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RAMON SOUSA BARROS FERREIRA

APROVEITAMENTO DE OKARA, RESÍDUO DA PRODUÇÃO DE EXTRATO DE SOJA, PARA EXTRAÇÃO DE ISOFLAVONAS USANDO *GREEN SOLVENTS*

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Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Engenharia de Alimentos

Thesis presented to the Faculty of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor, in Food Engineering.

Orientador: Prof. Dr. Eduardo Augusto Caldas Batista

ESTE TRABALHO CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELO ALUNO RAMON SOUSA BARROS FERREIRA, E ORIENTADA PELO PROF. DR. EDUARDO AUGUSTO CALDAS BATISTA

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Dedico este trabalho primeiramente a DEUS,
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RESUMO

O okara, resíduo sólido da extração de soja, é rico em fitoquímicos como ácidos fenólicos e isoflavonas, que são associadas a diversos benefícios à saúde. Este estudo buscou otimizar a extração de isoflavonas do okara usando Deep Eutectic Solvents (DES) e *Bio-based Solvents*. Na primeira etapa, diferentes DES com base em cloreto de colina ([Ch]Cl) foram investigados como solventes alternativos para a extração de flavonoides de soja e okara, utilizando o COSMO-SAC para seleção de solventes. O DES [Ch]Cl: ácido acético (1:2) com 30% de água destacou-se, resultando em elevados teores de flavonoides totais em soja e okara. As isoflavonas daidzeína, genisteína, gliciteína, daidzina, genistina e glicitina foram identificadas e quantificadas nos extratos. A segunda etapa avaliou os efeitos de diferentes condições na preparação de deep eutectic solvents (DES) com base em [Ch]Cl: ácido acético, [Ch]Cl: ácido fórmico e [Ch]Cl: ácido lático, focando na degradação desses DES. O estudo investigou os impactos da temperatura, tempo e concentração de água na esterificação do [Ch]Cl. Temperaturas elevadas, especialmente para o DES [Ch]Cl: ácido fórmico sem água adicional, intensificaram a reação de esterificação, apresentado aproximadamente 37 % em mol de ésteres a 80°C. A terceira etapa avaliou as condições operacionais de extração de isoflavonas, utilizando um planejamento fracionário e um delineamento composto central rotacional (DCCR) para otimização. O estudo alcançou a extração máxima de 1176,81 µg isoflavonas totais/g de soja seca e 450,9 µg/g de okara seco usando o DES ChCl: ácido acético. Após otimização, foi feita a análise dos precursores de DES na extração de isoflavonas e foi observado que o ácido acético + 70% de água foi 50% mais eficaz na extração de isoflavonas de okara do que o DES [Ch]Cl: ácido acético + 70% de água. A eficácia do ácido acético na extração de isoflavonas de okara abriu caminho para explorar outros solventes alternativos como os Bio-based Solvents formados por ácidos carboxílicos, polióis, gama-valerolactona e acetato de etila. No total, foram investigados 26 *Bio-based solvents* para extração de compostos fenólicos e isoflavonas de okara e as condições oprecaionais com o melhor solvente foram otimizadas. Glicerol adicionado de 40% de água de destacou como melhor Bio-based solvent extraindo aproximadamente 45.0 mg GAE/100 mL de extrato and 60 µg de isoflavonas totais/mL de extrato. Em conclusão, foi observado que o modelo COSMO-SAC foi uma ferramenta útil na seleção de solventes, poupando tempo e custos; com relação a degradação dos DES é necessária uma avaliação mais detalhada do impacto do teor de éster nas propriedades do DES, no desempenho da aplicação e noutros fatores relevantes; pode-se sugerir também, que o DES [Ch]Cl: ácido acético e o glycerol aquoso podem ser alternativas sustentáveis para a extração de isoflavonas de soja e okara, respectivamente.

Palavras-chave: Solventes Eutéticos Profundos, resíduo de soja, okara, solventes verdes, flavonoides, isoflavonas

ABSTRACT

The okara, a solid residue from soybean extraction, is rich in phytochemicals such as phenolic acids and isoflavones, which are associated with various health benefits. This study aimed to optimize the extraction of isoflavones from okara using Deep Eutectic Solvents (DES) and Bio-based Solvents. In the first stage, different DES based on choline chloride ([Ch]Cl) were investigated as alternative solvents for the extraction of flavonoids from soybean and okara, using COSMO-SAC model for solvent selection. The [Ch]Cl: acetic acid (1:2) DES with 30% water stood out, resulting in high levels of total flavonoids in soybean and okara. The isoflavones daidzein, genistein, glycitein, daidzin, genistin, and glycitin were identified and quantified in the extracts. The second stage evaluated the effects of different conditions on the preparation of Deep Eutectic Solvents (DES) based on [Ch]Cl: acetic acid, [Ch]Cl: formic acid, and [Ch]Cl: lactic acid, focusing on the degradation of these DES. The study investigated the impacts of temperature, time, and water concentration on the esterification of [Ch]Cl. High temperatures, especially for DES [Ch]Cl: formic acid without additional water, intensified the esterification reaction, yielding approximately 37% mol of esters at 80°C. The third stage evaluated the operational conditions of isoflavone extraction, using a Fractional Factorial Design and a Central Composite Rotatable Design (CCRD) for optimization. The study achieved maximum extraction of 1194.56 µg total isoflavones/g of dry soy and 450.9 µg/g of dry okara using the DES ChCl: acetic acid. After optimization, the analysis of DES precursors in isoflavone extraction revealed that acetic acid + 70% water was 50% more effective in extracting okara isoflavones than [Ch]Cl: acetic acid + 70% water DES. The effectiveness of acetic acid in okara isoflavone extraction paved the way to explore other alternative solvents such as Bio-based Solvents formed by carboxylic acids, polyols, gamma-valerolactone, and ethyl acetate. In total, 27 Bio-based Solvents were investigated for the extraction of phenolic compounds and isoflavones from okara, and the operational conditions with the best solvent were optimized. Glycerol with 40% water added stood out as the best Bio-based Solvent, extracting approximately 45.0 mg GAE/100 mL of extract and 60 µg of total isoflavones/mL of extract. In conclusion, it was observed that the COSMO-SAC model was a useful tool in solvent selection, saving time and costs; regarding DES degradation, a more detailed evaluation of the impact of ester content on DES properties, application performance, and other relevant factors is necessary. It can also be suggested that [Ch]Cl: acetic acid DES and aqueous glycerol may be sustainable alternatives for soy and okara isoflavone extraction, respectively.

Key words: Deep Eutectic Solvents, soybean residue, okara, flavonoids, green solvents, isoflavones.

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CAPÍTULO 1

-INTRODUÇÃO E OBJETIVOS GERAL E ESPECÍFICOS-

1.1 INTRODUÇÃO

O okara é o resíduo sólido obtido após a extração do extrato de soja que é utilizado para produção de bebidas à base de soja ou tofu (COLLETTI *et al.*, 2020; ZHANG *et al.*, 2021). Em 2018, o mercado global de extrato de soja foi avaliado em US$ 7,30 bilhões e projeções estimam que chegue em US$ 11,08 bilhões até 2025 (GVR, 2019a, 2019b). Esse crescimento significativo está ocorrendo devido ao aumento da conscientização sobre os benefícios que trazem para a saúde humana os alimentos e bebidas à base de soja (GVR, 2019b). Dados estatísticos da produção de extrato de soja são escassos na literatura. Em 2006, foi estimada que cerca de 1 milhão de toneladas de extrato de soja tenha sido produzida na Europa Ocidental, América do Norte e Japão (COLLETTI *et al.*, 2020). Segundo Colletti et al. (2020), aproximadamente 170 mil toneladas de okara podem ser produzidas a partir de 1 milhão de toneladas de extrato de soja com teor de proteína de 3,5 %.

O okara pode ser adicionado na alimentação humana (LEE; GAN; KIM, 2020; VOSS *et al.*, 2021)(JANKOWIAK *et al.*, 2014b), mas em sua totalidade, é destinado à alimentação animal ou descartado como resíduo, devido à sua alta umidade (70-80%), que o torna muito perecível e de baixo valor econômico (DE OLIVEIRA, 2016; VONG; LIU, 2016; YU; YANG, 2019). No entanto, esse resíduo contém nutrientes valiosos para a saudabilidade alimentar humana, que incluem, ácidos fenólicos e, principalmente, isoflavonas (YU; YANG, 2019).

As isoflavonas, estão relacionadas a alívios de várias doenças, como, alívio de sintomas da menopausa, redução do risco de morte por câncer de mama e próstata, redução dos níveis de colesterol, osteoporose, obesidade e diabetes (VITALE *et al.*, 2013)(ZAHEER; HUMAYOUN AKHTAR, 2017)(HSIAO; HO; PAN, 2020). Dessa forma, os compostos bioativos do okara, o tornam um resíduo interessante para extração de antioxidantes e aplicação em produtos que promovam o bem estar humano (ALVAREZ *et al.*, 2019; NKURUNZIZA *et al.*, 2019).

A inclusão desses fenólicos em produtos de alto valor agregado requerem técnicas de extração que sejam eficientes e econômicas. Diversos métodos de extração de compostos fenólicos em produtos, subprodutos e resíduos de soja foram otimizados por diversos pesquisadores (KUMAR *et al.*, 2021).

Dentre os métodos utilizados, os por extração convencionais, como extração em leito fixo e maceração com solventes orgânicos ainda são os mais utilizados, por possuir facilidade de operação e baixo custo. No entanto, contam com longos tempos de extração (2-12 h), degradação de compostos fenólicos e o uso de grandes quantidades de solventes perigosos, como metanol, acetona, hexano e acetonitrila, tornando os extratos fenólicos perigosos para que serem usados como ingrediente alimentar (KUMAR *et al.*, 2021)(YAHYA; ATTAN; WAHAB, 2018).

Devido às preocupações com o meio ambiente e a saúde humana, novas técnicas de extração estimuladas pela expansão do que é chamado de química verde têm surgido e estão sendo consideradas ecológicas para substituir os métodos de extração convencionais com solventes orgânicos tóxicos (SHARMILA *et al.*, 2016).

Do ponto de vista da sustentabilidade, um solvente favorável não deve apenas ter a alta capacidade para os compostos alvo, mas também, ser barato e seguro (YU; YANG, 2019). Os *Deep Eutetic Solvents* (DES, em português: solventes eutéticos profundos) são um novo tipo de solvente de fácil preparação, podem ser econômicos e renováveis (SHANG *et al.*, 2019). Além disso, a literatura os caracteriza como não tóxicos, pois partem de misturas contendo ingredientes seguros à base de colina, que é considerada um ingrediente seguro e desempenha importantes funções fisiológicas no organismo, e também, açucares, poliálcoois, ácidos carboxílicos e aminoácidos (BAJKACZ; ADAMEK, 2017; DAI *et al.*, 2013).

Dessa forma, quando se fala em DES obtidos a partir de fontes seguras, parece lógico que a sua toxicidade seja desprezível, no entanto, essa afirmação as vezes é baseada na avaliação isolada de cada componente que compõe o DES, não sendo levado em consideração os efeitos sinérgicos de tal combinação (GULLÓN *et al.*, 2020). Por essa razão, antes do desenvolvimento de um produto e ou; aplicação industrial, é fundamental que uma avaliação crítica de uma possível toxicidade desses solventes seja feita (HAYYAN *et al.*, 2015).

Até o momento, 5 pesquisas utilizando DES para extrair isoflavonas foram encontradas nas bases de dados *Scopus* e *Web of Science*, do quais, um envolve uso de produtos de soja (BAJKACZ; ADAMEK, 2017; CUI *et al.*, 2015; HUANG, Y. *et al.*, 2021; LI, L. *et al.*, 2016; SHANG *et al.*, 2019). No entanto, o estudo feito por Bajkacz e Adamek (2017) para extração de isoflavonas de produtos de soja foi voltado para o desenvolvimento de um método UHPLC-UV para determinação dos componentes e não para avaliar eficiência de extração. Até o momento, nenhum trabalho envolvendo o uso de DES e okara para extração de fenólicos foi relatado na literatura.

Com relação aos estudos sobre toxicidade de DES, são escassos os trabalhos e apenas Benlebna et al. (2018) avaliaram a influência de extrato rico em polifenóis em ratos Wistar. No entanto, os resultados não foram promissores, pois, segundo o autor, as doses de DES (betaína:glicerol, 1:2) fornecidas aos ratos foi muita alta, sendo fornecidas 6 mL de DES/dia/rato durante 14 dias. Dessa forma, este estudo buscou aprofundar os estudos para assim, encontrar um solvente que não só seja seguro para o meio ambiente, mas também para animais e humanos. Se isto se confirmar, o extrato não precisará ser purificado, reduzindo perdas nos componentes bioativos e no custo do processo.

Para atingir tais objetivos, a escolha do DES que possua alta afinidade com os compostos de interesse é necessária, para isso, um conjunto de DES foi avaliado para determinação do solvente com maior afinidade pelo analito, seguido da otimização utilizando Metodologia de Superfície de Resposta e análise da citotoxicidade do extrato.

1.2 OBJETIVOS

1.2.1 Objetivo Geral

Este trabalho avaliou e otimizou a extração de isoflavonas de okara (resíduo de soja da produção de extrato de soja e tofu) utilizando *Deep Eutectic Solvents* (DES) e *Bio-based Solvents*.

1.2.2 Objetivos específicos

Os objetivos específicos avaliados foram:

- Avaliou-se a eficiência de um conjunto de DES, com diferentes polaridades, viscosidades e composições na extração de isoflavonas de okara através de triagem computacional usando o modelo COSMO-SAC seguida de validação experimental;
- Foi Investigado o tempo de extração das isoflavonas com o melhor DES;
- Realizou-se uma avalição prévia das variáveis temperatura (°C), teor de água no DES (%), razão sólido:líquido (S/L, mg/mL) e velocidade de agitação (rpm) utilizando o Delineamento fracionário 2^{4-1};
- Foi avaliado os efeitos das variáveis significativas do planejamento anterior utilizando Delineamento Composto Central Rotacional (DCCR);
- Otimizou-se as variáveis independentes para a máxima extração de isoflavonas utilizando a análise de superfícies de resposta e validar experimentalmente o modelo no ponto ótimo;
- Avaliou-se a citotoxicidade do melhor DES e do extrato obtido na condição otimizada;
- Foi avaliado um conjunto de *Bio-based solvents* na extração de isoflavonas de okara otimizando as condições aplicando-se um DCCR (2^3 + 6 pontos axiais + 5 repetições no ponto central) com o melhor solvente obtido.

CAPÍTULO 2

-REVISÃO BIBLIOGRÁFICA-

2 REVISÃO BIBLIOGRÁFICA

2.1 Soja

O conhecimento referente à existência da soja (*Glycine max*) data de mais de 5.000 anos (QI *et al.*, 2021). Essa matéria-prima de milênios atrás eram plantas rasteiras que se desenvolviam ao longo de rios e lagos, diferindo bastante da soja atual. O processo de domesticação da soja aconteceu principalmente na região oriental do Norte da China no século XI A. C., a partir de cruzamentos naturais realizados por cientistas chineses (APROSOJA BRASIL, 2018).

No início do século XX, precisamente, durante as três primeiras décadas, a soja foi produzida em grande parte no Oriente; China, Indonésia, Japão e a antiga Coreia foram os maiores produtores. Porém, entre o final da década de 40 e o início dos anos 1950, os EUA ultrapassaram a China e todo o Oriente na produção de soja (HYMOWITZ, 1970).

A soja tornou-se uma cultura de importância econômica global e está listada como a principal cultura do continente americano em termos de área de plantio (QI *et al.*, 2021). Em 2019/2020, conforme mostrado na Tabela 2.1, a produção mundial de soja foi de 336,46 mil toneladas; e o Brasil, EUA e a Argentina são os três maiores produtores, nesta ordem. Juntos, os países respondem por 80,7 % da produção mundial de soja (USDA, 2021).

Tabela 2.1. Produção mundial de soja

Países	2018/19	2019/20	2020/21	Exportou
			Fevereiro	
Brasil	119.700	126.000	133.000	85.000
Estados Unidos	120.515	96.667	112.459	61.235
Argentina	55.300	48.800	48.000	7.000
China	15.967	18.100	19.600	-
Índia	10.930	9.300	10.500	-
Paraguai	8.512	9.900	10.250	6.300
Canadá	7.417	6.145	6.350	4.200
Outros	22.695	21.546	20.830	5.953
Total	361.036	336.458	360.989	169.688

Fonte: USDA (2021). Elaborado por: próprio autor.

A soja é comercializada na forma de farelo de soja, óleo de soja e grãos de soja e pode ser usada em muitos produtos industriais, incluindo sabão, cosméticos, resinas, plásticos, tintas, lápis de cor, solventes e roupa (CHEN *et al.*, 2012; MAPA, 2021).

No Brasil, 80 % do farelo de soja é destinado para compor a fabricação de ração animal. Já o óleo de soja é destinado para produção de óleo comestível e para a produção de biodiesel, onde cerca de 71 % da produção nacional vem da soja (APROSOJA, 2018; BIODIESELBR, 2019).

A soja pode ser utilizada para o desenvolvimento de vários alimentos. No oriente, ela é utilizada na forma fermentada para fazer missô (pasta de soja fermentada), shoyu (molho de soja), natto (queijo de soja fermentado) e tempeh (bolo de soja fermentado); na forma liquida, como extrato de soja, e na forma coalhada como o tofu (coalhada de soja). Além disso, pode ser comercializada em pó e usada em diversos outros alimentos que fazem parte da vida diária dos asiáticos por séculos (ZHANG *et al.*, 2021).

Já no Ocidente, especificamente no continente americano, além do óleo de soja; extrato de soja, shoyu, missô, proteína texturizada e tofu são os alimentos mais populares (CHEN, K. I. *et al.*, 2012). Alguns desses alimentos, como a proteína texturizada de soja e o tofu, tornaram-se uma fonte de substituição aos produtos cárneos e consumidos principalmente entre os vegetarianos devido ao seu alto teor de proteínas; 50% e 8%, respectivamente (ZHANG *et al.*, 2021).

Uma vez que, dentre todos os produtos obtidos a partir da soja, este projeto tem por objetivo trabalhar apenas com o subproduto ou resíduo da produção de extrato de soja chamado de okara, a próxima sessão se concentrará apenas em falar sobre a produção do resíduo.

2.2 Extrato de soja e okara

O aumento da conscientização sobre os benefícios para a saúde associados ao consumo de soja, o conhecimento de alergias associadas ao consumo de leite de vaca e um movimento relacionado à produção de alimentos mais sustentáveis fizeram a quantidade de produtos derivados de soja aumentar (HADJ SAADOUN *et al.*, 2021). O extrato de soja é um desses produtos e o seu consumo tem aumentando em muitos países e junto com eles têm aumentando também o seu principal subproduto, o okara (COLLETTI *et al.*, 2020; HADJ SAADOUN *et al.*, 2021).

Até o momento, foram feitas buscas extensas sobre dados estatísticos da produção de extrato de soja e essas informações ainda são escassas. No entanto, algumas informações foram encontradas na literatura em um artigo de revisão publicado por Colletti et al. (2020). Segundo estes autores, em 1993, aproximadamente 1 milhão de toneladas de extrato de soja foram produzidas mundialmente. Em 2006, considerando apenas a Europa Ocidental, América do

Norte e Japão, a produção foi estimada em cerca de 1 milhão de toneladas. O extrato de soja é geralmente produzido na proporção de soja para água de 1:5; bebidas adoçadas e aromatizadas podem ser feitas em uma proporção de grãos para água de até 1:20. Pode-se estimar que aproximadamente 170 mil toneladas de okara sejam produzidas a partir de 1 milhão de toneladas de extrato de soja com teor de proteína de 3,5 % considerando uma razão de grãos:líquido de 1:7. Segundo os autores, outros produtos obtidos a partir do extrato de soja não estão inclusos nesta estimativa, logo, eles afirmam, que é razoável concluir que vários milhões de toneladas de okara sejam produzidos todos os anos.

Um outro produto que possui uma grande aceitação devido ao seu alto teor de proteínas e que é obtido a partir do extrato de soja, é o tofu. Do ponto de vista histórico, é um produto que tem origem identificada por volta dos anos 965 d.C, quando foi registrado pela primeira vez na China por Tao Ku em Anedotas, Simples e Exótico (do inglês: *Anecdotes, Simple and Exotic*) (ZHANG *et al.*, 2021).

O tofu, é um produto que envolve a gelificação de proteínas de soja. Inicialmente, a soja é embebida por 7 h, e após esse período ela é moída com água. Em seguida, é filtrada para produzir o extrato de soja (LI *et al.*, 2021; SHEN; KUO, 2017). O extrato de soja cru é aquecido na faixa de 90 a 95 °C por 3 a 10 minutos, aproximadamente, para desnaturação das proteínas. E, logo depois, é resfriado até 30 °C. A coalhada de soja é gerada após o resfriamento com a adição de gluco-delta-lactona (GDL) ou sulfato de cálcio, que são os componentes comumente utilizados como coagulantes. Após a adição dos agentes coagulantes, o extrato de soja é reaquecido a 80 °C. Após a coalhada ser formada, ela é quebrada e transferida para uma forma para ser prensada e produzir o tofu (LIU; KUO, 2011; SHEN; KUO, 2017).

Como principal subproduto desse processo de extração se tem o okara, que é a parte insolúvel após produzir o extrato com água e contém predominantemente fibras, proteínas e gorduras. Da produção do tofu, para cada 1 kg de soja utilizado, são produzidos aproximadamente 1,1 kg de okara úmido. De acordo com CHEN *et al. (*2020), aproximadamente 0,5 milhões de toneladas de okara são produzidos como subprodutos todos os anos em Taiwan. Na China e Japão são produzidos aproximadamente 2,8 e 0,8 milhões de toneladas de okara oriundas da produção de tofu, respectivamente (CHEN, P. C. *et al.*, 2020).

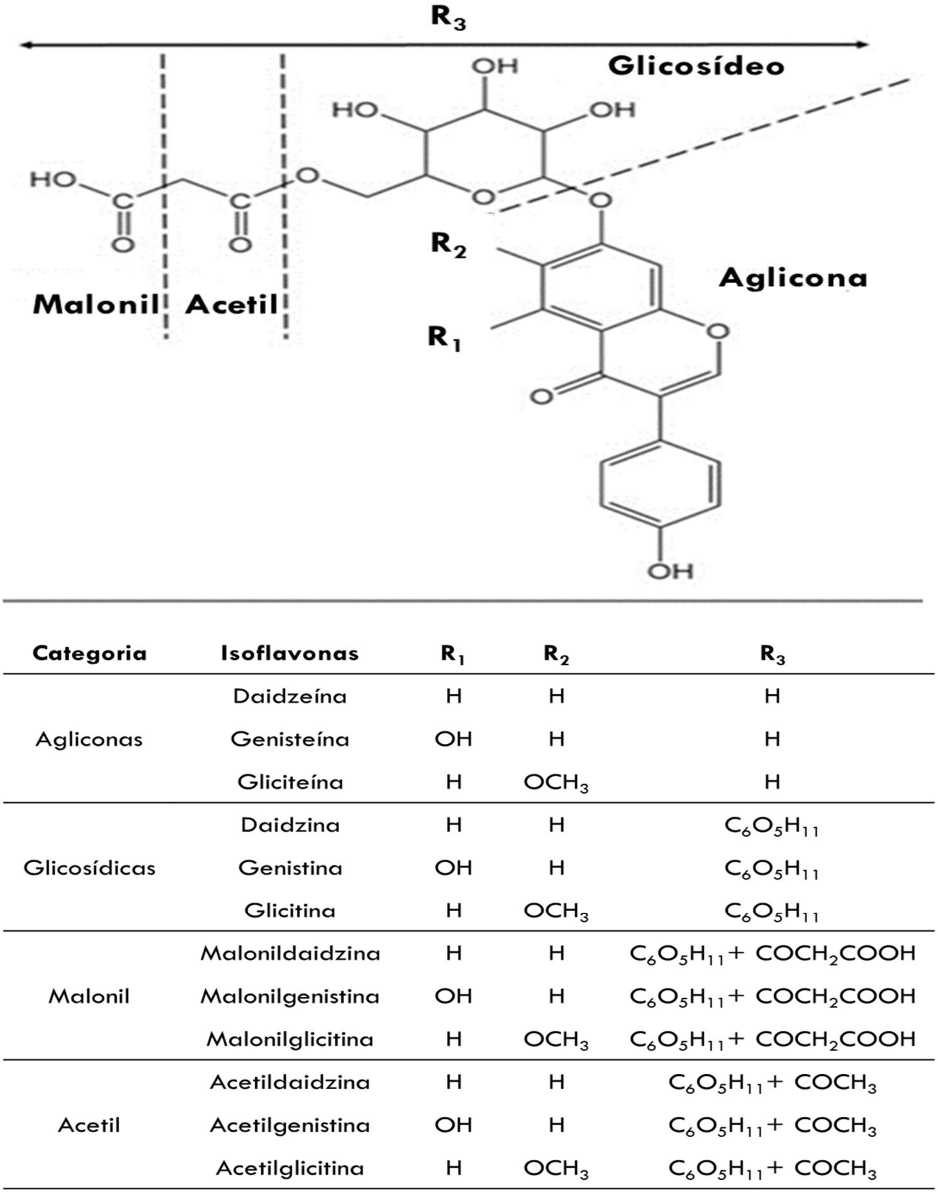
Várias tentativas já foram feitas objetivando adicionar esse subproduto em produtos alimentícios, como biscoitos, bebidas simbióticas, doces e salgadinhos de soja (LEE; GAN; KIM, 2020; VOSS *et al.*, 2021)(JANKOWIAK *et al.*, 2014b). Apesar de apresentar de 40-65% de fibras, 25 % de proteína e 10 % de óleo em base seca, o okara é pouco utilizado como fonte de alimento. Um problema comum é que as fibras de okara são, em sua maioria, insolúveis e têm efeitos adversos na textura dos alimentos (NAGANO *et al.*, 2020). Além disso, sua alta umidade o torna muito perecível e de baixo valor econômico (VONG; LIU, 2016). Dessa forma, a maior parte do okara gerado acaba sendo utilizada como ração animal, ou até mesmo, descartado como resíduo (JANKOWIAK *et al.*, 2014b).

No entanto, esse resíduo é rico em fitoquímicos, como as isoflavonas e ácidos fenólicos, que são compostos que apresentam benefício nutricional a alimentação humana se extraídos e aplicados em algum produto (ALVAREZ *et al.*, 2019; NKURUNZIZA *et al.*, 2019). Com isso, o okara torna-se um resíduo interessante para a extração de compostos bioativos para aplicação em produtos que promovam o bem-estar dos humanos.

2.3 Isoflavonas

As isoflavonas são compostos fenólicos pertencentes ao subgrupo dos flavonoides; são encontradas principalmente na soja e foram atribuídas a elas diversas funções de saudabilidade, alivio dos sintomas da menopausa, redução do risco de morte de câncer de mama e próstata, redução do colesterol e da perda óssea(JACKSON *et al.*, 2002; MANOUSI; SARAKATSIANOS; SAMANIDOU, 2019).

Foram identificados até doze tipos de isoflavonas na soja, que podem ser classificadas em formas não conjugadas, como agliconas (daidzeína, genisteína e gliciteína) e isoflavonas conjugas, classificadas como, glicosídicas (genistina, daidzina e glicitina), malonilglicosídicas (6”-O-malonilgenistina, 6”-O-malonildaidzina e 6”-O- malonilglicitina) e acetilglicosídicas (6”-O-acetilgenistina, 6”-O-acetildaidzina e 6”-O-acetilglicitina), conforme mostrado na Figura 2.1. As isoflavonas conjugadas, malonilgenistina, genistina e daidzina representam de 83 a 95% do total de isoflavonas presente na soja (JACKSON *et al.*, 2002; NKURUNZIZA *et al.*, 2019)



Categoria	Isoflavonas	R_1	R_2	R_3
Agliconas	Daidzeína	H	H	H
	Genisteína	OH	H	H
	Gliciteína	H	OCH_3	H
Glicosídicas	Daidzina	H	H	$C_6O_5H_{11}$
	Genistina	OH	H	$C_6O_5H_{11}$
	Glicitina	H	OCH_3	$C_6O_5H_{11}$
Malonil	Malonildaidzina	H	H	$C_6O_5H_{11}$+ $COCH_2COOH$
	Malonilgenistina	OH	H	$C_6O_5H_{11}$+ $COCH_2COOH$
	Malonilglicitina	H	OCH_3	$C_6O_5H_{11}$+ $COCH_2COOH$
Acetil	Acetildaidzina	H	H	$C_6O_5H_{11}$+ $COCH_3$
	Acetilgenistina	OH	H	$C_6O_5H_{11}$+ $COCH_3$
	Acetilglicitina	H	OCH_3	$C_6O_5H_{11}$+ $COCH_3$

Figura 2.1. Estrutura química das isoflavonas de soja. Fonte: (CARNEIRO *et al.*, 2020)

Como já foi dito anteriormente, as isoflavonas conjugadas dominam a concentração total de isoflavonas na soja e seus subprodutos, no entanto, são as agliconas que desempenham uma atividade biológica maior. As isoflavonas conjugadas dificilmente são absorvidas pelo epitélio intestinal e, dessa forma, têm atividades biológicas mais fracas (NKURUNZIZA *et al.*, 2019; VITALE *et al.*, 2013).

No entanto, o processamento de matérias-primas oriundas da soja; seja físico (imersão, fervura, tratamento com ácidos e bases em temperatura ambiente ou altas temperaturas), biológico (fermentação) e enzimático acabam afetando as características nutricionais desses produtos, convertendo os derivados de malonil e acetil glicosídeos em derivados de agliconas (COWARD *et al.*, 1998; JACKSON *et al.*, 2002; NKURUNZIZA *et al.*, 2019; WANG;

MURPHY, 1996; ZAHEER; HUMAYOUN AKHTAR, 2017). As alterações químicas mais comuns nas isoflavonas incluem a descarboxilação dos grupos malonil em acetil-glicosídeos e a hidrólise do éster das formas acetil ou malonil-glicosídeos em β-glicosídeos e a clivagem da ligação glicosídica leva a uma conversão em isoflavonas agliconas (JANKOWIAK *et al.*, 2014a).

Em um trabalho publicado em (2004) por Rostagno e colaboradores, foi demonstrado que a malonilgenistina era termicamente sensível a 100 °C, levando a formação de genistina devido à degradação térmica. No estudo de Benjamin et. al (2017) para recuperação de isoflavonas de farinha de soja, em temperaturas acima de 160 °C as formas malonil e outras isoflavonas conjugadas foram rapidamente degradadas, no entanto, as concentrações de agliconas aumentaram

Avaliando o efeito do pH na conversão de soluções concentradas contendo isoflavonas conjugadas, Ismael e Hayes (2005) observaram que as isoflavonas malonilgenistina, acetilgenistina e malonildaidzina e acetildaidzina não sofreram nenhuma alteração significativa após 2 horas de incubação a 37 °C em pH ácido (pH 2). Nenhuma degradação ou conversão significativa ocorreu.

Já Lopes Barbosa, Lajolo e Genovese (2006) estudando diferentes pH's em isolados de proteína de soja observaram que em pH > 8 e pH < 4 ocorria uma reação de desesterificação das isoflavonas. Eles observaram que as formas malonilglicosídicas caíram de 50-43 %, enquanto, as formas glicosídicas aumentaram proporcionalmente de 44 para 53 %. As formas acetil e agliconas mostraram pouca variação. Em seus estudos a aglicona genisteína foi a que apareceu em maior proporção e a porcentagem de gliciteína foi maior entre pH 3 e 5. Foi observado também que em temperaturas abaixo de 50 °C as isoflavonas glicosídicas eram hidrolisadas em agliconas, devido a atividade da enzima β-glicosidase que é ativa em temperaturas abaixo de 60 °C (LOPES BARBOSA; LAJOLO; GENOVESE, 2006).

As isoflavonas desempenham um papel fundamental na saúde humana, esses compostos bioativos são conhecidos como fitoestrógenos, devido à sua atividade estrogênica, e também, como antioxidantes (VITALE *et al.*, 2013)

Algumas revisões encontradas na literatura relacionam o uso das isoflavonas a alívios de várias doenças crônicas; como, alívio de sintomas da menopausa, redução do risco de morte por câncer de mama, câncer de próstata, redução dos níveis de colesterol e doenças cardiovasculares, osteoporose ou densidade mineral óssea, obesidade e diabetes (HSIAO; HO; PAN, 2020; VITALE *et al.*, 2013; ZAHEER; HUMAYOUN AKHTAR, 2017).

Considerando todos esses benefícios, há um interesse emergente no consumo dietico de isoflavonas por via oral, seja no consumo direto através de derivados de soja ou cápsulas. As indústrias de alimentos saudáveis usam normalmente extratos orgânicos enriquecidos por isoflavonas pró-nutricionais que podem ser obtidos utilizando solventes orgânicos ou tecnologias emergentes como extração com fluido supercrítico, ultrassom, água pressurizada e *Deep Eutectic Solvents* (DES).

2.4 Métodos de extração

A inclusão de compostos fenólicos em produtos de alto valor agregado requer estratégias de extração eficientes e econômicas. Dessa forma, diversos métodos baseados em extração de compostos fenólicos em matrizes vegetais como soja e seus subprodutos e resíduos foram otimizados por diversos pesquisadores (KUMAR *et al.*, 2021). Neste tópico, serão abordados métodos de extração de compostos fenólicos tradicional com solventes orgânicos (que envolve maceração, agitação e extração a quente com refluxo a base de solventes) e com DES.

O método de extração convencional ainda é amplamente utilizado para a extração de compostos fenólicos devido à facilidade de operação e baixo custo. No entanto, esses procedimentos contam com algumas desvantagens como longo tempo de extração (2-12 h), degradação de compostos fenólicos, uso de grandes quantidades de solventes e o uso de solventes perigosos (metanol, acetona ou hexano) tornam os extratos fenólicos perigosos para serem usados como ingrediente alimentar (KUMAR *et al.*, 2021). Dessa forma, diversos cientista usaram o etanol (reconhecido como seguro) e temperaturas próximas de 60 °C para obter extratos de boa qualidade para aplicação nos setores farmacêuticos, cosméticos e de alimentos. Tran *et al.* (2019), Jankowiak *et al.* (2014b) e Carneiro *et al.* (2020) estudaram o efeito do etanol na extração de compostos fenólicos, precisamente, isoflavonas de grãos de soja, okara e resíduos de soja (folhas, galhos e vargem), respectivamente.

Tran *et al.* (2019) conseguiram extrair 1932,44 μg/(g de matéria seca) de isoflavonas com etanol 80%, uma relação de solvente para soja seca de 26,5 mL/g, temperatura de 72,5 °C e 67,5 minutos de extração. Já Jankowiak *et al.* (2014b) encontraram um resultado ótimo de 907 μg/g de matéria seca usando etanol 50%, razão sólido-líquido de 1/10, temperatura ambiente, 2 horas de extração e agitação de 240 rpm. Carneiro *et al.* (2020) extraíram e identificaram seis isoflavonas (daidzina, genistina, glicitina daidzeína, genisteína e glicitina) com etanol adicionado de 20% de HCl aquoso 0,1 N. Somando as isoflavonas, foram extraídas 2,707 kg de isoflavonas/tonelada de folhas, 0,569 kg de isoflavonas/toneladas de galhos e 0,301 kg de isoflavonas/toneladas de vagem. Segundo estes autores, as folhas de soja apresentaram mais isoflavonas do que os próprios grãos, que continham 2,087 kg de isoflavonas/toneladas de grãos.

O processo de extração permite que os componentes desejados na matriz vegetal sejam isolados. Para que isso aconteça, a escolha de solventes apropriados se faz necessário para extrair os compostos bioativos de interesse. Esses solventes dependem da natureza específica dos compostos bioativos a serem isolados (ALCÂNTARA *et al.*, 2019; YAHYA; ATTAN; WAHAB, 2018).

Se o interesse for extrair solutos hidrofílicos a regra é que os solventes sejam polares; como metanol, etanol, acetona pura ou misturas de acetona/água, acetato de etila e acetronitila. Além disso, são necessários que esses solventes apresentem pontos de ebulição baixos, pois compostos fenólicos podem ser termicamente lábeis. De forma análoga, para a solubilização de solutos hidrofóbicos são necessários solvente apolares, como hexano e isopropanol, por exemplo (YAHYA; ATTAN; WAHAB, 2018).

Existem vários métodos de extração tradicionais, que incluem (i) extração líquido-líquido, (ii) extração sólido-líquido e (iii) microextração em fase sólida (YAHYA; ATTAN; WAHAB, 2018). No entanto, a extração sólido-líquido foi o método usado neste trabalho.

Como primeira etapa para extração sólido-líquido, tem-se o umedecimento da superfície da matéria-prima com o solvente, logo depois, a penetração do solvente nos interstícios do sólido. Já dentro do sólido, ocorre a dissolução dos solutos com maior afinidade com o solvente e esses solutos são transportados do interior do sólido até sua superfície e, por fim, dispersas na fase líquida externa de solvente (BERK, 2018). Além da dissolução, há casos em que ocorrem mudanças químicas na estrutura da matriz e do soluto, como, por exemplo, a hidrólise de biopolímeros insolúveis para a produção de moléculas solúveis e a conversão de fitoquímicos presentes incialmente na matriz em outras moléculas (BERK, 2018; NKURUNZIZA *et al.*, 2019).

Nos últimos anos, devido às preocupações com o meio ambiente, a expansão da química verde vem crescendo e estimulando o desenvolvimento de várias técnicas de extração que podem ser consideradas ecológicas para substituir métodos de extração convencionais que envolvem o uso de solventes orgânicos tóxicos (SHARMILA *et al.*, 2016). Na Tabela 2.2 são mostrados alguns dos trabalhos mais recentes envolvendo o uso de diferentes tecnologias para extração de isoflavonas de produtos, subprodutos e resíduos de soja.

Os *DES* são um novo tipo de solvente que, dependendo dos reagentes precursores, podem exibir propriedades benéficas que incluem procedimentos de preparação simples, podem ser econômicos e renováveis (SHANG *et al.*, 2019). Além disso, podem ser não tóxicos (BAJKACZ; ADAMEK, 2017), podendo servir como um bom solvente de substituição aos solventes orgânicos já utilizados.

Tabela 2.2. Trabalhos envolvendo extração de isoflovonas de soja

Matéria-prima	Método de extração	Solvente	Parâmetros	Isoflavonas identificadas	Métodos de análises	Melhores condições	Referência
okara	extração com água subcrítica (SWE)	água	temperatura: 120-160 °C; pressão (MPa): 2-5; razão S/L (mg/ml): 15:25 tempo: 5-275 min.	genistina; daidzina; genisteína e daidzeína	HPLC UV-VIS	temperatura: 140 °C; pressão: 3,75 MPa; razão S/L (mg/ml): 1:20; tempo: 125 min; CI:108,43 mg/100 g de okara seco	(NKURUNZIZA; PENDLETON; CHUN, 2019)
okara	extração com água em tubo com agitação	água	razão S/L: 1 para 20, 30, 40, 50, 60, 70, 80, 90 e 100; temperatura: 20, 35, 50, 65, 80 e 95 °C; pH: 2, 3, 4, 5, 6, 7, 8, 9 e 10	daidzina, glicitina, genistina, malonil-daidzina, malonil-glicitina, malonil genistina, daidzeína, gliciteína, acetil-genistina, genisteina	HPLC-PDA	temperatura: 20 °C; tempo: 1 h; razão S/L: 1:20 e ph 10; CI: 911 µg / g de okara	(JANKOWIAK *et al.*, 2014a)
okara	extração com etanol em tubo de agitação	etanol	concentração de etanol: 0, 10, 20, 30 40, 50, 60, 70, 80 e 90 %; razão S/L: 1 para 10, 12,17 e 35;	isoflavonas totais	HPLC-PDA	temperatura ambiente, 80 % de etanol; razão S/L: 1:17; CI: 900 µg / g	(JANKOWIAK *et al.*, 2014b)
okara	extração com água subcrítica	água	temperatura; 100-200 °C; pressão:2-5 MPa; razão S/L: 10-30 mg/ml; tempo: 5-275 min	genistina; daidzina; genisteína e daidzeína	HPLC UV-VIS	temperatura: 150 °C, pressão: 4 MPa e razão S/L (mg/ml): 1:20; tempo: 250 min; CI:~105 mg/100g de amostra em base seca	(NKURUNZIZA *et al.*, 2019)

okara de soja preta	extração com ultrassom	água	razão S/L (g/ml): 1 para 30, 50 e 60; frequência/potência: 40khz/300w; temperaturas: 30, 50 e 60 °C; tempo: 1, 2 e 3 h.	daidzina, genistina, daidzeína e genisteína	HPLC-DAD	temperatura: 60 °C; razão S/L(g/ml): 1:50; tempo: 3 horas; CI: 442,43 µg/g de okara seco	(YU; YANG, 2019)
soja em pó	extração com solvente em agitador orbital	40 a 90% de metanol	razão S/L: 1:10-30; temperatura: 25 °C; agitação: 250 rpm; tempo: 0,4 a 4 h; logo depois o extrato foi autoclavado a 121 °C/20 min.	malonil-daidzina; malonil-glicitina; malonil-genistina; daidzina; glicitina; genistina; daidzeína; genisteína e gliciteína.	HPLC UV-VIS	65% metanol; tempo: 0,5 h; razão S/L: 1:10; CI: 345 mg/100 g de soja	(HSU *et al.*, 2020)
galhos, folhas e vagem de soja	maceração dinâmica com solvente	acetonitrila, etanol e acetona	razão S/L (g/ml); 1:10; 120 rpm, 30°c e 2h;	daidzina; glicitina;genistina; daidzeína; genisteína e gliciteína	HPLC-ESI-MS	folhas: CI:271 mg/100g; galhos: CI: 57 mg/100g; vagens: CI: 30 mg/100g	(CARNEIRO *et al.*, 2020)
farinha de soja	Maceração	etanol (60-90%) (v/v)	razão S/L (g/ml): 1:15-1:25; temperatura: 50-70 °C; tempo: 45-75 min.; pH: 2,5, 7 e 9.	daidzina; glicitina;genistina; daidzeína; genisteína e gliciteína	HPLC-UV/DAD	etanol 80 %; temperatura: 72,5 °C, tempo: 67,5 min; razão S/L: 1:26,5; CI:1932,44 µg / g de matéria seca.	(TRAN *et al.*, 2019)
soja germinada	extração com solvente	tampão de citrato de sódio com cloreto de sódio	temperatura: 23-37 °C; pH: 3,6-6,4; tempo: 50 min. razão S/L (mg/mL): 100:1,5	agliconas	UHPLC-DAD	-	(YOSHIARA *et al.*, 2018)

brotos de soja preta	extração com ultrrassom	metanol 70%	razão S/L (g/ml): 1:20; frequência: 80 khz; temperatura: 30-60 °C; tempo: 1-30 min.	daidzina; glicitina;genistina; daidzeína; genisteína; gliciteína e acetil e malonil glicosídeos.	UHPLC-Q-TOF-MS E HPLC-DAD	no quarto dia de germinação dos brotos a concentração máxima de isoflavonas totais foi de 284 µg/g	(REN *et al.*, 2017)
extrato de soja	extração líquido-líquido assistida por *salting-out*	NaCl e acetonitrila	concentração de sal: 20 e 30%; razão solvente:amostra:1:1-12:1; agitação: 300 rpm; tempo: 10-120 min. pH: 2,5-7,5	daidzina, glicitina, genistina, malonil-daidzina, malonil-glicitina, malonil-genistina, acetil-daidzina, acetil-glicitina; acetil-genistina, daidzeína, gliciteína e genisteína	UHPLC-MS/MS	20% de NaCl; razão solvente:amostra: 6:1; tempo: 30 min; pH 2,5: CI: 210,8 mg/L de extrato de soja	(PARK; JUNG, 2017)
farelo de soja	extração com fluido supercrítico	co$_2$ e metanol como cossolvente	pressão: 30-60 MPa; temperatura: 313,15 – 343,15 K; taxa de fluxo CO$_{2:}$ 5,88 kg/h; tamanho de partícula: 0,68 mm; tempo de extração: 200-320 min.	daidzina, daidzeína, genistina e genisteína	simulação e HPLC-UV	temperatura: 323,15 k; pressão: 59,45 mpa; tempo: 283 min: CI: 0,901 g/g de farelo de soja	(LUMMAETEE *et al.*, 2017) e (ZUO *et al.*, 2008)
três suplementos dietéticos; produtos de soja: soja, farinha, macarrão, cereais, cubos de soja, nozes de soja e bebida de soja	extração com NADES assistida por ultrassom	solventes eutéticos naturais profundos (nades)	teor de água no NADES: 10-75%; razão S/L (mg/µl): 1:1; 1:2; 1:3; 1:4; 1:5 e 1:7,5; tempo de extração: 40-120 min.; temperatura de extração: 30-80 min.; potência: 264-616 W.	daidzina, daidzeína, genistina e genisteína	UHPLC-UV	teor de água: 30 %; razão S/L(mg/µl):1:3; tempo: 60 min; temperatura: 60 °C; potência ultrassônica: 616 W; as concentrações de isoflavonas variaram de 38,19 até 253000 µg/g para a	(BAJKACZ; ADAMEK, 2017)

						bebida de soja e suplementos de soja, respectivamente.	
farinha de soja e isolado proteico	extração com água subcrítica	água	temperatura: 60-200 °C; tempo estático: 4-14 min.; razão S/L: 1-7 % para 25 ml de solvente; pressão: 1500 psi;	daidzina, glicitina, genistina, malonil-daidzina, malonil-glicitina, malonil-genistina, acetil-daidzina, acetil-glicitina; acetil-genistina, daidzeína, gliciteína e genisteína	HPLC-UV	farinha de soja: temperatura: 122 °C; tempo: 14 min; razão S/L: 1 %; CI: 94,3 mg/100g isolado proteico de soja: temperatura: 144 °C; tempo: 2 min; razão S/L: 1 %; CI: 98 mg/100g	(BENJAMIN *et al.*, 2017)
farinha de soja	extração com ultrassom e hidrólise com HCl para conversão em agliconas	etanol (30, 50 e 70%)	tempo de extração: 10-30 min.; frequência: 24-56 khz; tempo de hidrólise: 20-60 min.; temperatura de hidrólise: 70-100 °C;	daidzeína e genisteína	HPLC-UV	tempo: 30 min; frequênca: 55 khz; tempo de hidrólise: 20 min; temperatura de hidrólise: 168 °C; CI: 3251,9 µg/g	(DA SILVA, B.; KUPSKI; BADIALE-FURLONG, 2019)

CI* = concentração total de isoflavonas

2.5 Deep eutectic Solvents

O “fenômeno” eutético profundo foi citado pela primeira vez por Abbott *et al.* (2003), quando misturaram cloreto de colina (ChCl) e ureia, que apresentam pontos de fusão de 302 °C e 133 °C, respectivamente. Ao misturar os dois componentes sólidos na proporção molar de 1:2 (ChCl:ureia) foi obtida um mistura eutética que era líquida à temperatura ambiente com um ponto de fusão de 12 °C com propriedades solventes consideradas interessantes (ABBOTT *et al.*, 2003; HUANG *et al.*, 2019). Abbott *et al.* (2003) observaram que esse método de formação de líquidos à temperatura ambiente não se limitava apenas ao cloreto de colina, logo listou-se uma variedade de sais de amônio quaternário com ureia.

