**UNIVERSIDADE ESTADUAL DE CAMPINAS**
**Faculdade de Engenharia de Alimentos**

**GABRIELA BOSCARIOL RASERA**

# MUSTARD GRAINS AS SOURCE OF COMPOUNDS WITH ANTIOXIDANT PROPERTIES: A STUDY BASED ON GERMINATION, EXTRACTION AND IDENTIFICATION PROCESSES

# GRÃOS DE MOSTARDA COMO FONTE DE COMPOSTOS COM PROPRIEDADES ANTIOXIDANTES: UM ESTUDO BASEADO NOS PROCESSOS DE GERMINAÇÃO, EXTRAÇÃO E IDENTIFICAÇÃO

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**Orientador:** Prof. Dr. Ruann Janser Soares de Castro

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**Comissão examinadora**

**Prof. Dr. Ruann Janser Soares de Castro**
**Orientador – DCA/FEA/UNICAMP**

**Prof. Dr. Severino Matias de Alencar**
**Membro Titular – ESALQ/USP**

**Dra. Juliana Campos Hashimoto**
**Membro titular – DTA/FEA/UNICAMP**

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**Resumo**

Germinação é um processo econômico e natural que oferece novas oportunidades para a melhoria da qualidade nutricional e do potencial biológico de grãos. As transformações bioquímicas que acontecem durante esse processo em mostarda ainda não foram elucidadas pela literatura, mas sabe-se que as condições de germinação influenciam diretamente a composição final dos germinados. Os grãos de mostarda apresentam grande potencial bioativo, e, além de baratos, são mundialmente conhecidos e consumidos. Desta forma, o presente projeto teve como objetivos avaliar a influência dos parâmetros (luz, temperatura e tempo) na germinação de sementes de mostarda; determinar o solvente mais adequado (água, acetona e metanol) para obtenção de extratos com maior teor de compostos fenólicos e atividade antioxidante e avaliar as propriedades antioxidantes dos extratos obtidos utilizando os métodos FRAP, ORAC, ABTS e DPPH, além da determinação do teor de compostos fenólicos totais (TPC), flavonoides totais e taninos condensados, das frações solúveis e insolúveis de grãos de mostarda germinados e não germinados. Adicionalmente, foi realizada a identificação dos compostos fenólicos por cromatografia líquida de alta eficiência nos extratos de grãos de mostarda não germinados. As análises foram realizadas utilizando-se grãos de mostarda branca (*Sinapsis alba*) e preta (*Brassica nigra*). As condições de germinação que permitiram a produção de extratos com maior conteúdo de compostos fenólicos e melhores propriedades antioxidantes para mostarda branca foram: 72 h de germinação, temperatura de incubação de 25°C no escuro. Para as amostras de mostarda preta, a condição definida como mais adequada foi: 48 h de germinação a 25°C, com períodos alternados de luz e escuro. A combinação de solventes selecionada para a extração de compostos fenólicos com propriedades antioxidantes para ambas as variedades foi acetona e água (50% v:v), condição em que os resultados foram no mínimo 20 vezes maiores quando comparados com acetona pura. Em relação ao estudo comparativo entre os extratos de mostarda não germinada com a germinada nas condições escolhidas, observou-se melhoria nas propriedades antioxidantes dos grãos. As propriedades antioxidantes avaliadas pelos métodos FRAP, DPPH, ABTS e ORAC apresentaram aumento de 68%, 43%, 66% e 45%, respectivamente, para a fração solúvel de mostarda branca, e 29%, 3%, 160% e 42%, respectivamente, para a fração solúvel de mostarda preta após a germinação. As transformações entre a fração solúvel e insolúvel foram perceptíveis durante o processo. Enquanto a atividade antioxidante da fração solúvel e insolúvel da mostarda branca aumentou durante o processo, para a mostarda preta, o comportamento foi inverso: a

atividade antioxidante para fração solúvel aumentou e para a insolúvel diminuiu. Este contexto exemplifica a complexidade e as peculiaridades do processo germinativo, visto que as transformações que nele ocorrerem podem alterar significativamente o poder antioxidante dos extratos obtidos. A priori, apenas o ácido sinápico foi identificado em ambas as variedades de mostarda não germinadas. A complexidade do processo germinativo em mostarda ainda não foi totalmente elucidada, mas o presente trabalho mostrou algumas justificativas para as mudanças positivas encontradas. Além disso, diante dos resultados obtidos podemos concluir que o processo de germinação se apresenta como uma alternativa de consumo para os grãos de mostarda, que são, até então, largamente consumidos na forma de molhos.

**Palavras-chave**: grãos de mostarda; germinação; propriedades antioxidantes.

**Abstract**

Germination is an economic and natural process that offers new opportunities to improve nutritional quality and biological potential of grains. The biochemical transformations that happen during this process in mustard grains were not elucidated by literature yet, but it is known that germination conditions influence directly the final composition of sprouts. Mustard grains present a bioactive potential, and besides being cheap, are consumed and known worldwide. Thus, the aim of this project was to evaluate the influence of parameters such as light, temperature and time, on germination of mustard seeds; determinate the most adequate solvent (water, acetone and methanol) to obtain extracts with the maximum of phenolic compounds and antioxidant activity; and evaluate the antioxidant activity of the extracts by FRAP, ORAC, ABTS and DPPH, besides the determination of total phenolic compounds (TPC), flavonoids and total condensed tannins of soluble and insoluble fractions of non-germinated and germinated mustard grains. Additionally, it was made the identification of phenolic compounds by high performance liquid chromatography of non-germinated mustard grains. All analysis were made with white (*Sinapsis alba*) and black (*Brassica nigra*) mustard grains. Germination conditions that allowed the production of extracts with higher content of phenolic compounds for white mustard were: 72h of germination at 25°C at dark and for black mustard 48h at 25°C with altered periods of light and dark. The combination of solvents selected for extraction of phenolic compounds with antioxidant activity as acetone and water (50% v/v), condition with results at least 20-folds higher when compared with pure acetone. Regarding the comparison between non-germinated and germinated mustard in the chosen conditions, it was observed an improvement of antioxidant properties of the grain. The antioxidant properties evaluated by FRAP, DPPH, ABTS and ORAC methods showed an increase of 68%, 43%, 66% and 45%, respectively, for soluble fraction of white mustard and 29%, 3%, 160% and 42%, respectively, for soluble fraction of black mustard. The transformation of bound and soluble fraction was noted during the process. While antioxidant activity of soluble and bound fraction of white mustard increased during the process, for black mustard this behavior was inverse: the antioxidant activity in soluble fraction increased and insoluble fraction decreased. This context exemplifies the complexity and peculiarity of germinative process, since transformations can change significantly antioxidant power of the extracts. Initially, only sinapic acid was found in both mustard species without germination. The complexity of germinative process in mustard was not elucidate yet, but the present word shows some justifications of the positive changes observed. Besides that, we can conclude that germination process is an

alternative of consumption of mustard grains, that are until now, largely consumed as sauces.

**Keywords**: mustard grains; germination; antioxidant properties

Sumário

## Introdução

Comum dos Estados Unidos, Reino Unido, Canadá, países Europeus, Índia, China e países da Ásia, a mostarda preta (*Brassica nigra*) e branca (*Sinapsis alba*) foram as variedades estudadas no presente trabalho. Utilizadas como item suplementar em diversos alimentos, principalmente na preparação de molhos, carnes e peixes, a mostarda atua como preservativo e fornece flavor pungente. Os grãos de mostarda apresentam uma grande variedade de minerais e ácidos graxos ômega 3, óleos essenciais, vitaminas do complexo B, vitamina E, glucosinolatos, flavonoides e carotenoides (López-Argüello, Pérez-Rodríguez, Bosch-Bosch, & Barrera-Vázquez, 1998; Divakaran & Babu, 2016; Morley, 2016; Rana, 2016; Bi, Lim, & Henry, 2017). Estudos na literatura têm demonstrado que as sementes de mostarda apresentam diversos compostos biologicamente ativos com potencial para diminuição da glicemia, redução do acúmulo de lipídeos e de colesterol, além de propriedades antioxidantes, anti-inflamatórias e anticancerígenas (Khan et al., 1996; Grover, Yadav & Vats, 2002, Grover, Yadav, & Vats, 2003; Thirumalai, et al., 2011; Divakaran & Babu, 2016).

Além do sabor e uso difundido mundialmente e dos benefícios para a saúde, o preço dos grãos de mostarda torna o seu consumo acessível para a população de classe social mais baixa, o que justifica mais uma vez, a importância de estudo deste grão.

A germinação é um processo eficiente e econômico que melhora a qualidade nutricional e a funcionalidade de sementes e leguminosas. Este processo aumenta a digestibilidade de carboidratos e proteínas, altera a composição de aminoácidos, aumenta o conteúdo de vitaminas e reduz fatores antinutricionais, além de promover o acúmulo de diversos compostos bioativos, como o ácido ɣ-aminobutírico (GABA) e polifenóis (Yeo & Shahidi, 2015; Mamilla & Mishra, 2017; Gan et al., 2017).

Adicionalmente, a germinação oferece novas oportunidades para melhoria da qualidade nutricional e do potencial funcional de grãos (Yeo & Shahidi, 2015; Mamilla & Mishra, 2017; Gan et al., 2017). Esta melhoria está associada à complexidade das transformações positivas que acontecem durante a formação do broto, mostrando o quão útil pode ser esse processo. Portanto, uma revisão bibliográfica foi realizada para apresentar os últimos dados presentes na literatura e para sugerir tendências dessa área de pesquisa. A revisão de literatura está apresentada no Capítulo 1.

Ainda, é válido ressaltar que o rendimento de extração de compostos fenólicos depende de fatores como a escolha da natureza química do solvente, visto que um solvente puro não é capaz de extrair todos os antioxidantes com diferentes polaridades e

estruturas presentes no material de estudo (Garcia-Salas et al., 2010). Apesar de ser um assunto amplamente abordado na literatura para diferentes matrizes vegetais, estudos de extração de compostos fenólicos utilizando diferentes solventes assim como suas combinações para obtenção de extratos de mostarda com propriedades antioxidantes ainda não foram relatados na literatura, assunto este, que foi abordado no Capítulo 2.

Associado às necessidades dos estudos de extração, sabe-se que as alterações geradas pelo processo de germinação dependem diretamente de alguns parâmetros como temperatura, embebição, tempo, umidade e luz, tornando-se necessária a identificação das melhores condições para acúmulo dos compostos de interesse. Considerando que os nutrientes e compostos bioativos presentes no mesmo tipo de grão podem aumentar, diminuir ou se manter durante a germinação, a otimização das condições desse processo para cada variedade é crucial para maximizar a composição fitoquímica e as funções biológicas (Cevallos-Casals & Cisneros-Zevallos, 2010; Mamilla & Mishra, 2017). A interferência dos parâmetros ambientais no processo de germinação de sementes de mostarda assim como a escolha da melhor condição de germinação foi abordada no Capítulo 3.

Por fim, devido à falta de informações na literatura sobre o perfil fenólico e as propriedades antioxidantes de mostarda germinada, o que indica que sua germinação é um assunto inovador e pioneiro, abordou-se no Capítulo 4, as propriedades antioxidantes dos extratos produzidos e a caracterização parcial dos compostos fenólicos presentes nos grãos de mostarda não germinados e germinados.

# Capítulo I: Como a germinação afeta o conteúdo de compostos fenólicos e as propriedades antioxidantes dos grãos?

Gabriela Boscariol Rasera[a*], Ruann Janser Soares de Castro[a]
Departamento de Ciência de Alimentos, Faculdade de Engenharia de Alimentos,
Universidade Estadual de Campinas.
Rua Monteiro Lobato, 80, Campinas – SP, Brasil
*Autor de correspondência:
E-mail: gabi_boscariol@hotmail.com

**Resumo**

Germinação é um processo fácil e econômico que melhora a qualidade nutricional e funcional dos grãos. Trata-se de um processo com transformações complexas e peculiares para cada grão. Ainda, as condições ambientais em que os grãos são expostos influenciam diretamente na composição final do broto e, consequentemente na sua bioatividade. É possível perceber uma correlação positiva entre o processo de germinação e o teor de compostos fenólicos presentes nos grãos, apesar da explicação para isso ser peculiar e complexa para cada processo de germinação. De maneira geral, sabe-se que os compostos fenólicos podem estar presentes nas forma solúvel e insolúvel, sendo que a primeira está normalmente armazenada no vacúolo da planta, e a segunda ligada a macromoléculas e constituindo a estrutura dos grãos. Durante o processo de germinação e consequente atuação das enzimas ativadas pela absorção de água, essas duas frações se transformam simultaneamente. A fração solúvel pode se tornar insolúvel devido ao crescimento da radícula e necessidade de formação estrutural e a insolúvel pode ser transformada à solúvel, devido a hidrólise das ligações com as macromoléculas de armazenamento da planta. Dessa forma, a compreensão das diversas vias e consequentes resultados se tornam alvo de estudo da literatura. Dentro desse contexto, o objetivo central dessa revisão é o de apresentar estudos recentes que investigam as alterações promovidas pelo processo de germinação e sua relação com as propriedades antioxidantes de diferentes grãos. Algumas vias bioquímicass para formação de compostos fenólicos já elucidadas foram abordadas, assim como tendências futuras para uso de germinação como um processo para melhoria de propriedades biológicas dos grãos.

**Palavras-chave:** Germinação; compostos fenólicos; transformações.

## 1. Introdução

Germinação significa o surgimento de uma nova planta. Isso inclui os eventos fisiológicos desde que as sementes são plantadas até a extensão da radícula que penetra as estruturas próximas. A absorção de água inicia as atividades metabólicas e celulares do grão. Temperatura, luz, umidade e tempo são fatores responsáveis para a transição do estado de dormência para metabolismo ativo por meio das enzimas hidrolíticas endógenas e a ativação de hormônios. Enzimas hidrolíticas iniciam a degradação de macromoléculas de armazenamento como carboidratos, proteínas e lipídios para gerar os produtos necessários para o desenvolvimento e crescimento da planta. Como dito anteriormente, a germinação é visualmente detectada com o aparecimento da radícula que aumenta o consumo de oxigênio e água, como consequência das necessidades para o crescimento da radícula (Bewley, 1997; Nonogaki & Nonogaki, 2017).

É importante a compreensão, de que durante a germinação, mudanças fitoquímicas são extensas, como resultado de um fluxo dinâmico e complexo de nutrientes, incluindo remobilização, degradação e acúmulo dos mesmos (Nelson et al., 2013). Como consequência, mudanças bioquímicas e nutricionais importantes acontecem nos grãos, modificando as características dos mesmos (Gan et al., 2017). Como resultado desse processo dinâmico, a diversidade da composição e, consequentemente, a composição da atividade biológica, são específicas para cada grão (Paucar-Menacho et al., 2010).

Compostos bioativos são encontrados em frutas, vegetais e grãos, e, apresentam efeitos em células específicas do corpo, resultando em melhores condições de saúde. Como eles não são essenciais, não são reconhecidos como nutrientes, apesar de exibirem efeitos benéficos como poder antioxidante (Gibney et al., 2009). A respeito disso, já se sabe que estes compostos podem aumentar, diminuir, e principalmente sofrerem transformações, de acordo com o uso da planta, sendo novamente específico para cada broto e grão (Nelson et al., 2013). Mesmo assim, já se sabe também que esse processo pode acumular diferentes compostos bioativos em brotos, como vitaminas, GABA (ácido γ-aminobutírico) e polifenóis (Gan et al., 2017). O objetivo central dessa revisão é relacionar o processo de germinação de diferentes grãos com as mudanças no perfil de compostos fenólicos, assim como de suas propriedades antioxidantes.

