), biológicos e biotecnológicos (ICH, 1997; ICH, 1999b). Para produtos derivados vegetais, o guia internacional do EMEA (2011a) reporta as principais diretrizes para a sua produção, enquanto que o guia EMEA (2011b) descreve as especificações de controle de qualidade destes produtos. Esse último menciona que se estiverem presentes impurezas em produtos fitoterápicos, essas devem ser avaliadas conforme guia do ICH que contempla a determinação de impurezas em produtos farmacêuticos (ICH, 2006b).

Dessa forma, levando-se em consideração que os extratos vegetais, bem como seus derivados, podem conter impurezas e que estas podem vir a causar algum dano toxicológico, é importante conduzir estudos de identificação, quantificação e qualificação dos demais constituintes dos derivados vegetais e não somente dos principais marcadores químicos e substâncias ativas.

Vale destacar que o termo qualificação remete a verificação da segurança biológica das impurezas que podem estar presentes em matérias-primas ou produtos farmacêuticos. A qualificação das impurezas pode ser realizada por meio de dados da literatura científica ou por meio de dados experimentais de estudos

toxicológicos que confirmem os níveis seguros da mesma (MELO *et al*., 2013). Segundo os guias do ICH de 2006 e 2012, é necessário avaliar o perfil do potencial genotóxico de novas substâncias farmacêuticas, bem como das eventuais impurezas presentes no produto, sendo que no mínimo devem ser realizados estudos para determinar as mutações genéticas, aberrações cromossômicas e danos ao DNA que tais compostos possam causar ao organismo.

Conforme relatado nos Capítulo 1 e 2, o processo de extração das sementes de soja com posterior hidrólise ácida consititui um processo clássico para a obtenção das formas bioativas das isoflavonas. Porém, concomitantemente à obtenção desses fitoestrógenos diversas impurezas podem ser geradas. Nesse contexto, torna-se de grande relevância científica a avaliação quali e quantitativa de prováveis impurezas que podem estar presentes nos extratos de soja.

Em estudos prévios do nosso grupo de pesquisa descrevemos a formação do 5-hidroximetilfurfural (HMF) e 5-etoximetil-2-furfural (EMF) a partir da hidrólise ácida das sementes de soja (NEMITZ *et al*., 2015). Essas substâncias são formadas a partir da degradação dos açúcares em condições ácidas, com elevadas temperaturas e em meio hidroetanólico (SHEN & WU, 2014; CHEN *et al*., 2014).

A maioria dos estudos que realizam hidrólise ácida dos extratos de soja tem por objetivo a obtenção das isoflavonas agliconas durante experimentos analíticos. Contudo, algumas patentes e também alguns estudos científicos relatam o uso da hidrólise ácida do extrato de soja para a fim de obter isoflavonas agliconas para desenvolver formulações ou avaliação das atividades biológicas (BRYAN & SHEN, 1995; MEREDITH & RAMOT, 2002; UTKINA *et al*., 2004; ZHANG *et al.,* 2007; DESHMUKH & AMIN, 2013). Cabe destacar que os autores descrevem métodos de purificação dos extratos hidrolisados de soja, porém não relatam a busca ou preocupação de identificação e análise quantitativa de possíveis impurezas que possam ser formadas durante o processo, e consequentemente, possam estar presentes nos produtos finais.

Considerando que guias internacionais (EMEA, 2011a; EMEA 2011b) preconizam que devem ser realizadas as análises de impurezas em produtos derivados vegetais conforme diretrizes do guia do ICH (2006b), e levando-se em conta o uso de hidrólise ácida para a produção de extratos enriquecidos em

isoflavonas agliconas a partir da soja, este capítulo remete-se a análise quantitativa e toxicológica *in vitro* das principais impurezas formadas durante o processo de obtenção de tais extratos. Para isso, primeiramente foi realizada a validação analítica de método cromatográfico para o doseamento de HMF e EMF no extrato de soja hidrolisado pela via ácida, e posteriormente foram realizadas buscas na literatura para verificar a segurança biológica das impurezas furânicas presentes no extrato. Uma vasta literatura para HMF foi encontrada, porém não foram encontrados muitos relatos para o EMF. Dessa forma, análises toxicológicas *in vitro* de genotoxicidade e mutagenicidade foram realizadas para esse composto.

Os dados experimentais deste capítulo estão apresentados na forma de artigo científico, redigido nas normas do periódico em que foi publicado.

4.2. ARTIGO

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DETERMINATION OF THE MAIN IMPURITIES FORMED AFTER ACID HYDROLYSIS OF SOYBEAN EXTRACTS AND THE *IN VITRO* MUTAGENICITY AND GENOTOXICITY STUDIES OF 5-ETHOXYMETHYL-2-FURFURAL

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ABSTRACT

Soybean acid hydrolyzed extracts are raw-materials widely used for manufacturing of pharmaceuticals and cosmetics products due to their high content of isoflavone aglycones. In the present study, the main sugar degradation products 5-hydroxymethyl-2-furfural (HMF) and 5-ethoxymethyl-2-furfural (EMF) were quantitatively determined after acid hydrolysis of extracts from different soybean cultivars by a validated liquid chromatography method. The furanic compounds determined in samples cover the range of 0.16 to 0.21 mg/mL and 0.22 to 0.33 mg/mL for HMF and EMF, respectively. Complementarily, due to the scarce literature regarding the EMF toxicology, this study also assessed the EMF mutagenicity by the *Salmonella*/microsome test and genotoxicity by the comet assay. The results revealed that EMF did not show mutagenicity at the range of 50 - 5000 µg/plate in *S. typhimurium* strains TA98, TA97a, TA100, TA102 and TA1535, but induced DNA damage in HepG2 cells at non-cytotoxic doses of 0.1 - 1.3 mg/mL, mainly by oxidative stress mechanisms. Based on literature of HMF genotoxicity, and considering the EMF genotoxicity results herein shown, purification procedures to remove these impurities from extracts are recommended during healthcare products development to ensure the security of the products.

Keywords: acid hydrolyzed extracts; genotoxicity; healthcare products; HMF and EMF; mutagenicity; soybean.

INTRODUCTION

Chemical hydrolysis is a process commonly used to obtain hydrolyzed compounds to simplify food routine analysis or to develop products enriched in bioactive compounds [1,2]. When an acid hydrolysis protocol is performed, the extreme pH and temperature conditions can break a large number of conjugated substances present in samples, resulting in different derivative and/or degradation products, causing then, a positive or negative effect on the final product.

Once carbohydrates usually make up a great portion of biomasses, their degradation is quite probable during hydrolysis process. Under acid and thermal conditions, the main degradation products formed from sugars are furfural and 5-hydroxymethyl-2-furfural (HMF) [3]. These compounds are classified as furanic derivatives and their production is very associated with the catalytic system and raw materials used during hydrolysis process. Their determination in processed foods is well-described, but their evaluation in acid hydrolyzed herb-drug preparations is poorly reported [4,5].

Acid hydrolysis protocol is a common process to obtain the isoflavone aglycones from natural sources. The soybean acid hydrolyzed extract is a plant derivative very attractive for pharmaceutical and cosmetics industries that wish to manufacture products containing bioactive phytoestrogens [1, 6]. However, the generation of possible impurities during the process is commonly neglected.

Recently Nemitz, Teixeira and von Poser [7] reported the occurrence of two major impurities in soybean hydroethanolic extracts after acid hydrolysis. These compounds have been isolated and identified as HMF and its derivative 5-ethoxymethyl-2-furfural (EMF). The same authors also published a chromatography study that allows the quantitative analysis of isoflavones even in the presence of these impurities; however, the chromatography method was not validated for the determination of HMF and EMF present in the samples [8].

According to the European guideline EMA/CPMP/QWP/2820/00 Rev 2 [9] when impurities, or degradation products, are present in herbal medicinal products it is recommended the assessment of identification, quantification and qualification of these compounds. So, since HMF and EMF are produced after acid hydrolysis of

soybean hydroethanolic extract, it is essential not only their identification, but also their quantification, and toxicology evaluation before manufacturing health products.

It is important to highlight that some furanic compounds have a consolidated approach concerning their toxicological effects. HMF at high concentrations is considered as cytotoxic, irritant to skin, eyes, upper respiratory tract and mucous membranes [10]. Many *in vitro* HMF genetic toxicity studies are reported in literature and they suggest that this compound has genotoxic effects at high doses [10]. Because of this, food and drug agencies have estimated HMF limit levels in some products. Moreover, some concerns have been raised for the potential properties of specific HMF derivatives, especially for the sulfoxymethylfurfural (SMF) a major metabolic product formed after sulfonation pathway [10]. Nevertheless, the toxic effects of EMF, an ether derivative from HMF, appear to be unknown until now.

Considering the context here exposed, the present study shows the simultaneous determination of HMF and EMF amounts in three different soybean acid hydrolyzed extracts by an ultra-fast liquid chromatography (UFLC) method. In addition, due to the scarce literature concerning toxic effects of EMF, this study also conducted the *in vitro* evaluation of EMF mutagenicity by the *Salmonella*/microsome test and genotoxicity by the comet assay using a metabolically active human hepatoma cell line, HepG2.

MATERIALS AND METHODS

Chemicals

Soybean cultivars BRS Taura RR, BRS 262 and BRS 154 were obtained from Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA). Furanic standards and 3-(4,5-dimethylthiazol- 2yl) 2,5-diphenyl-tetrazolium bromide salt (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Materials used in cell culture were purchased from Gibco (Panwell, TX, USA) and Invitrogen by Life Technologies (São Paulo, SP, Brazil). Formamidopyrimidine glycosylase (FPG) protein was purchased from New England Biolabs (Acton, MA, USA). *Salmonella typhimurium* strains TA98, TA97a, TA100, TA102 and TA1535 and S9 mix were purchase from Molecular Toxicology Incorporation (Boone, NC, USA).

Soybean extracts and UFLC analysis

Soybeans were previously grinded and defatted by exhaustive extraction with n-hexane in a Soxhlet apparatus. The extraction of 5.0 g of defatted soybean material was performed in a Soxhlet using 200 mL of ethanol 80% (v/v), during 4 h at 70 - 80 °C. Acid hydrolysis was performed as reported by Nemitz, Teixeira and von Poser [7]. Three independent extracts were obtained from each cultivar. The samples were neutralized, diluted and analyzed by UFLC method.

The UFLC analysis was performed using the same equipment and chromatography conditions previously described by Nemitz et al. [8]. A new validation process was performed according to the ICH guideline [11] for the new approach involving the analysis of HMF and EMF in the samples.

In vitro EMF toxicity studies

Salmonella/microsome mutagenicity assay (Ames test)

The *Salmonella*/microsome mutagenicity assay was performed according to Maron and Ames [12] by the plate incorporation method (with pre-incubation) in the presence or absence of metabolic activation (S9 mix). As recommended by OEDC guideline [13] the Ames test was carried out using five histidine-dependent auxotrophic mutants of S. typhimurium strains (TA100, TA98, TA97a, TA102, and TA1535).

EMF concentrations were determined according to a range finding experiment in TA100 strain, and no cytotoxicity was observed for this strain at concentrations up to 5000 µg/plate. For positive controls, aflatoxin B1 (1 µg/plate) was used for all strains in the presence of S9 mix, and in the absence it was used sodium azide (1 µg/plate) or 4-nitroquinoline-oxide (4-NQO, 0.5 µg/plate) for TA100 and TA1535, or TA97a, TA98 and TA102 strains, respectively. The solvent used to dilute EMF (DMSO:H_2O 1:1, v/v) was used as negative control. Independent experiments, in triplicate, were performed for each protocol.

The evaluation criterion was based on comparing the average number of revertant colonies appearing on the treated plates and control plates, using a

statistical tool. Besides, the mutagenic index (MI) was calculated for each concentration tested, as follows: MI = [(mean number of revertants per plate with the test compound / mean number of revertants per plate found with the negative control]. A test substance was considered positive when significant ANOVA variance was observed, and MI was at least twice higher than that observed in the negative control plates, or at least three times higher, for the TA1535 strain.

Cells culture and genotoxicity protocols

HepG2 cells were purchased from Rio de Janeiro Cell Bank (catalogue 0103; Brazil). The cells were maintained as a monolayer in 75 cm^2 flasks in DMEM supplemented with 15% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and antibiotics (1% penicillin plus streptomycin and 0.1% of the gentamycin) and kept in a humidified 5% CO_2 atmosphere at a temperature of 37 °C. For the experiments, $1x10^5$ cells were seeded in 24-well plates, incubated for 24 h at 37 ºC and 5% CO_2 atmosphere, in a complete DMEM, washed with DPBS, and then submitted to one of the protocol treatments: MTT or alkaline comet assay.

MTT assay

In order to determine the best conditions for genotoxicity assay, the viability of HepG2 front EMF was investigated by the MTT assay. Each protocol was performed in quadruplicate. The HepG2 cells were seeded as previously reported and then submitted to one of the following treatments: (a) negative control (DMEM with 1% DMSO); (b) positive control (DMEM with 20% DMSO); (c) screening of EMF cytotoxicity at concentrations of 0.325, 1.25 and 5 mg/mL during 3, 6 and 24 h; (d) EMF at concentrations of 0.1, 0.4, 0.7, 1.0, 1.3 mg/mL during 3 h of exposure. After, the viability of cells was performed by the MTT assay according to Mosmann [14].

Alkaline comet assay

The alkaline version of the comet assay was carried out to evaluate the genotoxic potential of EMF. For the experiments, the HepG2 cells were seeded as previously reported and then submitted during 3 h to one of the following treatments:

(a) negative control (DMEM with 1% DMSO); (b) positive control (hydrogen peroxide 1.2 mM); and (c) EMF at concentrations of 0.1, 0.4, 0.7, 1.0, 1.3 mg/mL. At the end of treatments, the comet assay was performed by the alkaline single-cell gel electrophoresis process according to Tice et al. [15] and stained with silver nitrate according to Nadin, Vargas–Roig and Ciocca [16]. Independent experiments, in quadruplicate, were performed for each concentration.

In order to suggest a mechanism involved in DNA damage, the comet assay was also performed in the cells exposed to EMF in the presence of repair endonuclease formamidopyrimidine DNA glycosylase (FPG), which recognizes the common oxidized purine 8-OHgua (7,8-dihydro-8-oxo-guanine) [17]. For the experiments, the HepG2 cells were seeded as previously reported in section "*Cells culture and genotoxicity protocols"* and then submitted during 3 h to one of the following treatments: (a) negative control (DMEM with 1% DMSO); (b) positive control (hydrogen peroxide 1.2 mM); and (c) EMF at 0.1 mg/mL. The comet assay was performed with some modifications as proposed by Collins [17]. Independent experiments, in quadruplicate, were performed for each concentration.

Visual scoring was chosen for measuring the DNA damage after comet assay [15]. The slides were visualized in an optical microscopic and the extent of DNA damage were evaluated classifying comets into five categories based on the length of migration and/or the perceived relative proportion of the DNA in the tail and size of head (nucleus): 0 representing comets with no tail (undamaged cells) and 1–4 representing increasing relative tail intensities and minor head size. Two parameters, damage index (DI) and damage frequency (DF), were used to evaluate DNA damage.

DI was calculated according to the formula: $DI = (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)$, and DF (%) was calculated as following: $DF = (100 - n_0)$. Where, n= number of cells in each class analyzed. Means were compared using the one-way ANOVA followed by Dunnet or Tukey test, with minimum of $p<0.05$ to be considered as statistically significant.

RESULTS AND DISCUSSION

Soybean extracts and UFLC analysis

The three different Brazilian soybean cultivars were extracted and subsequently submitted to acid hydrolysis. An UFLC method was validated to determine the amount of HMF and EMF present in the samples and the summary of results for all validation parameters are presented in **Table 4.1.** The chromatographic conditions used herein were similar as those used for isoflavones determination in different matrices, previously reported by Nemitz et al. [8]. The results showed that all validation parameters for HMF and EMF analysis were in accordance with the literature recommendations [11].

Table 4.1. Summary of method validation results.

Parameter	HMF	EMF
Specificity	Acceptable [a]	Acceptable [a]
Concentration range (µg/mL)	10 - 100	10 - 100
Regression equation[b]	y = 12919x + 9332	y = 8685x + 5376,6
Correlation coefficient (r)	0.9995	0.9994
Limit of detection (µg/mL)	0.96	0.98
Limit of quantitation (µg/mL)	2.91	2.98
Repeatability[c] (*RSD* %)	0.27 – 2.98	0.18 – 2.15
Intermediate precision[d] (*RSD* %)	1.38 – 4.90	0.73 – 4.14
Accuracy[e] (recovery %)	99.34 – 102.37	96.56 – 100.60
Matrix effects[f] (%)	1.82	0.76
Robustness	Acceptable [g]	Acceptable [g]

[a] peak purity of HMF and EMF present in soybean acid hydrolyzed extract
[b] $y = ax + b$, where x is the concentration (µg/mL) of the drugs and y is peak area.
[c] range of RSD of 6 determinations at each concentration (20, 40 and 80 µg/mL) in SAE
[d] mean of 18 determinations at each concentration (20, 40 and 80 µg/mL) in SAE
[e] range of recovery of determinations at each concentration (20, 40 and 80 µg/mL) in SAE
[f] calculated by comparison of slopes from analytical curve in solvent and in SAE
[g] the responses showed no differences in terms of percentage of HMF and EMF in SAE after changes made in the experimental conditions

In these conditions, it was determined the individual amount of furanic compounds in liquid soybean acid hydrolyzed extracts (SAE) and the capacity of furanic production from each defatted soybean powder (**Table 4.2**). The quantity of HMF is probably related to the amount of sugars present in the samples, and the presence of EMF is related with the etherification of HMF during acid hydrolysis in an ethanolic media. Chen et al. [4] reported the simultaneous determination of HMF and EMF in ethanolic extracts after acid hydrolysis of cranberry bean, quinoa, lentil, buckwheat seeds, apple, cucumber and carrot. The range found was 30.64 to 43.4 mg/ 100 g. So, comparing the sum of HMF and EMF here found with the values reported by Chen et al. [4], soybeans produce higher quantities of furanic compounds, which probably is associated with higher sugar amounts in samples.

The presence of furanic compounds in plant derivatives can cause a positive [4] or negative [5] effect in the extracts when the intention is their uses in pharmaceutical products. In this way, once the furanic compounds are present in soybean acid hydrolyzed extracts, the next step is to check whether the amounts are close to the toxic limits. For this approach, the data from literature can help to assess the HMF limits in healthcare products [10]. But, on the other hand, the literature concerning the toxic potential of EMF is scarce. Based on this, the present study conducted a preliminary assessment of EMF genetic toxicity. To that, the mutagenic effects were evaluated by the AMES assay, as recommended by OECD guideline [13], and the genotoxic effects were assessed by the comet assay with HepG2 cells, which presents phase I and II drug-metabolizing enzyme activities, playing a crucial role in the activation and detoxification of carcinogens that react with DNA [18].

In vitro EMF toxicity studies

Salmonella/microsome mutagenicity assay

The bacterial reverse mutation test is commonly employed as an initial screen for genotoxic activity and, in particular, for point mutation-inducing activity. The TA97a and TA98 strains can detect frameshift mutation in DNA target –C-C-C-C-C-C-; +1 cytosine, and –C-G-C-G-C-G-C-G-, respectively. The other strains TA100 and TA1535 detect base pair substitution (-G-G-G-), and TA102 can detect cross-linking mutagens and transitions/transversions TAA ochre [13].

Table 4.2. Furanic compounds present in soybean extracts from three different Brazilian cultivars after hydroethanolic extraction and acid hydrolysis process (1.3 M HCl, 2 h, 80 ºC), and the capacity of their production from each soybean defatted powder.

Soybean cultivar	mg/mL of liquid SAE (RSD,%)		g/100 g of defatted soybean powder (RSD,%)	
	HMF	EMF	HMF	EMF
EMBRAPA BRS 262	0.18 mg/mL (1.92%)	0.33 mg/mL (0.83%)	0.69 g/ 100 g (1.92%)	1.30 g/ 100 g (0.83%)
EMBRAPA BRS Taura RR	0.21 mg/mL (3.03%)	0.31 mg/mL (3.14%)	0.83 g/ 100 g (3.03%)	1.26 g/ 100 g (3.14%)
EMBRAPA BRS 154	0.16 mg/mL (1.11%)	0.22 mg/mL (1.18%)	0.63 g/ 100 g (1.11%)	0.88 g/ 100 g (1.18%)

RSD: relative standard deviation; Soybean acid hydrolyzed extract.

The results for the EMF mutagenic effects are shown in **Table 4.3.** As can be seen, EMF was not mutagenic to the S. typhimurium strains once the mutagenic index (MI) for all doses evaluated were below from 2.0 for TA100, TA97a, TA102 and TA98 strains or below from 3.0 for the TA1535 strain. Similar results were reported in previous literature [19] for the HMF mutagenicity analysis.

Although the results did not show high MI after EMF exposure, it is important to highlight that some number of revertant colonies on test plates were statistically higher from the results of the negative control, indicating a tendency to cause mutagenesis in some bacteria strains at high EMF doses. This situation can be observed in the EMF analysis without S9 mix at the doses of 2500 µg/plate and 5000 µg/plate for TA97a ($p<0.05$), 2500 µg/plate for TA1535 ($p<0.05$) and 5000 µg/plate for TA100 ($p<0.01$), as well as in the EMF analysis with S9 mix at the doses of 2500 µg/plate and 5000 µg/plate for TA97a ($p<0.01$), and 5000 µg/plate for TA100 ($p<0.001$). Besides, it is important to note that EMF at highest doses (5000 µg/plate) were not toxic for almost all strains, but in some cases the highest doses showed some toxicity, as observed by TA1535, TA102 and TA98 strains without S9 mix, and TA102 strain with S9 mix, in which the revertant colonies on test plates were considerably lower than the mean colonies of the negative control.

Cells viability

In the guideline reported by Tice et al. [15] it is recommended a minimum of 70% for cell viability to carry out the comet assay. So, in order to follow this recommendation, it was selected the MTT assay as the complementary cytotoxicity test.

During the EMF cytotoxicity screening at different exposure times and doses (**Fig. 4.1A**) it was possible to note that in the lowest EMF dose (0.325 mg/mL) this compound was not considered cytotoxic for HepG2 cells (viability > 70%) whatever of exposure time. At the medium EMF dose (1.25 mg/mL) the cells viability was higher than 80% after 3 h of treatment, but after 6 or 24 h the viability was reduced to less than 40%. High cytotoxicity (cells viability < 10%) was observed in the cells after the highest EMF dose (5 mg/mL) independently of the exposure time analyzed.

So, based on these screening results, and in view to perform the genotoxicity study at non-cytotoxicity conditions, it was chosen 3 h of treatment with EMF doses below or close to 1.25 mg/mL. In this way, it is possible to observe in **Fig. 4.1B** that in the EMF doses covering the range of 0.1 to 1.3 mg/mL after 3 h of treatment the cells showed high viability (> 90%).

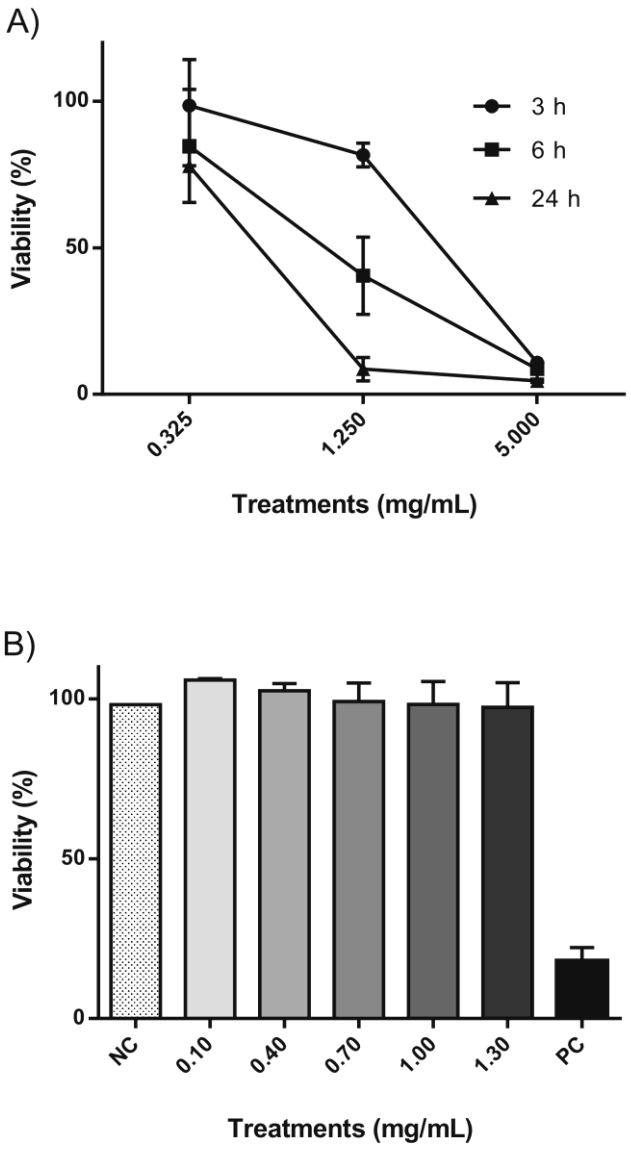

Figure 4.1. Evaluation of the viability of HepG2 cells using MTT assay after EMF exposure. Where: (A) screening of time (3, 6 and 24 h) and concentrations of EMF (0.325, 1.25 and 5 mg/mL); and (B) cells viability after 3h of treatment with EMF in the concentration range of 0.1 – 1.3 mg/mL. Negative Control (NC): DMEM with 1% DMSO, and Positive Control (PC): DMEM with 20% DMSO. Values are the mean ± standard deviation.

Table 4.3. Induction of *his*+ revertants in *S. typhimurium* strains by EMF with and without metabolic activation (S9 mix).

Substance	Concentration (µg/plate)	*S. typhimurium* strains									
		TA98		TA97a		TA100		TA1535		TA102	
		Rev/plate[a]	MI[b]	Rev/plate[a]	MI[b]	Rev/plate[a]	MI[b]	Rev/plate[a]	MI[b]	Rev/plate[a]	MI[b]
Without activation (-S9)											
NC[c]	-	27.7±5.0	-	96.7±7.1	-	128.3±14.2	-	17.7±10.3	-	450.0±19.0	-
EMF	50	23.0±2.6	0.83	122.0±4.4	1.27	118.7±4.5	0.92	16.7±3.5	0.94	430.7±9.6	0.96
	250	23.0±4.6	0.83	100.0±13.1	1.03	156.7±41.0	1.22	17.0±3.0	0.96	407.3±14.5	0.91
	500	28.0±3.6	1.01	105.7±30.7	1.09	151.3±23.7	1.18	31.0±11.4	1.76	416.7±5.5	0.93
	2500	28.0±4.6	1.01	138.7±6.0*	1.43	155.0±9.5	1.21	39.7±12.1*	2.25	385.0±27.9	0.86
	5000	0±0	-	138.0±7.0*	1.43	219.7±20.5**	1.71	6.0±2.6	0.34	295.0±42.0	0.65
PC[d]	0.5 (4NQO)	214.3±41.4***	7.75	279.0±7.0***	2.89					3173.3±292.9***	7.05
	1 (NaN_3)					3152.3±344.4***	24.56	1254.7±48.3***	71.02		
With activation (+S9)											
NC[c]	-	25.3±3.2	-	66.0±14.0	-	125.7±8.1	-	11.3±1.5	-	373.7±17.8	-
EMF	50	25.0±5.6	0.99	85.0±5.3	1.29	129.7±12.7	1.03	15.3±5.1	1.35	289.3±64.5	0.77
	250	21.7±5.0	0.86	86.7±13.9	1.31	119.3±11.2	0.95	11.3±7.1	1.00	273.7±57.8	0.73
	500	24.0±10.0	0.95	92.3±18.0	1.40	135.7±18.8	1.08	8.7±3.5	0.76	290.0±60.9	0.78
	2500	31.0±8.2	1.22	115.0±17.5**	1.74	156.7±14.3	1.25	5.7±1.5	0.50	206.3±20.1	0.55
	5000	23.3±3.8	0.92	124.3±7.1***	1.88	213.0±20.0***	1.70	14.3±2.9	1.26	172.7±41.0	0.46
PC[d]	1 (AFB_1)	101.0±12.5***	3.99	469.3±69.1***	7.11	726.0±65.8***	5.78	112.0±9.5***	9.89	1319.7±103.7***	3.53

[a]Number of revertants/plate: mean of three independent experiments ± SD; [b]MI: mutagenic index (nº. of *his*+ induced in the sample/nº. of spontaneous *his*+ in the negative control); [c]NC: negative control dimethylsulfoxide/distillated water (1:1) used as a solvent for the EMF. [d]PC: positive control (-S9) → NaN^3: sodium azide to TA100 and TA1535; 4NQO: 4-nitroquinoline-oxide to TA97a, TA98 and TA102; [d]PC (+S9) → AFB_1: aflatoxin B1; Significantly different in relation to the negative control: * p<0.05; ** p<0.01; *** p<0.001.

Alkaline comet assay

The DNA damage induced by EMF in HepG2 cells after 3 h of treatment is shown in **Fig. 4.2**. In all non-cytotoxic doses evaluated, EMF caused a significant increase in the damage index (**Fig. 4.2A**) and damage frequency (**Fig. 4.2B**) compared to the negative control (Dunnet test, $p<0.05$). Besides, the ANOVA analysis revealed that in the lowest doses of EMF (0.1, 0.4 and 0.7 mg/mL) the damage index was statistically different ($p<0.05$) from the damage caused by the highest dose (1.3 mg/mL), indicating a possibility of dose-response curve, however, to ensure this hypothesis more tests should be evaluated.

Genotoxicity assay can detect genetic damages directly or indirectly by several *in vitro* and *in vivo* tests. When positive results are finding during the tests, it is possible to predict some potential of the chemical to be human carcinogens and/or mutagens [15]. In the present study, the EMF genotoxic potential was evaluated using the alkaline comet assay which detects the DNA single strand breaks (SSB) and alkali-labile sites (ALS) after an electrophoresis migration of damaged DNA. The results showed that EMF induced DNA damage in all doses evaluated, with a damage index and frequency much higher than the negative control. Comparing the EMF effects here observed with the HMF cytotoxic and genotoxic potential previously reported by Severin et al. [19], it is possible to suggest that EMF at lower doses is more cytotoxic and apparently more genotoxic than HMF front the same cell line using a similar test procedure.

Comet assay with enzyme biomarkers has been performed to suggest genotoxic mechanisms of carcinogens. Based on the fact that furan causes oxidative damage in purine DNA bases [20] and a furan ring is present in the chemical structure of EMF, this damage mechanism was herein investigated by the addition of FPG protein on the genotoxicity assay. This endonuclease has been used to assess oxidative DNA base damage because it detects 8-OHgua and other oxidatively damaged purines [17].

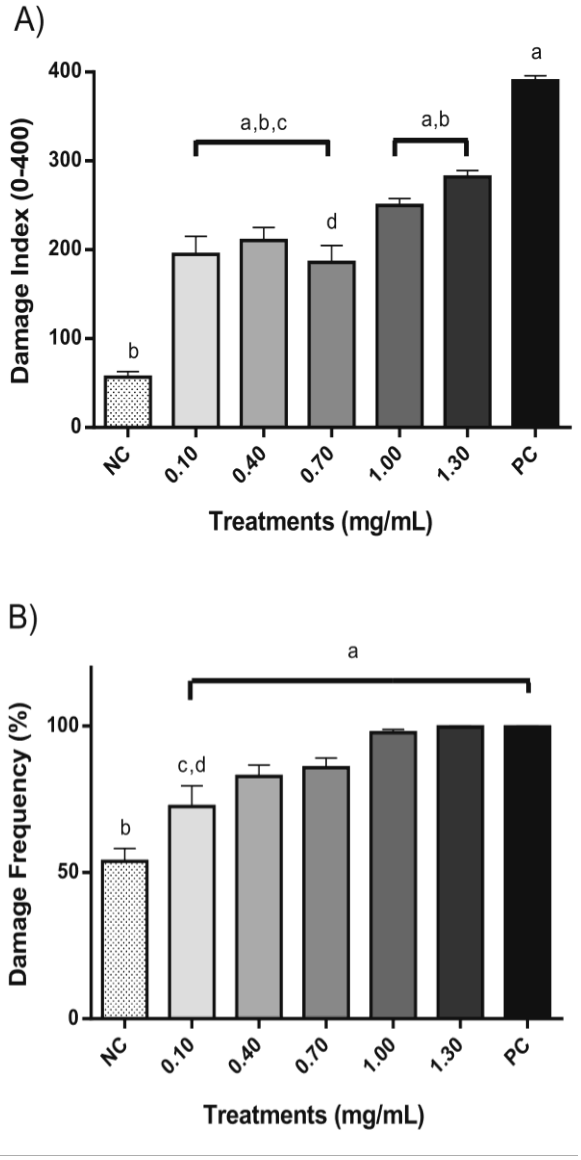

Figure 4.2. Evaluation of genotoxicity by the comet assay in HepG2 cells after 3 h of treatment at non-cytotoxic doses of EMF (0.1 – 1.3 mg/mL). Where: (A) DNA damage index (0-400), (B) DNA damage frequency (%). Negative control (NC): DMEM with 1% DMSO; and Positive Control (PC): hydrogen peroxide 1.2 mM. Values are the mean ± standard deviation. Letters represent the statistical analysis by one-way ANOVA, where: a= significantly different from the NC ($p<0.05$), b= significantly different from the PC ($p<0.05$), c= significantly different from the 1.3 mg/mL ($p<0.05$), d= significantly different from the 1.0 mg/mL ($p<0.05$).

The results of comet assay with FPG enzyme showed an increase in the damage index (**Fig. 4.3**) since the differences between the effects in the absence and the presence of the FPG protein were statistically different ($p<0.001$), and the

increase of damage index was higher than 70%. This situation could be associated with a DNA purine oxidation caused by the furan ring present in EMF.

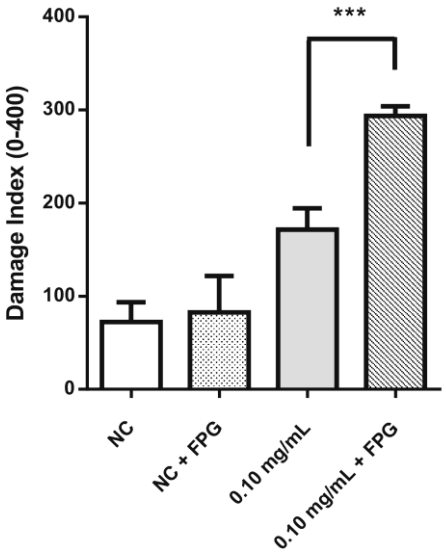

Figure 4.3. DNA damage index (0-400) in HepG2 cells after 3 h of EMF treatment (0.1 mg/mL) using the comet assay with or without FPG endonuclease. Negative control (NC): DMEM with 1% DMSO. FPG: Formamido pyrimidine glycosylase. Values are the mean ± standard deviation. ***significantly different ($p<0.001$).