Dessa forma, o termo *Deep Eutetic Solvents* foi utilizado para descrever misturas de amidas com sais de amônio quaternário, que tinham pontos de fusão muito mais baixos que os de seus componentes puros. Essas misturas de amidas são conhecidas como doadoras de ligações de hidrogênio (HBD, do inglês: *Hydrogen-Bond Donor*) e os sais de amônio quaternário como receptores de ligações de hidrogênio (HBA, do inglês: (Hydrogen-Bond Acceptor) (ABBOTT *et al.*, 2003; MARTINS; PINHO; COUTINHO, 2019). Mais tarde, além das amidas, ácidos carboxílicos e açucares também foram utilizados como HBD (ASHWORTH *et al.*, 2016).

Com os passar dos anos, diferentes definições do que seria um solvente eutético profundo foram surgindo. Em uma revisão publicada em 2014 por Smith, Abbott e Ryder (2014), eles acrescentaram que DES são sistemas formados a partir de uma mistura eutética de ácidos e bases de Lewis ou Bronsted que podem conter uma variedade de espécies aniônicas e/ou catiônicas.

Já em 2019, Martins, Pinho e Coutinho propuseram um novo conceito para DES. Em uma análise crítica da literatura, ele afirmaram que as definições citadas acima não eram suficientes para definir essa classe de solventes e que misturas com pontos eutéticos não poderiam ser definidos como DES, como relatado por Smith, Abbott e Ryder (2014).

Com base em diagramas de equilíbrio sólido-líquido (SLE) o abaixamento da temperatura em um solvente eutético profundo pode ser definido como a diferença ΔT_2 entre o ponto eutético ideal ($T_{E,ideal}$) e real (T_E) e não como a diferença ΔT_1 entre a combinação linear dos pontos de fusão dos componentes puros e do ponto eutético real (SMITH; ABBOTT; RYDER, 2014), conforme é mostrado na Figura 2.2 (MARTINS; PINHO; COUTINHO, 2019).

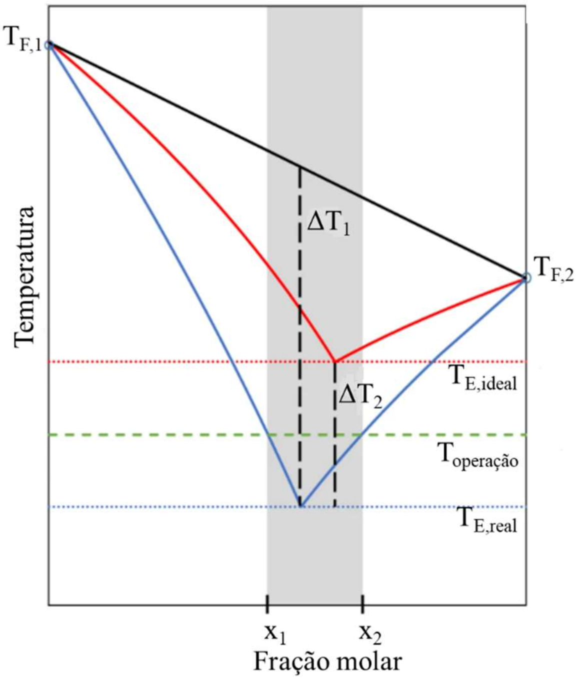


Figura 2.2. Comparação do diagrama de fases sólido-líquido de uma mistura eutética ideal simples (linha vermelha) e uma mistura eutética profunda (linha azul); $T_{F,1}$ e $T_{F,2}$ são as temperaturas de fusão dos componentes puros 1 e 2. Fonte: (MARTINS; PINHO; COUTINHO, 2019)

Na opinião de Martins, Pinho e Coutinho (2019), o solvente eutético profundo é uma mistura de dois ou mais compostos puros, onde a temperatura do ponto eutético é bastante diferente da temperatura do ponto eutético ideal e abaixo da temperatura de operação, e que apresenta desvios negativos significativos da idealidade. Caso contrário, apenas o termo "solvente eutético" poderia ser usado para descrever misturas que não atendem a esses critérios.

Os DES foram relatados como fáceis de preparar e podem ser obtidos a partir da mistura sólida de dois ou três componentes sob agitação e aquecimento até formação de um líquido homogêneo utilizando temperaturas que variam de 70 a 100 °C (BAJKACZ; ADAMEK, 2017; CUI *et al.*, 2015; SHANG *et al.*, 2019; XIA; LI; JIANG, 2021; XU *et al.*, 2019).

Atualmente, os DES são utilizados para extrair vários compostos bioativos como, isoflavonas (SHANG *et al.*, 2019), ácidos fenólicos (PONTES *et al.*, 2021), carotenóides (DA COSTA *et al.*, 2020; LI, H. *et al.*, 2019) e antocianinas (ASLAN TÜRKER; DOĞAN, 2021).

Sobre extração de isoflavonas utilizando DES, tema que envolve este trabalho, até o momento apenas 5 pesquisas foram encontradas na base de dados *Scopus* e *Web of Science* (busca realizada em março de 2021). Esses trabalhos estão mostrados na Tabela 2.3. Dos cinco estudos, um envolve uso de produtos de soja para desenvolvimento de método analítico.

Tabela 2.3. Trabalhos envolvendo o uso de DES para extração de isoflavonas

Matéria-prima	HBA	HBD	DES principal	Parâmetro ótimo de extração	Rendimento	Referência
Broto de grão de bico	ChCl, betaína, L-prolina	D-frutose, etilenoglicol, propilenoglicol, glicerol, 1,4-butanodiol, ác. DL-maílico, ác. Lático, malonato, ureia, xilitol, ácido cítrico, D-glicose e sacarose	Inicialmente foi feita uma varredura com 20 DES e escolhido um para otimização ChCl:propilenoglicol (1:1)	Teor de água no Des: 33 %; Tempo: 35 min.; Temperatura: 59 °C; Razão S/L: 40 mg/mL	6,22 mg/g de isoflavonas totais	(SHANG *et al.*, 2019)
Produtos de soja: soja, farinha, macarrão, cereais, bebida de soja, cubos de soja, nozes de soja e suplementos dietéticos	ChCl	Sacarose, D-glicose, D-xilose, ác. L-tartárico, ác. Cítrico, ureia e glicerol	Inicialmente foi feita uma varredura com 17 DES e escolhido um para otimização ChCl:ácido cítrico (1:1)	Teor de água no Nades: 30 %; Tempo de extração: 60 min.; temperatura: 60 °C; Potência ultrassônica: 616 W Razão S/L: 1:3 mg/µL	O conteúdo de isoflavonas utilizando a condição ótima para diferentes produtos variou de 38 a 253000 µg/g de produto seco.	(BAJKACZ; ADAMEK, 2017)
Raízes de feijão guandu	ChCl e glicerol	Sacarose, 1,2-propanodiol, glicose, sorbitol, L-prolina, ác. Lático, glicol,	11 DES foram varridos e escolhido um para otimização	Temperatura: 80 °C; Tempo: 11 min; Teor de água: 30 %;	0,449, 0,617 e 0,221 mg / g para genistina, genisteína e	(CUI *et al.*, 2015)

		glicerol, 1,3-butanodiol, 1,4-butanodiol, 1,6-hexanodiol	ChCl:1,6-hexanodiol (1:7)	Razão S/L: 1g/14 mL; potência do micro-ondas: 600 W	apigenina, respectivamente	
Folhas de *Dalbergia odorifera*	ChCl	Etilenoglicol, glicerol, 1,2-butanodiol, 1,3-butanodiol, 1,4-butanodiol, 2,3-butanodiol, 1,6-hexilenoglicol, ác. Lático, ácido cítrico, Glicose e sacarose	11 DES na proporção molar HBA:HBD de 1:2 foram varridos e escolhido um para otimização ChCl: etilenoglicol	Razão molar HBA:HBD: 1:2; Razão S/L: 1:20 g/mL; Tempo: 20 min.; Pressão negativa: -0,7 MPa; Temperatura: 45,38 °C; Teor de água: 26, 51 %	5,62 mg/g de massa seca	(LI, L. *et al.*, 2016)
Radix Pueraria	ChCl, betaína e L-prolina	glicose, maltose, sacarose, xilitol, glicerol, ácido lático, ácido málico, ureia, acetamida	25 DES foram varridos e um foi escolhido para otimização Lprolina:ácido málico (1:1)	Tempo: 30 min.; Temperatura: 40 °C; Razão S/L: 25 mg/g; Teor de DES: 60 % (ou 40 % de água); Frequência ultrassônica: 50 kHz	124,9 mg/g	(HUANG, Y. *et al.*, 2021)

2.6 Estudos sobre toxicidade de DES

Os DES podem ser preparados com uma variedade de componentes disponíveis, a maioria deles é formulada à base de cloreto colina, que é considerada um ingrediente seguro e desempenha importantes funções fisiológicas no organismo. Dessa forma parece lógico que DES à base de cloreto de colina tenham toxicidade desprezível. Porém, essa toxicidade as vezes é baseada na avaliação desses componentes separadamente, sem levar em consideração os efeitos sinérgicos das combinações (GULLÓN *et al.*, 2020). Por isso, antes do desenvolvimento de um produto e/ou uma aplicação industrial, é de bom senso e obrigatória uma avaliação crítica de uma possível toxicidade desses solventes (HAYYAN *et al.*, 2015).

Em um artigo de revisão publicado recentemente por Gullón et al.(2020), em uma busca sobre o tema na base de dados *Scopus*, 19 artigos foram encontrados até 2019. Dos 19 estudos, 4 envolveram o uso de animais para análise de toxicidade subaguda. O restante dos trabalhos foi relacionado a toxicidade e citoxicidade em células animais e humanas e bactérias e leveduras.

O estudo realizado por Hayyan *et al.*(2015) avaliou a citoxicidade em uma linha de células de câncer humano e células normais e toxicidade aguda em ratos usando DES baseado em cloreto de colina e glicerina, etilenoglicol, trietilenoglicol e ureia. Os solventes indicaram citoxicidade relativamente alta nas linhas celulares e foi verificado que a toxicidade celular é dependente da composição, viscosidade e concentração do DES. Em geral, segundo o autor, todas as linhas celulares foram suscetíveis à toxicidade de DES. Os estudos em camundongos também mostraram que os DES apresentaram um efeito tóxico nos animais. Os autores também observaram que a citoxicidade foi influenciada pelas diferentes combinações dos componentes, bem como pela razão molar e que os DES foram mais tóxicos do que os componentes individuais.

Radošević et al.(2015), estudando DES à base de cloreto de colina e glicose, glicerol e ácido oxálico como HBD avaliaram a citoxicidade *in vitro* em células de peixes e células humanas. Os autores observaram que os DES ChCl:glicose e ChCl:glicose apresentaram baixa citoxicidade e o DES ChCl:ácido oxálico apresentou citoxicidade moderada.

Faggian *et al.* (2016) avaliaram o DES prolina:ácido glutâmico (1:2) como veículo para administração oral de rutina em um estudo farmacocinético em camundongos Balb/c e eles observaram que a absorção de rutina via DES foi melhorada quando comparada com a absorção de routina via suspensão aquosa. Dessa forma, eles concluíram que o DES prolina:ácido glutâmico pode ser utilizado como uma ferramenta interessante para solubilização e administração de routina devido às valiosas propriedades farmacocinéticas. Neste estudo, os autores não investigaram o efeito de toxicidade do DES.

Em um estudo publicado por Mbous et al.(2017), eles avaliaram o impacto de DES baseados em ChCl:glicose, ChCl:frutose e N, N-dietiletanol cloreto de amônio:trietilenoglicol em ratos de laboratório. Foi observado que o DES N, N-dietiletanol cloreto de amônio:trietilenoglicol apresentou toxicidade menor em comparação com os demais. Os autores atribuíram essa diferença ao aumento de viscosidade dos DES formados com açucares que segundo eles, podem não ter circulado adequadamente nos camundongos e isso, pode ter interrompido o fluxo sanguíneo, pois os solventes injetados não foram diluídos em água, sendo injetados com uma alta viscosidade.

Por último, Benlebna et al. (2018) avaliaram a toxicidade por administração oral em ratos Wistar de extratos de DES (betaína:glicerol, 1:2) de grãos de café verde, rico em polifenóis. A administração de 3 mL do extrato fenólico de DES, fornecidas em duas refeições (com 28,01 mg de composto fenólico expresso em mg de ácido clorogênico e 5,89 mg de cafeína) induziu a mortalidade em dois ratos, de 6 utilizados; induziu o consumo excessivo de água, reduziu a ingestão alimentar causou a perda de peso, hepatomegalia (inchaço do fígado) e estresse plasmático associado a níveis elevados de lipídios no sangue. Os ratos foram alimentados com uma dieta granulada padrão e tiveram acesso livre a comida e água da torneira. Em conclusão, os autores demonstraram a toxicidade oral do DES em uma condição de curto prazo (14 dias, 6 mL/dia/rato). E que, os efeitos deletérios seriam decorrentes da grande quantidade de DES fornecida aos ratos e nesse caso, as doses deveriam ser adaptadas para diminuir seus efeitos colaterais até encontrar-se a dose adequada para uso na administração oral de biomoléculas.

Todos os estudos apresentados identificaram fatores relacionados à toxicidade e citoxicidade dos DES. Foi um consenso, que mais estudos devem ser realizados para melhorar as formulações de DES que não só sejam eficientes e seguras para o meio ambiente, mas também para animais e humanos. Dessa forma, combinações adequadas dos componentes que formam o DES, bem com razão molar e propriedades de viscosidade e pH, concentração do DES e dosagem em dietas devem ser melhor estudadas para reduzir ou remover os efeitos tóxicos e tornar esses solventes seguros, sustentáveis e ecológicos para extração de compostos de alto valor agregado de resíduos agroalimentares (BENLEBNA *et al.*, 2018; GULLÓN *et al.*, 2020).

CAPÍTULO 3

Deep eutectic solvents as an alternative for extraction of flavonoids from soybean and okara: An experimental and computational approach based on COSMO-SAC model.

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Deep eutectic solvents as an alternative for extraction of flavonoids from soybean (*Glycine* max (L) Merrill) and okara: An experimental and computational approach based on COSMO-SAC model

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Deep eutectic solvents as an alternative for extraction of flavonoids from soybean (*Glycine max* (L) Merrill) and okara: An experimental and computational approach based on COSMO-SAC model.

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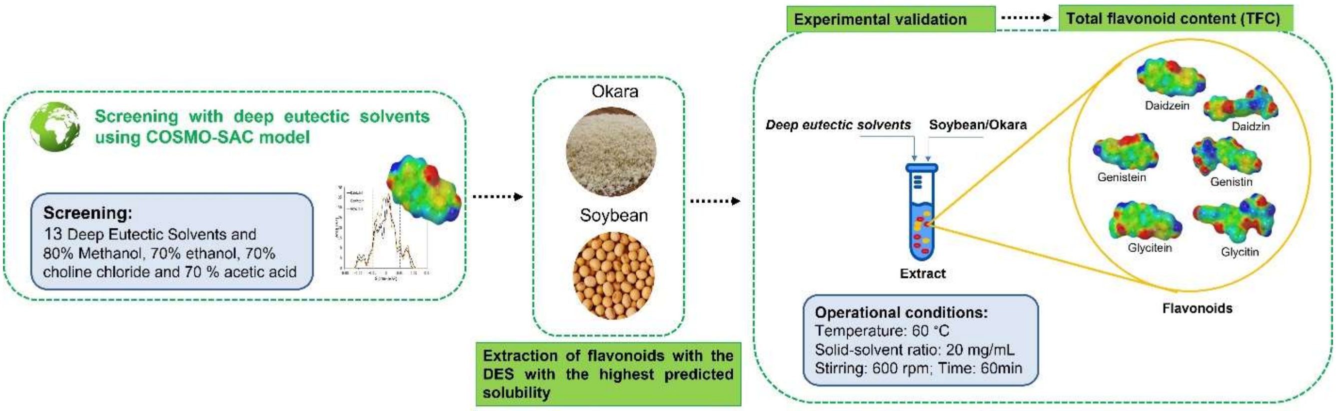
Abstract

In this study, different Deep Eutectic Solvents based on choline chloride ([Ch]Cl) with carboxylic acids, sugars, and glycerol, were investigated as alternative solvents for the extraction of flavonoids from soybean and okara. Initially, the COSMO-SAC was investigated as a tool in solvent screening for the extraction of flavonoids. Experimental validation was performed using total flavonoid analysis with the solvents that showed greater interaction with the solutes. The extracts obtained from soybean and okara using the DES [Ch]Cl:acetic acid added with 30 % water showed the highest total flavonoid content, 1.05 mg eq. of catechin/g dry soybean and 0.94 mg eq. of catechin /g dry okara, respectively. For phenolic compound extraction, [Ch]Cl: acetic acid DES extracted approximately 1.16 mg GAE/g of soybean and 0.69 mg GAE/g of okara. For antioxidant activity, soybean and okara extracts obtained with [Ch]Cl: acetic acid showed FRAP results of 0.40 mg Trolox/mL of extract and 0.45 mg Trolox/mL of extract, respectively. In addition, the isoflavones daidzein, genistein, glycitein, daidzin, genistin, and glycitin were identified and quantified in the soybean and okara extracts obtained with DES [Ch]Cl: acetic acid with 30% water, totaling 1068.05 and 424.32 μg total isoflavones/g dry sample. Therefore, The COSMO-SAC model was a useful tool in solvent screening, saving time and costs. Also, DES can be an alternative solvent for extracting flavonoids to replace conventional organic solvents, respecting current environmental and human health concerns.

Keywords: deep eutectic solvents, soybean residue, isoflavones, flavonoids, antioxidant activity.

Graphical abstract



3.1. Introduction

Soybean (*Glycine max* (L) Merrill) is a crop of global economic importance and is listed as the main crop of the American continent in terms of planted areas (Qi et al., 2021). In food, it is used in fermented form to make miso (fermented soybean paste), shoyu (soy sauce), natto (fermented soybean), and tempeh (fermented soybean cake); in liquid form, as soybean extract, and in curdled form, as tofu (soy curd) (Zhang et al., 2021).

Increased awareness of the health benefits associated with soy consumption, allergies related to cow's milk consumption, and the movement toward healthier and more sustainable food production has caused an increase in the number of soy-based products (Hadj Saadoun et al., 2021). Soybean extract is one of these products, and its consumption has increased in many countries, causing an increase in the waste, okara (Colletti et al., 2020; Hadj Saadoun et al., 2021).

Okara is the solid fraction obtained after the extraction of soybean extract, which is used to produce soybean-based drinks or tofu (Colletti et al., 2020; Zhang et al., 2021). Despite its potential applicability in food products (Jankowiak et al., 2014a; Lee et al., 2020; Voss et al., 2021), okara is usually discarded as waste due to its high moisture content (70-80%), which makes it very perishable and of low economic value (Vong & Liu, 2016; Yu & Yang, 2019). However, like soybean, this residue is rich in important nutrients for human health, which include phenolic acids and especially the isoflavone aglycones, daidzein, genistein, glycitein, and the conjugated isoflavones, daidzin, genistin, and glycitin (Yu & Yang, 2019). Generally, the isoflavone content in okara represents 12-40% of the initial isoflavone content in soybeans, corresponding to an isoflavone content in okara dry mass of 0.02-0.12%. (Jankowiak et al., 2014a).

Isoflavones are related to the treatment of several diseases, such as relief of menopausal effects, reduction of the risk of breast and prostate cancer, reduction of cholesterol, osteoporosis, obesity, and diabetes levels (Hsiao et al., 2020; Vitale et al., 2013; Zaheer & Humayoun Akhtar, 2017).

The recovery and reincorporation of these phenolic compounds into value-added products require efficient and economical extraction techniques. Different methods for extraction of phenolic compounds in soy products, by-products, and residues have been optimized by several researchers employing various types of organic solvents that are considered dangerous, such as methanol, acetone, hexane, and acetonitrile (Kumar et al., 2021; Yahya et al., 2018).

However, attempting environmental and human health concerns, new extraction techniques have emerged stimulated by the expansion of Green Chemistry, one of its principles being the use of safer solvents (Sharmila et al., 2016). In the last decades, the use of Deep Eutectic Solvents (DES) has attracted attention for being easy to prepare, economical, renewable, and non-toxic (Shang et al., 2019).

DES are described as mixtures of two or more compounds, where the strong interaction between hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD) results in a decrease in the melting temperature of the mixture, which is significantly lower than those of the pure components. Besides, these solvents are expected to remain liquid below the operating temperature (Abbott et al., 2003; Martins et al., 2019). In addition, DES precursors are recognized as safe by the FDA (Food and Drug Administration), making these solvents potential substitutes for conventional organic solvents (Bajkacz & Adamek, 2018; Dai et al., 2013). Natural and safe ingredients, such as quaternary ammonium salts, alcohols, carboxylic acids, sugars, and others, are commonly used to prepare DES. The choline chloride, the most important hydrogen bond acceptor to prepare DES, has important physiological functions in the body. In the same way, carboxylic acids, as hydrogen bond donors, such as acetic acid and lactic acid, can be obtained naturally by fermentation from renewable sources and are also considered acidulant, flavoring, antimicrobial, and pH control agents (Bajkacz & Adamek, 2018; Dai et al., 2013; Svetlitchnyi et al., 2022; Toledo Hijo et al., 2022). Therefore, phenolic extracts obtained with these solvents may be used as food additives with antioxidant and antimicrobial properties (Wojeicchowski et al., 2021).

In three previous works, DES have already been applied to extract isoflavones from soy products. Bajkacz and Adamek et al. (2017) applied several DES to the ultrasound-assisted extraction (UAE) of daidzein, genistein, daidzin, and genistin from soy products to implement an analytical method in UHPLC. This analytical method did not involve optimization of the extraction conditions. Among the DES evaluated, choline chloride ([Ch]Cl) and citric acid, added of 30 wt. % of water, presented the highest extraction efficiency. The process conditions were 3 h of extraction, 60 °C, and 616 W ultrasonic power.

Duru et al. (2022) extracted daidzein, genistein, and puerarin from kudzu roots and soybean molasses by applying DES [Ch]Cl: citric acid (1:2 molar ratio) coupled with UAE. The extractions were carried out for 2 h at 80 °C, 180 W ultrasonic power, and 37 kHz frequency. The water content in DES ranged from 10-30 wt. %, where 30 wt. % of water was most efficient in extracting puerarin from kudzu roots. For extraction of genistein and daidzein from soy molasses, 10 wt. % of water presented the best result. Higher amounts of water increase the polarity of the solvent, decreasing the interaction with the aglycone forms, which have greater interactions with less polar solvents, unlike the glycosidic forms that have a direct relationship with the polarity of the solvents (Duru et al., 2022).

Bragagnolo et al. (2022) applied pressurized liquid extraction using the DES choline chloride: citric acid: water (1:1:11) at 120 °C, 100 bar, and 20 min to extract phenolic compounds and total flavonoids from agro-soy by-products (stems, leaves, pods, and roots). The authors observed that soy leaf extracts had higher flavonoid levels than other parts, approximately 419.0 µg quercetin/g. These authors also identified the isoflavones daidzin and genistin in the extracts of all samples.

Considering those mentioned above and the gaps in the literature, this study focused on screening a set of DES for extracting flavonoids from soybean and okara using an *in-silico* approach. So, the affinity between each isoflavone and each solvent was evaluated through the infinite dilution coefficient calculated with the COSMO-SAC model. Solvents with the highest affinities for isoflavones globally were chosen for extraction experiments. This way, it was possible to select solvents and obtain original data using a methodology that saves time and expense.

3.2. Material and methods

3.2.1 Material

The soybean used in this study was grown in the north of Paraná (Brazil) and donated by its producer. This raw material was used to produce soybean extract and obtain the okara.

The reagents used for preparing the DES were choline chloride ([Ch]Cl) (purity>98.0%), glycerol (purity>99.0%), D-(+)-glucose (purity>99.5%), DL-lactic acid (purity>90.0%) and formic acid (purity>98.0%) obtained from Sigma-Aldrich (USA) and the glacial acetic acid (putity>99%) was from Exôdo (Brazil). For conventional extractions, ethanol (99%) and methanol (purity>99.9%), both from Merck (Germany), were used. Sodium nitrite (97 % purity, Ecibra, Brazil), aluminum chloride hexahydrate (95 % purity, Merck, Germany), sodium hydroxide (99 % purity, Merck, Germany), and catechin standard (purity >98 %) from Sigma-Aldrich were used for total flavonoid analysis.

The standards used for quantification of isoflavones by UHPLC (Ultra-High-Performance Liquid Chromatography) were daidzein (purity>98%), genistein (purity>99.0%) and glycitein (purity>99%) from Targetmol (USA) and, daidzin (purity>99.0%), genistin (purity>99.0%) and glycitin (purity>99.0%) from Extrasynthese (France). Methanol (HPLC grade, purity>99.9%) and acetonitrile (HPLC grade, purity>99.9%) were from Merck (Germany), and formic acid (HPLC grade, purity>99.9%) was from Sigma-Aldrich (USA).

3.2.2 Methods

3.2.2.1 Sample preparation

The okara was obtained according to Baú and Ida (2015). First, the soybeans were washed with distilled water for dirt removal and then submerged in a soybean: water ratio of 1:3 (m:v) at 5 °C for 14 h. After that, the water was drained, and the soybeans were rinsed. Distilled water was again added at a soaked soybean: water ratio of 1:8 (m:v), and the mixture was processed in an industrial blender for 2 min at 25 °C to obtain a paste. After that, the paste was filtered and freeze-dried (Liobras, model L101, Brazil) until constant mass at $T = (-50 \pm 1)$ °C, $p = (13 \pm 2)$ µHg during 48 h. This dry insoluble fraction was ground in a knife mill (Marconi, model MA 340, Brazil).

The soybean used in the extraction experiments was first crushed in an industrial blender, then milled in a knife mill, and freeze-dried until constant mass. Both samples were packed in polyethylene bags and stored in a vertical ultra-freezer at -80 °C (Nuaire, Glacier model, USA). The samples were unfrozen according to the need for the extraction experiments.

3.2.2.2 Characterization of the raw material

The freeze-dried raw materials were characterized in water content (%) by Karl Fischer titration in a KF Titrino Plus apparatus (model 870, Metrohm, Switzerland) equipped with a KF Thermoprep oven (model 832, Metrohm, Switzerland) according to AOCS Ca 23-55 method (AOCS, 2017). For total lipid content (%), samples were dried in an ANKOM dryer system (RDI Dryer, ANKOM Technology, USA) and posteriorly subjected to a hexane extraction in an ANKOM oil extractor (XT-15 Extractor, ANKOM Technology, USA) according to official procedure Am 5-04 AOCS (AOCS, 2017). Ashes were determined by method Ba 5a-49 (AOCS, 2009), and crude fiber analyses were performed according to the analytical method adopted by the Adolfo Lutz Institute (IAL, 2008). At last, the total carbohydrate content was determined by mass difference. The results of this section are presented in the supplementary material in Table S1.

3.2.2.3 Molecular geometry optimization

The molecular structures of the isoflavones were obtained from the PubChem Platform (https://pubchem.ncbi.nlm.nih.gov/) in .SDF format and then loaded into the free software Avogadro (https://avogadro.cc/). Their molecular conformation was changed to optimize the molecules until obtaining a minimum activation energy. After that, the molecules were loaded in .MOL format into the GAMESS Quantum Chemistry package (Schmidt et al., 1993) to generate their sigma profile (σ-profile).

3.2.2.4 COSMO-SAC modeling for solvent screening

For solvent screening, COSMO-SAC was applied to evaluate the interaction of the solvents with the isoflavone analytes daidzein, genistein, glycitein, daidzin, genistein, and glycithin using the activity coefficient at infinite dilution (ln γ_i^∞). For this, the JCOSMO software developed by Gerber and Soares (Gerber & Soares, 2010), with the GMHB1808 multi-hydrogen bond parameterization, freely available at (doi: https://doi.org/10.5281/zenodo.3613786) (Soares et al., 2017) was used. The sigma profiles of the molecules were obtained with the GAMESS Quantum Chemistry package (Schmidt et al., 1993), following the procedure described by Ferrarini et al. (2018). The activity coefficients at infinite dilution of the biomolecules at 333.15 K (60 °C) were determined in mixtures with the solvents shown in Table 1. The DES evaluation considered a simple mixture of Hydrogen Bond Acceptor (HBA) and Hydrogen Bond Donor (HBD) compounds. To decrease the viscosity of DES and increase the mass transfer of the biocompounds present in the matrix to the liquid phase, 30% water was added to the solvents, as shown in Table 3.1. Validation experiments were performed, and the extracts were analyzed for total flavonoid content following the methodology described in section 3.2.2.6.

Table 3.1. Solvent screening, followed by molar ratio, water content, chemical formula, and log K_{ow}.

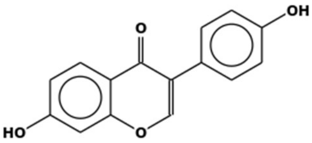
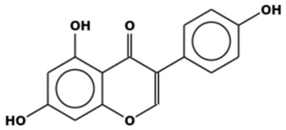
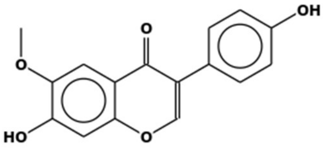
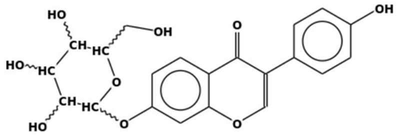
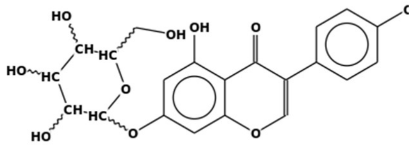
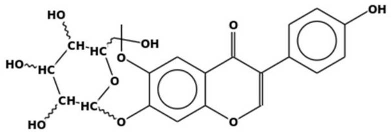
Solvents	HBA: HBD molar ratio	Water (wt. %)	HBD chemical formula	HBD log K_{ow}^{a}	References
[Ch]Cl:Formic acid	1:2	30 %	CH_2O_2	-0.54	-
[Ch]Cl: Acetic acid	1:2	30 %	$C_2H_4O_2$	-0.17	(Pontes et al., 2021)
[Ch]Cl: DL-Lactic acid	1:2	30 %	$C_3H_6O_3$	-0.72	(Crespo et al., 2018)
[Ch]Cl: Malic acid	1:1	30 %	$C_4H_6O_5$	-1.26	(Crespo et al., 2018)
[Ch]Cl: Malonic acid	1:1	30 %	$C_3H_4O_4$	-0.81	(Crespo et al., 2018)
[Ch]Cl: Tartaric acid	2:1	30 %	$C_4H_6O_6$	-1.9	(Crespo et al., 2018)
[Ch]Cl: Aconitic acid	2:1	30 %	$C_6H_6O_6$	-1.0	-
[Ch]Cl: Citric acid	2:1	30 %	$C_6H_8O_7$	-1.64	(Crespo et al., 2018)
[Ch]Cl: D(+)-glucose	1:1	30 %	$C_6H_{12}O_6$	-2.9	(Silva et al., 2018)
[Ch]Cl: D(+)-xylose	1:1	30 %	$C_5H_{10}O_5$	-2.5	(Silva et al., 2018)
[Ch]Cl: Arabinose	1:1	30 %	$C_5H_{10}O_5$	-2.5	(Silva et al., 2018)
[Ch]Cl: D(-)-Fructose	1:1	30 %	$C_6H_{12}O_6$	-3.2	(Silva et al., 2018)
[Ch]Cl: Glycerol	1:1	30 %	$C_3H_8O_3$	-1.8	(Abbott et al., 2007)
[Ch]Cl 70%	-	30 %	$C_5H_{14}ClNO$	-5.16	-
Acetic acid 70%	-	30 %	$C_2H_4O_2$	-0.17	-

Ethanol 70%	-	30 %	C_2H_6O	-0.31	-
Methanol 80%	-	20 %	CH_4O	-0.77	-

[a]$K_{ow:}$ Octanol-water partition coefficient

Table 3.2 presents the isoflavones and their molecular structures and log K_{ow}.

Table 3.2. Target biocompounds considered in the solvent screening.

Components	molecular structure	log K_{ow}
Daidzein		2.5
Genistein		2.84
Glycitein		2.4
Daidzin		0.7
Genistin		0.9
Glycitin		0.6

The octanol-water partition coefficient (Kow) of a molecule is defined as the ratio of its concentration in the octanol-rich phase to that in the water-rich phase and, therefore, the Kow value will be greater than unity if the concentration of the molecule is higher in the octanol-rich phase than in the water-rich phase. Kow is usually expressed as log Kow, meaning its value is positive for hydrophobic molecules and negative for hydrophilic ones. The larger the positive value, the more hydrophobic the molecule, or the smaller the negative value, the more hydrophilic the molecule.

3.2.2.5 Obtaining the extracts for experimental validation.

For flavonoid extraction, the pre-treated soybean and okara were weighed and mixed with the solvents in test tubes of 10 mL (solid-solvent ratio 20 mg/mL). The tube was placed inside 50 mL jacket containers containing water and connected to a thermostatic bath for temperature control. Extractions were performed by keeping the mixture at 60 °C, under constant magnetic stirring at 600 rpm for 60 min.

3.2.2.6 Total flavonoid content (TFC)

The total flavonoid content was determined according to Juan e Chou (2010). Firstly, 250 μL of the extract was added to a test tube and mixed with 1.25 mL of ultrapure water and 75 μL of 5 wt. % $NaNO_2$ aqueous solution. After six minutes, the mixture was combined with 150 μL of 10 wt. % $AlCl_3$ solution. After five minutes, 0.5 mL of 1 M NaOH solution and 275 μL of water were added to the test tube and vigorously stirred. The absorbances of the solutions were measured at 510 nm in a spectrophotometer (Thermo Scientific, Orion AquaMate, USA), and the results were expressed as mg catechin equivalent/g dry sample.

3.2.2.7 Total phenolic content (TPC)

The total phenolic content was measured using the Folin-Ciocalteu methodology described by Singleton and Rossi (1965) with modifications (Lazarin et al., 2020). In test tubes, 1.68 mL of ultrapure water, 20 µL of extract, and 100 µL of 10% (v/v) Folin-Ciocalteu reagent were added. After 3 min, 200 µL of 20 % (w/v) sodium carbonate solution was added to the tubes, and the mixture was vigorously agitated and allowed to react for 60 min. Then, the reading was performed in a spectrophotometer (Thermo Scientific, Orion AquaMate, USA) at 760 nm. The results were expressed as mg gallic acid equivalent (GAE)/g dry sample, calculated from a gallic acid standard curve.

3.2.2.8 Antioxidant activity

The antioxidant activity evaluations were determined using the FRAP and ORAC methods.

Ferric-reducing ability power (FRAP)

FRAP was performed according to the methodology proposed by Benzie & Strain (1996), with modifications. Initially, the FRAP solution was prepared from a mixture of 20 mL of acetate buffer (0.3 M), 2 mL of TPTZ solution (10 mM), and 2 mL of ferric chloride solution (20 mM). This mixture was prewarmed at 37 °C in the dark. In each microplate well, 175 µL of FRAP solution was added to 25 µL of the Sample, blank or standard (Trolox solution 0.01 - 0.06 mg/mL) and pre-incubated in the dark at 37 °C. After 30 min, the absorbance was read at 595 nm using a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Ortenberg, Germany) with Omega Mars 3.32R5 data analysis software. The results were expressed as mg trolox equivalent (TE) per mL of extract.

Oxigen radical absorbance capacity (ORAC)

The antioxidant activity determined by the hydrophilic ORAC method followed the methodology described by Ou et al. (2013) with modifications. To each well of the black microplate was added 25 µL of the Sample, blank (75 mM phosphate buffer, pH 7.4) and standard (Trolox solution 0.01 - 0.06 µg/mL), and was also added 150 µL of fluorescein working solution. The microplate was conditioned at 37 °C for 15 min in the shade of light. Immediately after, 25 µL of the AAPH solution was added. The fluorescence decrease (excitation at 485 nm; 510 nm) was measured in a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Ortenberg, Germany) for 100 min at 37 °C. Fluorescein solution was used as a positive control; the results were processed by Omega Mars 3.32R5 data analysis software and expressed as mg Trolox equivalent (TE) per mL of extract.

3.2.2.9 Quantification of isoflavones

Soybean and okara isoflavones were identified and quantified according to Huang Huang et al. (2021). The extracts were diluted 5 times in methanol and filtered through 0.22 µm membrane filters, and analyzed in a UHPLC system (Waters Acquity SQD/UPLC System, United States) equipped with a photodiode detector array (PDA), a single quadrupole MS system with electrospray ionization (ESI), an automatic injector, a quaternary pump, and a column oven. The compounds were separated on an Acquity Premier UPLC BEH C18 column (1.7 µm, 2.1 × 55 mm, Waters, USA). The samples were eluted at 0.2 mL/min with the column operating at 25 °C and a linear gradient consisting of Milli-Q water with 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B). The elution gradient was as follows: 0–1 min, 85% A; 1–12 min, 85%–20% A; 12–22 min, 20%–0% A; 22–26 min, 0% A; 26–28 min, 0%–85% A; 28–30 min, 85% A. The injection volume was 2 µl, and the UV absorption was monitored at wavelengths of 220:254 nm.

Six external standards were used for the quantification of the compounds. The six isoflavone standards, daidzein, genistein, glycitein, daidzin, genistein, and glycitein, were solubilized in methanol and, for the preparation of the curves were diluted in concentrations from 6.25 ng/mL to 10 µg/ml. The curves were plotted relating each isoflavone standard's peak area and concentration, and all showed an $R^2>0.99$. The acquired data were processed using MassLynx software (Waters, USA). The results were expressed as ug of the analyte per gram of dry soybean and okara (ug/ g dry sample).

The cone and capillary voltage were defined by injecting the mixture of isoflavone standards infused into the mass spectrometer and defined as the maximum intensity obtained. The capillary voltage was 3 kV, and the cone voltages were: 60 V for daidzein, genistein, and glycitein; and 40 V for daidzin, genistin, and glycitin.

The mass spectrum scan was operated in positive ESI mode in the 50-2000 m/z range. Nitrogen was used as the collision and desolvation gas. In addition, the extractor voltage was 5 V, the RF lens 0.3 V, the source temperature of 100°C, the desolvation temperature of 200°C, the desolvation gas flow rate of 600 L/h and the cone flow rate of 60 L/h. The ion fragments detected by mass spectrometry were identified by the mass charges of the six analyzed compounds.

3.3. Results and Discussion

3.3.1 Solvent screening by modeling in COSMO-SAC

There is a wide range of solvents that can be used in the extraction of bioactive compounds from plant matrices. These solvents can be classified as conventional solvents, such as methanol and ethanol, and alternative solvents, such as deep eutectic solvents.