## 2. O que acontece durante a germinação?

A germinação causa mudanças importantes nas características bioquímicas e na melhoria nutricional e sensorial dos grãos (Yang et al., 2017) devido aos efeitos na composição dos mesmos, que acontecem a fim de sustentar o crescimento da semente. Apesar de algumas transformações ocorrerem de maneira geral nos grãos, constituindo as fases de desenvolvimento, sabe-se que a diversidade de composição e consequentemente de bioatividade são marcantes e, um estudo detalhado do tipo de semente e das condições de germinação, é então, essencial para um conhecimento adequado (Paucar-Menacho et al., 2010).

As primeiras transformações (Figura 1) que ocorrem durante a germinação, iniciam-se com a embebição das sementes. Essencial para este processo, a absorção da quantidade de água necessária (A), como resposta do menor potencial de água das células, dá início às atividades celulares e metabólicas (Nonogaki & Nonogaki, 2017) e à reorganização das organelas celulares (Bewley, 1997).

A temperatura, luz e umidade adequadas fazem a transição do grão do estado de dormência para o início do metabolismo ativo, por meio da ativação de inúmeras enzimas e hormônios hidrolíticos, como a giberelina (B). Este hormônio, transita do embrião (ou germe, E) para a camada de aleurona, facilitando a liberação de enzimas hidrolíticas endógenas (C) degradadoras de macromoléculas de armazenamento (D), que quando degradadas produzem nitrogênio e o esqueleto de carbono necessários para o crescimento e fotossíntese da planta (Nelson et al., 2013) pertencentes aos eventos pós-germinativos e crescimento da radícula (Nonogaki & Nonogaki, 2017).

A germinação é detectada visualmente pelo rompimento do grão com o surgimento da radícula. Assim, o embrião gera uma pressão para a saída da mesma. O alongamento celular na radícula do embrião é provavelmente induzido pelo afrouxamento da parede celular através das proteínas modificadoras de parede celular, como as expansinas, que estão sob o controle da giberelina (B). Neste período há aumento no consumo de oxigênio. Essa taxa decai em seguida até que a radícula penetre na membrana, quando ocorre novamente aumento expressivo do consumo desse gás. A vias das pentoses fosfato, glicolítica e oxidativa retomam e as enzimas do ciclo de Krebs são ativadas. Vale ressaltar que os tecidos da semente seca e madura contêm mitocôndrias, enzimas do ciclo de Krebs e oxidases terminais suficientes para fornecer quantidades adequadas de ATP para apoiar o metabolismo durante várias horas após a embebição (Bewley, 1997). Após o afrouxamento da parede celular e aumento do consumo de oxigênio, uma fase lag  inicia-se, onde o conteúdo de água é estabilizado, eventos

bioquímicos acontecem e RNA e proteínas são sintetizados. Nota-se o enfraquecimento do endosperma vivo, evento essencial para a germinação de sementes e pré-requisito para a ruptura do endosperma pela radícula, que marca a conclusão da germinação. Os eventos pós-germinativos iniciam-se e são marcados pelo aumento da necessidade de consumo de água, devido ao surgimento da radícula, que absorve rapidamente a água e alonga. Além disso, a mobilização de reservas das sementes, como proteínas, lipídios e polissacarídeos é intensificada, como dito anteriormente. A degradação dos macronutrientes é bem coordenada e o crescimento das plântulas continuarão até que as verdadeiras folhas surjam e iniciem atividades fotossintéticas eficientes (Nonogaki; Nonogaki, 2017).

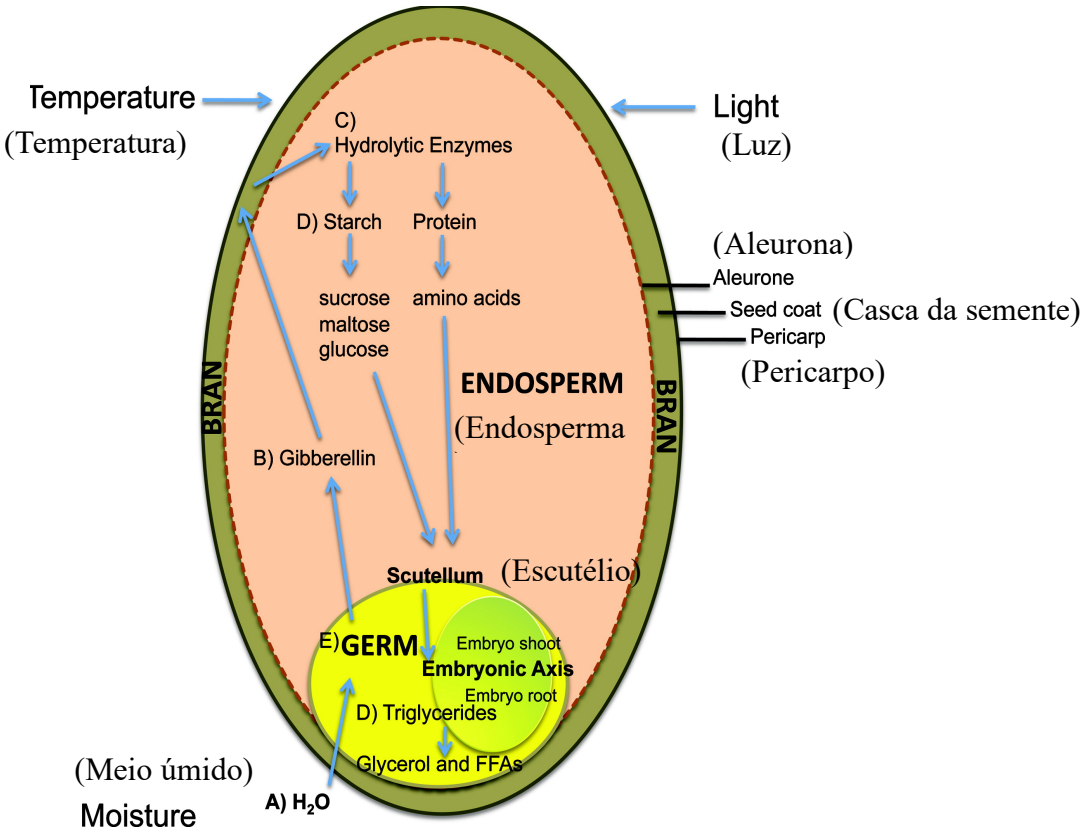


**Figura 1.** Esquema da semente e eventos germinativos, sendo que A: água, B: Giberilina, C: Enzimas hidrolíticas, D: Amido, proteínas e triglicerídeos degradados a sacarose, maltose, glicose, proteínas e ácidos graxos e glicerol, E: Embrião. Fonte: Traduzido de Nelson et al. (2013). Anexo I.

## 3. Condições ambientais de germinação, o que muda?

Sabe-se que as condições ambientais em que as sementes são germinadas influenciam diretamente na composição final do broto e consequentemente na sua

bioatividade. Cada semente tem sua especificidade em composição, e, os fatores ambientais devem ser então, combinados para que o ponto ótimo de germinação seja determinado (Facelli; Chesson & Barnes, 2005). Desta forma, fatores como luz, umidade, temperatura e tempo são primordiais para a determinação das condições mais adequadas de germinação. Uma breve descrição de como estes fatores atuam sobre a germinação de sementes e grãos é apresentada a seguir.

a) Luz:

Trata-se de um fator ambiental que afeta a fotossíntese da planta (Yan et al., 2013), sendo que a quantidade, a duração e a qualidade da luz desempenham um papel importante na regulação da dormência e germinação das sementes, o que é essencial para o crescimento e o desenvolvimento da planta (Barrero et al., 2014). A luz é detectada por diferentes fotorreceptores em plantas, como os fitocromos, que são os principais reguladores da germinação em resposta à luz. Os fitocromos são os responsáveis por perceber os sinais de luz e transformá-los em sinais bioquímicos, dependendo da qualidade, intensidade, comprimento de onda e duração da luz (Vieira; Rodrigues; Garcia, 2017).

A luz evoca mudanças foto-oxidativas nas plantas, como a produção de diferentes formas de espécies reativas de oxigênio (ROS), produzidas principalmente por cloroplastos. ROS podem iniciar uma série de alterações metabólicas, como a redução da atividade enzimática e diminuição da taxa fotossintética quando em excesso. Assim, para minimizar os possíveis danos oxidativos causados por estas espécies reativas, as plantas ativam seus mecanismos de desintoxicação constituídos de substâncias de baixo peso molecular como os antioxidantes e as enzimas antioxidantes (Simlat et al., 2016).

Portanto, a ausência ou presença de luz leva a formas de desenvolvimento morfologicamente distintos durante o crescimento da planta (Galvao et al., 2012).

b) Temperatura:

A temperatura é outro parâmetro fundamental para o crescimento e o desenvolvimento das sementes. O processo fotossintético parece ser sensível a esse fator, havendo impactos prejudiciais no desempenho e produtividade da planta quando exposta a temperaturas inadequadas, como enfraquecimento da absorção de água e nutrientes, mudança na taxa de crescimento e redução nas taxas de processos fotoquímicos, como reações enzimáticas e capacidade fotossintética (Yan et al., 2013).

Assim, temperaturas inadequadas tornam-se estresse abiótico para a planta, e, como mecanismo de proteção e defesa, as mesmas produzem os compostos fenólicos,

incluindo ácidos fenólicos, flavonoides e proantocianidinas, além de polifenóis presentes como glicosídeos, acilglicosídeos e outras formas conjugadas (Zhang & Tsao, 2016).

c) Tempo de germinação:

O tempo de germinação afeta diretamente a decomposição das macromoléculas. O prolongamento do tempo proporciona mais decomposição das moléculas de reserva das plantas e, portanto, maior nível de liberação dos componentes bioativos (Chungcharoen et al., 2015), o que, juntamente com alta atividade de beta-glucanase e alfa-amilase com o tempo, aumenta o teor de compostos fenólicos livres nos brotos (Farzaneh et al., 2017).

## 4. Germinação como uma estratégia para aumentar o conteúdo de compostos fenólicos e atividade antioxidante de grãos.

Um antioxidante pode ser definido como "uma substância que, quando presente em baixas concentrações em comparação com a de um substrato oxidável, atrasa significativamente ou impede a oxidação desse substrato" (Halliwell, 1990). Isso pode acontecer por: a) sequestro de radicais livres do meio; b) quelação de íons metálicos; c) inibição de enzimas produtoras de radicais livres; d) ativação de enzimas antioxidantes endógenas; e) prevenção de peroxidação lipídica; f) prevenção de danos no DNA; g) prevenção da modificação proteica e destruição de açúcares (Carocho; Morales & Ferreira, 2017).

O termo "compostos fenólicos" pode ser atribuído a compostos que possuem um anel aromático com grupamento hidroxila e "polifenóis", compostos que possuem um ou mais anéis aromáticos com um ou mais grupamentos hidroxilas, embora sejam termos sinônimos. Os compostos fenólicos podem ser divididos em diversos sub-grupos de acordo com as suas características estruturais (Figura 3), que são os fatores responsáveis pela atividade antioxidante de cada polifenol ou composto fenólico. Assim, o número e a posição de grupamentos hidroxilas nas moléculas, grau de hidroxilação, distância entre grupamento carbonil e anel aromático, assim como a quantidade de anéis aromáticos desempenham um papel importante na atividade antioxidante do composto. (Zhang & Tsao, 2016).

Dados de pesquisas abordados na literatura demonstram o potencial efeito positivo na composição e qualidade nutricional dos grãos germinados. A Tabela 1 apresenta alguns estudos publicados nos últimos 3 anos mostrando brevemente os efeitos positivos da germinação no conteúdo de compostos fenólicos e propriedades antioxidantes de diferentes sementes e grãos.

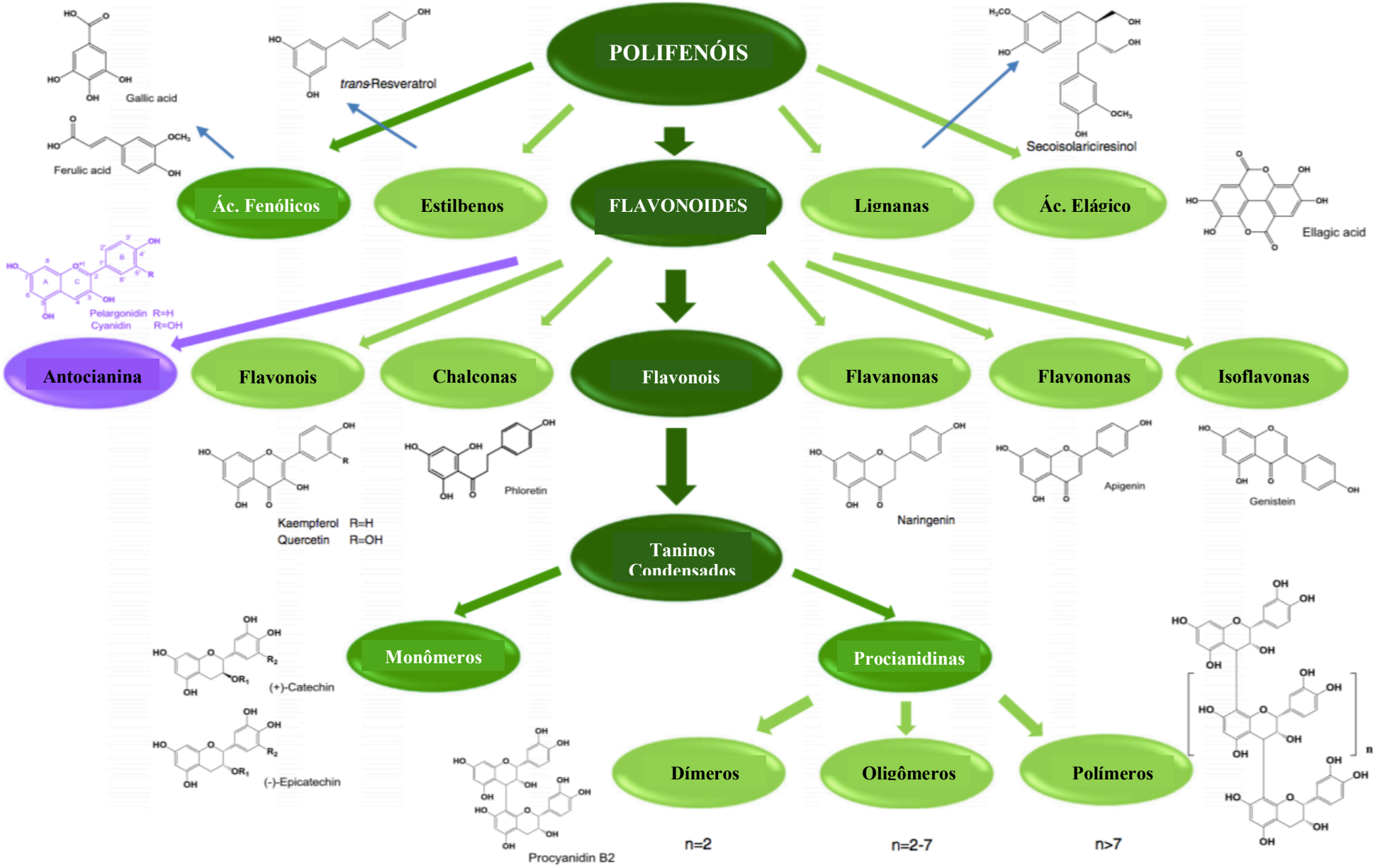


**Figura 2.** Classificação esquemática de polifenóis. Fonte: Traduzida de Zhang &Tsao, 2016. Anexo II

**Tabela 1.** Grãos germinados e mudanças observadas no conteúdo de compostos fenólicos e propriedades antioxidantes durante a germinação.