CONCLUSIONS

An UFLC method was successful validated and applied for simultaneous determination of HMF and EMF in soybean acid hydrolyzed extracts obtained from three different soybean cultivars. The furanic amount present in extracts was close to 0.5 mg/mL, expressed as a sum of HMF and EMF. Due to scarce literature regarding EMF toxic effects, the *in vitro* EMF mutagenicity and genotoxicity were evaluated. The findings suggest that this compound does not induce bacteria mutations, but induce DNA damage in HepG2 cells even at non-cytotoxic doses, mainly by oxidative stress mechanisms. Analyzing the literature data and the results herein showed, the furanic impurities present in samples were close to genotoxic limits of HMF and EMF. Because of this, purification procedures to remove these compounds are recommended during the development of drug products containing soybean acid hydrolyzed extracts.

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CAPÍTULO V

Obtenção das isoflavonas agliconas da soja por meio de processos hidrolíticos alternativos ao da hidrólise ácida

5.1. INTRODUÇÃO

Para fins analíticos ou produtivos envolvendo isoflavonas agliconas são descritos na literatura três tipos principais de hidrólise do extrato de soja: processos por via ácida, básica ou enzimática (ROSTAGNO *et al*., 2009). Os processos realizados pelas vias ácida e enzimática são os mais descritos e são caracterizados por meio da hidrólise das isoflavonas na ligação glicosídica das moléculas. Os métodos hidrolíticos que utilizam catalisadores ácidos possuem como principal vantagem o preço e a rapidez de processo, porém a maior desvantagem é a falta de seletividade por compostos específicos, podendo causar degradações de diversas substâncias presentes na amostra (SCHWARTZ & SONTAG, 2009; CHEN *et al*., 2014). Por outro lado, processos biotecnológicos envolvendo hidrólise enzimática possuem característica de seletividade por substratos específicos, podendo evitar a formação de produtos secundários tóxicos e sendo, por isso, altamente atrativos para processos industriais de produção de insumos para saúde (YOON *et al*., 2003; VISKUPIČOVÁ *et al*., 2009; PERVAIZ *et al.,* 2013).

Em estudos precedentes, demonstramos que após a realização de hidrólise ácida do extrato de soja são produzidos não somente isoflavonas agliconas, mas também impurezas furânicas geradas a partir da degradação ácida dos açúcares presentes na soja (NEMITZ *et al*., 2015). Para obter uma fração enriquecida em isoflavonas agliconas livre de impurezas furânicas foi descrito a necessidade de três procedimentos de purificação, sendo eles: partição líquido-líquido com acetato de etila, extração em fase sólida por coluna de sílica gel e precipitação das isoflavonas com clorofórmio. Esse processo de purificação é vantajoso em termos de obtenção de uma fração enriquecida com alta pureza em isoflavonas. Contudo, é um processo com muitas etapas, que utiliza diversos solventes orgânicos, tornando-o além de lento, oneroso e desfavorável na rotina industrial para sua produção em larga escala.

Nesse sentido, processos de hidrólise enzimática são promissores e vêm sendo visados durante o desenvolvimento de produtos para saúde contendo flavonoides (VISKUPIČOVÁ *et al*., 2009; PERVAIZ *et al.,* 2013). As enzimas mais conhecidas e utilizadas industrialmente para realizar a hidrólise de isoflavonas são as β-glicosidases (ISMAIL & HAYES, 2005; LEE *et al*., 2008). Essas enzimas são

capazes de catalisar a hidrólise da ligação β-glicosídica de oligo- e dissacarídeos, ou ainda de outros compostos conjugados a glicose, tais como isoflavonas. Diversas fontes de β-glicosidases são descritas, sendo as provenientes de amêndoas e de *Escherichia coli* as mais utilizadas na produção de isoflavonas em larga escala (ISMAIL & HAYES, 2005).

A presença de β-glicosidases é relatada em diversos fungos (SORENSEN *et al*., 2013) e devido a isso, muitos alimentos fermentados de soja possuem alto teor de isoflavonas agliconas em sua composição. Os produtos derivados de soja koji, tempeh, miso, natto, shoyu, tofu fermentado e leite de soja são exemplos de alimentos enriquecidos em isoflavonas agliconas obtidos após fermentação com diversos fungos, principalmente os dos gêneros *Aspergillus, Rhizopus, Mucor, Actinomucor, Monascus, Saccharomyces, Neurospora, Acetobacter, Bacillus* e *Lactobacillus* (ROSA *et al*., 2009; CHEN *et al*., 2012).

A hidrólise a partir de enzimas presentes em fungos do gênero *Aspergillus* é descrita na literatura científica e tecnológica para a produção de derivados de soja enriquecidos em isoflavonas agliconas visando o uso em produtos para saúde. A tecnologia protegida sob o número WO 2008070940 (YONG *et al*., 2008) descreve a adição de uma enzima β-glicosidase isolada a partir de *Aspergillus oryzae* em um concentrado de isoflavonas da soja para obtenção de um produto enriquecido nestas substâncias. Handa e colaboradores (2016) descreveram um processo fermentativo em meio sólido utilizando como substrato a farinha de soja e como biocatalisador os fungos *Monascus purpureus* ou *A. oryzae*, a fim de aumentar a biotransformação das isoflavonas como um processo anterior à realização de extração das mesmas. Estudo similar foi relatado por Georgetti e colaboradores (2009), onde sementes desengorduradas de soja foram fermentadas em meio sólido contendo os fungos *A. awamori* (ATCC 22342), *A. niger* e *A. niveus* para a produção de extratos enriquecidos em isoflavonas agliconas e polifenóis livres.

Vale destacar ainda, que algumas leveduras, além de possuírem a enzima β-glicosidase, possuem capacidade fermentativa que pode levar a transformação dos carboidratos até alcoóis (VAN DIJKEN *et al*., 1993; PÉREZ-GREGORIO *et al*., 2011; REIS *et al*., 2014). A fermentação da glicose é realizada através do processo de glicólise, produzindo duas moléculas de ácido pirúvico, que são convertidas em

etanol, com a liberação de duas moléculas de CO_2 e a formação de duas moléculas de ATP (HAGMAN & PIŠKUR, 2015). Tal fato é interessante quando se almeja o consumo prévio dos oligossacarídeos das amostras a fim de gerar um produto enriquecido em compostos bioativos sem a presença de compostos tóxicos provenientes da degradação dos açúcares formados durante processos catalíticos (DUEÑAS *et al*., 2012). Dentre algumas leveduras, o uso de *Saccharoyces cerevisiae* para consumo dos açúcares presentes na soja vem sendo descrito na literatura (LONG & GIBBONS, 2012; LUJAN-RHENALS *et al*., 2015), especialmente com ênfase na produção do bioálcool.

É possível observar que existem diferentes vias que podem ser escolhidas para realizar a hidrólise das isoflavonas conjugadas. Contudo, além da escolha pela via hidrolítica a ser utilizada, é necessária também a correta seleção das condições do método a ser empregado, uma vez que mudanças no tempo de reação, pH, temperatura, e concentração do catalisador (ou biocatalisador) são fundamentais para o rendimento final dos compostos bioativos de interesse. Dessa forma, a otimização dos processos através de ferramentas estatísticas, tais como planejamentos e desenhos fatoriais, pode vir a auxiliar durante a escolha do método de hidrólise a ser utilizado (KASHYAP *et al.*, 1997; CHIANG *et al*., 2001; TIPKANON *et al*., 2010).

Assim, esta etapa do trabalho objetivou a realização de processos hidrolíticos alternativos ao da hidrólise ácida para a obtenção de isoflavonas agliconas da soja sem a presença de impurezas possivelmente tóxicas. Para isso, foi realizada a otimização dos processos hidrolíticos enzimático (β-glicosidase) e fermentativo (*S. cerevisae*) através de ferramentas estatísticas. Complementarmente, foi realizada uma etapa de purificação dos extratos e a fração de maior pureza foi avaliada quanto ao seu potencial para aplicação cutânea por meio de ensaio de viabilidade celular *in vitro* utilizando cultura de queratinócitos.

Os dados experimentais deste capítulo estão apresentados na forma de artigo científico, redigido nas normas do periódico em que foi submetido à publicação.

5.2. ARTIGO

Submetido à publicação

SOYBEANS ISOFLAVONE AGLYCONE-RICH EXTRACTS: OPTIMIZATION BY DIFFERENT BIOPROCESSES AND PRODUCTION OF A PURIFIED FRACTION WITH PROMISING WOUND HEALING PROPERTY

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ABSTRACT

In this study, extracts containing isoflavone aglycones (IA) were obtained from defatted soybeans (DS) by different hydrolytic mechanisms, including acid, enzymatic and fermentation processes. The analyses of IA, sugars, furanic compounds, saponins and triterpenes were performed and compared for each hydrolyzed extract obtained. All procedures were carried out using a sample corresponding to an extract obtained from 1.0 g of DS. The acid hydrolysis was accomplished by a classical process; however, the conditions used during enzymatic hydrolysis (EH) and fermentative process (FP) were firstly screened by Plackett-Burman design, and subsequently optimized by Box Behnken design. The optimum conditions to enhance IA content were obtained by EH using 838 units of β-glucosidase during 4.5 h at pH 6.0 and 37 ºC, and by FP using 1500 mg of commercial bakery yeast (*Saccharomyces cerevisiae)* during 24 h at pH 7.6 and 33 ºC. All hydrolyzed extracts were partitioned with ethyl acetate to obtain IA-rich fractions. In general, the fractions showed high IA content, but in some cases exhibited the presence of furanic compounds and/or triterpenes. The most pure and easily obtained fraction was the one from enzymatic hydrolyzed extract. This fraction was considered non-cytotoxic for keratinocytes after 24 and 48 h of treatment at a range of concentrations (0.1 to 1.0 µg of total IA/mL). Moreover, this fraction showed proliferative effects at 0.1 µg of total IA/mL suggesting its potential as an ingredient for skin regeneration during wound healing.

Keywords: Enzymatic and fermentation processes; Isoflavone aglycone-rich fractions; Keratinocytes; Plackett-Burman and Box Behnken designs.

INTRODUCTION

Soybeans are a rich source of proteins, carbohydrates, lipids, and other phytochemicals, such as sterols, isoflavones and saponins (Cederroth et al., 2012). The consumption of this legume, or derived products, is associated with many health benefits, mostly linked to the presence of isoflavones (Barnes, 2010). These compounds play a key role in several health effects due to their estrogen receptors binding ability (Morito et al., 2001; Barnes, 2010). Some estrogen-like properties exhibited by isoflavones are the stimulation of hyaluronic acid and collagen synthesis by keratinocytes and fibroblasts, respectively, resulting in skin repair processes that include reduction of wrinkles and wound healing (Miyazaki et al., 2002; Sudel et al., 2005).

Isoflavones naturally occur in soybeans as glucosidic conjugates (Albulescu and Popovici, 2007). In this way, generally acidic, basic, and enzymatic hydrolysis processes are applied to obtain their bioactive forms from soy products. These procedures are responsible for the breakdown of the conjugated isoflavones resulting in the aglycone units of genistein, daidzein and glycitein (Schwartz and Sontag, 2009). The acid hydrolysis has advantages related to process cost and speed. However, the greatest disadvantage is the lack of specificity for a specific target resulting in undesired degradation products (Schwartz and Sontag, 2009; Chen et al., 2014). Recently, it was demonstrated that acid hydrolysis of soybeans not only transform the conjugated isoflavones into their aglycones, but also degraded the soybean oligosaccharides to furanic compounds (Nemitz et al., 2015a). The main sugar degradation products formed are hydroxymethylfurfural (HMF) and ethoxymethylfurfural (EMF), both considered genotoxic compounds when at high amounts (Nemitz et al., 2016).

In order to obtain an isoflavone aglycones-rich fraction (IAF) intended to healthcare products development, our research group recently established a purification method to remove furanic impurities from soybean acid hydrolyzed extracts (Nemitz et al., 2015a). However, although this method has allowed an IAF with high purity, it cannot be considered a good candidate for scaling up during industrial routine, since it requires several steps making the process expensive and

slow. Taking this into account, when the intention is the large production of IA from soybeans, it is recommended to seek alternatives using more selective methods of hydrolysis. Therewith, fewer impurities probably would be formed, and the purification process would be simpler and more appropriate for industrial application.

In this context, the use of enzymes can be a very interesting hydrolysis alternative (Singh et al., 2016). Regarding to isoflavone biotransformation, the use of β-glucosidases is largely reported in literature (Ismail & Hayes, 2005; Lee et al., 2008). Besides, this kind of process has been used by some industrial companies [14,15]. The enzymatic hydrolysis can be carried out using purified β-glucosidases isolated from natural sources, or using microorganisms that expressed this enzyme (Shen and Bryan, 1997; Shen and Bryan, 1998). Considering the last option, several fungal and yeasts containing β-glucosidases are applied during food industrial processes to obtain fermented soy derivatives rich in IA for dietary products. Some microorganisms used for this purpose are the strains of species from the genera *Aspergillus*, *Rhizopus*, *Mucor*, *Actinomucor*, *Monascus, Saccharomyces, Neurospora, Acetobacter, Bacillus* and *Lactobacillus* (Rosa et al., 2009; Chen et al., 2012).

Several yeasts have high β-glucosidase activity resulting in the breakdown of all sugar β-conjugated compounds present in soybeans during fermentation process. However, yeasts that have low enzymatic activity cannot carry out the hydrolysis process with high performance producing an incomplete biotransformation of isoflavones (Dueñas et al., 2012). In these cases, it is important to highlight that yeast fermentation process could be performed not only intended to IA production, but also to promote complementary steps for further processes (Rekha and Vijayalakshmi, 2010). These practices are mainly reported by food engineering.

From the context here presented, this study was conducted in order to cover two main goals: (1) to obtain an IAF with high purity through a simple purification process, and (2) to obtain an IAF to be used as ingredient of dermal products, especially for wound treatments. To achieve these purposes, different hydrolysis procedures of soybean extracts, including acidic, enzymatic and fermentative processes were investigated. The hydrolysis methods mediated by biocatalysts were optimized by factorial experimental designs to maximize the IA content. The chemical

compositions of hydrolyzed extracts were compared not only for IA, but also for sugars, furanic compounds, saponins and triterpens. Purification process with ethyl acetate partition was performed, and the chemical compositions of IA-rich fractions were also analyzed and compared. Finally, to suggest the application of an IAF for wound treatment, keratinocytes viability and proliferation after IAF treatment were evaluated by the MTT and Ki-67 assays.

MATERIALS AND METHODS

Chemicals

Soybeans from EMBRAPA BRS 262 cultivar were obtained by donation of SEMEL seeds (São Paulo, Brazil). Isoflavone standards, daidzein, glycitein and genistein, as well as the β-glucosidase enzyme were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Glucose, furanic standards HMF and EMF were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fresh baker's yeast of the species *Saccharomyces cerevisiae* (Fleischmann®) was acquired from a local supermarket. The UFLC solvents were purchased from Merck (Darmstadt, Germany). Celite® resin was supplied by Merck Millipore (Darmstadt, Germany). Hydrochloric acid (HCl), ethylenediaminetetraacetic acid (EDTA), and dimethyl sulfoxide (DMSO) were acquired from Nuclear (Diadema, SP, Brazil). For cell culture procedures, Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA solution and 3-(4,5-dimethylthiazol- 2yl) 2,5-diphenyl-tetrazolium bromide salt (MTT) were supplied by Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). Ki-67 antibody (sc-23900 PE) was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Hydroethanolic soybean extraction

Soybeans seeds were grinded with an analytical mill. Then, they were defatted by Soxhlet extraction with *n*-hexane. The defatted soybean (DS) powder was extracted in a Soxhlet apparatus using a protocol of 10.0 g of DS and 200 mL of ethanol 80% (v/v), during 8 h at 70 - 80 °C. Several extractions were performed, pooled, filtered and stored at - 4 ºC.

Hydrolytic procedures

Before performing the hydrolysis processes, the ethanol present in the hydroethanolic soybean extract (HSE) was removed by evaporation under reduced pressure. Each 200 mL of HSE was reduced to approximately 20 mL, and then, the volume was adjusted to 40 mL with purified water. The process was repeated several times, pooled and stored at – 20 ºC. Each 4 mL of aqueous extract corresponds to the content that has been previously extracted from 1.0 g of DS.

Controls – acid hydrolyzed and non-hydrolyzed extracts

For negative control, a non-hydrolyzed extract was used, in which 4 mL of the aqueous extract were diluted with ethanol up to a final volume of 50 mL. For positive control, an acid hydrolysis protocol was used, in which 4 mL of the aqueous extract were diluted with ethanol and HCl was added up to a final acid concentration of 1.3 M. The mixture was adjusted to a volume of 40 mL with ethanol and refluxed at 80 °C for 2 h. At the final of the process, the acid hydrolyzed extract was diluted up to a final volume of 50 mL with ethanol.

Enzymatic hydrolysis – Screening protocols

Initially, an enzymatic hydrolysis procedure was performed as suggested by Liggins et al. (1998), with minor modifications. An aliquot of 10 mL of the aqueous extract was diluted with β-glucosidase solution (pH 6.8) to a final enzyme concentration of 100 units (U). The mixture was maintained overnight at 37 ºC, ethanol was added to stop the enzyme activity, and then, diluted to 50 mL with this same solvent. In these conditions, the hydrolysis was not satisfactory. So, the conditions were adjusted, and a screening study by Plackett-Burman design was performed to detect the relevant factors for the enzymatic hydrolysis. The Minitab 17® software was used to generate and analyze the experimental design. The twelve-run design is presented in **Table 5.1** and the assay was accomplished to select the most critical factors during conversion of isoflavone glucosides to aglycones. The protocol was carried out with 4 mL of the aqueous extracts and the independent variables (-1; +1) were: x_1: enzyme concentration (200 U; 800 U), x_2: pH (5.8 ; 7.8); x_3: time (2 h; 8 h), x_4: temperature (25 ºC; 50 ºC). After knowing which

factors were significant for enzymatic hydrolysis, the process optimization was determined by Box-Behnken design (BBD).

Fermentation process – Screening protocols

Preliminary, assays using a classical one-variable-at-a-time method, in which different concentrations of yeast and different period of time were analyzed. The protocol accomplished was: an aliquot of 4 mL of the aqueous extract was diluted up to 20 mL with phosphate buffer solution at pH 6.8 and 100, 250, 500, 1000, 1500 and 2000 mg of yeast were added. The mixture was maintained overnight at 37 ºC, ethanol was added to stop the fermentative process; the extracts were filtered through Celite® resin, and diluted up to 50 mL with ethanol. Complementary analyses were carried out using the same protocol, with the maximum amount of yeast and 24 or 48 h. The fermentative process was not satisfactory using all preliminary conditions. So, the factors which could affect the process were screened by a Plackett-Burman design, using the Minitab 17® software. The twelve-run design is presented in **Table 5.1** and the assay was performed to select the most critical factors during fermentation process. The independent variables (-1; +1) were: x_1: yeast concentration (300 mg; 900 mg), x_2: pH (5.8 ; 7.8), x_3: time (2 h; 8 h), x_4: temperature (25 ºC; 50 ºC). After this step, the optimization of fermentation process was accomplished by BBD.

BBD optimizations

For optimization of enzymatic hydrolysis (EH), a design with three factors in three different levels was used, as shown in **Table 5.2**. A total of 15 experiment trials were conducted in randomized runs and with three center points to estimate the pure error. The protocol was carried out at 37 ºC with 4 mL of the aqueous extract. The BBD independent variables (-1; 0; +1) were: x_1: enzyme concentration (200 U; 600 U; 1000 U), x_2: pH (5.8; 6.8; 7.8), x_3: time (2 h; 4 h; 6 h).

For optimization of fermentation process (FP), a design with four factors in three different levels was used, as shown in **Table 5.3.** A total of 27 experiment trials were conducted in randomized runs and with three center points to estimate the pure error. The protocol was performed at 37 ºC with 4 mL of the aqueous extract. The

BBD independent variables (-1; 0; +1) were: x_1: yeast concentration (500 mg; 1000 mg; 1500 mg), x_2: pH (5.8; 6.8; 7.8), x_3: time (6 h; 15 h; 24 h); x_4: temperature (25 ºC; 37 º C; 49 ºC).

The data obtained from each BBD was analyzed by response surface methodology (RSM) on isoflavone aglycones content, and were subject to analysis of variance (ANOVA). The experimental results of RSM were fitted by response surface regression procedure, using the following second order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{K} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + e \quad (1)$$

Where, Y is the predicted response, β_0, β_i, β_{ii}, and β_{ij} represent the regression coefficients of constant, linear, quadratic, and interactions terms, respectively; while x_i, x_j, and x_k represent the independent variables, and k is the number of variables (Montgomery, 2001).

For both EH and FP optimizations, ANOVA was conducted to confirm the fitted mathematical models. The fitness of the second-order polynomial model was expressed by the lack of fit, coefficient of determination (r^2), and significance of the regression coefficients. The interaction and influence of variables on the yield of IA was represented as three-dimensional response surface plots and contour plots, in which the optimal extraction condition was observed. The Minitab 17® software was used to generate the experimental design, statistical analysis and regression model.

The optimal conditions of EH and FP were established by response surface plots, targeting the maximal attainable response for each independent variable. The predictive capacity of the mathematical models was assessed by comparison between responses predicted and new experimental responses, as suggested by Bezerra et al. (2008). For this purpose, five independent experiments were performed on the same day using the optimal conditions estimated by mathematical models.

Fermentation followed by acid hydrolysis

The optimized extract obtained after FP was submitted to an additional step of acid hydrolysis. The protocol carried out was: an aliquot of 4 mL of aqueous extract

was diluted up to 20 mL with phosphate buffer solution at pH 7.6 and 1500 mg of yeast. The mixture was maintained at 33 ºC for 24 h. At the end of process, ethanol was added to stop the fermentative process; the extracts were filtered by Celite® resin, and submitted to acid hydrolysis (1.3 M HCl, 2 h under reflux). At the final of process, the hydrolyzed extracts were diluted to 50 mL with ethanol.

Isoflavone aglycones-rich fractions obtaining

In this step it was obtained different isoflavone aglycones-rich fractions. Firstly, the ethanol present in the soybean hydrolyzed extracts was evaporated under reduced pressure. The aqueous remaining extracts were submitted to liquid-liquid partition with ethyl acetate. The organic phase was then subsequently evaporated under reduced pressure, and the fractions were kept in a dehumidified environment.

Analyses of isoflavone aglycones and furanic compounds

The quantitative analysis of IA and the qualitative analysis of furanic compounds in soybean extracts and fractions were performed using an ultra-fast liquid chromatography method using the same conditions as described by Nemitz et al. (2015b).

Analyses of sugars, triterpenes and saponins

The quantitative analysis of water-soluble sugars present in extracts was performed by phenol-sulfuric acid assay (Dubois et al., 1956). Prior to analysis, the soybeans extracts were diluted (1:40) in water. In a test tube, 0.3 mL of water, 0.2 mL of diluted sample, 0.5 mL of 5% phenol solution and 2 mL of concentrated sulfuric acid were added. The reaction mixture was allowed to cool at room temperature for 30 min, and absorbance was measured at 490 nm. In parallel, a blank tube was prepared, using the same protocol, but with water instead of the diluted sample. A calibration curve was obtained from glucose concentrations ranging from 3.3 to 11.7 µg/mL. The amount of total carbohydrates (µg/mL) in extracts was expressed as glucose content.

The qualitative analyses of saponins and triterpenes present in extracts and fractions were carried out using thin-layer chromatography (TLC). The diluted samples were applied on aluminum-backed plates pre-coated with silica gel F_{254} (20 x20 cm, 200 mm, 60 Å, Merck) and submitted to the TLC procedures. The saponins' evaluation was performed as reported by Krishnamurthy et al. (2012) with minor modifications. The chromatographic run was carried out with the lower phase of chloroform: methanol: water system (65: 35: 10, v/v). Plates were developed in mobile phase, dried at 100 ºC for 10 min, sprayed with anisaldehyde sulfuric, and dried again. The triterpenes' evaluation was performed as reported by Muffler et al. (2011) with modifications. The chromatographic run was carried out with dichloromethane: ethyl acetate system (80: 20, v/v). Plates were developed in mobile phase, dried at 100 ºC for 10 min, sprayed with anisaldehyde sulfuric, and dried again. After evaluation of chemical composition of all fractions, the most IA enriched and easily obtained was chosen to perform the biological assays.

Biological assays

Cells culture

Immortalized Human Keratinocytes (HaCaT) cell line was provided by kind donation of Luisa L. Villa PhD (ICESP, School of Medicine, University of São Paulo) and Silvya S. Maria-Engler PhD (Faculty of Pharmaceutical Sciences, University of São Paulo). Cells were grown in DMEM high glucose, supplemented with 10% FBS and maintained at 37 °C, in 5% CO_2 humidified atmosphere.

MTT assay

In order to evaluate cell viability, MTT assay was performed. For that, HaCaT keratinocytes were seeded in 96-well plates at a density of $8x10^3$ cells per well and incubated for 24 h before treatment. The IA-rich fraction was solubilized in DMSO, which was diluted with DMEM to obtain final concentrations of 0.1, 0.5 and 1.0 µg of total IA/mL. The diluted samples used for cells treatment were prepared with a final DMSO concentration lower than 0.5% (v/v). Cells were treated with the diluted samples and incubated for 24 h and 48 h. For negative control, the cells were treated only with culture medium (DMEM/10% FBS) during the same times. After, the

treatment was withdrawn and the MTT solution (0.5 mg/mL) was added and the plates were incubated for 3 h at 37 °C. The formazan crystals formed were solubilized with DMSO and the absorbances were measured using a micro-plate reader at 570 nm with background subtraction at 630 nm. The assay was executed in three independent tests, with four replicates for each concentration. Cell viability was calculated as percentage in relation to control.

Ki-67 proliferation assay

The IA-rich fraction was submitted to Ki-67 assay to evaluate its proliferative potential. HaCaT keratinocytes were seeded in 24-well plates (2.5 $x10^4$ cells/well) and incubated for 24 h. Then, the cells were treated for 48 h with diluted samples of IA-rich fraction prepared as reported during MTT assay. For negative control, the cells were treated only with culture medium (DMEM/10% FBS). After the incubation time, the treatment was removed and the cells adhered to plates were trypsinized and transferred to a tube and centrifuged. The pellets of cells were fixed with ethanol 70% and permeabilized with a phosphate-buffered saline (PBS) solution containing 1% FBS and 0.09% NaN_3. Subsequently, it was also added 10 µL of Ki-67 antibody to each tube. The mixture was allowed to stand at room temperature, protected from light. After 20 min, the cells were centrifuged and resuspended in PBS. In parallel, control cells were processed in the same way, with or without Ki-67 antibody for labeled and unlabeled controls, respectively. The fluorescence intensity of the samples was analyzed using a FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Results were expressed as mean ± standard deviation of at least three independent experiments, and statistical analysis was performed by one-way ANOVA followed by Tukey or Dunnett tests. Both analysis were achieved using the GraphPad Prism version 6.1 ® software. Only for the factorial designs, Minitab 17® software was used.

RESULTS AND DISCUSSION

Selection of soybean cultivar

The first goal of this study was to obtain a soybean fraction with high IA purity through a simple process. However, to attain this, it is necessary an adequate selection of raw-material, an efficient extraction protocol, a satisfactory hydrolysis procedure and an easy purification step. So, primarily, the sample was chosen after an extensive literature search to select the soybean cultivar. Among the analyzed data, Ávila et al. (2011) reported that the Brazilian cultivar EMBRAPA BRS 262 represented a good source of isoflavones and, for this reason, this cultivar was chosen for the development of the present study.

Selection of hydrolysis processes

During soybean extraction and hydrolysis, many factors such as pH, temperature, time, and type of catalyst can influence these processes. Besides, the levels of these factors can also impact them. Several studies have reported the optimization of hydrolysis process using a different soybean derived samples. Most of them have reported the optimization of hydrolysis conditions to obtain IA during analytical assays, mainly applied for quality control methods of food products (Rostagno et al., 2009). Nevertheless, a few studies have reported the hydrolysis processes of soybeans as a way to obtain an IA-rich fraction to be used in the pharmaceutical or cosmetic applications (Nemitz et al., 2015c).

Acid hydrolysis of soybean extract from EMBRAPA BRS 262 seeds was previously optimized by one-variable-at-a-time by our research group to obtain an extract enriched in IA (Nemitz et al., 2015a). The optimized conditions were 1.3 M HCl in a hydroethanolic extract, under reflux during 2 h. However, we reported that this method led to the production of furanic impurities that hinder the purification process to obtain IA-rich fractions. So, in the present study, more selective hydrolysis processes, such as those using enzymes and yeasts as biocatalysts, were selected to optimize the production of IA rich fractions from the same soybean cultivar.

Enzymatic hydrolysis (EH) of isoflavones is normally carried out with β-glucosidase enzymes (Nemitz et al., 2015c). The present study performed the EH based in a method reported by Liggins et al. (1998). In turn, soybean fermentation procedures are generally performed by solid state methods with yeasts from genus of *Aspergillus* (Handa et al., 2014; Silva et al., 2011; Handa et al., 2016). Differently to literature data, the present study chose to obtain IA from soybeans by a fermentation process using a liquid state method with *Saccharomyces cerevisiae*. This process is more applied to break oligosaccharides and, consequently, to obtain bioethanol from soybeans, as well as for production of derived fermented foods and dietary supplements (Siqueira et al., 2008; Tudor et al., 2013; Wang et al., 2014; Yeh et al., 2014; Hassaan et al. 2015).

According to Schmidt et al. (2011), *S. cerevisiae* presents β-glucosidase able to hydrolyze conjugated flavonoids. Because of this, *S. cerevisiae* fermentation can be an alternative not only for biotransformation of conjugated isoflavones, but also for the breakdown of other sugars present in soybean extracts. In this sense, few studies were found. Among them, Romero et al. (2004) performed the fermentation of soybean seeds with *S. cerevisiae* to obtain an extract with high antioxidant properties. However, these authors did not evaluate the isoflavones content in the final product. So, as it can be seen, this kind of process is poorly explored for obtaining IA demonstrating the importance of studies covering this matter.

Screening of hydrolysis conditions

As previously reported, the enzymatic hydrolysis of soybeans was firstly tested usingthe protocol reported by Liggins et al. (1998). In these conditions, the results showed that the IA content was significantly lower when compared with the content present in acid hydrolyzed extract ($p < 0.05$). Regarding to fermentation, due to the scarce literature about the use of *S. cerevisiae* during the process, the preliminary conditions were performed with modifications by one-variable-at-a-time. The main results are presented in the supplementary material (**Supplementary material**).

From these preliminary results, it was observed that more tests were necessary to evaluate which factors impact during the bioprocesses. Thus, Plackett-Burman design was chosen to perform more detailed screening studies, and to

observe what kind of factors impacts during IA obtaining from soybeans. Four factors (enzyme or yeast concentrations, pH, incubation temperature, and incubation time) were screened. The concentrations (μg/mL) of IA at various processing conditions are shown in **Table 5.1**. According to the obtained results, all treatments of EH or FP showed a significant increase in IA content when compared with non-hydrolyzed extracts, but most of the results are lower when compared with those of acid hydrolyzed extract. The main results obtained from the screening tests are shown in **Fig. 5.1**. Factors were considered statistically significant when the calculated t-value was greater than 2.365 at $\alpha = 0.05$. The results showed that the effects of enzyme concentration, pH, and time were significant during EH, while all factors were significant during FP.

The significant effects of time on the rate of isoflavones conversion during EH were also reported by Tipkanon et al. (2010) and Xie et al. (2003). Besides, the last authors reported that pH and enzyme concentration could influence during IA obtaining from soybeans. Ismail and Hayes (2005) stated that enzyme concentration will depend of conjugated forms of isoflavones present in the sample. These authors showed that β-glucosidase was very effective at low concentrations for hydrolyzing glucoside forms, but the same was not observed for acetyl-glucosides or malonyl-glucosides, in which increased enzyme levels were necessary during the hydrolysis process.

BBD optimizations for EH and FP

After knowing the impacting factors that influence the biotransformation of isoflavones during EH and FP, a BBD optimization was performed for each process. This experimental design is a multivariate technique, based on RSM, applied to optimize the conditions during a process development. This technique is classified as a rotatable or nearly rotatable second-order design based on three-level incomplete factorial projects that aims to build a regression model (approximation) that is closer to the true model (Ferreira et al., 2007).

Table 5.1. Plackett-Burman Design for selection of significant factors affecting isoflavone aglycones content during different biocatalysis systems (enzymatic or fermentation processes)

Factors	Treatments and levels											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A (biocatalyst[1])	+	+	-	+	+	+	-	-	-	+	-	-
B (pH)	5.8	7.8	7.8	5.8	7.8	7.8	7.8	5.8	5.8	5.8	7.8	5.8
C (Temperature, ºC)	50	25	50	50	25	50	50	50	25	25	25	25
D (Time, h)	2.0	8.0	2.0	8.0	8.0	2.0	8.0	8.0	8.0	2.0	2.0	2.0
E (Dummy)	-	-	+	-	+	+	-	+	+	+	-	-
F (Dummy)	-	-	-	+	-	+	+	-	+	+	+	-
G (Dummy)	+	-	-	-	+	-	+	+	-	+	+	-
H (Dummy)	+	+	-	-	-	+	-	+	+	-	+	-
J (Dummy)	+	+	+	-	-	-	+	-	+	+	-	-
K (Dummy)	-	+	+	+	-	-	-	+	-	+	+	-
L (Dummy)	+	-	+	+	+	-	-	-	+	-	+	-
Isoflavone Aglycone Content[2]												
Enzymatic hydrolysis	36.53	27.46	9.45	36.31	27.19	18.99	10.01	31.02	27.55	28.16	8.15	14.61
Fermentation process	20.92	13.25	8.75	29.47	13.82	14.59	15.04	26.66	15.98	14.43	6.38	8.74
Negative control[3]	5.38 ± 0.115											
Positive control[4]	37.55 ± 0.69											

[1] Biocatalyst: Units of commercial β-glucosidase Enzyme (low level: 200 U, high level: 800 U), or milligrams of commercial yeast containing *Saccharomyces cerevisae* (low level: 300 mg, high level: 900 mg).
[2] Expressed as µg/mL for the sum of daidzein, glycitein and genistein (using 4 mL of aqueous extract for all treatments, and at final of each hydrolysis process the extracts were diluted to 50 mL)
[3] Negative control: 4 mL of soybean aqueous extract diluted until 50 mL with ethanol
[4] Positive control: 4 mL of soybean aqueous extract after acid hydrolysis (HCl 1.3 M, 2 h under reflux) diluted until 50 mL with ethanol
(E - L) Dummy variables: dummy variables: + indicates a high level; - indicates a low level.