The numerous solvents available, as well as the wide range of HBA and HBD compounds capable of preparing DES, to be applied in extraction processes, makes their screening a long and costly step, sometimes carried out by a "trial and error" approach. In this ambit, predictive models, such as the COSMO-SAC model, have been used to evaluate the solute affinity for the solvent before experimental steps. The interaction of each isoflavone, daidzein, genistein, glycitein, daidzin, genistin, and glycitin, with the solvent was investigated through the prediction of the activity coefficient at infinite dilution of each isoflavone in the solvent.

The screening by the COSMO-SAC model allows a better understanding of the extraction processes and solute-solvent interaction (Toledo Hijo et al., 2022). It permits saving time and financial resources. COSMO-based models have already been successfully applied for DES screening and solute-solvent interaction studies in extracting phenolic compounds from vegetable matrices (Liu & Zhang, 2022; Toledo Hijo et al., 2022).

The σ-profile of the molecules and solvents are shown in Figs. 3.1 and 3.2 as a tool to explain the polarity differences. The σ-profile of a molecule is located between ±0.02 $e/Å^2$. Values less than -0.01 $e/Å^2$ and greater than +0.01 $e/Å^2$ are considered hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD), respectively, *i.e.*, the polar regions. The range between -0.01 and + 0.01 $e/Å^2$ is considered the nonpolar region of the molecule (Mussagy et al., 2022; G. Oliveira et al., 2020). The colors of the 3D molecules are also a representation of the polarization charge density. The deep blue and red colors correspond to the strongly negative polarization charge density (positive parts of the molecules) and strongly positive polarization charge density (negative part of the molecules), respectively. The parts in green are the neutral or nonpolar regions of the molecules.

For the σ-profile of isoflavone molecules (Fig. 3.1A and 3.1B), it is observed that all molecules show a nonpolar region. But they also show peaks in the HBA and HBD polar regions. Thus, the isoflavones appear to have the ability to interact with both nonpolar and polar solvents.

In Fig. 3.2A, the $[Ch]^+$ cation of choline chloride exhibits strong attraction to HBD regions and with nonpolar groups, while Cl^- anion exhibits strong attraction behavior to HBA regions. Ethanol and methanol have similar characteristics and are predominantly nonpolar but with peaks in the polar regions.

The carboxylic acids (Fig. 3.2C and 3.2D) exhibit strong attraction abilities for both HBA and HBD groups and nonpolar groups, as well as the monosaccharides and glycerol (Fig. 3.2B). It is important to note that for DES, as the solvent, the σ-profile was evaluated for the HBD and HBA individually.

It is already well established that understanding solute-solvent interaction is important in extraction processes. In general, according to the σ-profile evaluation, polar and nonpolar species attract the isoflavones, and the evaluated compounds are potential solvents to extract them. Therefore, this affinity can be better understood by calculating the activity coefficient at infinite dilution ($\ln \gamma_i^\infty$) of the solutes at 333.15 K (60 °C), using an *in silico* approach based on thermodynamic models from quantum chemistry.

The values of $\ln \gamma_i^\infty$ of each isoflavone in all evaluated solvents are shown in Fig. 3.3, that the most negative are the values of $\ln \gamma^\infty$, the highest solute-solvent affinity.

Among the 17 solvents evaluated in the COSMO-SAC screening, 8 were selected for experimental screening and model validation. Considering the values of $\ln \gamma_i^\infty$, the solute-solvent affinity follows the order: methanol 80 % > ethanol 70 % > acetic acid 70 %>[Ch]Cl 70 % > [Ch]Cl: Acetic acid > [Ch]Cl: Formic acid > [Ch]Cl: Glycerol > [Ch]Cl: DL-lactic acid.

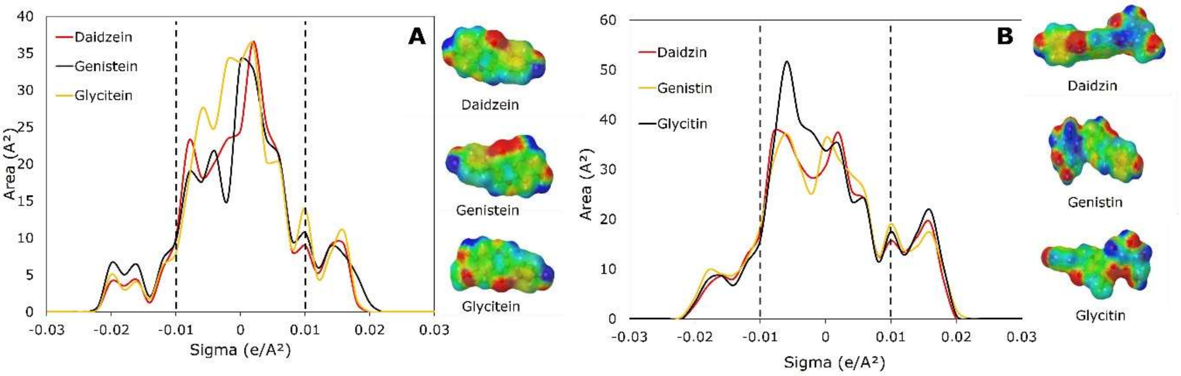


Fig. 3.1. σ-profile of isoflavone aglycone and glycoside molecules

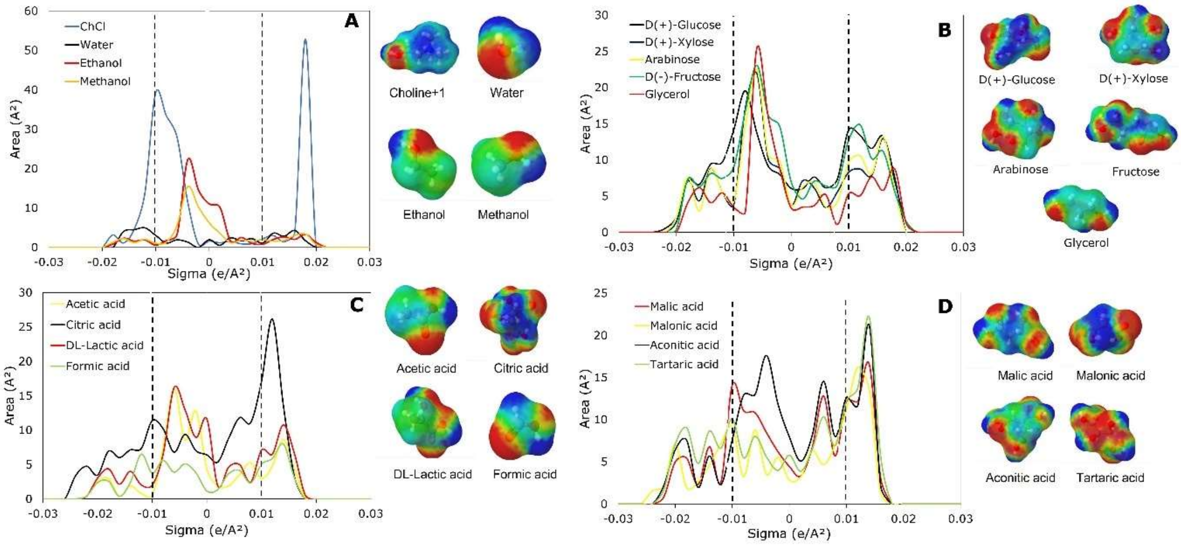


Fig. 3.2. σ-profile of alcohols, [Ch]Cl, water, mono, di, and tricarboxylic acids

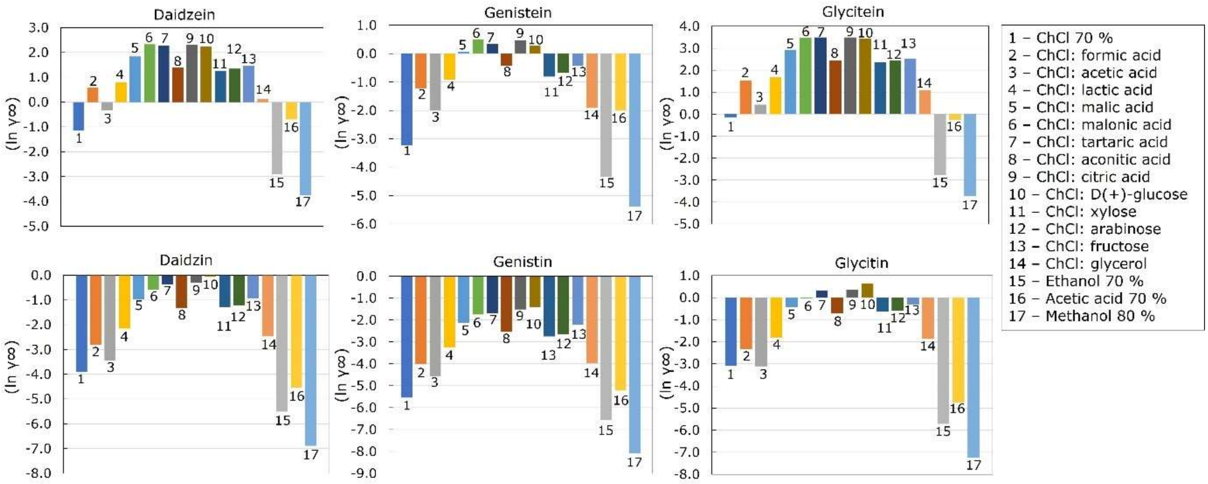


Fig. 3.3. Evaluation of the activity coefficient at infinite dilution of the isoflavones at 333.15 K in different solvents

Analyzing the log Kow data from Table 3.1 (for solvents) and Table 3.2 (for solutes), it can be observed that, although most of the chosen solvents (acetic, formic and lactic acids, methanol, and ethanol) present the lowest negative log Kow values (less hydrophilic) for the extraction of isoflavones (positive log Kow or hydrophobic behavior), this information should not be the only one to be considered, since [Ch]Cl is more hydrophilic and even so has a strong attraction for isoflavones represented by the values of the activity coefficients at infinite dilution ($\ln \gamma_i^\infty$).

3.3.2 Experimental validation

After computer screening with COSMO-SAC model prediction, the solvents with the highest interaction with the analytes were: methanol 80 % (v/v), ethanol 70 % (v/v), acetic acid 70 % (v/v), [Ch]Cl 70 % (w/v) and the DES [Ch]Cl: Acetic acid, [Ch]Cl: DL-lactic acid, [Ch]Cl: glycerol, [Ch]Cl: Formic acid. The DES [Ch]Cl: D(+)-glucose presented the worst performance in the computational modeling and was also used for experimental validation.

The total flavonoid content (TFC) of the extracts obtained from soybean and okara using the selected solvents was determined according to Juan e Chou (2010).

Fig. 4 contains the extraction data of total flavonoid content (TFC) from soybean and okara with the best solvents obtained from screening in COSMO-SAC. Extractions with conventional solvents, methanol, and ethanol, were performed for comparison since studies already use both solvents (Carneiro et al., 2020; Hsu et al., 2020; Kim et al., 2020).

The ethanol-water and methanol-water mixture extracted flavonoids from soybean in amounts equivalent to those reported in the literature. Kim et al. (2020) extracted approximately 0.8 mg equivalent of catechin/g of dry soybean using ethanol 70% (v/v) at room temperature. Zhu et al. (2018), using 18 varieties of soybean (6 black soybeans and 12 yellow soybeans), extracted 0.39-0.9 mg equivalent of rutin/g of dry soybean using methanol 80%.

For both raw materials, the DES [Ch]Cl: Acetic acid extracted the highest amount of flavonoids, 1.05 mg eq. of catechin/g dry soybean and 0.94 mg eq. of catechin /g dry okara. Although for soybean, the extract obtained with [Ch]Cl: Acetic showed no significant differences ($\alpha<0.05$) in total flavonoid content with those obtained with acetic acid 70% and ethanol 70%.

Flavonoids were not detected in the extracts obtained with DES [Ch]Cl: D(+)-glucose by the analytical method used, corroborating the prediction of COSMO-SAC as the worst flavonoid extraction solvent.

For soybean, the order of total flavonoid extraction was [Ch]Cl: Acetic acid ≈ ethanol 70% ≈ acetic acid 70% > methanol 80 % > [Ch]Cl 70 % ≈ [Ch]Cl: DL-lactic acid > [Ch]Cl: Glycerol ≈ [Ch]Cl: Formic acid > [Ch]Cl: D(+)-glucose.

For extraction of flavonoids from okara, the best solvents were [Ch]Cl: acetic acid > methanol 80% ≈ [Ch]Cl 70 % > [Ch]Cl: DL-lactic acid > acetic acid 70 % ≈ ethanol 70 % > [Ch]Cl: formic acid > [Ch]Cl:glycerol > [Ch]Cl:D(+)-glucose.

In addition to the intermolecular forces between solute and solvent, the solid-liquid extraction process of flavonoids considers the structure of the solid matrix and its modifications during the process, the amount of solute, and the solute-solute intermolecular forces. This justifies the difference in the solvent order by evaluating the activity coefficients at infinite dilution and the experimental results of total flavonoids, as well as concerning the soybean and okara raw materials.

Comparing the results of total flavonoids between soybean and okara, it is evident that soybean has a higher content of flavonoids. This difference was already expected as part of the flavonoids present in soybeans is extracted by water during the process of obtaining soy milk, resulting in okara with a lower content of flavonoids (Jankowiak et al., 2014a; Jankowiak et al., 2014b).

Pontes et al. (2021), evaluating DES composed of [Ch]Cl and different carboxylic acids, also obtained better extraction yields of phenolic compounds from olive leaves using the DES [Ch]Cl: Acetic acid (50 wt. % water) when compared to ethanol (50 % wt. % water). According to the authors, the higher extraction capacity was also attributed to the rupture of cells in the plant matrix, which after extraction with DES, showed more pronounced damage than with ethanol.

Toledo Hijo et al. (2022) reported good performance of DES [Ch]Cl: Acetic acid added with 50-75 wt. % water for extraction of phenolic compounds from yerba mate leaves when compared with [Ch]Cl-water and ethanol-based systems([Ch]Cl: ethanol and ethanol: water). Similar behavior was observed in this study, where DES [Ch]Cl: Acetic acid showed better performances than [Ch]Cl 70 % and ethanol 70 %.

For the extractions of total flavonoids from soybean and okara, DES composed of [Ch]Cl: Acetic acid proved to be an outstanding extraction solvent. In addition, it is considered environmentally friendly and non-toxic by the literature. It has equivalent prices ([Ch]Cl, ~USD

1.21 kg^{-1} and acetic acid, ~USD 0.6 - 1.0 kg^{-1}) to ethanol (~USD 0.82-0.98 L^{-1})(Toledo Hijo et al., 2022).

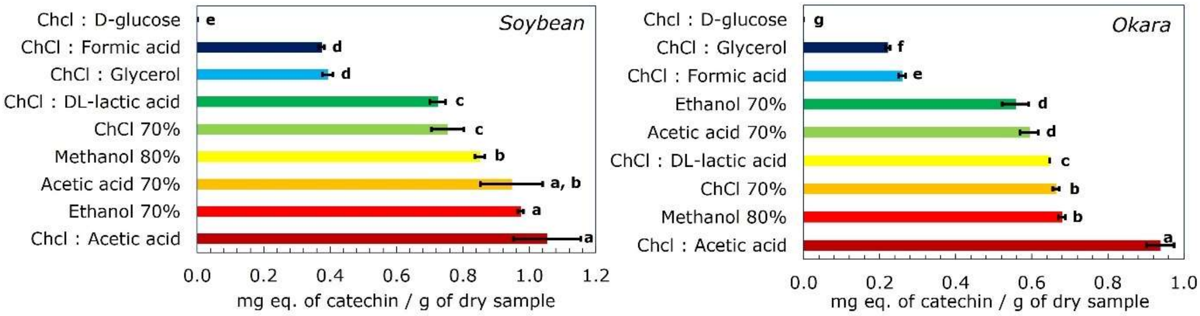


Fig. 4. Total flavonoid extraction using different solvents. Bars followed by the same letter have no statistical difference by the Tukey test ($\alpha \leq 0.05$)

To assess the relationship between the predicted responses by the COSMO-SAC model and the experimental results of TFC, we used Pearson's correlation coefficient (r). This coefficient provides an idea of the degree of relationship between the predicted and experimental responses. This relationship should be inversely proportional between the two variables. As the value of $\ln \gamma^{\infty}$ decreases, the solute-solvent interaction increases, and therefore, in theory, the total flavonoid content should be higher.

Inverse correlations were found between the total flavonoid contents of soybean and okara and the values of $\ln \gamma^{\infty}$ of each analyzed isoflavone. These relationships were statistically verified by Pearson's correlation coefficient (r). In this test, the r value equals -1, indicating the existence of a perfectly negative relationship, i.e., the variables are inversely related; zero indicates no relationship, and +1, a perfectly positive relationship, i.e., the variables are directly correlated. An r value >±0.5 indicates a high level of correlation (Wojeicchowski et al., 2021). The r values, shown in Table 3.3, were < -0.5, confirming the negative correlations. The correlations were higher for soybean, with r values < -0.7 for glycitein, daidzin, genistin, and glycitin and r values≈ -0.7 for daidzein and genistein.

Table 3.3. Pearson's correlation coefficient (r) between isoflavones and total flavonoids content from soybean and okara

	r	
Isoflavones	**Soybean**	**okara**
Daidzein	-0.699	-0.555
Genistein	-0.639	-0.520
Glycitein	-0.708	-0.551
Daidzin	-0.762	-0.612
Genistin	-0.718	-0.589
Glycitin	-0.763	-0.595

As complementary analyses, the total phenolic content and the antioxidant activity were determined for the soybean and okara extracts obtained with the 4 best solvents.

3.3.3 Total phenolics extraction

The phenolic compounds, which include phenolic acids and flavonoids (Canaan et al., 2022), were determined by the Folin-Ciocalteu method for soybean and okara extracts obtained with the 4 best solvents (Fig 3.5).

The total phenolic content for the soybean extracts (Fig 5) ranged from 1.17 mg GAE/g dry sample to 6.47 mg GAE/g dry sample. For the okara extracts, the range was from 0.0 mg GAE/g dry sample to 0.91 mg GAE/g dry sample.

According to Fig 3.5, the solvent that extracted the highest amount of phenolic compounds for soybean was acetic acid 70 %, followed by ethanol 70 %, methanol 70 %, and the DES [Ch]Cl: Acetic acid. In addition, all extracts are significantly different by the Tukey test ($\alpha \leq 0.05$).

For okara, the best solvent for extraction of phenolic compounds was methanol 80 % and, subsequently, DES [Ch]Cl: Acetic acid and [Ch]Cl: DL-lactic acid, both with no significant differences in the Tukey test ($\alpha \leq 0.05$). Phenolic content was not detected in the extract obtained with [Ch]Cl 70% by the analytical method used.

The total phenolic content analysis is not very selective because it includes all phenyl group molecules(Oliveira et al., 2022). This may have been the cause of the non-equivalence of the TPC and TFC results because other molecules such as gallic acid, chlorogenic acid, p-hydroxybenzoic acid, caffeic acid, p-coumaric acid, and ferulic acid may be present in soybean and okara samples (Zhu et al., 2018).

For soybean, Canaan et al. (2022) obtained 5.8 mg GAE/g dry sample using aqueous ethanol 70% (v/v) at room temperature for 24 h. Davy and Vuong (2021) obtained 0.86 mg GAE/g dry sample for freeze-dried okara by ultrasound-assisted extraction with methanol 50%. These results are close to those reported in this study.

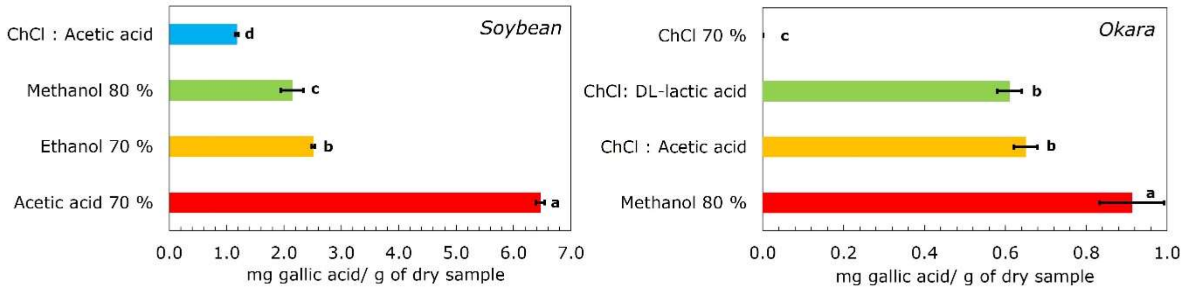


Fig. 3.5. Total phenolic content with different solvents. Bars followed by the same letter have no statistical difference by the Tukey test ($\alpha \leq 0.05$)

3.3.4 Antioxidant activity

FRAP and ORAC analyses were carried out to evaluate the antioxidant activity of soybean and okara extracts obtained with the 4 best solvents. Fig. 3.6 shows the results.

The antioxidant activity of the soybean extract obtained with [Ch]Cl: Acetic acid by the FRAP method was approximately 100% higher than that obtained with methanol 80 %. The okara extracts obtained with methanol and [Ch]Cl: Acetic acid did not show significant differences in the Tukey test (α ≤ 0.05).

DES can stabilize the extracted solutes due to molecular interactions resulting from hydrogen bonding between solutes and solvents. This interaction can reduce the availability of solutes to react with atmospheric oxygen(Dai et al., 2016). Adding to the DES's viscosity, such interactions reduce the biomolecules' mobility capacity and, therefore, their contact with oxygen at the air-solvent interface. (Dai et al., 2014). As a consequence, compared to conventional solvents, a reduction in oxidative degradation occurs, even in high-temperature processes(Dai et al., 2014, 2016).

For ORAC method, methanol 80 % showed the highest value for the soybean and okara samples. It was not possible to obtain results by this method for extracts obtained with DES [Ch]Cl: Acetic acid and Acetic acid 70% due to the loss of signal intensity.

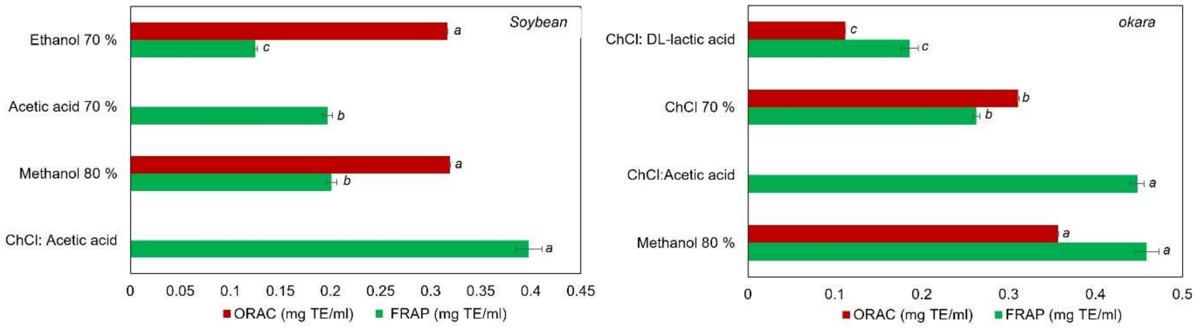


Fig. 3.6. Antioxidant activity of extracts obtained from soybean and okara. Bars followed by the same letter in the same column color have no statistical difference by the Tukey test (α ≤ 0.05).

3.3.4 Isoflavone content

The isoflavones of the extracts obtained from soybean and okara using the DES [Ch]Cl: acetic acid with 30% water were identified and quantified in a UHPLC-MS system, and the results are shown in Table 3.4.

For both soybean and okara, the total isoflavone contents are close to those reported in the literature (Hsu et al., 2020; Lummaetee et al., 2017; Muliterno et al., 2017; Ren et al., 2017; Tran et al., 2019; Yu & Yang, 2019).

Table 3.4. Isoflavone content obtained with DES [Ch]Cl: acetic acid with 30% water.

	Concentration (µg of isoflavone/g dry sample)						
Samples	**Daidzein**	**Genistein**	**Glycitein**	**Daidzin**	**Genistin**	**Glycitin**	**Total**
Soybean	116.60±8.34	97.03±5.7	8.73±0.45	317.01±7.9	434.20±18.1	94.48±4.55	1068.05
Okara	37.87±2.72	165.39±3.63	30.33±2.38	60.61±2.57	100.32±3.26	29.80±0.78	424.32

For soybean and okara, the total isoflavone contents were 1068.05 and 424.32 µg of TIC/g dry sample, respectively. For soybean, according to Table 3.4, the highest isoflavone contents were for genistin (40.65%), daidzin (29.68%), and daidzein (10.92%). For okara, the majority of isoflavones were genistein (38.98%), genistin (23.64%), and daidzin (14.28%).

3.4. Conclusions

This study evaluated the extraction of flavonoids from soybean and okara using different DES based on [Ch]Cl, as HBA with various HBD, acetic acid, formic acid, lactic acid, glycerol, malic acid, malonic acid, tartaric acid, aconitic acid, citric acid, xylose, arabinose, fructose, and glucose added with 30 % water as alternatives for sustainable replacement of toxic and volatile solvents such as methanol and ethanol, respectively.

The DES [Ch]Cl: Acetic acid added with 30 % water showed the highest extraction capacity for total flavonoids from soybean and okara among the solvents chosen in the screening step. For soybean, acetic acid 70 % was an excellent extraction solvent, equivalent to 70% ethanol and [Ch]Cl: Acetic acid. For okara, DES [Ch]Cl: Acetic acid extracted significantly more flavonoids than all other solvents. Furthermore, adding 30 % water to [Ch]Cl makes this mixture a good alternative for flavonoid extraction since it extracts a similar amount as methanol 80% and the DES [Ch]Cl: lactic acid.

In addition, the total phenolic content of soybean extracts was higher when acetic acid 70% was used. For okara, the total phenolic contents of the extracts obtained with [Ch]Cl: acetic acid and [Ch]Cl: DL-lactic acid were similar and lower than that obtained with methanol at 80%. Unfortunately, methanol represents a risk to the environment and human health.

Although it was not possible to quantify the antioxidant activity by the ORAC method for extracts containing acetic acid, the FRAP method showed that, for soybean, the extract obtained with DES [Ch]Cl: Acetic acid presented a statistically higher iron reduction capacity when compared with those of the extracts obtained with methanol 80%, ethanol 70% and acetic acid 70%. For okara, the extract obtained with methanol 80% showed the highest absolute result, but it was not statistically different than with [Ch]Cl: Acetic acid.

In addition, the presence of the isoflavones daidzein, genistein, glycitein, daidzin, genistin, and glycitin in the soybean and okara extracts obtained with DES [Ch]Cl: acetic acid with 30% water was validated through their identification and quantification using a UHPLC-MS system. The total isoflavone contents were 1068.05 and 424.32 μg total isoflavones/g dry sample for soybean and okara, respectively.

Therefore, the COSMO-SAC model can be used as a powerful tool to assist in screening solvents for extraction processes, saving time and expense. Besides, DES proved to be an alternative solvent for the extraction of isoflavones, replacing conventional solvents such as ethanol and methanol.

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Supplementary Material

Deep eutectic solvents as an alternative for extraction of flavonoids from soybean (*Glycine max* (L) Merrill) and okara: An experimental and computational approach based on COSMO-SAC model.

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1 Characterization of the raw material

In this study, the content of moisture, ashes, lipids, proteins, total fiber, and carbohydrates were determined. The results for ashes, lipids, proteins, and fiber were expressed in relation to the dry mass of the soybean and okara and, are presented in Table S1.

Table S1. Physical-chemical composition of soybean and okara

	This study				Literature(d)	
Composition	Soybean(a)	RSD(b), %	Okara(a)	RSD(b), %	Soybean	Okara
Water %	2.22±0.21	9.46	7.97±0.34	4.26		
Ashes %	4.43±0.03	0.68	3.0±0.03	1.0	4.6-4.7	2.8-3.18
Lipids %	20.91±0.05	0.24	13.46±0.25	1.86	20.89-21.2	13.0-14.72
Proteins %	34.7±0.3	0.86	27.5±1.2	4.36	37.3-46.97	32.29-37.0
Carb % ©	37.74	-	48.07	-		
Fibers %	37.27±0.27	0.72	43.96±0.82	1.87		

(a) Average ± standard deviation; –(b) relative standard deviation; (c) Carbohydrates % (d) Bowles et al. (2006) and Espinosa-Martos et al. (2009)

As shown, the moisture contents after the freeze-drying process were 2.22 % and 7.97 % for soybean and okara, respectively. The contents of ashes, lipids and proteins agree with those reported in the literature by Bowles et al. (2006) and Espinosa-Martos et al. (2009). Bowles et al. (2006) reported 4.7 and 2.8 % ashes, 37.3 and 37.0 % proteins and 21.2 and 13.0 % lipids for soybean and okara, respectively. Espinosa-Martos et al. (2009), on the other hand, found 46.97 and 32.29 % protein, 20.89 and 14.72 % lipids, and 4.6 and 3.18 % ash for soybean and okara, respectively. This variation in soybean and okara composition results may be influenced by several factors including climate, soil, soybean variety, and processing conditions of the soybean and okara.

CAPÍTULO 4

Influence of carboxylic acids on cholinium chloride-based deep eutectic solvents: a comprehensive study on ester formation and stability

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Abstract

Deep eutectic solvents (DES), especially those derived from cholinium chloride ([Ch]Cl), play a crucial role as alternative solvents in various chemical processes. Despite their widespread use in research, the understanding of the possible formation of impurities - such as esters - in these solvents is still incomplete. In this study, the thermal stability, long-term performance and the impact of water addition on [Ch]Cl-based DES were investigated, which have not yet been studied in the literature. Specifically, three [Ch]Cl-based DES with carboxylic acids - acetic acid, formic acid and lactic acid - were investigated. The results show that the DES formed with [Ch]Cl:formic acid has a higher temperature sensitivity and a lower long-term performance, however, the addition of water to this DES reduces significatively the rate of ester formation. The DES [Ch]Cl:lactic acid was found to be one of the most long-term stable, as was the [Ch]Cl:acetic acid DES. However, the [Ch]Cl:lactic acid has the disadvantage that, in contrast to acetic acid, lactic acid already contains a considerable amount of lactide ester. It is noteworthy that the esterification in the preparation of the DES [Ch]Cl:acetic acid only took place at temperatures up to 40 °C and no further impurities were formed, making it the most suitable of the DES studied for processes at this temperature. In summary, the choice of acid appears to influence ester formation, with temperature accelerating this reaction and the addition of water playing a crucial role in slowing down the rate of ester formation. In the long term, however, ester formation appears to be unavoidable, as esterification continues even after its production. Therefore, a comprehensive investigation of [Ch]Cl-carboxylic acid combinations with a focus on the ester content and its influence on the process is essential. A thorough investigation of the potential (re)use of these solvents is crucial for sustainable long-term applications.

Keywords: esters, esterification, acetic acid, lactic acid, formic acid, green solvents, RMN

4.1. **Introduction**

Deep eutectic solvents (DES) are a new class of alternative solvents formed from the mixture of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) (Abbott et al., 2003; Martins et al., 2019). According to Martins et al. (2019), DES are defined as mixtures of two or more pure compounds whose eutectic point temperature deviates significantly from the ideal temperature, and remains constantly below the operating temperature. Among the HBA, cholinium chloride ([Ch]Cl) stands out as HBA, while various compounds, including carboxylic acids, amides and sugars, serve as HBD (Ashworth et al., 2016).

Among DES classes, those based on [Ch]Cl and carboxylic acids have attracted considerable research interest due to their versatility and low toxicity. However, the assessment of toxicity can be controversial and depends on the DES rather than the class as a whole (De Morais et al., 2015; Macário et al., 2018, 2019). These solvents are used in various chemical processes, including extraction of bioactive compounds (Farias et al., 2023; Ferreira et al., 2023), metal recovery (Chen et al., 2023; Luo et al., 2023), purification and solubilization of compounds (Bowen et al., 2022; Uka et al., 2023; Xu et al., 2023), lignocellulosic biomass processing (Sun et al., 2023), removal of microplastics from water (Hunter et al., 2023), production and purification of biodiesel (Abed et al., 2023; Chinh Nguyen et al., 2023; Mamtani et al., 2023), among various other applications.

Nevertheless, DES based on [Ch]Cl-carboxylic acids lead to the formation of secondary compounds by esterification reactions, which can occur independently of the preparation method, as reported by Rodriguez-Rodriguez et al. (2019). Thermal stability studies using different preparation methods, such as heating with stirring and stirring at room temperature, showed that while heating led to a lower viscosity of DES, the use of high temperatures increased ester formation. This behavior was attributed to the esterification reaction between the carboxylic acid and the OH group of the cholinium cation (Florindo et al., 2014; Rodriguez Rodriguez et al., 2019). This behavior was first observed by Florindo et al. (2014), who reported impurity levels of 5-30 wt.% with the heating method, while the grinding method yielded pure mixtures without ester formation. The evaluation of the heating, grinding, and freeze-drying preparation methods were also evaluated in the preparation of DES based on L-proline, glycolic acid, and DL-malic acid (Van Den Bruinhorst et al., 2018). The authors also observed that in these DES the esterification reaction occurred independently of the preparation method, with the grinding method having the lowest amount of esters. However, it should be kept in mind that, as shown by Van Den Bruinhorst et al. (2018) and Rodriguez-Rodriguez et al. (2019) water can be used to reduce the esterification reaction rate of DES. Since the esterification reaction is an equilibrium process, the gradual production of water during the reaction is expected to reduce the reaction rate until a limit is reached over time.

The aim of this work was to investigate ester formation in DES based on [Ch]Cl-carboxylic acid, in particular [Ch]Cl: acetic acid, [Ch]Cl: formic acid and [Ch]Cl: lactic acid, all in a molar ratio of 1:2. In the study, DES esterification was observed, focusing on the percentage of [Ch]Cl esterification by nuclear magnetic resonance (NMR) under different DES preparation conditions, including temperature (20 °C to 80 °C), time (up to 14 days) and water addition (10 wt.% to 70 wt.%).

4.2. Material and methods

4.2.1. Material

The reagents used to prepare the DES were cholinium chloride ([Ch]Cl) from Sigma-Aldrich (USA, purity > 98 %), formic acid from Carlo Erba Reagents (France, purity > 99 %); acetic acid from Honeywell (Germany, purity > 99.99 %); L-lactic acid from Sigma-Aldrich (France, purity > 88-92 %). In this work, ultrapure water, which was double distilled, passed through a reverse osmosis system and treated in the Milli-Q plus 185 water purification system, and dimethyl sulfoxide D6 (DMSO) from Eurisotop (USA, purity > 99.9 %) were used.

4.2.2. Methods

4.2.2.1. Preparation of the deep eutectic solvents

The DES were prepared on magnetic stirrers (Heidolph, MR HEI-Tec, Germany) at 600 rpm, with a conduction heating jacket at a fixed temperature until a homogeneous transparent liquid was achieved. The HBA-HBD mixtures evaluated in this study were [Ch]Cl: acetic acid (molar ratio 1:2), [Ch]Cl: formic acid (molar ratio 1:2) and [Ch]Cl: lactic acid (molar ratio 1:2). The individual compounds were accurately weighed within $\pm 10^{-4}$ g on an analytical balance (Sartorius, BCE623I-1S, Germany).

Three key parameters were investigated in the preparation of DES: temperature, time and water addition. Specifically, first DES were prepared at temperatures of 20 °C, 40 °C, 60 °C and 80 °C for 2 h, both without and with 10 wt.% water addition, in order to evaluate the temperature effect. Then, to evaluate the time-dependent aspects, a kinetic study was carried out over 14 days at intervals of 2 h, 48 h, 168 h and 336 h at 20 °C for DES without added water. Finally, the effects of water addition were investigated, focusing on water contents of 10 wt.%, 30 wt.% and 70 wt.% over a period of 2 h at 20 °C.

4.2.2.2. Esterification calculation

The esterification reaction was evaluated using a NMR spectrometer (Bruker Ascend 300, USA) operating at 300 MHz according to Rodriguez-Rodriguez et al. (2019) to record the ^{1}H NMR spectrum. All the samples (DES and compounds used in the formation of DES) were dissolved in DMSO.

The percentage of [Ch]Cl esterification was determined by analyzing the ^{1}H NMR spectrum of DES according to the method described by Rodriguez-Rodriguez et al. (2019). Before performing the NMR analysis, it is important to consider the possible esterification reactions for the DES under study, shown in Fig. 4.1, to enable a comprehensive analysis.

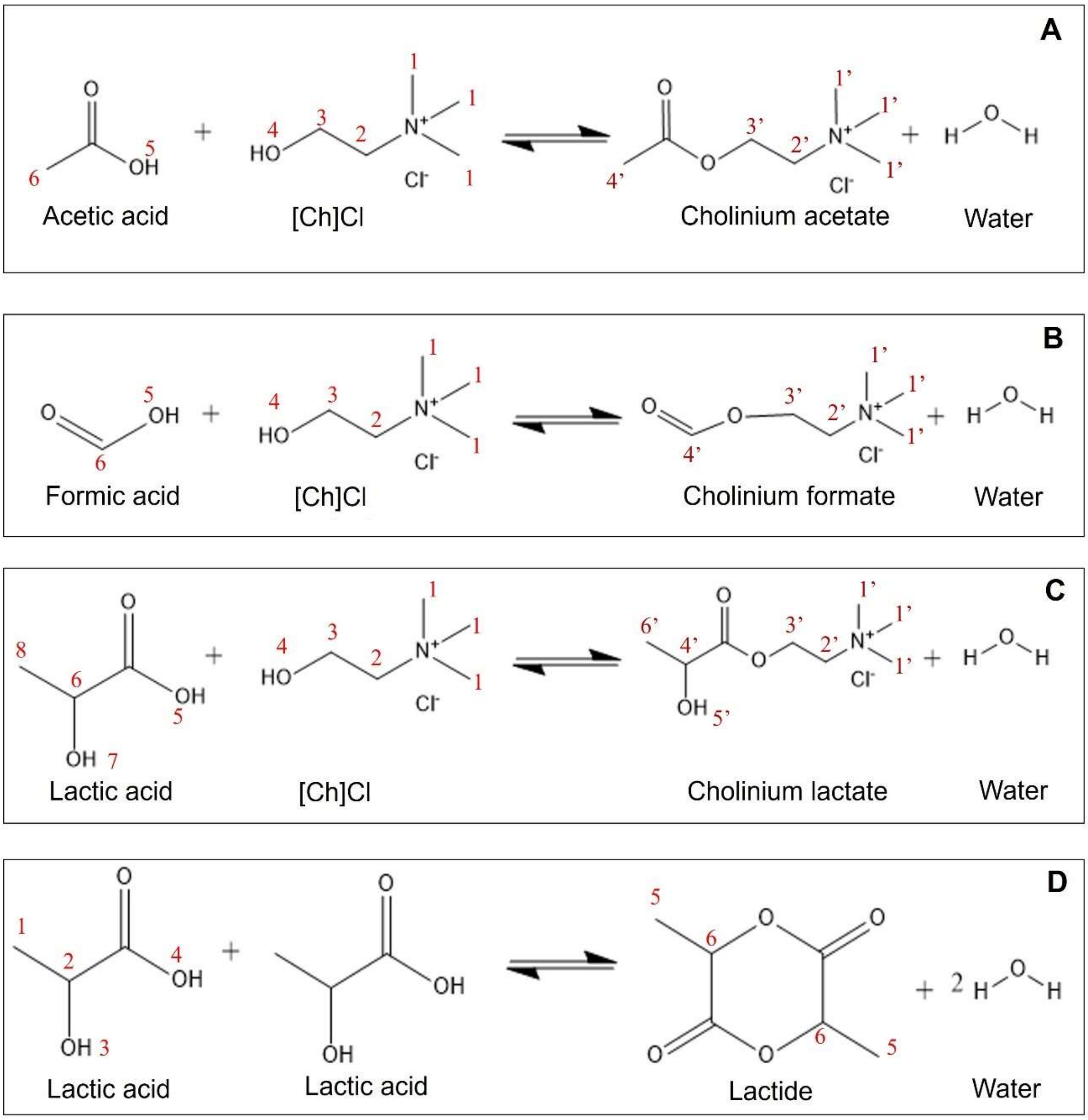

Figure 4.1. Esterification reactions for the systems formed by (A) [Ch]Cl: acetic acid, (B) [Ch]Cl: formic acid and (C) [Ch]Cl: lactic acid and (D) lactide.

Fig. S1 in Supplementary Material illustrates an example of a ^{1}H NMR spectrum integration to determine the percentage of [Ch]Cl esterification. First, the characteristic peak of the [Ch]Cl methyl groups (labeled 1) was identified, and the integration was normalized to 9 based on the sum of protons associated with the three methyl groups. Subsequently, the esterification signal became clear when the hydrogen atoms of the [Ch]Cl methyl groups (peak 1) shifted upon ester formation (peak 1'). Similarly, the hydrogen atoms of the ethylene group of [Ch]Cl (peak 3) partially split during esterification (peak 3'). The hydrogen atoms of the carboxyl group of formic acid (peak 6) were also shifted during ester formation (peak 4’). After integration of the ^{1}H NMR spectrum, the molar percentage of [Ch]Cl esterification ($E_{[\mathrm{Ch}]\mathrm{Cl}}$) was calculated by relating the peak area of the ester methyl group (peak 1') to the sum of the areas of the ester methyl groups (peak 1') and [Ch]Cl (peak 1) according to Eq. 1. A similar concept was applied for ethylene group of [Ch]Cl and carboxyl group of formic acid during the ester formation as represented in Eq. 1.

$$E_{[\mathrm{Ch}]\mathrm{Cl}}\ (\mathrm{mol\%}) = \frac{(\mathrm{A1'})}{(\mathrm{A1'+A1})} \times 100 = \frac{(\mathrm{A2'})}{(\mathrm{A2'+A2})} \times 100 = \frac{(\mathrm{A3'})}{(\mathrm{A3'+A3})} \times 100 \qquad \text{Eq. (1).}$$

In Eq. 1, A1’ represents the area of the methyl group of esters and A1 the area of the methyl group of [Ch]Cl, A2’ and A3’ represents the area of the ethylene group of esters and A2 and A3 the area of ethylene group of [Ch]Cl, as shown in Fig. S1 in Supplementary Material.

For the [Ch]Cl: lactic acid system, in addition to the formation of cholinium lactate esters, there was the formation of lactide ester, already present in commercial lactic acid (Fig.1D). To calculate the molar percentage of lactide esterification ($E_{[\mathrm{Ch}]\mathrm{Cl}}$), the peak of the methyl group of lactic acid (peak 1) and that of lactide (peak 5) were considered. The calculation was carried out according to Eq. 2:

$$E_{\mathrm{Lactide}}\ (\mathrm{mol\%}) = \frac{(\mathrm{A5})}{(\mathrm{A1+A5})} \times 100 \qquad \text{Eq. (2).}$$

where, A5 represents the area of the methyl group of lactide and A1 the area of the methyl group of lactic acid, as shown in Fig. S2 in Supplementary Material.