| Grãos e sementes germinados | Determinações | Mudanças observadas (não germinadas para germinadas) | Referência |
|---|---|---|---|
| Oliva (*Olea europaea* L.) seeds | Conteúdo de fenólicos totais (TPC); DPPH; ABTS; FRAP | Fenólicos totais aumentaram14 vezes em média na oliva germinada. Atividade antioxidante dos brotos (8 a 16 dias) foi 30 vezes maior que das sementes não germinadas. | (Falcinelli et al., 2018) |
| Cultivares de arroz | Conteúdo de fenólicos totais; flavonoides totais; taninos totais; ácido fítico; DPPH; FRAP; ensaio de atividade de eliminação de radicais hidroxila; poder redutor; tocoferol; ascorbato total. | Três, das dez variedades de arroz estudadas (IET-23466, IET-23463 e PR-123), apresentaram nível maior de compostos bioativos e atividade antioxidante após 48 horas de germinação. | (Kaur, Asthir, & Mahajan, 2017) |
| Grão de bico | Conteúdo de fenólicos totais; DPPH; ABTS (TEAC) | Germinação aumentou duas vezes o teor de compostos fenólicos totais; 4 vezes o valor de DPPH e 2 vezes o valor de TEAC. | (Hayta & İşçimen, 2017) |
| Milho roxo (*Zea mays* L.) | Compostos fenólicos totais; determinação quantitativa e qualitativa de compostos fenólicos totais por HPLC-DAD-ESI/MS; compostos não antocianinas; antocianinas; ORAC | Ácidos fenólicos livres diminuíram de1679,72 mg GAE/100 g a 1042,82 mg GAE/100 g.<br>Os valores de ORAC também diminuíram de 4837,061 mg TE/100 g a 2060,83 mg TE/100 g.<br>Antocianinas foi a classe de compostos fenólicos de maior abundância nos brotos de milho roxo com 63 horas de germinação. | (Paucar Menacho et al., 2017) |
| Favas (*Vicia faba* L.) Tremoço (*Lupinus albus*) Grão de bico (*Cicer arietinum* L.) Lentilha (*Lens culinaris*) Feno-grego (*Trigonella foenum-graecum* L.) Feijão (*Phaseolus vulgaris*) | Conteúdo de fenólicos totais; flavonoides totais; DPPH | Fenólicos totais, flavonoides totais, e atividade antioxidante das leguminosas aumentaram proporcionalmente ao tempo de germinação (6 dias máximos) | (Saleh et al., 2017) |

| | | | |
|---|---|---|---|
| Arroz marrom | Conteúdo de fenólicos totais; ORAC | Germinação a 34°C por 96 horas aumentou em 3 vezes o conteúdo de compostos fenólicos totais e duas vezes a atividade antioxidante avaliada pelo método ORAC. | (Cáceres et al., 2017) |
| Arroz marrom (*Oryza sativa* L.) | DPPH; ABTS; FRAP | 36 horas de germinação aumentou as proporções de compostos antioxidantes bioacessíveis. | (Xia et al., 2017) |
| Soja, grão de bico, feijão mungu, lentilhas vermelhas e feijão roxo | Compostos fenólicos totais (TPC); flavonoides totais. Quantificação de ácidos fenólicos por HPLC e DPPH | O conteúdo de polifenóis totais dos extratos variou de 0,45 a1,62 g/kg para grãos não germinados e de 0,68 a 2,45 g/kg e 0,65–2,53 g/kg em germinação a 30 °C e 40 °C, respectivamente. Atividade antioxidante dos extratos aumentou proporcionalmente com o aumento de TPC. Ácido ferúlico foi o ácido fenólico mais predominante detectado nos grãos germinados. | (Mamilla & Mishra, 2017) |
| Feijão-arroz (*Vigna umbellata*) | Fenólicos totais; composição de flavonoides e ácidos fenólicos por HPLC-MS; DPPH e FRAP | Germinação por 24 horas aumentou o conteúdo total de fenólicos e atividade antioxidante aproximadamente 2 vezes. Sete compostos fenólicos (dois ácidos fenólicos e cinco flavonoides) foram encontrados nos brotos de feijão-arroz. | (Sritongtae et al., 2017) |
| Linhaça (*Linum usitatissimum*L.) | Conteúdo de fenólicos totais (TPC); conteúdo de flavonoides; ORAC; análises atividade antioxidante celular (CAA). | Linhaça germinada por 10 dias aumentou 55 vezes o conteúdo de flavonoides e 6 vezes o conteúdo de TPC quando comparada com a não germinada. Os valores de ORAC aumentaram 7 vezes. CAA mostrou um aumento de aproximadamente 4 vezes. | (Wang et al., 2016) |

## 5. Transformação de compostos fenólicos durante a germinação

Os compostos fenólicos podem estar presentes nas células em suas formas livres e solúveis, esterificadas e insolúveis (Nayak et al., 2017) e, de maneira geral, a germinação resulta em mudanças no nível de fenólicos totais da semente germinada, atuando diferentemente em suas frações (Cevallos-Casals & Cisneros-Zevallos, 2010). A maioria dos compostos fenólicos é sintetizada no espaço intracelular, especialmente no retículo endoplasmático e é armazenada no vacúolo celular como fração solúvel (Agati et al., 2012). Já os compostos insolúveis estão normalmente ligados nos componentes estruturais da parede celular como celulose, hemicelulose, lignina, proteínas estruturais e carboidratos (Figura 2) por meio de ligações covalentes e, devido a funções potencialmente fisiológicas, esta fração vem sendo explorada nos últimos anos (Chen et al., 2016).

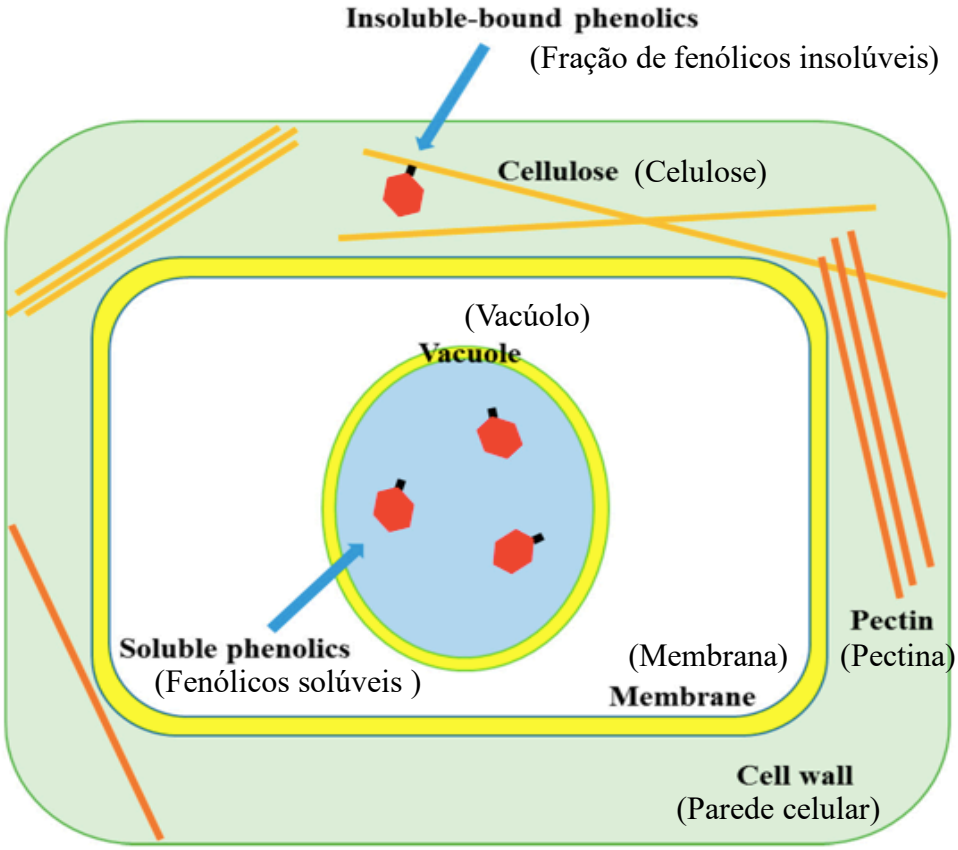


**Figura 3.** Localização dos compostos fenólicos solúveis e insolúveis na célula de plantas. Fonte: Traduzida de Shahidi; Yeo, 2016. Anexo III.

Quando há aumento do conteúdo de fenólicos totais atribui-se isso às transformações que ocorrem durante o processo de germinação e a síntese *de novo*, que normalmente acontece como resposta de proteção contra mudanças ambientais e para formação estrutural (Cevallos-

Casals & Cisneros-Zevallos, 2010). A síntese de compostos fenólicos tem como precursor original a glicose, e, várias vias de sinalização molecular importantes, incluindo as vias das pentose fosfato, glicólise, via do acetato/malonato e via de taninos hidrolisáveis, estando estas envolvidas na síntese e transformação dos diferentes compostos fenólicos (Gan et al., 2017). Alguns exemplos de vias bioquímicas de síntese de compostos fenólicos estão exemplificados nos esquemas 4, 5 e 6.

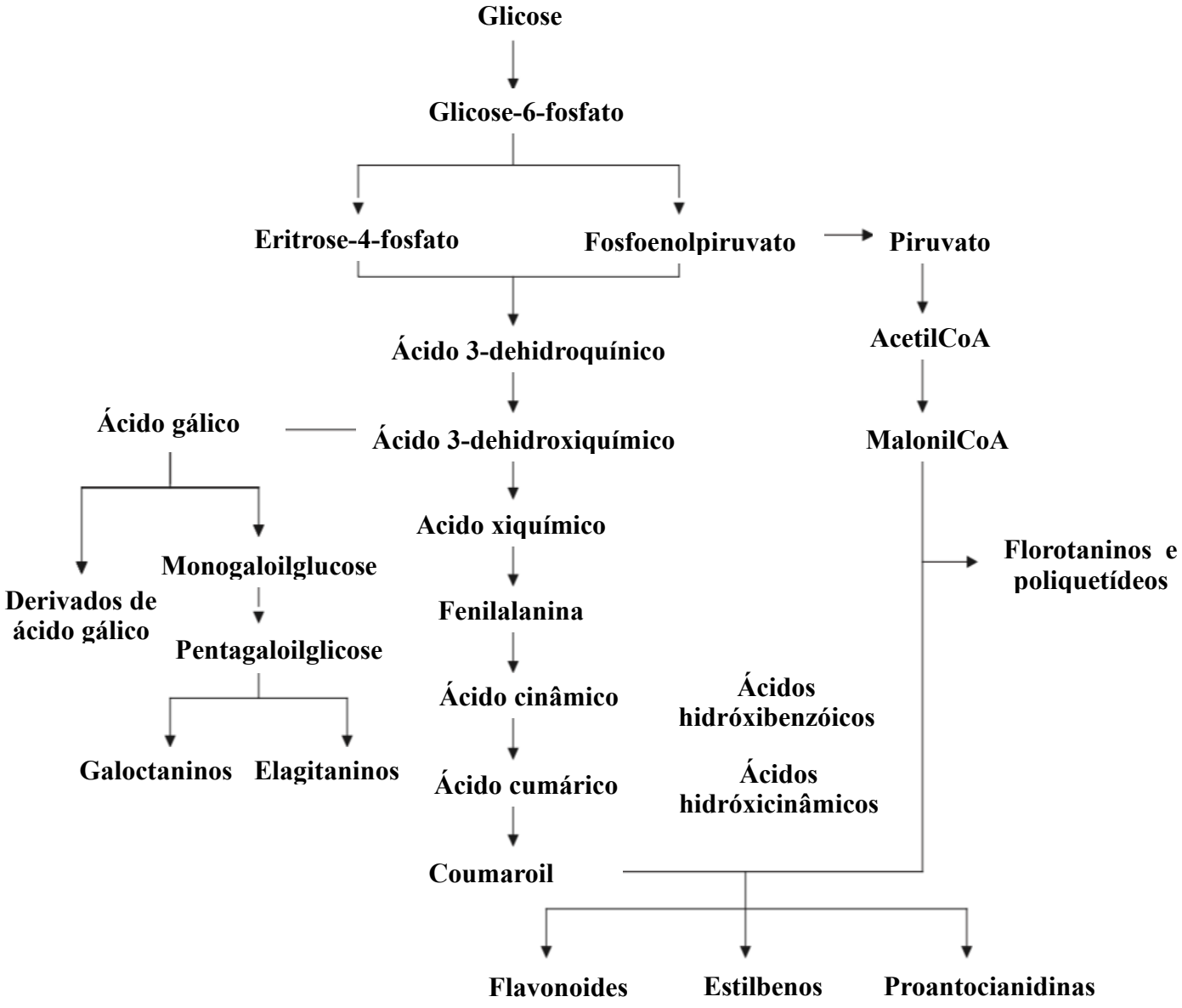


**Figura 4.** Via proposta para síntese e transformação de compostos fenólicos. Fonte: Traduzida de Gan at al., (2017). Anexo IV.

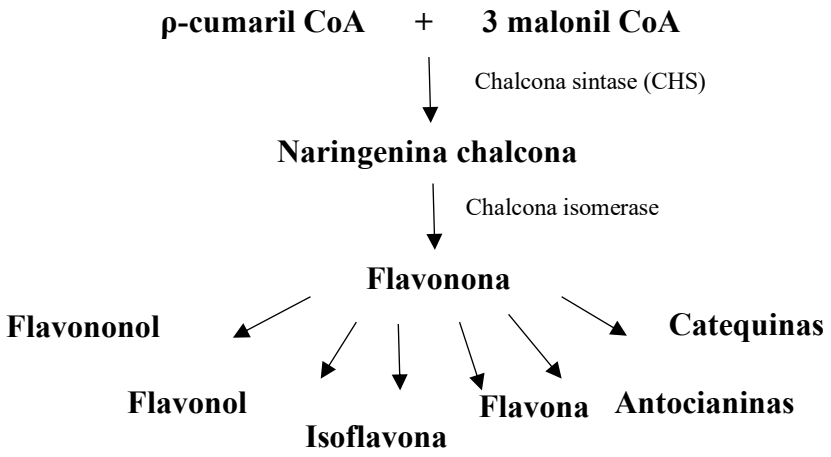


**Figura 5**. Via proposta para síntese de flavonoides. A formação do composto Naringenina chalcona se dá pela junção de ρ-cumaril CoA com 3 moléculas de malonil CoA por meio da ação da enzima chalcona sintase. A naringenina chalcona é convertida a flavonona pela ação da chalcona isomerase. Flavonona é convertida às demais formas de flavonoides por diversas vias enzimáticas. Baseado em Shahidi & Yeo (2016).