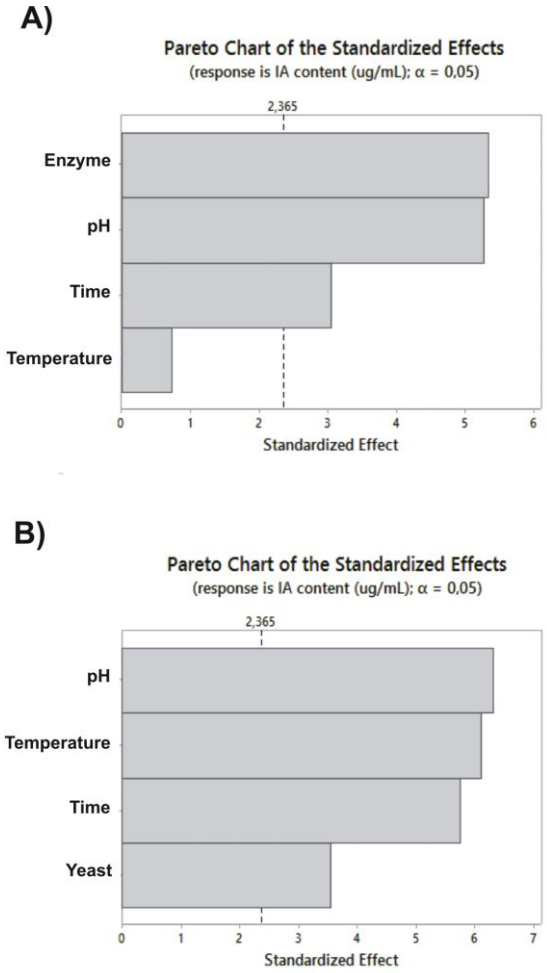

Figure 5.1. Plackett-Burman experimental designs for (A) enzymatic hydrolysis of soybean extract by β-glucosidase, and (B) fermentation process of soybean extract by *S. cerevisiae*. Bar charts representing the *t*-calculated for IA content in each bioprocess for the investigated factors (enzymatic or yeast concentration, pH, time and temperature) and their *t*-critical, represented by the vertical lines.

To perform the EH and FP optimizations, the factors and levels were chosen based on screening protocols. For EH optimization, **Table 5.2** shows the results obtained during BBD assay. Based on multiple regression analysis of the experimental data, the predicted response value was expressed by the following second-order polynomial equation using coded variables:

$$y = -60.9 - 0.0056\,x_1 + 29.6\,x_2 + 4.82\,x_3 - 0.00002\,x_1^2 - 2.961\,x_2^2 - 0.645\,x_3^2 + 0.00657\,x_1x_2 - 0.000073\,x_1x_3 + 0.185\,x_2x_3 \quad (2)$$

The fit of the model to the data was evaluated statistically by ANOVA. For any of the terms in the model, a large regression coefficient and a small *p*-value would indicate a more significant effect on the respective response variables (Montgomery, 2001). According to the ANOVA of regression model, both linear and quadratic terms were significant ($p < 0.01$). The high coefficient of determination values for the response evaluated ($r^2 = 0.98$) demonstrated the suitability of BBD for EH assay. Additionally, the mathematical model obtained showed to be suitable for the analysis of response surface, since no evidence of lack-of-fit was detected. The regression coefficients of the predicted quadratic polynomial model were obtained for the coded variables and the significance of each coefficient was determined using Student's *t*-test and the *p*-value, in which a larger *t*-value and smaller p-value show the significance of the corresponding coefficient (Montgomery, 2001). It was observed that the enzyme concentration was significant in both linear and quadratic terms, whereas the pH was verified to be significant only for the linear term. The other term coefficients were not significantly influential on the model.

For fermentation process, **Table 5.3** shows the results obtained during BBD assay. The second-order polynomial equation using coded variables were:

$$y = -75.2 - 0.0212\,x_1 + 17.0\,x_2 + 1.211\,x_3 + 2.346\,x_4 - 0.000004\,x_1^2 - 1.789\,x_2^2 - 0.0203\,x_3^2 - 0.02135\,x_4^2 + 0.00498\,x_1x_2 - 0.000206\,x_1x_3 + 0.000026\,x_1x_4 + 0.172\,x_2x_3 - 0.0394\,x_2x_4 - 0.02738\,x_3x_4 \quad (3)$$

According to the ANOVA of regression model, both linear and quadratic terms were significant ($p < 0.01$). The high coefficient of determination values for the response evaluated ($r^2 = 0.91$) demonstrated the suitability of BBD for FP. Additionally, the mathematical model obtained showed to be suitable for the analysis of response surface, since no evidence of inadequacy was detected by the lack-of-fit test. The regression coefficients of the predicted quadratic polynomial model were analyzed, and it was observed that the time was significant in linear term, while temperature was significant in quadratic term. The other term coefficients were not significantly influential on the model.

Table 5.2. Box-Behnken Design arrangement and the experimental value for response variables for production of isoflavone aglycones from soybean extract using different enzymatic conditions

Test run number	Hydrolysis Condition*			Total Isoflavone Aglycones (µg/mL)**
	Enzyme (U)	pH	Time (h)	
1	200	5.8	4	33.52
2	1000	5.8	4	22.78
3	200	7.8	4	33.09
4	1000	7.8	4	14.25
5	200	6.8	2	18.72
6	1000	6.8	2	34.79
7	200	6.8	6	32.13
8	1000	6.8	6	35.60
9	600	5.8	2	33.55
10	600	7.8	2	36.22
11	600	5.8	6	29.98
12	600	7.8	6	19.25
13	600	6.8	4	23.04
14	600	6.8	4	33.39
15	600	6.8	4	29.57

* β-glucosidase as biocatalyst, Temperature: 37 ºC
** expressed as µg/mL for the sum of daidzein, glycitein and genistein (using 4 mL of aqueous extract for all treatments, and at final of each hydrolysis process the extracts were diluted to 50 mL)

The optimized conditions denoted by RSM were: EH using 838 units of β-glucosidase, pH 6.0 and 4.5 h; whereas for FP using 1500 mg of commercial bakery yeast (*Saccharomyces cerevisiae)*, pH 7.6, 24 h and 33 ºC. The conditions were also visualized by individual response surface plots for each variable (Supplementary material). Moreover, to estimate the validity of the developed mathematical model equation, five replicates of each process (EH or FP) were performed under the optimal conditions as predicted by the models. The average value of the experiments and the predicted values of the developed model are shown in **Table 5.4**, demonstrating a good predictive capacity of models.

Table 5.3. Box-Behnken Design arrangement and the experimental value for response variables for production of asoflavone aglycones from soybean extract using different fermentation conditions

Test run number	Fermentation Conditions				Total Isoflavone Aglycones (µg/mL)**
	Yeast* (mg)	pH	Time (h)	Temperature (ºC)	
1	500	5.8	15	37	28.35
2	1500	5.8	15	37	24.22
3	500	7.8	15	37	21.30
4	1500	7.8	15	37	27.13
5	1000	6.8	6	25	15.85
6	1000	6.8	24	25	30.23
7	1000	6.8	6	49	22.17
8	1000	6.8	24	49	24.72
9	500	6.8	15	25	19.50
10	1500	6.8	15	25	24.13
11	500	6.8	15	49	23.16
12	1500	6.8	15	49	28.41
13	1000	5.8	6	37	20.11
14	1000	7.8	6	37	17.76
15	1000	5.8	24	37	27.87
16	1000	7.8	24	37	31.71
17	500	6.8	6	37	17.05
18	1500	6.8	6	37	22.77
19	500	6.8	24	37	29.45
20	1500	6.8	24	37	31.46
21	1000	5.8	15	25	23.69
22	1000	7.8	15	25	18.29
23	1000	5.8	15	49	28.41
24	1000	7.8	15	49	21.12
25	1000	6.8	15	37	28.22
26	1000	6.8	15	37	26.64
27	1000	6.8	15	37	28.63

* commercial yeast (*S. cerevisiae*); **expressed as µg/mL for the sum of daidzein, glycitein and genistein (using 4 mL of aqueous extract for all treatments, and at final of each hydrolysis process the extracts were diluted to 50 mL)

Table 5.4. Predicted and experimental values of isoflavone aglycone (IA) content in soybean extracts at optimum response values obtained by Box-Behnken Design for enzymatic hydrolysis and fermentation process

Hydrolzyed extract	Total IA (µg/mL)*	
	Predicted value	Experimental value
Enzymatic hydrolysis	37.53	36.28 ± 0.84
Fermentation process	32.26	32.16 ± 1.38

* Expressed as a sum of genistein, daidzein and glycitein

Chemical composition of hydrolyzed extracts

As shown in **Table 5.5**, all hydrolyzed extracts presented higher IA content when compared with non-hydrolyzed extracts ($p < 0.05$). However, when comparing IA content of extracts hydrolyzed by bioprocesses (SE_{EH} and SE_{FP}) with acid hydrolyzed extract (SE_{AH}), only the soybean extract obtained after enzymatic hydrolysis has a similar IA content ($p > 0.05$). Because of this, the soybean extract obtained after fermentation was submitted to a complementary acid hydrolysis process. After the additional step, the acid hydrolyzed fermented extract (SFE_{AH}) showed a similar IA content with both SE_{EH} and SE_{AH} ($p > 0.05$).

Besides, other chemicals were assessed in the hydrolyzed extracts. The quantification of sugars, as well as the qualitative investigation of furanic compounds, saponins and triterpenes were performed (Supplementary material), and the summary of results are shown in **Table 5.5**. These analyses were carried out to visualize if the processes degrade other compounds or only transform the conjugated isoflavones into their aglycones.

As it can be seen in **Table 5.5**, the negative control (non-hydrolyzed soybean extract) showed high presence of saponins, high sugar content, low presence of triterpenes, absence of furanic compounds and some content of IA. This composition was in accordance with extracts obtained from soybean without any hydrolysis treatment (Kim et al., 2006; Rostagno et al., 2009).

On the other hand, the positive control (acid hydrolyzed extract) showed high IA content, as well as presence of triterpenes and furanic compounds resulted from

degradation of saponins and sugars during the process, respectively. This result is in accordance with the expected effects that acid conditions normally causes in the compounds present in soybean extract (Amin et al., 2011; Nemitz et al., 2015a).

In the extract obtained after enzymatic hydrolysis (SE_{EH}) it was observed high presence of saponins, low presence of triterpenes, absence of furanic compounds and high sugar content, being these chemical composition very similar with the negative control. The difference it was the increase of IA contents, demonstrating the selectivity of enzymatic process, which only transforms β-glucosides conjugated chemicals present in the extract, such as isoflavones.

In the fermented extract (SE_{FP}) it was observed high content of IA and absence of sugars or furanic compounds, demonstrating that sugars were consumed in the process and no degradation products such as HMF and EMF were produced. Besides, the results showed that *S. cerevisiae* did not cause hydrolysis of saponins during the fermentation process. Comparing these results with other studies concerning fermentation processes with *Aspergillus* (Amin et al., 2011; Amin et al., 2013), the present process became very interesting because it shows the selectivity of *S. cerevisiae* in only break the isoflavones conjugates, and not the saponin compounds.

Instead, when acid hydrolysis was carried out in the fermented extract, the new hydrolyzed product (SFE_{AH}) showed an increase of triterpenes, which are formed by the extreme pH condition during the process that promote the hydrolysis of saponins. Considering the results for the SFE_{AH} it is also important to note that the acid hydrolysis performed in the fermented extract did not produce the sugar degradation products (furanic compounds), differently from the classical acid method (positive control). This is related to the previous sugar consumption led by *S. cerevisiae* during the fermentation process.

Table 5.5. Total isoflavone aglycones content (TIA), total sugar content, presence or absence of saponins, triterpenes and furanic compounds in different soybean extracts

Sample	TIA (µg/mL)[1]	Total Sugar Content (%)[2]	Saponins[4]	Triterpenes[4]	Furanic compounds[5]
Negative control (SE)	5.38 ± 0.11[a]	171.86 ± 4.34	Presence (++)	Presence (+)	Absence
Positive control (SE_{AH})	37.55 ± 0.70[b]	N.D.[6]	Absence	Presence (++)	Presence (++)
SE_{EH}	36.28 ± 0.84[b]	173.15 ± 6.95	Presence (++)	Presence (+)	Absence
SE_{FP}	32.16 ± 1.38[c]	L.L.D.	Presence (++)	Presence (+)	Absence
SFE_{AH}	37.15 ±0.48[b]	L.L.D.	Absence	Presence (++)	Absence

[1] Expressed as µg/mL for the sum of daidzein, glycitein and genistein present in 50 mL of extract
[2] Expressed as the amount of total carbohydrates (µg/mL) regarding to glucose content
[3] Presence or absence visualized by TLC method
[4] Presence or absence visualized by TLC method
[5] Presence or absence visualized by UFLC method
[6] Not determined by the method of sugar quantification, but according to the literature data it can be considered that all sugars are degraded during extreme pH and high temperature conditions
+: low presence
++: high presence
SE: non-hydrolyzed soybean extract; SE_{AH}: soybean extract after acid hydrolysis; SE_{EH}: soybean extract after enzymatic hydrolysis; SE_{FP}: soybean extract after fermentation hydrolysis; SFE_{AH}: soybean fermented extract after acid hydrolysis.
L.L.D: lower than the limit of detection
Different letters in the same column represent statistical differences ($p<0.05$).

Obtaining fractions and their chemical composition

The purification of IA from the hydrolyzed extracts was performed by ethyl acetate partitions. This solvent has high affinity with IA (Guanjun et al., 2014), but also with triterpenes and furanic compounds (Nemitz et al. 2015a; Ogwuche et al., 2014). Therefore, the chemical composition of hydrolyzed extracts significantly affects the obtaining of fractions, resulting in a greater or lesser IA purity. As shown in **Table 5.6**, the fraction obtained after purification of acid hydrolyzed extract (F-SE_{AH}) showed low IA purity (<10%), probably due to the high presence of triterpenes and furanic compounds. Each mg of this fraction had 98.05 ± 1.44 µg of total IA, in which the individual content of genistein, daidzein and glycitein was 49.27 ± 1.08, 44.32 ± 2.08 and 4.41 ± 0.23 µg/mg, respectively.

On the other hand, in the fractions obtained by purification of enzymatic (F-SE_{EH}) or fermented extracts (F-SE_{FP}), it was observed high IA purity without presence of triterpenes or furanic compounds. The fraction obtained from enzymatic extract showed the highest IA purity. Each mg of this sample presented 898.17 ± 9.83 µg of total IA, in which the individual content of genistein, daidzein and glycitein was 466.96 ± 10.54, 391.61 ± 5.42 and 39.60 ± 1.88 µg/mg, respectively. The fraction obtained from fermented extract has 851.65 ± 12.11 of total IA/mg, in which the individual content of genistein, daidzein and glycitein was 418.93 ± 14.79, 388.47 ± 7.48 and 44.24 ± 1.71 µg/mg, respectively. Lastly, in the fraction obtained by purification of acid hydrolyzed fermented extract (F-SFE_{AH}) it was observed absence of furanic compounds, but presence of triterpenes, decreasing the IA purity of fraction for < 60%. Each mg of this fraction had 597.72 ± 7.57 µg of total IA, in which the individual content of genistein (316.79 ± 8.87), daidzein (257.02 ± 11.41) and glycitein (23.91 ± 0.87 µg/mg).

In summary, the conditions of biotransformation of isoflavones were optimized, and one-step purification process was implemented. When the intention is to obtain soybean extracts enriched in IA, it is recommended to perform enzymatic hydrolysis with β-glucosidase or fermentation with *S. cerevisiae* followed by acid hydrolysis. However, when the intention is the production of an IA-enriched fraction with high purity by a fast hydrolysis method and simple purification process, it is recommended the ethyl acetate partition of soybean extract hydrolyzed by enzymatic process.

Table 5.6. Total isoflavone aglycones content (TIA), presence or absence of saponins, triterpenes and furanic compounds in dry fractions obtained from purification of different soybean hydrolyzed extracts

Sample	TIA (µg/mg)[1]	Saponins[2]	Triterpenes[3]	Furanic compounds[4]
F-SE$_{AH}$	98.05 ± 1.44	Absence	Presence	Presence
F-SE$_{EH}$	898.17 ± 9.83*	Absence	Absence	Absence
F-SE$_{FP}$	851.65 ± 12.11	Absence	Absence	Absence
F-SFE$_{AH}$	597.72 ± 7.57	Absence	Presence	Absence

[1] Expressed as µg/mg for the sum of daidzein, glycitein and genistein present in dry fractions
[2] Presence or absence of saponins visualized by TLC
[3] Presence or absence of triterpens visualized by TLC
[4] Presence or absence of HMF and EMF visualized by UFLC method
F-SE$_{AH}$: fraction from soybean acid hydrolyzed extract; F-SE$_{EH}$: fraction from soybean enzymatic extract; F-SE$_{FP}$: fraction from soybean fermented extract; F-SFE$_{AH}$: fraction from acid hydrolyzed fermented soybean extract
* represents the higher TIA content between all fractions ($p<0.05$)

Biological assays

Based on the aforementioned results, the fraction selected to evaluate the *in vitro* keratinocytes proliferative potential was the F-SE$_{EH}$. Keratinocytes are skin cells responsible for tissue epithelization, causing a positive effect during cutaneous wound healing (Pastar et al., 2014).

Before assessing proliferation effects by the Ki-67 assay, the viability of HaCaT cells after treatment with this fraction at final concentrations of 0.1, 0.5 and 1.0 µg of total IA/mL during 24 and 48 h was assessed by MTT assay. These concentrations were chosen based on the data reported by Zhang et al. (2007) which stated that a soybean product showed an estrogenic activity at 0.4 µg of total IA/mL in MCF-7 cells (estrogen-sensitive cell line). Moreover, these concentrations is in accordance with data reported by Nemitz et al. (2015c), which stated that the most evident estrogenic effects of genistein and daidzein are described at very low concentrations, generally under 1 µM, corresponding then, to amounts below to 0.25 µg/mL for daidzein and 0.27 µg/mL for genistein.

The results obtained by MTT assay are shown in **Fig. 5.2**, and as it can be seen, the fraction could be considered non-cytotoxic for HaCaT cells at the concentrations investigated. These data suggest that F-SE_{EH} could be used as raw-material in skin care products with no keratinocytes cytotoxicity. Besides, an increase of cells viability was observed at 0.1 µg of total IA/mL (107 ± 2.38%) after 24 h of treatment, and at 0.1 and 0.5 µg of total IA/mL after 48 h (115.21 ± 4.98 and 111.06 ± 0.20, respectively). Therefore, the proliferation effects could possibly occur, mainly after 48 h of treatment.

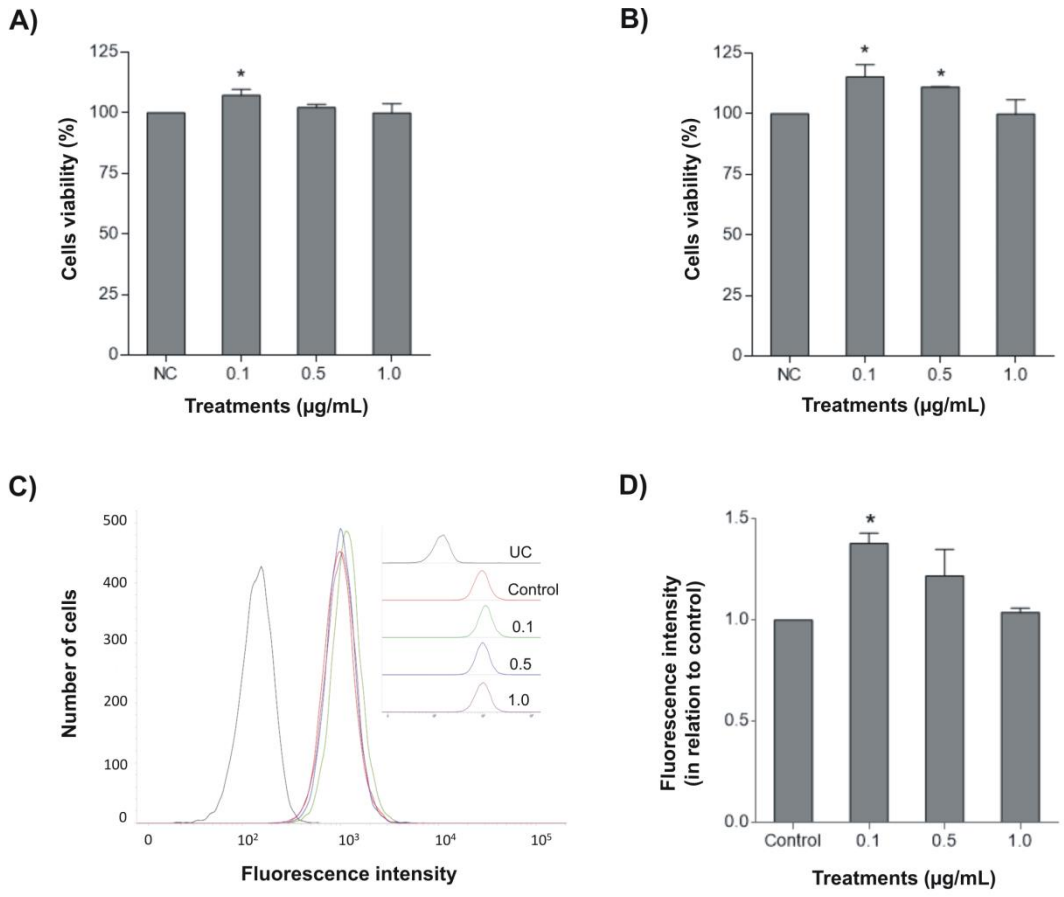

Figure 5.2. Cells viability of HaCaT keratinocytes measured by MTT assay after (A) 24 h and (B) 48 h of treatments with F-SE_{EH} at concentrations of 0.1, 0.5 and 1 µg of TIA/mL. Where, NC: negative control. As well as, the Ki67 assay after 48 h of cells treatment with F-SE_{EH} at 0.1, 0.5 and 1 µg of TIA/mL. Where: (C) is the flow cytometry histogram; and (D) shows the increase of fluorescence intensity compared to the control. UC indicates the untreated and unlabeled cells during Ki67 assay, and control is the untreated but labeled cells during Ki67 assay. F-SE_{EH}: fraction from soybean enzymatic extract; *statistically higher than controls ($p<0.05$).

To confirm the proliferative effects of F-SE_{EH}, this fraction was submitted to the Ki-67 proliferation assay, a gold standard method to evaluate *in vitro* proliferative activity. The protocol was performed after 48 h of cells treatment, using the same concentrations of the MTT assay. The results are also shown in **Fig. 5.2.** The lowest tested concentration of this fraction (0.1 µg of total IA/mL) showed significant proliferative effects on keratinocyte cells when compared with the control, indicating then, the potential of F-SE_{EH} to be used as a raw-material to develop wound healing products. However, more assays may be conducted to confirm the wound healing capacity, as well as *in vivo* studies.

CONCLUSIONS

In this study, different hydrolytic procedures, including acid, enzymatic and fermentation processes were evaluated to obtain different soybean extracts with high content of isoflavone aglycones. The acid hydrolysis was carried out with a classical protocol, and the enzymatic and fermentative processes were performed with β-glucosidase and *S. cerevisiae,* respectively. The bioprocesses conditions were optimized by Plackett-Burman and Box Behnken designs. All hydrolyzed extracts were analyzed in terms of sugars, furanic compounds, isoflavone aglycones, saponins and triterpenes presence. The type of hydrolysis had considerably altered the chemical composition of the extracts. Consequently, the composition influenced the purity of fractions obtained after purification with ethyl acetate partitions. The fraction that showed the higher isoflavone aglycones purity and that was obtained by the easier hydrolysis process was the one obtained from the enzymatic hydrolyzed extract. This fraction was considered non-cytotoxic for keratinocytes after 48 h of exposure at concentrations ranging from 0.1 to 1.0 µg of total IA/mL, and also showed a proliferative cell effects at the lowest concentration assessed, suggesting its potential as an ingredient for skin regeneration during wound healing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found to complement the results.

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Supplementary material

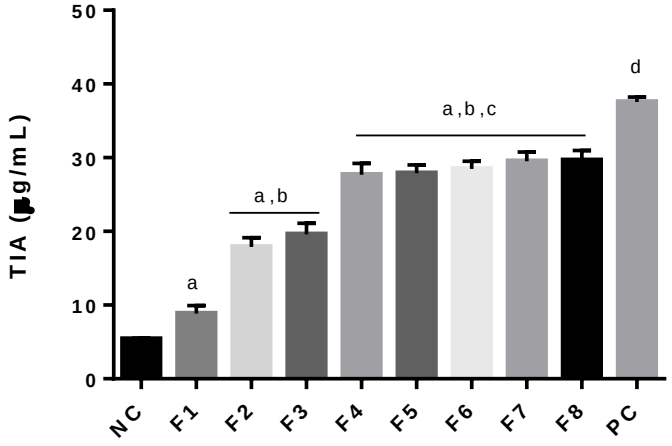

Figure S1. Total isoflavone aglycones (TIA) present in different soybean extracts during screening tests of fermentation process (*S. cerevisiae*). The results are expressed as sum of genistein, daidzein and glycitein. All extracts were obtained from 1.0 g of defatted soybean seeds, and diluted up to 50 mL. Moreover, all fermentation processes (F1 – F8) were performed at pH 6.8 and 37 ºC. The abbreviations represent: NC: negative control (non-hydrolyzed extract); F1: fermentation process during 12 h with 100 mg of yeast; F2: fermentation process during 12 h with 250 mg of yeast; F3: fermentation process during 12 h with 500 mg of yeast; F4: fermentation process during 12 h with 1000 mg of yeast; F5: fermentation process during 12 h with 1500 mg of yeast; F6: fermentation process during 12 h with 2000 mg of yeast; F7: of yeast; fermentation process during 24 h with 2000 mg of yeast; F8: of yeast; fermentation process during 48 h with 2000 mg of yeast; PC: positive control (acid hydrolyzed extract). Letters represent: (a) statistically different from NC at α=0.05; (b) statistically different from F1 at α=0.05; (c) statistically different from F2 and F3 at α=0.05; (d) higher TIA content when compared with all groups at α=0.05.

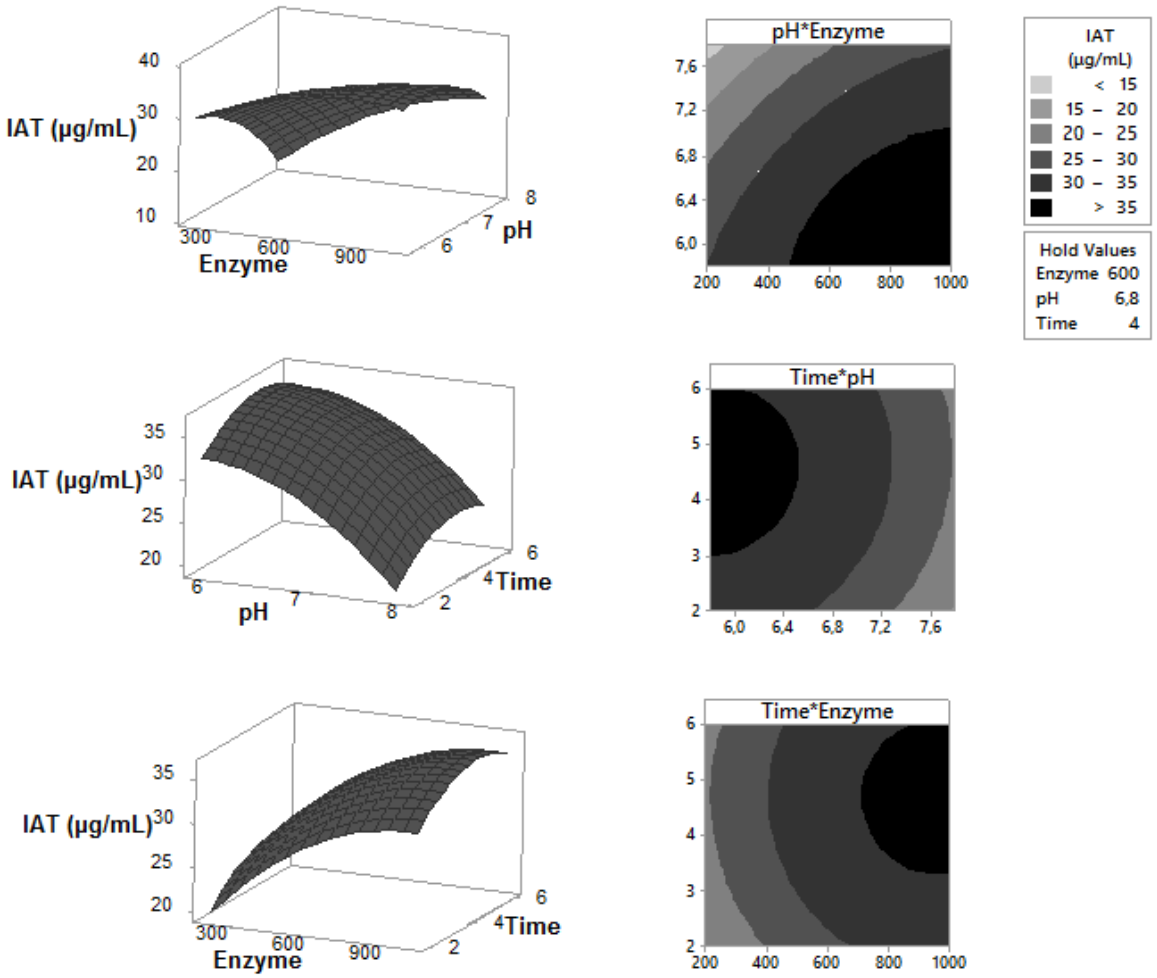

Figure S2. Contour plot and response surface of IA content in soybean extract hydrolyzed by enzymatic process. Where: IAT = total isoflavone aglycone expressed as sum of genistein, daidzein and glycitein.

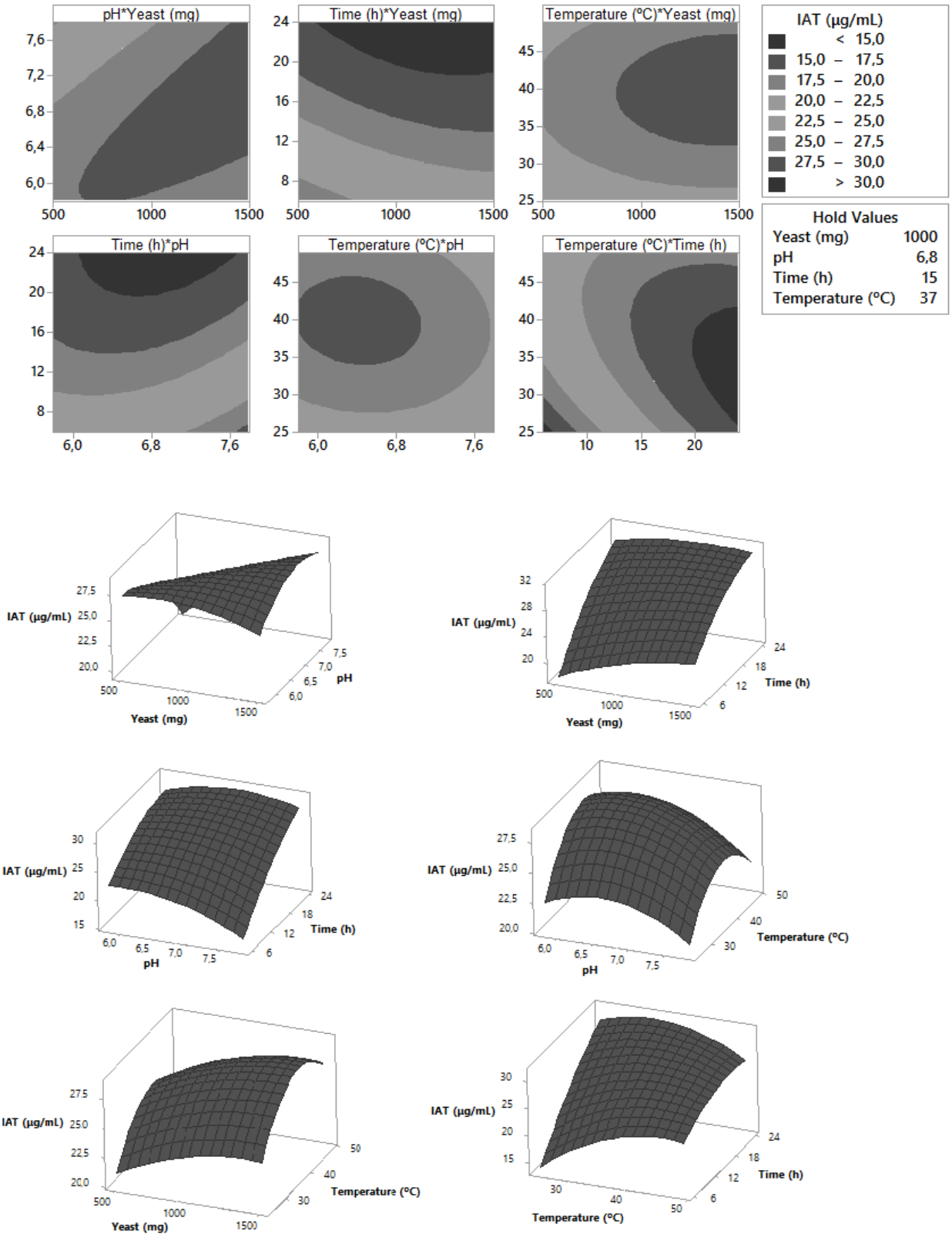

Figure S3. Contour plot and response surface of IA content in soybean extract hydrolyzed by fermentation process. Where: IAT = total isoflavone aglycone expressed as sum of genistein, daidzein and glycitein

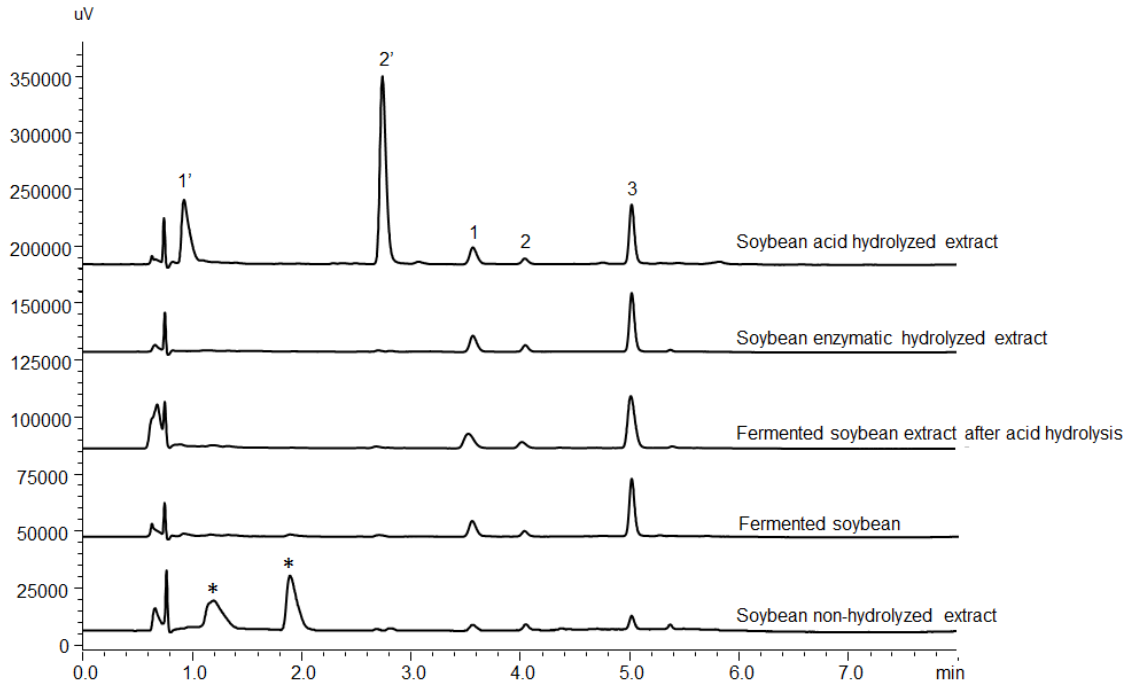

Figure S4. Representative UFLC chromatograms of soybean extracts after different hydrolysis processes. Where, peaks with * representing conjugated isoflavones; and the peaks with other numbers representing: 1=daidzein; 2=glycitein; 3=genistein; 1'= hydroxymethylfurfural, 2'= ethoxymethylfurfural.