4.3. Results and Discussion

In this study, we investigated the influence of temperature, time and water concentration on the esterification reaction in three different DES. Each DES contained [Ch]Cl as HBA and either acetic acid, formic acid or lactic acid as HBD. The evaluation of the esterification reaction was based on the observed partial ^{1}H chemical shift in the NMR spectrum, as described in detail in the experimental section.

4.3.1. Effect of the temperature in deep eutectic solvents preparation

To evaluate the effects of temperature, DES preparations were carried out at constant temperatures of 20 °C, 40 °C, 60 °C and 80 °C, during 2 h for DES without and with 10 wt.% of water. The ^{1}H NMR spectra for DES [Ch]Cl: acetic acid are shown in Fig. 4.2, further spectra for the DES studied can be found in Figs. S3 and S4 in Supplementary Material. The quantitative results of the esterification results for all DES studied are shown in Fig. 4.3 (see Tables S1 and S2 in Supplementary Material for more details).

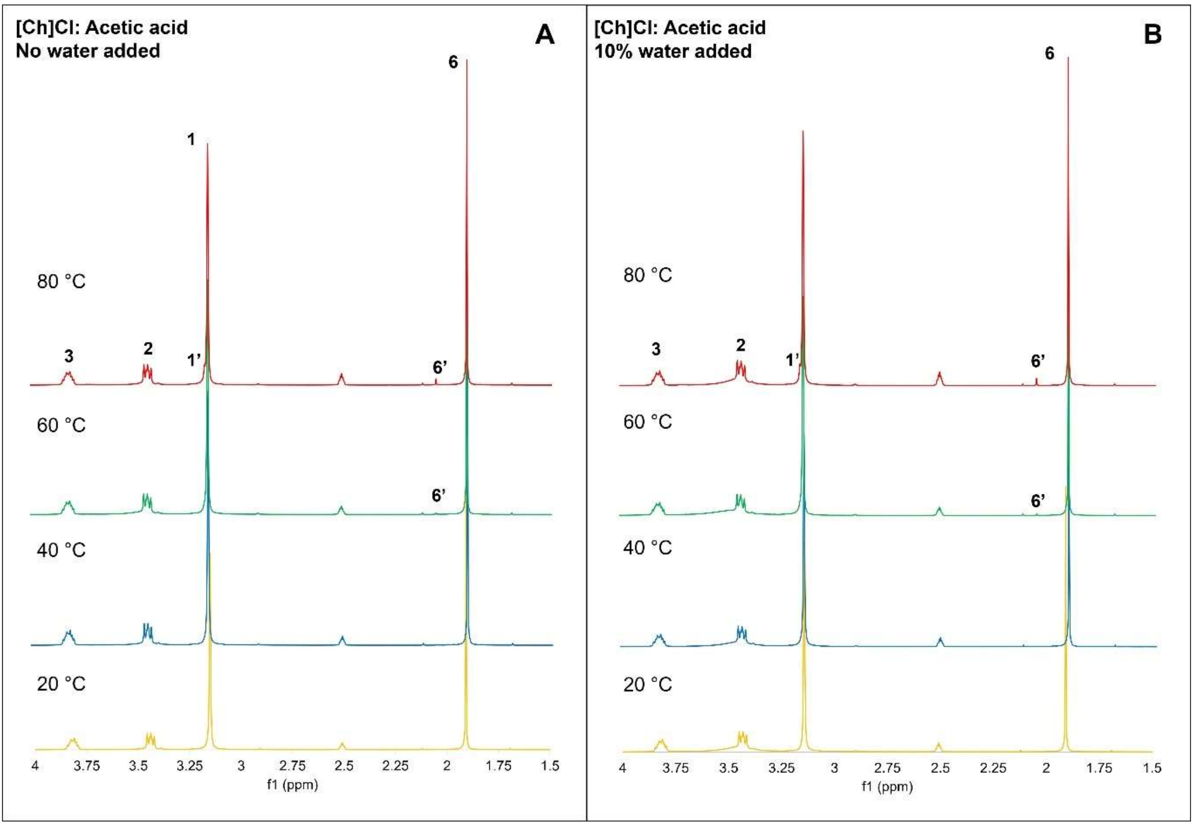

Figure 4.2. H-NMR spectra for the DES (A) without addition and (B) with addition of 10 wt.% water at different preparation temperatures, during 2h.

Fig. 4.2 shows that for the DES of [Ch]Cl: acetic acid, without and with 10 wt.% water, no clear peak indicating the esterification reaction could be observed up to a temperature of 40 °C at a preparation time of 2 h. When the DES is heated to 60 °C, the methyl group peaks of [Ch]Cl (peak 1) show a broadening of the base and the appearance of a protrusion characteristic of a peak that begins to split off (peak 1') and belongs to the methyl group of the cholinium acetate ester. This phenomenon can be observed in DES with and without the addition of water. Looking at the ^{1}H NMR of DES heated to 80 °C, a clear increase in the signal intensity of the prominence associated with the peak of the ethyl group can be observed (peak 1'). Moreover, the acetic acid methyl group peak at 1.91 ppm (peak 6) gradually disappears when the temperature increases from 60 °C to 80 °C, accompanied by the appearance of peaks at 2.05 ppm (peak 6'). These observations emphasize the significant influence of the preparation temperature on the stability of the DES. These results are also confirmed by the esterification values of [Ch]Cl, which reached 4 mol% at 80 ºC, while they were 0 mol% at 20 ºC for both the DES without and for the DES with added water (see Fig. 4.3). Additionally, it is worth mentioning that [Ch]Cl: acetic acid esterification results observed exclusively results from the cholinium acetate without the formation of any other esters.

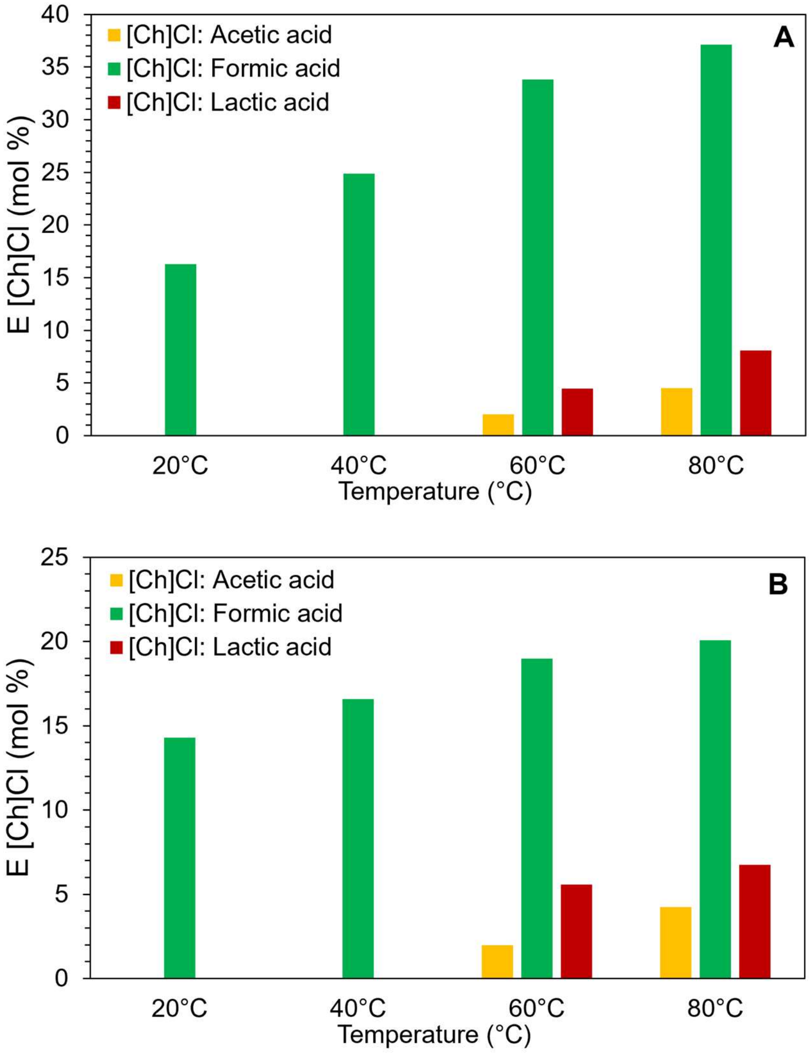

Figure 4.3. Molar percentage of [Ch]Cl esterification ($E_{[Ch]Cl}$) for DES (A) without addition and (B) with addition of 10 wt.% water at different preparation temperatures, during 2 h.

Looking now at the results related to the preparation of [Ch]Cl: formic acid DES without addition of water and with 10 wt.% addition of water at different temperatures over a period of

2 h, the ^{1}H NMR spectra (Fig. S3 in the Supplementary Material) show a separation of the peaks characterizing the methyl group (3.14 ppm, peak 1) of [Ch]Cl and the methyl group of the cholinium formate ester (3.17 ppm, peak 1'). These results were observed for all temperatures studied in the preparation [Ch]Cl: formic acid DES. Similarly, it was also observed that the peaks of the ethylene groups of [Ch]Cl at 3.43 ppm (peak 2) and 3.80 ppm (peak 3) split into 3.73 ppm (peak 2') and 4.54 ppm (peak 3') upon ester formation. Moreover, the intensity of the peaks corresponding to ester formation gradually increases with temperature, as shown in Fig. S3 in the Supplementary Material for the peaks labeled 1', 2', 3' and 4'. This observation is confirmed by the determination of the percentage of [Ch]Cl esterification (see Fig. 4.3, please).

For [Ch]Cl: formic acid without the addition of water, an increase in the cholinium formate ester content of about 131% was observed when DES was prepared at 80 °C compared to 20 °C (from 16 mol% at 20 °C to 37 mol% at 80 °C, Fig. 3A). For [Ch]Cl: formic acid with 10 wt.% water added, the ester content also increases, albeit to a lesser extent, by about 43% when DES is prepared at 80 °C compared to 20 °C (from 14 mol% at 20 °C to 20 mol% at 80 °C, Fig. 3B). Consequently, the presence of water reduces the formation of esters by approximately 46% when the DES is produced at 80 °C. However, a more detailed discussion on the effect of water will be provided later. In addition, in the case of [Ch]Cl: formic acid, also no esters other than cholinium formate were formed.

Looking now at the results of the preparation of [Ch]Cl: lactic acid without and with the addition of water (i.e. 10 wt.%) for up to 2 h at different temperatures, the ^{1}H NMR results are shown in Fig. S4 in the Supplementary Material. It should be noted that, as mentioned in the experimental section, in this case the lactic acid used for DES formation already contained an ester, namely lactide ester formed from the condensation of two molecules of lactic acid (see Fig. S2 in the Supplementary Material, where the peaks number 5 and number 6 correspond to chemical shifts of the hydrogen protons of the lactide molecule). This observation was also made by Morais et al. (2021) when using DES from [Ch]Cl: lactic acid (1:10 molar ratio). They suggested that the formation of lactide could confirm the polymerization of lactic acid, as lactide is a common intermediate in this process (Morais et al., 2021). Concerning the ester content of the lactic acid remained almost constant at all temperatures studied, i.e. at about 19 mol% (see Tables S1 and S2 in the Supplementary Material). The only exception was the [Ch]Cl: lactic acid composition with 10 wt.% water at 80 ºC, where the ester value was slightly lower, namely 17 mol%. No evidence of decomposition mechanisms other than lactide was found when evaluating degradation of lactic acid.

Finally, regarding the results of the [Ch]Cl: lactic acid, as shown in Fig. S4 in the Supplementary Material, no characteristic peaks of the esterification reaction were observed up to a temperature of 40 °C, beyond the lactide esters already present. However, at temperatures of 60 °C and 80 °C, chemical shifts corresponding to the hydrogens of the methyl groups of the ester at 3.17 ppm (number 1') and the OH group at 4.48 ppm (number 5') were observed. Both groups were associated with the formation of cholinium lactate when the mixture was heated above 40 °C. Specifically, [Ch]Cl esterification increased from 4 mol% at 60 °C to 8 mol% at 80 °C, an increase of approximately 81%, as shown in Fig. 4.3A for the system without added water. It should be noted that these values do not take the lactide into account. When examining the [Ch]Cl esterification values for the [Ch]Cl: lactic acid with the addition of 10 wt.% water, only a 21% increase was observed (from 5.56 mol% at 60 ºC to 6.74 mol% at 80 ºC, see Figure 4.3B and Table S2 in the Supplementary Material). Thus, the addition of 10 % water at a temperature of 80 °C led to a reduction in the formation of cholinium lactate, although the specific influence of water will be investigated in more detail later. Moreover, it should have in account when evaluating the degradation of [Ch]Cl: lactic acid, in contrast to the other DES, two mechanisms must be taken into account, as two esters are presented/formed, namely lactide and cholinium lactate.

In summary, the ester content of all DES increased with increasing temperature. In particular, [Ch]Cl: formic acid showed the highest sensitivity to esterification, with [Ch]Cl esterification increasing from 16 mol% at 20 °C to 37 mol% at 80 °C without added water and from 14 mol% at 20 °C to 20 mol% at 80 °C with 10 wt.% added water. Our results agree with those of Rodriguez-Rodriguez et al. (2019), who observed that for all [Ch]Cl-based DES and various acids (lactic acid, glycolic acid, levulinic acid, malic acid, oxalic acid, glutaric acid and malonic acid) the degrees of esterification were directly proportional to the temperature increase. With their results ranged from 1 mol% for the DES [Ch]Cl: glutaric acid (1:1) at 60 °C to 34 mol% for the DES [Ch]Cl: oxalic acid (1:1) at 100 °C after 2 hours of preparation. According to the authors, the experimental data show the formation of esters when heat is applied. In contrast, we observed no esterification of [Ch]Cl in the [Ch]Cl-based DES prepared with acetic and lactic acid up to a temperature of 40 °C. It is worth noting that acetic acid does not contain an ester, whereas lactic acid itself already contains an ester. In contrast to our results, Van Den Bruinhorst et al. (2018) reported ester formation in proline-based DES with glycolic and malic acid, which occurs even at room temperature.

4.3.2. Effect of the time in deep eutectic solvents

Considering the different time intervals at which researchers prepare and use DES and their reusability, we investigated the temporal effects on DES esterification. The esterification reaction for the three DES studied was observed over different times (time storage) - 2 h, 48 h, 168 h and 336 h - at a constant temperature of 20 ºC. Fig. 4.4 shows the results the percentage of [Ch]Cl esterification over time for DES without added water at 20 ºC.

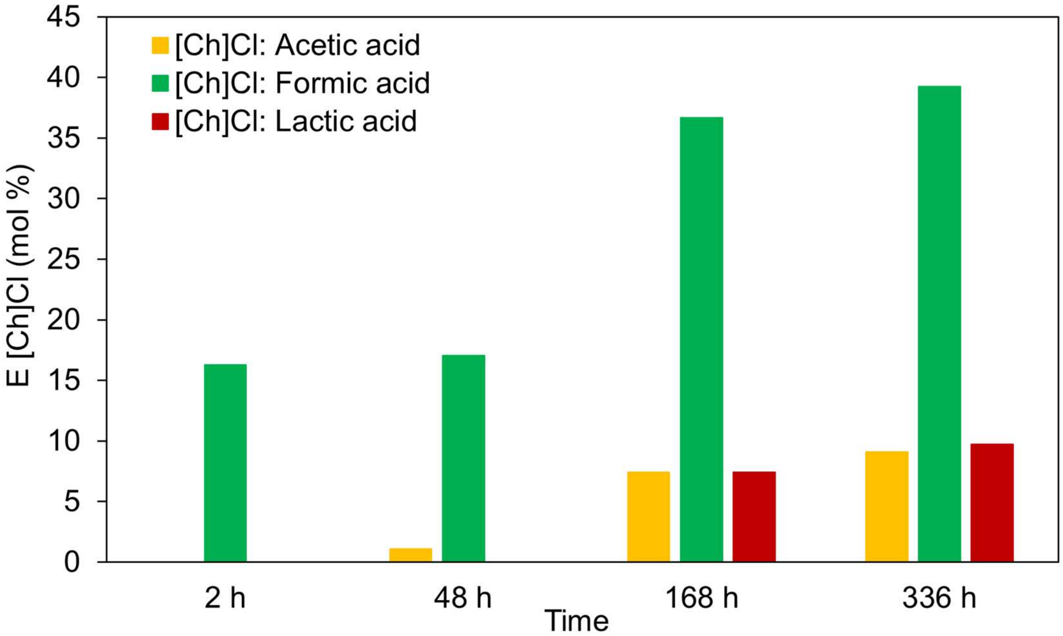

Figure 4.4. Molar percentage of [Ch]Cl esterification ($E_{[Ch]Cl}$) for DES prepared without addition of water at 20°C, measured at different preparation times.

The [Ch]Cl: acetic acid and [Ch]Cl: lactic acid DES exhibited [Ch]Cl esterification values of 0 mol% after 2 h of preparation, which increased to 9 mol% and 10 mol%, respectively, after 336 h of storage. It is noteworthy that the lactide content in [Ch]Cl: lactic acid DES remained almost constant during the storage period (see Table S3 in the Supplementary Material). In contrast, the [Ch]Cl: formic acid mixture showed a significant increase in ester content by about 141%, rising from 16 mol% to 39 mol% during the 336-h storage period.

For all DES tested, the increase in [Ch]Cl esterification within the first 48 h of storage was not significant (e.g., [Ch]Cl: acetic acid content was 0 mol% after 2 h and 1 mol% after 48 h). A notable increase in esterification occurred after 168 h (e.g., [Ch]Cl: acetic acid content was 1 mol% after 48 h and 7 mol% after 168 h). Comparing esterification values between 168 and 336 hours showed no significant difference (e.g., [Ch]Cl: acetic acid content was 7 mol% after 168 h and 9 mol% after 336 h). Therefore, seems that 168 h period is critical for ester formation. Van Den Bruinhorst et al. (2018) also investigated the effect of time on the esterification reaction of the DES formed by [Ch]Cl: glycolic acid. They observed that the esterification reaction continues even after preparation, yielding a proline ester content of approximately 5.5 mol%. Rodriguez-Rodriguez et al. (2019) also observed that the ester content of [Ch]Cl with glycolic acid and [Ch]Cl with lactic acid increased to 14 mol% and 11 mol%, respectively, after 11 months of preparation. Both the authors concluded that, as the esterification reaction produces water and is an equilibrium reaction, it is expected that the production of water gradually suppresses the reaction rate, reaching a limit over time.

In summary, the stability of [Ch]Cl: acetic acid and [Ch]Cl: lactic acid was generally higher from the three DES tested. In particular, [Ch]Cl: acetic acid showed a clear advantage, as acetic acid did not exhibit ester formation. This property emphasizes its superior stability compared to the other DES, making it suitable for post-preparation storage and possible reuse in multiple cycles. However, additional parameters need to be carefully considered when we are considering the reuse of the solvent.

4.3.3. Effect of the water in deep eutectic solvents

The impact of water addition in DES preparation was investigated with varying concentrations: 0 wt.%, 10 wt.%, 30 wt.%, and 70 wt.%. The selected amounts of water were based on experiments conducted for the extraction of bioactive compounds (Ferreira et al., 2023; Pontes et al., 2021; Toledo Hijo et al., 2022), in which the formation of esters has not yet been taken into account, an aspect that has been overlooked. The experiments were conducted for a preparation time of 2 h at 20 °C. The [Ch]Cl esterification results are summarized in Table 4.1.

Table 4.1. Molar percentage of [Ch]Cl esterification ($E_{[Ch]Cl}$) and of lactide esterification ($E_{Lactide}$) for DES prepared with different water content, at 20°C, during 2 h.

		% of water added			
	Solvents	***NWA**	**10 wt.%**	**30 wt.%**	**70 wt.%**
$E_{[Ch]Cl}$ (mol %)	[Ch]Cl: Acetic acid	0.00	0.00	0.00	0.00
	[Ch]Cl: Formic acid	16.28	14.29	11.85	11.07
	[Ch]Cl: Lactic acid	0.00	0.00	0.00	0.00
$E_{Lactide}$ (mol %)	[Ch]Cl: Lactic acid	18.75	18.87	19.34	19.26
	Lactic acid pure	21.26			

NWA: No water added

Looking now at the results for [Ch]Cl-acetic acid, no solvent degradation was observed when different amounts of water were added to this DES, as evidenced by the absence of chemical shifts of the ions in the ^{1}H NMR spectra (see Fig. S5 to S8 in the Supplementary Material).

In contrast to the [Ch]Cl: acetic acid DES, the [Ch]Cl: formic acid system showed esterification up to 16 mol%. It is noteworthy that, the addition of water enhances the stability of the DES, leading to a significant decrease of the esterification reaction up to 32% when 70 wt.% water is added (percentage of [Ch]Cl esterification: 16 mol% for DES without water and 11 mol% for DES with 70 wt.% water).

For [Ch]Cl: lactic acid, no degradation effect was observed beyond the secondary compounds that were already present in the "pure" lactic acid - the acid used in DES preparation as received from the producer - prior to DES preparation (as shown in Figure S2 in Supplementary Material and discussed previously). However, the addition of water led to a significant decrease in lactide content, which dropped from 21 mol % (no water added) to about 19 mol% (70 wt.% water added). This decrease can be attributed to the hydrolysis of lactide in the presence of water, producing two molecules of lactic acid (Morais et al., 2021) (Castro-Aguirre et al., 2016; Dusselier et al., 2013).

In summary, the results indicate a reduction in the degree of esterification of the DES investigated with increasing water addition, as expected taking in account what has been suggested by Van Den Bruinhorst et al. (2018) and Rodriguez-Rodriguez et al. (2019). In situations where the presence of esters is undesirable, the addition of water proves to be a crucial factor in controlling their formation. Since esterification is a reversible equilibrium reaction, the equilibrium must be maintained in favor of the reactant. Although water can alter the properties of DES, as reported by (Rodriguez Rodriguez et al., 2019), who found that the addition of more than 51 wt.% water to DES-urea:[Ch]Cl (2:1) can turn the mixture into an aqueous solution. However, it is important to consider the specific objectives and carefully weigh the advantages and disadvantages of adding water. Although many researchers prefer to dry the reagents prior to DES preparation, this study shows that such a practice compromises DES stability and shifts the reaction equilibrium towards product formation (esters and water). Moreover, this approach increases the cost and complexity of the work. Another point that should have been in account is that many studies in the literature have used DES for the extraction of various compounds such as phenolic compounds (Barbieri et al., 2020), metals (Wang et al., 2022) and lignins (Ceaser et al., 2023; Li et al., 2021) without considering the effects of DES esterification in these processes. In addition, these solvents were used in simulation procedures with models such as COSMO-SAC and COSMO-RS to predict activity coefficients at infinite dilution ($\ln \gamma_i^\infty$) without considering the formation of secondary compounds. Not considering these compounds in the modeling may affect the prediction accuracy of the model in solvent screening procedures, especially when extracting bioactive compounds.

4.4. Conclusions

In this work, we investigated the effects of different conditions for the preparation of DES based on [Ch]Cl: acetic acid, [Ch]Cl: formic acid and [Ch]Cl: lactic acid on DES degradation. In particular, we investigated the effects of temperature, time and water concentration on [Ch]Cl esterification. Elevated temperatures, especially for [Ch]Cl: formic acid-DES without added water, intensified the esterification reaction. In contrast, [Ch]Cl: acetic acid proved promising for processes up to 40 ºC and showed no esterification or impurity formation. Over a period of 336 h or 14 days at 20 ºC, an increase in esters was observed for all three DES, which was particularly pronounced for [Ch]Cl: formic acid. However, for [Ch]Cl: lactic acid and [Ch]Cl: acetic acid, the increase in esters was minimal, with the added advantage that [Ch]Cl: acetic acid showed no ester formation, unlike lactic acid, which formed lactide. Although the addition of water could not completely prevent ester formation, it effectively slowed down the esterification process. Therefore, ester formation in [Ch]Cl-carboxylic acid-based DES is an inevitable long-term outcome, but the strategic addition of water can effectively slow down this process. Nevertheless, a more nuanced assessment of the impact of ester content on DES properties, application performance and other relevant factors is critical. Further research is needed to expand the practical applications of these solvents. The knowledge gained in this study contributes to both basic research and applications in processes where DES are used.

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Author contributions

Ramon S. B. Ferreira – Conceptualization, methodology, formal analysis, investigation, writing – original draft, writing – review and editing. **João A. P. Coutinho** – Conceptualization, writing - review and editing, supervision, project administration, funding acquisition. **Eduardo A. C. Batista** – Conceptualization, writing - review and editing, supervision, project administration, and funding acquisition.

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Supplementary Material

Influence of Carboxylic Acids on [Ch]Cl-Based DES: A Comprehensive Study on Ester Formation and Stability

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FIGURES

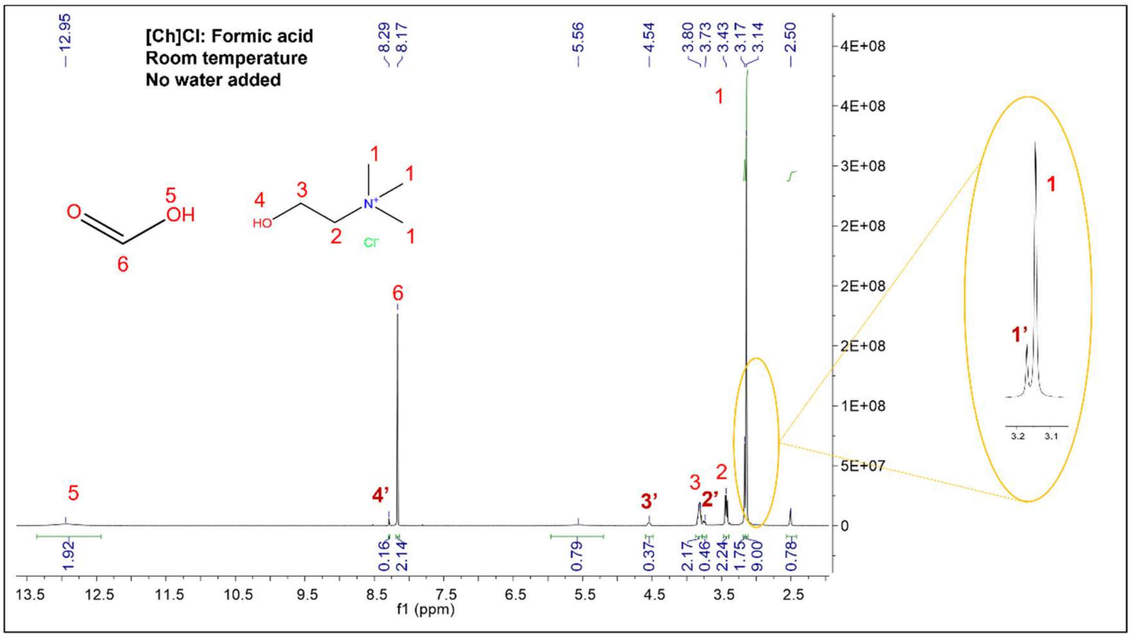

Figure S1. ^{1}H-NMR spectra of DES [Ch]Cl: Formic acid prepared at 20°C for 2h without the addition of water.

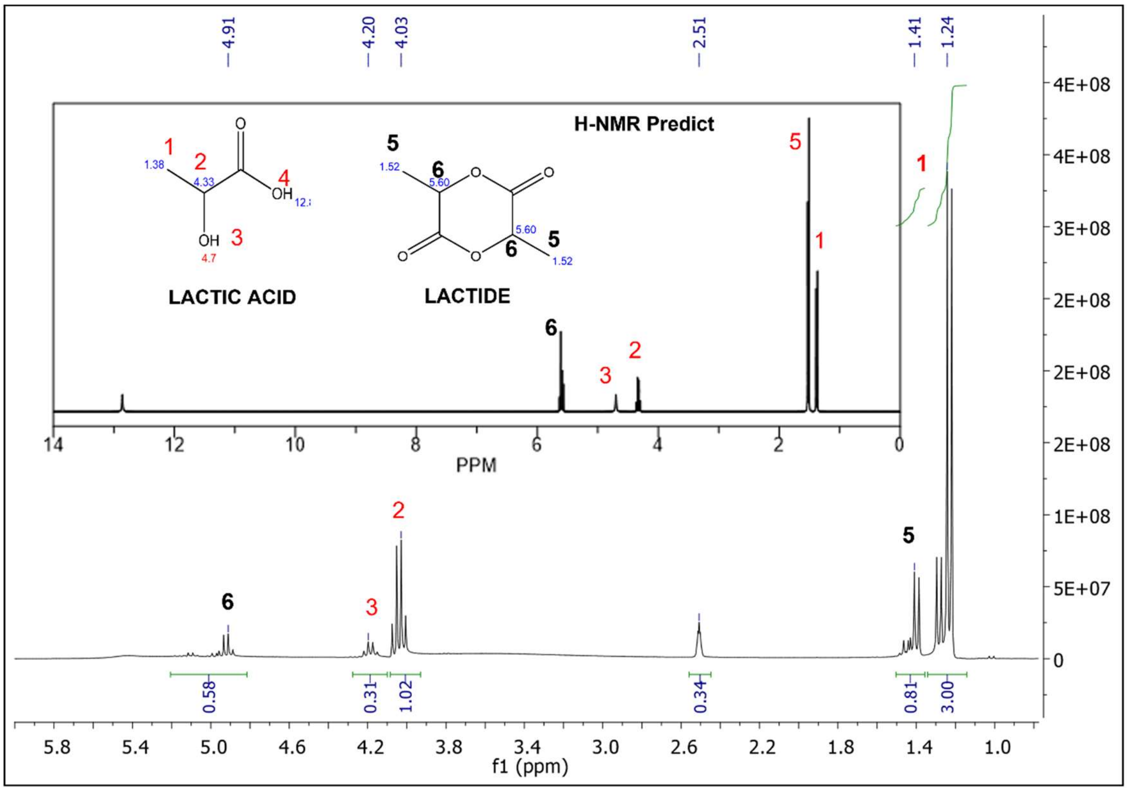

Figure S2. ^{1}H-NMR spectrum of "pure" lactic acid (purity > 88-92 %). The predicted H-NMR was generated in CHEMDRAW Software (PerkinElmer, USA).

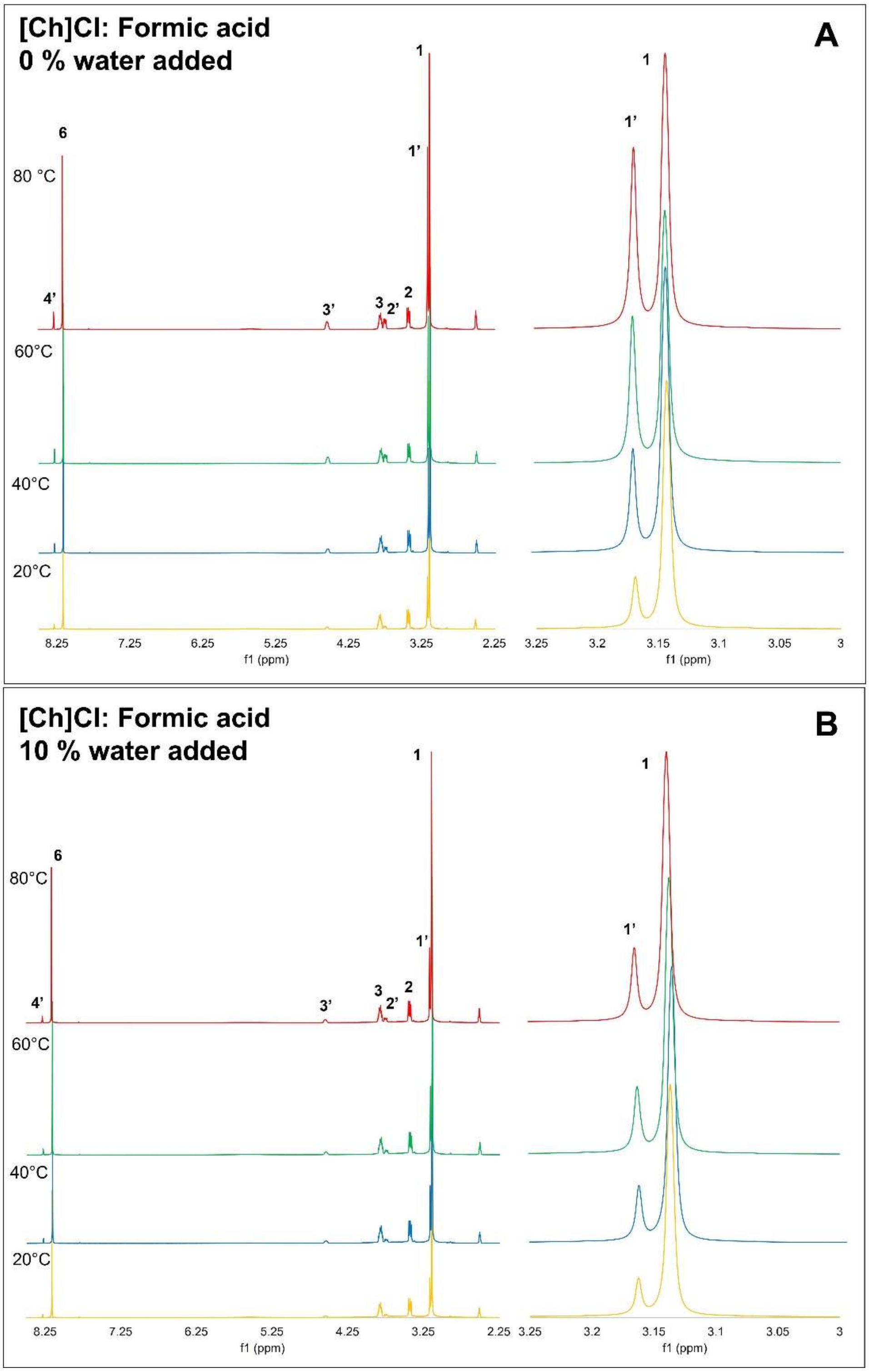

Figure S3. H-NMR spectra for DES (A) without added water and (B) with 10% water.

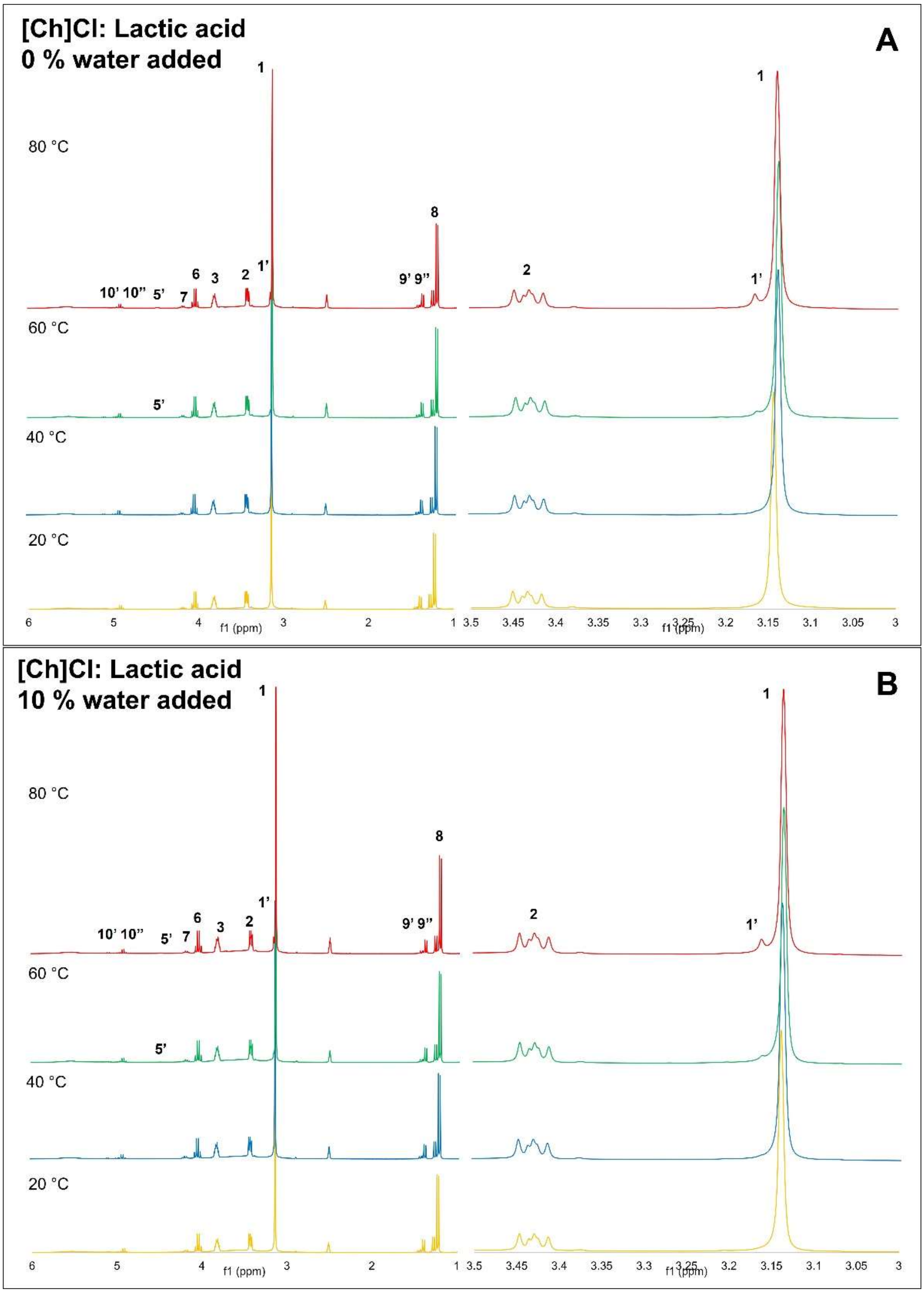

Figure S4. H-NMR spectra for DES (A) without added water and (B) with 10% water.

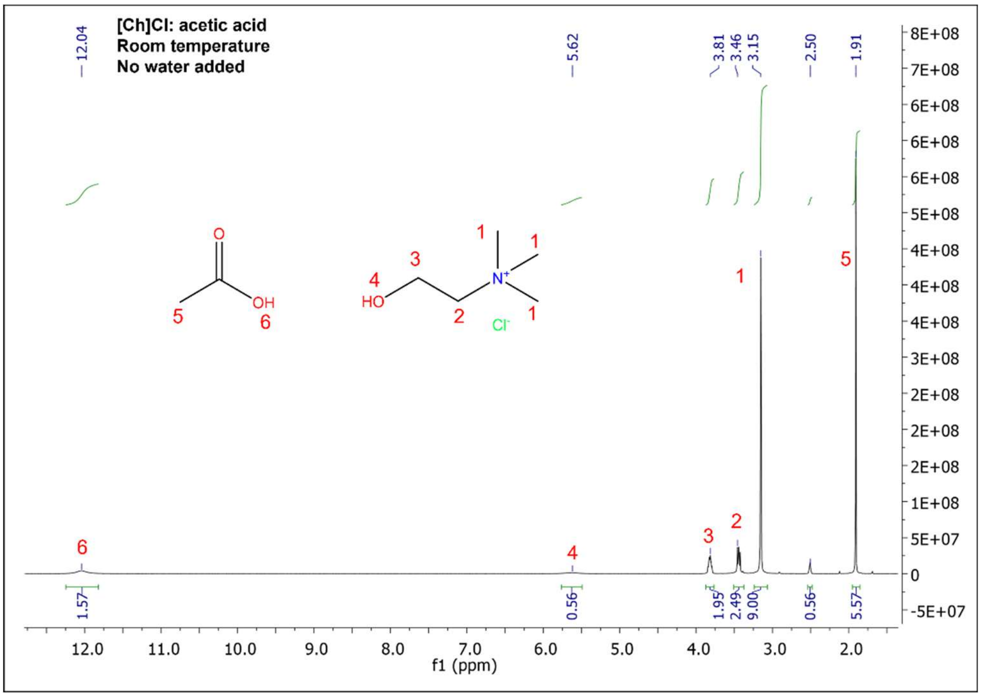

Figure S5. ^{1}H-NMR spectra of DES [Ch]Cl: acetic acid prepared at 20°C for 2 h without the addition of water. No degradation effect was observed.

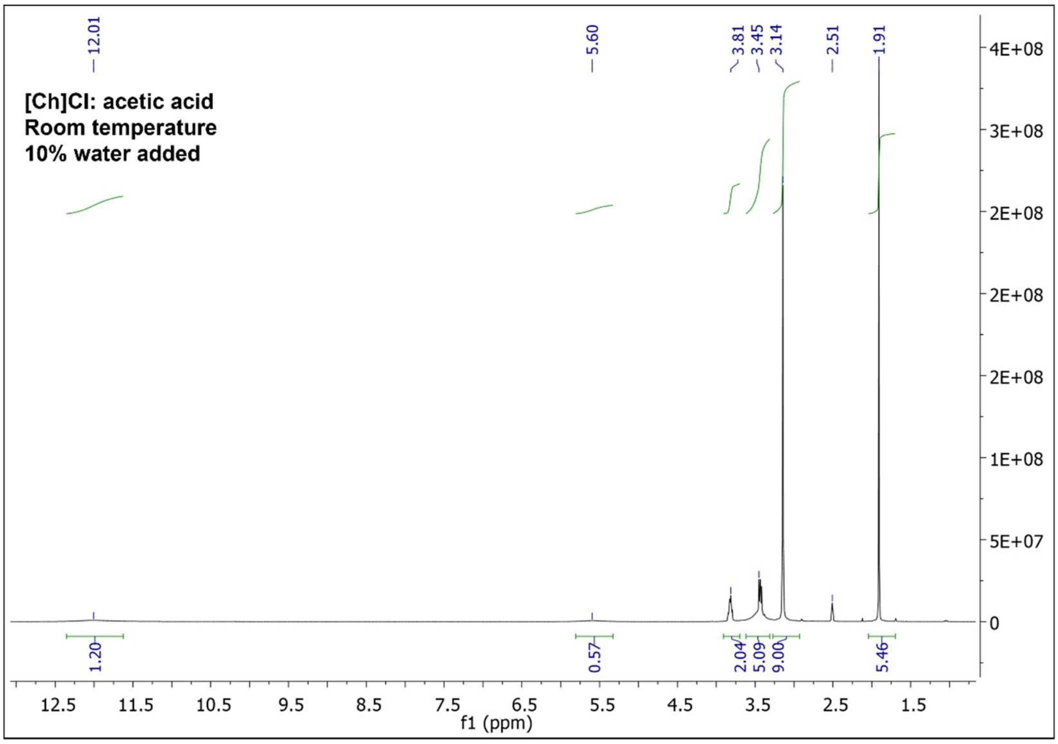

Figure S6. ^{1}H-NMR spectra of DES [Ch]Cl: acetic acid prepared at 20°C for two hours with the addition of 10% water. No degradation effect was observed.