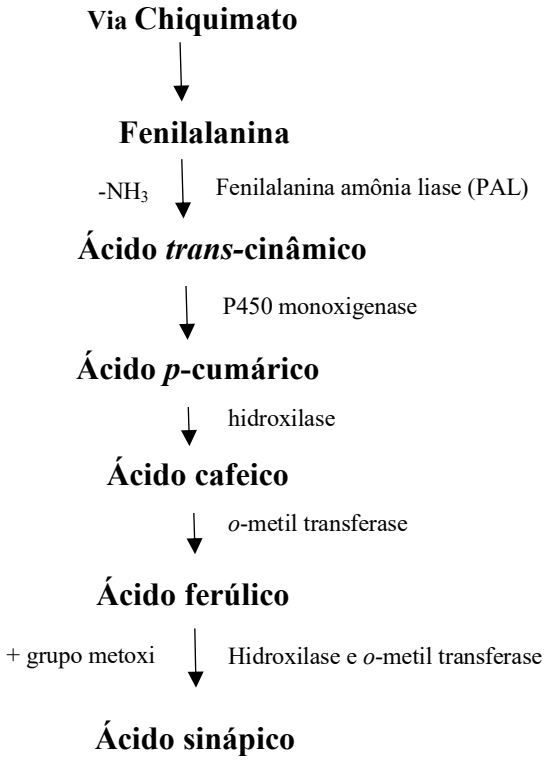


**Figura 6.** Via proposta de síntese de fenólicos totais. O aminoácido essencial fenilalanina, derivado da via bioquímica chamada de Chiquimato, por meio da ação da enzima fenilalanina amônia liase (PAL), perde uma amônia e gera ácido trans-cinâmico. O mesmo, após ação da enzima P450 monoxigenase é convertido a ácido p-cumárico, que pela ação da hidroxilase, gera ácido cafeico. Esse último, pela ação da enzima o-metil transferase é convertido a ácido ferúlico que é convertido a ácido sinápico pela ação da hidroxilase ou o-metil transferase. Baseado em Shahidi & Yeo (2016).

Além disso, nos períodos iniciais de germinação, os carboidratos, proteínas e lipídios são degradados e acompanhados consequentemente de um aumento de açúcares simples e aminoácidos livres (Nelson et al., 2013) e as frações de fenólicos insolúveis ligadas à parede celular também são liberadas. Por outro lado, com a formação de novas estruturas devido ao crescimento da planta durante a germinação, os compostos fenólicos solúveis podem se ligar à carboidratos e proteínas para a formação de novas paredes celulares, diminuindo sua quantidade na fração solúvel (Gan et al., 2017). Assim, a síntese e transporte de fenólicos para uso na parede celular ocorrem ao mesmo tempo (Yeo & Shahidi, 2015) o que faz com que a taxa de liberação e conjugação varie de acordo com a semente que está germinando (Gan et al., 2017). Trata-se, portanto, de um processo complexo, contínuo e peculiar de cada semente, e, com grande potencial de estudo. De fato, a literatura mostra diferentes pesquisas com o objetivo de entender e explicar as transformações de compostos fenólicos que ocorrem durante a germinação para a semente estudada.

Chen et al., (2017) observaram que diferentes variedades de trigo apresentaram mudanças diferenciadas na composição fenólica durante a germinação. A taxa de compostos fenólicos insolúveis e solúveis mudou positivamente de acordo com o tempo de germinação e variedade. Consequentemente, com maior teor de compostos fenólicos, a atividade antioxidante (medida pelos métodos ABTS e DPPH) também apresentou mudança positiva. Ácido ferúlico, ρ-cumárico e sinápico foram os principais ácidos fenólicos encontrados em grãos de trigo germinados. Ácido sinápico livre esteve presente em todas as variedades germinadas (4 dias) e para a fração insolúvel, um aumento de 60,4% foi detectado na variedade Jimai22 enquanto reduções de 29,9% e 13,5% foram reportadas para as variedades Sumai188 e Luyuan502, respectivamente.

O arroz marrom nativo (BR) foi germinado por 4 dias a 30°C. Os autores descreveram mudanças na composição fenólica e na atividade antioxidante no endosperma e no embrião. Os ácidos ferúlico, *p*-cumárico e sinápico foram os compostos mais encontrados, com uma variação interessante dos valores em cada parte do grão quando germinado. Existem muito mais ácidos fenólicos no embrião (especialmente ácido ferúlico e *p*-cumárico, que aumentaram 27 e 14 vezes, respectivamente, em relação ao grão não germinado e depois diminuíram) do que no endosperma. Além disso, a maior parte do ácido fenólico presente no grão é na forma insolúvel. A germinação pode alterar essa composição por meio da ação enzimática, causando mudanças na bioatividade (Cho & Lim, 2018).

Diferenças quantitativas e qualitativas notáveis foram identificadas em compostos fenólicos em comparação feita entre grãos de amaranto germinados e crus. Compostos não-flavonoides (ácidos hidroxibenzóicos e hidroxicinâmicos) foram os mais encontrados no grão

e no broto de amaranto. A germinação otimizada (26 °C por 63 h) aumentou significativamente ($p<0,05$) a concentração de compostos hidroxinâmicos, representando 97% do conteúdo total de fenólicos, sendo os ácidos 4-o-cafeiolquínico e o 4-o-feruloilquínico os principais compostos desse grupo nos brotos, e que não foram encontrados nos grãos sem germinação. Ainda, este processo causou aumento de 50% no conteúdo total de compostos hidroxibenzóicos, sendo o ácido vanílico o único detectado no brotos de amaranto (Paucar-Menacho et al., 2017).

Apesar das mudanças de diferentes formas de isoflavonas serem complexas durante a germinação, esse processo pode aumentar o conteúdo total de agliconas na soja (Huang, Cai, & Xu, 2014) . Em análise realizada por Cromatografia Líquida de Alta Eficiência relatou-se que o conteúdo total de agliconas, incluindo daidzeína, genisteína e gliciteína de grãos de soja foi significativamente maior ($p < 0,05$) em grãos germinados do que não germinados, o que significa que a germinação aumentou isoflavona na sua forma livre (Huang, Cai, & Xu, 2017). Outras pesquisas com resultados similares explicaram que isso pode ser atribuído às glicosidases ativadas e associadas à conversão de monômeros de isoflavona através das vias de malonato e fenilpropanoide (Wang et al., 2015).

Resultados mais específicos foram observados por Chen et al., (2016) que mostraram as mudanças causadas pela germinação em alpiste (na temperatura de 20°C variando tempo de germinação de 24 a 120 horas), onde o conteúdo de ácido gálico foi encontrado apenas em sua forma livre e aumentou gradualmente com o tempo de germinação. O ácido fotocatecuico foi encontrado apenas em sua forma insolúvel que foi mantida em conteúdo durante a germinação. O ácido hidroxibenzóico foi detectado na forma livre e insolúvel e ambas as formas foram modificadas durante o processo. O ácido vanílico foi encontrado apenas na forma insolúvel e a forma livre somente foi detectada em sementes não germinadas. Isso pode ser explicado pelo uso da fração solúvel durante a germinação para a formação da parece celular. O ácido cafeico foi detectado em alpiste apenas na forma insolúvel e foi mantido sem alterações durante a germinação. O ácido siríngico, por sua vez, foi detectado apenas em sementes não germinadas na forma livre. Já o ácido *p*-cumárico estava presente principalmente na forma insolúvel e apresentou aumento durante a germinação. O ácido ferúlico foi observado nas frações livres e insolúvel com aumento em ambas durante a germinação. Apesar do conhecimento dos mecanismos de síntese e regulação serem limitados durante a germinação, esse artigo mostra resultados consistentes do que acontece com cada composto durante a germinação de alpiste.

Ti et al. (2014) também elucidaram as diferentes mudanças durante a germinação de arroz marrom (a 20°C, no escuro por 17, 24, 30, 35 e 48 horas). Os autores observaram que o ácido fotocatecuico foi encontrado apenas em sua forma livre nos grãos germinados e cru, apresentando aumento após 48 horas de germinação. Os ácidos clorogênico e cafeico foram

detectados na forma livre com o primeiro mantendo-se estável durante o processo e o segundo apresentando aumento durante as 48 horas de germinação. O ácido siríngico, presente nas frações livre e insolúvel aumentou durante as 48 horas de germinação. Já o ácido cumárico, apenas detectado na forma insolúvel também aumentou durante todo o tempo de germinação. Por último, o ácido ferúlico foi observado principalmente na forma insolúvel e aumentou durante todo o tempo de processo.

Em termos de classes de fenólicos totais, outro estudo observou o comportamento do composto tocoferol durante a germinação. Sementes de *Chamaerops humilis* var. *humilis* mostraram altos níveis de tocoferol, incluindo α-tocotrienol quando em dormência. Durante a germinação, os autores observaram a síntese de α-tocoferol no embrião, que foi associada à proteção de lipídeos contra peroxidação lipídica durante a germinação. Os brotos apresentaram, portanto, conteúdos de α-tocotrienol (principalmente devido à presença no embrião e endosperma) e α-tocoferol com efeitos antioxidantes (Siles et al., 2015).

Claramente, cada grão, com condições específicas de germinação, tem mudanças peculiares de transformação, demonstrando que é impossível generalizar as transformações fenólicas que ocorrem durante a germinação e a necessidade da realização de estudos específicos para cada semente e grão.

## 6. Mostarda, por quê?

As especiarias despertam interesse desde tempos passados na humanidade e são amplamente utilizadas em produtos farmacêuticos, nutracêuticos, perfumaria, indústria alimentícia e cosméticos (Aruna & Baskaran, 2010). A mostarda, em especial, é conhecida mundialmente por séculos como um condimento (Fahey, 2016) sendo comercialmente importante em diversas partes do mundo, com grande variedade de uso e aplicações. É comum dos Estados Unidos, Reino Unido, Canadá, países Europeus, Índia, China e países da Ásia (Rana, 2016).

A mostarda é classificada em três diferentes espécies: *Brassica nigra, Sinapis alba* e *Brassica junceae*, popularmente conhecidas como mostarda preta, branca e amarela, respectivamente (Sforza; Prandi, 2016). A mostarda preta (*Brassica nigra)* pertence à família *Brassicaceae* e ao gênero *Brassica L.* e é a mais pungente das três; a mostarda branca (*Sinapis alba*) pertence à mesma família e ao gênero *Sinapis L.* ("Cover Crops | USDA PLANTS", 2017) e apresenta pungência média. A pungência presente nos grãos é devido à presença de AITC (alil isotiocianato), composto solúvel em óleo (Morley, 2016), que tem potencial de ser usado como antibacteriano, antifúngico, antifermentativo e pode evitar escurecimento na indústria de alimentos (Divakaran & Babu, 2016).

De maneira geral, os vegetais *Brassica* são importantes fontes alimentares de moléculas nutritivas e benéficas, como glucosinolatos, carotenoides, flavonoides, vitamina C e tocoferóis, que possuem propriedades antioxidantes e anticancerígenas (Li, et al., 2017). Os grãos de mostarda apresentam uma grande variedade de minerais e ácidos graxos ômega 3, óleos essenciais, vitaminas do complexo B e vitamina E (Divakaran & Babu, 2016). Ainda, estudos na literatura têm demonstrado que as sementes de mostarda apresentam diversos compostos biologicamente ativos com potencial para diminuição da glicemia, redução do acúmulo de lipídeos e de colesterol, além de propriedades antioxidante, anti-inflamatória e anticancerígena (Khan et al., 1997; Grover et al., 2002, 2003; Thirumalai et al., 2011; Divakaran & Babu, 2016).

Com base na análise de relatórios estratégicos produzidos por institutos de referência mundiais, a FIESP e o ITAL elaboraram o relatório Brasil Food Trends 2020, que aponta as tendências e exigências recentes dos consumidores mundiais de alimentos, agrupadas em 5 categorias: 1) Sensorialidade e prazer; 2) Saudabilidade e bem-estar; 3) Conveniência e praticidade; 4) Confiabilidade e qualidade e 5) Sustentabilidade e ética (Barbosa et al., 2017).

O grão de mostarda encaixa-se na categoria saudabilidade e bem-estar, visto que além dos benefícios para a saúde já descritos anteriormente, por ser um condimento diferente, pode diminuir o teor de sódio presente nos alimentos, outra grande tendência da alimentação. No caso, a germinação seria um processamento natural e clean label para sua aplicação, o que também é bem visto pelos consumidores segundo as tendências reportadas pelo relatório Brasil Food Trends 2020, no quesito inovação e facilidade.

## 7. Tendências futuras de pesquisa

Já é de conhecimento da literatura que a germinação causa mudanças estruturais nos compostos fenólicos das sementes e grãos, e isso pode mudar as propriedades biológicas e de bioacessibilidade dos compostos bioativos. Assim sendo, utilizar testes biológicos *in vitro* (com linhagens celulares ou digestão simulada, por exemplo) ou mesmo testes *in vivo* são propostas de continuação dos estudos, visto que avaliam uma perspectiva biológica dos ensaios mais próxima de situações reais.

Wang et al. (2016) por exemplo, realizaram o primeiro estudo focado na cinética da atividade antioxidante de linhaça e brotos durante a germinação (8 a 10 dias) por atividade antioxidante celular (CAA). Para tal, utilizaram células humanas de câncer de fígado (HepG2). Os resultados obtidos sugeriram que a linhaça e seus brotos são uma fonte alternativa de antioxidantes celulares para aplicações futuras. Essa metodologia é útil para examinar a biodisponibilidade de antioxidantes alimentares, porque leva em consideração diversos fatores

como absorção celular e metabolismo, possibilitando a obtenção de uma perspectiva biológica mais realista (Kellett, Greenspan, & Pegg, 2018).

Ainda, por meio da indução de estresse oxidativo em culturas celulares, Wang et al. (2015) observaram que o extrato de soja chinesa germinada foi capaz de proteger as células contra estresse oxidativo sem morte celular, mostrando outro exemplo de estudo *in vitro* com culturas celulares.

Além disso, a digestão *in vitro* é outa tendência para estudos em grãos germinados devido à sua abordagem fisiológica. Apesar de ser comum, poucos estudos utilizaram dessa técnica para analisar grãos germinados e os produtos derivados destes, como extratos. Trata-se de um teste biológico *in vitro* que pode mostrar a bioacessibilidade de compostos fenólicos e eficiência antioxidante dos brotos (Pérez-Burillo; Rufián-Henares & Pastoriza, 2018). Świeca, Sęczyk, & Gawlik-Dziki, (2014) simularam o processo de digestão *in vitro* e observaram a liberação dos compostos fenólicos presentes no broto de lentilha apesar dos flavonoides estarem pouco bioacessíveis. A complexidade da digestão e a interação com outros compostos também foi discutida.

A falta de pesquisas com esse foco para produtos germinados mostra o alto potencial de estudo nessa área. A figura 7 ilustra o caminho proposto para obtenção de resultados seguros e consolidados, onde a combinação e sequência adequada de métodos é crucial.