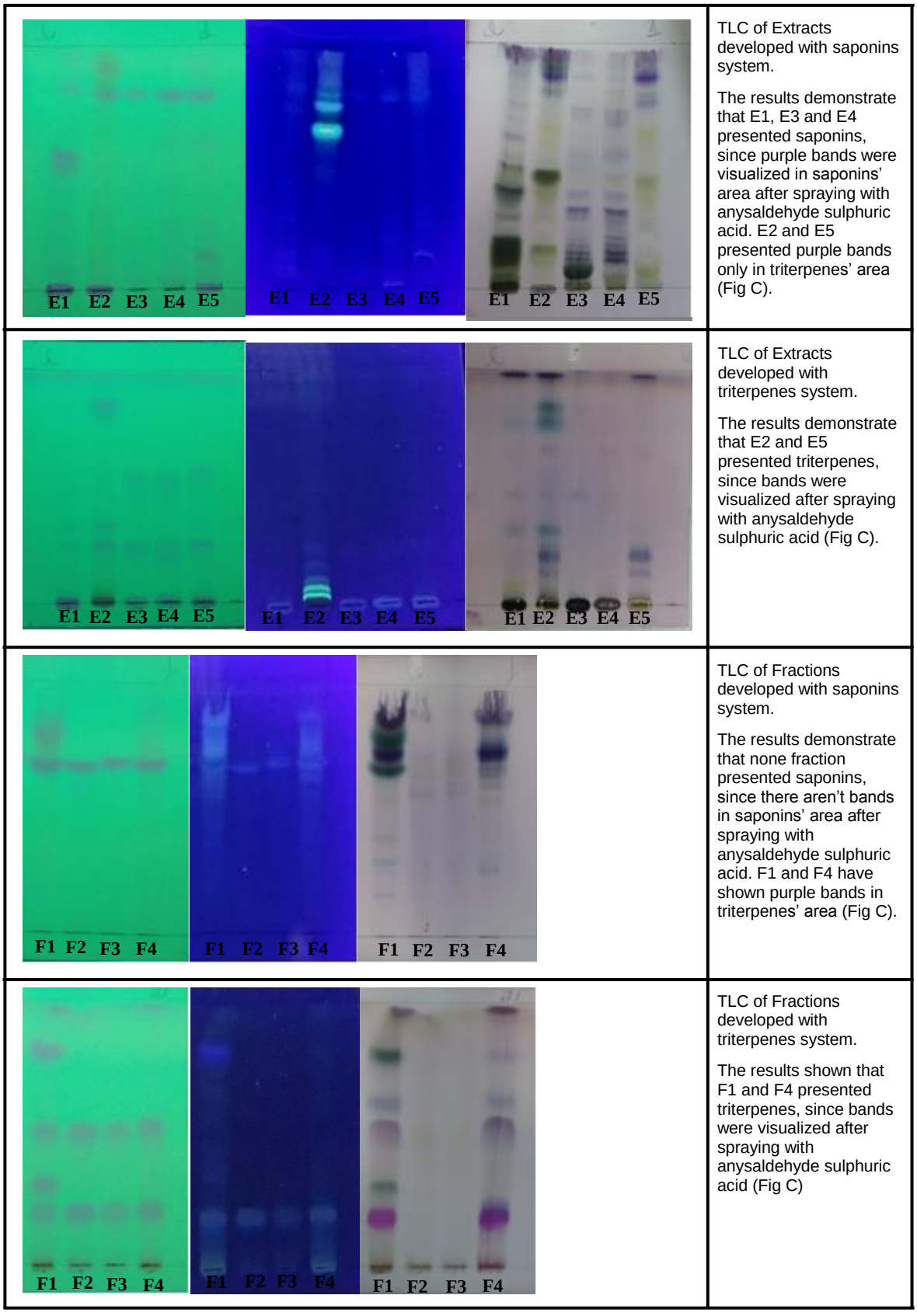

Figure S5. TLC of soybean extracts (E1 - E5) and fractions (F1 – F4) developed with saponins and triterpenes systems. For saponins the chromatographic run was carried out with the lower phase of chloroform: methanol: water (65: 35: 10). For triterpens the chromatographic run was carried out with dichloromethane: ethyl

acetate (80: 20). All plates were visualized by UV lamp at (A) 254 nm, (B) 360 nm, or (C) normal light after sprayed with anysaldehyde sulphuric acid. The analyzed samples were (E1) soybean non-hydrolyzed extract, (E2) soybean acid hydrolyzed extract, (E3) soybean enzymatic hydrolyzed extract, (E4) fermented soybean extract, (E5) fermented soybean extract after acid hydrolysis, (F1) fraction from soybean acid hydrolyzed extract, (F2) fraction from soybean enzymatic hydrolyzed extract, (F3) fraction from fermented soybean extract, (F4) fraction from fermented soybean extract after acid hydrolysis. Bands visualized in short and long UV lights were representative of isoflavones, and bands visualized after spraying with anysaldehyde sulphuric acid were representative of other compounds. In saponins system, the purple bands below to the middle of plate were representative of saponins, while the purple bands upper to the middle of plate were representative of triterpenes. In triterpenes system, all bands in the plate were representative of these compounds.

CAPÍTULO VI

Espessamento de nanoemulsões contendo fração de isoflavonas agliconas da soja com ácido hialurônico e estudos de permeação cutânea

6.1. INTRODUÇÃO

Diversos estudos sugerem os efeitos benéficos do uso das isoflavonas da soja na pele, incluindo as atividades antioxidante (WEI *et al*., 1995; WEI *et al*., 2002; GEORGETTI *et al*., 2006; GEORGETTI *et al*., 2009; GEORGETTI *et al*., 2013), anti-inflamatória (GEORGETTI *et al*., 2008; KHAN *et al*., 2012), anti-envelhecimento e fotoprotetora (SUDEL *et al*., 2005; HUANG *et al*., 2008; LIN *et al*., 2008; HUANG *et al*., 2010; IOVINE *et al*., 2012; IOVINE *et al*., 2014).

Dentre as potencialidades das isoflavonas, estudos recentes destacam o efeito desses compostos na cicatrização de feridas cutâneas decorrente da sua reconhecida atividade estrogênica. Estudos *in vitro* apontam que isoflavonas, especialmente nas formas agliconas, aumentam a síntese de ácido hialurônico, elastina e colágeno em fibroblastos e queratinócitos presentes nas camadas da pele (JURZAK & ADAMCZYK, 2013; KIM et al., 2015; ZHAO *et al*., 2015). Além disso, um estudo *in vivo* realizado por Hwang e colaboradores (2001) demonstrou que o uso tópico de genisteína em ratos (1 mg/200g e 4 mg/200g) aumentou a produção de colágeno em lesões após 14 dias de tratamento, melhorando o processo cicatrizante das feridas cutâneas.

Alguns estudos descrevem, ainda, que o uso simultâneo das isoflavonas pode potencializar a ação estrogênica e fotoprotetora das mesmas (RANDO *et al*., 2009; IOVINE *et al*., 2011). Essa premissa vem impulsionando pesquisadores a desenvolver produtos contendo concomitantemente genisteína e demais isoflavonas agliconas, a fim de obter produtos com melhor desempenho farmacológico. Recentemente, nesse sentido, Renda e colaboradores (2013) relataram que extratos de diferentes espécies de *Trifolium* L. (contendo principalmente as isoflavonas genisteína, daizeína, formononetina e biochanina A) apresentaram potencial atividade cicatrizante *in vivo* após aplicação diária de 0,5 g de pomadas contendo 1% de extrato das plantas em feridas cutâneas de camundongos.

Devido às potencialidades do uso dérmico das isoflavonas agliconas, o desenvolvimento de produtos tópicos de base nanotecnológica torna-se promissor não apenas para contornar reduzida solubilidade desses compostos, mas também para aumentar a sua capacidade de permeação, retenção, ou ainda, absorção

percutânea. A velocidade e extensão da absorção percutânea está relacionada com o reduzido tamanho dos sistemas nanoestruturados, apresentando como vantagem a possibilidade de formação de um depósito mais uniforme sobre a pele e uma maior superfície de contato em comparação a sistemas convencionais (PURI *et al.,* 2009; ZHAI & ZHAI, 2014).

Dentre alguns sistemas lipídicos nanométricos utilizados para aumentar a permeação/retenção de substâncias ativas, encontram-se as nanoemulsões. Essas formulações são caracterizadas como dispersões de gotículas oleosas nanométricas em uma fase aquosa externa, estabilizada por um sistema tensoativo. Apresentam-se como líquidos de aspecto leitoso, onde geralmente as substâncias hidrofóbicas encontram-se solubilizadas no núcleo oleoso e/ou adsorvidas na interface das gotículas (BOUCHEMAL *et al.*, 2004; ANTON & VANDAMME, 2011).

Estudos prévios realizados pelo nosso grupo de pesquisa relataram a preparação, caracterização e permeação cutânea da isoflavona genisteina a partir de nanoemulsões (SILVA *et al.,* 2009, VARGAS *et al.,* 2012; ARGENTA et al., 2014). Primeiramente, dois tipos de óleos foram avaliados para o preparo das gotículas, triglicerídeos de cadeia média e octidodecanol, sendo estas estabilizadas com lecitina de gema de ovo. Posteriormente também foi avaliada a produção das formulações contendo tensoativos catiônicos. Todas as formulações desenvolvidas possibilitaram um aumento na retenção *in vitro* de genisteína nas diferentes camadas da pele (quando comparado com genisteína não incorporada a nenhum carreador), demonstrando, portanto, a boa perspectiva destes sistemas visando o uso tópico desta isoflavona.

Neste contexto, esta parte do trabalho objetivou a continuidade dos trabalhos do grupo com isoflavonas e nanoemulsões, porém agora contemplando a incorporação de uma fração enriquecida em isoflavonas agliconas da soja (FIA) visando um produto tópico com potencial de aplicação durante processos de cicatrização de feridas da pele. A FIA utilizada nesta parte do trabalho foi obtida pela purificação de um extrato de soja hidrolisado pela via enzimática e demonstrou, conforme estudos relatados no capítulo anterior, uma promissora atividade proliferativa *in vitro* de queratinócitos, células importantes durante processo de cicatrização de feridas cutâneas. Além disso, devido ao interesse de desenvolver um

produto visando à regeneração dérmica de feridas, as nanoemulsões foram espessadas com ácido hialurônico. Este polímero foi escolhido uma vez que o seu uso acelera o fornecimento de nutrientes e solutos para o tecido lesado, aumentando a motilidade celular do processo proliferativo durante a cicatrização (XU *et al*., 2012).

Os dados experimentais deste capítulo estão apresentados na forma de artigo científico em fase de elaboração, o qual será oportunamente submetido à publicação.

6.2. ARTIGO

IN VITRO SKIN PERMEATION/RETENTION OF DAIDZEIN, GENISTEIN AND GLYCITEIN FROM A SOYBEAN ISOFLAVONE RICH FRACTION-LOADED NANOEMULSIONS AND DERIVED HYDROGELS

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ABSTRACT

Positive skin effects have been reported for isoflavones, mainly for their aglycone forms. In this way, the present study addresses the development of products containing isoflavone aglycone-rich fraction (IAF) aimed for dermal application. The formulations investigated were IAF nanoemulsions (NE_{IAF}) and derivative semisolid hydrogels composed of hyaluronic acid ($H\text{-}NE_{IAF}$). The IAF used during this study contained high purity of isoflavone aglycones (IA), obtained after purification of a soybean extract previously hydrolyzed by an enzymatic process. Nanoemulsions (NE) containing 1.0 mg of IA/mL (expressed as sum of genistein, daidzein and glycitein) were prepared by spontaneous emulsification procedure. At the end of process, hyaluronic acid (3%) was added into NE to obtain the hydrogels. Formulations exhibited mean droplet size below to 300 nm and negative ζ-potential. A significant decrease of IA release from formulations was observed when compared to an isoflavone propylene glycol suspension (control). The IA release followed the order: control > NE_{IAF} > HNE_{IAF}. Moreover, after 8 h of permeation/retention *in vitro* study with porcine ear skin, a significant increase of IA retention was observed from formulations as compared to the control. The distribution of IA in skin layers was evaluated, and the highest IA retention was found in dermis. These results demonstrate the potential of both formulations for topical skin applications of IA.

Keywords: isoflavone aglycone-rich fraction; nanoemulsions; hydrogels; permeation/retention study.

INTRODUCTION

Phytoestrogens are plant compounds that present health-enhancing properties. They are categorized in some groups, such as lignans, isoflavones, coumestans, and phytoesterols (Albertazzi and Purdie, 2002; Liu et al., 2010). Isoflavones are a subclass of flavonoids that has attracted much attention in dermatological field mainly due to their estrogenic and antioxidant properties (Shmid and Zulli, 2002). Because of this, skincare products containing these compounds (isolated or isoflavones-rich plant extracts) are available in the market (Nemitz et al., 2015a; Nemitz et al., 2016a).

The mostly impacting effects caused by isoflavones on the skin are often related to the stimulation of fibroblast proliferation, reducing of collagen breakdown, protein tyrosine kinase inhibition, and photoprotection against ultraviolet (UV) damage (Shmid and Zulli, 2002; Varani et al., 2004; Sudel et al., 2005, Lin et al., 2008, Huang et al., 2008). Studies have described that these effects are mainly related to the unconjugated forms of isoflavones, also namely aglycones. Moreover, literature data have reported that extracts containing more than one isoflavone have higher beneficial effects when compared to the isolated forms (Kao & Chen, 2005; Chiu et al., 2009; Rando et al., 2009; Huang et al., 2010).

High isoflavone content extracts can be obtained from several plants. Legumes from Fabaceae family are the most important sources, in which soybeans are considered the greatest one (Messina, 1999). In soybean seeds, isoflavones mainly occur as beta-glucoside compounds (Barnes, 2010), and the extraction followed by hydrolysis process is a generally process applied to obtain their bioactive forms (Nemitz et al. 2015a). Our research group recently optimized the production of an enzymatic hydrolyzed soybean extract, which was purified leading to an isoflavone aglycone-rich fraction (IAF) containing high content of isoflavone aglycones (IA) genistein, glycitein, and daidzein. Such a fraction was considered non-cytotoxicity for keratinocytes, and showed *in vitro* proliferative cell effects, suggesting benefits during skin repair (Nemitz et al., 2016b).

Due to the limited water solubility of IA, the development of topical products containing these substances is a great challenge. Normally, their incorporation in

traditional systems is facilitated applying alcohol-based systems, or ointment base and water-in-oil vehicles (Nemitz et al., 2015a). However, oily products are less patient-acceptable for topical application, and organic solvents can cause skin irritation (Weiss, 2011). Therefore, to overcome these drawbacks, nanostructured delivery systems have been considered as a promising strategy to develop alcohol free hydrophilic systems containing IA. In this way, our research group recently patented four nanocarriers containing IAF in order to improve hydrophilic products with potential to be used in several healthcare products (Nemitz et al., 2015b).

Moreover, nanotechnology products have been also considered as a promising strategy for skin delivery. Among them, nanoemulsions (NEs) have been reported as advantageous dermal delivery systems of genistein (Silva et al., 2009; Vargas et al., 2012; Argenta et al., 2014). NEs are dispersions of oily droplets in an external aqueous phase, stabilized by a suitable surfactant system within nanometric size ranged generally between of 10 - 1000 nm (Jaiswal et al., 2015). They can increase the skin permeation rate and enhance the topical effect of drugs by prolonged residence time in the uppermost skin layers due to both the large surface area and the low surface tension of the nanodroplets (Abolmaali et al., 2011; Chellapa et al., 2015; Jaiswal et al., 2015). However, such systems are obtained as liquid oil in water dispersions, exhibiting low viscosity. In consequence, their incorporation into a semisolid dosage form is required for a proper topical application (Chellapa et al., 2015).

To promote viscosity and increase the spreadability capacity, different polymers have been used as thickening agents of NEs (Chellapa et al., 2015). Within this purpose, normally nontoxic, biocompatible, and biodegradable hydrophilic polymers are applied (Marin et al., 2013; Bassyouni et al., 2015). Among them, the most used are hydroxyethylcellulose, polyacrilic acid derivates (carbomers) and chitosan, which can present non-ionic, ionic or cationic characteristics, respectively (Marin et al., 2013). Another ionic polymer that has been used as gelling agent of medical and cosmetic products is hyaluronic acid (HA) (Brown and Jones, 2005). This anionic polymer is a high molecular weight biopolysacharide, naturally occurring in the skin, composed of unbranched repeating units of glucuronic acid and N-acetyl glucosamine linked by β1–3 and β1–4 glycosidic bonds. It is characterized by

viscoelastic nature, biocompatibility and non-immunogenicity, leading to its use in a number of clinical applications, including in topical wound healing products (Brown and Jones, 2005; Necas et al., 2008). The use of exogenous HA in topical formulations is mentioned to reduce the time of wound repair and improve the quality of the scar due to its capacity to remodel extracellular matrix by keratinocytes and fibroblast proliferation (Price et al., 2005; Price et al., 2007).

In this context, the present study investigated the feasibility of an IAF to be incorporated into topical formulations comprising nanoemulsions, tickened or not with hyaluronic acid, intended to enhance the IA penetration and retention through the skin.

MATERIALS AND METHODS

Chemicals

Isoflavone standards, daidzein, glycitein and genistein (≥95%, ≥98% and ≥98% of purity, respectively) were acquired from Cayman Chemical Company (Ann Arbor, MI, USA). Methanol and acetonitrile liquid chromatography grade were supplied from Merck (Darmstadt, Germany). Egg-lecithin (Lipoid E-80®) and medium chain triglycerides (MCT) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Hyaluronic acid sodium salt with a high molecular weight was acquired from Carbosynth (Berkshire, United Kingdom). Ultrapure water was obtained from a Milli-Q apparatus (Millipore, Billerica, USA). The soybeans (cultivar BRS 262) were obtained from *Empresa Brasileira de Pesquisa Agropecuária* (EMBRAPA), kindly donated by SEMEL seeds (São Paulo, Brazil). Porcine ears were obtained at a local abattoir (Slaughterhouse Ouro do Sul, Harmony, RS, Brazil).

Chromatographic analysis of isoflavones

The simultaneous determination of genistein (GEN), daidzein (DAID), and glycitein (GLY) in IAF, formulations and porcine ear skin samples was carried out by using a validated ultra-fast liquid chromatography (UFLC) method (Nemitz et al., 2015c). The system consisted of a Shimadzu Prominence system device coupled to a photodiode array (PDA) detection with an automatic injector controlled by LC-

Solution Multi-PDA software (Kyoto, Japan). The stationary phase was a Shim-pack XR ODS column (Shimadzu, 100 x 2.0 mm i.d.; particle size, 2.2 µm) guarded by pre-column. The mobile phase consisted of (A) trifluoroacetic acid 0.1% (v/v) and (B) acetonitrile. The gradient elution was 20 - 25% B (0 - 2 min), 25 - 30% B (2 - 3 min), 30 - 35% B (3 - 4 min), and 35 - 20% B (4 - 6 min) and 20 % B (6 - 8 min). The flow rate was a gradient of 0.35 mL/min (0 - 1.5 min), 0.35 - 0.5 mL/min (1.5 - 2 min), 0.5 mL/min (2 - 3 min), 0.5 - 0.35 mL/min (3 – 4 min), and 0.35 mL/min up to 8 minutes. The analysis was carried out at 55 °C, with 3 µL of injection volume and wavelength at 260 nm.

Obtaining isoflavone aglycone rich fraction (IAF)

Soybeans were grinded and defatted by exhaustive extraction with *n*-hexane in a Soxhlet apparatus. The extraction of 10.0 g of defatted soybean material was performed using 200 mL of ethanol 80% (v/v), during 8 h at 70 - 80 °C. The ethanol in hydroethanolic soybean extract (HSE) was evaporated under reduced pressure. A volume of 200 mL of HSE was reduced to approximately 20 mL, and then, this amount was adjusted up to 40 mL with purified water. Various processes were performed, pooled, and stored at – 20 ºC. Each 4 mL of aqueous extract corresponds to the content that has previously extracted from 1.0 g of DS. The enzymatic procedure was performed in a water bath at 37 °C with 4 mL of aqueous extract and 838 units of β-glucosidase. The volume was adjusted up to 20 mL with phosphate buffer pH 6.0, and the reaction was performed during 4.5 h. Then, liquid-liquid partitions were performed with ethyl acetate, and the organic phase was evaporated under reduced pressure.

Preparation of formulations

Nanoemulsions containing IAF (NE_{IAF}) were prepared by the spontaneous emulsification technique. The oil phase components (egg-lecithin, MCT and IAF) were solubilized in ethanol and injected into an aqueous phase containing polysorbate 80, under constant stirring. Subsequently, the organic solvent and the solvent excess were removed under reduced pressure until the desired volume. In turn, the hydrogels containing nanoemulsions (HNE_{IAF}) were obtained by adding 3%

of hyaluronic acid into nanoemulsions. The final composition of formulations is presented in **Table 6.1**. The chemical markers were genistein, daidzein and glycitein, and the formulations were developed with a final content of 1 mg/mL expressed as the sum of these isoflavones. Blank formulations (NE_B and HNE_B) were prepared under the same conditions in the absence of IAF.

Table 6.1. Final composition of formulations (%)

Components (%)	Formulations			
	NE_B	**HNE_B**	**NE_{IAF}**	**HNE_{IAF}**
MCT	8	8	8	8
Egg lecithin	2	2	2	2
Polysorbate 80	1	1	1	1
Isoflavone aglycones	-	-	0.1	0.1
Hyaluronic acid	-	3	-	3
Water qsp	100	100	100	100

Where, NE_{IAF}: nanoemulsion containing isoflavone aglycone-rich fraction; NE_B: blank nanoemulsion; HNE_{IAF}: nanoemulsion based hydrogel containing isoflavone aglycone-rich fraction; HNE_B: blank nanoemulsions based hydrogel.

Characterization of formulations

pH determination

The analysis of pH was determined directly in nanoemulsions, and with a 10% solution of hydrogels in water, using a calibrated potentiometer (Model 21 pH, Hanna, Brazil). The results were expressed as the average of three independent determinations.

Size, polydispersity index, and Zeta potential determination

The formulations were characterized by dynamic light scattering analysis with monochromatic laser diffusion, which crosses the colloidal dispersion, using Zetasizer (Zetasizer 3000HS, Malvern Instruments, Malvern, England). This analysis was made by observing the 90^o scattering after diluting the samples (1:1000) in 0.1 mM NaCl solution previously filtered through a 0.22 µm membrane. The results were expressed as the average of three independent determinations. Zeta potential was

determined by electrophoretic mobility of the droplets in Zetasizer (Zetasizer 3000HS, Malvern Instruments, Malvern, England). All analyzes were performed after diluting the samples (1:1000) in 1 mM NaCl previously filtered through a 0.22 µm membrane. The results were expressed as the average of three independent determinations.

Isoflavone content

For chromatographic analysis, the formulations were diluted in the proportion 1:100 in a diluent of acetonitrile: water (50:50, v/v), filtered through a 0.22 µm membrane, and analyzed by the UFLC method.

In vitro release assay

For release studies, Franz diffusion cells were used, with an internal circular interface area of 1.77 cm^2 and receptor compartment with 10 mL of volume. The assays were performed using cellulose ester membranes (50 nm pore diameter, Milipore®) between the donor compartments and the cell receptors. The membranes were pre-hydrated with pH 7.4 phosphate buffer and the bath temperature was adjusted to 32 ± 1.0 °C. The receptor compartment was filled with a mixture of pH 7.4 phosphate buffer and ethanol (70:30 v/v) and remained under constant stirring at 450 rpm. The hydrogels (400 µg), nanoemulsions (400 µL) or control suspensions (400 µL) were added on membranes. The experiment was carried out keeping *sink* conditions, using the same concentration of isoflavones and fluid receptor as reported by Nemitz et al. (2015c). Aliquots were removed every hour and replaced by same amount of fresh receptor media. The total duration of the experiment was 8 h. Control release profile was analyzed through a suspension of IAF in propylene glycol containing the equivalent of 1 mg/mL of isoflavone aglycones. The aliquots were filtered with a 0.22 µm membrane and analyzed by the UFLC method.

Skin permeation studies

For permeation/skin retention studies, Franz diffusion cells were also used. Porcine ears were obtained from a local slaughterhouse. The ears were withdrawn from the animals before the scalding procedure, cleaned, and the full-thickness skin

was excised from the outer region of the ear with a scalpel. The subcutaneous tissue was removed and the skin was stored in aluminum foil for one month at the lasted in a -20 °C freezer. Before usage, the skin was left in contact with pH 7.4 phosphate buffer for 15 minutes. Then, the skin was placed to a Franz cell, maintaining the dermis in contact with the receptor fluid. The bath temperature was adjusted to 32 ± 1.0 °C and the acceptor compartment was filled with fluid (pH 7.4 phosphate buffer with 30% of ethanol) and remained under constant stirring at 450 rpm. The hydrogels (400 µg), nanoemulsions (400 µL) or control suspensions (400 µL) were added on skins. The experiment was carried out keeping *sink* conditions. After 8 h of experiment, a fluid sample was collected from the acceptor compartment and the skin was removed from the Franz cell. The excess of formulation was removed from each skin, which was subsequently submitted to the tape stripping process for analysis of non-viable epidermis (stratum corneum). The first stripped tape was discarded, while the following 14 tapes were placed in a test tube. The remaining layers were separated (viable epidermis and dermis), reduced to tiny pieces and placed in different test tubes. It was added 2 mL of methanol in all tubes, and then they were kept in an ultrasound bath for 30 minutes for the extraction of isoflavones from skin layers. The samples were then filtered with a 0.22 µm membrane and analyzed in UFLC method.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 6.1. Data were analyzed by parametric test one-way ANOVA and complemented by a Tukey test for multiple comparisons. The significance level was $\alpha = 0.05$.

RESULTS AND DISCUSSION

Preparation and characterization of formulations

To obtain an isoflavone aglycones-rich fraction (IAF) it was performed hydroethanolic extraction of defatted soybeans, enzymatic hydrolysis and acetyl acetate partition as method previously optimized by our research group (Nemitz et al., 2016b). The IAF showed high purity of isoflavone aglycones and it was

incorporated into NEs to achieve an isoflavone final content of 1 mg/mL, expressed as a sum of GEN, DAID, and GLY. The main physicochemical properties of NEs are show in **Table 6.2**. As can be seen, spontaneous emulsification procedure yielded monodisperse droplets (PDI < 0.2) exhibiting an average size in a 200 nm-range. The IAF incorporation into nanoemulsions has increased significantly ($p<0.05$) the negative value of ζ-potential of formulation (from -24 to -50 mV) possible due to the presence of negative-charged compounds from the IAF at o/w interface. Isoflavones seem to be associated with the oil droplets, since a recovery close to 100% was notice. Such results are in agreement with the expected, once isoflavone aglycones (IA) are low water solubility compounds with partition coefficients (logP) close to 2.98, 2.78 e 2.57 for GEN, DAID, and GLY, respectively (estimated by online ACD/I-Lab service), favoring then, their incorporation into the oil phase of NEs.

However, although NEs have shown good properties, they possess the drawback of low viscosity to topical application. So, to improve their topical application, the NEs were herein thickened as a semisolid form. To that, HA was used as gelling agent of system. The properties of HA, including specific viscoelasticity, biocompatibility, hydration and lubrication, make this polysaccharide potentially very useful for development of topical products (Jin et al., 2010; Xu et al., 2012). The effect of HA addition on NEs was evaluated comparing the physicochemical properties of nanodroplets before and after thickening. As can be seen in **Table 6.2**, NE_B and HNE_B did not show difference between the size and ζ-potential ($p>0.05$). According to Jenning et al. (2000), this result suggests no aggregation of droplets is occuring between charged groups of nanoemulsion surfactants and gel-forming polymer. On the other hand, comparing NE_{IAf} and HNE_{IAF}, it was observed a significant increase on size and ζ-potential ($p<0.05$). However, once it is not observed changes in these properties after thickening blank nanoemulsions, the increase of NE_{IAF} droplet size, after the addition of HA, is probably caused by the interaction of this polymer with other chemicals present in IAF that may located in the surface of droplets, and not because the aggregation of them. Similar findings were reported by Arora et al. (2014) and Gohel et al. (2014), in which an increase of size was observed after the addition of an anionic polymer in nanoemulsions containing ketoprofen or *Boswellia serrata* extract, respectively.

Table 6.2. Physicochemical properties of formulations.

	Droplet size (nm)	PDI	ζ- potential (mV)	Isoflavone content (mg/mL)			
				Daidzein	Glycitein	Genistein	Total
NE_B	187.0 ± 6.4 [a]	0.101 ± 0.02	- 24.5 ± 1.1 [a]	-	-	-	-
NE_{IAF}	134.7 ± 2.7 [b]	0.170 ± 0.03	- 50.3 ± 2.6 [b]	0.39 ± 0.010	0.11 ± 0.001	0.50 ± 0.010	1.01 ± 0.020
HNE_B	185.7 ± 11.6 [a]	0.187 ± 0.02	- 24.4 ± 2.1 [a]	-	-	-	-
HNE_{IAF}	295. 2 ± 26.2 [c]	0.205 ± 0.02	- 59.3 ± 0.9 [c]	0.38 ± 0.014	0.10 ± 0.001	0.50 ± 0.009	0.99 ± 0.027

Legend: NE_B: nanoemulsion blank; NE_{IAF}: isoflavone aglycone-rich fraction loaded nanoemulsion; HNE_B: nanoemulsion blank thickened in hydrogel; NE_{IAF}: nanoemulsion containing isoflavone aglycone rich fraction thickened in hydrogel; PDI = Polydispersity index. Values expressed by the mean of triplicate analysis ± standard deviation. Different letters in same column are statistically different ($p<0.05$).

In vitro release study of isoflavones from formulations

Studies regarding the release of interesting compounds from formulations are an important step during the development of new products. Because of this, the profile of DAID, GLY and GEN releases from formulations over time were herein evaluated.

Figure 6.1 shows the release of each isoflavone aglycone from nanoemulsions and hydrogels. As can be seen, the incorporation of IAF into nanoemulsions reduces the release profile of all isoflavones when compared to a control (propylene glycol suspension). Approximately 95% of each isoflavone was released after 8 h of assay for control, whereas 78.08 ± 3.45 %, 83.05 ± 3.46 % and 61.14 ± 2.26 % was noticed for DAID, GLY and GEN, respectively. The decrease of compounds release is in agreement with literature, which reports that NEs normally decrease the release of flavonoids, due to their interaction with the oil core or phospholipids of formulations (Silva et al., 2009; Bonifácio et al., 2014; Vinardell and Mitjans, 2015; Argenta et al., 2016). Besides, comparing each isoflavone released from NE_{IAF} after 8 h, the DAID and GLY presented a similar result ($p>0.05$), and both showed a higher release when compared with GEN ($p<0.05$). These results are in accordance with the partition coefficients of isoflavones, demonstrating that GEN, which has the higher logP, probably possess a higher interaction with the oil core of NE, making its release more difficult from formulations.

Figure 6.1 also shows the isoflavones release from hydrogels. As can be seen, thickening formulations reduces the release profile of all isoflavones when compared to NEs. After 8 h of assay the released amounts were 49.79 ± 1.50 %, 61.61 ± 2.95 %, 36.40 ± 0.43 % for DAID, GLY and GEN, respectively. This is in accordance with literature data, in which studies have report that hydrogels could retain the oil droplets of nanoemulsions in the polymer network, increasing the resistance to compounds release (Argenta et al., 2016; Balestrin et al. 2016).