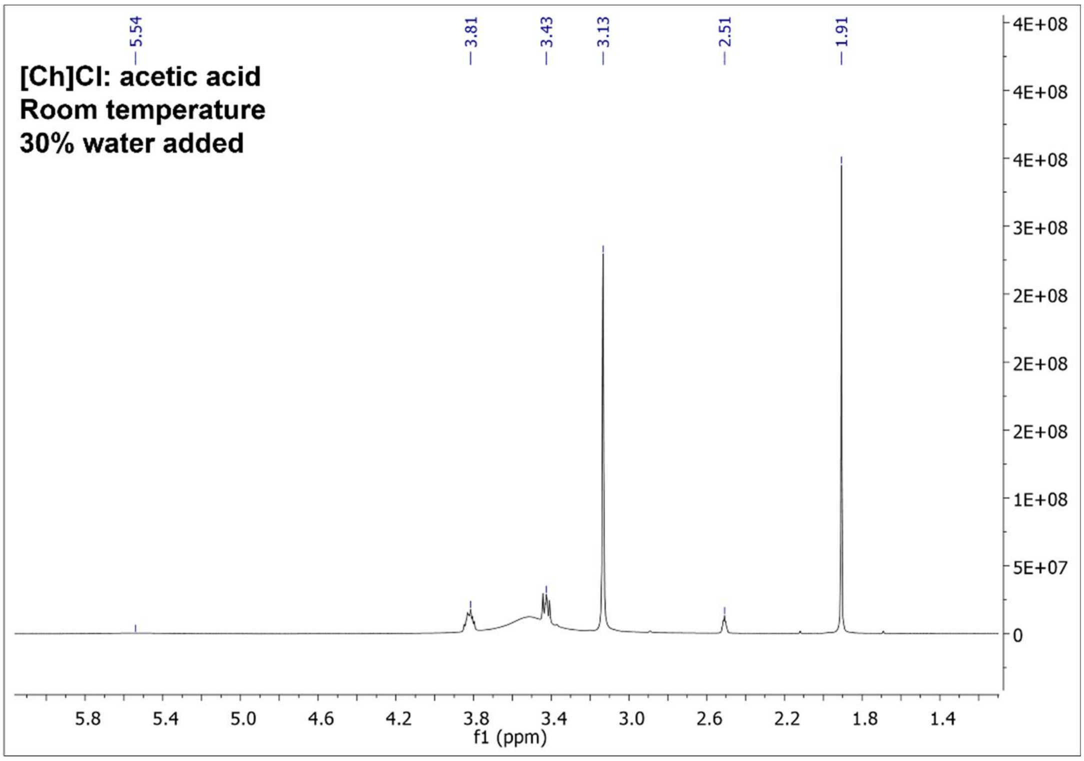

Figure S7. ^{1}H-NMR spectra of DES of [Ch]Cl: acetic acid prepared at 20°C for two hours with the addition of 30% water. No degradation effect was observed.

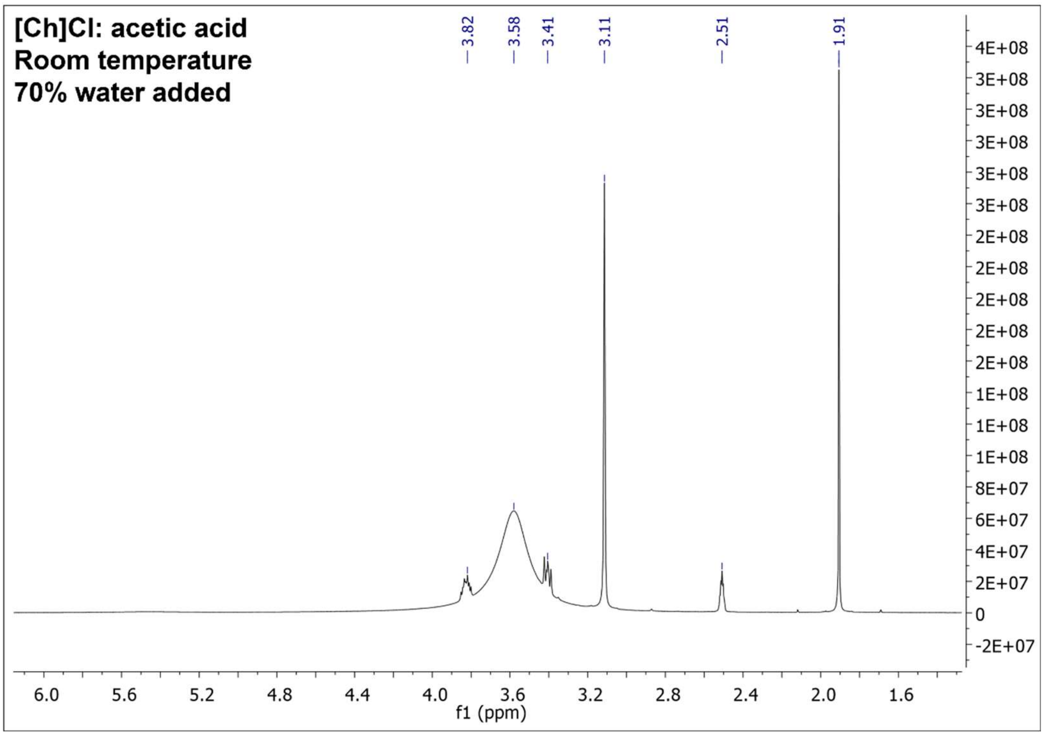

Figure S8. ^{1}H-NMR spectra of DES [Ch]Cl: acetic acid prepared at 20°C for two hours with the addition of 70% water. No degradation effect was observed.

TABLES

Table S1. The effect of temperature on the esterification of DES without added water.

		%E [Ch]Cl (mol%)			
DES	**$x_{HBA:HBD}$**	**20°C**	**40°C**	**60°C**	**80°C**
[Ch]Cl: Acetic acid	01:02	0.00	0.00	2.00	4.50
[Ch]Cl: Formic acid	01:02	16.28	24.87	33.82	37.11
[Ch]Cl: Lactic acid	01:02	0.00	0.00	4.46	8.07
Lactide in [Ch]Cl:Lactic acid		18.75	19.16	18.54	18.53

Table S2. The effect of temperature on the esterification of DES with 10% added water.

		%E [Ch]Cl (mol%)			
DES	**$x_{HBA:HBD}$**	**20°C**	**40°C**	**60°C**	**80°C**
[Ch]Cl: Acetic acid	01:02	0.00	0.00	1.98	4.24
[Ch]Cl: Formic acid	01:02	14.29	16.59	18.99	20.07
[Ch]Cl: Lactic acid	01:02	0.00	0.00	5.56	6.74
Lactide in [Ch]Cl:Lactic acid		18.88	19.92	19.2	16.72

Table S3. The effect of storage time on DES prepared without added water at 20°C and stored at room temperature.

		%E [Ch]Cl (mol%)			
DES	**$x_{HBA:HBD}$**	**2 h**	**48 h**	**168 h**	**336 h**
[Ch]Cl: Acetic acid	01:02	0.00	1.09	7.41	9.09
[Ch]Cl: Formic acid	01:02	16.28	17.05	36.66	39.23
[Ch]Cl: Lactic acid	01:02	0.00	0.00	7.41	9.73
Lactide in [Ch]Cl:Lactic acid		18.47	18.78	20.59	18.64

CAPÍTULO 5

Optimizing the operational conditions for the extraction of phytoestrogens from soybean and okara using deep eutectic solvents (DES)

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Artigo em elaboração

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Optimizing the operating conditions for the extraction of phytoestrogens from soybean and okara using Deep Eutectic Solvents (DES)

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Abstract

Soy extract waste, okara, is a potential source of bioactive compounds such as isoflavones, which in turn are phytoestrogens with potential health benefits. With the aim of developing a green approach to recovering these compounds and valorizing okara, a study is presented to screen variables for the extraction of isoflavones from okara and soy (for comparison) using Deep eutectic solvents (DES) formed by choline chloride ([Ch]Cl) and acetic acid (AA) ([Ch]Cl: AA, 1:2). To do this, a fractional design (24-1) was applied to evaluate the variables temperature, % water, solid-liquid ratio (S/L ratio, mg/mL) and stirring speed (rpm) in the extraction of the isoflavones daidzein, genistein, glycitein, daidzin, genistin and glycitin followed by optimization applying a Central Composite Rotatable Design (CCRD) for cases where two or more variables were significant. In addition, cytotoxicity tests using CACO-2 cells and scanning electron microscopy (SEM) were carried out with the extracts obtained in the optimum condition and their solids, respectively. The study showed that 1194.56 μg TIC/g of dried soybeans were extracted using [Ch]Cl: AA with 70% water added at low temperature (25 °C), 500 rpm, S/L ratio of 10mg/mL and extraction time of 2 hours. For okara, after the selection of the variables, a CCRD was applied using the variables % water and temperature for optimization based on the maximum extraction of isoflavones. At the optimum point, 450.9 µg TIC/g of dry okara were extracted at 40 °C, 500 rpm, 61.5% water in DES, S/L ratio of 10 mg/mL and extraction time of 1 hour. In addition, the SEM images showed that the solvents caused damage to the cell walls of soybeans and okara, favoring extraction. In the cytotoxicity tests, it was observed that the neutralization step of the extracts is necessary to reduce the cytotoxic effects caused by the high acidity of the solvents. In summary, this work has shown that aqueous mixtures of DES can be used as sustainable alternative techniques for recovering isoflavones from soybean residue, and can replace toxic conventional solvents.

Keywords: Isoflavone, choline chloride, flavonoids, phenolic compounds, alternatives solvents

5.1. Introduction

Soybean extract residue, also called okara, is the insoluble fraction obtained from the extraction of soybean extract (Hu et al., 2022). With the increasing development of the soy-based products industry, the okara accumulation has become a problem; as it is a highly perishable residue, with an undesirable flavor, strong "raw beans" odor. Therefore, its use is still small despite the nutritional benefits it presents (Wang et al., 2022). Since okara is rich in protein, presenting 25-28%, 9-11 % lipids, 52-58 % dietary fiber, contains vitamins, such as thiamine, riboflavin, and nicotinic acid, and isoflavone aglycones such as daidzein, genistein, glycitein and isoflavone glycosides such as daidzin, genistin and glycitin (Gupta et al., 2018).

Isoflavones, which are the key components in our research, are phytoestrogens that are structurally like 17-estradiol and bind to estrogen receptors β and α (Messina, 2016). These compounds have been shown to contribute to healthy blood sugar levels, and due to their metabolic functions can inhibit adverse effects of cardiovascular diseases, diabetes, osteoporosis, prostate cancer, breast cancer, and especially reduction of menopausal symptoms (Messina, 2016; Nile et al., 2022).

Due to the advantages that these bioactive compounds can provide, the recovery and inclusion of these flavonoids in food products can add value to okara and reduce its environmental impact.

For recovery of these phenolic compounds, methods using solvents such as methanol, acetone and acetonitrile are still widely used. However, these solvents have sufficient levels of toxicity to cause damage to human health and the environment. Due to growing concerns about the environment and human well-being, techniques considered safer are being applied for the recovery of these compounds. As an example, we have the application of ethanol (recognized as safe) which was studied by Tran *et al.* (2019), Jankowiak *et al.* (2014b) and Carneiro *et al.* (2020) for extraction of isoflavones from soybeans, okara, and soybean residues (leaves, twigs, and pod), respectively.

Tran *et al.* (2019) extracted 1932.44 µg/g dry soybean isoflavones with 80% ethanol, a liquid-solid ratio of 26.5 mL/g, a temperature of 72.5 °C, and 67.5 minutes of extraction. Jankowiak *et al.* (2014b), on the other hand, found an optimum result of 907 µg/g dry okara using 50% ethanol, 1/10 solid-liquid ratio, 20 °C, 2 hours extraction time, and 240 rpm stirring.

Due to the polar characteristic of isoflavone molecules, water has also been applied as a more ecological and safer alternative for extracting them from soy products and especially okara (Benjamin et al., 2017; Jankowiak, Kantzas, et al., 2014; Nkurunziza, Pendleton, & Chun, 2019; Nkurunziza, Pendleton, Sivagnanam, et al., 2019; Yu & Yang, 2019). Different extraction methods were applied using water, such as ultrasound-assisted extraction (UAE) (Yu & Yang, 2019), subcritical water extraction (SWE) (Nkurunziza, Pendleton, & Chun, 2019) and water with modified pH (Jankowiak, Kantzas, et al., 2014).

As a new alternative considered safe and environmentally friendly in the literature, we are proposing the application of deep eutectic solvents (DES) to extract isoflavones from okara and soybean. DES are a new class of solvents formed from the mixture of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) (Abbott et al., 2003; Martins et al., 2019). For DES formation, choline chloride is usually the most used as HBA, and different carboxylic acids, amides, and sugars as HBD (Ashworth et al., 2016). These solvents are easy to prepare, can be economical and renewable, and some are considered by the literature to be non-toxic (Shang et al., 2019).

DES were applied only two times for isoflavone extraction. Bajkacz and Adamek applied a set of deep eutectic solvents in ultrasound-assisted extractions (UAE) of daidzein, genistein, daidzin and genistein from different soy-based products. Among them, the DES consisting of choline chloride and citric acid ([Ch]Cl: CA, 1:2) plus 30% water showed the highest extraction efficiency. The process conditions were 3 h of extraction, 60 °C, and 616 W ultrasonic power. Duru et al. (2022) extracted daidzein, genistein, and puerarin from kudzu roots and soybean molasses by applying DES [Ch]Cl: CA (1:2) coupled with UAE. The extractions were performed for 2 h at 80 °C, ultrasonic power 180 W, and a frequency of 37 kHz. The water content in DES ranged from 10-30 % and the results showed that water had a positive influence on extraction, where 30 % water showed the highest efficiency for extracting puerarin from kudzu roots. For extraction of genistein and daidzein from soy molasses, 10 % water showed the best result.

In a previous work, we screened a set of DES for total flavonoid extraction from soybean and okara and we found that the DES consisting of Choline Chloride: Acetic Acid ([Ch]Cl: AA, HBA: HBD) in the molar ratio of 1:2 extracted more total flavonoids than all the DES analyzed and the conventional solvents methanol and ethanol (Ferreira et al., 2023).

Choline chloride is a compound that has important functions for the body, being one of the B-complex vitamins. Acetic acid, on the other hand, can be obtained naturally by fermentation from renewable sources and is considered an acidulant agent and pH controller in foods. In this study, we are proposing an alternative solvent, not yet reported in the literature, for the extraction of isoflavone daidzein, genistein, glycitein daidzin, genistin, and glycitin using DES [Ch]Cl: AA (1:2). In addition, extractions with pure water, acetic acid + water, and choline chloride + water were performed to evaluate the interaction of the individual components of the DES with the solutes of interest.

5.2. Material and methods

5.2.1 Material

The soybean used to obtain the okara was cultivated and donated by an agricultural producer in the north of the state of Paraná (Brazil).

The reagents used for preparing the DES mixtures were choline chloride ([Ch]Cl) (purity>98.0%) and acetic acid (AA), both from Exôdo (Brazil). For conventional extractions, ethanol (99%) and methanol (purity>99.9%) were purchased from Merck (Germany).

The standards used for quantification of isoflavones by UHPLC (Ultra-High-Performance Liquid Chromatography) were daidzein (purity > 98%), genistein (purity > 99.0%) and glycitein (purity > 99%) from Targetmol (USA) and, daidzin (purity > 99.0%), genistin (purity > 99.0%) and glycitin (purity > 99.0%) from Extrasynthese (France). Methanol (HPLC grade, purity > 99.9%) and acetonitrile (HPLC grade, purity > 99.9%) were from Merck (Germany), and formic acid (HPLC grade, purity > 99.9%) was from Sigma-Aldrich (USA).

5.2.2 Methods

5.2.2.1 Sample Preparation

The okara was obtained according to Baú and Ida (2015). First, the soybeans were washed with distilled water for dirties removal and then submerged in a soybean: water ratio of 1:3 (m:v) at 5 °C for 14 h. After that, the water was drained, and the soybeans were rinsed. Distilled water was again added to soaked soybeans: water ratio of 1:8 (m:v) and the mixture was processed in an industrial blender for 2 min at 25 °C to obtain a paste. After that, the paste was filtered and freeze-dried (Liobras, L101 model, Brazil) until constant mass at $T = (-50 \pm 1)$ °C, $p = (13 \pm 2)$ µHg for 48 h. The dry insoluble fraction was then triturated in a knife mill (Marconi, model MA 340, Brazil).

The soybean used was first crushed in an industrial blender, then milled in a knife mill, and freeze-dried until constant mass. Both samples were packed in polyethylene bags and stored in a vertical ultra-freezer at -80 °C (Nuaire, Glacier model, USA). The samples were unfrozen according to the amount needed for the extraction experiments.

5.2.2.2 Preparation and characterization of the Deep Eutectic Solvent

In a previous work published by our research group (Ferreira et al., 2023), several solvents were used in screening for the extraction of total flavonoids from soybean and okara. The DES composed of [Ch]Cl) as hydrogen bonding acceptor (HBA) and acetic acid as hydrogen bonding donor (HBD) showed the highest extraction efficiency and was used to optimize the extraction of six isoflavones: daidzein, genistein, glycitein, daidzin, genistin, and glycitin.

To prepare the DES [Ch]Cl: AA (1:2), the HBA and HBD samples were weighed in Schott flasks on an analytical balance (Precisa, model XT 220^{a}, Brazil, precision±0.0001g) and shaken at 240 rpm in an orbital shaker (Benchtop shaker incubator, new dynamics, model NT 712, Brazil) heated to 60 °C for 30 min until the formation of a clear and homogeneous liquid.

5.2.2.3 Extraction method and determination of time

For isoflavone extraction, initially, soybean and okara were weighed and mixed with the solvents in test tubes of 10 mL. The tube was placed inside 50 mL containers containing water and jacketed for circulating water from a thermostatic bath to maintain the temperature. Extractions were performed by heating the mixture, under constant magnetic stirring.

Initially, extraction times for soybean and okara were determined using a 3-hour kinetics. For these experiments, the variables temperature was set at 40 °C, water content in the DES at 25.4%, S/L ratio at 35 mg/mL, and 500 rpm of agitation. These conditions were those of the central point of the fractional design presented in Table 5.1 (section 5.2.2.5). The results were analyzed by UHPLC to determine the content of the isoflavones daidzein, genistein, glycitein, daidzin, genistin, and glycitin according to the method described in section 5.2.2.4.

5.2.2.4 Quantification of isoflavones

The identification and quantification of soybean and okara isoflavones were according to Ferreira et al. (2023). The extracts were all diluted 5 times in methanol and filtered on 0.22 µm membrane filters and analyzed on a UHPLC system (Waters Acquity SQD/UPLC System, United States) equipped with a photodiode detector array (PDA), a single quadrupole MS system with electrospray ionization (ESI), an automatic injector, a quaternary pump, and a column oven. The separation of the compounds was performed on an Acquity Premier UPLC BEH C18 column (1.7 µm, 2.1 × 55 mm, Waters, USA). The samples were eluted at 0.2 mL/min with the column operating at 25 °C and a linear gradient consisting of Milli-Q water with 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B). The elution gradient was as follows: 0–1 min, 85% A; 1–12 min, 85%–20%A; 12–22 min, 20%–0%A; 22–26 min, 0% A; 26–28 min, 0%–85%A; 28–30 min, 85% A. The injection volume was 2 µl and the UV absorption was monitored at wavelengths of 220:254 nm.

Six external standards were used for the quantification of the compounds. The six isoflavones standards daidzein, genistein, glycitein, daidzin, genistein, and glycitein were solubilized in methanol and for the preparation of the curves were diluted in concentrations from 6.25 ng/mL to 10 µg/mL. The curves were plotted relating the peak area and concentration of each isoflavone standard and all showed an $R^2 > 0.99$. The acquired data were processed using MassLynx software (Waters, USA). The results were expressed as ug of the analyte per gram of dry soybean or okara (ug/ g dry sample).

5.2.2.5 Experimental design

Fractional factorial design (FFD)

To evaluate the effects of the independent variable temperature (°C) (x_1), water content in DES (%) (x_2), solid-liquid ratio (S/L) (x_3) and stirring speed (x_4) a 2^{4-1} fractional design was applied using total isoflavone content as the response variable. This design is used when there are many variables, usually over 3, and you want to perform a screening to analyze their effects. Table 5.1 summarizes the experimental conditions used in the factorial design. In total, 12 experiments were carried out with 4 repetitions at the central point to determine the variability of the results and to evaluate the experimental errors. The extraction time was previously evaluated and fixed as described previously in section 5.2.2.3.

Table 5.1. Levels and factors of fractional factorial design 2^{4-1}

Factors	Levels		
	-1	**0**	**1**
Temperature (°C) (x_1)	25	40	55
Water (%) (x_2)	0.40	25.40	50.40
S/L ratio (mg/mL) (x_3)	10	35	60
Stirring (rpm) (x_4)	200	500	800

After analyzing the effects of the variables, a Central Composite Rotatable Design (CCRD) was applied in cases where 2 or more variables were significant. All data obtained were processed using Statistica 14.0 software (Statsoft Inc., USA).

5.2.2.6 Scanning electron microscopy (SEM)

SEM analyses were performed on freeze-dried soybeans and okara before and after DES extractions under optimized conditions. The analyses were carried out at the Biomass Characterization, Analytical Resources, and Calibration Laboratory (LRAC) at the School of Chemical Engineering (FEQ/UNICAMP). After extraction, the samples were dried in a vacuum oven at 50 °C until a constant weight was reached, then fixed onto a sample holder with double-sided carbon tape and gold-metallized using a Sputter Coater (Brand: EMITECH, Model: K450, United Kingdom). The samples were then analyzed using a High-Resolution Scanning Electron Microscope with Energy Dispersive X-ray Detector (TermoFisher Scientific, Quattro S, Czech Republic). Images were obtained using a voltage of 2 kV, a current of 3.6 pA, a spot size of 2.0, and magnifications of 200, 400, and 1200x.

5.2.2.7 Biological Analysis

Cell culture

The Caco-2 cell line (human colorectal adenocarcinoma cells) was cultured in RPMI 164 medium (Gibco, UK) supplemented with 10% fetal bovine serum (Gibco, UK) and 1% penicillin/streptomycin (Gibco, UK) and kept under a humid atmosphere at 37 °C and 5% CO_2 (Stove Panasonic Healthcare, USA). The cells were maintained until the 34th passage. Furthermore, cells were subcultured after treatment with 0.1% trypsin (Sigma-Aldrich, USA) every 2 or 3 days.

Cytotoxicity assay

The cytotoxicity assay was determined by the reduction of tetrazolium salts to formazan crystals - MTT assay ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) (Mosmann, 1983). Briefly, cells were seeded in a 96-well plate (5 x 103 cells/well) and cultured for 24 h to form a cell monolayer. Afterwards, the soy extracts and [Ch]Cl: AA added to 70% and 30% water at the initial state (acidic pH) and neutralized (pH 7.0) were diluted in a culture medium free of fetal bovine serum and antibiotics and added to the cells. The concentrations chosen were 50, 20, 8, 3.2, 1.28, 0.51, 0.20 and 0.081% to determine half the maximum inhibitory concentration (IC50). After 24 h of treatment, the medium was replaced with an MTT solution (1mg/mL) and after 3 h the medium was removed and the MTT crystals were dissolved in 100 µL of DMSO. Finally, the plate was homogenized for 5 min and absorbance was measured on a Cytation 5 Imaging Multi-Mode reader (Biotek, USA) at 570 nm. Cell viability data were determined by three independent experiments (n = 3). The IC50 values were determined by non-linear regression curve and fitted using GraphPad Prism 8.0.2 and were expressed as the mean ± SD, and the values were normalized to the untreated control.

5.3. Results and discussion

5.3.1 Determining the extraction time

The effect of extraction time was investigated for recovery of the isoflavones daidzein, genistein, glycitein, daidzin, genistin, and glycitin from two different raw materials, soybean and okara, using DES [Ch]Cl: AA (1:2) added with 25.4 % water. Fig. 5.1 shows the extraction data of the isoflavones that were grouped into their respective categories: isoflavone aglycones (daidzein, genistein, and glycitein) and isoflavone glycosides (daidzin, genistin, and glycitin). The time setting was based on the maximum extraction for total isoflavone content (TIC).

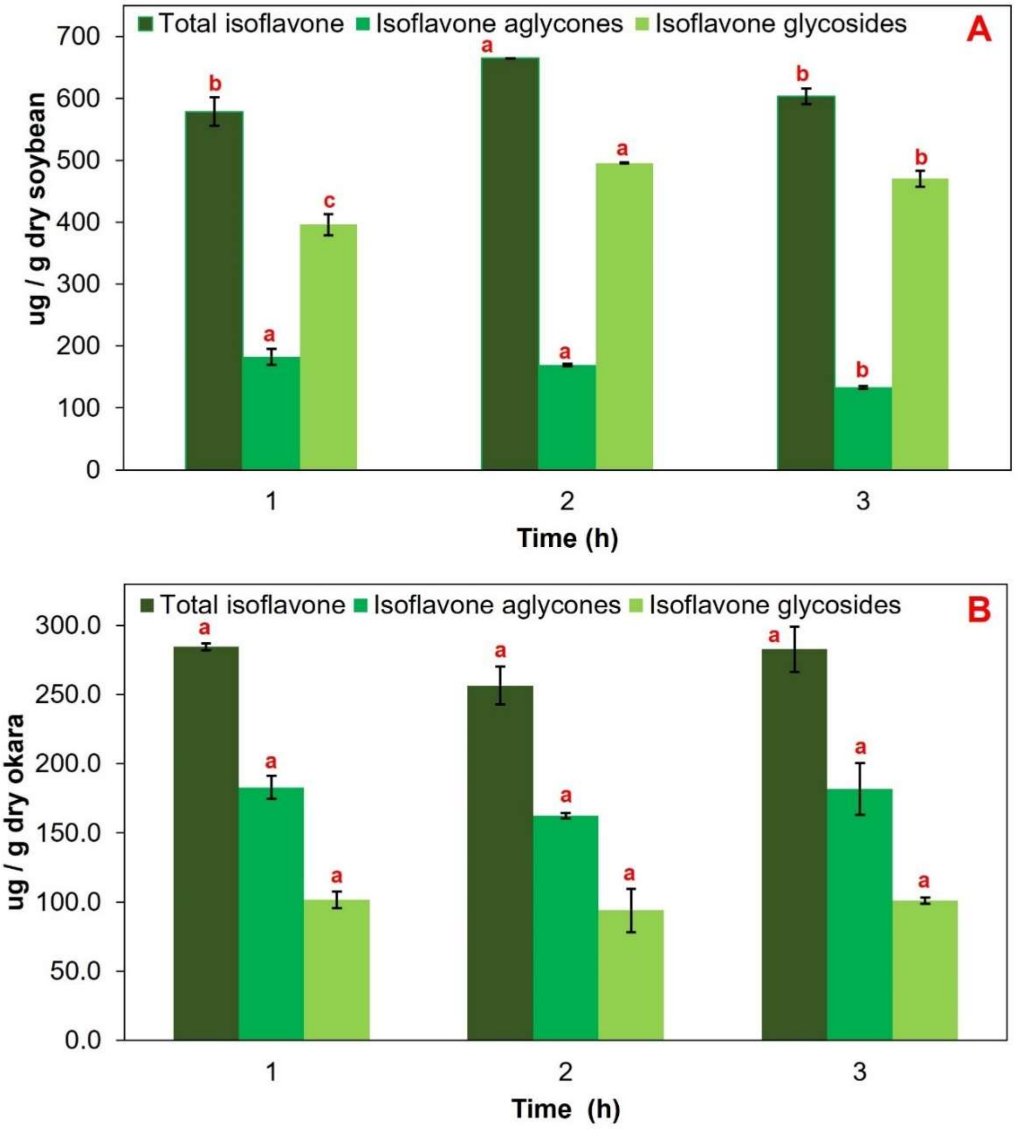

Fig. 5.1. Extraction time of (A) soybean and (B) okara isoflavones. Bars with equal colors followed by the same letter have no statistical difference by the Tukey test ($\alpha \leq 0.05$)

For soybean isoflavone extraction, it is noted that two hours was sufficient to obtain higher isoflavone contents in the different classes. The isoflavone contents with two hours of extraction were significantly different from the others applying Tukey's test at 5 % significance ($p<0.05$). When increasing the time from one to two hours an increase of approximately 15 % in TIC is observed; increasing from 578.69 to 665.04 µg/g dry soybean. With three hours of extraction, a decrease of approximately 10% is observed in the TIC (603.42 µg/g dry soybean). Longer exposure times may be favoring the extraction of other major components such as carbohydrates, proteins and lipids that may compete with the analytes, decreasing the concentration of isoflavones.

The total content of the glycosides forms daidzin, genistin, and glycitin was higher than the isoflavone aglycones daidzein, genistein, and glycitein in the three soybean extracts analyzed.

For okara, lower isoflavone contents were observed. Usually, soybean contains 0.3 % isoflavones, and okara contains about 0.1 % (Jankowiak, Jonkman, et al., 2014). In other words, the production of soy extract removes approximately 0.2 % of the isoflavones from soybean. With only one hour of extraction, 284.62 µg of total isoflavones/g dry okara were recovered, higher than the content found for the subsequent times, which decreased slightly. Unlike soybean, the okara extracts showed a higher concentration of isoflavone aglycones in the three extracts.

5.3.2 Variable screening

Screening of variables for the extraction of isoflavones from okara

For the screening of the variables temperature (°C), water in DES (%), S/L ratio, and agitation (rpm), the fractional design strategy 2^{4-1} with four repetitions at the central point was applied. This strategy is appropriate for analyzing of previous effects and helps in the decision to define the next full factorial design (Rodrigues & Iemma, 2015).

The results of the extraction of isoflavone from okara are presented in Table 5.2. To analyze the effects, statistically significant variables were defined as those with p-value < 0.05, as shown in Tables 5.3.

The results showed that the process was affected by temperature (p=0.004), water content in DES (%) (p=0.002), and S/L ratio (p=0.020). An increase in temperature from 25 °C to 55 °C (level -1 to +1) indicated an average increase in isoflavone content of 90.38 µg TIC / g dry okara. Increasing the water content in DES from 0.4% to 50.5% caused an average increase of 122.57 µg TIC/g dry okara, being variable with the highest effect on the extraction process. Whereas, increasing the S/L ratio from 10 mg/mL to 60 mg/mL resulted in an average reduction of 40.01 µg TIC/g dry okara.

Although we found a significant effect of the S/L ratio on the extraction process, we decided to set the ratio at 10 mg/mL, as this value is considered low. Although the S/L ratio provides a high extraction yield, it results in an extract that is extremely diluted in isoflavones. Therefore, the use of lower S/L ratios in another experimental design is not justified (Huang et al., 2021; Li et al., 2016; Nkurunziza, Pendleton, & Chun, 2019; Shang et al., 2019; Yu & Yang, 2019).

Agitation was not a statistically significant variable with a p-value<0.05. From experimental observations, 500 rpm kept the system sufficiently agitated. Therefore, we set this variable at 500 rpm for the DCCR. In addition, working with an S/L ratio of 10 mg/mL and water contents above 40 % in the complete design the resistance to mass transfer would already be lower compared to the conditions studied in the fractional design.

Observing the results obtained in Table 5.2, according to the experiment condition we have isoflavone extraction results ranging from 168.33 to 403.65 µg TIC/g dry okara.

Compared to the maximum value observed here (403.65 µg TIC/g dry okara), it is relevant to mention, that in this first experimental design, an increase of approximately 40 % was already observed in relation to the previous condition obtained with the kinetics at a time of 1 h (284.62 µg TIC/g of dry okara).

Table 5.2. Experimental values for extraction of isoflavones from okara using fractional design 2^{4-1}

Test	Temp. (°C)	Water (%)	S/L ratio (mg/mL)	Stirring (rpm)	Daidzein	Genistein	Glycitein	Daidzin	Genistin	Glycitin	Total
1	25 (-1)	0.4 (-1)	10 (-1)	200 (-1)	43.82	60.55	10.20	24.50	25.86	11.75	176.69
2	25 (-1)	0.4 (-1)	60(+1)	800 (+1)	40.30	59.50	7.58	23.94	26.75	10.26	168.33
3	25 (-1)	50.4 (+1)	10 (-1)	800 (+1)	116.74	121.22	24.65	54.08	47.63	20.48	384.79
4	25 (-1)	50.4 (+1)	60 (+1)	200 (-1)	92.33	108.59	20.95	55.27	49.41	20.80	347.34
5	55 (+1)	0.4 (-1)	10 (-1)	800 (+1)	107.79	129.74	21.77	52.42	44.27	16.81	372.79
6	55 (+1)	0.4 (-1)	60 (+1)	200 (-1)	76.94	103.22	14.79	39.55	44.22	16.24	294.96
7	55 (+1)	50.4 (+1)	10 (-1)	200 (-1)	119.54	130.35	22.85	59.25	50.55	21.12	403.65
8	55 (+1)	50.4 (+1)	60 (+1)	800 (+1)	96.51	112.91	22.49	60.73	49.96	24.66	367.25
9	40 (0)	25.4 (0)	35 (0)	500 (0)	82.76	95.14	18.07	32.71	26.67	12.20	267.55
10	40 (0)	25.4 (0)	35 (0)	500 (0)	87.37	95.90	21.62	21.55	16.32	8.52	251.27
11	40 (0)	25.4 (0)	35 (0)	500 (0)	74.74	88.10	16.01	38.55	29.90	12.82	260.12
12	40 (0)	25.4 (0)	35 (0)	500 (0)	88.95	102.91	20.15	43.99	37.36	17.96	311.32

Table 5.3. Results from fractional design 2^{4-1} effects for Okara

			Levels			
Variable	**Unity**	**-1**	**0**	**1**	**Effect**	**p-value**
Mean					314.48	0.000
Curvature					-109.67	0.010
Temperature	**°C**	25	40	55	90.38	0.004
Water	**%**	0.40	25.40	50.40	122.57	0.002
S/L ratio	**mg/mL**	10	35	60	-40.01	0.020
Stirring	**rpm**	200	500	800	17.63	0.092

Screening of variables for the extraction of isoflavones from soybean

The results of the extraction of isoflavone from soybean are presented in Tabel 5.4. To analyze the effects, statistically significant variables were defined as those with p-value < 0.05, as shown in Tables 5.5.

In the analysis of the effects of the variables, only the water content in DES was statistically significant (p-value = 0.00). Adding 50 % water to the DES caused an average increase of 516.48 µg TIC/g dry soybean. In the next step, we chose to evaluate only the water content in DES (%) in the extraction of isoflavones from soybean, varying the water content from 50-100% and setting the temperature at 25 °C, S/L ratio at 10 mg/mL, stirring at 500 rpm, and 2 h of extraction. Analyzing the results in Table 5.4, the concentration of isoflavones ranged from 366.70 to 1089.58 µg TIC/ g dry soybean. It is important to note that here too, only with the use of fractional design there was an increase of approximately 64% in the isoflavone content compared to the previous conditions, performed in the extraction kinetics (item 5.3.1), where the value of 665.04 µg/g dry soybean was obtained at a time of 2h (Fig 5.1A).

Table 5.4. Experimental values for extraction of isoflavones from soybean using fractional design 2^{4-1}

Test	**Temp. (°C)**	**Water (%)**	**S/L ratio (mg/mL)**	**Stirring (rpm)**	**Daidzein**	**Genistein**	**Glycitein**	**Daidzin**	**Genistin**	**Glycitin**	**Total**
1	25 (-1)	0.4 (-1)	10 (-1)	200 (-1)	40.02	46.81	2.68	89.48	174.97	12.74	366.70
2	25 (-1)	0.4 (-1)	60 (+1)	800 (+1)	37.73	45.49	3.23	90.92	176.17	13.92	367.47
3	25 (-1)	50.4 (+1)	10 (-1)	800 (+1)	136.78	109.38	18.08	340.66	408.02	76.67	1089.58
4	25 (-1)	50.4 (+1)	60 (+1)	200 (-1)	101.54	86.90	14.73	345.73	371.25	79.38	999.53
5	55 (+1)	0.4 (-1)	10 (-1)	800 (+1)	76.15	71.86	6.95	143.98	227.87	20.15	546.96
6	55 (+1)	0.4 (-1)	60 (+1)	200 (-1)	65.44	65.64	6.52	149.13	214.98	30.77	532.49
7	55 (+1)	50.4 (+1)	10 (-1)	200 (-1)	119.09	92.71	12.17	304.63	279.65	73.93	882.17
8	55 (+1)	50.4 (+1)	60 (+1)	800 (+1)	106.56	86.82	16.83	318.82	299.72	79.53	908.27
9	40 (0)	25.4 (0)	35 (0)	500 (0)	79.03	74.34	9.09	190.49	202.12	31.15	586.21
10	40 (0)	25.4 (0)	35 (0)	500 (0)	81.86	67.87	8.67	214.34	220.48	50.32	643.53
11	40 (0)	25.4 (0)	35 (0)	500 (0)	88.11	74.64	8.41	223.42	214.76	48.58	657.91
12	40 (0)	25.4 (0)	35 (0)	500 (0)	86.04	71.56	8.97	229.35	221.61	64.20	681.73

Table 5.5. Results of fractional design 2^{4-1} effects for soybean

		Levels				
Variable	**Unidade**	**-1**	**0**	**1**	**Effect**	**p-value**
Mean					711.646	0.000
Curvature					-138.597	0.069
Temperature	**°C**	25	40	55	11.651	0.712
Water	**%**	0.40	25.40	50.40	516.483	0.000
S/L ratio	**mg/mL**	10	35	60	-19.416	0.547
Stirring	**rpm**	200	500	800	32.852	0.336

5.3.3 Optimizing extraction of isoflavones from okara

The Central Composite Rotatable Design (CCRD) strategy was applied from the conditions analyzed in the previous fractional design fixing the extraction time at one hour, agitation at 500 rpm, and S/L ratio at 10 mg/mL. The effects and interactions of temperature and water content in DES on isoflavone extraction were investigated. The proposed design was the CCRD with two levels and two factors (2^2), 4 axial points, and 4 repetitions at the central point to calculate the experimental error, totaling 12 experiments. Table S1 summarizes the experimental conditions applied (please, see Supplementary Material). The extraction results using temperature and % water as independent variables are shown in Table 5.6. For statistical analysis of the CCRD, the total content of isoflavones was used as the response variable.

As the first step of the statistical analysis, as is being shown in Table S2 (Supplementary Material), through the Central Composite Design (CCD) analysis, the curvature was observed to be statistically significant ($p\text{-value}<0.05$). Thus, a first-order model was not sufficient to describe the results, and it was necessary to add axial points to obtain a second-order model.

The analysis of variance (ANOVA) of the Central Composite Rotatable Design (considering the axial points) is presented in Supplementary Material in Tables S3 and S4.

From the analysis of the Pareto diagram, shown in Fig. 1S in Supplementary material, it was found that only the variable water content in DES was statistically significant ($p\text{-value}<0.05$).

From the coded matrix and the experimental results, a second-order polynomial model was reparametrized. Table S5 in Supplementary Material shows the ANOVA after model reparameterization. The reparametrized second-order model is shown in Eq. (1):

$$Y = 428.48 - 22.73X - 24.02X^2 \qquad \text{Eq. (1)}$$

where Y is the total isoflavone content (µg TIC/ g dry okara), X is the water content in DES.

With the reparametrized model, the predicted values, the absolute deviation and the relative deviation were calculated, which are shown in Table 5.6. The low relative deviation indicates a good fit of the model to the experimental data. From the reduced ANOVA analysis (Table 5.7) with 5 % significance ($\alpha = 0.05$), the regression showed a Fcalc (15.69) greater than the Ftab (4.26), indicating that the model is statistically significant. For the lack of fit analysis, the Fcalc (1.45) was less than the Ftab (6.94), indicating that the lack of fit was not statistically significant and therefore the model fits the experimental data. Furthermore, the model showed a coefficient of determination of the explained variable (R^2) of approximately 78 %. Thus, it is concluded that the model is statistically significant and can explain the experimental data; therefore, it can be used to generate response surfaces and contour curves to optimize the operating conditions. However, since only one significant variable was obtained, a quadratic curve was generated for the prediction of the optimized condition, as presented in Fig. 5.2.

The model was experimentally validated at the condition of maximum isoflavone extraction. The predicted value of 433.73 µg TIC/ g dry okara was found using 61.5 % water in DES and 40 °C. The predicted value was compared to the experimental value, as shown in Table 5.8. Although there is a difference between the predicted value (433.73 µg TIC/ g dry okara) and the experimental (450.9 µg TIC/ g dry okara), the absolute and relative deviations (17.17 and 3.81, respectively) are within the ranges seen in Table 5.6, confirming the model validation.

Table 5.6. Experimental extraction values of extraction of isoflavones from okara, predicted ones with the model and deviations for CCRD

Ensaios	Temp. (°C)	Water (%)	Daidzein	Genistein	Glycitein	Daidzin	Genistin	Glycitin	Observed value	Predicted value	Absolute deviation[b]	Relative deviation[c] (%)[c]
1	44.4 (-1)	48.79 (-1)	124.21	138.99	26.09	48.88	58.06	19.98	416.21	427.19	10.98	2.64
2	44.4 (-1)	91.21 (+1)	123.64	95.07	27.92	62.59	44.11	24.25	377.58	381.73	4.15	1.10
3	65.6 (+1)	48.79 (-1)	119.10	131.22	25.28	52.13	56.82	19.37	403.91	427.19	23.28	5.76
4	65.6 (+1)	91.21 (+1)	115.71	97.18	24.39	61.00	44.54	19.37	362.20	381.73	19.53	5.39
5	40 (-1.41)	70 (0)	136.18	132.93	28.42	59.28	56.92	21.80	435.54	428.48	7.06	1.62
6	70 (+1.41)	70 (0)	130.36	133.53	28.21	64.13	59.27	23.56	439.06	428.48	10.58	2.41
7	55 (0)	40 (-1.41)	127.64	145.13	25.18	51.84	58.24	22.77	430.80	412.58	18.22	4.23
8	55 (0)	100 (+1.41)	185.74	123.19	50.12	0.00	0.00	0.00	359.04	348.30	10.75	2.99
9 (CP) [a]	55 (0)	70 (0)	136.72	135.53	29.43	64.99	56.51	27.20	450.39	428.48	21.91	4.86
10 (CP)	55 (0)	70 (0)	129.07	130.95	26.73	67.65	57.67	20.98	433.06	428.48	4.58	1.06
11 (CP)	55 (0)	70 (0)	131.58	125.40	25.64	64.36	51.40	21.30	419.69	428.48	8.79	2.09
12 (CP)	55 (0)	70 (0)	128.17	123.44	26.36	67.56	50.95	25.64	422.12	428.48	6.36	1.51

[a] CP= central point.

[b] Absolute deviation = |Observed value – Predicted value|.

[c] Relative deviation (%) = 100.Absolute deviation/Observed value.

Table 5.7. ANOVA of the model for isoflavone extraction from okara

Variation source	[b]SS	[c]DF	[d]MS	[e]F_{cal}	[f]F_{tab}
Regression	7979.44	2	3989.72	15.69	4.26
Residual	2289.02	9	254.34		
Lack of fit	1702.54	6	283.76	1.45	6.94
Pure error	586.48	3	195.49		
Total	10268.46	11			
[a]R^2	77.71%				

[a]R^2 = % explained variation; [b]SS = Sum of Squares; [c]DF = Degree of Freedom;

[d]MS = Mean of Squares; [e]Fcalc. = calculated F-value; [f]Ftab. = tabulated F-value.