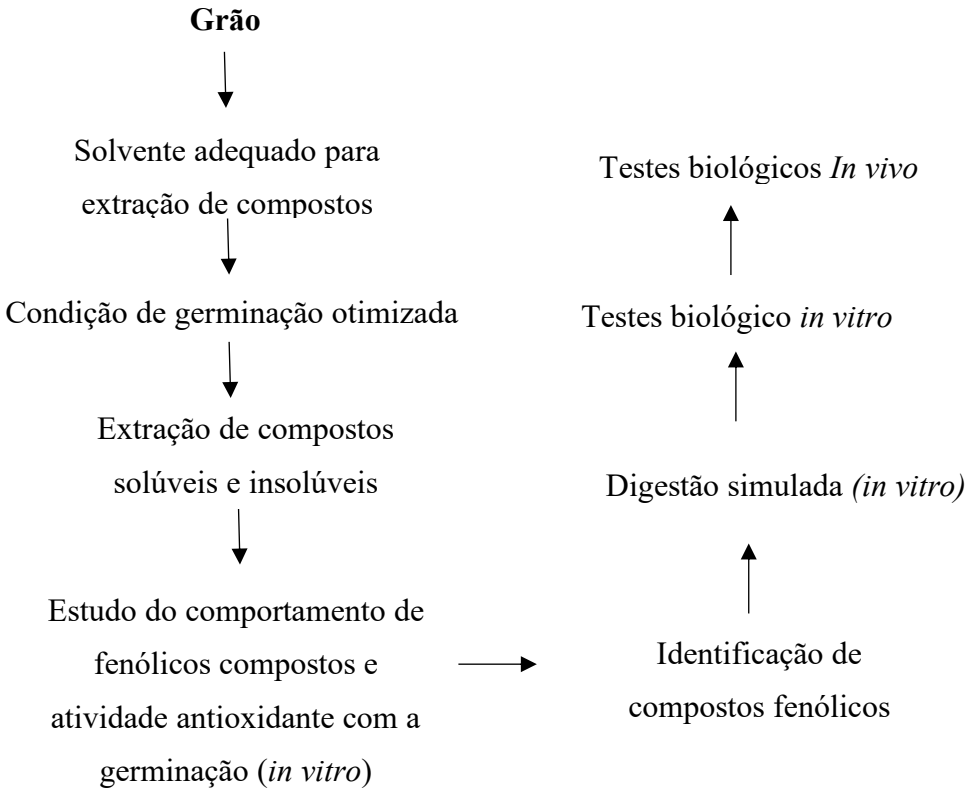


**Figura 7.** Caminho proposto para pesquisa de grãos germinados

## 8. Conclusão

O processo de germinação mostra-se como uma excelente alternativa para melhorar a capacidade antioxidante de sementes e grãos, como discutido nessa contribuição. Os compostos fenólicos nas duas frações, livre e insolúvel, são transformados positivamente durante o processo de germinação melhorando a composição fitoquímica das sementes e consequentemente suas propriedades antioxidantes. Apesar de algumas mudanças serem similares durante a germinação, cada grão deve ser estudado de forma específica para definição dos tratamentos mais adequados. A revisão de literatura aqui apresentada mostrou claramente a necessidade de identificação e caracterização de compostos fenólicos, além da realização de ensaios que simulem de forma mais próxima dos eventos reais no organismo, como o processo de digestão e/ou testes *in vivo*, no sentido de dar a dimensão factual da atuação destes compostos em sistemas complexos.

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# Capítulo II: Sinergy between different solvents increased the extraction of antioxidant compounds from white (*Sinapsis alba*) and black (*Brassica nigra*) mustard grains

Gabriela Boscariol Rasera[a*], Ruann Janser Soares de Castro[a]
Department of Food Science, School of Food Engineering, University of Campinas.
80 Rua Monteiro Lobato, Campinas – SP, Brazil
*Corresponding author:
E-mail address: gabi_boscariol@hotmail.com

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**Abstract**

Mustard grains are worldwide consumed as an important condiment. These grains are interesting source of bioactive substances, among them, phenolic compounds with antioxidant properties deserve to be highlighted. To recover these compounds, it is known that the solvents used for extraction must be studied. Thus, this work aimed to determine the best solvent mixture for extraction of antioxidant compounds from two varieties of mustard grains (white - *Sinapsis alba* and black - *Brassica nigra*) using a simplex centroid mixture design. For this, the experiments were performed using pure, binary or ternary solvent mixtures containing water, acetone and methanol. All extracts were analyzed for total phenolic compounds (TPC), DPPH- and ABTS-radical scavenging activities. The binary mixture of water and acetone, in equal proportions, was the best solvent combination to obtain an extract with higher TPC content and antioxidant properties. At this condition, white mustard extract showed 19.39 mg of gallic acid equivalents per g (mg GAE $g^{-1}$) of dry and defatted mustard for total phenolic compounds, 109.25 and 51.25 μmol of Trolox equivalents per g of sample (μmol TE $g^{-1}$) for ABTS and DPPH, respectively. The extraction of antioxidant compounds with water/acetone resulted in increases of 23-folds for TPC, 48-folds for ABTS and 25-folds for DPPH compared to pure acetone. For black mustard, TPC was 12.16 mg GAE $g^{-1}$, ABTS and DPPH 36.22 and 26.24 μmol TE $g^{-1}$. The extract obtained with this solvent combination was 19- 31- and 27-folds higher than that produced with pure acetone for TPC, ABTS and DPPH, respectively. Results showed the antioxidant potential of mustard grains not explored by literature yet. Despite, it was elucidated the best solvent for extraction of phenolic compounds and obtaining of samples with better antioxidant properties from mustard grains.

**Keywords:** mustard grains; phenolic compounds; simplex centroid mixture design.

## 1. Introduction

Consumer interest in spices is present in humanity since the past. They are also widely used in pharmaceuticals, nutraceuticals, perfumery, toiletry, cosmetics industries and for centuries in Indian traditional medicinal systems (Aruna and Baskaran, 2010). Mustard, in special, is known for centuries as an important commercially condiment in all of the world with a huge variety of use and applications (Fahey, 2016). Mustard grains are classified in three different species: *Brassica nigra, Sinapis alba* and *Brassica junceae*, popular know as black mustard, white mustard and yellow mustard, respectively (Sforza & Prandi, 2016). In general, the *Brassica* vegetables are sources of glucosinolates, carotenoids, flavonoids, vitamin C and tocopherol that have antioxidant and anticancer properties (Li et al., 2017). Mustard grains are also rich in minerals, Omega-3 fatty acid, essential oils, vitamins B and E (Divakaran and Babu, 2016).

Fruits, legumes, spices, herbs and grains are the major sources of phenolic compounds. The high intake of them is associated to lower risks of chronic and degenerative disease's development caused by oxidative stress. Known as secondary plant metabolism, phenolic compounds are the front-line defense of plants and can be divided into several sub-groups according to their structural characteristics. The three main sub-groups most commonly found in plant food are phenolic acids, flavonoids and non-flavonoids. In addition, the antioxidant properties of these compounds are strictly related with their molecular structure. Thus, some characteristics showed recognized relevance in their antioxidant properties, such as: the number and the position of hydroxyl groups in the molecule, the hydroxylation degree, the distance between carbonyl group and the aromatic ring besides the number of aromatic rings (Zhang and Tsao, 2016).

The extract yield of phenolic compounds can be affected by several factors as the solvent chemical nature, solvent concentration, solvent:solid ratio, extraction time, temperature and particle size of the plant material. Besides that, pure solvents cannot extract all the antioxidants with their different polarities and structures (Garcia-Salas et al., 2010). Therefore, it is convenient the use of a solvent's mixture, that can be binary, ternary or even multicomponent mixture. In this system, it is possible to observe the solvent-solvent interaction in the solute's solvation competition and the synergistic effect between them (Marcus, 2002). In addition, it is already known the importance of that, solvent optimization to obtain extracts rich in antioxidant compounds from mustard grains is not found in literature yet.

In this work, a simplex centroid mixture design was used to study the most adequate solvent mixture for extraction of antioxidant compounds from two varieties of mustard grains (*Brassica nigra* and *Sinapsis alba*). The total phenolic compounds and the antioxidant

properties evaluated by DPPH and ABTS-radical scavenging were used as responses to select the best extractor.

## 2. Material and Methods

### 2.1 Material

Samples of two varieties of mustard grains, namely black (*Brassica nigra*) and white (*Sinapsis alba*), were purchased in a local market from Piracicaba (Sao Paulo, Brazil). The Folin and Ciocalteau's phenol reagent, sodium carbonate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), potassium persulphate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the HPLC standarts were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were purchased in the grade commercially available.

### 2.2 Simplex centroid mixture design

The experimental mixture design was used to obtain the optimum mixture of the different solvents for maximum extraction of phenolic compounds, antioxidant activities and to investigate the presence of either synergistic or antagonistic effects in a blend of the components. A three-component augmented simplex centroid design was employed in which each component was studied at six levels, namely 0 (0%), 1/6 (16.67%), 1/3 (33%), 1/2 (50%), 2/3 (66.67%) and 1 (100%) (Table 1).

Quadratic or special cubic regression models were fitted for the variations of all the responses studied as a function of significant ($p \leq 0.10$) interaction effects between the proportions, thereby obtaining acceptable determination coefficients ($R^2 \geq 0.70$). Equation 1 represents these models as follows:

$$Yi = \sum_{i=1}^{q} \beta iXi + \sum\sum_{i<j}^{q} \beta ij\, XiXj + \sum \sum_{i<j<k}^{q} \sum \beta ijkXiXjXk$$

where '$Y_i$' is the predicted response; '$q$' represents the number of components in the system; '$X_i$, $X_j$, $X_k$' are the coded independent variables; '$\beta_i$' is the regression coefficient for each linear effect term; and '$\beta_{ij}$' and '$\beta_{ijk}$' are the binary and ternary interaction effect terms, respectively. Statistica® 13.3 software from TIBCO (Palo Alto, California, USA) was employed for the experimental design, data analysis and model building.

**Table 1** – Matrix of the simplex centroid mixture design for extraction of antioxidant compounds from mustard grains using different solvents and their mixtures.

| Run | Independent variables | | |
|---|---|---|---|
| | **Water** | **Acetone** | **Methanol** |
| | $x_1$ | $x_2$ | $x_3$ |
| **1** | 1 | 0 | 0 |
| **2** | 0 | 1 | 0 |
| **3** | 0 | 0 | 1 |
| **4** | 1/2 | 1/2 | 0 |
| **5** | 1/2 | 0 | 1/2 |
| **6** | 0 | 1/2 | 1/2 |
| **7** | 2/3 | 1/6 | 1/6 |
| **8** | 1/6 | 2/3 | 1/6 |
| **9** | 1/6 | 1/6 | 2/3 |
| **10** | 1/3 | 1/3 | 1/3 |

To confirm the validity of the models, three assays were performed under the most adequate condition according to the mixture design and the experimental values were compared with the predicted values by the models within a 95.0% confidence interval.

The recovering of antioxidant compounds was performed according to each assay showed in the Table 1. For this, defatted (with hexane, four times) and dry samples were mixed with the solvent (25 mg/mL) and maintained under stirring (150 rpm) for 20 minutes at 25°C (Yeo and Shahidi, 2017). After extraction, the solution was centrifuged (17 000 x g) for 15 minutes, and the supernatant was collected and stored at -18°C for further analysis. Ten extracts were obtained and analyzed to their total phenolic compounds (TPC) content and antioxidant properties using two methods (ABTS- and DPPH-radical scavenging). Test were executed in triplicate.

### 2.3 Total phenolic content (TPC)

The total phenolics were estimated according to the method of Swain and Hillis (1959) with a modified version as described by de Camargo et al. (2012). The Folin and Ciocalteau's phenol reagent (0.5 mL) was mixed with 0.5 mL of extracts in a tube, and 4 mL of distilled water. After 3 minutes of incubation, 1 mL of saturated sodium carbonate solution (0.3 g $mL^1$) was added to each tube. The reaction mixtures were allowed to stand for 2 hours at room temperature in the dark. The absorbance was read at 760 nm. The total amount of phenolic

compounds was expressed as mg of gallic acid equivalents (GAE) per gram dry weight of defatted sample (mg GAE $g^{-1}$)

### 2.4 ABTS radical cation scavenging activity

The ABTS assay (Al-Duais et al., 2009) was performed using a microplate reader Spectra Max M3 (Molecular Devices, LLC, Sunnyvale, CA, USA). The ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] radical cation (generated by oxidation with potassium persulphate) was prepared in 75 mM potassium phosphate buffer saline solution (PBS) (pH 7.4). At the time of analysis, the working solution of ABTS radical cation (potassium persulphate (140 mM, 0.088 mL) and ABTS (7 mM, 10 mL) in PBS) was prepared by diluting its stock solution in PBS to reach an absorbance value of 0.70 ± 0.20 (734 nm).

Mustard extracts were diluted in PBS to reach a final concentration of 2.5 mg $mL^{-1}$. Aliquots of 20 µL of each extract were added to 220 µL of ABTS radical cation solution and the absorbance was read at 734 nm after 6 min. The control assay was made with distilled water in place of the samples. A standard curve was prepared using different concentrations of Trolox (2.5-200 µM) and the results were expressed as µmol of Trolox equivalents per g of sample (µmol TE $g^{-1}$).

### 2.5 DPPH radical scavenging activity

DPPH radical scavenging activity was carried out according to the method described by Al-Duais et al. (2009). Briefly, 134 µL of 150 µM DPPH radical solution, which was freshly made in ethanol, was added to 66 µL of appropriately diluted extracts (2.5 mg $mL^{-1}$) or standards. After 45 min of incubation at room temperature in the dark, the absorbance was measured at 517 nm using a microplate reader Spectra Max M3 (Molecular Devices, LLC, Sunnyvale, CA, USA). Ethanol was used as blank. A standard curve was prepared with different concentrations of Trolox (20-140 µM) and the results were expressed as µmol of Trolox equivalents per g of sample (µmol TE $g^{-1}$).

### 2.6 Identification and quantification of phenolic compounds by reversed-phase high performance liquid chromatography (RP-HPLC)

High performance liquid chromatography (HPLC) analyses (Melo et al., 2015) were carried out using an analytical HPLC unit, equipped with Shimadzu ODS-A column (4.6 mm × 250 mm, 5 µm) and photodiode array detector (SPD-M10AVp, Shimadzu Co., Kyoto, Japan). Mustard extracts (20 µL) diluted (3%) with methanol were injected in the HPLC system at a flow rate of 1.0 mL $min^{-1}$. For the analyses of all phenolic compounds, the mobile phase consisted of water/formic acid (99.9/0.1, v/v) (A) and acetonitrile/formic acid (99.9/0.1, v/v)

(B), starting with 5% B and increasing to 7% B (7 min), 20% B (50 min), 45% B (70 min), 100% B (85 min), held at 100% B for 10 min, and decreasing to 5% B (100 min). The column was maintained at a constant temperature of 28 °C. The chromatograms were analyzed using Class-VP® software. The following authentic standards (Sigma–Aldrich, St. Louis, MO, USA) were examined: galic acid, caffeic acid, ferulic acid, p-coumaric acid, synapic acid, siringic acid, vanillic acid, trans-cinnamic acid, catechin, epicatechin, epicatechin gallate, chrysin, hesperidin, hesperitin, luteolin, mirecetin, polydatin, procyanidin B1, procyanidin B2, quercetin, rutin, resveratrol and vitexin. In addition, to confirm the identity of the compound, standard co-chromatography was also performed.