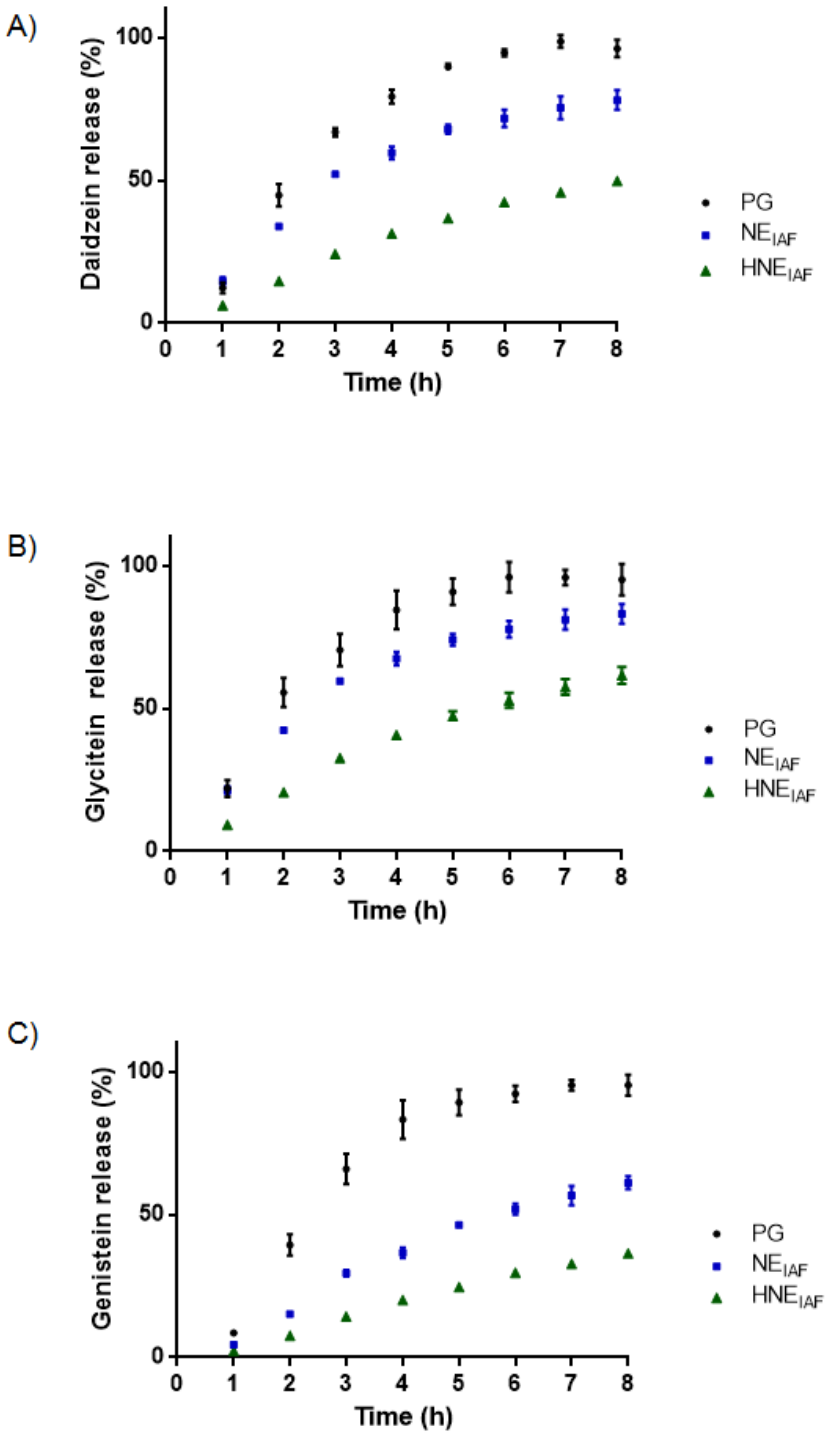

Figure 6.1. Release profile of (A) DAID; (B) GLY; (C) GEN after 8 hours from a propylene glycol control suspension (PG) and NE_{IAF} and HNE_{IAF} through synthetic cellulose membranes in Franz Difusion Cells. The values are means ± SD of three experiments.

Besides of release profiles, it was also calculated the fluxs of each isoflavone from formulations. The DAID fluxs were 9.33 ± 0.41, 6.20 ± 0.19 μg/cm^2/h, and 11.56 ± 0.36 from NE_{IAf} HNE_{IAF}, and PG control, respectively, showing a statically difference between them ($p<0.05$). The GLY fluxs were 3.09 ± 0.12, 1.96 ± 0.09 μg/cm^2/h, and 3.03 ± 0.17 from NE_{IAf}, HNE_{IAF}, and PG control, respectively, showing a similarity for PG and NE_{IAF} ($p>0.05$), and both are statistically higher when compared with HNE_{IAF} ($p<0.05$). The GEN fluxs were 9.51 ± 0.35, 5.86 ± 0.07 μg/cm^2/h, and 14.83 ± 0.57 from NE_{IAf}, HNE_{IAF}, and PG control, respectively, showing a statically difference between them ($p<0.05$). Thus, the release fluxs for all isoflavones followed the order: PG > NE_{IAf} > HNE_{IAf}. Overall results are in agreement with the previously discussed for the profiles in terms of percentage released after 8 h of assay. Besides, as can be noted, the amount released over the time for each isoflavone is in accordance with the correspondent content present in the formulations, in which followed the order: GEN > DAID > GLY, whatever of formulation analyzed.

Permeation/retention assay

The ability of compounds to penetrate the skin may be influenced by several factors. Among them, the formulation composition play a key role, considering that the compound have to diffuse out of the vehicle to the skin surface and then it must permeate the stratum corneum (SC) barrier on the way to the site of action (Kriwet and Miiller-Goymann, 1995). Since isoflavones have been reported as estrogenic compounds, their penetration in SC followed by retention in deep layers, such as viable epidermis and dermis, become necessary to bind with estrogenic receptors of fibroblast and keratinocyte cells and exert their skin beneficial effects.

Taking this into account, it was performed an assay with porcine ear skin in Franz diffusion cells in order to evaluate the skin permeation/retention ability of isoflavones from formulations. The receptor fluid was analyzed, as well as the skin layers of stratum corneum, viable epidermis, and dermis. It is important to highlight that the present study shows for the first time a simultaneously study regarding the distribution of DAID, GLY, and GEN during skin permeation/retention assays.

The results were herein showed encompassing two main approaches. Firstly, it was analyzed the sum of all isoflavones retained in the skin from each formulation (**Table 6.3**), and then, the distribution of DAID, GLY, and GEN in skin layers from each formulation was demonstrated in **Figure 6.2**. Whatever the formulation, no isoflavone was found in receptor fluid. Differently, isoflavone aglycones were quantified in the skin layers, demonstrating that they have penetrated and kept retained in the cutaneous tissues after 8 h of skin treatments with formulations.

Data presented in **Table 6.3** showed that both NE_{IAF} and HNE_{IAF} led a higher IA skin retention when compared with a PG control ($p<0.05$). These results demonstrate that the incorporation of isoflavones in NEs systems is a very attractive alternative to enhance their penetration on the skin. Besides, it was observe that both formulations allowed IA penetration and retention in all skin layers, with highest amount in dermis. However, when the results of NE_{IAF} and HNE_{IAF} were compared, the IA retained was higher from NE_{IAF} ($p<0.05$) in total skin, as well as in all skin layers.

Table 6.3. Total isoflavone aglycones (expressed as a sum of DAID, GLY, GEN) retained in skin layers from each formulation.

Formulations	Total isoflavones (µg/cm^2 ± SD, n = 6)			
	Stratum corneoum	**Epidermis**	**Dermis**	**Total retained**
PG control	0.199 ± 0.024	0.050 ± 0.007	0.181 ± 0.031	0.430 ± 0.054
NE_{IAF}	0.373 ± 0.041	0.551 ± 0.094	0.746 ± 0.082	1.671 ± 0.077
HNE_{IAF}	0.386 ± 0.022	0.403 ± 0.061	0.597 ± 0.040	1.386 ± 0.083

Where PG: propylene glycol; NE: nanoemulsion; HNE: nanoemulsion based hydrogel; IAF: isoflavone aglycone-rich fraction; SD: standard deviation

A possible explanation for the lower IA skin retention from hydrogels when compared with nanoemulsions can be attributed to the droplet size of formulations, since HNE_{IAF} showed higher diameter than NE_{IAF}. According to Shakeel et al. (2012)

the size is a key property during the dermal delivery of compounds from NEs and its derivate products. Besides, such a result could be also attributed to the lower amount of IA released for HNE_{IAF} after 8 h of kinetics. In fact, IA were released faster and in a greater extent from nanoemulsions (NE_{IAF}) than from the derivative hydrogel (HNE_{IAF}), causing then a decrease in the amount available to its penetration into the skin. Similar result was reported by Balestrin et al. (2016), in which NE containing *Achyroclines satureoides* extract enabled higher flavonoid retention on the skin as compared to its NE-based hydrogels.

Data from **Figure 6.2** showed the distribution of isoflavones in skin layers after different skin treatments. Firstly, it was analyzed the behavior of isoflavones without nanocarriers (PG control suspension). In this case, it can be observed that DAID and GEN showed more retention in both stratum corneum and dermis. In turn, GLY retention did not could be quantified in any layer, since their amount was very low. This could be associated with the very low GLY content in the IAF, which is 3 times lower than DAID and 5 times lower than GEN. Considering the sum of the three skin layers, GEN from PG control suspension showed the highest amount retained, which could be associated with its higher amount in IAF, or with its higher LogP in comparison with the other isoflavones.

Analyzing the effect of NEs and hydrogels in the skin distribution of DAID, it can be observed that both formulations increased the penetration and retention of this isoflavone in comparison with a PG control. In the stratum corneum DAID had a higher retention amount from HNE_{IAF}. However, for the other skin layers (epidermis and dermis) the DAID amount was higher from NEs. This could be related with the release of DAID from formulations, in which is higher from NEs, allowing then, a better penetration until deepest skin layers. When the retained amount from each formulation was summed (taking all layers together) the total retained amount of DAID was 0.163 ± 0.011 μg/cm^2 from PG control, 0.837 ± 0.084 μg/cm^2 from NE_{IAF}, and 0.685 ± 0.065 μg/cm^2 from HNE_{IAF}.

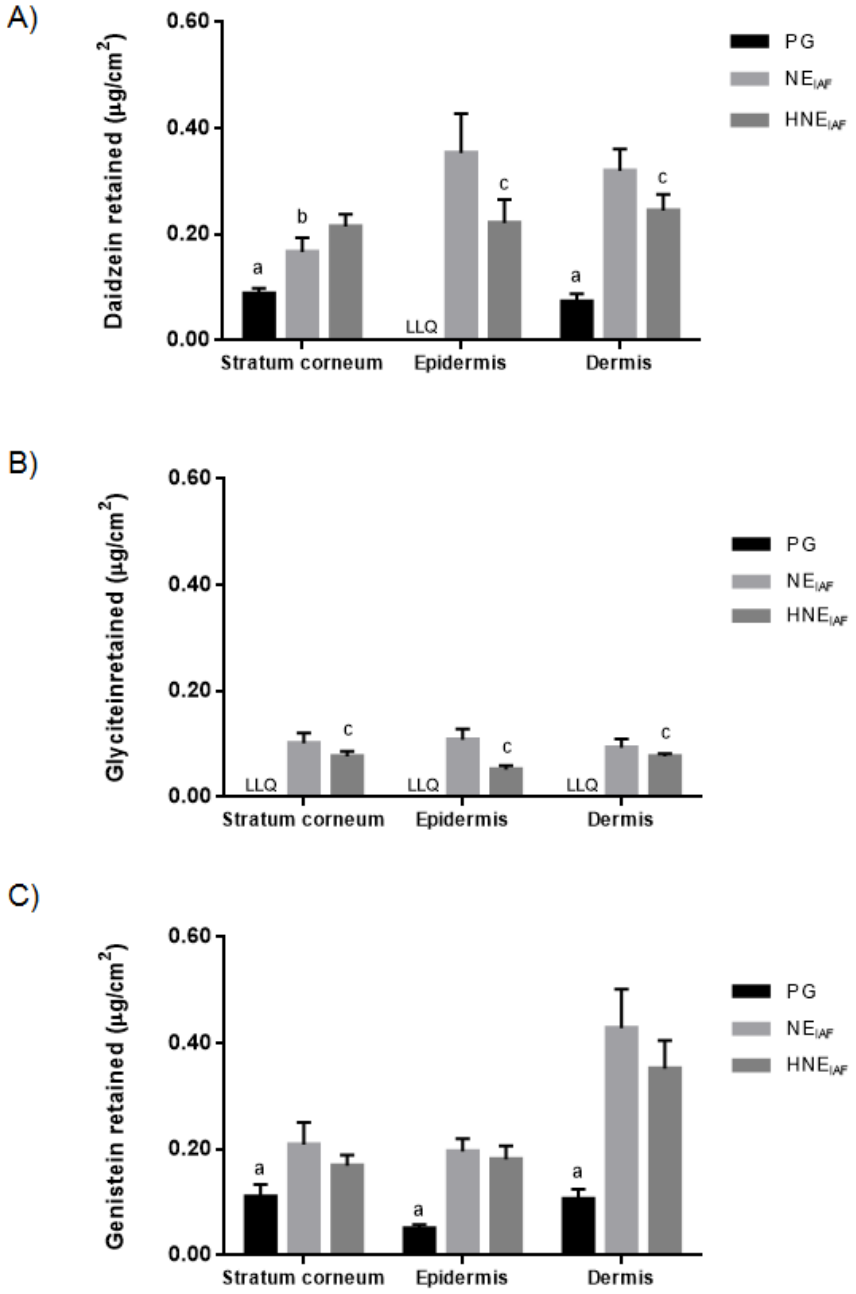

Figure 6.2. Distribution profile of (A) DAID; (B) GLY; (C) GEN into porcine ear skin layers from a propylene glycol control suspension (PG) and NE_{IAF} and HNE_{IAF} after 8 hours of permeation/retention study in Franz Diffusion Cells. The values are means ± SD of six experiments. Notes: The GLY amount was lower than limit of quantification (LLQ) in the skin layers from PG. (a) significantly lower ($p<0.05$) comparing the column of PG with other columns in the same group; (b) significantly lower ($p<0.05$) comparing NE_{IAF} with HNE_{IAF}; (c) significantly lower ($p<0.05$) comparing HNE_{IAF} with NE_{IAF}; (d) represent that the columns have statistical similarity ($p>0.05$). Abbreviations: PG, propylene glycol; NE, nanoemulsion, HNE: nanoemulsion based hydrogel, IAF: isoflavone aglycone-rich fraction.

Analyzing the effect of NEs and hydrogels in the skin distribution of GLY, it can be observed that both formulations increased the penetration and retention of this isoflavone in comparison with a PG control. It can also be notice that in all skin layers the higher retention amount had occurred from NE_{IAF}. This is probably related to the release profile of GLY from formulations, in which is higher from NEs, allowing then, a higher penetration and retention in the skin. When the retained amount from each formulation was summed (taking together all layers) the total retained amount of GLY was 0.302 ± 0.025 μg/cm^2 from NE_{IAF}, and 0.202 ± 0.016 μg/cm^2 from HNE_{IAF}.

As the other isflavones, when the effect of NEs and hydrogels was analyzed during the skin distribution of GEN, it can be observed that both formulations increased the penetration and retention of this isoflavone in comparison with a PG control. But, differently of DAID and GLY, it was observed a similar amount of GEN in each skin layer from both NE_{IAF} and HNE_{IAF}. Besides, the highest GEN amounts from these two formulations were found in dermis. Such result could be related with at least two situations (1) a positive effect that the carriers cause during the penetration of GEN until the deeper skin layer, and (2) the higher LogP of genistein in comparison of other isoflavones, promoting then a better skin penetration. When the retained amount from each formulation was summed (taking together all layers) the total retained amount of GEN was 0.207 ± 0.041 μg/cm^2 from PG control, 0.834 ± 0.057 μg/cm^2 from NE_{IAF}, and 0.702 ± 0.074 μg/cm^2 from HNE_{IAF}.

CONCLUSIONS

In this study, nanoemulsions and nanoemulsions-based hydrogels containing an isoflavone aglycones-rich fraction were prepared. The fraction was obtained from defatted soybeans. Isoflavones (genistein, daidzein and glycitein) were efficiently incorporated into formulations at a final content of 1 mg/mL. The incorporation of this fraction into nanoemulsions has the potential to bypass the problems associated with poor water solubility of these compounds. Hyaluronic acid was successfully used as thickening agent of nanoemulsions. Regardless of formulation, isoflavones were retained in stratum corneum, epidermis and dermis, demonstrating the potential of these formulations as topical delivery systems of isoflavone aglycones.

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DISCUSSÃO GERAL

O Brasil é atualmente o segundo maior produtor mundial de soja (EMBRAPA, 2016a). Essa ampla produção se deve, dentre muitos fatores, ao esforço dispensado pela Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), especialmente no desenvolvimento de novas cultivares de soja que possibilitam o incremento no teor produtivo das substâncias presentes no seu grão (EMBRAPA, 2016a). Dentre diversas cultivares que essa empresa vem desenvolvendo destaca-se a EMBRAPA BRS 262 (EMBRAPA, 2016b). Essa cultivar possui abrangência de plantio nos estados do Paraná, São Paulo e Santa Catarina, sendo relatada como uma promissora fonte de isoflavonas (ÁVILA *et al.,* 2011).

As sementes de soja são uma fonte de geração de produtos e subprodutos para diferentes áreas industriais, tais como alimentícia, farmacêutica, cosmética e química (CHEN *et al*., 2012). Dentre as aplicações, destaca-se o desenvolvimento de produtos para aplicação cutânea contendo isoflavonas na forma de aglicona, especialmente para a isoflavona mais abundante na soja, a genisteina. Tal interesse justifica-se pelos diversos relatos relacionados com as atividades biológicas das isoflavonas, tais como antioxidante (WEI *et al*., 1995; WEI *et al*., 2002; GEORGETTI *et al*., 2006; GEORGETTI *et al*., 2009; GEORGETTI *et al*., 2013), anti-melanoma (PARK *et al*., 2011a; DANCIU *et al*., 2013), anti-inflamatória (GEORGETTI *et al*., 2008; KHAN *et al*., 2012), cicatrizante (HWANG *et al*., 2001; EMMERSON *et al*., 2010; PARK *et al*., 2011b; KIM *et al*., 2015), anti-herpética (ARGENTA *et al*., 2015), anti-envelhecimento e fotoprotetora (SUDEL *et al*., 2005; HUANG *et al*., 2008; LIN *et al*., 2008; HUANG *et al*., 2010; IOVINE *et al*., 2012; IOVINE *et al*., 2014).

Considerando que essas atividades biológicas são geralmente descritas para mais de uma isoflavona presente nos grãos da soja (genisteína, daidzeína e/ou gliciteína), os principais objetivos desta tese incluíram a obtenção e caracterização de uma fração enriquecida nas principais isoflavonas da soja e a sua incorporação em produtos tópicos de base nanotecnológica para aumentar o potencial de retenção cutânea destes compostos. A primeira etapa da tese foi dedicada à revisão bibliográfica acerca da literatura científica e tecnológica (**Capítulos I e II**) envolvendo processos e produtos contendo as isoflavonas agliconas da soja. Essa etapa foi importante na tomada de decisões sobre os experimentos a serem posteriormente realizados.

O **Capítulo I** apresenta uma revisão de estudos que descrevem os principais efeitos das isoflavonas agliconas na pele, as diferentes técnicas de obtenção desses compostos e o interesse na sua incorporação em sistemas tópicos. Na busca por literatura referente aos efeitos biológicos, diversos estudos descrevem a atividade estrogênica, antioxidante e fotoprotetora desses compostos (**Tabela 1.1**). Atividades promissoras são relatadas para as formas isoladas das isoflavonas, extratos de soja, bem como produtos tecnológicos contendo estas matérias-primas. Destaca-se que alguns estudos contemplam a ação cicatrizante da genisteína. Porém, o principal enfoque comercial para produtos tópicos contendo isoflavonas é a ação antienvelhecimento como uma terapia de reposição hormonal tópica, sendo o público-alvo principalmente as mulheres na menopausa ou pós-menopausa. Estudos clínicos para esse tipo de produtos já foram conduzidos e os resultados obtidos demonstraram melhora significativa da elasticidade e tonicidade cutânea após o uso.

A partir da análise de estudos da literatura (**Tabela 1.2 e 1.3**) foi possível observar que o rendimento extrativo das isoflavonas é muito correlacionado com o método de extração empregado e com o tipo de amostra. Diferentes técnicas de extração são descritas e a escolha sempre deve ser baseada no real objetivo do processo. Por exemplo, quando objetiva-se a obtenção de isoflavonas agliconas, é necessária uma etapa complementar de hidrólise. Essa pode ser realizada por meio de catalisadores químicos ou biológicos, conforme apresentado na **Tabela 1.4**, sendo a hidrólise ácida e a hidrólise enzimática as mais descritas.

Por fim, o **Capítulo I** apresenta uma compilação de propostas de formulações tópicas contendo isoflavonas. Durante o desenvolvimento tecnológico de produtos tópicos, a incorporação das isoflavonas pode ocorrer em suas formas isoladas e sintéticas, ou ainda a partir de extratos de materiais vegetais conforme esquema ilustrativo proposto na **Figura 1.3.** Devido à reduzida hidrossolubilidade das isoflavonas bioativas, já foram propostos lipossomas contendo as formas isoladas de genisteína e daidzeína, nanoemulsões e nanocápsulas contendo genisteína isolada, microcápsulas contendo extrato de soja, nanopartículas lipídicas sólidas (NLS) contendo fração de isoflavonas, bem como complexação de genisteina, daidzeína ou extratos enriquecidos em isoflavonas a ciclodextrinas, e ainda, a complexação de dadzeína a dendrímeros (**Tabela 1.5**).

O **Capítulo II** foi redigido após a realização de buscas de literatura tecnológica sobre patentes envolvendo isoflavonas. Tal análise de dados é relevante para um diagnóstico crítico do cenário atual sobre a temática de interesse e então prever o impacto que novas tecnologias podem vir a causar no contexto industrial e comercial. Dessa forma, foi realizada uma busca na base de dados Espacenet e Espacenet Latipat contemplando uma janela de tempo entre 1994 e 2014, utilizando as seguintes palavras-chaves no título das patentes: isoflavonas, genisteína, daidzeína, gliciteína, biochanina, formononetina, e respectivas palavras em inglês. Essa base de dados foi escolhida por ser uma fonte altamente confiável, gratuita e de ampla cobertura internacional, contemplando uma busca de patentes em mais de 90 países. O intervalo de tempo aplicado foi escolhido tendo em vista que os direitos dos titulares sobre as invenções são de vinte anos. Com isso, a busca aplicando esse período possibilita visualizar um cenário bastante atual sobre a temática.

O primeiro objetivo do trabalho foi realizar a busca de patentes contemplando isoflavonas de uma forma geral. Assim, a soma dos dados encontrados com as palavras-chaves isoflavone* e isoflavona* foi de 968 patentes (**Figura 2.1a**). Numa segunda etapa, o objetivo foi dividir essas patentes em *clusters*, conforme assunto de interesse, sendo eles (1) os processos de obtenção de frações enriquecidas a partir de materiais vegetais, (2) o uso de isoflavonas em cosméticos e (3) a incorporação em sistemas micro e nanoestruturados. Esses três *clusters* foram escolhidos a fim de dar continuidade ao estudo de revisão previamente realizada durante elaboração do **Capítulo I**. Das 968 patentes encontradas, 705 foram compiladas na **Tabela 2.1** e divididas nos respectivos *clusters*. As 705 patentes dessa tabela são relacionadas na verdade a 346 tecnologias. Isso ocorre devido ao depósito de uma mesma tecnologia poder ocorrer em diferentes países, originando diferentes números de patentes. As tecnologias pertencentes a cada *cluster* foram avaliadas quanto aos principais titulares, país, e tipo de depositante (independente, empresa ou universidade). Com isso, foi possível descrever os países, empresas e universidades que mais investem em tecnologias envolvendo isoflavonas, para visualização de possíveis parcerias de pesquisa ou concorrências de mercado.

Por fim, o trabalho objetivou a busca de patentes por tipo específico de isoflavonas. Assim, a soma das buscas do Espacenet com as palavras-chaves em

inglês e do Espacenet Latipat com as palavras-chaves em português foi de 162 para genisteina, 58 para daidzeina, 28 para formononetina e 7 para biochanina (**Figura 2.1b**). Destaca-se que apenas não foram encontradas patentes com as palavras-chaves de título glycitein* no Espacenet e gliciteina* no Espacenet Latipat. Do total de 255 patentes, 122 estavam compreendidas nos *clusters* de interesse (**Tabela 2.2).** Como era de se esperar, o maior número de patentes foi encontrado para a genisteína, uma vez que essa isoflavona é a que apresenta maior número de relatos científicos que comprovam a sua atividade biológica. Essas patentes também foram divididas nos *clusters* para visualizar os tipos de depositante.

Após essa etapa de avaliação da literatura, a parte experimental da tese foi dividida em quatro eixos principais, como segue:

- Quantificação das isoflavonas agliconas da soja em diferentes matrizes
- Avaliação das impurezas presentes no extrato de soja hidrolisado pela via ácida
- Obtenção de frações enriquecidas em isoflavonas agliconas da soja por diferentes vias hidrolíticas
- Desenvolvimento e caraterização de formulações de uso tópico contendo a fração enriquecida em isoflavonas agliconas da soja

Quantificação das isoflavonas agliconas da soja em diferentes matrizes

Na pesquisa e desenvolvimento de produtos da saúde e para os ensaios analíticos e bioanalíticos dos mesmos é de extrema importância a utilização de métodos quantitativos que permitam a confiança dos resultados e a praticidade nas rotinas laboratoriais (BRASIL, 2003; BRASIL, 2012). Para análise das isoflavonas da soja, o nosso grupo de pesquisa utilizava um método de CLAE desenvolvido e validado por Yatsu e colaboradores (2014). No entanto, tal método possui tempo total de análise cromatográfica de 23 minutos, o que dificulta a rotina de trabalho quando utilizado para ensaios bioanalíticos longos, tal como estudos de permeação e liberação. Dessa forma, objetivou-se desenvolver um novo método que permitisse uma análise mais rápida e eficiente das isoflavonas da soja.

Nesse sentido, foi escolhida a cromatografia líquida de ultraeficiência (CLUE). Esse tipo de técnica permite trabalhar a uma pressão superior à da CLAE, admitindo, o uso de colunas cromatográficas de menor tamanho de partícula interna. Isso permite um processo separativo com maior eficiência de separação e menor tempo de análise e consumo de solventes (GAIKWAD *et al*., 2010).

No desenvolvimento do método de CLUE (**Capítulo III**) foi utilizado o equipamento com detecção de arranjo de diodos (DAD). A amostra utilizada para avaliação da separação das substâncias de interesse foi um extrato de soja hidroetanólico obtido por meio da extração em Soxhlet seguido de processo de hidrólise ácida, tendo como justificativa o fato da amostra ser a mais complexa do trabalho. O extrato foi analisado em diferentes condições cromatográficas. Inicialmente, foram avaliadas algumas alterações metodológicas tendo como base o método de CLAE previamente descrito por Yatsu e colaboradores (2014). A fase estacionária utilizada durante os estudos foi uma coluna C18 Shim-pack XR ODS e a fase móvel foi constituída de um gradiente entre (A) água acidificada com 0,1% de ácido trifluoracético e (B) acetonitrila. Alterações de fluxo, gradiente de fase móvel, volume de injeção e temperatura de forno foram efetuadas durante os testes.

A condição mais adequada para realizar a separação das substâncias de interesse foi estabelecida por meio, não somente de um gradiente de fase móvel, mas também de fluxo. O comprimento de onda foi de 260 nm, pois é onde ocorre a maior intensidade dos picos das isoflavonas. A análise foi realizada a 55 ºC a fim de diminuir a pressão do sistema para um valor distante do limite do equipamento. A temperatura estava dentro dos limites estabelecidos pelo fornecedor da coluna. O cromatograma obtido com a condição escolhida está apresentado na **Figura 3.1** e os parâmetros de adequabilidade do método mostraram-se apropriados para análise das isoflavonas agliconas (**Tabela 3.1**).

Após desenvolvimento do método, este foi validado conforme guias internacionais (ICH, 2005; FDA, 2013) para análise quantitativa das isoflavonas agliconas em diferentes matrizes. As matrizes utilizadas foram o extrato de soja hidrolisado pela via ácida, a fração obtida após purificação deste extrato, as nanoemulsões contendo a fração e espessadas, ou não, com carbopol, mucosa esofágica suína e as camadas de pele suína para os estudos de permeação.

Essas matrizes foram avaliadas durante as análises de especificidade. As matrizes brancas, ou seja, sem presença de isoflavonas, representadas pelas formulações placebo, mucosa e camadas de pele mostraram ausência de picos nos mesmos tempos de retenção das isoflavonas (**Figura 3.3a**). Nas amostras onde não foi possível a ausência de isoflavonas, tais como extrato e fração, foi realizada a análise de pureza dos picos das isoflavonas presentes nessas matrizes. Os picos de daidzeína, gliciteína e genisteína mostraram-se puros, independente da matriz avaliada. Para a análise de especificidade, foram ainda avaliados os produtos oriundos de degradação forçada das isoflavonas. Dessa forma, após realizar estudos em condições de estresse ácido, básico, oxidativo e térmico, os produtos de degradação formados de daidzeína e genisteína não co-eluiram com os picos dessas substâncias e nem com a gliciteína (**Figura 3.3B**). Alem disso, foram realizados estudos de espectrometria de massas para avaliação das substâncias químicas de referência das isoflavonas e também para análise dos compostos formados após degradação para propor as possíveis estruturas desses produtos.

O método de CLUE para quantificação de isoflavonas agliconas da soja apresentou regressão linear na faixa de 0,1 a 5,0 µg/mL para daidzeína, gliciteína e genisteína, em todas as matrizes avaliadas. As curvas de calibração não apresentaram desvio da linearidade após análise de ANOVA ($p>0,05$) e apresentaram coeficiente de correlação adequado para a determinação analítica e bionalítica das isoflavonas. Para a determinação do efeito de matriz, os coeficientes angulares (*slopes*) de cada curva obtida após contaminação de isoflavonas nas soluções de matrizes foram comparados com os slopes das curvas obtidas pelas isoflavonas sem a presença das matrizes. Os resultados obtidos foram analisados conforme proposto por Niessen e colaboradores (2006), e baixo efeito de matriz foi demonstrado durante todas as análises.

O método foi considerado preciso, pois os desvios padrões relativos (DPR) das análises relacionadas à precisão intradia ficaram abaixo de 3,96% para as análises analíticas, 8,40% para as bionalíticas, e 6,21% para as análises biotecnológicas, e os DPR relacionados à precisão intradia ficaram abaixo de 3,96% para as análises analíticas, 8,40% para as bioanalíticas, e 6,21% para as análise biotecnológica. O método também foi considerado exato, uma vez que os resultados

de exatidão encontraram-se entre 90.37% e 104.81% nas análises analíticas, bioanalíticas, e biotecnológicas. Para as amostras bioanáliticas, ainda foi realizada a análise de recuperação, sendo que essa foi efetuada pela extração das isoflavonas presentes na matriz contaminada previamente. Os resultados mostraram recuperação entre 93,24% e 102,0%. A robustez do sistema cromatográfico foi determinada utilizando-se o delineamento experimental de Plackett-Burman, por meio do guia descrito por Heyden e colaboradores (2001). Modificações de fatores tais como proporção de solvente orgânico, fluxo de fase móvel, temperatura de forno e concentração de agente acidificante foram realizadas durante o experimento. De acordo com os resultados obtidos, as modificações nos fatores estudados não interferiram na determinação de isoflavonas agliconas nas diferentes matrizes avaliadas.

O método validado foi utilizado para a quantificação das isoflavonas nas diferentes aplicações propostas. Dessa forma, foi encontrado um teor total de isoflavonas agliconas aproximado de 203 mg a cada 100 g de soja desengordurada, doseado a partir de extratos hidrolisados. O extrato hidrolisado foi purificado conforme Nemitz e colaboradores (2015), e a fração obtida após processos de purificação apresentou um teor total de isoflavonas agliconas aproximado de 0,89 mg/mg. Nanoemulsões foram preparadas por emulsificação espontânea, sendo incorporado durante o processo a fração a uma concentração final de 0,1%. O teor de isoflavonas agliconas encontrado nas nanoemulsões foi de aproximadamente 0,86 mg/mL. As formulações foram espessadas com 0,5% de carbômero 940 conforme descrito por Vargas e colaboradores (2012), e o teor total de isoflavonas agliconas encontrado nos hidrogéis foi de aproximadamente 0,86 mg/g.

Por fim, as nanoemulsões contendo a fração foram submetidas a estudos de permeação em pele de orelha suína e mucosa de esôfago suíno a fim de avaliar a real aplicação do método bionalítico e verificar a repetibilidade do processo. Nenhuma isoflavona foi detectada no fluído receptor após o estudo de permeação cutânea. Os resultados do teor retido na pele foram representados pelo somatório de genisteína e daidzeína, sendo que os valores foram de aproximadamente 0,60 $\mu g/cm^2$ para o estrato córneo e para as demais camadas da pele (epiderme+derme). Apenas a isoflavona gliciteína não pôde ser doseada na pele após os experimentos

de permeação cutânea, pois a quantidade retida nas matrizes foi inferior ao limite de quantificação (0,1 µg/mL). Já para as análises de permeação em mucosa esofágica, todas as isoflavonas puderam ser quantificadas, e os resultados do teor total de isoflavonas agliconas encontrado foi de aproximadamente 6,42 µg/cm^2 e 11,69 µg/cm^2 para a mucosa e fluído receptor, respectivamente.

Comparando o método aqui descrito com estudos da literatura que descrevem o uso de CLUE para análise de isoflavonas, é importante destacar que, até o momento, o nosso método é o único desenvolvido e validado para quantificação simultânea de isoflavonas agliconas da soja em amostras bioanalíticas de pele e mucosa (KLEDJUS *et al*., 2005; KLEJDUS *et al*., 2007; FIECHTER *et al*., 2010; KISS *et al*., 2010; MANCHÓN *et al*., 2010; TORO-FUNES *et al*., 2012). Além disso, o método foi desenvolvido com o objetivo de poder ser utilizado para verificação de impurezas furânicas que possam estar presentes nos produtos derivados de soja e esse tipo de enfoque não é dado em nenhum outro trabalho da literatura, o que demonstra o impacto científico do nosso estudo.

O isolamento e identificação das impurezas furânicas hidroximetilfurfural (HMF) e etoximetilfurfural (EMF) em extratos de soja foram previamente descritos em estudos realizados pelo nosso grupo de pesquisa (NEMITZ *et al*., 2015). Essas substâncias são formadas a partir da degradação ácida dos açúcares presentes nos extratos de soja quando for realizado o protocolo de hidrólise ácida em meio hidroetanólico com elevadas temperaturas.

Avaliação das impurezas presentes no extrato de soja hidrolisado pela via ácida

Conforme comentado, o método de CLUE (**Capítulo III**) permitiu não apenas a separação dos picos cromatográficos referentes às isoflavonas agliconas daidzeína, gliciteína e genisteína, mas também a separação das impurezas HMF e EMF presentes no extrato de soja hidrolisado pela via ácida. Durante a realização dos estudos, foi observado que os picos cromatográficos referentes ao HMF e EMF apresentavam áreas muito superiores ao das isoflavonas agliconas. Dessa forma, foi percebida a necessidade de quantificação dessas substâncias, dando início à proposta de trabalho referente ao **Capítulo IV** desta tese.