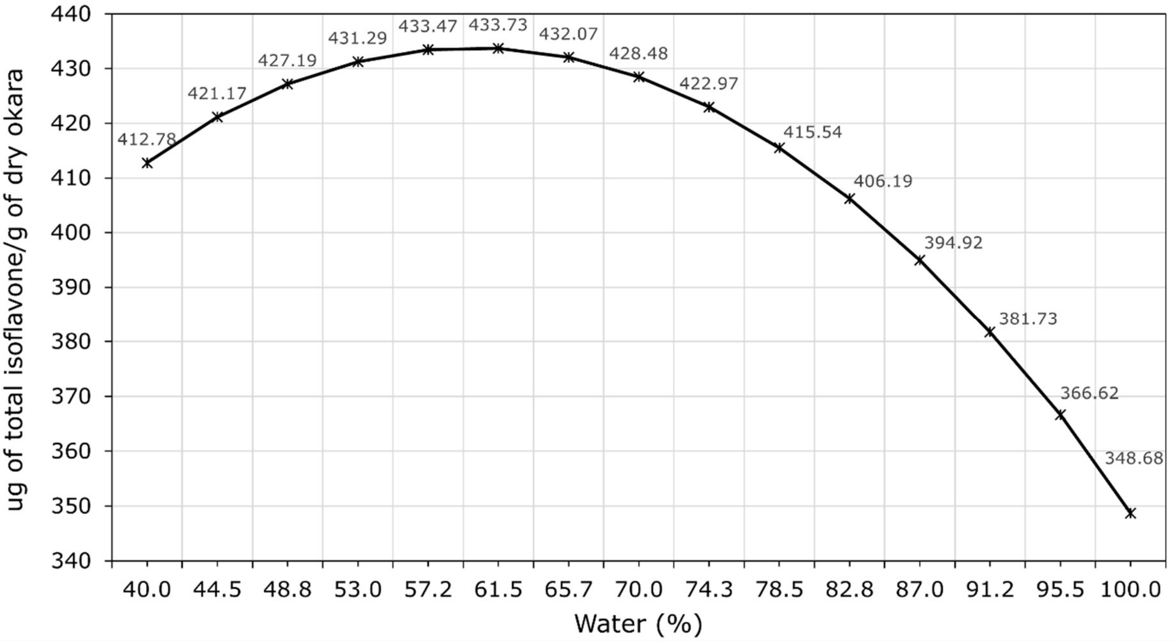

Fig. 5.2. The predicted curve obtained from the reparametrized model for isoflavone extraction from okara.

Table 5.9. Optimized conditions for extraction of isoflavones from okara

Optimum Condition		**ug/g of dry okara**		**Absolute deviation**	**Relative deviation (%)**
		Predicted value	**Observed value**		
Temperature	40 °C	433.73	450.90	17.17	3.81
Stirring	500 rpm				
Water	61.5 %				
S/L ratio	10 mg/mL				

Analyzing the data from Table 5.6, temperature appears to influence the concentrations of daidzein and daidzin. Looking at the data, the levels of daidzein decrease from 124.21 to 119.10 ug/g of sample when the temperature is increased from 44.4 °C to 65.6 °C, while keeping the water content in the DES fixed at 48.79% (assay 1 and 3, respectively). The opposite was observed for daidzin, which increased from 48.88 to 52.13 ug/g of sample. The same behavior was observed for assays 2 and 4, with a water content of 91.21%, and for assays 5 and 6, when the temperature was increased from 40 to 70 °C, respectively. The decrease in daidzein levels is likely due to thermal degradation. Muliterno et al. (2017). observed a significant reduction in isoflavone content when using a temperature of 70 °C.

The most plausible explanation for the increase in daidzin levels is the conversion of the malonyl daidzin and acetyl daidzin forms into their glycosides form, daidzin. This behavior was observed by Rostagno et al. (2005) while studying the stability of isoflavones from 25 to 40 °C. According to the authors, the malonyl forms of genistin, daidzin, and glycitin were converted into their β-glycosides when stored at 40 °C for 7 days. Corresponding increases in daidzin, genistin, and glycitin were observed without altering the total isoflavone content, confirming the conversion of malonyl and acetyl isoflavones (Góes-Favoni et al., 2010; Rostagno et al., 2005).

5.3.4 Optimizing extraction of isoflavones from soybean.

Optimization the extraction isoflavones from soybean was based on a preliminary analysis of the fractional design. The operating conditions temperature, stirring, S/L ratio, and time were set at 25 °C, 500 rpm, 10 mg/mL, and 2 hours of extraction, respectively, as described previously. Since only water showed a significant effect, only its effect was studied by varying the concentration from 50-100 %, as shown in Fig. 5.3.

Analyzing the data in Fig. 5.3 it was observed that the maximum extraction of 1194.56 μg TIC/ g dry soybean occurred with the addition of 70% water in DES and after that, it was seen a decline from 70% to 100% (pure water).

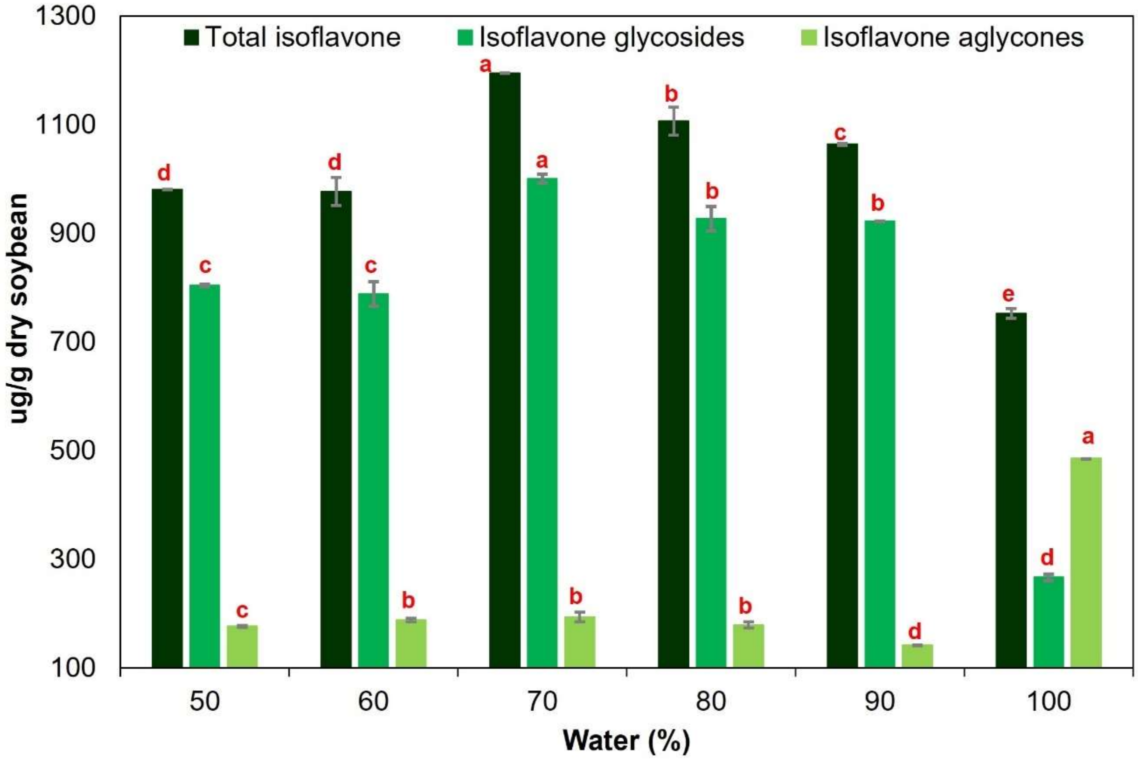

Fig. 5.3. Aglycones, glycosides, and total isoflavones extracted from soybeans. Bars followed by the same letter in the column of the same color show no statistical difference by the Tukey's test ($\alpha \leq 0.05$).

It appears that a transition mechanism in the solubilization of the isoflavone glycosides occurs from 70% water content, which decreases until 266.87 µg TIC/g dry soybean when pure water is used.

For the isoflavones aglycones, however, this change occurs with the addition of 60% water by mass to the DES. Pure water proved to be a good extracting solvent for the isoflavones daidzein, genistein, and glycitein, and the addition of DES impaired the solute-solvent interactions for this class of solutes.

Analyzing the extraction data of the isoflavone aglycones, it was observed that from the addition of 70% water in DES, the content of the isoflavones daidzein, genistein, and glycitein decreased up to 90% water. However, extraction with pure water obtained expressively more aglycone isoflavones (Table S6, Supplementary Material). For the isoflavones glycosides daidzin, genistin and glycitin, pure water proved not to be a good extracting solvent for these forms.

Solubility and viscosity are important parameters in solid-liquid extraction studies. The high viscosity, resulting from the extensive hydrogen bonds between HBA and HBD, compromises the mass transfer, reducing the diffusion rate of the solute present in the solid phase (Wojeicchowski et al., 2021). Therefore, water is added to reduce the viscosity of the solvent and increase the extraction efficiency. However, the solubility of the analytes in DES may be impaired due to the weakening of the intermolecular interactions between HBA and HBD, because water is able to form hydrogen bonds or ionize the HBA and HBD components, decreasing the solubility of the isoflavones(Chen et al., 2023). Chen et al. (2023) in their study of the solubility of soy genistin in acidic NADES observed that the solubility of genistin decreases with increasing water content in the system formed by [Ch]Cl: malic acid, where the maximum solubility found was 1.6 g/L at 40 °C and by adding 5 % water in the DES.

Our studies are in accordance with the observations of Chen et al. (2023) . We clearly observed that pure water is not a good extracting solvent for the glycosides forms. As can be seen in Figure 3, pure water drastically decreased the levels of daidzin, genistin, and glycitin. The same was observed for okara, where the glycosides forms could not be extracted. It should be taken into consideration that the levels of isoflavones in okara are lower. This, combined with the fact that water is not a good solvent for this class of solutes, resulted in the absence of these bioactive compounds in the extract. However, our system is more complex and involves the presence of other solutes, so the addition of 70% water was necessary to obtain a better result and considering the total content of isoflavones, a significant difference was observed in relation to the other % of water by applying the Tukey's test at 5% significance.

5.3.5 Effect of the solvent on the matrix structure

After optimization of the conditions for extraction of isoflavones from soybean and okara, SEM analyses were performed to evaluate the effect of the solvent on the matrix structure and compared to the raw material before extraction, as shown in Fig. 5.4.

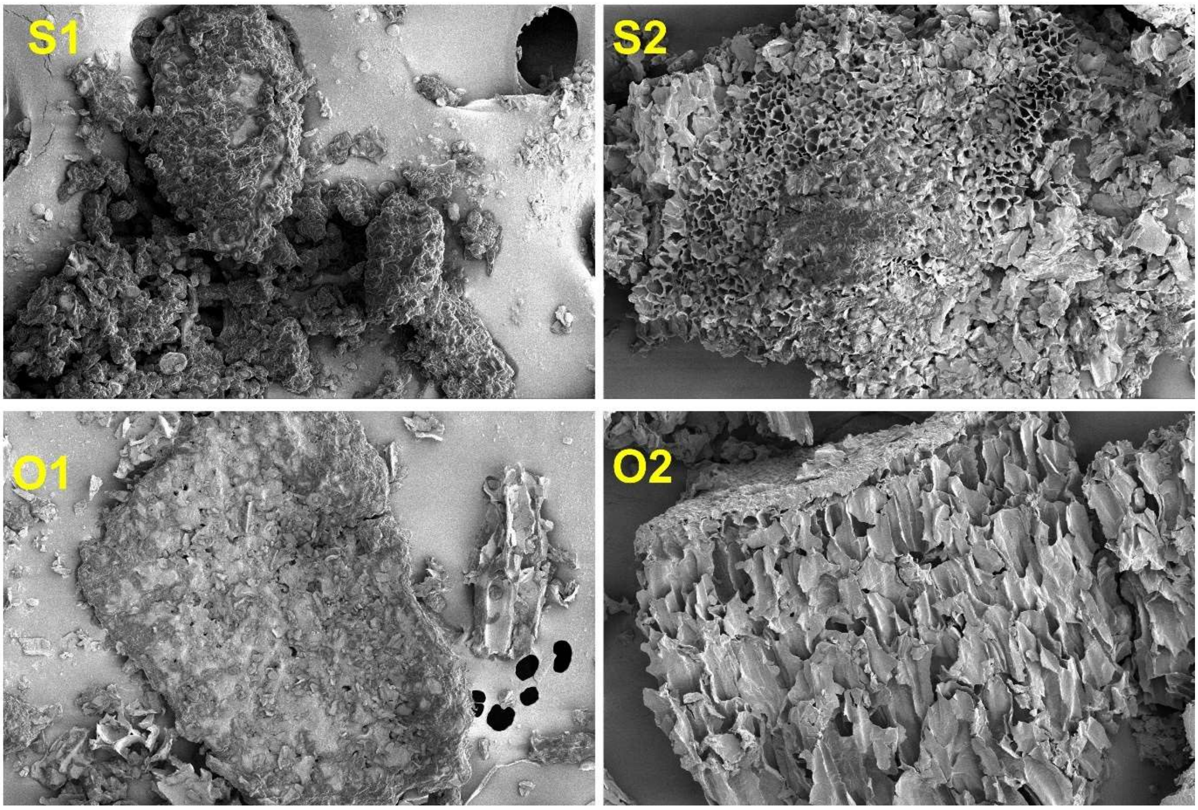

Fig. 5.4. Scanning electron microscopy images before and after the extraction of isoflavones from soybean (S1 and S2, respectively) and from okara (O1 and O2, respectively).

The solvent caused damage to the biomass surface for both raw materials. For the freeze-dried soybean, the surfaces presented massive and rounded characteristics (Fig. 5.4S1). After extraction with aqueous DES, the surfaces presented significant pores and flocculated characteristics. For the okara, both samples showed a ruptured cellular structure. In Fig. 5.4O1, okara presents a flocculated structure, different from freeze-dried soybean. This is because, for obtaining okara, there is an extraction process using water to remove soluble solids that compose the soy milk. But when the aqueous DES mixture is applied, there is an increase in the proportion of ruptured cells with the appearance of pores. DES, compared to water, seems to cause more cellular damage to the biomass. This allows for better access to the bio compounds in the matrix. Images obtained with 400x and 1200x magnifications are available in the Supplementary Material, in Figs. S2 and S3. These observations are in accordance with those made by Pontes et al. (2021), who, after extracting phenolic compounds from olive leaves using the DES [Ch]Cl: AA with 50% added water, found that the DES caused more damage to the biomass than ethanol. Other authors (Xie et al., 2019; Zhou et al., 2018) have also suggested that DES may be more efficient in breaking the cell wall, facilitating the release of bioactive compounds.

5.3.6 Effect of DES precursors on isoflavone extraction

After optimization, we decided to enrich the study by evaluating the effect of DES precursors on isoflavone extraction. The objective of this part was to evaluate whether the extractions were benefiting from the hydrogen bonding formed from the interaction of [Ch]Cl and acetic acid or if the extraction was being favored by only one compound in the mixture.

For soybean, extractions were performed using acetic acid + 70% water and choline chloride + 70% water for comparison with extraction using DES + 70% water (Fig. 5.5A). It is noted that the DES had a positive effect on isoflavone extraction, extracting more than their individual components. In this case, the DES [Ch]Cl: AA + 70% water extracted 1194.56 μg TIC/ g dry soybean, approximately 45% more than the acetic acid + 70% water which extracted 834.12 μg TIC/ g dry soybean and approximately 150% more than [Ch]Cl+ 70% water which extracted 481.72 μg TIC/ g dry soybean.

For okara, extractions were performed adding 61.5% water to the acetic acid and choline chloride under the optimized condition. It was observed, that in this case, the extractions were favored by the presence of acetic acid in the mixture. Whereas the presence of [Ch]Cl caused a negative effect, decreasing the extraction efficiency as shown in Figure 5.5B. In this case, acetic acid + 61.5 % water extracted 673.13 µg TIC/ g dry okara, approximately 50% more than the DES [Ch]Cl: AA which extracted 450.9 µg TIC/ g dry okara and approximately 100% more than [Ch]Cl+ 61.5% water which extracted 333.71 µg TIC/ g dry okara.

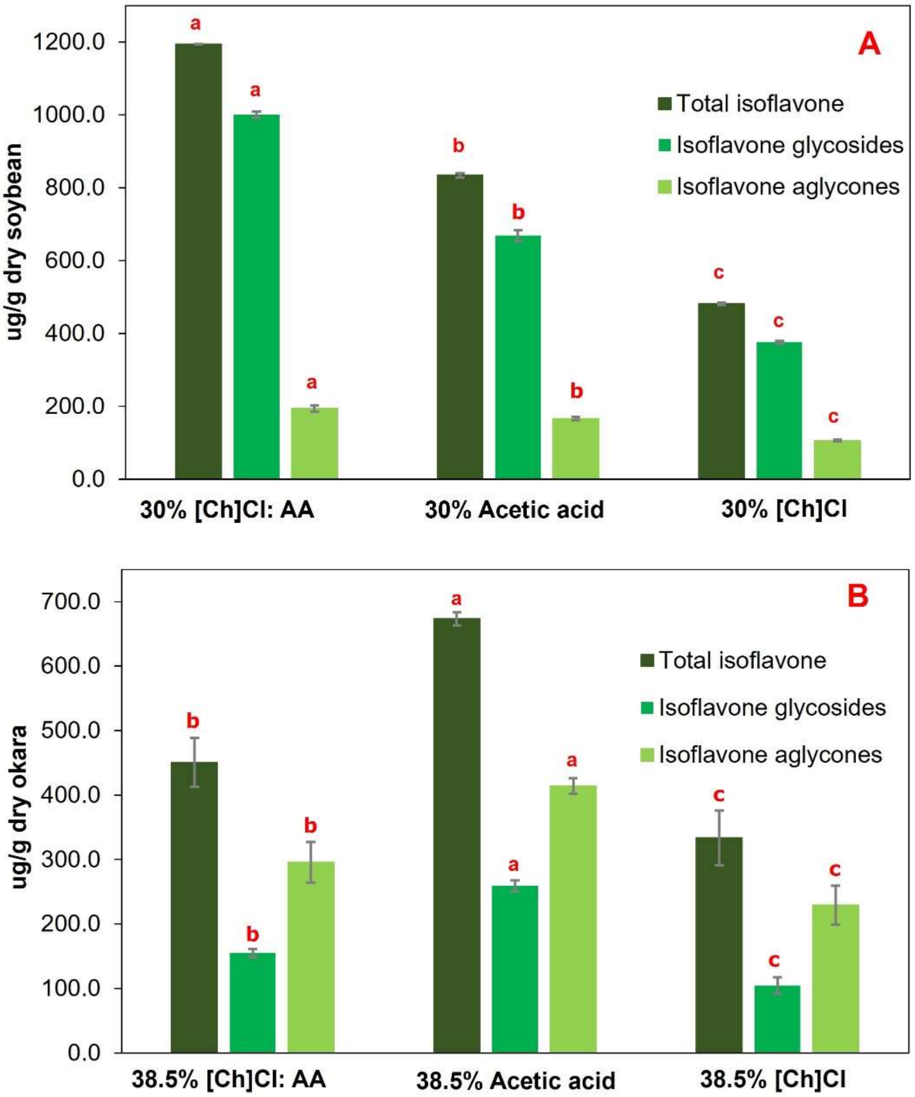

Fig. 5.5. DES and DES precursors with 70% water and 61.5% water in the extraction of isoflavones from (A) soybean and (B) okara, respectively. Bars with equal colors followed by the same letter have no statistical difference by the Tukey test ($\alpha \leq 0.05$)

Both for soybean and okara, the total isoflavone contents are close to those reported in the literature (Hsu et al., 2020; Lummaetee et al., 2017; Muliterno et al., 2017; Ren et al., 2017; Tran et al., 2019; Yu & Yang, 2019).

According to Tables S7 and S8 (Supplementary Material) for okara, from the total isoflavone content (673.13 μg TIC/g dry okara) obtained with acetic acid aqueous mixture, 61.5% correspond to isoflavone aglycones (19.13% daidzein, 35.6% genistein, and 6.77% glycitein) and 38.5% to isoflavone glycosides (19.41% daidzin, 12.63% genistin, and 6.46% glycitin).

The results of the isoflavone profile found in this study are the opposite of that reported by Muliterno et al. (2017) which used a mixture of water, ethanol and acetone (1:1:1, v/v/v) for 1 h at 25 °C to extract isoflavones from okara, where of the 50 mg of isoflavones/100 g of solids, 66% corresponded to the glycosidic forms (42.2% genistin, 12.1% glycitin, and 11.7% daidzin). This difference may be associated with the way okara was obtained, which in our study was different from that reported by Muliterno et al. (2017). Muliterno et al. (2017) obtained the byproduct from a manufacturer in which soybeans were cooked in hot water at 80-90 °C, and due to the high temperatures, malonyl forms may have been converted to β-glycosides through decarboxylation or deesterification processes (reactions that convert malonyl and acetyl forms into β-glycosides)(Muliterno et al., 2017; Privatti et al., 2022). In addition, differences in solvents can also cause this change in the isoflavone profile, as already observed in this work when using pure water and different DES compositions (see Fig. 5.3 and Fig. 5.4).

For soybean, according to Tables S9 and S10 (Supplementary Material), from the total isoflavone content (1176.81 μg TIC/g dry soybean) obtained with [Ch]Cl: AA aqueous mixture, 83.52% of isoflavones were in their glycosides form (32.68% daidzin, 44.91% genistin, and

5.93% glycitin) and only 16.48% in the aglycones form (8.14% daidzein, 7.58% genistein, and 0.76% glycitein).

5.3.7 Cytotoxicity test

Cytotoxicity tests were carried out using soybean extract and the solvents [Ch]Cl: AA added to 70 % and 30 % water. In this study, only the soybean extract was used because it presented the highest content of total isoflavones. In addition, both the extracts and the solvents were neutralized to pH 7.0 in order to assess cytotoxicity under different conditions for possible food, industrial and/or pharmaceutical applications.

To determine half of the maximum inhibitory concentration, IC_{50}, the Caco-2 cell line was used. The choice of the Caco-2 cell lineage was motivated by its extensive use as a model for intestinal permeability, due to its morphological and functional similarity to the mucosa of the small intestine, thus representing the intestinal barrier most appropriately responsible for absorption and secretion to test the cytotoxicity of these tissues, with the aim of applying these solvents for the development of new products intended for human consumption (Czaikoski et al., 2020; Rasera et al., 2023).

Fig. 5.6 shows the cytotoxicity to Caco-2 cells of the extracts and solvents in their initial state (acidic pH) and after neutralization at pH 7.0. For the acid and neutralized soybean extracts, the IC_{50} values were 2.37 and 4.67 %, respectively. For the solvent formed by [Ch]Cl: AA + 70% water, the IC_{50} values were 1.22 and 3.66 % for the acid and neutralized solvents, respectively. With the solvent [Ch]Cl:AA added with 30 % water, the IC_{50} was 0.6 and 3.36 % for the acid and neutralized solvents, respectively.

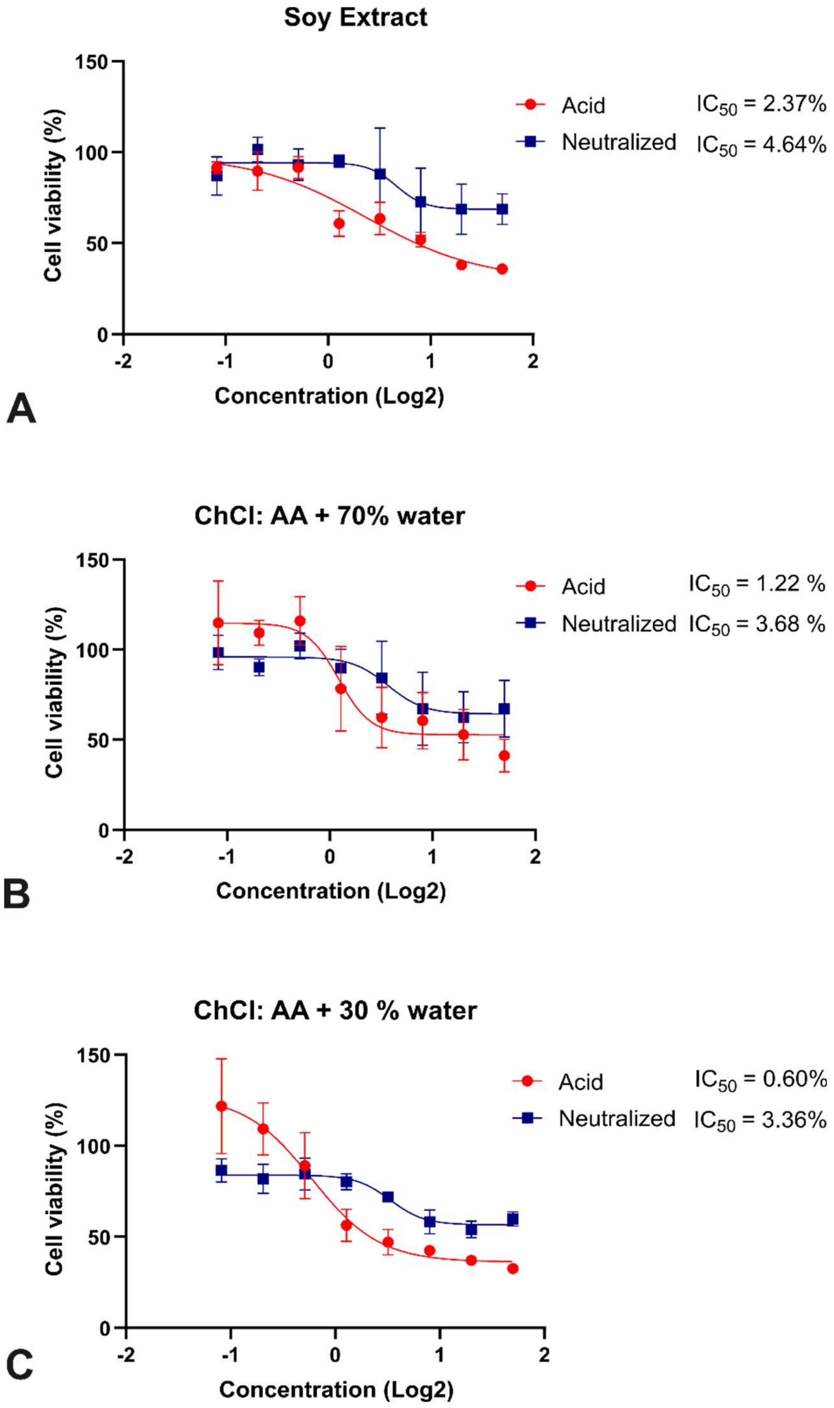

Fig. 5.6. Cytotoxicity tests performed with the soybean extracts obtained in the optimized condition and the solvents [Ch]Cl: AA added to 70 and 30 % in Caco-2 colorectal adenocarcinoma cells in 24 hours. Dose-response curve of soy extract (A), [Ch]Cl:AA added to 70% water (B) and [Ch]Cl:AA added to 30% water (C), red line represents initial state (acidic pH) and blue line the neutralized pH.

For both extracts and the solvents, a greater cytotoxic effect is observed in acidic systems. Furthermore, increasing the water concentration in these systems slightly reduces the cytotoxicity effect on Caco-2 cells. Therefore, for industrial applications involving human use, it is recommended to neutralize the studied extracts and mixtures beforehand.

5.4 Conclusion

This study evaluated the extraction of isoflavones from soybean and okara using DES based on [Ch]Cl and acetic acid, and aqueous mixtures based on [Ch]Cl: water and acetic acid: water as sustainable alternatives in substitution to toxic and volatile solvents such as methanol, acetone, and acetonitrile.

Applying fractional design, a screening of the variables temperature, water content in DES, S/L ratio and stirring speed was performed on the extraction of the isoflavones daidzein, genistein, glycitein, daidzin, genistein and glycitin from soybean and okara using DES [Ch]Cl: AA (1:2) as an alternative solvent.

For soybean, 1176.81 µg TIC/ g dry soybean were extracted using [Ch]Cl: AA added with 70 % water at low temperature (25 °C), 500 rpm, S/L ratio of 10mg/mL and 2 hours extraction time. For okara, after screening the variables, a complete design (CCRD) was applied using the variables water content in DES and temperature for optimization based on maximum extraction of isoflavones. At the optimum point, 450.9 µg TIC/ g dry okara was extracted using 40 °C, 500 rpm, 61.5 % water in DES, S/L ratio of 10 mg/mL and 1 hour of extraction.

For the case of extraction of isoflavone from okara, the 70% acetic acid aqueous mixture extracted 673.13 µg TIC/ g dry okara, approximately 50% more than the studied DES.

In addition, the SEM images showed that the solvents caused damage to the cell walls of soybeans and okara, favoring extraction. And, in the cytotoxicity tests, it was observed that the neutralization step of the extracts is necessary to reduce the cytotoxic effects caused by the high acidity of the solvents.

Therefore, DES [Ch]Cl: AA and aqueous acetic acid mixture can be applied as an alternative for extraction of isoflavones from soybean and okara, respectively Clearly, more studies need to be conducted to optimize the extraction of isoflavones from okara using the mixture of acetic acid and water.

Acknowledgments

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Supplementary Material

Optimizing the operating conditions for the extraction of phytoestrogens from soybean and okara using Deep Eutectic Solvents (DES)

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TABLES

Central composite rotatable design (CCRD)

For okara, in addition to fractional design, the effects and interactions of temperature and water content in DES on isoflavone extraction were investigated. The proposed design was the CCRD with two levels and two factors (2^2), 4 axial points, and 4 repetitions at the central point to calculate the experimental error, totaling 12 experiments. Table S1 summarizes the experimental conditions applied.

Table S1. Levels and independent variables of the CCRD.

	-α	**Levels**			**+α**
Factors	-1.41	-1	0	1	1.41
Temperature (°C) (x_1)	40	44.39	55	65.61	70
Water (%) (x_2)	40	48.79	70	91.21	100

The curvature effect was first investigated by applying a central composite design (CCD)(2^2) to evaluate if a first-order model is sufficient to explain the response.

All data obtained were processed using Statistica 8.0 software (Statsoft Inc., USA). A polynomial model was fitted to the responsesand evaluated by analysis of variance (ANOVA). The F-test was applied for regression analysis and the lack of fit of the model. The quality of the model fit was evaluated by the coefficient of determination R^2 and the regression was used to calculate the predicted results. In addition, the relative and absolute deviations of the observed and predicted values were calculated. For model validation, the point of maximum extraction of total isoflavones was selected.

Table S2 shows the evaluation of curvature, variable effects. and interactions of the effects of a Central Composite Design (2^2). As shown in Table S2 the curvature was statistically significant (p-value<0.05). This shows that a 1st order model is not enough to describe the results. Being necessary the addition of axial points is to obtain a 2nd order model.

Table S2. Analysis of Variance (ANOVA) for Central Composite Design (CCD)

	^{a}SS	^{b}DF	^{c}MS	^{d}F	^{e}P
Curvature	3417.84	1	3417.84	17.48	0.025
(1) Temperature (°C)	191.51	1	191.51	0.98	0.4
(2) Water (%)	1613.86	1	1613.86	8.26	0.06
1 by 2	2.38	1	2.38	0.01	0.92
Pure Error	586.48	3	195.49		
Total SS	5812.06	7			

^{a}SS = Sum of Squares; ^{b}DF = Degree of Freedom; ^{c}MS = Mean of Squares; ^{d}Ftab. = tabulated F-value. ^{e}p = p-value used to verify the significance of variables.

Table S3. Analysis of Variance (ANOVA) for Central Composite Rotatable Design (CCRD)

Variables	**SS**	**DF**	**MS**	**F**	**p**
(1) Temperature (°C) (L)	64.36	1	64.365	0.32924	0.606301
(1) Temperature (°C) (Q)	80.27	1	80.269	0.41060	0.567255
(2) Water (%) (L)	4132.82	1	4132.816	21.14033	0.019337
(2) Water (%) (Q)	3913.74	1	3913.742	20.01971	0.020808
1L by 2L	2.38	1	2.377	0.01216	0.919161
Lack of Fit	1555.53	3	518.509	2.65230	0.222144
Pure Error	586.48	3	195.494		
Total SS	10268.46	11			

Table S4. Reduced ANOVA

	SS	**DF**	**MS**	**Fcal**	**Ftab**
Regressão	8126.45	5	1625.29	4.55	4.39
Resíduos	2142.01	6	357.00		
Falta de Ajuste	1555.53	3	518.51	2.65	29.46
Erro puro	586.48	3	195.49		
SQ total	10268.46	11			
R²	79.14%				

Table S5. ANOVA obtained after model reparameterization.

Variables	**SS**	**DF**	**MS**	**F**	**p**
(2) Water (%) (L)	4132.82	1	4132.816	21.14033	0.019337
(2) Water (%) (Q)	3846.62	1	3846.620	19.67637	0.021296
Lack of Fit	1702.54	6	283.756	1.45148	0.408909
Pure Error	586.48	3	195.494		
Total SS	10268.46	11			

Table S6. Content of soy isoflavones extracted with DES ChCl: acetic acid with different concentrations of water and pure water.

% water	**Daidzein**	**Genistein**	**Glycitein**	**Daidzin**	**Genistin**	**Glycitin**	**IA**	**IG**	**TIC**
50.0	86.49±1.54	79.06±0.7	11.14±0.17	299.18±4.33	385.54±0.97	119.49±21.42	176.70	804.20	980.90
60.0	91.73±1.37	89.26±3.64	7.59±0.22	279.46±5.19	439.63±17.59	69.14±4.55	188.58	788.24	976.81
70.0	95.81±2.85	89.19±5.78	8.92±0.45	384.58±17.89	546.22±17.14	69.84±4.59	193.91	1000.64	1194.56
80.0	91.37±2.18	78.83±3.61	9.14±0.14	372.62±7.91	500.34±3.75	54.13±4.89	179.34	927.10	1106.44
90.0	79.47±6.73	55.53±5.45	6.97±0.38	398.97±1.68	448.09±0.72	75.09±1.58	141.97	922.16	1064.13
100.0	258.04±5.44	179.27±3.64	48.45±4.98	128.23±6.24	103.54±5.49	35.11±2.83	485.76	266.87	752.63

*IA: Isoflavone aglycones; IG: isoflavone glycosides; TIC: Total isoflavone content

Table S7. Isoflavone composition of extracts from okara obtained with DES and DES precursors with 61.5% water.

	Concentration (ug of isoflavone/ g dry okara)								
Solvents	Daidzein	Genistein	Glycitein	Daidzin	Genistin	Glycitin	IA*	IG*	TIC*
38.5% ChCl:AA	116.61	151.31	27.98	52.00	74.74	28.26	295.90	155.00	450.90
38.5% AA	128.75	239.66	45.56	130.69	85.00	43.50	413.97	259.19	673.13
38.5% ChCl	80.49	130.41	18.37	32.54	55.39	16.50	229.27	104.44	333.71

*IA: Isoflavone aglycones; IG: isoflavone glycosides; TIC: Total isoflavone content

Table S8. Mass fraction (w) of each analyte in okara relative to the total extracted isoflavones

	w*100 (ug / ug total)								
Solvents	Daidzein	Genistein	Glycitein	Daidzin	Genistin	Glycitin	IA*	IG*	TI*
38.5% ChCl: AA	25.86	33.56	6.21	11.53	16.58	6.27	65.62	34.38	100.00
38.5% AA	19.13	35.60	6.77	19.41	12.63	6.46	61.50	38.50	100.00
38.5% ChCl	24.12	39.08	5.51	9.75	16.60	4.94	68.70	31.30	100.00

*IA: Isoflavone aglycone; IG: isoflavone glycosidic; TIC: Total isoflavone content

Table S9. Isoflavone composition of extracts from soybean obtained with DES and DES precursors with 70% water in the extraction of isoflavones from soybean.

	Concentration (ug of isoflavone/ g dry soybean)								
Solvents	Daidzein	Genistein	Glycitein	Daidzin	Genistin	Glycitin	IA*	IG*	TIC*
30% ChCl: AA	95.81	89.19	8.92	384.58	546.22	69.84	193.91	1000.64	1194.56
30% AA	69.37	80.78	15.72	309.70	311.61	46.95	165.87	668.25	834.12
30% ChCl	51.92	46.98	6.75	142.67	212.57	20.84	105.65	376.08	481.72

*IA: Isoflavone aglycones; IG: isoflavone glycosides; TIC: Total isoflavone content

Table S10. Mass fraction (w) of each analyte in soybean relative to the total extracted isoflavones

	w*100 (ug / ug total)								
Solvents	Daidzein	Genistein	Glycitein	Daidzin	Genistin	Glycitin	IA*	IG*	TIC
30% ChCl: AA	8.02	7.47	0.75	32.19	45.73	5.85	16.23	83.77	100.00
30% AA	8.32	9.68	1.88	37.13	37.36	5.63	19.89	80.11	100.00
30% ChCl	10.78	9.75	1.40	29.62	44.13	4.33	21.93	78.07	100.00

*IA: Isoflavone aglycones; IG: isoflavone glycosides; TI: Total isoflavone content

FIGURES

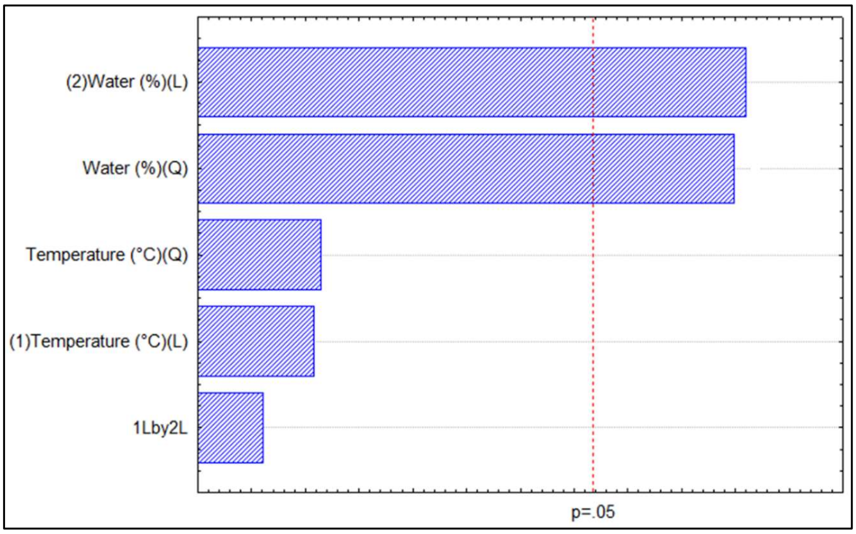

Fig. S1. Pareto diagram for okara variable

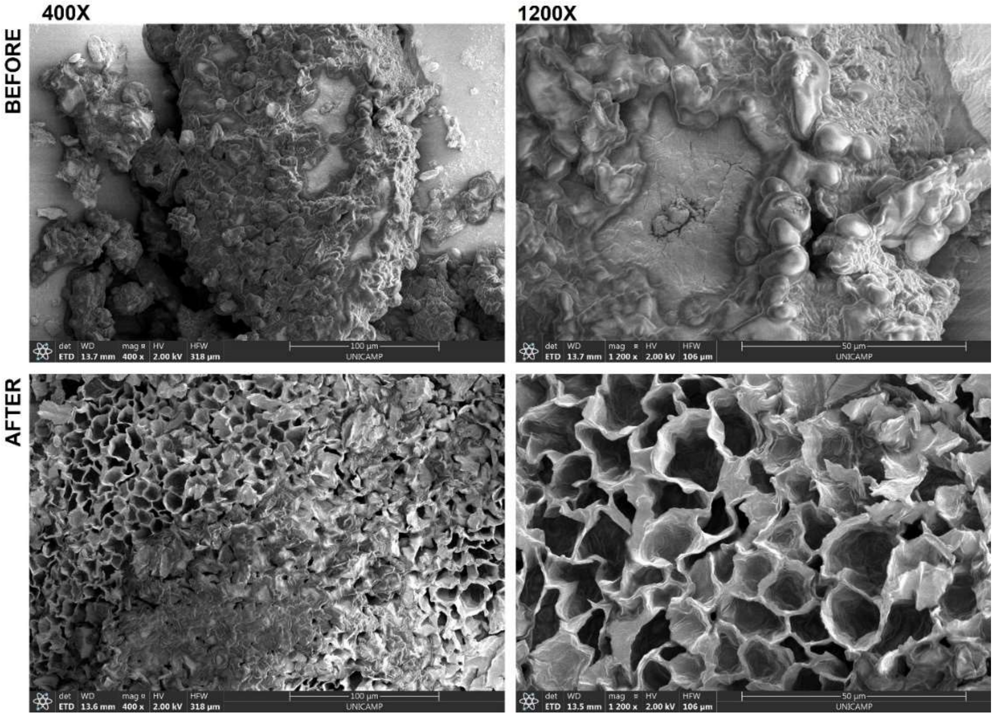

Fig. S2. SEM analyses for soybean before and after extraction process

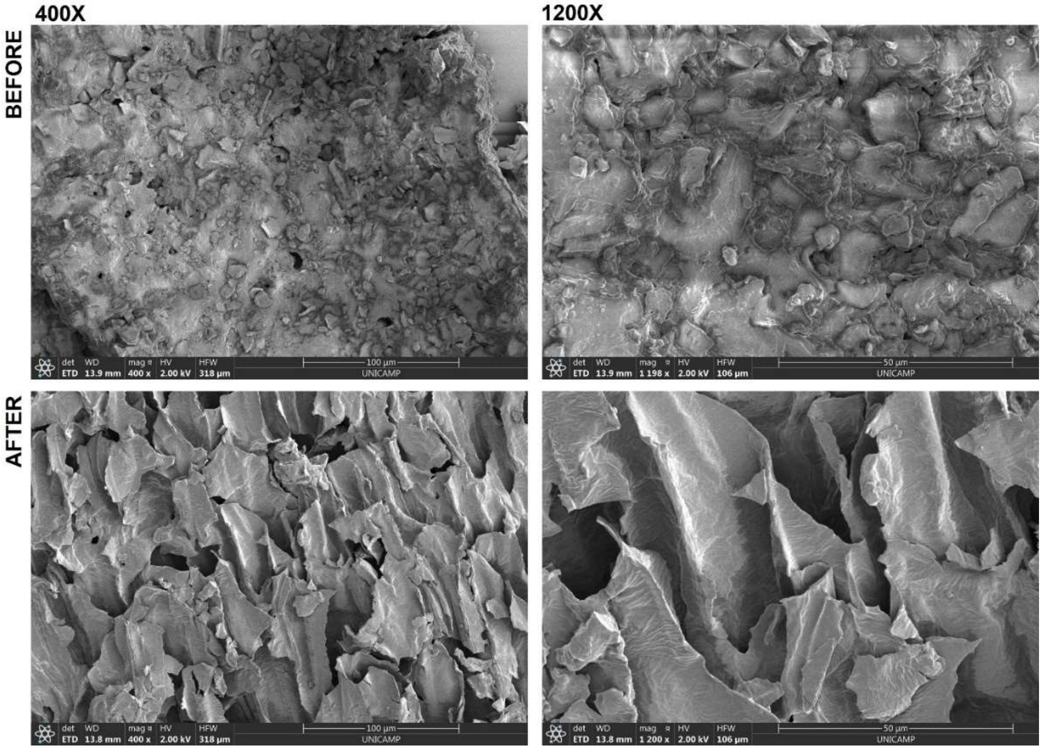

Fig. S3. SEM analyses for okara before and after extraction process

CAPÍTULO 6

Exploring Bio-based Solvents for the Recovery of Phenolic Compounds and Isoflavones from Okara: Solvent Screening and Optimization

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Abstract

Okara is a solid residue resulting from the extraction of soy milk and contains fibers, proteins, lipids, carbohydrates, and phenolic compounds, particularly isoflavones, which act as phytoestrogens and are mainly used in the treatment of menopausal symptoms. Approximately 30% of soy isoflavones are retained in okara, making it an interesting source for the extraction of bioactive compounds. Therefore, this study aimed to analyze 26 *Bio-based Solvents* for the extraction of total phenolic compounds (TPC) and total isoflavones (TI), followed by optimization using a Central Composite Rotatable Design (CCRD 2^3). The screening was carried out using a set of carboxylic acids+water/ethanol and polyols+water/ethanol based on the maximum extraction of total phenolic compounds (TPC), quantified by the Folin-Ciocalteu methodology, and the isoflavones daidzein, genistein, daidzin, and genistin, quantified by HPLC. From the screening, it was found that the solvent glycerol+70% water extracted the highest content of phenolic compounds from freeze-dried okara, approximately 11.0 mg GAE/g DS of extract and 1056. 8 ug of TI/g DS. Based on the CCRD, the operational conditions of temperature, percentage of water, and solid-liquid ratio (mg/mL) were optimized. For TPC and TI, the best condition was achieved at 66 °C, with 40% water added to glycerol and a solid-liquid ratio of 60 mg/mL, using fixed agitation and time of 500 rpm and 45 min, respectively. Under these conditions, an extract was obtained with approximately 45.0 mg GAE/100 mL of extract and 60 μg of TI/mL of extract. Therefore, the use of glycerol+40% water as solvent achieved high extraction of phenolic compounds and isoflavones from okara residue, providing the replacement of conventional toxic solvents and enabling the valorization of a residue, which is normally discarded due to its high perishability.