Limits of detection and quantification (LOD and LOQ) were calculated using the following equations, respectively:

$$(3)\ LOD = 3.3 \times s \div S$$

$$(4)\ LOQ = 10 \times s \div S$$

where $s$ is the estimated standard deviation of the linear coefficient of the equation and $S$ is the slope of the analytical curve.

## 3 Results and Discussion

The results for each assay of the mixture design to TPC, DPPH- and ABTS-radical scavenging of black and white mustard extracts are presented in Tables 2 and 3. The extracts obtained from white mustard showed TPC ranging from 0.82 to 20 mg GAE $g^{-1}$, ABTS-radical scavenging ranging from 2.25 to 109.25 µmol TE $g^{-1}$ and DPPH-radical scavenging ranging from 2.01 to 58.7 µmol TE $g^{-1}$. Extracts from black mustard showed values of TPC ranging from 0.65 to 12.16 mg GAE $g^{-1}$, ABTS-radical scavenging ranging from 1.14 to 36.22 µmol TE $g^{-1}$ and DPPH-radical scavenging ranging from 0.95 to 26.24 µmol TE $g^{-1}$. It is important to note that this great variation observed between the values shows the importance of the study to determinate the best solvent combination to extract antioxidant compounds. Further, choose the most appropriated solvent for each plant material is one of the most important factors to obtain a higher bioactive compounds content (González-Montelongo et al., 2010).

**Table 2.** Responses for TPC, ABTS- and DPPH-radical scavenging from the mixture design used to study the most adequate solvent to obtain extracts rich in phenolic compounds and high antioxidant properties from white mustard grain.

| Run | TPC (mg GAE $g^{-1}$) | ABTS (μmol TE $g^{-1}$) | DPPH (μmol TE $g^{-1}$) |
|---|---|---|---|
| **1** | 12.48 ± 0.65 | 72.24 ± 4.18 | 43.89 ± 0.32 |
| **2** | 0.82 ± 0.27 | 2.25 ± 0.18 | 2.02 ± 0.14 |
| **3** | 10.48 ± 0.29 | 61.75 ± 3.55 | 30.91 ± 1.09 |
| **4** | 19.39 ± 0.66 | 109.25 ± 4.61 | 51.25 ± 0.08 |
| **5** | 18.49 ± 0.61 | 105.86 ± 0.68 | 50.18 ± 0.71 |
| **6** | 7.89 ± 0.48 | 74.79 ± 1.67 | 44.78 ± 0.08 |
| **7** | 20.00 ± 0.33 | 97.13 ± 5.14 | 47.69 ± 1.03 |
| **8** | 19.52 ± 0.61 | 99.52 ± 1.76 | 57.44 ± 0.55 |
| **9** | 19.41 ± 0.65 | 93.97 ± 4.25 | 53.14 ± 0.31 |
| **10** | 16.84 ± 0.02 | 108.05 ± 1.45 | 58.70 ± 0.63 |

**Table 3.** Responses for TPC, ABTS- and DPPH-radical scavenging from the mixture design used to study the most adequate solvent to obtain extracts rich in phenolic compounds and high antioxidant properties from black mustard grain.

| Run | TPC (mg GAE $g^{-1}$) | ABTS (μmol TE $g^{-1}$) | DPPH (μmol TE $g^{-1}$) |
|---|---|---|---|
| **1** | 5.32 ± 0.08 | 6.21 ± 0.82 | 8.88 ± 0.08 |
| **2** | 0.65 ± 0.00 | 1.14 ± 0.03 | 0.95 ± 0.18 |
| **3** | 4.56 ± 0.02 | 13.30 ± 1.00 | 15.40 ± 0.62 |
| **4** | 12.16 ± 0.71 | 36.22 ± 1.10 | 26.24 ± 0.28 |
| **5** | 8.02 ± 0.03 | 20.61 ± 1.80 | 17.24 ± 0.83 |
| **6** | 3.05 ± 0.04 | 8.62 ± 0.50 | 7.47 ± 0.36 |
| **7** | 9.60 ± 0.54 | 25.09 ± 0.59 | 20.17 ± 0.85 |
| **8** | 10.00 ± 0.15 | 24.63 ± 0.38 | 24.00 ± 2.60 |
| **9** | 6.21 ± 0.13 | 14.15 ± 0.25 | 11.16 ± 0.04 |
| **10** | 7.84 ± 0.33 | 16.73 ± 1.23 | 15.05 ± 0.75 |

The analysis of variance (ANOVA) test indicated that the *p*-values for the assays were less than 0.05, demonstrating that the proposed models showed high significance at a 95% confidence level. The coefficient of determination value ($R^2$) of the model could be used to check the variability of the experimental data. Values of $R^2$ ranged from 0.71 to 0.98 indicated that the models were able to explain 71 to 98% of the experimental data variability. The computed *F*-values for regressions were greater than the tabulated *F*- values, reflecting the

statistical significance of the models. Equations in Table 4 represent the models with significant factors for experimental data. Statistical analysis showed that the models were predictive for TPC, ABTS and DPPH-radical scavenging of the mustard extracts.

The variations in the TPC and antioxidant properties of the extracts obtained from black and white mustards were also depicted using mixture contour plots (Figures 1 and 2). A contour plot furnish a dimensional view where all the points that have the same results are linked to create contour lines of constant responses (Rao and Baral, 2011). On the response surfaces, each factor (pure mixture component) is represented in the corner of an equilateral triangle, and each point within this triangle refers to a different proportion of the components in the mixture. Besides, each ingredient in maximum percentage is placed at corresponding corner, and with the minimum is positioned at the middle of the opposite side of the triangle (Martinello et al., 2006).

Contour plot evaluation indicated that the best extraction solvent was the binary combination between water and acetone for both, white and black mustard. The highest efficiency of the water:acetone mixture in the phenolic compounds extraction was also observed for others authors (González-Montelongo et al., 2010; Meneses et al., 2013). Besides that, the use of ethanol and acetone is recommended, mainly because they are less toxic than other organic solvents as methanol and have high extraction efficiency (Socaci et al., 2018).

TPC, ABTS- and DPPH-radical scavenging contour plots interpretation for both mustard varieties showed similar profiles for each solvent and mixtures of them, indicating that the mixture of water and acetone was the most appropriate combination to recover antioxidant compounds. In addition, it is possible to observe a clear relationship between the content of phenolic compounds and the antioxidant properties of the extracts.

Validation essays were done, in genuine triplicate, to confirm the predictive capacity of proposed models and the results are showed in Table 4. The experimental values agreed with the values predicted by the models within a 95% confidence interval, confirming the models validity for the evaluated responses (Table 5).

In general, more polar phytochemicals compounds can be extract with water, while phenolic compounds with high hydroxylated aglycones are soluble in alcohols like ethanol and methanol (Arts and Hollman, 1998). Acetone, as a pure solvent, was inefficient for the recovering of the interest compounds. Thus, knowing that less polar solvents, like ethyl acetate, acetone and chloroform have more affinity by compounds with low polarity (Lafka et al., 2007), it is assumed that a few compounds with these chemical characteristics were found in the mustard extracts evaluated. On the other hand, the combination of different solvents resulted in interesting synergistic effects. Although the binary combination of water and methanol had an

important synergistic effect, considering a common condition of extraction for the two varieties of mustard, the mixture of water and acetone, in equal proportions, resulted in the highest values for TPC and antioxidant properties, an indicative that the majority antioxidant compounds of white and black mustard have high affinity for these two solvents. According to some authors, the mixture of water and organic solvents is able to create a more polar condition, which can facilitate the water and/or organic solvents extraction of soluble phenolic compounds (Do et al., 2014; Meneses et al., 2013). Socaci et al. (2018) elucidated that extraction yield is reduced because phenolic compounds in general are more soluble in organic solvents less polar than water, and, that's the reason why suitable proportion study between water and an organic solvent is a decisive factor in the phenolic compounds extraction.

Alcântara et al. (2019) investigated the efficiency of the extraction of phenolic compounds from seeds of chia, using a simplex-lattice design. The solvents used were acetone, ethanol and water. The authors concluded that the addition of water to organic solvents improved the extraction efficiency, since mixtures with moderate polar solvents were highly efficient in the extraction. It was found TPC, DPPH and FRAP values of 58.44 mg GAE/g, 250.20 μmol TE/g and 720.15 μmol TE/g extract of chia seeds, respectively, when the extraction was performed with the binary mixture of water (1/3) and acetone (2/3).

Moreover, Fernández-Agulló et al. (2013) also demonstrated that the addition of water to the organic solvents improved the extraction efficiency of bioactive compounds from green husk. They used water, methanol, ethanol and 50% aqueous solutions of methanol and ethanol and measured total phenols content and antioxidant activities by reducing power assay and DPPH-radical scavenging. The solvents used showed a significantly different extraction capacity and the extract with higher phenolic content and antioxidant capacity was 50% aqueous solution of ethanol.

Boulekbache-Makhlouf et al. (2013) determined that for the phenolic compounds extraction of eggplant by-product, the most suitable solvent was 70% acetone that extracted the highest content of phenolics, flavonoids and tannins, when compared to 70% methanol and 70% ethanol. The authors also analyzed the efficiency of antioxidant activity (ferric reducing power, DPPH-radical scavenging, scavenging capacity of hydrogen peroxide and metal chelating activity). Results showed that for all methods, except DPPH, the phenolic extract with acetone 70% was the more adequate solvent.

**Table 4.** ANOVA and codified mathematic models for total phenolic compounds (TPC and antioxidant activity (ABTS and DPPH) from white and black mustard extracts with different solvents.

| Response | Model | Equations | *F calculated* | *Ftabulated* | $R^2$ | *p-value* |
|---|---|---|---|---|---|---|
| | | **White mustard** | | | | |
| TPC | Quadratic | $14.73x_1+3.29x_2+15.02x_3+58.06\ x_1x_2$ | 4.92 | 3.37 | 0.71 | 0.04 |
| ABTS | Quadratic | $87.76x_1+5.2x_2+78.69x_3+272.33x_1x_2+168.30\ x_2x_3$ | 6.07 | 3.48 | 0.82 | 0.03 |
| DPPH | Quadratic | $48.22x_1+3.96x_2+37.35x_3+113.68x_1x_2+117.94x_2x_3$ | 6.34 | 3.48 | 0.83 | 0.03 |
| | | **Black mustard** | | | | |
| TPC | Quadratic | $5.21x_1+0.68x_2+5.06x_3+36.97x_1x_2+13.52x_1x_3$ | 75.85 | 3.48 | 0.98 | <0.001 |
| ABTS | Quadratic | $6.71x_1+0.16x_2+13.34x_3+123.58x_1x_2+38.85x_1x_3$ | 43.18 | 3.48 | 0.97 | <0.001 |
| DPPH | Quadratic | $12.14\ x_1-0.67x_2+16.77x_3+85.70x_1x_2$ | 21.19 | 3.37 | 0.91 | 0.001 |

**Table 5.** Predicted and experimental results obtained with the most suitable conditions from white and black mustard extracts for models' validation

| | **White mustard** | | | | |
|---|---|---|---|---|---|
| **Response** | **Independent variables (validation assays)** | | | **Predicted values** | **Experimental values** |
| | **$x_1$ (water)** | **$x_2$ (acetone)** | **$x_3$ (methanol)** | | |
| TPC | 0.5 | 0.5 | 0 | 23.51[a] | 23.03 ± 4.22[a] |
| ABTS | 0.5 | 0.5 | 0 | 114.56[a] | 123.28 ± 12.73[a] |
| DPPH | 0.5 | 0.5 | 0 | 54.49[a] | 57.82 ± 4.79[a] |
| | | | **Black mustard** | | |
| TPC | 0.5 | 0.5 | 0 | 12.18[a] | 11.21 ±1.74[a] |
| ABTS | 0.5 | 0.5 | 0 | 34.32[a] | 34.78 ± 0.98[a] |
| DPPH | 0.5 | 0.5 | 0 | 27.16[a] | 26.22 ± 0.60[a] |

Identical letters in the same line were not significantly different ($p > 0.05$).

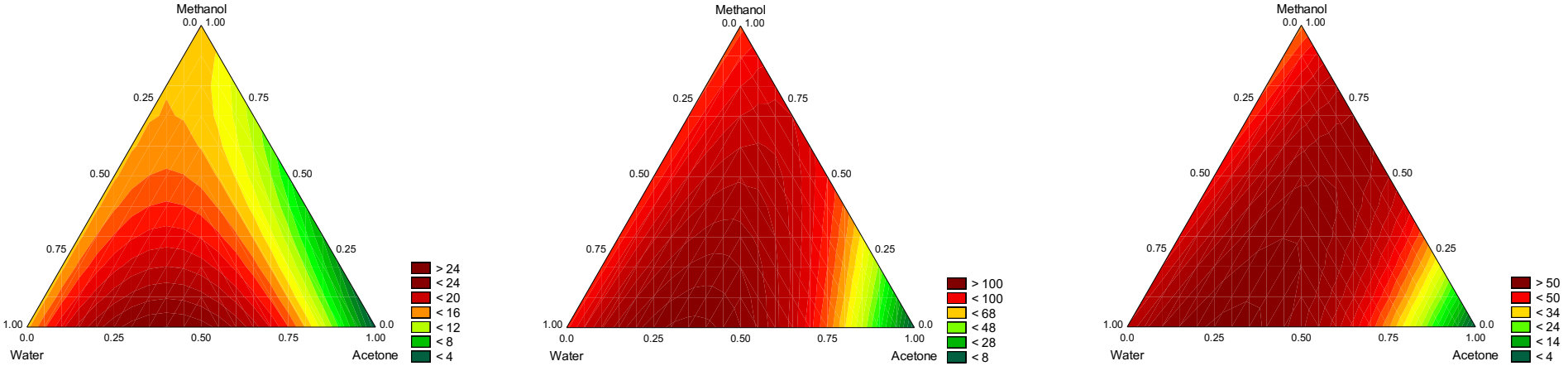


**Figure 1.** Contour plots for total phenolic compound (TPC) and antioxidant activity analyzed by ABTS and DPPH, respectively, from white mustard extracts

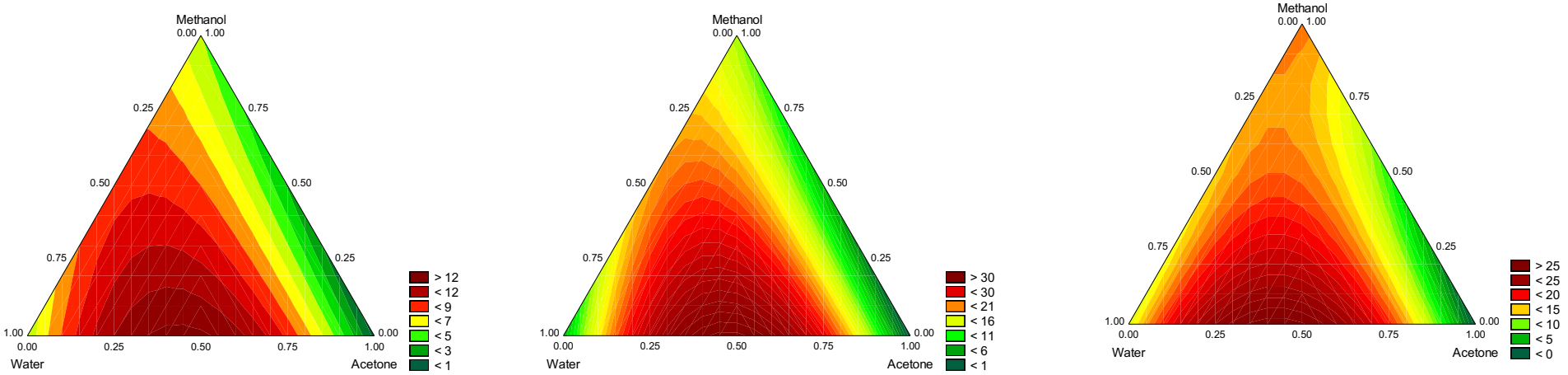


**Figure 2.** Contour plots for total phenolic compound (TPC) and antioxidant activity analyzed by ABTS and DPPH, respectively, from black mustard extracts.