Para a fabricação de produtos destinados à saúde é recomendado identificar, quantificar e qualificar as impurezas presentes nos produtos ou insumos farmacêuticos (ICH 2006a, ICH 2006b). A qualificação é o processo de aquisição e avaliação de dados que estabelece a segurança biológica de impurezas. Para realizar a qualificação de um produto de degradação, é necessário avaliar dados da literatura, e muitas vezes, conduzir estudos de segurança e eficácia. Dessa forma, estudos de toxicidade e genotoxicidade devem ser realizados sempre que houver presença de possíveis contaminantes ou impurezas nos insumos e medicamentos (MELO *et al*., 2013).

Uma vez que o extrato hidrolisado de soja é enriquecido em isoflavonas agliconas, e que essas são substâncias com interesse terapêutico, esse derivado vegetal é muito utilizado para elaboração de produtos alimentares, farmacêuticos e cosméticos. Porém, quando o extrato é obtido por meio de hidrólise ácida, escassa literatura é encontrada sobre a presença de compostos furânicos formados após degradação dos açúcares da soja durante o processo hidrolítico e a consequência desta situação. O primeiro relato abordando tal temática foi efetuado pelo nosso grupo de pesquisa, em estudos precedentes recentemente publicados (NEMITZ *et al.,* 2015). No entanto, apesar de identificados, o HMF e EMF presentes nos extratos hidrolisados de soja não haviam sido quantificados simultaneamente por um método validado, e nem tampouco havia sido avaliado se as concentrações encontravam-se em níveis tóxicos.

Neste contexto, os estudos do **Capítulo IV** foram iniciados referentes à validação do método de CLUE para análise quantitativa das impurezas furânicas HMF e EMF presentes no extrato hidrolisado de soja obtido pela via ácida. Além disso, foi realizada uma busca na literatura a respeito da toxicidade desses compostos. Apenas foram encontrados estudos referentes à toxicidade e genotoxicidade do HMF, porém para o EMF não foi encontrado relato algum sobre tais aspectos. Assim, procedeu-se à avaliação experimental *in vitro* da mutagenicidade e genotoxicidade desse composto. Os teores de HMF e EMF encontrados nos extratos hidrolisados de três diferentes cultivares de soja foram comparados com os valores de genotoxicidade dos mesmos para avaliação crítica do uso desses extratos na elaboração de produtos para saúde.

Para realizar a validação do método de análise de compostos furânicos presentes no extrato hidrolisado de soja, primeiramente, foi verificada a especificidade da análise de HMF e EMF nessa matriz. Dessa forma, a análise da pureza dos picos cromatográficos mostrou a não coeluição de outras substâncias no mesmo tempo de retenção que esses compostos. O método apresentou regressão linear na faixa de concentração de 10 a 100 µg/mL para HMF e EMF. As curvas de calibração não apresentaram desvio da linearidade por análise ANOVA ($p>0,05$) e apresentaram coeficiente de correlação adequado para determinação desses compostos no extrato. Um baixo efeito de matriz foi observado após comparação dos *slopes* das curvas de HMF e EMF na presença ou ausência de matriz. O método foi considerado preciso, pois os DPR das análises relacionadas à precisão intradia ficaram abaixo de 2,98% e as relacionadas à precisão intradia ficaram abaixo de 4,90%. O método também foi considerado exato, uma vez que os resultados encontraram-se entre 96,56% e 102,37%. A robustez do sistema cromatográfico foi confirmada utilizando o delineamento experimental de Plackett-Burman.

O método validado foi utilizado para a quantificação do HMF e EMF em extratos referentes a três diferentes cultivares de soja (EMBRAPA BRS 262, EMBRAPA BRS Taura RR, EMBRAPA BRS 154). A avaliação de três diferentes amostras foi efetuada para comprovar que, independentemente da cultivar de soja, pode ocorrer uma formação de HMF e EMF após hidrólise ácida em meio hidroetanólico. Os extratos foram obtidos por meio da extração em Soxhlet (5,0 g de soja desengordurada em 200 mL de etanol 80%) seguido de hidrólise ácida. Os teores individuais de cada composto furânico foram: 0,18 mg/mL de HMF e 0,33 mg/mL de EMF para o extrato da EMBRAPA BRS 262; 0,21 mg/mL de HMF e 0,31 mg/mL de EMF para extrato de EMBRAPA BRS Taura RR; e 0,16 mg/mL de HMF e 0,22 mg/mL de EMF no extrato de EMBRAPA BRS 154, representando valores de aproximadamente 10 vezes superiores aos teores das isoflavonas agliconas presentes nos mesmos extratos.

A presença desses compostos furânicos pode causar, ou não, efeitos benéficos quando a intenção é o uso para produção de medicamentos. Chen e colaboradores (2014) demonstraram que a presença de baixas concentrações desses compostos pode aumentar a atividade antioxidante dos extratos de plantas.

Porém, quando esses compostos encontram-se em altas concentrações nos produtos, podem apresentar efeitos nocivos ao organismo devido ao potencial de genotoxicidade relatado para o HMF (SEVERIN *et al.,* 2010).

Para conferir se a quantidade de HMF presente nos extratos hidrolisados estava próxima dos níveis genotóxicos desse composto, uma revisão da literatura foi realizada. Muitos estudos foram encontrados abrangendo a avaliação da toxicidade deste derivado furânico. Isso se deve ao fato de este composto ser um subproduto da reação de Maillard em amostras que contenham açúcares e passem por processos envolvendo calor. Estando presente, portanto, em diversos produtos alimentares. Em elevadas concentrações o HMF é considerado citotóxico para diversas células, irritante para os olhos, pele, trato respiratório superior e membranas de mucosas (SCCNFP, 2004; MORALES, 2009). Além de diversos estudos científicos, o Programa Nacional de Toxicologia (NTP) dos Estados Unidos relata que o HMF é considerado carcinogênico para camundongos B6C3F1 após dois anos de tratamento oral, causando adenomas hepatocelulares e carcinomas em doses de 175 mg/kg (NTP, 1990; NTP 2010).

As agências internacionais e nacionais de alimentos e medicamentos estipulam os valores limítrofes desta substância nesses produtos. Para administração oral do HMF, uma dose de 132 mg/pessoa é tolerada com ampla margem de segurança conforme relatado por Zaitzev e colaboradores (1975), enquanto que para administração intravenosa o limite é de 10 mg/L em soluções parenterais que contenham hexose (ULBRICHT *et al*. 1984). Além disso, caso o HMF seja administrado topicamente, ou venha a entrar em contato com a pele por algum motivo, a concentração deve ser baixa, uma vez que o HMF quando aplicado topicamente em camundongos causou papilomas cutâneos após administração de doses entre 10 – 25 mM (MORALES, 2009).

Quanto aos efeitos genotóxicos, muitos estudos são descritos na literatura para verificação dos danos que o HMF pode causar. Nish e colaboradores (1989) avaliaram a atividade clastogênica de células V79 após tratamento com HMF e relataram presença de aberrações cromossômicas após exposição das células a esse composto na concentração de 2 mg/mL e redução de atividade mitótica na faixa de 1 a 2 mg/mL. Glatt e colaboradores (2005) relataram indução da

degradação de cromátides irmãs em células V79 expressando CYP2E1 e SULT1A1 após exposição ao HMF nas concentrações de 0,025 a 4,8 mg/mL. Durling e colaboradores (2009) realizaram ensaio cometa em diferentes culturas celulares e observaram dano ao DNA após exposição ao HMF na concentração de 12,6 mg/mL em Caco-2 e HEK293 e de 3,15 mg/mL para V79. Severin e colaboradores (2010) realizaram o ensaio cometa em células HepG2 e observaram dano ao DNA com efeito concentração-resposta após exposição ao HMF na faixa de concentração de 0,67 a 4,6 mg/mL. Portanto, como pode ser observado, diversos estudos apontam que em elevadas concentrações o HMF pode ser potencialmente genotóxico para diferentes culturas celulares.

A partir desses dados da literatura, foi verificado que as quantidades de HMF presentes nos extratos de soja, após processo de hidrólise ácida, encontram-se numa faixa que pode ser considerada genotóxica. Porém, diferentemente do HMF, nenhum dado sobre segurança biológica foi encontrado para o EMF. Dessa forma, durante a elaboração do **Capítulo IV** foram realizados estudos *in vitro* de mutagenicidade e genotoxicidade por meio de teste de AMES e ensaio cometa com cultura de células hepáticas humanas (HepG2), respectivamente. A escolha de efetuar o ensaio cometa com cultura celular do tipo HepG2 deu-se pela capacidade de metabolização dessas células, e também pelo fato de existirem estudos semelhantes com o HMF nessas células, o que permitiria a comparação da genotoxicidade desses compostos furânicos.

O teste de AMES, também denominado de teste de *Salmonella*/ microssoma, é um ensaio bacteriano de curta duração que busca identificar substâncias que causem danos genéticos que podem evoluir até mutações. Esse ensaio baseia-se na indução de mutações reversas em linhagens de *Salmonella typhimurium* auxotróficas para o aminoácido histidina que provocam prototrofia nas mesmas. Cada linhagem celular utilizada no experimento é mutada de forma diferente no operon da histidina, o que permite que tenham especificidade na detecção de determinado tipo de mutágeno (MORTELMANS & ZEIGER, 2000). As linhagens recomendadas conforme o guia internacional da OECD (1997) são as linhagens de *S. typhimurium* TA 97a, TA98, TA100, TA1535 e TA 102.

As linhagens TA97a e TA98 detectam mutágenos que causam erro de leitura de DNA (*frameshifts)* apresentando como ponto preferencial oito resíduos repetitivos com sequências de guanina (C) e citosina (C), tais como –C-C-C-C-C-C-; +1 citosina, e –C-G-C-G-C-G-C-G-. As linhagens TA100 e TA1535 detectam mutágenos que causam substituição dos pares de bases do DNA nos sítios de ponto preferencial GC. Já a linhagem TA102 detecta mutágenos que causam trocas dos pares de bases (transição/transcrição) e pontes do DNA (*cross-linking*) nos sítios preferenciais de adenina (A) e timina (T) (MORTELMANS & ZEIGER, 2000). De acordo com os guias internacionais, os protocolos de teste de AMES devem ser realizados com e sem indução microssomal externa devido ao fato dessas células procarióticas não possuírem sistema de metabolização mitocondrial. Assim, é recomendado que durante os experimentos seja, ou não, adicionado um sistema enzimático extraído de células de fígado de ratos, chamado de “S9 mix”.

Basicamente, a execução do ensaio é feita por meio de contagem do crescimento bacteriano em placas contendo meio de cultura específico. As cepas das linhagens utilizadas durante o teste são incapazes de crescer em meio de cultura mínimo, sem histidina, a menos que ocorram mutações que restaurem a síntese deste aminoácido. A frequência de mutação reversa é facilmente medida pela contagem do número de colônias que crescem em meio mínimo após a exposição de uma população de células a um agente mutagênico (MORTELMANS & ZEIGER, 2000).

No presente trabalho, o ensaio de AMES foi realizado pelo método de incorporação seguido de pré-incubação, conforme descrito por Maron e Ames (1983) na presença e ausência de ativação metabólica (S9 mix), utilizando as linhagens recomendadas pelo guia da OEDC (1997). As concentrações do EMF utilizadas durante o ensaio foram determinadas por teste preliminar de toxicidade em cepa TA100, sendo determinada a faixa de 50 a 5000 µg/poço. Os resultados do ensaio de mutagenicidade do EMF estão apresentados na **Tabela 4.3.** Conforme pode ser observado, não ocorreu mutação nas cepas avaliadas, uma vez que o índice de mutagenicidade (MI) foi inferior a 2,0 para as cepas TA100, TA97a, TA102 e TA98, e inferior a 3,0 para a cepa TA1535. No entanto, quando comparou-se o número de colônias revertentes do controle negativo com as colônias revertentes após

exposição ao EMF, foi verificada certa tendência de mutagenicidade nas concentrações superiores testadas. Os ensaios realizados com indução metabólica não apresentaram significância em comparação à ausência de adição do S9 mix, demonstrando que a mutagenicidade do EMF não metabolizado não apresenta diferença do EMF após metabolização.

O ensaio cometa é um teste realizado para avaliar a genotoxicidade de substâncias químicas e pode ser executado por meio de modelos *in vitro* e *in vivo*. Neste estudo, optou-se pela realização de um protocolo *in vitro* conforme descrito por Tice e et al. (2000), empregando tratamento alcalino. Esse ensaio é rápido, de baixo custo e sensível para mensurar e analisar lesões reversíveis ao DNA de células expostas a agentes genotóxicos. Os danos mais facilmente mensurados são quebras simples, diretas ou induzidas por lesões nas bases do DNA, tais como metilação, oxidação e formação de adutos, cujo procedimento transforma essas anomalias em lesões álcali-lábeis (COLLINS, 2004). Essa técnica também vem sendo utilizada com adição de enzimas específicas para detectar o mecanismo de ação das quebras. Dentre os marcadores utilizados encontra-se a proteína formamido pirimidina glicosilase (FPG), que detecta quebras do tipo oxidativas nas bases purínicas do DNA (COLLINS *et al*., 1996).

Basicamente, o ensaio cometa consiste na passagem de uma corrente elétrica em lâminas contendo as células lisadas da amostra após tratamento com a substância a ser estudada (TICE *et al*., 2000; COLLINS, 2004). A corrente elétrica faz com que ocorra uma migração dos segmentos de DNA livres, resultantes de quebras, para fora do núcleo. Após a eletroforese, as células que apresentam núcleo redondo são identificadas como normais, sem dano detectável no DNA. Já as células lesadas são identificadas visualmente por uma espécie de cauda, similar a um cometa, formada pelos fragmentos de DNA que foram “arrastados” pela corrente elétrica da eletroforese. O tamanho da cauda é proporcional à dimensão do dano que foi causado no material genético, e a visualização dos núcleos é feita após as lâminas serem coradas e analisadas ao microscópio (TICE *et al*., 2000).

No presente trabalho, para poder efetuar a análise de genotoxicidade, primeiramente foram avaliadas quais as concentrações do EMF poderiam ser utilizadas no ensaio cometa. Tice e colaboradores (2000) preconizam que para

executar o ensaio deve haver viabilidade celular a cima de 70% durante o ensaio. Para isso, foram testadas várias concentrações do EMF e tempos de exposição por meio de ensaio de citotoxicidade colorimétrico empregando sal de tetrazólio (MTT), e os resultados estão apresentados na **Figura 4.1**. Para a realização do ensaio, que permitisse uma viabilidade adequada das células HepG2, foram escolhidas as seguintes condições: concentração do EMF entre 0,1 e 1,3 mg/mL e tempo de exposição de 3 h. Os resultados mostraram que o EMF foi capaz de induzir danos ao DNA em todas as doses avaliadas, com um índice e frequência de dano superior ao do controle negativo, conforme apresentado na **Figura 4.2**. Adicionalmente, foi realizado um ensaio cometa com adição do marcador enzimático FPG. Os resultados apresentados na **Figura 4.3** mostram um aumento significativo do dano ao DNA após a adição desse marcador, o que sugere que um dos mecanismos de ação do dano que o EMF causa nas células possa possivelmente estar correlacionado com a oxidação das bases purínicas do DNA.

É importante destacar que a faixa de concentração de EMF presente nos extratos hidrolisados de soja foi entre 0,22 a 0,33 mg/mL. De acordo com os dados experimentais apresentados no **Capítulo IV**, esses valores não são considerados indutores de mutagenicidade em cepas de *Salmonella*, porém são capazes de induzir alta genotoxicidade em células do tipo HepG2. Esses dados demonstram que a presença do EMF nos extratos de soja hidrolisados pela via ácida configura um risco de segurança dos produtos desenvolvidos a partir dessa matéria-prima.

Além disso, foi possível realizar uma comparação entre o potencial mutagênico e genotóxico do EMF e do HMF. A partir dos resultados obtidos com o teste de AMES foi observado que o EMF não mostrou-se altamente mutagênico. Resultados semelhantes foram encontrados por Severin e colaboradores (2010) para o HMF utilizando o mesmo tipo de ensaio e as mesmas cepas de *Salmonella*. No entanto, quando avaliaou-se os danos causados em células HepG2, o EMF foi considerado mais citotóxico e genotóxico que o HMF. Os autores relataram que o HMF apresentou IC_{50} de 38 mM (4,79 mg/mL) após 20 h de tratamento, enquanto que no nosso trabalho demonstramos que o EMF foi citotóxico numa concentração bastante próxima (5 mg/mL) após apenas 3 h de tratamento, e ainda altamente citotóxico a partir de 1,25 mg/mL após 6 ou 24 h de tratamento. A genotoxicidade do

HMF nessas células foi relatada por Severin e colaboradores (2010) como sendo a partir de 25 mM (3,15 mg/mL), enquanto que nossos estudos mostraram que o EMF foi genotóxico a partir de 0,1 mg/mL, demonstrando que a presença do grupamento etila na molécula aumenta consideravelmente o potencial de dano ao DNA nessas células testadas.

Destaca-se que o foco da presente tese é o uso de derivados da soja para administração cutânea. No entanto, o trabalho do **Capítulo IV** foi realizado em células hepáticas (HepG2). Esse protocolo foi escolhido a fim de poder comparar os resultados obtidos com o EMF com dados da literatura obtidos para o HMF, que já estavam elucidados para esse tipo celular (SEVERIN *et al*., 2010). A partir disso, para poder aproximar os resultados do **Capítulo IV** para o real objetivo desta tese, ampliamos nossos resultados para uma possibilidade de comparação entre o HMF e EMF a fim de sugerir um potencial risco de administração cutânea do EMF. Dessa forma, ponderando que o EMF foi consideravelmente mais citotóxico que o HMF quando avaliado frente a uma mesma cultura celular, e levando-se em consideração que o HMF possui potencial de causar irritação cutânea (MORALES, 2009), pode-se sugerir que muito provavelmente o EMF também pode possuir potencial irritante para a pele. Porém, para confirmar essa hipótese, estudos abordando esta temática devem ser realizados.

Obtenção de frações enriquecidas em isoflavonas agliconas da soja por diferentes vias hidrolíticas

Durante os estudos precedentes de mestrado foi descrita a obtenção de uma fração contendo as três principais isoflavonas agliconas da soja (NEMITZ *et al*., 2015). O protocolo desenvolvido para obter essa fração foi baseado na extração das sementes desengorduradas de soja com posterior hidrólise ácida e processos de purificação para retirada dos compostos furânicos e demais substâncias indesejáveis. Por fim, a FIA obtida por este método apresentou alta pureza em isoflavonas agliconas.

Destaca-se, no entanto, que embora o nosso grupo tenha desenvolvido um método de obtenção de FIA altamente vantajoso em termos de pureza de isoflavonas, foi decidido não dar continuidade aos demais estudos com tal fração.

Isso ocorreu devido ao fato da FIA ser obtida por meio de um método de obtenção que necessita três diferentes etapas de purificação para retirada das impurezas furânicas contidas no extrato de soja após a hidrólise ácida, sendo elas: partição líquido-líquido com acetato de etila, seguida de extração em fase sólida por coluna de sílica gel com diferentes solventes orgânicos e precipitação das isoflavonas com clorofórmio. O processo foi considerado desprendioso, com alto consumo de solventes orgânicos, tornando-o além de lento, caro e desfavorável na rotina laboratorial para desenvolvimento e avaliação de novos produtos contendo a fração, bem como para uma futura rotina industrial de sua produção em larga escala.

Dessa forma, levando-se em consideração tal limitação, deu-se início aos estudos do **Capítulo V**. O objetivo desta parte do trabalho foi realizar a hidrólise do extrato de soja por vias alternativas a da hidrólise ácida a fim de evitar a formação dos produtos de degradação dos açúcares (HMF e EMF) e facilitar os processos de purificação até obtenção de uma fração enriquecida em isoflavonas agliconas. A escolha dos métodos alternativos foi baseada na literatura, que atesta que os processos de hidrólise enzimática com β-glicosidases puras, ou ainda, os processos de fermentação com microrganismos que expressam atividade de β-glicosidases, são considerados métodos específicos e eficientes para o processamento hidrolítico de polifenóis conjugados a açúcares até as respectivas formas livres (VISKUPIČOVÁ *et al*., 2009).

O **Capítulo V** descreve a obtenção de um extrato de soja por meio de extrações sequenciais utilizando o seguinte protocolo: extração de 10 g de soja desengordurada em 200 mL de etanol 80% em Soxhlet durante 8 h. O etanol presente no extrato foi evaporado sob pressão reduzida, resultando em um extrato aquoso concentrado. Cada 20,0 mL do extrato aquoso correspondiam a 100,0 mL de extrato hidroetanólico, que consequentemente, correspondiam ao extraído a partir de 5,0 g de soja desengordurada. A maioria dos protocolos hidrolíticos foi realizado a partir de 4,0 mL do extrato aquoso, que corresponde ao extraído de 1,0 g de soja desengordurada. Durante os estudos, foram efetuados protocolos de hidrólises ácida, enzimática e fermentativa. É importante ressaltar que o extrato de soja sem passar por nenhum processo hidrolítico foi considerado o controle negativo e o extrato após processo de hidrólise ácida foi considerado o controle positivo. Todos

os extratos tiveram seus volumes ajustados até 50 mL ao final dos processos hidrolíticos para poder efetuar as comparações das substâncias presentes em cada amostra.

Estudos da literatura indicam que durante a realização de procedimentos hidrolíticos, diversos fatores podem influenciar o rendimento final do processo, dentre eles destacam-se tipo de catalisador, tempo de reação, pH e temperatura (SUN & CHENG, 2002; SALWANE *et al.,* 2013). Além disso, é relatado que os níveis escolhidos para estes fatores, bem como o tipo de amostra a ser hidrolisada, causam impactos consideráveis no processo. Sendo assim, alguns pesquisadores relataram a necessidade de otimização desses fatores durante processos hidrolítcos compreendendo produtos de soja (CHIANG *et al*., 2001; TAYLOR *et al*., 2005; LEE & CHOUNG, 2011; CSUPOR *et al*., 2015). Porém, os objetivos dos estudos geralmente são voltados para fabricação de alimentos, ou ainda, para melhorar o desempenho de procedimentos analíticos nas avaliações de controle de qualidade das sementes de soja ou seus derivados (ROSTAGNO *et al*., 2009). Escassa literatura foi encontrada sobre otimizações de processos visando à fabricação de produtos farmacêuticos e cosméticos.

Dentre as diversas ferramentas existentes para a otimização de processos, as metodologias de superfície de resposta (MSR) ganham destaque devido à sua capacidade de explorar a relação entre diferentes variáveis envolvidas no processo catalítico. Essas metodologias são baseadas na utilização de planejamentos experimentais por meio de princípios estatísticos (MONTGOMERY, 2001). Diferentes tipos de MSR são descritos, sendo o desenho experimental do tipo Box-Behnken (BBD) o mais amplamente utilizado para otimização de extrações a partir de materiais vegetais (SAID & AMIN, 2015). Este tipo de experimento é realizado abrangendo três níveis de cada fator, sendo sempre avaliados níveis alto, médio e baixo, com diferenças equidistantes entre eles (MONTGOMERY, 2001). Segundo recomendações de Bezerra e colaboradores (2008), é importante a escolha adequada dos fatores que serão avaliados durante os procedimentos de BBD, bem como dos seus níveis. Nesse sentido, estudos de triagem são comumente realizados para visualizar quais fatores são relevantes para o processo a ser avaliado. Os estudos de triagem podem ser realizados por meio de experimentos

individuais, avaliando-se uma variável por vez, ou também por meio de ferramentas estatísticas, tal como desenhos experimentais do tipo Plackett-Burman.

O processo de hidrólise enzimática efetuada no presente trabalho baseou-se no método descrito por Liggins e colaboradores (1998), em que 100 unidades de β-glicosidase são utilizadas para realizar a hidrólise de uma amostra correspondente ao extraído de 2,5 g de soja em pH de 6,8, temperatura de 37 ºC durante 12 horas. No entanto, após efetuar este processo, foi averiguada a necessidade de modificações do processo enzimático, uma vez que o teor encontrado de isoflavonas agliconas no extrato foi baixo. A partir disso, escolheu-se realizar uma triagem mais apronfundada das varíaveis que poderiam estar interferindo significativamente no processo hidrolítico de transformação das isoflavonas conjugadas até suas formas livres de agliconas. As variáveis de entrada escolhidas foram concentração de enzima, pH, tempo e temperatura, e a variável de saída foi a concentração de isoflavonas agliconas (expressa como somatório de daidzeína, gliciteína e genisteína). A ferramenta estatística utilizada para essa avaliação foi o desenho experimental do tipo Plackett-Burman (**Tabela 5.1**). A partir dos resultados apresentados na **Figura 5.1**, foi possível observar que a concentração de enzima, pH e tempo estavam interferindo significativamente durante o processo, e apenas a temperatura não causou efeito significativo.

Os efeitos significativos de tempo de reação enzimática durante a obtenção de isoflavonas agliconas em amostras de soja também foram verificados por Tipkanon e colaboradores (2010) e Xie e colaboradores (2003). Esses últimos autores relatam, ainda, que a concentração da enzima influencia significativamente o processo hidrolítico. Além disso, Ismail e Haydes (2005) enfatizaram que a concentração da enzima depende das formas conjugadas das isoflavonas presentes na amostra, pois a β-glicosidase em baixas concentrações é efetiva para hidrólise das formas glicosiladas, mas não para clivagem das demais formas conjugadas, tais como malonil-glicosídeo e acetil-glicosídeo.

Devido à escassez de literatura abordando a biotransformação das isoflavonas por meio de fermentação com *Saccharomyces cerevisiae*, o processo utilizado no presente estudo baseou-se no método enzimático, sendo que adaptações foram realizadas por meio de modificações avaliando-se uma variável

por vez. A quantidade de fermento comercial adicionado para realizar o processo hidrolítico foi avaliada em uma faixa de 100 a 2000 mg, mantendo-se a reação à 37 ºC, com pH de 6,8 durante 12, 24 ou 48 horas. No entanto, após realizar a comparação dos teores de isoflavonas agliconas nos extratos obtidos com o teor de isoflavonas agliconas presentes no controle positivo foi averiguada a necessidade de modificações do processo fermentativo. De forma semelhante ao processo enzimático, escolheu-se realizar uma triagem das varíaveis que poderiam estar interferindo significativamente no processo hidrolítico por meio de desenho experimental do tipo Plackett-Burman. As variáveis escolhidas foram as mesmas da triagem enzimática, porém com diferentes níveis em alguns fatores e trocando-se o tipo de biocatalisador (**Tabela 5.1**). A partir dos resultados apresentados na **Figura 5.1**, foi possível observar que todas as variáveis pesquisadas impactaram significativamente no processo fermentativo de *S. cerevisae* para obtenção das isoflavonas agliconas a partir dos extratos de soja.

Geralmente, todos esses fatores são considerados impactantes nos processos fermentativos para obtenção de isoflavonas agliconas a partir de amostras de soja. No entanto, as condições descritas na literatura envolvem fermentação em meio sólido com fungos do gênero *Aspergillus* (HANDA *et al*., 2014; SILVA *et al*., 2011; HANDA *et al*., 2016). Esse tipo de processo é realizado pela adição das cepas do microrganismo diretamente na amostra sólida de soja, com posterior extração das isoflavonas agliconas utilizando um solvente de polaridade adequada. Diferentemente desse tipo de método, o presente estudo realizou a fermentação como etapa posterior à etapa de extração, e utilizou fermento comercial contendo *S. cerevisiae.* Procedimentos com esse tipo de microrganismo são descritos para obtenção de bioetanol a partir da soja (TUDOR *et al*., 2013). No entanto, Schmidt e colaboradores (2011) relataram que *S. cerevisae* possui β-glicosidase com capacidade para realizar ruptura de moléculas conjugadas de flavonoides com açúcares na posição beta. Por causa desse relato, a fermentação por *S. cerevisiae* foi aqui avaliada e otimizada.

Após efetuar as triagens e conhecer os fatores que impactam durante os processos mediados pelos biotalizadores, foram então realizadas as otimizações propriamente ditas. Os procedimentos hidrolíticos foram otimizados por meio de

desenho do tipo Box-Behnken. Para efetuar a otimização da hidrólise enzimática os fatores e níves avaliados estão apresentados na **Tabela 5.2**, e para otimização do processo fermentativo na **Tabela 5.3.** A varíavel de resposta foi o conteúdo de isoflavonas totais. O protocolo enzimático foi efetuado com 4 mL de extrato aquoso de soja (correspondente ao extraído de 1,0 g de soja desengordurada) a 37 ºC. Após análise dos resultados da MSR, os fatores otimizados para maximização do rendimento de isoflavonas agliconas foram: 838 unidades de enzima, durante 4,5 h e pH 6,0. O protocolo de fermentação foi efetuado também com 4 mL de extrato aquoso de soja (correspondente ao extraído de 1,0 g de soja desengordurada) e após análise dos resultados da MSR, os fatores otimizados para maximização do rendimento de isoflavonas agliconas foram: 1500 mg de fermento comercial, pH 7,6 durante 24 h a 33 ºC.

Os extratos controles, bem como os extratos otimizados, foram analisados para quantificação dos teores de isoflavonas agliconas. Conforme apresentado na **Tabela 5.5**, todos os extratos hidrolisados apresentaram diferenças estatísticas significativas em relação aos conteúdos de isoflavonas agliconas quando comparado com o do controle negativo. No entanto, comparando-se os extratos hidrolisados apenas por biocatalisadores com o controle positivo, somente o extrato obtido por hidrólise enzimática apresentou semelhança estatística para o teor de isoflavonas agliconas. Isso demonstra que apesar de o processo de fermentação ter causado aumento significativo dessas substâncias, o método não foi capaz de transformar todas as formas conjugadas das isoflavonas em suas formas livres. Devido a esse fato, optou-se por realizar um processo adicional de hidrólise ácida no extrato previamente fermentado. Essa estratégia fez com que o novo extrato apresentasse semelhança estatística com o controle positivo para o teor de isoflavonas agliconas.

Além disso, foi realizado o doseamento de açucares solúveis totais e verificação da presença de saponinas, triterpenos, HMF e EMF em cada um dos extratos (controles e otimizados). A análise do teor de açúcares foi efetuada nos extratos para poder relacionar a sua degradação, ou não, em compostos furânicos. Já a presença de saponinas e triterpenos foi avaliada uma vez que as saponinas são substâncias altamente complexas presentes na soja, caracterizadas por serem conjugadas a diferentes açúcares, sendo que em meio ácido geram triterpenos. As

saponinas são solúveis em meio aquoso, enquanto que os triterpenos, assim como os compostos furânicos, possuem maior afinidade com solventes orgânicos. Com isso, a presença de triterpenos e compostos furânicos em extratos de soja possivelmente resultam em processos complexos para obtenção de uma fração altamente enriquecida e purificada em isoflavonas agliconas. Os resultados das análises referentes à avaliação desses compostos também estão apresentados na **Tabela 5.5**. A análise dos extratos controles demonstrou que o extrato não hidrolisado de soja apresentou alto teor de açúcares solúveis, presença de saponinas e ausência de compostos furânicos. Estes resultados estão de acordo com a composição química das sementes de soja (KIM *et al*., 2006). Já o extrato hidrolisado pela via ácida apresentou altos teores de produtos de degradação dos açúcares (HMF e EMF), além de triterpenos, que também estão de acordo com o relatado em condições ácidas (AMIN *et al*., 2011; NEMITZ *et al*., 2015).

Quanto aos extratos obtidos por meio dos bioprocessos otimizados, o extrato obtido por hidrólise enzimática apresentou alto teor de açúcares, presença de saponinas e ausência de compostos furânicos, demonstrando a seletividade desse processo catalítico em apenas clivar as moléculas que apresentam β-glicosídeos, tais como isoflavonas. O extrato obtido após fermentação com *S. cerevisae* apresentou presença de saponinas, ausência de compostos furânicos e baixo teor de açúcares. Isso demonstra que a enzima β-glicosidase presente neste microrganismo possuiu capacidade de hidrolisar as isoflavonas até suas formas agliconas de uma forma seletiva, não hidrolisando as saponinas. Além disso, destaca-se que o processo de fermentação consumiu os açúcares presentes no extrato de soja, transformando-os possivelmente em bioetanol e gás carbônico, que ocorre durante procedimentos de fermentaçao com este microrganismo.

O extrato fermentado com posterior hidrólise ácida apresentou, por sua vez, baixo teor de açúcares, ausência de compostos furânicos e presença de triterpenos. A diferença entre o extrato fermentado e o extrato fermentado hidrolisado pela via ácida está basicamente na transformação das saponinas até as suas formas livres, ou seja, triterpenos. Isso ocorre devido ao processo ácido, em que as saponinas presentes no extrato fermentado foram hidrolisadas, aumentando o teor de triterpenos da amostra. A não formação do HMF e EMF está relacionada com a

prévia transformação dos açúcares até gás carbônico e etanol durante o processo de fermentação, não possibilitando a formação de compostos furânicos durante a hidrólise acida posteriormente efetuada na amostra fermentada.

Geralmente, a hidrólise de saponinas pode ocorrer por ação de ácidos e bases. Porém, enzimas que clivam ligação a β-glicosídica não possuem capacidade de hidrolisar estruturas complexas, tais como as saponinas, que apresentam outros tipos de ligação com os açúcares. Portanto, para que ocorra hidrólise enzimática, é necessária a presença de hidrolases específicas para tais compostos. Destaca-se que essas enzimas podem ou não estar presentes em alguns microrganismos. No caso das saponinas da soja, Amin e colaboradores (2013) relataram a sua biotransformação por ação de enzimas presentes em cepas do genêro *Aspergillus*. Diferentemente, no presente trabalho foi demonstrado que a fermentação com *S. cerevisae* não foi capaz de provocar a hidrólise das saponinas presentes nos extratos de soja, podendo ser considerado, portanto, um processo mais seletivo para hidrólise de isoflavonas do que a fermentação com microrganismos do gênero *Aspergillus*.

A fim de obter frações enriquecidas em isoflavonas agliconas, a purificação dos extratos hidrolisados foi realizada através de uma partição líquido-líquido com acetato de etila, já que as isoflavonas agliconas são altamente solúveis neste solvente. Contudo, conforme previamente relatado, neste solvente também são solúveis os compostos furânicos e os triterpenos. Por causa disso, as frações obtidas pela partição dos diferentes extratos muito possivelmente possuiriam composições químicas diferentes, prejudicando a pureza em isoflavonas agliconas. Dessa forma, foi realizada a verificação do teor de isoflavonas agliconas e a presença ou ausência de triterpenos e compostos furânicos em cada fração obtida (**Tabela 5.6**).