Keywords: green solvents, bioactive compounds, extraction, sustainability

6.1. Introduction

Global food waste is a worldwide issue, with adverse impacts on the economy, environment and society. According to the Food and Agriculture Organization (FAO), more than a third of global food production, totaling approximately 1.3 billion tons, is lost. Fruits, vegetables, roots, and tubers emerge as the staple foods with the highest wastage rates, at around 40-50% (Jenkins et al., 2016; Silva et al., 2023). It is important to highlight that when discarded, these wastes take with them many valuable components, such as phenolic acids, flavonoids, polyphenols that have a wide range of biological properties with anti-inflammatory, antioxidant and antimicrobial activities (Privatti and Rodrigues, 2023).

Soy extract residue, also known as okara, is the insoluble fraction obtained from the extraction of soy extract, which is widely used to produce tofu and "soy milk", as it is called commercially (Hu et al., 2022). With the continuous advancement of the soy-based products industry, the accumulation of okara has become a significant environmental issue. Due to its high susceptibility to spoilage, undesirable taste and strong "raw bean" odor, its use is still limited compared to the nutritional benefits it offers (Wang et al., 2022). It is estimated that the annual world production of okara is around 14 million tons (Privatti and Rodrigues, 2023). Rich in bioactive compounds such as the vitamins thiamine, riboflavin and nicotinic acid and isoflavones such as daidzein, genistein, daidzin and genistein (Gupta et al., 2018), the waste of okara represents a loss of bioactive compounds of high nutritional value.

Isoflavones are phytoestrogens that are structurally similar to 17-estradiol and bind to the β and α estrogen receptors (Messina, 2016), contributing to the maintenance of healthy blood sugar levels and can inhibit the adverse effects of cardiovascular diseases, diabetes, osteoporosis, prostate cancer, breast cancer and, especially, the reduction of menopause symptoms (Messina, 2016; Nile et al., 2022). Given these advantages, the recovery and inclusion of these compounds in food products can add value to okara, avoiding the waste of these bioactive compounds.

Methods using solvents such as methanol, acetone and acetonitrile are still widely used to recover these bioactive compounds (Kumar et al., 2021; Yahya et al., 2018). However, these solvents have levels of toxicity sufficient to cause damage to human health and the environment. Due to growing concern for the environment and human well-being, techniques considered safer are being applied to recover these compounds. As an example, we have the application of ethanol (recognized as safe), which was studied by Tran et al. (2019), Jankowiak et al. (2014b) and Carneiro et al. (2020), for the extraction of isoflavones from soybeans, okara and soybean residues (leaves, branches and pods), respectively. More recently, Deep Eutectic Solvents have been used to extract flavonoids from soybeans and okara (Ferreira et al., 2023) and isoflavones from soybean branches, leaves, pods, roots and beans (Bragagnolo et al., 2024, 2022).

Following the same path as Deep Eutectic Solvents and Ionic Liquids, *Bio-based Solvents* are considered sustainable solvents derived from renewable sources such as alkanediols, cirene and gamma-valerolactone (GVL). These solvents, which are less toxic than volatile organic compounds, are viable options for extracting bioactive compounds from natural sources (Clarke et al., 2018). Aqueous solutions of alkanediols and glycerol were effective in extracting phenolic compounds from walnut leaves (*Juglans regia* L.)(Vieira et al., 2020). Alkanediols, cyrene and GVL have also been successfully used to extract phenolic compounds from kiwi waste (Silva et al., 2023).

Despite their potential, these *Bio-based Solvents* have been little explored in the extraction of phenolic compounds from natural sources and have never been tested to extract phenolic compounds and isoflavones from soybean residues. This work sought to explore a set of *Bio-based Solvents* composed of carboxylic acids, polyols, GVL and ethyl lactate to extract phenolic compounds and isoflavones from okara. In this way, this study aimed to contribute to sustainable techniques for recovering bioactive compounds from soy waste, thus contributing to the formation of an economy based on renewable resources and valuing agri-food waste.

6.2. Material and methods

6.2.1 Reagents

Table 6.1 summarizes the list of chemical compounds used in this work, both for the extraction of phenolic compounds and isoflavones from okara and the reagents and standards used in the methods of analysis and quantification of bioactive compounds. The water used was double distilled, passed through a reverse osmosis system and then treated with a Milli-Q plus 185 water purification device.

Table 6.1. List of compounds used in this work accompanied by purity and supplier.

Compound and abbreviation	Purity (wt.%)	Supplier
Bio-based Solvents		
1,2,4-Butanotriol	98.0	Sigma-Aldrich
Acetic acid	99.9	Honeywell
Benzoic acid	99.0	Acros Organics
Butyleneglycol	99.5	Sigma-Aldrich
Citric acid	99.6	Acros Organics
Ethanol	99.0	Carlo Erba Reagents
Ethyl lactate	>98.0	Sigma-Aldrich
Formic acid	>99.0	Carlo Erba Reagents
Gamma-valerolactone (GVL)	98.0	ThermoScientific
Glycerol	>98.0	Biochem - Frilabo
Lactic acid	>88-92%	Sigma-Aldrich
Malic acid	98.0	Alfa Aesar
Malonic acid	>98.0	Fluka
Methanol	99.0	Fisher Scientific
Propylene glycol	>99.0	Sigma-Aldrich
Tartaric acid	99.0	Acros Organics
Conventional Solvents		
Ethanol	99.0	Carlo Erba Reagents
Methanol	99.0	Fisher Scientific
Isoflavones standards		
Daidzein	>98.0	TCI
Daidzin	>98.0	TCI
Genistein	>98.0	TCI
Genistin	>98.0	TCI
Phenolic compound, antioxidant and isoflavones assays		
[2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate)] (ABTS)	98.0	Panreac
Acetonitrile	99.9	Fisher Chemical
Folin-Ciocalteu	n.a	Panreac
Gallic acid	99.5	Merck
Trolox	97.0	Acros Organics
2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH)	n.a	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	99.8	Euriso-top

6.2.2 Raw material

The soybeans used to make okara were purchased at a local market and processed in the Path laboratory at the University of Aveiro.

The okara was obtained according to Baú and Ida (2015). First, the soybeans were washed with distilled water for dirt removal and then submerged in a soybean: water ratio of 1:3 (m:v) at 5 °C for 14 h. After that, the water was drained, and the soybeans were rinsed. Distilled water was again added at soaked soybean: water ratio of 1:8 (m:v) and the mixture was processed in an industrial blender for 2 min at 25 °C to obtain a paste. After that, the paste was filtered and freeze-dried (Liobras, L101 model) until constant mass at $T = (-50 \pm 1\ °C)$, $p = (13 \pm 2)$ μHg for 48 h. The dry insoluble fraction was then triturated in a knife mill (Marconi, model MA 340). The okara was packed in polyethylene bags and stored in a vertical ultra-freezer at -80 °C (Nuaire, Glacier model, USA). The samples were unfrozen according to the amount needed for the extraction experiments.

6.2.3 Bio-based solvents screening

The *Bio-based Solvents* were prepared on magnetic stirrers (Heidolph, MR HEI-Tec, Germany) at 25 °C, 600 rpm for 30 min. In total, 28 binary mixtures were prepared with 70% water or 70% ethanol added by mass. The choice of the amount of water/ethanol was based on previous work carried out by our research group where it was found that the addition of 70% water in the Deep Eutectc Solvent based on Choline Chloride: Acetic acid (1:2) showed greater extraction efficiency of soy isoflavones. Table 6.2 summarizes the list of *Bio-based Solvents* used in the experimental screening.

Table 6.2. List of *Bio-based Solvents* used for experimental screening.

Solvents	Water (%)	Solvents	Ethanol (%)
Formic acid + Water	70	Formic acid + Ethanol	70
Acetic acid + Water	70	Acetic acid + Ethanol	70
Lactic acid + Water	70	Lactic acid + Ethanol	70
Malic acid + Water	70	Malic acid + Ethanol	70
Malonic acid + Water	70	Malonic acid + Ethanol	70
Tartaric acid + Water	70	Citric acid + Ethanol	70
Citric acid + Water	70	Benzoic acid + Ethanol	70
Glycerol + Water	70	Glycerol + Ethanol	70
1,2,4-Butanotriol + Water	70	1,2,4-Butanotriol + Ethanol	70
Propyleneglycol + Water	70	Propyleneglycol + Ethanol	70
Butyleneglycol + Water	70	Butyleneglycol + Ethanol	70
GVL + Water	70	GVL + Ethanol	70
Ethyl lactate + Water	70	Ethyl lactate + Ethanol	70
Ethanol + Water	30	-	
Methanol + Water	30	-	

The extractions of bioactive compounds from the biomass were carried out in a heated reactor with a dry bath at 40 °C and magnetic stirring at 500 rpm for 1 hour, using a solid-liquid ratio of 10 mg/mL. For comparison, extractions were also performed with conventional solvents methanol and ethanol, as shown in Table 6.2.

After the extractions, the mixtures were centrifuged at 10000 rpm, 25 °C, for 10 min to separate the biomass from the extract. The supernatants were filtered and stored in an ultra-freezer at -80°C for subsequent analysis of total phenolic compounds, isoflavones and antioxidant activity. The *Bio-based Solvents* that obtained the okara extract richest in bioactive compounds was chosen for the optimization stage by applying a Central Composite Rotatable Design (CCRD).

6.2.4 Determination of extraction time

The extraction time was determined by evaluating the TPC content using a short kinetics of 180 min. The best solvent obtained from the screening was used for this study. The extractions of the bioactive compounds from the biomass were carried out using the same conditions as in the screening stage, i.e. 40 °C, magnetic stirring at 500 rpm, a solid-liquid ratio of 10 mg/mL and 70% water added to the solvent. The time was determined based on the point where the extraction curve no longer sloped. The quantification of phenolics was determined according to item 6.2.6.

6.2.5 Design of experiments

After screening, the effects and interactions of temperature, S/L ratio and percentage of water on the extraction of total phenolic compounds and isoflavones from okara with the best solvent were evaluated. In total, 4 responses were optimized: 1) the concentration of total phenolic compounds (TPC) in the extract; 2) the concentration of total isoflavones (TI) in the extract; 3) the extraction yield of total phenolic compounds (Y_1) and, 4) the extraction yield of total isoflavones (Y_2). The proposed factorial design was a CCRD with two levels and three factors (2^3 factorial design), six axial points and five repetitions at the central point, to determine the variability of the results and evaluate the experimental errors, totaling 19 experiments. This type of design was chosen to investigate a larger area but with fewer experiments (Rodrigues and Iemma, 2015). In addition, the experiments were randomized to avoid statistical distortions in the results (Barros Neto et al., 2010). Table 6.3 summarizes the levels and experimental conditions employed.

Table 6.3. Levels and factors of the CCRD factorial design (2^3).

	-α	Levels			**+α**
Variables	**-1.68**	**-1**	**0**	**1**	**1.68**
Temperature (°C)	29.98	38.10	50.00	61.90	70.02
S/L ratio (mg/mL)	10.0	20.13	35.0	49.87	60.0
Water (%)	40.0	52.16	70.0	87.84	100.0

Firstly, curvature was investigated in the central composite design (2^3) to evaluate if a first order model is sufficient to explain the responses.

The parameters of the polynomial model were fitted to the experimental results using Statistica 14.0 (Statsoft Inc., United States). The results were then evaluated by analysis of variance (ANOVA), and the significance of the regression and lack of fit of the model was evaluated using the F-test. The quality of the fit of the second-order equation was assessed using the coefficient of determination R^2. The absolute and relative deviations between the observed and predicted values were also calculated. After checking the quality of the model to describe the experimental data, the response surfaces and contour curves were generated in Statistica 14.0 (Statsoft Inc., USA). The points of maximum extraction of TPC and TI and maximum yield Y1 and Y2 were chosen to validate the models.

The extraction time was set as previously described (Section 6.2.4). In addition, the stirring speed was also set at 500 rpm based on previous studies, as mentioned above in Section 6.2.3.

6.2.6 Total phenolic compound (TPC)

The total phenolic compounds were measured using the Folin-Ciocalteu methodology described by Singleton and Rossi (1965) with modifications (Lazarin et al., 2020). In test tubes, 1.68 mL of ultrapure water, 20 μL of extract, and 100 μL of 10% (v/v) Folin-Ciocalteu reagent were added. After 3 min, 200 μL of 20 % (w/v) sodium carbonate solution was added to the tubes, and the mixture was vigorously agitated and allowed to react for 60 min. The absorbance was measured at 760 nm in a microplate reader (UV–visible spectroscopy SYNERGY|HT microplate reader from BioTek). The results were expressed as mg gallic acid equivalent per g dry sample (mg GAE/g DS) and mg gallic acid equivalent per mL of extract (mg GAE/mL of extract), calculated from a gallic acid standard curve.

6.2.7 Antioxidant capacity

The antioxidant activity was determined using the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and DPPH (2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl) methods. The results were expressed in mg of Trolox/g of sample from Trolox calibration curves.

The antioxidant activity using the ABTS radical was carried out according to the methodology proposed by Re et al. (1999). The ABTS solution was diluted in ethanol until the absorbance reached 0.7 at 734 nm. After, 30 µL of extract and 2970 µL of ABTS radical were added to test tubes. The mixture was homogenized and, after 6 min of reaction, the absorbance was measured at 734 nm in a microplate reader (UV–visible spectroscopy SYNERGY|HT microplate reader from BioTek). The analyses were realized in quintuplicate, and the results were expressed in µmol of equivalent in Trolox per liter (µmol TE/L) using a Trolox equivalent standard curve from 25 to 500 µM.

The antioxidant activities by DPPH method followed the protocol described by Brand-Williams et al. (1995) with modifications. The method consists in the reduction of the stable free radical DPPH by an antioxidant. Firstly, each extract was diluted 5 times in the ethanol. Then, 50 µL of each sample was added with 250 µL of a DPPH 100 µM ethanolic solution in a microplate, which was kept in absence of light for 30 min at 25 ºC. After this time, the absorbance was measured at 517 nm in a microplate reader described in this section. The analyses were realized in quintuplicate and accompanied by a control (50 µL of diluted solvent, 250 µL DPPH 100 µM) and the blank for each sample (50 µL of sample, 250 µL of ethanol). The results were expressed in µmol of equivalent in Trolox per liter (µmol TE/ L) using a Trolox equivalent standard curve from 5 to 200 µM.

6.2.8 Quantification of isoflavones

The analysis and quantification of okara isoflavones was carried out according to Chen et al. (2023). After the extractions, the extracts were diluted 5 times in methanol and filtered through 0.45 µm membrane filters and analyzed on a Hitachi HPLC system equipped with a DAD detector (Diode Array Detector). The compounds were separated on an Acquity Premier UPLC BEH C18 column (1.7 µm, 2.1 × 55 mm, Waters, USA). The samples were eluted at 0.9 mL/min with a column oven operating at 40 °C and a linear gradient consisting of acetonitrile (mobile phase A), methanol (mobile phase B) and 0.1% aqueous acetic acid solution (mobile phase C). The elution gradient started at 20% A and 80% C, with a linear increase to 30% A in 6.5 min, followed by another linear increase to 40% A in 5 min. The proportion of A increased to 60% in 1 min and then to 80% in 1 min. This was followed by a linear increase of up to 100% of the proportion of A in 2.5 min. This was maintained for 2 min, then a linear decrease to 70% of A in 1 min. Another linear decrease to 50% A in 1 min. The mobile phases were changed to 20% A and 80% B in 2 min to stop the analysis. The injection volume was 20 µL and UV absorption was monitored at a wavelength of 260 nm.

Four external standards were used to quantify the compounds. The daidzein, genistein, daidizin and genistein standards were solubilized in methanol+5% DMSO and curves were prepared at concentrations of 0.05-10.0 µg/mL for each isoflavone standard, and all showed an $R^2 > 99.0\%$. The results were expressed as µg of analyte/g of dry sample (DS) and µg of analyte/mL of extract.

6.3. Results and Discussion

6.3.1 Experimental solvent screening

There is a wide range of solvents that can be used to extract bioactive compounds from plant matrices. These solvents can be classified as conventional solvents, such as methanol and ethanol, and alternative solvents, such as ionic liquids, deep eutectic solvents and, lastly and most recently, *Bio-based Solvents*. In a previous study carried out by our research group, we optimized the extraction of isoflavones from soy and okara using Deep Eutectic Solvent formed by [Ch]Cl: acetic acid (1:2). It was also analyzed the effect of the DES precursors on isoflavone extraction, i.e. acetic acid+water and [Ch]Cl+water, and for okara the solvent composed of acetic acid+water showed the highest extraction efficiency. It was then found that [Ch]Cl seemed to prevent the extraction of isoflavones. Based on this, we decided to explore other compounds that could be used as components of DES (Table 6.2), recently named *Bio-based Solvents*. Figure 6.1 shows the results of the extraction of TPC (Fig. 6.1A) and TI (Fig. 6.1B) from okara using the 28 solvents.

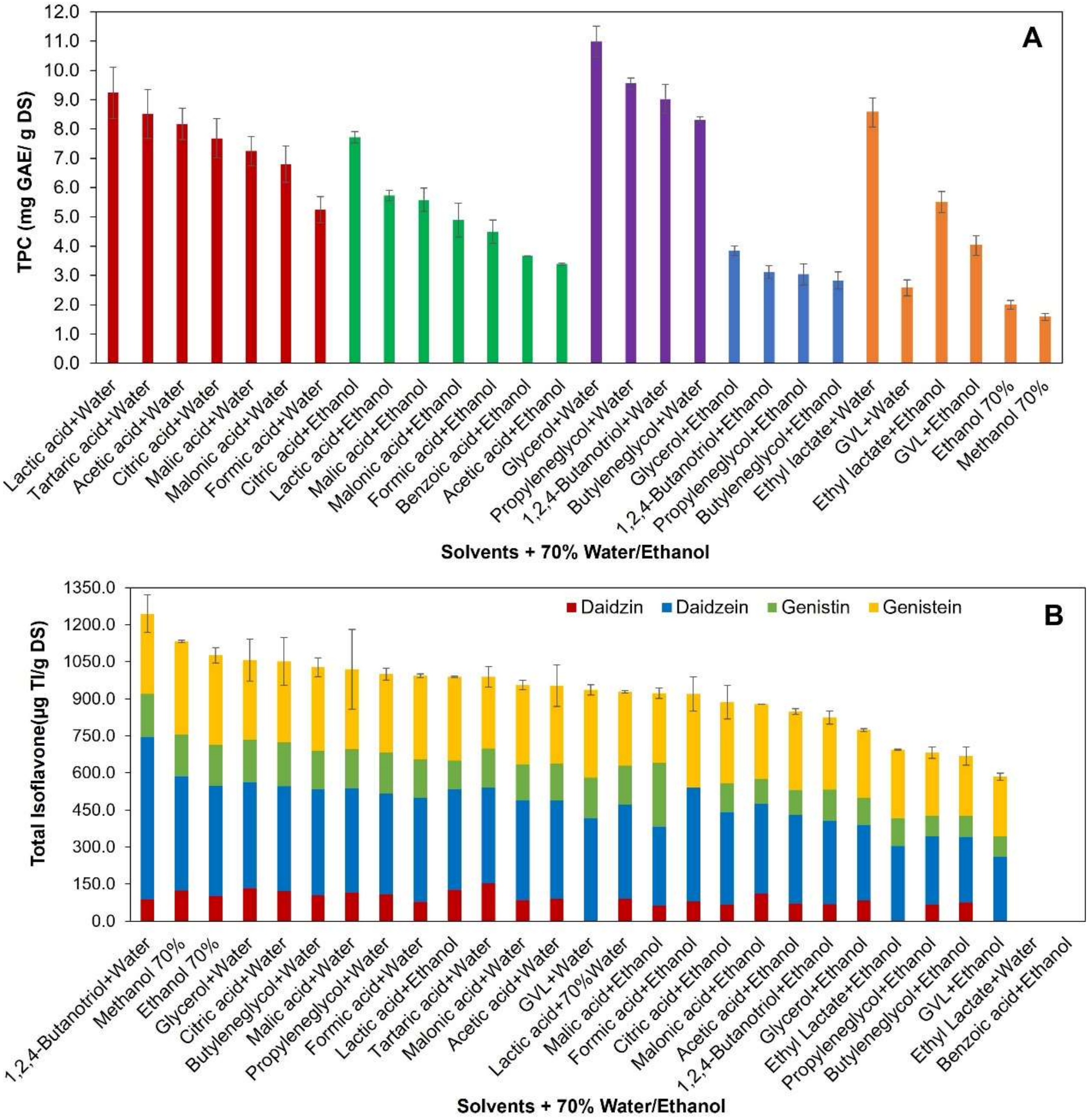

Fig. 6.1. Solvents used in the screening to extract **(A)** total phenolic compounds (TPC) and **(B)** total isoflavones (TI) from okara.

Analyzing Fig. 6.1A, it is possible to see a greater affinity of phenolic compounds for water-containing solvents, with emphasis on the polyols glycerol, propyleneglycol, 1,2,4-butanetriol and butyleneglycol, which had the highest TPC contents (11.0 mg GAE/g DS, 9.56 mg GAE/g DS, 9.03 mg GAE/g DS, and 8.31 mg GAE/g DS, respectively). The presence of ethanol in the polyols+ethanol solvents drastically decreased the affinity for the phenolic compounds, as shown in Fig. 6.1A. Ethyl lactate ester + water also proved to be a good option for extracting TPC, recovering approximately 8.57 mg GAE/g DS. Evaluating the effect of *Bio-based Solvents* consisting of carboxylic acids, it was also observed that the presence of water had a greater effect on the extraction of TPC. It is important to note that the conventional solvents methanol and ethanol showed the worst performance in recovering TPC from okara, extracting approximately 1.6 and 2.0 mg GAE/g DS, respectively. The results found here are similar to those reported by Ferreira et al. (2023) who extracted approximately 0.9 mg GAE/g DS using 80% methanol at an S/L ratio of 20 mg/mL, a temperature of 60 °C and constant stirring at 600 rpm for 60 min. In addition, the authors observed that when soybeans were used for TPC extraction, acetic acid+30% water extracted more bioactive compounds than the DES formed by [Ch]Cl: acetic acid (1:2) + 30% water.

Looking at Fig. 1B, it can be seen that 1,2,4-Butanetriol showed the highest extraction efficiency of total isoflavones (1244.5 ug TI/g DS), with emphasis on the isoflavone daidzein which showed the highest affinity for this solvent, corresponding to approximately 50% of the total isoflavones (657.34 ug Daizein/g DS). Here, unlike the TPC results, ethanol and methanol had a high capacity for extracting the isoflavones (extracting approximately 1131.80 ug of TI/g DS and 1076.4 ug of TI/g DS, respectively). It can therefore be assumed that these solvents had a greater affinity for isoflavones, since the analysis of total phenolic content is not very selective because it includes all molecules in the phenyl group, including molecules such as gallic acid, chlorogenic acid, p-hydroxybenzoic acid, caffeic acid, p-coumaric acid and ferulic acid, which may be present in okara samples. (Ferreira et al., 2023; Oliveira et al., 2022; Zhu et al., 2018).

Analyzing the *Bio-based Solvent* composed of glycerol+70% water, which was the best solvent for phenolic compound extraction, for isoflavones it was the fourth position among the best solvents, extracting approximately 1056. 8 ug of TI/g DS. The solvent citric acid+70% water also proved to be a good option for recovering isoflavones, extracting approximately 1051.0 ug of TI/g DS. These results are superior to those observed by Ferreira et al. (2023) who extracted approximately 424.42 ug TI/g DS using DES formed by [Ch]Cl: acetic acid (1:2) added to 30% water at an S/L ratio of 20 mg/mL, a temperature of 60 °C and constant stirring at 600 rpm for 60 min. The difference in results can be attributed to three factors (1) difference in the origin of the raw material; (2) solvent used and (3) extraction operating conditions.

As can be observed, the solvents Glycerol+water and 1,2,4-Butanetriol+water showed the best results for extracting TPC and TI, respectively. The choice of the best solvent was based on the richness of the bioactive compounds in the extract. It was therefore decided to continue with the solvent composed of glycerol+water for the optimization stage, as the extract obtained with this solvent had isoflavone contents close to that obtained with 1,2,4-Butanetriol+water and higher TPC contents, proving to be richer in other bioactive compounds. To confirm these results, antioxidant activity analyses were carried out with the 5 best solvents observed from the TI results (Fig 6.1B)

6.3.2 Antioxidant activity

The antioxidant activity of okara extracts obtained with the solvents Glycerol+water, 1,2,4-Butanetriol+water, citric acid+water, methanol 70% and ethanol 70% was monitored using the DPPH and ABTS tests, as shown in Fig. 6.2.

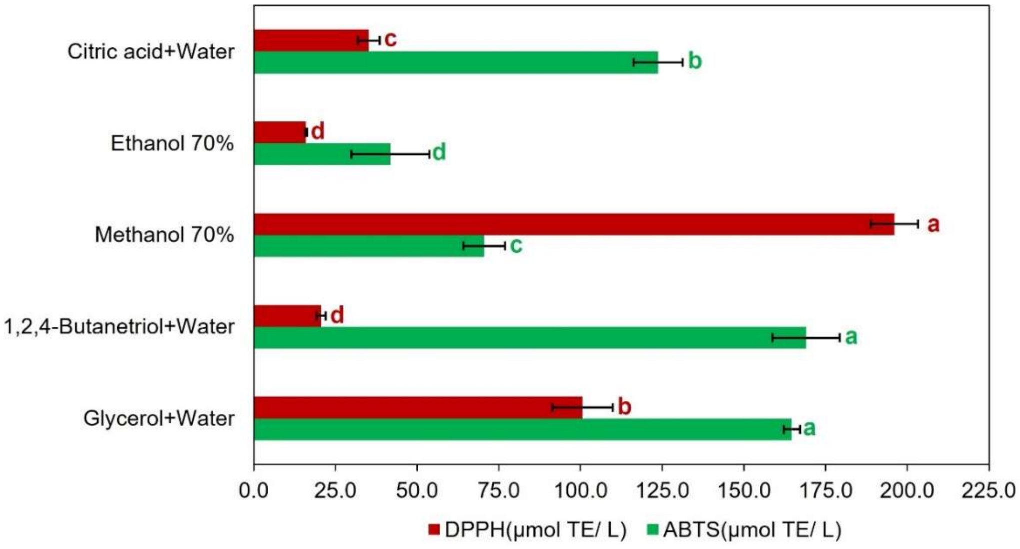

Fig. 6.2. Antioxidant activity of okara extracts measured by DPPH (in red) and ABTS (in green).

Evaluating the results of antioxidant activity by DPPH, methanol 70% showed the highest antioxidant activity (196.0 umol/L) followed by glycerol+water (100.56 umol/L). Looking at the ABTS data, 1,2,4-Butanoltriol+Water showed the highest antioxidant activity (169.0 umol TE/L) followed by glycerol+water (164.67 umol TE/L). It is important to note that both solvents showed no significant difference in the Tukey test at 5% significance for the ABTS test. These results suggest that glycerol+water is a solvent rich in bioactive compounds, with a high content of phenolic compounds, isoflavones and a high antioxidant capacity when compared to the other solvents.

6.3.3 Determination of extraction time

The effect of extraction time was investigated for the recovery of TPC from okara using the Bio-based solvent Glycerol+water. The extractions were conducted at 40 °C, 500 rpm magnetic stirring, S/L ratio of 10 mg/mL and 70% water added to the solvent. Fig. 6.3 shows the phenolic compound extraction data. The definition of the time was based on the time when the slope of the curve became minimal.

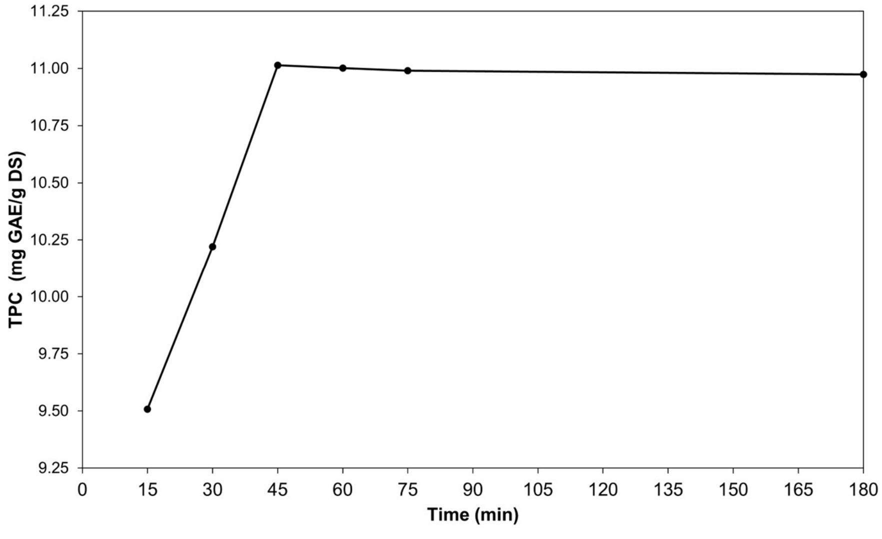

Fig. 6.3. Evaluation of the effect of extraction time of TPC from okara using glycerol+70% water.

According to Fig. 6.3, it was observed that after 45 min, the extraction of TPC stabilizes, and this time was fixed for the next steps. This time already represents an improvement in the extraction of bioactive compounds from okara compared to the screening experiments and studies carried out previously by our research group. The TPC content found after 45 min of extraction was 11.01 mg GAE/g DS, practically the same result observed after 60 min (11.0 mg GAE/g DS).

6.3.4 Optimizing the extraction conditions.

Considering the results mentioned above, the composition of the glycerol+water binary mixture and the experimental conditions were optimized for the extraction of total phenolic compounds and total isoflavones from okara using Central Composite Rotatable Design (CCRD).

From the point of view of sustainability and application, glycerol shows promise in recovering bioactive compounds, since glycerol is generally recognized as safe by the FDA. In addition, the Scientific Committee on Food approved the conclusion of the 1976 Joint FAO/WHO Expert Committee on Food Additives of "unspecified acceptable daily intake (ADI) for man". The committee concluded that glycerol has low acute toxicity and that the local irritant effects of glycerol in the gastrointestinal tract reported in some gavage studies were probably due to the hygroscopic and osmotic effects of glycerol. Glycerol did not raise concerns with regard to genotoxicity and was not a cause for concern with regard to carcinogenicity, since from the available chronic toxicity and carcinogenicity studies, glycerol was not carcinogenic in mice and rats and showed no evidence of adverse effects in a 2-year chronic toxicity study. The committee noted that no adverse effects were reported in rats given doses of up to 10,000 mg/kg body weight (bw) per day for 1 year, the highest dose tested. The Panel also noted that there was no increase in the incidence of tumors in rats given doses of up to 5,000 mg/kg bw per day for 2 years, the highest dose tested. Thus, the committee concluded that there is no need for a numerical ADI and no safety concerns regarding the use of glycerol as a food additive (Mortensen et al., 2017).

Based on this, glycerol extracts can be applied in food formulations without the need for purification, which can bring health benefits, added value to different products and cost savings since it is possible to reduce a step in the process. To date, no research using glycerol to extract isoflavones has been reported in the literature, making it a novelty for bioactive compound extraction technology using green solvents.

Here, we applied the Response Surface Methodology (RSM) to evaluate the relationship between the independent variables (temperature, S/L ratio and % water in glycerol) and the responses TPC, TI, Y_1 and Y_2, optimizing the operating conditions for each response.

The results obtained through RSM and the effects of the independent variables on each response are shown in Fig. 6.4A, Fig. 6.4B, Fig. 6.5A and Fig. 6.5B. The experimental conditions for the 19 CCRD extractions, and their respective experimental results for TPC, TI, Y_1 and Y_2 are presented in Supplementary Material in tables S1 and S2.

The results for TPC, TI, Y_1 and Y_2 are presented in Tables S1 and S2 (Supplementary Material).

From the coded matrix and experimental results, the coefficients of the second-order polynomial models were obtained using Statistica 14.0 software (Statsoft Inc., United States).

Analysis of variance (ANOVA) was used to evaluate the statistical significance of the models for TPC, TI, Y_1 and Y_2 and the adjustment of the models to the experimental data. Tables S3, S4, S5 and S6 show the ANOVA's for the TPC, Y1, TI and Y2 models, respectively (Supplementary Material). The first stage of the ANOVA analysis consisted of assessing the statistical significance of the models at a 10% significance level (p-value ≤ 0.1). All the models indicated that the Fcal of the regression was higher than the Ftab, showing that all the regressions were significant (Tables S3, S4, S5 and S6 in Supplementary Material). With regard to the lack of fit, all the Fcal were lower than the Ftab, indicating a non-significant lack of fit, which statistically confirms the fit of the models to the experimental data.

Table S7 (Supplementary Material) shows the reparametrized models, i.e. only the significant terms with a p-value ≤ 0.1 were considered. The relative deviations between the observed and predicted values were low (Tables S1 and S2 in Supplementary Material), indicating a good fit of the model to the experimental data. This fit can also be confirmed by looking at Figs. S1A-S1D in Supplementary Material. In addition, the percentage of explained variation (R^2) was considered high for all the models, with 97.25% for TPC, 96.08% for Y1, 98.35% for TI and 87.75% for Y2, as shown in Table S7.

Thus, it can be concluded that all the models are significant and can predict and explain the experimental data; therefore, the response superficies methodology can be applied to optimize the variables for the maximum extraction of bioactive compounds from okara.

The response surfaces were generated using Statistica 14.0 software (Statsoft Inc., United States) and are shown in Fig. 6.4 and Fig. 6.5.

Response surface analysis for TPC

Analyzing the results for TPC in Table S1 (Supplementary Material), the results ranged from 12.92 (test 11) to 38.05 mg GAE/100 mL of extract (test 12). The only difference between the two tests is in the S/L ratio, which is higher in test 12 (60 mg/mL) compared to 10 mg/mL in test 11. The solid S/L ratio was the variable that had the greatest effect on TPC extraction, as shown in the Pareto Diagram in Fig. S2A (Supplementary Material). Fig. 6.4A shows that the higher the S/L ratio, higher is the TPC. Fig. 6.4 also shows a strong influence on TPC extraction when we increase the S/L ratio and decrease the % water. Less intensely, an increase in TPC is also observed at lower %water and higher temperatures. Based on the analysis of the response surfaces and contour curves, the optimum conditions determined for maximum TPC extraction were at levels 1.34 for temperature, +1.68 for S/L ratio and -1.68 for %water, which correspond to 66 °C, 60 mg/mL and 40% water in glycerol, respectively. At this point, the predicted value by the model was 41.98 mg GAE/100 mL of extract. An experiment was then carried out to validate the model for TPC in this condition and the experimentally observed value was 43.2 mg GAE/100 mL of extract, the relative deviation was 2.82%, considered low, validating the model experimentally.

Response surface analysis for Y1

Evaluating the results based on extraction yield of TPC (Y1), as shown in Table S1 (Supplementary Material), the results ranged from 5.72 mg GAE/g DS (test 8) to 12.93 mg GAE/g DS (test 11). Again, according to the pareto diagram in Fig. S2B (Supplementary Material), the S/L ratio had the greatest effect on extraction yield of TPC. However, unlike the previous result, the S/L ratio had an inverse effect on the yield, i.e. the lower the S/L ratio, the higher the extraction yield. This can be seen from the analysis of the response surfaces and contour curves in Fig. 6.4B, which indicate an increase in yield with increasing S/L ratio. As the proportion of solvent is higher at lower S/L ratios, the solvent is able to deplete the solute from the solid, providing greater extraction efficiency. According to Fig. 6.4B, the interaction between increasing temperature and decreasing % water also caused an increase in extraction yield. Therefore, based on Fig. 6.4B, the optimum condition determined here was at level +1 for temperature, -1.68 for the S/L ratio and -1.68 for %water; these values correspond to a temperature of 61.9 °C, an S/L ratio of 10 mg/mL and 40% water in the glycerol. At this point, the value predicted by the model was 12.7 mg GAE/g DS and that observed experimentally was

14.64 mg GAE/g DS. The relative deviation was 13.25 %, in this case considered high. However, as the observed value was higher than that predicted by the model and higher than all the values found in Table S1, we conclude that the objective was achieved and therefore the model can be considered validated under the condition of maximum extraction yield.

Fadil et al. (2024) used a ternary mixture of glycerol:ethanol:water to optimize TPC, DPPH, FRAP and total antioxidant capacity (TAC) results of extracts obtained from Moroccan *Cannabis sativa.* Contrary to what was observed in our work, Fadil et al. (2024) observed that the optimum condition for TPC was using the ratio 45% water: 0% glycerol: 55% ethanol using an S/L ratio of 30 mg/mL, 42 °C and 24 min of extraction, obtaining a value of 90 mg GAE/g of sample. The authors' justification for this was based on the lower density of ethanol (0.789 g cm^{-3}), allowing it to easily permeate solid particles, leading to a rapid dissolution of the compounds and, consequently, faster transfer of the solute-solvent complexes to the extract. On the other hand, the high density of glycerol (1.261 g cm^{-3}) could potentially hinder the infiltration of the water/glycerol mixture into solid particles, thus reducing extractability. However, this was not observed in our work, since ethanol extracted significantly less TPC than the glycerol+water mixture, as mentioned in section 6.3.1. This difference in solvent behavior can only be attributed to the difference in the raw material and the phenolic compounds present in it. However, the success of glycerol as the extracting solvent in this work can be attributed to the hydrogen bonds that can exist between the solvent and the phenolic compounds, influencing the solubility of them in glycerol+water mixtures (Fadil et al., 2024; Galanakis et al., 2013).

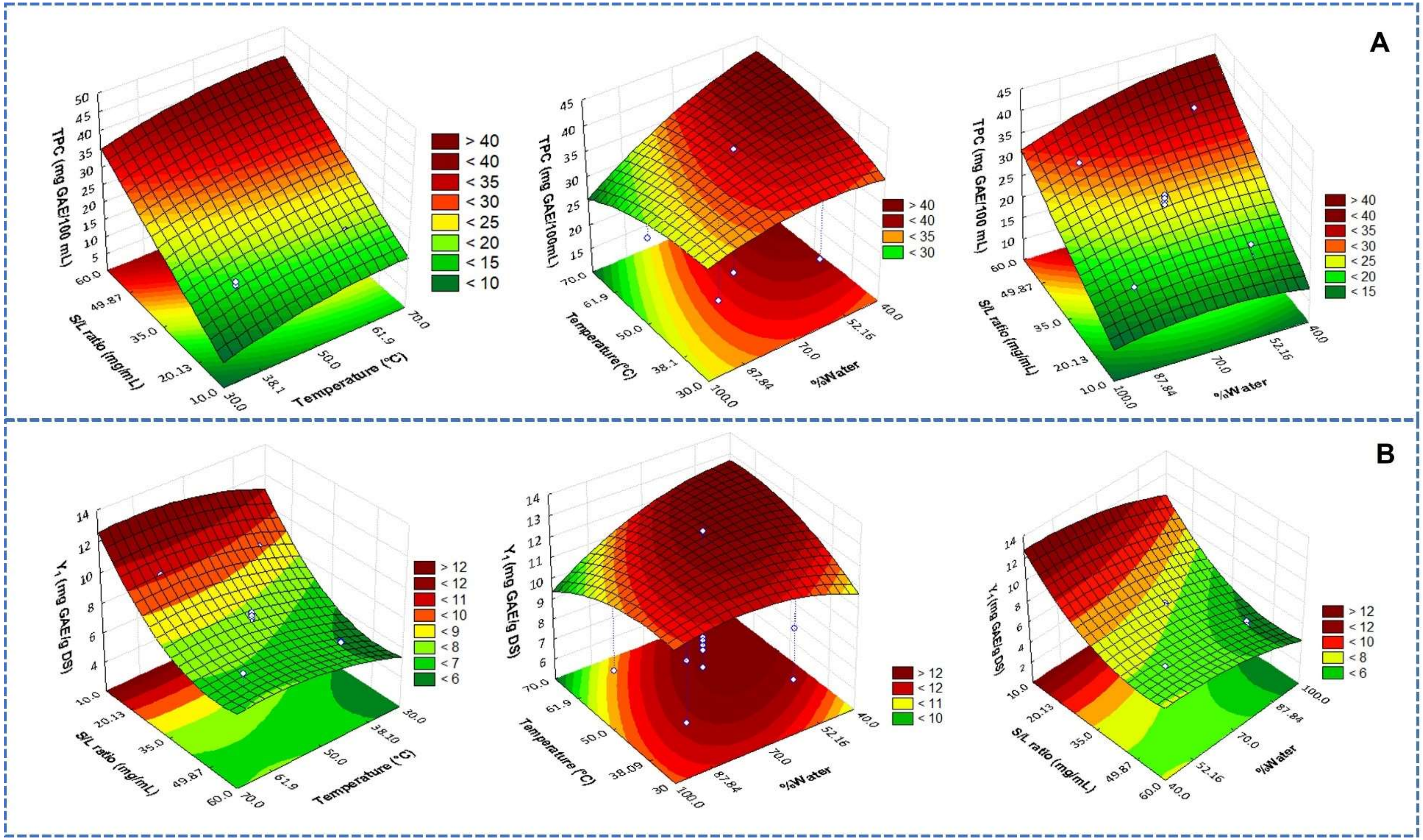

Figure 6.4. Response surface and contour curves obtained to evaluate the effect of temperature, S/L ratio and % water on (A) the concentration of TPC in the extracts and (B) the extraction yield of TPC.