Meneses et al. (2013) also corroborated with the results found in the present work. The authors studied antioxidant extraction from brewer's spent grains with different solvents such as methanol, ethanol, acetone, hexane, ethyl acetate, water, and mixtures of methanol:water, ethanol:water and acetone:water. The results suggested that the extract provided with acetone 60% (v:v) had the higher values for total phenolic compounds and antioxidant capacity measured by FRAP and DPPH.

The identification of phenolic compounds was performed using the extract obtained with water and acetone. For all standards analyzed, it was only possible to identify the compound sinapic acid that exhibited 1.93 and 1.91 mg per gram of extract (mg $g^{-1}$) for white and black mustard, respectively (Table 6).

**Table 6.** Sinapic acid content detected in white and black mustard extracts.

| | Sinapic Acid (mg $g^{-1}$ of extract) |
|---|---|
| White mustard | 1.93 ± 0.006 |
| Black mustard | 1.91 ± 0.15 |

Sinapic acid seems to be one of the phenolic acids responsible for the high antioxidant activity of *Cyclea gracillima* Diels extracts measured by DPPH-radical scavenging, TEAC, reducing power, $Fe^{2+}$ ion chelation, inhibition of human LDL oxidation, inhibition of human erythrocyte hemolysis, and scavenging of oxygen radicals in human blood. The herb can potentially be used as a natural antioxidant source for health (Lin et al., 2018).

In addition, Yao et al. (2012) also found sinapic acid in rice beans. They analyzed thirteen varieties of rice beans and twelve exhibit this phenolic acid in their composition. Besides, all of the varieties showed antioxidant activity. Phenolics acids are considered the major compounds that contribute to the total antioxidant activities of the beans.

## 4 Conclusion

Considering the diversity of composition of each grain variety in nature, individually designed and optimized studies for solvent extraction of bioactive compounds are extremely necessary. In this case, the extraction of antioxidant compounds from mustard grains showed that the combination between water and acetone, in equal proportions, was the most adequate solution. These results can support the future researches with mustard grains, since this is the first study that reported the optimization of the extraction of mustard antioxidant compounds.

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# Capítulo III: Mustard seeds germination to increase their antioxidant properties: is it a good option?

Gabriela Boscariol Rasera[a*], Ruann Janser Soares de Castro[a]
Department of Food Science, School of Food Engineering, University of Campinas.
80 Rua Monteiro Lobato, Campinas – SP, Brazil
*Corresponding author:
E-mail address: gabi_boscariol@hotmail.com

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**Abstract**

Germination is an efficient, economical and natural process that offers new opportunities to improve the nutritional quality and biological potential of grains. Mustard grains have great bioactive potential, are cheap and consumed worldwide. The biochemical transformations that occur during mustard grains germination have not been elucidated in the literature, but it is known that the germination conditions influence the final composition of the sprouts. The aim of this study was to evaluate the influence of the parameters (light, temperature and time) on the total phenolic compounds content and antioxidant properties (ABTS and DPPH) of extracts obtained from the germinated grains of black (*Brassica nigra*) and white (*Synapsis alba*) mustard. Although each germination condition is a peculiar case of study, the conditions recommended to obtain extracts with higher contents of phenolic compounds and antioxidant properties were the following: 1) 48 h of germination, temperature incubation of 25°C and equally alternating periods of light and dark; 2) 72 h of germination, 25°C as incubation temperature and light throughout the incubation period and 3) 72 h of germination at 30°C and light/darkness, for black mustard. At these conditions, the results showed values 15%, 105% and 82% higher for ABTS, DPPH and TPC, respectively, when compared to non-germinated sample. For the white mustard extracts, the best conditions were: 1) 24 h, 25°C and light; 2) 48 h, 30°C and light and 3) 72h, 25°C and darkness. The results for these conditions were 43%, 148% and 27% higher for ABTS, DPPH and TPC, respectively, when compared to non-germinated sample.

**Keyword:** mustard grains; germination conditions; phenolic compounds

## 1. Introduction

Germination means to sprout. When used for seeds, it means the emergence of a new plant including the physiological events that happen since the seeds are soaked until the germ appearance (Nonogaki and Nonogaki, 2017). It is already known that environmental conditions for germination change the final composition of the sprout and consequently its bioactivity. Because each seed has an specific composition, these external factors must be combined for high intake of bioactive compounds (Facelli et al., 2005).

The presence or absence of light, for example, can induce a morphologically different development during plant growing (Galvao et al., 2012). This is an environmental factor that affects plant photosynthesis (Yan et al., 2013) and evoque photooxidatives changes in plants, like production of reactive oxygen species (ROS) produced mainly by chloroplasts. This reactive species, when in excess, can initiate a series of metabolic alterations, like enzymatic activity reduction and decrease of photosynthetic rate. As a defense mechanism, to minimize oxidative damage, plants active their detoxification system constituted by substances with low molecular weight (some phenolic compounds, for example) and antioxidant enzymes (Simlat et al., 2016).

Temperature is another important factor that alters seed growing and development. Inadequate temperatures are abiotic stress for plant and as a defense and protective mechanism, plants can also produce phenolic compounds (Simlat et al., 2016). In addition, germination time affects directly macromolecules decomposition. With more germination time, higher is the activity of hydrolytic enzymes such as beta-glucanase and alfa-amylase, that degrade complex polysaccharides of cell wall (Farzaneh et al., 2017) releasing bioactive compounds that were linked before (Chungcharoen et al., 2015).

Despite it is already known by literature that the environmental conditions influence on germination and final composition of sprouts, there is no consensus on germination conditions for grains. In general, each seed is studied individually with specific time, temperature and light exposure, to define the most adequate way for germination and obtaining the products of interest. Rice beans were germinated at 30°C until 24 hours (Sritongtae, et al., 2017), linseed was germinated for ten days on darkness and 25°C (Wang et al., 2016) and common beans were germinated at 25°C, until 72 hours on a circle of light and dark (12 hours each) (de Souza Rocha et al., 2015). All of them were made to evaluate germination as a process to improve antioxidant activity and bioactive compounds. No studies were found to determine the most suitable conditions for germination of mustard grains in order to obtain a higher content of antioxidant compounds.

Moreover, some studies showed clearly that germination changed positively the content of phenolic compounds and the antioxidant properties in rice bean sprouts (*Vigna umbellata*) (Sritongtae et al., 2017), soybean (*Glycine* max), chickpea (*Cicer arietinum)*, mung bean (*Vigna radiata*), red lentils (*Lens culinaris)* and kidney bean (*Phaseolus vulgaris* var. *humilis)* (Mamilla; Mishra, 2017), linseed (*Linum usitatissimum L.)* (Wang et al., 2016), canary seed (Chen et al., 2016), brown rice (*Oryza sativa* L. *indica* SLF09) (Cáceres et al., 2017) and kiwicha (*Amaranthus caudatus*) (Paucar-Menacho et al., 2017a).

Mustard grains contain a great variety of minerals and fatty acids, essential oils, B-complex vitamins and E vitamin (Divakaran and Babu, 2016). Also, the grains have showed several bioactive compounds with potential to decrease blood glucose, lipid and cholesterol accumulation as well as antioxidant, anti-inflammatory and anticancer activities (Khan et al., 1997; Grover et al., 2003; Thirumalai et al., 2011; Divakaran and Babu, 2016). Finally, used mainly as a condiment, the germination of mustard seeds may be an innovative option for their consumption with a possible improvement on their nutritional and functional properties. In this sense, the main objective of this research was to investigate the most adequate conditions for germination of two varieties of mustard grains (black - *Brassica nigra* and white - *Sinapsis alba*) in order to increase their content of total phenolic compounds and antioxidant properties.

## 2. Material and methods

### 2.1 Reagents

The Folin and Ciocalteau's phenol reagent, sodium carbonate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), potassium persulphate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were purchased in the grade commercially available.

### 2.2 Substrate and germination process

Samples of two varieties of mustard grains, namely black (*Brassica nigra*) and white (*Sinapsis alba*), were purchased in a local market from Piracicaba (Sao Paulo, Brazil).

To study the effects of different parameters of process (incubation time, temperature and photoperiod) on germination of mustard and on their antioxidant properties, the grains were, initially, soaked for 12 h with water (1g/100 mL). Subsequently, the samples were placed in a piece of paper filter that was curled up and placed in each adequate germination condition according to the assays showed in Table 1. After the specific time of germination, the samples

were collected, lyophilized, ground and subsequently defatted using n-hexane. Defatted and dried seeds were storage at -18°C for further analysis.

### 2.3 Obtaining of the mustard extracts

To obtaining the soluble fraction from mustard grains (SFM), 1g of defatted samples were mixed with 30 mL of a solution of water/acetone (1:1 v/v) over 20 min under stirring (150 rpm) at 25°C. After extraction, the solution was centrifuged (17 000 x g) for 15 minutes, and the supernatant was collected and stored at -18°C for further analysis. The extracts were analyzed to their total phenolic compounds (TPC) content and antioxidant properties using two methods (ABTS- and DPPH-radical scavenging).

### 2.4 Determination of total phenolic content

The total phenolics were estimated according to the method of Swain and Hillis (1959) with a modified version as described by de Camargo et al., (2012). The Folin and Ciocalteau's phenol reagent (0.5 mL) was mixed with 0.5 mL of extracts in a tube, and 4 mL of distilled water. After 3 minutes of incubation, 1 mL of saturated sodium carbonate solution (0,3 g $mL^{1}$) was added to each tube. The reaction mixtures were allowed to stand for 2 hours at room temperature in the dark. The absorbance was read at 760 nm. The total amount of phenolic compounds was expressed as mg of gallic acid equivalents (GAE) per gram dry weight of defatted sample (mg GAE $g^{-1}$)

### 2.5 Determination of ABTS radical cation scavenging activity

The ABTS assay (Al-Duais et al., 2009) was performed using a microplate reader Spectra Max M3 (Molecular Devices, LLC, Sunnyvale, CA, USA). The ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6- sulphonic acid)] radical cation (generated by oxidation with potassium persulphate) was prepared in 75 mM potassium phosphate buffer saline solution (PBS) (pH 7.4). At the time of analysis, the working solution of ABTS radical cation (potassium persulphate (140 mM, 0.088 mL) and ABTS (7 mM, 10 mL) in PBS) was prepared by diluting its stock solution in PBS to reach an absorbance value of 0.70 ± 0.20 (734 nm).

Mustard extracts were diluted in PBS to reach a final concentration of 2.5 mg $mL^{-1}$. Aliquots of 20 µL of each extract were added to 220 µL of ABTS radical cation solution and the absorbance was read at 734 nm after 6 min. The control assay was made with distilled water in place of the samples. A standard curve was prepared using different concentrations of Trolox (2.5-200 µM) and the results were expressed as µmol of Trolox equivalents per g of sample (µmol TE $g^{-1}$).

## 2.6 Determination of DPPH radical scavenging activity

DPPH radical scavenging activity assay was carried out according to the method described by Al-Duais, et al. (2009). Briefly, 134 µL of 150 µM DPPH radical solution, which was freshly made in ethanol, was added to 66 µL of appropriately diluted extracts (2.5 mg $mL^{-1}$) or standards. After 45 min of incubation at room temperature in the dark, the absorbance was measured at 517 nm using a microplate reader Spectra Max M3 (Molecular Devices, LLC, Sunnyvale, CA, USA). Ethanol was used as blank. A standard curve was prepared with different concentrations of Trolox (20-140 µM) and the results were expressed as µmol of Trolox equivalents per g of sample (µmol TE $g^{-1}$).

## 2.7 Calculations and statistics

The results were statistically analyzed according to the Tukey test, using the software Minitab® 18 from Minitab Inc. (USA). The values were expressed as the arithmetic mean and were considered statistically different when the *p*-value $\leq 0.05$.

## 3. Results and Discussion

Tables 1 and 2 show the results for TPC, ABTS- and DPPH- radical scavenging of the extracts obtained from the two varieties of mustards submitted to different germination conditions as well as for the non-germinated and soaked samples, totaling 20 trials for each variety of mustard studied.

The extracts produced using non-germinated and soaked mustard (for both varieties) were not statistically different ($p \leq 0.05$) for TPC, ABTS- and DPPH-radical scavenging, an indicative that biochemical transformations after water entering were minimal and have not changed the content of phenolic compounds and the antioxidant properties of the extracts. Moreover, these compounds were not lost during soaking process as presented by Paucar-Menacho et al. (2017b) that observed a decrease in phenolic compounds during the germination of purple corn and justified this with the leaching during the soaking period prior to germination.

The results observed for extracts obtained from white mustard indicated that germination process, regardless of the conditions, positively affected the TPC, ABTS- and DPPH-radicals scavenging. For TPC, the values ranged from 18.53 (soaked sample) to 28.26 GAE $g^{-1}$ (germinated sample for 72h at 30°C in the presence of light), from 80.35 (soaked sample) to 137.71 µmol TE $g^{-1}$ (germinated sample for 72h at 25°C in the presence of light) for ABTS and from 19.7 (soaked sample) to 60.13 µmol TE $g^{-1}$ (germinated sample for 48h at 30°C in the presence of light) for DPPH. Black mustard extract changed from 8.82 to 16.6 GAE $g^{-1}$

for TPC, from 41.07 to 71.8 μmol TE $g^{-1}$ for ABTS and from 16.3 7 to 36.95 μmol TE $g^{-1}$for DPPH.

The results obtained showed a clearly difference between the lowest, in general detected for the extracts produced from the soaked or non-germinated mustard, and the highest values, most common for the germinated samples, for each determination. This can be explained in function of the changes that occur during germination, mainly because the reactions of synthesis and transport of phenolic compounds for cell wall formation are simultaneous (Yeo and Shahidi, 2015). It is well stablished that during germination, extensive phytochemical alterations may occur including remobilization, degradation and accumulation of nutrients, showing a continuous, complex and dynamic process (Nelson et al., 2013) that is peculiar for each seed (Gan et al., 2017).

It is important to note that inferior values observed to extracts obtained from germinated samples compared to non-germinated samples, such as in the assays 3, 5, 6, 8, 11, 13 and 14 for ABTS-radical scavenging of black mustard, do not mean that there was loss of compounds with antioxidant properties but it is an indicative that biochemical transformations are occurring constantly with soluble and bound fractions. During germination, due the plant growing, new cell compounds are synthetized and soluble phenolic can link with carbohydrates and proteins to form new cell walls, explaining the decrease in soluble phenolic compounds and consequently in the antioxidant properties of the extracts (Gan et al., 2017). Therefore, bound phenolic extraction is recommended for the next steps.