A fração obtida após a partição do extrato hidrolisado pela via ácida apresentou uma baixa pureza em isoflavonas agliconas. Esse resultado está de acordo com o esperado, pois o extrato que foi particionado possuía considerável presença de triterpenos e compostos furânicos, que também se solubilizam no acetato de etila. As frações obtidas após a partição dos extratos hidrolisados pela via enzimática e fermentativa foram as de maior pureza. Isso ocorreu devido ao fato de

que estes extratos não apresentaram compostos furânicos ou elevada taxa de triterpenos, fazendo com que a fração ficasse, portanto, com maior teor de isoflavonas agliconas após a partição com acetato de etila. Já a fração obtida após a partição do extrato fermentado que foi hidrolisado posteriormente pela via ácida apresentou uma pureza intermediária quando comparada com as demais frações. Isso ocorreu devido ao fato de que nesse extrato não estavam presentes compostos furânicos, mas sim triterpenos, que passaram para a fase acetato de etila durante o processo de partição.

O **Capítulo V** descreve, ainda, a viabilidade de queratinócitos após tratamento com a fração mais promissora em termos de pureza de isoflavonas agliconas e rapidez de processo, ou seja, fração obtida após purificação do extrato de soja hidrolisado pela via enzimática. O ensaio foi realizado com células do tipo HaCaT após tratamento com a fração em concentrações de 0,1 a 1,0 µg de isoflavonas agliconas totais/mL, durante 24 ou 48 h. Essa concentração foi escolhida baseada nos estudos de Zhang e colaboradores (2007) que descreveram efeitos proliferativos após o tratamento de células sensíveis ao estrogênio, com uma fração enriquecida em isoflavonas da soja na concentração de 0,4 µg de isoflavonas agliconas totais/mL.

Durante a realização dos estudos biológicos do **Capítulo V**, a avaliação da viabilidade celular dos queratinócitos foi realizada pelo ensaio colorimétrico com sal de tetrazólio (MTT). Esse método mensura a atividade mitocondrial de células vivas e representa um parâmetro de avaliação da atividade metabólica. Durante o ensaio, o sal de MTT de coloração amarela é adicionado nas células previamente tratadas, e as células que estiverem vivas realizarão (em suas mitocôndrias) a redução deste sal até cristais de formazam de coloração violeta. Após solubilização desses cristais, a intensidade da cor é mensurada em espectrofotômetro, sendo proporcional à viabilidade celular (MOSMANN, 1983).

Todas as concentrações testadas da fração apresentaram alta taxa de viabilidade celular após tratamento por 24 e 48 h nas concentrações testadas. Além disso, destaca-se um aumento significativo da viabilidade celular nas concentrações de 0,1 µg de isoflavonas agliconas/mL após 24 h, e de 0,1 e 0,5 µg de isoflavonas agliconas/mL após 48 h (**Figura 5.2**). Esses dados podem ser considerados um

indicativo promissor de atividade proliferativa em queratinócitos, pois envolve a proliferação de células envolvidas nos processos de cicatrização de feridas cutâneas.

Com isso, na sequência do trabalho foi escolhido realizar um ensaio imunológico confirmatório de atividade proliferativa. O fundamento do teste baseia-se em utilizar um anticorpo Ki-67 que reage com um antígeno nuclear específico envolvido em fases ativas de divisão celular, e com isso, as células que se encontrarem nas fases G1 – G2, S e mitose serão marcadas e apresentarão fluorescência que pode ser medida em citômetro de fluxo (KLEIN *et al*., 2000). O ensaio foi realizado por meio de tratamento das células HaCaT com a fração de interesse durante 48 h nas mesmas concentrações utilizadas no ensaio de MTT. Ao final do processo, as células foram tripsinizadas e tratadas com o anticorpo Ki-67 para avaliação da densidade de células marcadas. O resultado obtido da concentração mais baixa testada de fração, concentração equivalente a 0,1 µg de isoflavonas agliconas/mL, evidenciou que houve um aumento significativo na proliferação quando comparado com o valor de queratinócitos basais (células não tratadas, porém marcadas), comprovando que nestas condições a fração apresentou atividade proliferativa de queratinócitos.

Desenvolvimento e caraterização de formulações de uso tópico contendo a fração enriquecida em isoflavonas agliconas da soja

O último capítulo da tese (**Capítulo VI**) apresenta o preparo e a avaliação de nanoemulsões espessadas, ou não, contendo fração de isoflavonas agliconas da soja. As formulações foram desenvolvidas com o objetivo de uma futura avaliação da aplicação cutânea durante processos de cicatrização de feridas. Para isso, foi utilizada a fração enriquecida em isoflavonas agliconas da soja que apresentou promissora atividade proliferativa de queratinócitos. As nanoemulsões foram produzidas pelo método de emulsificação espontânea, empregando como principais componentes da formulação os triglicerídeos de cadeia média (8%), lecitina de gema de ovo (2%) e polissorbato 80 (1%) em água (qsp 100%). O espessamento das nanoemulsões foi realizado pela adição do ácido hialurônico (3%).

A fração enriquecida foi incorporada na nanoemulsão (NE_{FIA}) na concentração final de isoflavonas correspondente a 1,0 mg/mL, expressa como somatório de genisteína, daidzeína e gliciteína. As nanoemulsões apresentaram-se monodispersas (IPD < 0,2) com diâmetro médio abaixo de 200 nm e potencial zeta negativo. Nesses sistemas, provavelmente, as isoflavonas encontram-se solubilizadas no núcleo oleoso e/ou adsorvidas na interface das gotículas da fase interna. Isso se deve basicamente ao LogP apresentado por tais compostos (>2,5), tendo possivelmente uma maior afinidade com a parte oleosa da formulação.

Analisando os resultados das nanoemulsões descritas no **Capítulo VI**, apresentados na **Tabela 6.2**, o tamanho das gotículas das nanoemulsões contendo a franção foi menor do que o das nanoemulsões brancas ($p<0,05$). Situação inversa ocorreu com o potencial zeta, onde um maior valor em módulo foi encontrado após a incorporação da fração na nanoemulsão quando em comparação com a formulação branca. Ambas as situações podem ser explicadas pela complexidade na composição da fração, que quando adicionada na formulação alterou significativamente estes fatores. Muito provavelmente algumas substâncias presentes na FIA podem estar adsorvidas na interface da gotícula, interagindo com os tensoativos (lecitina e polissorbato) e modificando o potencial zeta. Resultados semelhantes foram encontrados por Zorzi et al (2016), quando as nanoemulsões, após a incorporação de um extrato contendo flavonoides, tiveram seu potencial zeta aumentado em módulo quando comparado com as formulações brancas.

A fim de adequar a viscosidade das formulações para uso tópico, as nanoemulsões foram espessadas com ácido hialurônico. Este polímero aniônico é um polissacarídeo linear e natural pertencente à classe dos glicosaminoglicanos não-sulfatados. É composto por unidades dissacarídicas repetidas do ácido D-glucurônico e N-acetilglucosamina através de ligações β-1,4 glicosídicas. Pode atingir altos pesos moleculares e possui alto poder higroscópico, sendo umectante, hidratante e visco-elástico, biocompatível e de baixa toxicidade. Devido a isso, é amplamente utilizado na área clínica, principalmente em produtos médicos, farmacêuticos e cosméticos. Dentre as diversas aplicações, já foram descritos os efeitos benéficos causados pelo ácido hialurônico durante processos de cicatrização de feridas cutâneas (BROWN & JONES, 2005).

As características físico-químicas das gotículas das nanoemulsões foram avaliadas antes e após seu espessamento com ácido hialurônico. A análise dos resultados mostrou que o espessamento das formulações brancas não alterou significativamente ($p>0,05$) o tamanho médio ou o potencial zeta das gotículas. De acordo com Jenning e colaboradores (2000), estes resultados indicam a não ocorrência de agregação das gotículas após adição do polímero.

Por outro lado, o espessamento das nanoemulsões contendo a fração alterou significativamente ($p<0,05$) tanto o tamanho quanto o potencial zeta das gotículas dessas formulações. Porém, muito provavelmente essas diferenças não estão relacionadas com a agregação das gotículas, já que isso não ocorreu nas formulações brancas, mas sim com uma provável interação do polímero com alguns componentes da fração que possam estar presentes na interface das gotículas das formulações. Situação semelhante foi relada por Gohel e colaboradores (2014) para nanoemulsões contendo extrato de *Boswellia* que foram espessadas com um polímero aniônico.

Estudos de liberação são muito importantes na pesquisa e desenvolvimento de novas formulações, uma vez que as substâncias ativas precisam ser liberadas da formulação para estarem disponíveis e exercerem suas funções nos sítios biológicos de interesse. Assim, a liberação das isoflavonas a partir das formulações foi avaliada por meio de células de difusão do tipo Franz, com membranas sintéticas de celulose e utilizando como meio receptor uma mistura de tampão fosfato pH 7,4 e etanol (70:30, v/v). O perfil de liberação das IA está representado na **Figura 6.1.** A quantidade adicionada de cada formulação no compartimento doador das células de Franz foi calculada para não ultrapassar as condições *sink* do experimento e o ensaio foi realizado durante 8 h. A cada intervalo de uma hora a quantidade liberada de IA foi sendo avaliada. Como controle foi utilizada uma suspensão em propilenoglicol contendo a fração na mesma concentração de IA das demais formulações. Os percentuais liberados de daidzeína, gliciteína e genisteína ao final do experimento seguiram a seguinte ordem de liberação: suspensão controle > nanoemulsões > hidrogéis, demonstrando a influência das composições das formulações durante a liberação das isoflavonas.

Estes resultados estão de acordo com a literatura, em que é descrito que nanoemulsões geralmente retardam a liberação de flavonoides devido a uma possível interação com os fosfolipídeos e o núcleo oleoso das gotículas (SILVA *et al*., 2009; BONIFÁCIO *et al*., 2014; ARGENTA *et al*., 2016). Além disso, a menor liberação das IA ocorreu a partir das nanoemulsões espessadas com ácido hialurônico. A redução desta cinética de liberação a partir de hidrogéis se deve provavelmente a barreira formada pela rede tridimensional da matriz polimérica em água, a qual geralmente pode reduzir a liberação das substâncias. Resultados semelhantes de liberação dos flavonoides genisteina e 3-O-metilquercetina foram descritos a partir de nanoemulsões espessadas com diferentes polímeros (ARGENTA *et al*., 2016; BALESTRIN *et al*., 2016).

Avaliando-se a liberação das isoflavonas de forma individual, foram observados valores percentuais estatisticamente semelhantes para a daidzeína e a gliciteína a partir das nanoemulsões, e ambas foram superiormente liberadas quando comparadas com a genisteína. A partir dos hidrogéis, os valores percentuais liberados foram estatisticamente diferentes e seguiram a seguinte ordem: gliciteína > daidzeína > genisteína. Destaca-se que estes resultados podem estar relacionados com os coeficientes de partição das isoflavonas, uma vez que a gliciteína é a que possui um menor LogP, seguido da daidzína e genisteína. Levando, provavelmente a uma maior interação da genisteína com o núcleo oleoso, diminuindo a sua liberação quando comparada com as demais isoflavonas.

A fim de avaliar as potencialidades de uso dessas formulações para aplicação tópica, foi realizado um estudo de permeação/retenção cutânea das isoflavonas em pele suína. O protocolo experimental foi muito semelhante ao dos estudos de liberação, porém trocando-se a membrana sintética por pele de orelha suína não escaldada. Uma suspensão em propilenoglicol contendo a fração na mesma concentração de IA das demais formulações foi utilizada como controle a fim de investigar o efeito promotor de penetração das formulações. Tanto no ensaio com o controle como nos ensaios com as formulações, a quantidade detectada de IA no fluído receptor ficou abaixo do limite de quantificação, evidenciando a pouca ou praticamente ausente taxa de permeação através da pele.

No entanto, ao avaliar a quantidade de IA totais retida na pele (**Tabela 6.3**), foi observado que houve um incremento considerável na penetração e retenção cutânea dessas substâncias a partir das formulações em comparação com o controle, seguindo a seguinte ordem: NEs (1,671 ± 0.077 μg/cm^2) > NEs espessadas em hidrogéis (1,386 ± 0.083 μg/cm^2) > controle (0,430 ± 0.054 μg/cm^2). Tais resultados demonstram que a incorporação da fração nestas formulações aumentou significativamente o potencial de penetração das substâncias nas diferentes camadas da pele. Além disso, ao avaliar a distribuição individual de cada isoflavona nas diferentes camadas da pele (estrato córneo, epiderme viável e derme), foi evidenciado que a incorporação em nanocarreadores permitiu uma penetração das isoflavonas até as camadas mais profundas da pele (**Figura 6.2**). Esse resultado é muito promissor, uma vez que as isoflavonas necessitam chegar até os receptores estrogênicos dos fibroblastos e queratinócitos da epiderme e derme para poderem exercer seus efeitos benéficos.

Uma explicação plausível para o aumento da penetração cutânea das isoflavonas a partir das formulações pode estar relacionada com a presença dos demais componentes lipídicos da formulação, que possivelmente promovem uma maior interação com os componentes da pele, e consequentemente possibilitam uma maior penetração e retenção das isoflavonas (GUPTA *et al*., 2013). Quando os resultados de retenção cutânea a partir das nanoemulsões e hidrogéis foram comparados, observou-se um maior teor de IA na pele a partir das nanoemulsões. Tal fato pode estar relacionado com o menor tamanho das gotículas lipídicas d esta formulação. De acordo com Shakeel e colaboradores (2012), o tamanho das gotículas é uma propriedade importante para facilitar a penetração cutânea de nanoemulsões e seus produtos derivados. Além disso, a maior retenção também pode estar relacionada com a maior taxa de liberação que as IA obtiveram a partir das nanoemulsões quando comparadas com o comportamento a partir do hidrogel.

Complementarmente, vale mencionar que a retenção cutânea das IA a partir de nanoemulsões mostrou-se diferente ao compararmos os valores obtidos no **Capítulo VI** com os valores obtidos nos estudos de validação bionalítica descritos no **Capítulo III**. Assim, foi evidenciado que o valor de IA retido nas camadas da pele foi estatisticamente superior a partir de nanoemulsões preparadas pela incorporação de

uma fração obtida após hidrólise enzimática (1,671 ± 0.077 µg/cm^2) do que a partir de nanoemulsões preparadas com uma fração obtida após hidrólise ácida (aprox. 1.26 µg/cm^2). Provavelmente, essa diferença se deve ao tipo de fração utilizada na fabricação de nanoemulsões, que dependendo dos demais constituintes presentes em sua composição pode causar um efeito positivo, ou não, durante a penetração cutânea das isoflavonas.

Comparando-se a abordagem dos estudos apresentados nos **Capítulos III** e **Capítulo VI** desta Tese com os demais trabalhos científicos encontrados na literatura, é importante destacar que a maioria dos pesquisadores realiza a avaliação da permeação cutânea individual das isoflavonas, principalmente genisteína, a partir de diferentes veículos e formulações (MOTLEKAR *et al*., 2003; SILVA *et al.,* 2009; KANG *et al*., 2010; KITAGAWA *et al*., 2010; CHADHA *et al*., 2011; MAIONE-SILVA *et al*., 2012; VARGAS *et a*l., 2012; ZAMPIERI *et al*., 2013; ARGENTA *et al*., 2014; ANDRADE et al., 2014). Poucos trabalhos foram encontrados abordando a avaliação simultânea da permeação cutânea das isoflavonas. Dentre esses, Huang e colaboradores (2008) estudaram o comportamento *in vitro* da permeação de genisteína e daidzeína (isoladas ou em mistura) em pele de orelha suína em função da taxa de ionização desses compostos (pH 6,0 e 10,8). Minghetti e colaboradores (2006) estudaram o comportamento *ex vivo* da permeação simultânea de genisteína e daidzeína em pele humana a partir de um extrato de soja solubilizado em diferentes veículos. Georgetti e colaboradores (2008) desenvolveram formulações contendo extrato de soja e avaliaram o potencial de retenção *in vitro* das isoflavonas em pele de orelha suína; no entanto, os resultados foram expressos em função da atividade antioxidante e não por teor quantificado na pele. Dessa forma, destaca-se que os estudos efetuados no último capítulo da presente Tese apresentam uma abordagem inédita envolvendo a avaliação da distribuição simultânea das isoflavonas genisteína, daidzeína e gliciteína em diferentes camadas da pele (estrato córneo, epiderme e derme), além de realizar a comparação da capacidade de penetração desses compostos a partir de diferentes formulações.

De uma forma geral, o conjunto dos resultados de todos os capítulos experimentais demonstra que diferentes hidrólises podem ser efetuadas em extrato de sementes desengorduradas de soja para obter as formas bioativas das

isoflavonas. Dependendo do procedimento efetuado, diferentes compostos podem estar presentes no produto final, impactando fortemente em sua eficácia, qualidade e segurança. Quando o procedimento de hidrólise ácida é efetuado, compostos furânicos são formados. Tais substâncias apresentam potencial genotóxico, e comprometem as etapas de purificação do extrato até um produto mais puro em isoflavonas e seguro para administração biológica. Diferentemente, as hidrólises enzimática e fermentativa com β-glicosidase e *S. cerevisiae*, respectivamente, são procedimentos mais seletivos que não causam a degradação dos açúcares até compostos furânicos. A fração acetato de etila obtida após partição do extrato hidrolisado pela via enzimática mostrou ser um insumo promissor para aplicação tópica, pois promove a proliferação de queratinócitos, importantes células envolvidas durante as etapas de cicatrização e regeneração cutânea. A incorporação da fração em nanoemulsões aumentou a capacidade de penetração e retenção das isoflavonas agliconas até camadas profundas da pele, tal como epiderme, caracterizada por apresentar queratinócitos em sua composição, demonstrando ser, portanto, um sistema promissor para administração tópica durante processos de regeneração cutânea.

Por fim, cabe mencionar que paralelamente aos estudos aqui apresentados, o nosso grupo de pesquisa realizou a incorporação da fração enriquecida em isoflavonas agliconas da soja (FIA) em outros nanocarreadores, além das nanoemulsões, e as tecnologias e produtos envolvidos nesta etapa foram recentemente protegidos por meio de depósito de patente no Instituto Nacional de Proteção Industrial do Brasil. A tecnologia em questão encontra-se no **Anexo 1**. A patente descreve a incorporação da FIA em lipossomas, nanopartículas lipídicas sólidas, carreadores lipídicos nanoestruturados, além das já citadas nanoemulsões. A escolha desses sistemas foi baseada nos dados obtidos durante as reviões bibliográfica e tecnológica descritas nos primeiros capítulos desta tese. Destaca-se que nenhum produto ou processo igual ao nosso havia sido previamente descrito, e dessa forma, foi escolhido realizar a proteção intelectual da tecnologia desenvolvida.

CONCLUSÕES

- Um método cromatográfico de ultraeficiência foi desenvolvido para realizar a análise de isoflavonas agliconas e principais impurezas furânicas formadas após etapas de hidrólise do extrato de soja;

- O método desenvolvido foi validado para realizar a quantificação das isoflavonas agliconas (daidzeína, gliciteína e genisteína) em diferentes matrizes e mostrou-se específico, linear, preciso, exato, robusto e indicativo de estabilidade para análise destes compostos em extrato hidrolisado de soja, fração enriquecida, nanoemulsões, hidrogéis, mucosa de esôfago suíno e camadas de pele de orelha suína;

- O método desenvolvido foi adicionalmente validado para a quantificação de compostos furânicos e mostrou-se específico, linear, preciso, exato e robusto para análise de hidroximetilfurfural (HMF) e etoximetilfurfural (EMF) em extrato de soja hidrolisado pela via ácida;

- O extrato de soja obtido após hidrólise ácida apresentou teores de HMF e EMF cerca de 10 vezes superiores ao teor de isoflavonas agliconas. Baseado nos relatos da toxicidade genética do HMF descritos na literatura, bem como nos dados experimentais obtidos para o EMF, esses compostos furânicos foram classificados como impurezas possivelmente tóxicas, e processos de purificação são recomendados antes de aplicações farmacológicas deste extrato;

- O EMF não foi considerado mutagênico nas concentrações avaliadas no ensaio de AMES, porém demonstrou ser genotóxico nas concentrações entre 0,1 e 1,3 mg/mL no ensaio cometa realizado em células hepáticas;

- Procedimentos de biotransformação das isoflavonas presentes nos extratos de soja foram realizados pelos métodos enzimático (β-glicosidase) e fermentativo (*Saccharomyces cerevisiae)*. Ambos os processos apresentaram maior seletividade hidrolítica quando comparados à hidrólise ácida, não causando degradação dos açúcares presentes na soja até compostos furânicos;

- Os bioprocessos enzimáticos e fermentativos foram otimizados por meio de desenhos experimentais do tipo Box-Benhken. Ambos os processos foram realizados com um extrato equivalente ao extraído de 1,0 g de soja desengordurada. As condições ótimas determinadas para hidrólise enzimática das isoflavonas foram de 838 unidades de β-glicosidase, em pH 6,0, durante 4,5 h a 37 ºC. Já as condições ótimas determinadas para hidrólise fermentativa das isoflavonas foram de 1500 mg de fermento comercial contendo *Saccharomyces cerevisiae,* em pH 7,6, durante 24 h a 33 ºC;

- Uma fração pura em isoflavonas agliconas foi obtida a partir do extrato de soja hidrolisado pela via enzimática realizando-se apenas uma etapa de purificação. A fração apresentou atividade proliferativa em cultura de queratinócitos, demonstrando ser um promissor candidato de matéria-prima para regeneração de tecido cutâneo;

- Nanoemulsões contendo a fração enriquecida em isoflavonas agliconas foram obtidas por emulsificação espontânea. Estes sistemas foram espessados até hidrogéis pela adição de 3% de ácido hialurônico. Ambas as formulações (nanoemulsões e hidrogéis) demonstraram ser promissoras para a administração tópica de isoflavonas agliconas da soja, uma vez que a retenção das isoflavonas agliconas em pele de orelha suína a partir das formulações foi superior quando comparado a de uma fração não incorporada a carreador algum.

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ANEXO

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Protocolo

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Número

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Dados do requerente ou interessado:

Tipo de formulário enviado:	DIRPA-FQ001 v.006
Referência interna:	ND000267
Primeiro requerente ou interessado:	UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CNPJ do primeiro requerente ou interessado:	92.969.856/0001-98
Número de requerentes ou interessados:	1
Título do pedido:	CARREADORES LIPÍDICOS DE TAMANHO NANOMÉTRICO COMPREENDENDO FRAÇÃO ENRIQUECIDA DE ISOFLAVONAS AGLICONAS DA SOJA, PROCESSO DE OBTENÇÃO DOS MESMOS E FORMULAÇÕES COMPREENDENDO OS MESMOS

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		Procedimento: Não Aplicável	

Ao Instituto Nacional da Propriedade Industrial:

O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas

1. Depositante (71):

1.1	Nome:	UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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2. Natureza: [x] Invenção [ ] Modelo de Utilidade [ ] Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

CARREADORES LIPÍDICOS DE TAMANHO NANOMÉTRICO COMPREENDENDO FRAÇÃO ENRIQUECIDA DE ISOFLAVONAS AGLICONAS DA SOJA, PROCESSO DE OBTENÇÃO DOS MESMOS E FORMULAÇÕES COMPREENDENDO OS MESMOS

4. Pedido de Divisão: do pedido Nº: —

5. Prioridade:

Declaração na forma do item 3.2 da Instrução Normativa nº 17/2013:

[ ] Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

O depositante reivindica a(s) seguinte(s) prioridade(s):

País ou Organização do depósito	Número do depósito	Data do depósito	Tipo
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6.8	E-mail:	helder.teixeira@ufrgs.br

7. Declaração de divulgação anterior não prejudicial:

[ ] Artigo 12 da LPI - Período de Graça.

8. Procurador (74):

8.1	Nome:	—
8.2	Qualificação:	—
8.3	CPF:	—
8.4	API:	—
8.5	OAB:	—
8.6	Endereço Completo:	—
8.7	CEP:	—
8.8	Telefone:	—
8.9	Fax:	—
8.10	E-mail:	—

9. Listagem de sequências biológicas: (documentos anexados, se houver)

[ ] Código de controle alfanumérico no formato de código de barras (arquivo em formato eletrônico PDF).

[ ] Código de controle alfanumérico no formato de código de barras (arquivo em formato eletrônico XML).

[ ] Listagem de sequências (arquivo em formato eletrônico TXT).

[ ] Declaração relativa à listagem de sequências (arquivo em formato eletrônico PDF).

10. Material biológico: (se houver)

10.1	Identificação:	—
10.2	Instituição depositária:	—
10.3	Endereço completo:	—
10.4	Número de adesão:	—

11. Declaração de igualdade de conteúdo técnico entre os arquivos eletrônicos em formato PDF e TXT (texto):

[x] Declaro que todos os documentos de especificação em formato texto têm conteúdo igual ao dos documentos de especificação anexados em formato PDF. Caso haja alguma divergência entre os arquivos PDF e TXT (texto), será considerado o conteúdo do arquivo PDF para fins de exame.

12. Dados gerais do conteúdo técnico do depósito (caso aplicável):

Sugestão de desenho a ser publicado com o resumo: —
Número de desenhos: —
Cor dos desenhos: [ ] Preto e branco [ ] Colorido / Escala de cinza

13. Documentos Anexados: (assinale e indique também o número de folhas)

X	Relatório descritivo em formato eletrônico PDF	20 p.
X	Reivindicações em formato eletrônico PDF	3 p.
X	Resumo em formato eletrônico PDF	1 p.
X	Relatório descritivo em formato eletrônico texto	— p.
X	Reivindicações em formato eletrônico texto	— p.
X	Resumo em formato eletrônico texto	— p.
X	Guia de Recolhimento da União (GRU) paga com comprovante de pagamento em formato eletrônico PDF [Código de serviço: 200, Número: 00.000.2.2.15.0635585.9, Nome do sacado: UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL]	1 p.
X	Documentos de qualquer outra natureza em formato eletrônico PDF [Portaria de Competência]	3 p.
X	Documentos de qualquer outra natureza em formato eletrônico PDF [Diário Oficial da União]	1 p.

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[x] Declaro que me comprometo a não realizar qualquer modificação capaz de alterar os arquivos automaticamente criados pelo sistema e-Patentes/Depósito, tendo ciência de que qualquer alteração poderá invalidar o depósito ou o peticionamento eletrônico.

[x] Declaro ciência de que eventuais exigências formais e/ou técnicas serão posteriormente publicadas na Revista Eletrônica da Propriedade Industrial (RPI) disponibilizada no portal do INPI.

[x] Declaro, sob penas da Lei, que todas as informações prestadas neste formulário eletrônico são completas e verdadeiras.

21 de setembro de 2015
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Assinatura (Requerente ou Procurador)

CARREADORES LIPÍDICOS DE TAMANHO NANOMÉTRICO COMPREENDENDO FRAÇÃO ENRIQUECIDA DE ISOFLAVONAS AGLICONAS DA SOJA, PROCESSO DE OBTENÇÃO DOS MESMOS E FORMULAÇÕES COMPREENDENDO OS MESMOS

Campo da Invenção

[001]A presente invenção descreve carreadores lipídicos de tamanho nanométrico (< 1,0 micrômetro) compreendendo uma fração enriquecida em isoflavonas agliconas da soja, processos de obtenção dos mesmos e formulações que compreendem tais carreadores. A tecnologia pertence ao campo da nanotecnologia e consiste em formulações aquosas que podem ser utilizadas na área alimentar, farmacêutica e cosmética.

Antecedentes da Invenção

[002] A soja, *Glycine max* L. Merr, é uma leguminosa amplamente cultivada em diversos países, e apresenta, devido a isso, grande impacto na economia mundial. O seu cultivo está voltado principalmente para a produção de alimentos, contudo diversas áreas industriais tem mostrado interesse crescente nos subprodutos que seus grãos podem originar (CHEN, K.I., et al.; "Soyfoods and soybean products: from traditional use to modern applications". Applied Microbiology and Biotechnology 2012, v. 96, p. 9–22).

[003] As sementes da soja são constituídas basicamente por óleo, proteínas, carboidratos, minerais, e metabólitos secundários tais como polifenóis, destacando-se as isoflavonas e saponinas. Dentre as diversas substâncias químicas encontradas nesta planta, as isoflavonas apresentam amplo interesse clínico, devido principalmente à semelhança estrutural com o hormônio 17-β-estradiol (NEMITZ, M.C., et al.; "Bioactive soy isoflavones: extraction and purification procedures, potential dermal use and nanotechnology-based delivery systems". Phytochemistry Reviews (*In press*), 2014, DOI: 10.1007/s11101-014-9382-0).

[004] As isoflavonas constituem um grupo de fitoestrógenos de amplo

interesse na área médica e nutricional. Tais compostos são encontrados em maior quantidade nos grãos e produtos de soja, podendo ser obtidos por processos de extração e purificação, ou ainda, adquiridos comercialmente nas suas formas sintéticas.

[005] As agliconas genisteína, daidzeína e gliciteína são as formas das isoflavonas consideradas com maior capacidade de absorção tanto no trato gastro-intestinal quanto na pele, além de apresentarem maior potencial terapêutico quando comparadas às formas conjugadas. A obtenção destas substâncias pode ser realizada por diferentes maneiras, tais como aquisição comercial das suas formas isoladas e sintéticas, ou ainda obtidas a partir de extratos de materiais vegetais. A extração de grãos seguida de procedimento de hidrólise e purificação tem sido a metodologia mais descrita para a obtenção das principais isoflavonas agliconas a partir da soja (NEMITZ, M.C., et al.; "Bioactive soy isoflavones: extraction and purification procedures, potential dermal use and nanotechnology-based delivery systems". Phytochemistry Reviews (*In press*), 2014, DOI: 10.1007/s11101-014-9382-0; ROSTAGNO, M., et al.; "Sample preparation for the analysis of isoflavones from soybeans and soy foods". J Chromatogr A, 2009, v. 1216, p. 2–29).

[006] Produtos das áreas alimentar, farmacêutica e cosmética contendo isoflavonas isoladas, ou ainda extratos de soja, são relatados na literatura científica, bem como encontrados no portfolio de indústrias da área da saúde. No entanto, estudos apontam que o extrato de soja possui maior capacidade de inibição do crescimento de tumores quando comparado a isoflavonas isoladas, e que o uso simultâneo das diferentes formas de isoflavonas pode potencializar a ação estrogênica e fotoprotetora das mesmas. Desta forma, extratos de soja e seus derivados, tais como frações purificadas contendo diferentes isoflavonas, são altamente promissores para a fabricação de produtos da saúde (IOVINE, B., et al.; "Synergic effect of genistein and daidzein on UVB-induced DNA damage: an effective photoprotective combination". Journal of Biomedicine and Biotechnology, 2011, v. 2011, p. 1–8; KIM, H-A., et

al.; "Soy extract is more potent than genistein on tumor growth inhibition". Anticancer Research, 2008, v. 28, p. 2837–2842; HSU, A., et al.; "Differential effects of whole soy extract and soy isoflavones on apoptosis in prostate cancer cells". Experimental Biology and Medicine (Maywood), 2010, v. 235, n. 1, p. 90–97; RANDO, G., et al.; "A. Differential effect of pure isoflavones and soymilk on estrogen receptor activity in mice". Toxicology and Applied Pharmacology, 2009, v. 237, p. 288–297).

[007] Contudo, quando os extratos apresentam as isoflavonas nas suas formas de agliconas, alguns desafios podem ser encontrados durante a fabricação de produtos terapêuticos ou alimentares devido à baixa hidrossolubilidade das isoflavonas agliconas e a alta taxa de amargor das mesmas (ALDIN, E. et al.; "Bitterness of soy extracts containing isoflavones and saponins". Journal of food Science, 2006, v. 71, n. 3, p. S211–S215; LARKIN, T.A, et al.; "The key importance of soy isoflavone bioavailability to understanding health benefits". Critical Reviews in Food Science and Nutrition, 2008, v. 48, n. 6, p. 538–552).

[008] Para contornar as limitações de incorporação de princípios ativos de caráter amargo ou hidrofóbicos em formulações aquosas, algumas alternativas são encontradas. Neste sentido, a nanotecnologia aplicada a ingredientes vegetais tem experimentado um rápido crescimento, com diversas aplicações inovadoras na área da saúde. Dentre algumas vantagens, destaca-se que o uso de nanocarreadores lipídicos permite a incorporação de substâncias hidrofílicas e/ou hidrofóbicas em formulações aquosas e possibilita o mascaramento de sabores desagradáveis de certas substâncias ativas, além de aumentar a estabilidade das substâncias incorporadas, e potencializar a permeabilidade dos ativos nas barreiras biológicas (FATHIA, M., et al.; "Nanoencapsulation of food ingredients using lipid based delivery systems". Trends in Food Science & Technology, 2012, v. 23, p. 13–27).

[009] Desta forma, considerando o sabor amargo e a baixa hidrossolubilidade das isoflavonas agliconas, juntamente com as vantagens

do uso de sistemas nanométricos na administração de substâncias de origem vegetal, e as potencialidades biológicas da administração simultânea de genisteína, daidzeína e gliciteína, a presente invenção refere-se a formulações aquosas compreendendo fração enriquecida em isoflavonas agliconas da soja em carreadores lipídicos de diâmetro médio de gotícula/partícula inferior a 1,0 micrômetro. Os nanocarreadores lipídicos da presente invenção são caracterizados por compreenderem nanoemulsões, lipossomas, nanopartículas lipídicas sólidas e carreadores lipídicos nanoestruturados. O processo de fabricação dos produtos é caracterizado por compreender uma etapa de homogeneização a alta pressão, a fim de produzir carreadores lipídicos nanométricos de tamanhos uniformes e por não apresentar em sua formulação final a presença de solventes orgânicos.

[0010]O uso de nanocarreadores lipídicos contendo isoflavonas agliconas é descrito na literatura científica e em algumas patentes. Porém, conforme descrito logo abaixo, nenhuma tecnologia se assemelha ao conteúdo da presente invenção.

[0011]A incorporação da daidzeína a lipossomas foi realizada por Dwiecki et al. (2009) pelo método de hidratação de filme lipídico (DWIECKI, K., et al.; "Antioxidant activity of daidzein, a natural antioxidant, and its spectroscopic properties in organic solvents and phosphatidylcholine liposomes". Journal of Photochemistry and Photobiology B: Biology, 2009, v. 96, p. 242–248). A presente invenção emprega um método distinto de preparo dos lipossomas e incorpora uma fração enriquecida em isoflavonas da soja.

[0012]A incorporação da genisteína a nanoemulsões foi realizada por Silva et al. (2009) e Argenta et al. (2014) pelo método de emulsificação espontânea (SILVA, A.P.C., et al.; "Development of topical nanoemulsions containing the isoflavone genistein". Die Pharmazie, 2009, v. 64, p. 32–35; ARGENTA, D. F., et al.; "Factorial design applied to the optimization of lipid composition of topical antiherpetic nanoemulsions containing isoflavone genistein". International

Journal of Nanomedicine, 2014, v. 9, p. 4737–4747). A presente invenção emprega um método distinto de preparo das nanoemulsões e incorpora uma fração enriquecida em isoflavonas da soja.