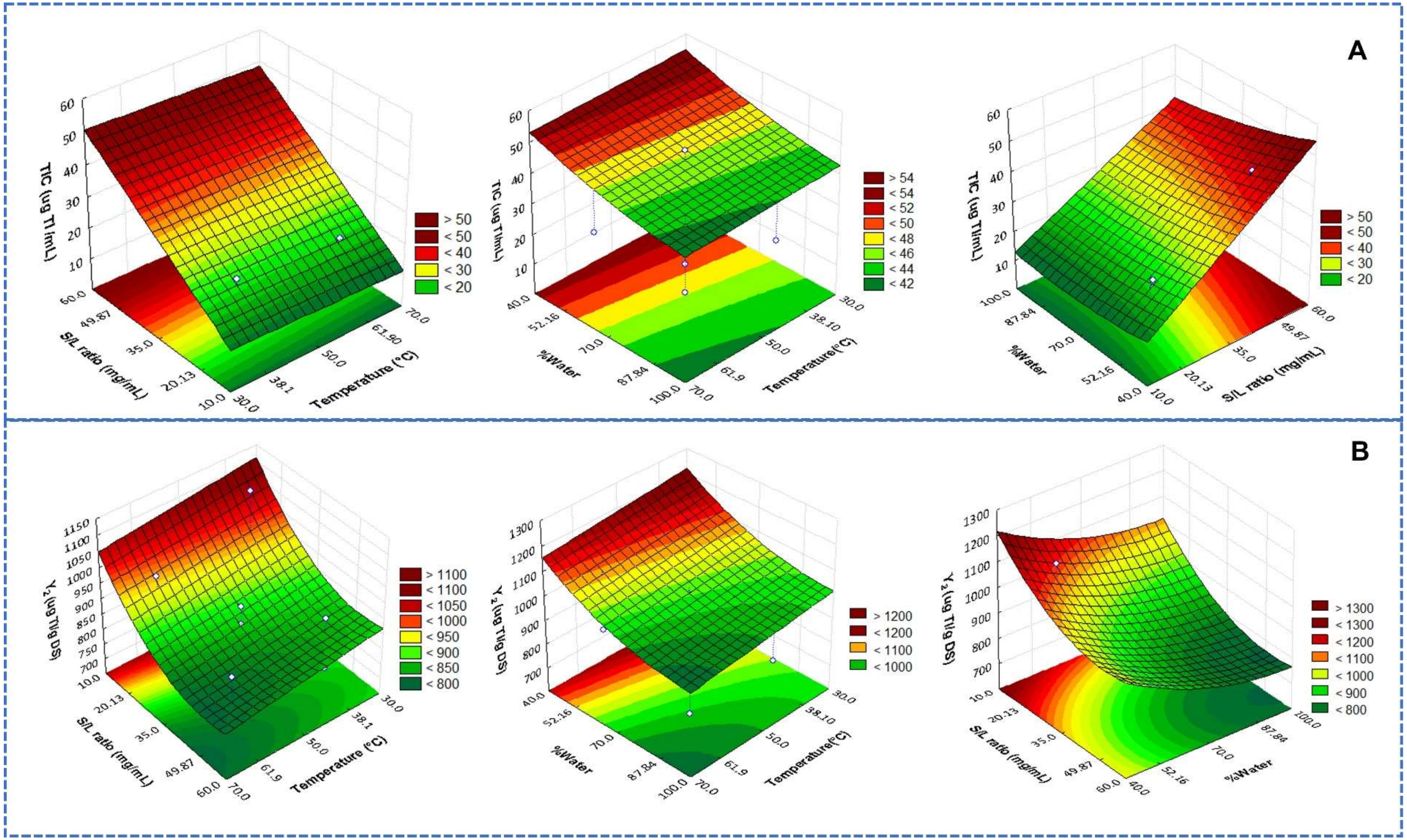

Figure 6.5. Response surface and contour curves obtained to evaluate the effect of temperature, S/L ratio and % water on (A) the concentration of TI in the extracts and (B) the extraction yield of TI.

Response surface analysis for TI

To analyze the isoflavone results, the sum of the four isoflavones analyzed (TI = daidzein + genistein + daidzin + genistin) was taken as the response. The results are shown in Table S2 (Supplementary Material). It was observed that the results ranged from 10.05 (test 11) to 47.54 µg TI/mL of extract (test 12), similar to what was previously observed for TPC. Again, the S/L ratio followed by % water had the greatest effect on TI extraction, as shown in the Pareto Diagram in Fig. S2C (Supplementary Material). The effect of the S/L ratio was positive and the effect of % water on the response was negative, as can be seen also from the analysis of the response surfaces and contour curves. Analyzing the response surfaces for the S/L ratio vs. temperature and %water vs. temperature (Fig. 6.5A), it is possible to see a slight increase in the TI content as the temperature decreases. Based on this, the point of maximum TI recovery was determined for the levels -1.68 for temperature, +1.68 for S/L ratio and -1.68 for % water, which correspond to a temperature of 30 °C, an S/L ratio of 60 mg/mL and 40% water in glycerol.

The results predicted by the model and that observed experimentally under these conditions were 55.05 µg TI/mL of extract and 59.18 µg TI/mL of extract, respectively, with a relative deviation of 6.98%, validating the model experimentally.

Response surface analysis for Y_2

According to Table S2 (Supplementary Material), the results of the TI extraction yield ranged from 712.57 (test 8) to 1109.05 µg TI/g DS (test 1). No longer a novelty, the S/L ratio followed by % water were again the most influential variables in the extraction yield of TI (Table (Fig. S2 in Supplementary Material). Decreasing the S/L ratio caused an increase in the extraction yield (Fig 4B) for the same reasons mentioned for the response Y1. Decreasing the S/L ratio and temperature caused an increase in extraction yield. The same behavior was observed for % water vs. temperature; decreasing both variables caused an increase in extraction yield. Based on the analysis of the response surfaces, the levels chosen for the optimum condition were -1.68 for temperature, -1.68 for S/L ratio and -1.68 for % water, which correspond to a temperature of 30 °C, an S/L ratio of 10 mg/mL and a water percentage of 40%. Under these conditions, the value predicted by the model was 1217.95 µg TI/g DS and the observed value was 1112.5 µg TI/g DS, with a relative deviation of 9.48 %, validating the model experimentally in the condition of maximum extraction yield of total isoflavones from okara.

6.3.5 Discussion

It is true that, according to the previous observations, the S/L ratio and % water are variables that strongly influence the extraction of phenolic compounds and isoflavones from okara. Although temperature is a variable that also had a significant effect, in the range studied it was less potent in the extractions. Thinking about obtaining extracts with the maximum concentration of bioactive compounds, two aspects should be taken into consideration with regard to the S/L ratio: (1) exhausting the sample in order to extract the maximum number of compounds or (2) obtain extracts that are more concentrated in bioactive compounds.

The first point leads to high extraction yields, due to the small S/L ratio used, which results in more diluted extracts, which makes their direct applications in food formulations difficult. To solve this problem, consecutive extractions can be carried out using the same solvent and simply changing the solid for a new solid, as done by Ferreira et al. (2021) maintaining the same aqueous ionic liquid solution to extract caffeine from freshly spent coffee under optimized operating conditions. After six extraction cycles, the total yield of caffeine in the solution increased from 3.29 to 13.10% by weight, corresponding to an increase in caffeine concentration from 1.74 to 6.94 g/L.

The second point involves the use of a higher S/L ratio, which provides extracts richer in phenolic compounds and isoflavones, however low extraction yields are observed, and this can generate waste of bioactive compounds if the raw material is discarded. One solution might be to apply extractions using a simulated moving bed or Shanks System, which allows the solute to be exhausted from the sample while concentrating the extract using a system of 5 or 6 columns.

Table S8 (Supplementary Material) summarizes all the optimum conditions obtained from the experimental design, followed by the values observed and predicted by the model and the relative deviations. Table S8 also contains information on the characterization of the extracts obtained under the optimum conditions. As the main objective of this work is to obtain an extract with the maximum concentration of bioactive compounds for possible application in food formulations, we decided to evaluate the TPC and TI results and carry out extractions with conventional solvents under the same conditions for comparison. The only difference between the TPC and TI optimum points is in temperature, as shown in Table S8. Under these conditions the extraction yields of TPC (Y1) were low, which shows that more than 50% of the phenolic compounds were still retained in the sample. This shows that more studies need to be carried out to exhaust the solid. Regarding the extraction yield of TI (Y2), the observed values were 966.41 and 980.88 μg TI/g DS for the temperatures of 66 °C and 30 °C, respectively, being, respectively, only 13.13% and 11.83% lower than the yield observed in the optimized condition for Y2 (1112.5 μg TI/g DS) (Table S8 in Supplementary Material).

Fig. 6.6 shows the results of extractions carried out with glycerol added to 40% water (glycerol 60%) and ethanol and methanol added to 40% water. The experiments were carried out using an S/L ratio of 60 mg/mL and the TPC and TI content was evaluated at temperatures of 30 °C and 66 °C.

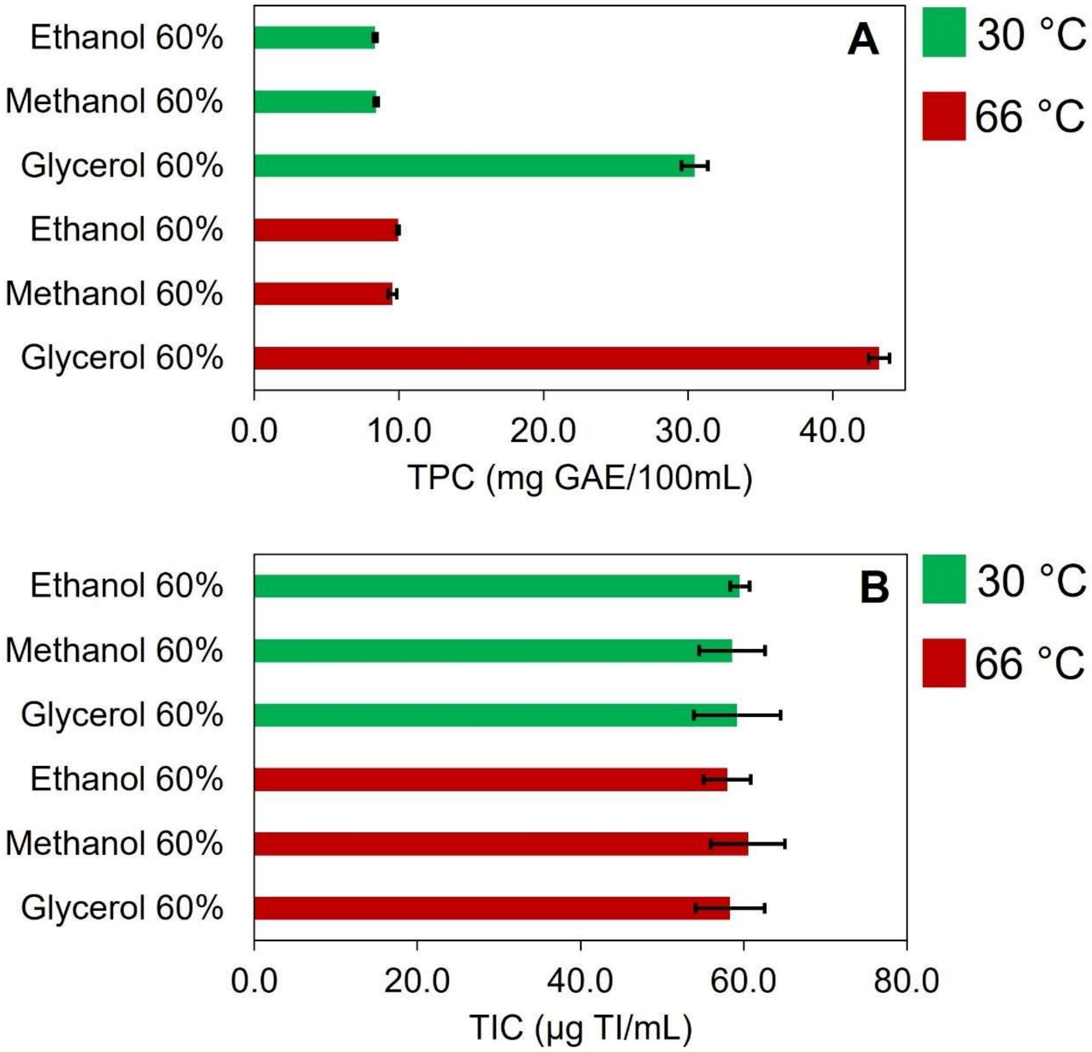

Fig. 6.6. Comparative results between glycerol 60% and conventional solvents (ethanol and methanol) obtained under optimal conditions for (A) TPC and (B) TI

Analyzing Fig. 6.6A, the TPC contents for ethanol 60% and methanol 60% were lower compared to the extracts obtained with glycerol 60%. Thus, glycerol 60% is an excellent solvent for recovering phrnolic compounds from okara. The TPC content of glycerol 60% extract obtained at 66 °C was approximately 42% higher than the extract obtained at 30 °C.

Analyzing the isoflavone content in Fig. 6.6B, all the solvents extracted similar amounts of TI. It can therefore be concluded that methanol and ethanol are more selective for isoflavones, while glycerol is able to extract other phenolic compounds in addition to isoflavones, providing a richer extract. Furthermore, temperature had little effect on the solubility of isoflavones in the solvent. If isoflavones are the focus, 30 °C is sufficient for high levels of these bioactive compounds in the extract.

Thus, the best condition found was an S/L ratio of 60 mg/mL, 66 °C, 40% water added to glycerol, 500 rpm agitation for 45 min. This condition provided an extract with 43.2 mg GAE/100mL of extract, 58.31 µg TI/mL of extract and extraction yields of 7.16 mg GAE/g DS and 966.41 µg TI/g DS.

Aqueous solutions of glycerol and alkanediols (1,2-ethanediol, 1,2-propanediol, 1,3-propanediol, 1,3-butanediol, 1,2-pentanediol, 1,5-pentanediol and 1,2-hexanediol) added to 20°% water were used to extract phenolic compounds from *Juglans regia* L. leaves, detaching the propanediols (isomers 1,2 and 1,3) with high extraction yields and high bioactivity (Vieira et al., 2020).

Analyzing all the results, it can be seen that pure water did not show good results for the extraction of phenolic compounds and isoflavones. Previous studies have shown that glycerol works differently in various plant raw materials. The results ranged from 90% glycerol for eggplant peels (Philippi et al., 2016) and two species of Artemisia (Shehata et al., 2015), 80% glycerol for walnut leaves (Vieira et al., 2020), 19, 47% and 15.9% glycerol for rice bran (Aalim et al., 2019; Huang et al., 2019), 10% glycerol for *Hypericum perforatum* (St. John's Wort) (Karakashov et al., 2015), 9.3% glycerol for olive leaves (Apostolakis et al., 2014) and 3.6% glycerol for filtered coffee residue (Michail et al., 2016).

6.4. Conclusions

This work explored a set of solvents integrating an experimental screening approach using 26 *Bio-based Solvents* and optimization of operating conditions, evaluating four responses: total phenolic compound in the extract (TPC), total isoflavones in the extract (TI), extraction yield of TPC (Y1) and extraction yield of TI (Y2). Glycerol, propyleneglycol, 1,2,4-butanetriol, butyleneglycol, ethyl lactate and lactic acid added to 70% water proved to be good options for extracting phenolic compounds from okara. 1,2,4-butanetriol, glycerol, citric acid, butyleneglycol and malic acid added to 70% water are good options for extracting isoflavones from okara. Ethanol and methanol are more selective for extracting isoflavones than *Bio-based Solvents*.

Glycerol+70% water showed excellent ability to extract phenolic compounds and isoflavones from okara, providing an extract rich in bioactive compounds. Glycerol was selected as the best bio-based solvent for extracting bioactive compounds. All the final extraction conditions were statistically optimized for the 4 responses. The best condition found was using an S/L ratio of 60 mg/mL, 66°C, 40% water added to glycerol, 500 rpm agitation for 45 min.

As a result, glycerol 60% proved to be an alternative solvent for extracting phenolic compounds and isoflavones from okara and could serve as a potential substitute for conventional solvents such as methanol and ethanol. In addition, by using a solvent generally recognized as safe by the FDA, these extracts can be used in food formulations without the need of purification, reducing process time and costs.

With the increase in biodiesel production, excess glycerol has been burned in furnaces and boilers to generate heat, making it an underused by-product for large industries. This work therefore provides an alternative way of using the glycerol produced. With this in mind, this study could serve as an additional incentive to expand studies using glycerol and soybean residues in extraction procedures, or even to apply glycerol to extract bioactive compounds from other plant matrices.

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Author contributions

Ramon S. B. Ferreira – Conceptualization, validation, visualization, formal analysis, investigation, resources, writing- original draft, writing – review and editing. **João A. P. Coutinho** – Supervision, Project administration, and funding acquisition, writing - review and editing. **Eduardo A. C. Batista** - Conceptualization, writing - review and editing, visualization, supervision, project administration, and funding acquisition.

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Supplementary Material

Exploring *Bio-based Solvents* for the Recovery of Phenolic Compounds and Isoflavones from Okara: Solvent Screening and Optimization

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TABLES

Table S1. Experimental and model-predicted extraction values obtained for TPC and Y_1 and their respective relative and absolute deviations.

				TPC (mg GAE/100 mL)				Y_1 (mg GAE/g DS)			
Test	Temperature (°C)	S/L ratio (m/mL)	%Water	Observed	Predicted	Absolute deviation	Relative deviation (%)	Observed	Predicted	Absolute deviation	Relative deviation (%)
1	-1	-1	-1	17.525	17.42	0.11	0.61	8.71	9.50	0.80	9.16
2	-1	-1	1	18.471	18.60	0.13	0.70	9.18	9.44	0.26	2.86
3	-1	1	-1	30.819	32.72	1.90	6.16	6.18	6.14	0.04	0.60
4	-1	1	1	30.770	30.68	0.09	0.30	6.17	6.08	0.09	1.50
5	1	-1	-1	20.291	19.93	0.36	1.76	10.08	10.22	0.14	1.34
6	1	-1	1	18.433	16.09	2.35	12.73	9.16	8.73	0.43	4.70
7	1	1	-1	35.816	35.23	0.59	1.64	7.18	6.85	0.33	4.55
8	1	1	1	28.514	28.16	0.35	1.23	5.72	5.37	0.35	6.16
9	-1.68	0	0	24.463	24.38	0.08	0.32	6.99	6.88	0.11	1.52
10	1.68	0	0	23.663	24.38	0.72	3.05	6.76	6.88	0.12	1.81
11	0	-1.68	0	12.916	14.78	1.87	14.46	12.93	12.47	0.46	3.56
12	0	1.68	0	38.048	37.80	0.24	0.64	6.34	6.82	0.48	7.50

13	0	0	-1.68	27.326	26.60	0.72	2.64	7.81	7.46	0.34	4.40
14	0	0	1.68	20.291	21.65	1.36	6.72	5.80	6.16	0.36	6.20
15	0	0	0	27.083	26.29	0.79	2.92	7.74	7.57	0.16	2.12
16	0	0	0	25.967	26.29	0.33	1.26	7.42	7.57	0.15	2.09
17	0	0	0	27.496	26.29	1.20	4.37	7.86	7.57	0.28	3.59
18	0	0	0	26.792	26.29	0.50	1.86	7.65	7.57	0.08	1.05
19	0	0	0	25.220	26.29	1.07	4.26	7.21	7.57	0.37	5.11

TPC (mg GAE/100 mL) = Total phenolic compounds (mg of GAE/ 100 mL of extract);
Y_1 (mg GAE/g) = Extraction yield of total phenolic compounds (mg of GAE/g DS).

Table S2. Experimental and model-predicted extraction values obtained for TI and Y1 and their respective relative and absolute deviations.

				TI (ug TI/mL of extract)				Y_2 (μg TI/g DS)			
Test	Temperature(°C)	S/L ratio (m/mL)	%Water	Observed	Predicted	Absolute deviation	Relative deviation (%)	Observed	Predicted	Absolute deviation	Relative deviation (%)
1	-1	-1	-1	23.29	20.86	2.43	10.43	1109.05	1020.90	88.15	7.95
2	-1	-1	1	18.15	18.45	0.29	1.62	864.32	898.05	33.73	3.90
3	-1	1	-1	44.03	43.36	0.67	1.53	886.46	875.23	11.24	1.27
4	-1	1	1	35.86	37.27	1.41	3.94	722.02	752.37	30.35	4.20
5	1	-1	-1	20.65	19.66	0.99	4.79	983.37	982.52	0.85	0.09
6	1	-1	1	17.75	17.25	0.51	2.85	858.98	859.67	0.69	0.08

7	1	1	-1	42.60	42.16	0.44	1.04	857.68	836.85	20.83	2.43
8	1	1	1	35.39	36.07	0.68	1.93	712.57	713.99	1.42	0.20
9	-1.68	0	0	30.02	29.76	0.26	0.86	857.75	848.85	8.90	1.04
10	1.68	0	0	28.09	27.75	0.34	1.22	802.56	784.31	18.26	2.27
11	0	-1.68	0	10.05	11.38	1.33	13.26	1005.00	1030.83	25.84	2.57
12	0	1.68	0	47.54	46.13	1.41	2.96	792.28	785.84	6.44	0.81
13	0	0	-1.68	31.73	34.11	2.37	7.48	906.63	972.01	65.37	7.21
14	0	0	1.68	28.40	26.96	1.44	5.06	811.36	765.39	45.97	5.67
15	0	0	0	28.13	28.76	0.63	2.22	803.71	816.58	12.86	1.60
16	0	0	0	29.64	28.76	0.88	2.97	846.75	816.58	30.17	3.56
17	0	0	0	28.14	28.76	0.61	2.18	804.05	816.58	12.53	1.56
18	0	0	0	28.07	28.76	0.68	2.42	802.14	816.58	14.44	1.80
19	0	0	0	27.41	28.76	1.35	4.93	783.01	816.58	33.57	4.29

TI (ug TI/mL) = Total isoflavones (mg of TI/mL of extract);
Y_2 (ug TI/g) = Extraction yield of total isoflavones (mg of TI/g DS).

Table S3. Analysis of variance for the TPC model.

Variation source	SS	DF	MS	Fcalc.	Ftab.
Regression	699.95	6	116.66	70.82	3.00
Residual	19.77	12	1.65		
Lack of fit	16.43	8	2.05	2.46	6.04
Pure error	3.34	4	0.83		
Total	719.72	18			
R^2 (%)	97.25%				

R^2 = % explained variation; SS = Sum of Squares; DF = Degrees of Freedom; MS = Mean Square; $F_{calc.}$ = calculated F-value; $F_{tab.}$ = tabulated F-value.

Table S4. Analysis of variance for the Y_1 model.

Variation source	SS	DF	MS	Fcalc.	Ftab.
Regression	52.22	6	8.70	48.99	3.00
Residual	2.13	12	0.18		
Lack of fit	1.86	8	0.23	3.41	6.04
Pure error	0.27	4	0.07		
Total	54.35	18			
R^2 (%)	96.08%				

R^2 = % explained variation; SS = Sum of Squares; DF = Degree of Freedom; MS = Mean Square; $F_{calc.}$ = calculated F-value; $F_{tab.}$ = tabulated F-value.

Table S5. Analysis of variance for the TI model.

Variation source	**SS**	**DF**	**MS**	**Fcalc.**	**Ftab.**
Regression	1536.27	5	307.25	154.82	3.03
Residual	25.80	13	1.98		
Lack of fit	23.11	9	2.57	3.82	6.00
Pure error	2.69	4	0.67		
Total	1562.07	18			
R^2 (%)	98.35%				

R^2 = % explained variation; SS = Sum of Squares; DF = Degree of Freedom; MS = Mean Square; $F_{calc.}$ = calculated F-value; $F_{tab.}$ = tabulated F-value.

Table S6. Analysis of variance for the Y_2 model.

Variation source	**SS**	**DF**	**MS**	**Fcalc.**	**Ftab.**
Regression	146585.26	5	29317.05	18.62	3.03
Residual	20467.95	13	1574.46		
Lack of fit	18274.18	9	2030.46	3.70	6.00
Pure error	2193.77	4	548.44		
Total	167053.20	18			
R^2 (%)	87.75%				

R^2 = % explained variation; SS = Sum of Squares; DF = Degree of Freedom; MS = Mean Square; $F_{calc.}$ = calculated F-value; $F_{tab.}$ = tabulated F-value.

Table S7. Reparametrized models obtained for optimizing the concentration of total phenolic compounds and total isoflavones (models 1 and 3, respectively) and optimizing the extraction yields (models 2 and 4, respectively).

Model 1	TPC (mg GAE/100mL) = 26.29 + 6.84*R - 1.47*W - 0.68*T^2 - 0.77*W^2 - 1.26*T*W - 0.8*R*W	**R^2=97.25%**
Model 2	Y_1 (mg GAE/g) = 7,57 – 1,68*R -0,39*W – 0,24*T^2 + 0,73*R^2 - 0,27*W^2 – 0,36*T*W	**R^2=96.08%**
Model 3	TI (µg TI/mL) = 28.76 - 0.6*T + 10.33*R – 2.12*W + 0.63*W^2 - 0.92*R*W	**R^2=98.35%**
Model 4	Y_2 (µg TI/g) = 816.58 – 19.19*T – 72.84*R – 61.43*W + 32.43*R^2 + 18.43*W^2	**R^2=87.75%**

TPC (mg GAE/100 mL) = Total phenolic compounds (mg of GAE/ 100 mL of extract);
Y_1 (mg GAE/g) = Extraction yield of total phenolic compounds (mg of GAE/g DS);
TI (µg TI/mL) = Total isoflavones (µg of TI/mL of extract);
Y_2 (µg TI/g) = Extraction yield of total isoflavones (µg of TI/g DS)
T (Temperature), R (Solid-liquid ratio) and W (Water%) are linear terms of the model, T^2, R^2, W^2 are the quadratic terms of the model and T*W and R*W are the terms of interactions

Table S8. Optimal conditions for each response, observed and predicted values, relative deviations and characterization of extracts obtained under optimal conditions.

	Optimal condition			**Values**			***Other results**			
Response	**Temperature (°C)**	**S/L ratio**	**Water%**	**Observed**	**Predicted**	**Relative deviation (%)**	**TPC**	**Y1**	**TI**	**Y2**
TPC (mg GAE/100 mL)	66.0	60	40	43.2	41.98	2.82	-	7.16	58.31	966.41
Y1 (mg GAE/g DS)	61.9	10	40	14.64	12.7	13.25	14.88	-	11.64	1164.26
TI (µg TI/mL)	30	60	40	59.18	55.05	6.98	30.46	4.94	-	980.88
Y2 (µg TI/g DS)	30	10	40	1112.5	1217.95	9.48	11.02	11.02	11.12	-

*Other results – relating to other analyses for the characterization of extracts obtained under optimal conditions.

FIGURES

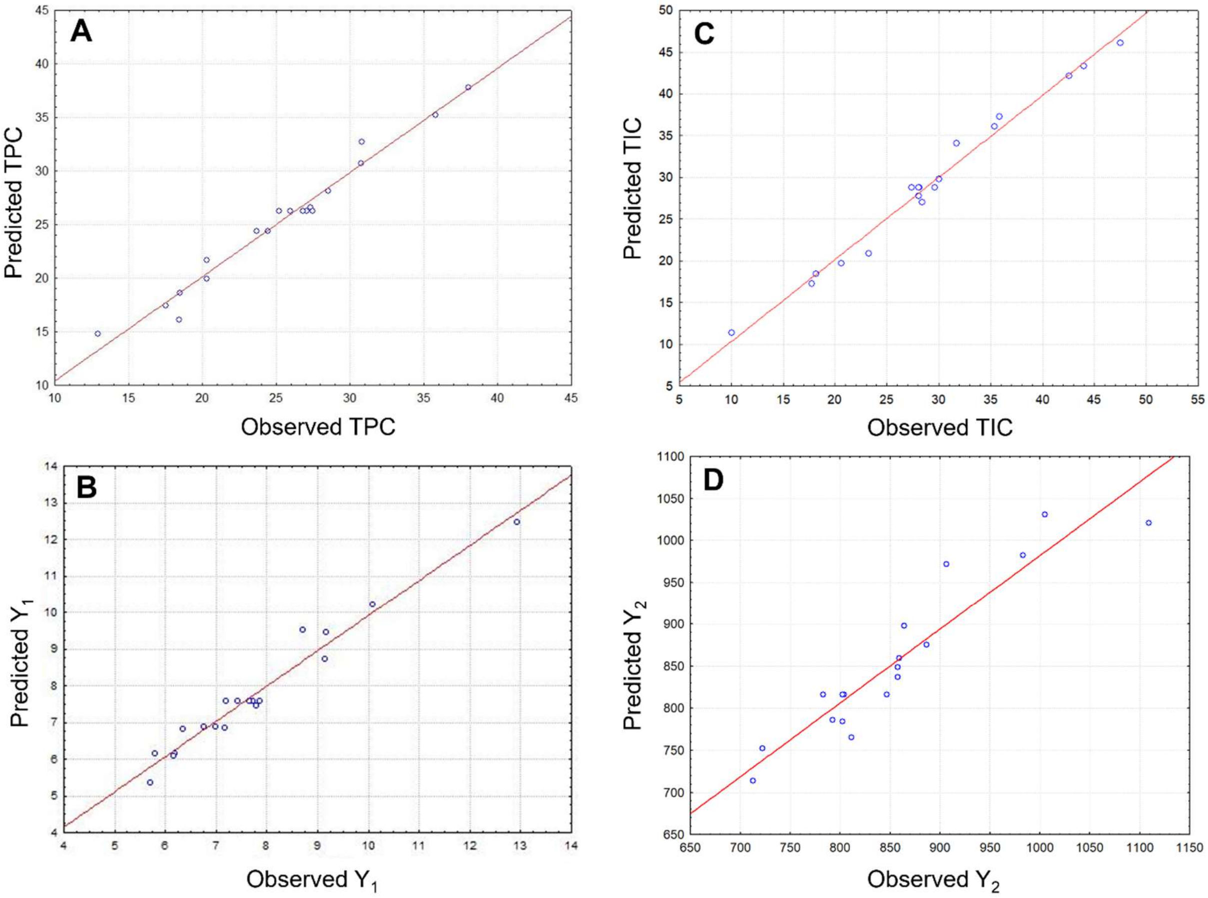

Figure S1. Observed and predicted values for the responses of (A) total phenolic compounds (TPC) in the extracts (B) extraction yield of TPC (C) total isoflavones (TI) in the extracts (D) extraction yield of TI

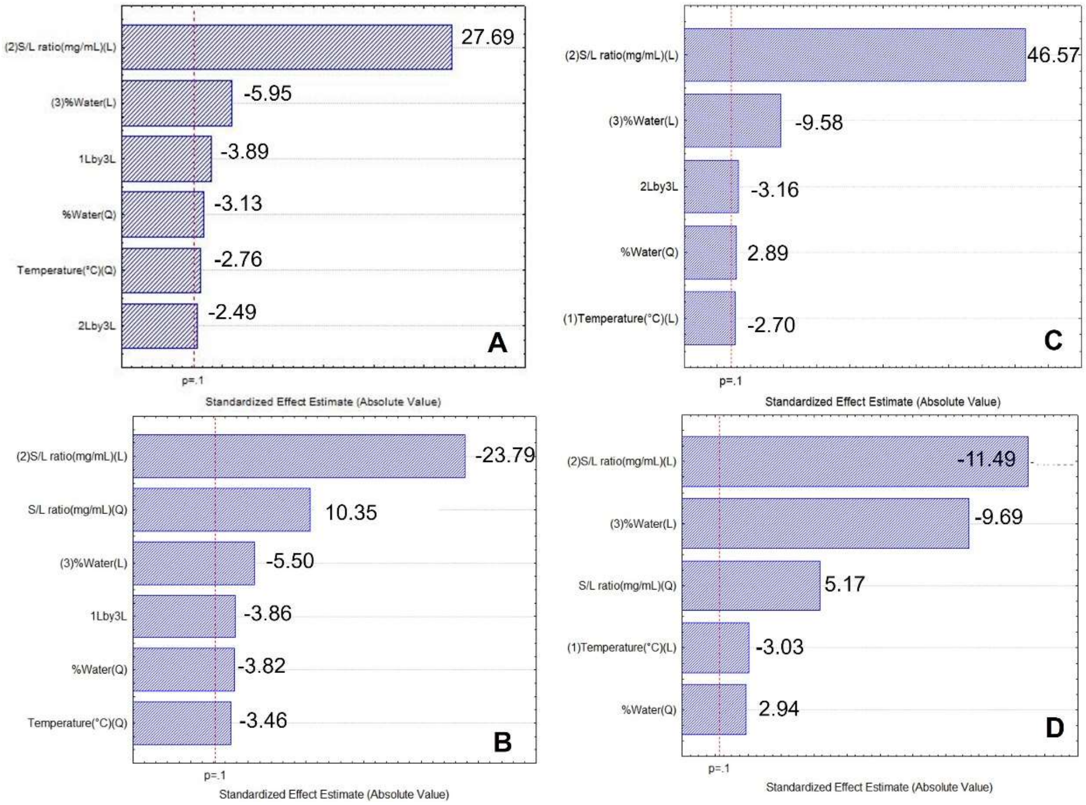

Figure S2. Pareto diagrams for the significant terms of the models for the results of (A) extraction of total phenolic compounds in the extract (B) extraction yield of TPC content (C) total isoflavone content in the extract (D) extraction yield of TI

CAPÍTULO 7

DISCUSSÃO GERAL

7.1. Discussão Geral

O Capítulo 3 da Tese de Doutorado abordou a utilização de *Deep Eutectic Solvents* (DES) como alternativa na extração de flavonoides de soja e okara. Inicialmente, uma triagem computacional do solvente foi conduzida usando o modelo COSMO-SAC para avaliar a interação soluto-solvente através do cálculo do coeficiente de atividade à diluição infinita (ln γ_i^{∞}), economizando tempo e recursos. Diferentes DES baseados em cloreto de colina ([Ch]Cl), como HBA, e vários HBD, foram avaliados, visando substituir solventes tóxicos como metanol e etanol. O DES [Ch]Cl: ácido acético com 30% de água destacou-se como o mais eficiente na extração de flavonoides tanto da soja quanto do okara.

No Capítulo 4, as condições operacionais para extração de isoflavonas de soja e okara utilizando o DES [Ch]Cl: ácido acético foram otimizadas utilizando planejamento fracionário (2^{4-1}) para triagem de variáveis, seguido de otimização aplicando um planejamento experimental completo (DCCR 2^3). Através do planeamento fracionário, analisaram-se as variáveis temperatura, % de água no DES, razão sólido/solvente (S/L) e velocidade de agitação na extração de isoflavonas totais de soja e okara. Na soja, obteve-se uma extração eficaz de 1176,81 μg isoflavonas totais (TI)/g de soja seca sob condições específicas de temperatura (25 °C), 70% de água no DES, 500 rpm, razão S/L de 10 mg/mL e 2 horas de extração. Para o okara, após otimização, foi obtido uma extração de 450,9 μg TIC/g nas condições de 40 °C, 500 rpm, 61,5% de água no DES, relação S/L de 10 mg/mL e 1 hora de extração. Após a otimização, foram avaliados os precursores do DES isolados na extração de isoflavonas de soja e okara e, surpreendentemente, o ácido acético + 70% de água extraiu mais isoflavonas do que o DES [Ch]Cl: ácido acético. Neste caso, o [Ch]Cl pode ter prejudicado a extração das isoflavonas. O ácido acético + 70% de água extraiu 673,13 μg de TIC/g de okara seco, aproximadamente 50% mais do que o DES estudado. Esse resultado, possibilitou explorar outros solventes que foram apresentados no Capítulo 6.

No Capítulo 5, a pesquisa explorou os efeitos de diferentes condições na preparação de *Deep Eutectic Solvents* (DES) com base em [Ch]Cl: ácido acético, [Ch]Cl: ácido fórmico e [Ch]Cl: ácido lático, focando na degradação desses DES. O estudo investigou os impactos da temperatura, tempo e concentração de água na esterificação do [Ch]Cl. Temperaturas elevadas, especialmente para o DES [Ch]Cl: ácido fórmico sem água adicional, intensificaram a reação de esterificação. Em contraste, [Ch]Cl: ácido acético mostrou-se promissor para processos até 40 ºC, sem esterificação ou formação de impurezas. Ao longo de 14 dias a 20 ºC, observou-se um aumento nos ésteres nos três DES, sendo mais pronunciado no [Ch]Cl: ácido fórmico.

Embora a adição de água não tenha impedido completamente a formação de ésteres, ela retardou efetivamente o processo de esterificação. No geral, a formação de ésteres em DES à base de [Ch]Cl: ácido carboxílico é inevitável a longo prazo, mas a adição estratégica de água pode retardar esse processo. No entanto, uma avaliação mais detalhada é fundamental para entender o impacto do teor de ésteres nas propriedades dos DES, no desempenho de aplicações e em outros fatores relevantes.

Por fim, baseado nos resultados do Capítulo 4, onde a mistura aquosa de ácido acético se mostrou mais promissora do que o DES ChCl: ácido acético para extrair isoflavonas de okara, no Capítulo 6 foi decidido expandir a análise e estudar um conjunto de 26 solventes formados por ácidos carboxílicos, polióis, gama-valerolactona e acetato de etila denominados de *Bio-Based Solvents*. Os solventes foram avaliados quanto ao teor de compostos fenólicos totais, isoflavonas totais e atividade antioxidante dos extratos obtidos. Após essa análise, o *Bio-based Solvent* formado por glicerol+água foi escolhido como o melhor solvente e as condições operacionais foram otimizadas aplicando um DCCR 2^3 para avaliar quatro respostas: teor de compostos fenólicos totais no extrato (TPC), isoflavonas totais no extrato (TI), rendimento de extração de TPC (Y1) e rendimento de extração de isoflavonas (Y2). As condições finais de extração foram estatisticamente otimizadas para as quatro respostas, destacando-se a utilização de uma razão S/L de 60 mg/mL, 66 °C, 40% de água adicionada ao glicerol e 500 rpm de agitação durante 45 minutos como a melhor condição para investigações futuras. Como resultado, o glicerol 60% provou ser um solvente alternativo para a extração de compostos fenólicos e isoflavonas do okara e pode servir como um potencial substituto para solventes convencionais como o metanol e o etanol. Além disso, ao utilizar um solvente geralmente reconhecido como seguro pela *Food and Drugs Administration* (FDA), esses extratos podem ser utilizados em formulações alimentícias sem a necessidade de purificação, reduzindo o tempo e os custos do processo. Com o aumento da produção de biodiesel no Brasil, o excesso de glicerol tem sido queimado em fornos e caldeiras para gerar calor, tornando-se um subproduto subutilizado por grandes indústrias. Assim, este trabalho apresenta uma alternativa de utilização do glicerol produzido. O Brasil é um grande produtor de biodiesel (que consequentemente gera oito vezes mais glicerol do que a demanda) e de soja, com grandes quantidades de subprodutos agrícolas a serem tratados. Com isso, este estudo pode servir como um incentivo adicional para ampliar os estudos utilizando glicerol e resíduos de soja em procedimentos de extração, ou mesmo para aplicar o glicerol na extração de compostos bioativos de outras matrizes vegetais.

Este estudo abordou a utilização da soja e do resíduo okara para extração de compostos bioativos com alto valor agregado. É importante destacar, que o resíduo de soja, após as extrações, continuará sendo um resíduo, que aponta para a necessidade de pesquisas futuras no âmbito da biorrefinaria com foco no aproveitamento integral desse resíduo. Os resultados deste estudo abrem caminho para criação de oportunidades de negócio e valorização de subprodutos não somente da soja, mas também do subproduto da produção de biodiesel, o glicerol. A junção destes dois subprodutos subutilizados, o glicerol e o okara, para a extração de isoflavonas e compostos fenólicos reduz impactos ambientais e ainda gera um produto que pode ser aplicado integralmente em formulações que buscam benefícios à saúde humana.

A avaliação de diferentes solventes e o estudo das condições de extração incluindo a otimização das variáveis operacionais demonstraram um esforço na busca por processos mais eficientes e sustentáveis. Além disso, as pesquisas sobre citotoxicidade e degradação dos solventes e a busca por estratégias para minimizar esse problema, mostraram um compromisso com o desenvolvimento de técnicas de extração que sejam não somente eficientes, mas seguras a nível ambiental e de saúde humana.

Pesquisas futuras precisam também ser realizadas com foco em testes de toxicidade desses extratos, já que o foco principal deste trabalho foi obter um extrato utilizando um solvente que pudesse ser aplicado diretamente em formulações alimentícias. E, pensando em formulações alimentícias, pesquisas voltadas para o desenvolvimento de formulações alimentícias contendo esses extratos podem reforçar a ideia de oportunidades de negócio e avanço científico.

CAPÍTULO 8

CONCLUSÃO GERAL

8.1. Conclusão geral

O presente estudo destacou a busca por solventes alternativos menos tóxicos e sustentáveis para a extração de isoflavonas de soja e okara. Este estudo proporcionou avanços na seleção de solventes e otimização da extração desses compostos, destacando a eficácia do COSMO-SAC na triagem inicial do solvente e o entendimento da formação de ésteres nos DES compostos por [Ch]Cl e ácido carboxílicos.

Por fim, as descobertas feitas nesse trabalho não apenas contribuem para práticas mais sustentáveis na extração sólido-líquido de isoflavonas de soja e okara, mas também oferecem uma alternativa para o aproveitamento de subprodutos industriais no contexto brasileiro, especialmente o excesso de glicerol gerado pela produção de biodiesel. Essa abordagem pode inspirar futuras pesquisas e práticas industriais mais conscientes, alinhadas com os princípios da sustentabilidade. Além disso, essa pesquisa pode contribuir no âmbito da biorrefinaria para aproveitamento mais eficiente do okara e do glicerol, ambos, resíduos subutilizados pela indústria.

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ANEXO

Termo de autorização para publicação

Deep eutectic solvents as an alternative for extraction of flavonoids from soybean (Glycine max (L) Merrill) and okara: An experimental and computational approach based on COSMO-SAC model

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