[0013]A incorporação de genisteína a nanopartículas lipídicas sólidas (NLS) foi realizada por Zhang et al. (2013) pelo método de emulsificação por fusão combinado com a técnica de ultra-sonicação, e por Andrade et al. (2014) pelo método de microemulsão (ZHANG, W., et al.; "Design, characterization, and in vitro cellular inhibition and uptake of optimized genistein-loaded NLC for the prevention of posterior capsular opacification using response surface methodology". International Journal of Pharmaceutics, 2013, v. 454, p. 354–366; ANDRADE, L.M., et al.; "Impact of lipid dynamic behavior on physical stability, in vitro release and skin permeation of genistein-loaded lipid nanoparticles". European Journal of Pharmaceutics and Biopharmaceutics, 2014, v. 88, p. 40–47). A presente invenção emprega um método distinto de preparo das NLS e incorpora uma fração enriquecida em isoflavonas da soja.

[0014]A incorporação de genisteína em nanocápsulas foi realizada por Zampieri et al. (2013) pelo método de deposição interfacial de polímero pré-formado (ZAMPIERI, A.L.T.C., et al.; "Biodegradable polymeric nanocapsules based on poly(DL-lactide) for genistein topical delivery: obtention, characterization and skin permeation studies". Journal of Biomedical Nanotechnology, 2013, v. 9, p. 527–534). A presente invenção não utiliza polímeros, diferindo, portanto, de tal documento.

[0015]A incorporação de daidzeína em nanocarreadores lipídicos foi previamente descrita por Zhang et al. (2011), onde a substância foi complexada a um fosfolipídio e, após, o complexo foi encapsulado a NLS pelo processo de homogeneização de filme (ZHANG, Z., et al.; "Daidzein–phospholipid complex loaded lipid nanocarriers improved oral absorption: in vitro characteristics and in vivo behavior in rats". Nanoscale., 2011, v. 3, n. 4, p. 1780–1787). A presente invenção incorpora fração enriquecida em isoflavonas e não utiliza uma etapa de complexação, diferindo, portanto, de tal documento.

[0016]As diferentes tecnologias protegidas pelos números US8551530 (B2), 15/11/2010, "Nanoparticle isoflavone composition & methods of making the same", WO2007000193 (A1), 31/01/2006, "Isoflavone nanoparticles and use thereof" e CN103211750 (A), 04/01/2013, "Method of preparing soy isoflavone nanoparticles by precipitation with compressed antisolvent (PCA) using a supercritical fluid" descrevem nanopartículas sólidas de genisteína vinculadas ou não a veículos. Contudo, os veículos apresentados em tais documentos não são compostos por nanocarreadores lipídicos e tensoativos, diferindo da presente invenção.

[0017]As diferentes tecnologias protegidas pelos pedidos CN102652736 (B), 16/04/2012, "Method for preparing soybean isoflavone sustained-release microspheres", CN101947251 (A), 21/09/2010, "Method for preparing chickpea isoflavone microcapsules", CN103211769 (A), 04/01/2013, "Method of preparing a controlled release particle of soy isoflavone with biodegradable polymer using a supercritical fluid extraction of emulsion (SFEE) process" e CN103099798 (A), 29/01/2013, "Preparation method of soybean isoflavone-chitosan slow-release microcapsules", descrevem a incorporação de isoflavonas a sistemas micro ou nanométricos que contêm em suas formulações polímeros ou ciclodextrinas. A presente invenção difere de tais documentos pelo fato de não utilizar tais excipientes em seus processos.

[0018]A tecnologia protegida pelo número CN102258475 (B), 28/05/2010, "Daidzein solid lipid nanoparticles and preparation method thereof" descreve a incorporação de daidzeína a NLS pelo método de dispersão de película fina e pelo método de dispersão a quente. A presente invenção emprega um método distinto de preparo das NLS e incorpora uma fração enriquecida em isoflavonas da soja.

[0019]A obtenção de uma fração enriquecida em isoflavonas agliconas após hidrólise e purificação do extrato de soja com posterior incorporação a carreadores nanoestruturados foi descrita em alguns artigos científicos. A produção de nanoemulsões contendo fração de isoflavonas da soja foi descrita

por Yatsu et al. (2014) e Nemitz et al. (2015), onde os sistemas foram produzidos pelo método de emulsificação espontânea, porém os trabalhos não apresentam as características quanto ao tamanho das estruturas e o potencial zeta (YATSU, F.K.J., et al.; "A new simplified and stability indicating liquid chromatography method for routine analysis of isoflavones aglycones in different complex matrices". Food Analytical Methods, 2014, v. 7, p. 1881–1890; NEMITZ, M.C., et al.; "A versatile,stability-indicating and high-throughput ultra-fast liquid chromatography method for the determination of isoflavone aglycones in soybeans, topical formulations, and permeation assays". Talanta, 2015, v. 134, p. 183–193). Destaca-se que a presente invenção utiliza um processo de obtenção das nanoemulsões distinto de tais documentos.

[0020]Nanoestruturas lipídicas sólidas contendo fração de isoflavonas da soja foram produzidas por Deshmukh & Amin (2013), onde os sistemas foram produzidos pelo método de fusão, inversão de fases e precipitação na água gelada (DESHMUKH, K.; AMIN, P. "Formulation and evaluation of solid–lipid nanoparticle based 0.1% Soy isoflavone dermal gels". Journal of Pharmaceutical and BioSciences, 2013, v. 1, p. 7–18). Destaca-se que a presente invenção utiliza um processo de obtenção das nanopartículas distinto de tal documento.

[0021]Nanocápsulas contendo extrato de soja foram produzidas pelo método de mistura de excipientes e posterior secagem por spray-drying (SANSONE, F., et al.; "Enhanced technological and permeation properties of a microencapsulated soy isoflavones extract". Journal of Food Engineering, 2013 v. 115, p. 298–305). A presente invenção difere de tal documento pelo fato de não utilizar polímeros na formulação.

[0022]A tecnologia protegida sob o número US2002160064 (A1), 31/10/2012, "Cosmetics containing isoflavone aglycones", propõe um produto cosmético contendo isoflavonas incorporadas a lipossomas. Tal tecnologia descreve o preparo dos sistemas pelo método de homogeneização a alta pressão com incorporação de genisteína e/ou daidzeína. Ao final do processo também é

descrita a incorporação de um extrato de alga de *Spirulina platen*sis. Alternativamente a tecnologia descreve a utilização de uma fração enriquecida em isoflavonas agliconas obtidos por procedimentos de hidrólise da soja. Contudo, é importante destacar que tal tecnologia não apresenta a etapa de evaporação do solvente orgânico durante a produção dos lipossomas, resultando em um produto final contendo etanol, o que pode causar irritação da pele dos usuários durante o uso. Diferindo da tecnologia US2002160064 (A1), a presente invenção compreende a produção de quatro diferentes tipos de carreadores lipídicos nanométricos aquosos, incluindo lipossomas, contendo a fração de isoflavonas agliconas da soja e livres de solventes orgânicos nos produtos finais.

[0023]O documento protegido sob o número KR100482355 (B1), 07/11/2003, "Liposome or nanoemulsion containing dipalmitoylhydroxy and soy isoflavones and production of cosmetic composition containing the same", descreve uma tecnologia para incorporar isoflavonas e dipalmitoilhidroxiprolina em lipossomas e nanoemulsões para uso em cosméticos. A tecnologia compreende uma formulação composta por 0,01 a 10% de dipalmitoilhidroxiprolina e isoflavonas da soja, 1 a 5% lecitina, 1 a 5% de tensoativo e 20 a 50% de óleo TCM, glicerina e álcool, resultando em nanoemulsões ou lipossomas contendo alto teor de solvente orgânico. Tal tecnologia descreve um produto contendo simultaneamente isoflavonas e aminoácidos, diferindo da presente invenção que utiliza fração de isoflavonas agliconas da soja. Além disso, a presente invenção difere do documento anterior por compreender formulações aquosas com ausência de solvente orgânico no final do processo, além de utilizar o homogeneizador à alta pressão para a produção de quatro diferentes tipos de carreadores lipídicos manométricos aquosos.

[0024] O pedido de patente de número PI0805156-9 (A2), 20/11/2008, "Nanoestrutura compreendendo extratos vegetais, processo de produção de nanoestrutura compreendendo extratos vegetais e composições compreendendo as mesmas" descreve a obtenção de nanopartículas lipídicas

tais como nanoemulsões, nanopartículas, nanoagregados e lipossomas contendo extratos vegetais, preferencialmente de *Achyrocline satureioides*. O processo se dá por dissolução dos componentes oleosos em uma fase orgânica, adição desta fase a uma fase aquosa contendo tensoativos, sob agitação, com posterior evaporação do solvente orgânico. Contudo, não está descrito nas reivindicações do documento a etapa de homogeneização a alta pressão durante o processo de fabricação das nanoestruturas e nem o uso de uma fração enriquecida a partir de um extrato vegetal. Portanto, a presente invenção não apresenta conflitos de interesse com tal documento.

[0025] Portanto, como pode ser observado, o estado da arte envolvendo nanotecnologia e isoflavonas não conflite com a tecnologia descrita na presente invenção.

Sumário da invenção

[0026]É um objeto da presente invenção carreadores lipídicos de diâmetro médio de gotícula/partícula inferior a 1,0 micrômetro compreendendo fração enriquecida de isoflavonas agliconas da soja.

[0027]Em uma realização preferencial, as isoflavonas agliconas da soja são enriquecidas em genisteína, gliciteína e daidzeína.

[0028]É um objeto adicional da presente invenção os processos de obtenção das nanoemulsões e lipossomas, caracterizados por:

a) Dissolver em uma solução orgânica composta por etanol, acetona e/ou mistura etanol:acetona (1:1):

i) de 2,0% p/p a 10,0% p/p de fase lipídica;

ii) 1,0% p/p de fração de isoflavonas agliconas da soja, em relação à fase lipídica;

b) Dissolver em uma solução aquosa:

iii) 1,0% p/p a 5,0% p/p de tensoativo;

c) Adicionar a solução orgânica na solução aquosa, sob agitação;

d) Evaporar o solvente orgânico;

e) Homogeneizar a formulação em homogeneizador à alta pressão, com 10 ciclos de 500 a 750 bar cada.

[0029]É um objeto adicional da presente invenção os processos de obtenção das nanoestruturas lipídicas sólidas e carreadores lipídicos nanoestruturados, caracterizados por:

a) Dissolver a uma temperatura entre 30º C a 80º C:

i) de 2,0% p/p a 10,0% p/p de fase lipídica;

ii) 1,0% p/p de fração de isoflavonas agliconas da soja, em relação à fase lipídica;

b) Dissolver em uma solução aquosa, com temperatura de 30º C a 80º C:

iii) de 1,0% p/p a 5,0% p/p de tensoativo;

c) Adicionar a solução aquosa na solução oleosa, sob agitação e com temperatura de 30º C a 80º C;

d) Agitar no ultra-turrax, sob aquecimento de 30º C a 80º C, durante período compreendido de 30 segundos a 5 minutos;

e) Homogeneizar a formulação em homogeneizador à alta pressão, com 10 ciclos de 500 a 750 bar cada.

[0030]É um objeto adicional da presente invenção o uso farmacológico, terapêutico e tecnológico de formulações compreendendo as nanoestruturas lipídicas compreendendo fração de isoflavonas agliconas da soja, nas áreas das ciências da saúde, ciências farmacêuticas, da química e da físico-química, como medicamento e/ou suplemento alimentar.

[0031]Em uma realização preferencial, a fração foi obtida por extração em meio hidro-etanólico, com posterior hidrólise ácida e etapas de purificação (NEMITZ, M.C., et al.; "A new approach for the purification of soybean acid extract: simultaneous production of an isoflavone aglycone-rich fraction and a furfural derivative-rich by-product". Industrial Crops and Products, 2015, v. 67, p. 414–421).

Descrição Detalhada da Invenção

[0032]Os exemplos descritos são meras concretizações preferenciais da presente invenção, não podendo ser compreendidos como limitantes da invenção. Variações ou concretizações similares devem ser consideradas dentro do escopo da presente invenção.

Fração de isoflavonas agliconas da soja:

[0033]A fração de isoflavonas agliconas da soja para uso na presente invenção pode ser obtida a partir de qualquer via de obtenção, ou seja, sintética, biossintética ou natural. A fração deve apresentar majoritariamente as isoflavonas daidzeína, genisteína e gliciteína.

[0034]Em uma realização especial a presente invenção compreende uma fração de isoflavonas agliconas da soja obtida da seguinte forma:

[0035]As sementes de soja são primeiramente moídas. O pó é desengordurado através da extração em aparelho de Soxhlet com *n*-hexano durante 9 horas. O pó desengordurado é submetido à extração em aparelho de Soxhlet utilizando etanol 80% em água (v/v) durante 4 horas na proporção droga-solvente 1:10 (p/v). O extrato hidroetanólico é submetido a processo de hidrólise com ácido clorídrico (1,3 M), durante 2 horas a 80 ºC. O solvente orgânico é então rotaevaporado, e a parte aquosa restante é particionada com acetato de etila por 4 vezes. A fração orgânica obtida após particionamento é submetida à coluna cromatográfica de sílica gel e a eluição é realizada com solventes de polaridade crescente. A fração obtida após eluição com clorofórmio:acetato de etila 75:25 (v/v) é coletada, rotaevaporada e as isoflavonas agliconas são precipitadas com clorofórmio. O pó obtido é recolhido por filtração e posterior secagem. A fração final é caracterizada por compreender um teor total de 92% de isoflavonas agliconas, expressas como somatório de genisteína, daidzeína e gliciteína.

[0036]Em especial a fração de isoflavonas agliconas da soja está presente na formulação compreendendo uma faixa de concentração de 0,2 a 1,0 mg/mL.

Fase lipídica:

[0037]A fase lipídica adequada para a presente invenção é constituída por tensoativos lipofílicos, óleos, lipídeos sólidos e/ou mistura desses.

[0038]Os tensoativos lipídicos incluem, mas não se limitam a, lecitina e fosfolipídeos. Lecitinas são conhecidas como glicerofosfolipídeos os quais são formados a partir de ácidos graxos, glicerol, ácido fosfórico e colina por esterificação. As lecitinas são frequentemente referenciadas como fosfatidil colinas. Fosfolipídeos adequados para o uso na presente invenção incluem, mas não se limitam, a fosfolipídeos encontrados na gema de ovo e na soja.

[0039]Substâncias oleosas adequadas para o uso na presente invenção incluem, mas não se limitam a, oleato de decila, isohexadecano, ésteres do ácido esteárico e/ou oléico, etanolamida de ácido graxo de coco, óleos naturais, como o óleo de milho, amendoim, sésamo, oliva, jojoba, soja, álcool graxo, parafina, triglicerídeos de cadeia média, palmitatos, miristatos e octildodecanol.

[0040]Lipídeos sólidos adequados para o uso na presente invenção incluem, mas não se limitam a, triglicerídeos (triestearina, tricaprina, trilaurina, trimiristina, tripalmitina), ácidos graxos (ácido esteárico), álcoois graxos (álcool cetílico, álcool estearílico), ceras (mateiga de cacau, cera de carnaúba, cera de abelhas, palmitato de cetila), glicídeos parciais (monoestearato de glicerila, behenato de glicerila, palmitoestearato de glicerila, tripalmitato de glicerila, trimiristato de glicerila, triestearato de glicerila) e/ou mistura destes.

[0041]Em uma realização preferencial os lipossomas descritos na presente invenção compreendem o uso de fosfatidilcolina de ovo (2,0% p/p a 10,0% p/p).

[0042]Em uma realização preferencial as nanoemulsões descritas na presente invenção compreendem o uso de uma mistura na proporção de 1:4 de lecitina de ovo e triglicerídeos de cadeia média (2,0% p/p a 10,0% p/p).

[0043]Em uma realização preferencial as nanopartículas lipídicas sólidas descritas na presente invenção compreendem o uso de monoestearato de glicerila (2,0% p/p a 10,0% p/p).

[0044]Em uma realização preferencial os carreadores lipídicos nanoestruturados descritos na presente invenção compreendem o uso de uma mistura na proporção 7:3 de monoestearato de glicerila e triglicerídeos de cadeia média (2,0% p/p a 10,0% p/p).

Tensoativos hidrofílicos:

[0045]Os tensoativos hidrofílicos adequados para o uso na presente invenção incluem os surfactantes aniônicos, não aniônicos, catiônicos e anfóteros.

[0046]Preferencialmente, os surfactantes da presente invenção podem ser escolhidos de grupo que compreende, sem, contudo limitar, surfactantes não iônicos como polissorbato 20, polissorbato 40, polissorbato 80, monoestearato de sorbitano 20, monoestearato de sorbitano 40, monoestearato de sorbitano 60, monoestearato de sorbitano 80, emulsificantes colato de sódio, deoxicolato de sódio, glicolato de sódio, poloxâmeros, taurocolato de sódio, taureodexicolato de sódio, e/ou mistura destes.

[0047]Em uma realização preferencial as formulações descritas na presente invenção compreendem o uso de polissorbarto 80 (1,0% p/p a 5,0% p/p).

Solventes orgânicos:

[0048]Solventes orgânicos adequados para o uso na presente invenção incluem, mas não se limitam a, solventes orgânicos polares próticos e apróticos, como por exemplo etanol, acetona e/ou mistura desses.

[0049]Em uma realização preferencial os lipossomas e as nanoemulsões da presente invenção compreendem o uso de etanol para a solubilização dos componentes da fase lipídica.

Obtenção das nanoestruturas lipídicas:

[0050]Os processos de obtenção das nanoemulsões e lipossomas compreendem as etapas de:

a) Dissolver, em uma solução orgânica composta por um solvente orgânico polar prótico, um solvente orgânico polar aprótico e/ou mistura solvente orgânico polar prótico:solvente orgânico polar aprótico (1:1):

i) de 2,0% p/p a 10,0% p/p de fase lipídica;

ii) 1,0% p/p de fração de isoflavonas agliconas da soja, em relação à fase lipídica;

b) Dissolver em uma solução aquosa:

iii) 1,0% p/p a 5,0% p/p de tensoativo;

c) Adicionar a solução orgânica na solução aquosa, sob agitação;

d) Evaporar o solvente orgânico;

e) Homogeneizar a formulação em homogeneizador à alta pressão, com 10 ciclos de 500 a 750 bar cada.

[0051]O processo de obtenção das nanoestruturas lipídicas sólidas e carreadores lipídicos nanoestruturados compreende as etapas de:

a) Dissolver a uma temperatura entre 30º C a 80º C:

i) de 2,0% p/p a 10,0% p/p de fase lipídica;

ii) 1,0% p/p de fração de isoflavonas agliconas da soja, em relação à fase lipídica;

b) Dissolver em uma solução aquosa, com temperatura de 30º C a 80º C:

iii) de 1,0% p/p a 5,0% p/p de tensoativo;

c) Adicionar a solução aquosa na solução oleosa, sob agitação e com temperatura de 30º C a 80º C;

d) Agitar no ultra-turrax, sob aquecimento de 30º C a 80º C, durante período compreendido de 30 segundos a 5 minutos;

e) Homogeneizar a formulação em homogeneizador à alta pressão, com 10 ciclos de 500 a 750 bar cada.

Formulações compreendendo os carreadores lipídicos

[0052]As formulações da presente invenção são formulações que compreendem as nanoestruturas lipídicas, associadas a excipientes adequados, úteis nas áreas alimentar, farmacêutica e cosmética.

[0053]Os carreadores da presente invenção podem estar sob a forma de nanoemulsões, lipossomas, nanopartículas lipídicas sólidas ou carreadores lipídicos nanoestruturados.

Caracterização das nanopartículas lipídicas:

[0054]A formação de nanopartículas foi primeiramente confirmada pela evidência de caráter homogêneo (sem separação de fases) e pela não ocorrência de precipitados. A seguir, as formulações foram caracterizadas de acordo com o diâmetro médio de gotícula/partícula, índice de polidispersão, potencial zeta, e teor.

[0055]Determinação do diâmetro de gotícula/partícula e do índice de polidispersão (IPD):

[0056]As formulações foram caracterizadas através do espalhamento de luz dinâmico pela difusão de raio laser monocromático que atravessa a dispersão coloidal. Essa determinação foi realizada observando-se o espalhamento a 173º C após diluição das amostras em água purificada, previamente filtrada em membrana de 0,22 µm. Os resultados foram expressos como média de três determinações independentes.

Determinação do potencial zeta:

[0057]O potencial zeta foi determinado através da mobilidade eletroforética das gotículas. As medidas foram realizadas após calibração com uma solução padrão a – 55 mV (látex poliestireno carboxilato). Todas as análises foram realizadas após a diluição das amostras em água purificada, previamente filtrada em membrana de 0,22 µm. Os resultados foram expressos como média de três determinações independentes.

Teor e taxa de associação:

[0058]O teor de isoflavonas agliconas (expressos como somatório de genisteína, gliciteína e daidzeína) foi efetuado por cromatografia líquida de alta eficiência. O método utilizado realiza a separação das isoflavonas em coluna cromatográfica Phenomenex RP-18 (Synergi fusion 150 x 4,6 mm diâmetro interno; tamanho de partícula 4 µm), conectada a uma pré-coluna C18. A fase

móvel é constituída por uma Fase A (acetonitrila acidificada com ácido trifluroacético 0,01%) e uma Fase B (água acidificada com ácido trifluroacético 0,1). A eluição é realizada em um sistema gradiente de: 20 – 25% B (0 – 10 min), 25 – 30% B (10 – 15 min), 30 – 35% B (15 – 23 min), 35 – 100% B (23 – 26 min), 20% B (26 – 30 min). O fluxo é mantido a 1,0 mL/min, com volume de injeção de 10 µL, detecção a 260 nm e a temperatura da coluna é mantida a 40º C. O método foi validado conforme guias internacionais e apresentou-se linear na faixa de 0,1 a 10 µg/mL, além de preciso e exato. Todos os componentes das formulações foram avaliados para análise de especificidade, e o método mostrou-se específico para quantificação das isoflavonas agliconas em todas as condições avaliadas.

[0059]A taxa de associação foi realizada através da ultrafiltração/centrifugação das formulações e posterior avaliação de teor de isoflavonas presente na fase aquosa inferior do ultrafiltrado. Para tanto uma amostra foi submetida ao processo de filtração em membranas de ultrafiltração de 10 kDa durante 30 minutos de centrifugação a uma força de 5000 g.

Exemplo 1: Nanocarreador lipídico consistindo de uma nanoemulsão.

Composição final:

[0060]Fase lipídica

a. 8,0% p/p Triglicerídeos de cadeia média
b. 2,0% Lecitina de ovo
c. 1,0 mg/mL Fração de isoflavonas agliconas da soja

[0061]Fase aquosa

a. 1,0% de Polissorbato 80

Procedimento:

[0062]Primeiramente, os componentes da fase lipídica são pesados e dissolvidos em etanol absoluto, com agitação constante. Os componentes da fase aquosa são pesados e dissolvidos em água purificada, com agitação constante. A proporção fase aquosa: fase orgânica deve ser 2:1. A fase

orgânica é vertida lentamente com auxílio de uma seringa sobre a fase aquosa, mantendo-se uma agitação constante durante 15 minutos. A formulação é rota evaporada, para eliminação do solvente orgânico e até a redução ao volume final desejado. Ao final do processo, a formulação é então homogeneizada em homogeneizador a alta pressão com 10 ciclos de 500 bar cada, a fim de manter os diâmetros de partícula da fase oleosa o menor possível e com menor índice de polidispersão.

[0063]Produto obtido: Nanoemulsão.

Resultados:

[0064]Tamanho: 166 nm

[0065]IPD: 0,10

[0066]Zeta: -30,1 mV

[0067]Teor: 0,92 mg/mL de isoflavonas agliconas totais, apresentando uma taxa de associação de 100%.

Exemplo 2: Nanocarreador lipídico consistindo de um lipossoma.

Composição:

[0068]Fase orgânica

a. 2% p/p Fosfatidilcolina de ovo
b. 0,2 mg/mL Fração de isoflavonas agliconas da soja

[0069]Fase aquosa

a. 1,0% p/p Polissorbato 80

Procedimento:

[0070]Primeiramente, os componentes da fase lipídica são pesados e dissolvidos em etanol absoluto, com agitação constante. Os componentes da fase aquosa são pesados e dissolvidos em água purificada, com agitação constante. A proporção fase aquosa: fase orgânica deve ser 2:1. A fase orgânica é vertida lentamente com auxílio de uma seringa sobre a fase aquosa, mantendo-se uma agitação constante durante 15 minutos. A formulação é rota evaporada, para eliminação do solvente orgânico e até a redução ao volume

final desejado. Ao final do processo, a formulação é então homogeneizada em homogeneizador a alta pressão com 10 ciclos de 500 bar cada, a fim de manter os diâmetros de partícula da fase oleosa o menor possível e com menor índice de polidispersão.

[0071]Produto obtido: Lipossoma.

Resultados:

[0072]Tamanho: 94 nm

[0073]IPD: 0,017

[0074]Zeta: -4,05 mV

[0075]Teor: 0,156 mg/mL de isoflavonas agliconas totais.

Exemplo 3: Nanocarreador lipídico consistindo de uma nanopartícula lipídica sólida.

Composição:

[0076]Fase orgânica

a. 2% p/p Monoestearato de glicerila
b. 0,2 mg/mL Fração de isoflavonas agliconas da soja

[0077]Fase aquosa

a. 1,0% p/p Polissorbato 80

Procedimento:

[0078]Primeiramente, os componentes da fase lipídica são pesados e dissolvidos a uma temperatura de 80º C, com agitação constante. Os componentes da fase aquosa são pesados e dissolvidos para um volume final de 100 mL de água purificada, com agitação constante a uma temperatura de 80º C. A fase aquosa é vertida sobre a fase lipídica, mantendo-se a temperatura a 80º C e com agitação constante durante 15 minutos. A formulação é misturada em ultra-turrax durante 1 minuto, a uma velocidade de 13.500 rpm e temperatura de 80º C. Ao final do processo, a formulação é então homogeneizada a quente em homogeneizador a alta pressão com 10 ciclos de 750 bar cada, a fim de manter os diâmetros de partícula da fase oleosa o

menor possível e com menor índice de polidispersão. O produto é deixado em repouso a uma temperatura ambiente por no mínimo 10 minutos.

[0079]Produto obtido: Nanopartícula lipídica sólida.

Resultados:

[0080]Tamanho: 396 nm

[0081]IPD: 0,60

[0082]Zeta: -22,20 mV

[0083]Teor: 0,166 mg/mL de isoflavonas agliconas totais.

Exemplo 4: Nanocarreador lipídico consistindo de um carreador lipídico nanoestruturado.

Composição:

[0084]Fase orgânica

a. 1,4% p/p Monoestearato de glicerila
b. 0,6% p/p Triglicerídeos de cadeia média
c. 0,2 mg/mL Fração de isoflavonas agliconas da soja

[0085]Fase aquosa

a. 1,0% p/p Polissorbato 80

Procedimento:

[0086]Primeiramente, os componentes da fase lipídica são pesados e dissolvidos a uma temperatura de 80º C, com agitação constante. Os componentes da fase aquosa são pesados e dissolvidos para um volume final de 100 mL de água purificada, com agitação constante a uma temperatura de 80º C. A fase aquosa é vertida sobre a fase lipídica, mantendo-se a temperatura a 80º C e com agitação constante durante 15 minutos. A formulação é misturada em ultra-turrax durante 1 minuto, a uma velocidade de 13.500 rpm e temperatura de 80º C. Ao final do processo, a formulação é então homogeneizada a quente em homogeneizador a alta pressão com 10 ciclos de 750 bar cada, a fim de manter os diâmetros de partícula da fase oleosa o

menor possível e com menor índice de polidispersão. O produto é deixado em repouso a uma temperatura ambiente por no mínimo 10 minutos.

[0087]Produto obtido: Carreador lipídico nanoestruturado.

Resultados:

[0088]Tamanho: 220 nm

[0089]IPD: 0,451

[0090]Zeta: -13,36 mV

[0091]Teor: 0,156 mg/mL de isoflavonas agliconas totais.

Reivindicações

1. Carreadores lipídicos de tamanho nanométrico **caracterizados por** compreenderem uma fração enriquecida de isoflavonas agliconas da soja incorporadas em carreadores lipídicos de tamanho nanométrico.

2. Carreadores lipídicos de acordo com a reivindicação 1, **caracterizados pelo** diâmetro de gotícula/partícula médio estar compreendido na faixa que vai de 0,01 a 1,0 micrômetro.

3. Carreadores lipídicos, de acordo com a reivindicação 1, **caracterizados pela** fração enriquecida de isoflavonas agliconas da soja compreender majoritariamente daidzeína, genistéina e gliciteína.

4. Carreadores lipídicos, de acordo com qualquer uma das reivindicações de 1 a 3, **caracterizados pela** fração enriquecida de isoflavonas agliconas da soja ser obtida a partir de extrato de soja com posterior etapas de hidrólise e purificação.

5. Carreadores lipídicos, de acordo com qualquer uma das reivindicações de 1 a 4, **caracterizados pela** fração enriquecida de isoflavonas agliconas da soja ser obtida comercialmente.

6. Processo de produção de carreadores lipídicos **caracterizado por** compreender etapas de:

a) Dissolver, em um solvente orgânico:

i) de 2,0% p/p a 10,0% p/p de fase lipídica;

ii) 1,0% p/p de fração de isoflavonas agliconas da soja, em relação à fase lipídica;

b) Dissolver, em uma solução aquosa,:

iii) 1,0% p/p a 5,0% p/p de tensoativo;

c) Adicionar a solução orgânica da etapa a) na solução aquosa da etapa b), sob agitação;

d) Evaporar o solvente orgânico; e

e) Homogeneizar a formulação em homogeneizador à alta pressão, com 10 ciclos de 500 a 750 bar cada.

7. Processo de produção de carreadores lipídicos **caracterizado por** compreender as etapas de:

a) Dissolver a uma temperatura entre 30º C a 80º C,:

i) de 2,0% p/p a 10,0% p/p de fase lipídica;

ii) 1,0% p/p de fração de isoflavonas agliconas da soja, em relação à fase lipídica;

b) Dissolver, em uma solução aquosa, com temperatura de 30º C a 80º C:

iii) de 1,0% p/p a 5% p/p de tensoativo;

c) Adicionar a solução aquosa da etapa a) na solução oleosa da etapa b), sob agitação e com temperatura de 30º C a 80º C;

d) Agitar no ultra-turrax, sob aquecimento de 30º C a 80º C, durante período compreendido de 30 segundos a 5 minutos; e

e) Homogeneizar a formulação em homogeneizador à alta pressão, com 10 ciclos de 500 a 750 bar cada.

8. Processo de produção, de acordo com as reivindicações 6 ou 7, **caracterizado pela** fração de isoflavonas agliconas da soja compreender daidzeína, genistéina e gliciteína.

9. Processo de produção, de acordo com a reivindicação 8, **caracterizado pela** fração de isoflavonas agliconas da soja compreender uma faixa de concentração de 0,2 a 1,0 mg/mL.

10. Processo de produção, de acordo com a reivindicação 8, **caracterizado pela** fração de isoflavonas agliconas da soja ser obtida a partir de extrato de soja com posterior etapas de hidrólise e purificação.

11. Processo de produção, de acordo com a reivindicação 8, **caracterizado pela** fração de isoflavonas agliconas da soja ser obtida comercialmente.

12. Processo de produção, de acordo com as reivindicações 6 ou 7, **caracterizado pelo** solvente orgânico ser escolhido do grupo que compreende solventes orgânicos polares próticos, solventes orgânicos polares apróticos e mistura dos mesmos.

13. Processo de produção, de acordo com a reivindicação 12, **caracterizado pelo** solvente orgânico polar prótico ser etanol, e o solvente orgânico polar aprótico ser acetona.

14. Processo de produção, de acordo com as reivindicações 6 ou 7, **caracterizado pela** fase lipídica compreender de 2,0% p/p a 10,0% p/p de lipídeos sólidos, líquidos e/ou mistura dos mesmos

15. Processo de produção, de acordo com a reivindicação 14, **caracterizado pela** fase lipídica ser escolhida do grupo que compreende:

a) lipídeos sólidos tais como triestearina, tricaprina, trilaurina, trimiristina, tripalmitina, ácido esteárico, álcool cetílico, álcool estearílico, mateiga de cacau, cera de carnaúba, cera de abelhas, palmitato de cetila, monoestearato de glicerila, behenato de glicerila, palmitoestearato de glicerila, tripalmitato de glicerila, trimiristato de glicerila, triestearato de glicerila e/ou mistura destes;

b) lipídeos líquidos tais como oleato de decila, isohexadecano, ésteres do ácido esteárico e/ou oléico, etanolamida de ácido graxo de coco, óleos naturais, como o óleo de milho, amendoim, sésamo, oliva, jojoba, soja, álcool graxo, parafina, triglicerídeos de cadeia média, palmitatos, miristatos e octildodecanol; e

c) tensoativos lipofílicos tais como lecitinas e fosfolipídeos e/ou mistura dos mesmos.

16. Processo de produção, de acordo com as reivindicações 6 ou 7, **caracterizado pela** fase aquosa compreender de 1,0% p/p a 5,0% p/p de tensoativo hidrofílico.

17. Processo de produção, de acordo com a reivindicação 16, **caracterizado pelo** tensoativo hidrofílico ser escolhido do grupo que compreende polissorbatos, monoestearatos de sorbitano, colato de sódio, deoxicolato de sódio, glicolato de sódio, poloxâmeros, taurocolato de sódio, taureodexicolato de sódio, e/ou mistura destes.

18. Formulação compreendendo carreadores lipídicos caracterizada pelo carreador lipídico ser conforme definido nas reivindicações 1 a 5.

Resumo

CARREADORES LIPÍDICOS DE TAMANHO NANOMÉTRICO COMPREENDENDO FRAÇÃO ENRIQUECIDA DE ISOFLAVONAS AGLICONAS DA SOJA, PROCESSO DE OBTENÇÃO DOS MESMOS E FORMULAÇÕES COMPREENDENDO OS MESMOS

A presente invenção descreve carreadores lipídicos de diâmetro médio de gotícula/partícula inferior a 1,0 micrômetro compreendendo uma fração enriquecida de isoflavonas agliconas da soja para uso alimentar, farmacêutico e/ou cosmético. A presente invenção também descreve os processos de obtenção das nanoestruturas por processo de mistura de uma fase orgânica e uma fase oleosa sob agitação, com posterior evaporação do solvente orgânico, e etapa final de homogeneização à alta pressão. As formulações compreendem tais nanoemulsões, lipossomas, nanopartículas lipídicas sólidas ou carreadores lipídicos nanoestruturados.