There are no literature data regarding to the influence of germination on TPC content or antioxidant properties of mustard seeds. However, it has been reported that temperature affected time to germination and total germination of mustards. Russo et al. (2010) studied time to maximum germination and maximum germination percent over temperatures for mustard varieties. They presented that, in general, long times was required to germination for all varieties (*Brassica juncea* and *Sinapisis alba*) when the incubation temperature was 4 and 10°C. At higher temperatures (16 to 32°C), maximum germination occurred by 2 days.

Besides, it is already known the influence of time and temperature in antioxidant activity and TPC content for other grains. Paucar-Menacho, et al. (2017a) observed a positive effect on germination time and temperature on total phenolic compounds in kiwicha grains. Besides, total phenols, total flavonoids and antioxidant activities of Broad beans (*Vicia faba* L.), lupine seeds (*Lupinus albus*), chickpea seeds (*Cicer arietinum* L.), lentil seeds (*Lens culimaris*), fenugreek seeds (*Trigonella foenum-graecum* L.) and common beans (*Phaseolus vulgaris*) increased as germination time progressed (6 days maximum) (Saleh et al., 2017).

In order to determine the most adequate germination condition between those analyzed, the mustard extracts with the highest antioxidant activities and TPC values were selected. In the background, for samples that do not differ statistically ($p \leq 0.05$), smaller germination time and light/darkness condition were prioritized by simulating conditions closer to the real ones.

Thus, to black mustard, the assays 10 (48 h of germination, temperature incubation of 25°C and equally alternating periods of light and dark;), 15 (72 h of germination, 25°C as incubation temperature and light throughout the incubation period) and 19 (72 h of germination at 30°C and alternation of light and dark periods) would be the recommended. The extracts obtained at these conditions showed values 15%, 105% and 82% higher than non-germinated sample for ABTS, DPPH and TPC, respectively. .

On the other hand, for white mustard, the assays 3 (24 h of germination at 25°C and light), 12 (48 h of germination at 30°C and light for all incubation period) and 17 (72 h of germination at 25°C in the dark) were selected as the most appropriate to obtain extracts with high antioxidant properties. The results for these conditions were 43%, 148% and 27% higher than non-germinated sample for ABTS, DPPH and TPC, respectively. .

This increase on antioxidants properties were also observed in linseed (*Linum usitatissimum* L.) germinated for 10 days with values of flavonoid content, TPC and ORAC 55, 6 and 6-fold, respectively, higher than non-germinated samples (Wang et al., 2016). Rice beans (*Vigna umbellata*) sprouts also showed this behavior with a 2-fold increase in TPC during germination for 24 hours (Sritongtae et al., 2017). Also, olive (*Olea europaea* L.) sprouts (germinated from 8 to 16 days) presented increases of 14- and 30-fold in the total phenolic content and antioxidant activity compared to non-germinated olive (Falcinelli et al., 2018).

**Table 1**. Germination conditions, TPC and antioxidant properties (ABTS- and DPPH-radicals scavenging) results for extracts from white mustard grains.

| Assay | Germination parameters | | | TPC and antioxidant properties | | |
|---|---|---|---|---|---|---|
| | Germination time (h) | Temperature (°C) | Photoperiod | TPC mg GAE $g^{-1}$ | ABTS µmol TE $g^{-1}$ | DPPH µmol TE $g^{-1}$ |
| **1 (non-germinated)** | 0 | Room temperature | - | $21.61 \pm 1.11^{cdef}$ | $86.64 \pm 1.26^{hi}$ | $24.22 \pm 0.89^{kl}$ |
| **2 (soaked)** | | | - | $18.53 \pm 0.89^{f}$ | $80.35 \pm 1.7^{i}$ | $19.7 \pm 0.53^{l}$ |
| **3** | 24 | 25 | Light | $26.45 \pm 0.58^{bcdef}$ | $124.44 \pm 2.62^{abcd}$ | $39.16 \pm 1.26^{fg}$ |
| **4** | | | Light + darkness | $24.71 \pm 0.36^{abcde}$ | $120.09 \pm 2.91^{bcd}$ | $35.69 \pm 0.53^{gh}$ |
| **5** | | | Darkness | $25.41 \pm 0.19^{abcd}$ | $127.34 \pm 0.57^{abcd}$ | $44.95 \pm 2.48^{cde}$ |
| **6** | | 30 | Light | $20.97 \pm 0.35^{cdef}$ | $94.29 \pm 1^{gh}$ | $14.22 \pm 0.52^{m}$ |
| **7** | | | Light + darkness | $23.79 \pm 0.77^{abcdef}$ | $105.75 \pm 3.51^{efg}$ | $48.15 \pm 2.9^{c}$ |
| **8** | | | Darkness | $20.66 \pm 0.63^{def}$ | $103.23 \pm 0.58^{fg}$ | $26.54 \pm 0.42^{jk}$ |
| **9** | 48 | 25 | Light | $24.72 \pm 1.03^{abcde}$ | $121.82 \pm 0.58^{bcd}$ | $55.54 \pm 1.14^{b}$ |
| **10** | | | Light + darkness | $23.48 \pm 0.86^{abcdef}$ | $96.83 \pm 1.72^{gh}$ | $29.45 \pm 0.1^{ij}$ |
| **11** | | | Darkness | $19.2 \pm 0.58^{ef}$ | $133.26 \pm 5.2^{ab}$ | $47.57 \pm 1.75^{c}$ |
| **12** | | 30 | Light | $23.12 \pm 0.65^{abcdef}$ | $124.95 \pm 8.42^{abcd}$ | $60.13 \pm 1.08^{a}$ |
| **13** | | | Light + darkness | $22.47 \pm 0.22^{abcdef}$ | $118.24 \pm 0.87^{cde}$ | $43.92 \pm 1.35^{cde}$ |
| **14** | | | Darkness | $25.95 \pm 0.57^{abcd}$ | $129.56 \pm 4.39^{abc}$ | $22.1 \pm 1.05^{jl}$ |
| **15** | 72 | 25 | Light | $26.54 \pm 1.44^{abc}$ | $137.71 \pm 2.42^{a}$ | $32.99 \pm 0.64^{hi}$ |
| **16** | | | Light + darkness | $24.03 \pm 0.81^{abcdef}$ | $117 \pm 7.23^{cdef}$ | $42.45 \pm 1.78^{def}$ |
| **17** | | | Darkness | $27.55 \pm 0.05^{ab}$ | $132.99 \pm 9.53^{ab}$ | $46.23 \pm 2.94^{cd}$ |
| **18** | | 30 | Light | $28.26 \pm 1.49^{a}$ | $122.1 \pm 8.61^{bcd}$ | $40.90 \pm 0.32^{ef}$ |
| **19** | | | Light + darkness | $24.79 \pm 0.16^{abcde}$ | $128.42 \pm 3.59^{abc}$ | $46.48 \pm 1.35^{cd}$ |
| **20** | | | Darkness | $21.37 \pm 0.71^{cdef}$ | $113.86 \pm 3.7^{def}$ | $46.38 \pm 1.77^{cd}$ |

Light + darkness means 12 hours in the light and 12 hours in darkness per day. Values in the same column with the same letters are not significantly different ($p \leq 0.05$).

**Table 2.** Germination conditions, TPC and antioxidant properties (ABTS- and DPPH-radicals scavenging) results for extracts from black mustard grains.

| Assay | Germination parameters | | | TPC and antioxidant properties | | |
|---|---|---|---|---|---|---|
| | Germination time (h) | Temperature (°C) | Photoperiod | TPC mg GAE $g^{-1}$ | ABTS µmol TE $g^{-1}$ | DPPH µmol TE $g^{-1}$ |
| **1 (non-germinated)** | 0 | Room temperature | - | $8.82 \pm 0.09^{f}$ | $57.36 \pm 0.46^{cde}$ | $18.03 \pm 0.6^{lm}$ |
| **2 (soaked)** | | | - | $9.24 \pm 0.36^{f}$ | $56.95 \pm 0.82^{de}$ | $17.92 \pm 0.77^{lm}$ |
| **3** | 24 | 25 | Light | $9.58 \pm 0.52^{f}$ | $51.91 \pm 1.74^{ef}$ | $23.68 \pm 0.63^{hij}$ |
| **4** | | | Light + darkness | $11.69 \pm 0.18^{de}$ | $64.21 \pm 2.2^{bc}$ | $27.19 \pm 0.42^{fg}$ |
| **5** | | | Darkness | $10.06 \pm 0.44^{ef}$ | $50.99 \pm 0.88^{ef}$ | $23.48 \pm 0.05^{ij}$ |
| **6** | | 30 | Light | $9.66 \pm 0.39^{ef}$ | $48.15 \pm 1.16^{fg}$ | $24.62 \pm 1.2^{hi}$ |
| **7** | | | Light + darkness | $11.81 \pm 0.1^{de}$ | $63.19 \pm 3.4^{bcd}$ | $31.2 \pm 0.3^{cd}$ |
| **8** | | | Darkness | $11.68 \pm 0.1^{de}$ | $51.37 \pm 2.01^{ef}$ | $29.41 \pm 1.32^{def}$ |
| **9** | 48 | 25 | Light | $11.53 \pm 0.58^{de}$ | $67.74 \pm 0.29^{ab}$ | $29.32 \pm 0.68^{def}$ |
| **10** | | | Light + darkness | $12.72 \pm 0.04^{cd}$ | $66.41 \pm 3.34^{ab}$ | $32.56 \pm 1.05^{bc}$ |
| **11** | | | Darkness | $9.84 \pm 0.26^{ef}$ | $43.51 \pm 1.91^{gh}$ | $26.11 \pm 0.53^{gh}$ |
| **12** | | 30 | Light | $12.4 \pm 0.4^{cd}$ | $71.8 \pm 1.31^{a}$ | $34.14 \pm 0.16^{b}$ |
| **13** | | | Light + darkness | $13.33 \pm 0.08^{cd}$ | $41.07 \pm 0.91^{h}$ | $19.4 \pm 0.16^{kl}$ |
| **14** | | | Darkness | $9.31 \pm 0.39^{f}$ | $52.11 \pm 0.99^{ef}$ | $28.51 \pm 0.78^{efg}$ |
| **15** | 72 | 25 | Light | $16.6 \pm 0.38^{a}$ | $56.12 \pm 2.93^{e}$ | $36.95 \pm 0.42^{a}$ |
| **16** | | | Light + darkness | $12.28 \pm 0.45^{cd}$ | $71.73 \pm 2.61^{a}$ | $16.37 \pm 1.21^{m}$ |
| **17** | | | Darkness | $13.99 \pm 0.43^{bc}$ | $56.75 \pm 1.31^{de}$ | $27.22 \pm 1.31^{efg}$ |
| **18** | | 30 | Light | $9.27 \pm 0.18^{f}$ | $56.28 \pm 1.74^{de}$ | $29.74 \pm 0.93^{de}$ |
| **19** | | | Light + darkness | $16.05 \pm 0.03^{ab}$ | $68.65 \pm 5.66^{ab}$ | $21.38 \pm 1.21^{jk}$ |
| **20** | | | Darkness | $11.48 \pm 0.36^{de}$ | $67.82 \pm 2.59^{ab}$ | $33.08 \pm 0.57^{bc}$ |

Light + darkness means 12 hours in the light and 12 hours in darkness per day. Values in the same column with the same letters are not significantly different ($p \leq 0.05$)

## 4. Conclusion

Based on the results obtained and the positive changes on the antioxidant properties promoted by the germination of black and white mustard grains, the following conditions were determined as the most suitable for the process: 1) 72h, 25°C and darkness; or 2) 72h, 30°C and light/darkness or 3) 72h, 30°C and darkness for black mustard and 1) 48h, 25°C and light or 2) 72h, 30°C and light/darkness or 3) 72h; 25°C at darkness for the white mustard.

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# Capítulo IV: Free and insoluble-bound phenolics: how does the variation of these compounds affected the antioxidant properties of mustard grains during germination?

Gabriela Boscariol Rasera[a*], Ruann Janser Soares de Castro[a]
Department of Food Science, School of Food Engineering, University of Campinas.
80 Rua Monteiro Lobato, Campinas – SP, Brazil
*Corresponding author:
E-mail address: gabi_boscariol@hotmail.com

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**Abstract**

Germination is an efficient and economical bioprocess, characterized by dynamic and complex positive changes in bioactive compounds and nutritional quality in grains. White (*Synapsis alba*) and black mustard (*Brassica nigra*) were studied by monitoring their phenolic profiles and antioxidant properties of extracts obtained from free and bound fractions. To characterize the mustard grain and the sprouts, determination of total phenolic compounds, total flavonoids and condensed tannins were performed to characterize the extracts from mustard grain and their sprouts. In addition, the antioxidant properties of the extracts were measured by ABTS- and DPPH-radicals scavenging, ORAC capacity, and FRAP assay. Free and bound fraction of phenolic compounds changed during germination, showing a complex process of synthesis and degradation. Soluble extract of phenolic compounds of germinated white mustard (72 hours at 25°C at darkness) was nearly 1.5-folds higher for all analyses (TPC, total flavonoids, condensed tannins, ABTS, DPPH, FRAP and ORAC) when compared with non-germinated extract. Insoluble fraction of the same germinated mustard, presented values that raised from 0.89 to 2.35-folds higher for the same analyses when compared again to non-germinated sample. Soluble phenolic compounds extract of germinated black mustard (48 hours, at 25°C alternating dark and light periods) augmented 1 to 2-folds when compared to non-germinated mustard for the same analyses. Insoluble fraction of this grain presented an inverse behavior, with a decrease or values maintenance during mustard germination. Results raised from 0.7 to 0.9-folds lower than non-germinated mustard. This work elucidate how complex and peculiar is germination. Besides, it is a health option of mustard grains consumption.

**Keywords:** mustard grains; free and insoluble-bound fraction; germination.

## 1. Introduction

Nowadays, people's lifestyle induces production of free radicals. Stress, pollution, smoke, drugs, xenobiotics, ionizing radiation can incite production of Reactive Oxigen Species (ROS) (Carocho, Morales, & Ferreira, 2018). ROS are intermediary formed during normal metabolic process and are neutralized by endogenous antioxidant system, constituted by antioxidants enzymes. Any imbalance at this process can causes oxidative stress, reported as one of the causes of diseases as Alzheimer, cardiovascular disorders and cancer (Sarangarajan, Meera, Rukkumani, Sankar, & Anuradha, 2017).

This imbalance could be minimized by antioxidants present in fruits and legumes, like phenolic acids, flavonoids, vitamins, carotenoids, tannins and anthocyanin (Carocho, Morales, & Ferreira, 2018). Known as a secondary metabolite of plants, phenolic compounds occur in both insoluble and soluble-bound form. The majority of soluble phenolic compounds are stored in the vacuoles of plant cells, and, insoluble-bound phenolics are linked through covalent bounds via ester, ether and carbon-carbon bound, to macromolecules such as structural protein, pectin and cellulose (Shahidi & Yeo, 2016).

Literature shows a wide diversity of studies related to natural food antioxidant capacity, as well as by-products as a source of this beneficial compounds such as peanut skins (de Camargo, Vidal, Canniatti-Brazaca, & Shahidi, 2014) and wine and grape juice by-products (de Camargo, Regitano-d'Arce, Biasoto, & Shahidi, 2014).

It is important to know that the antioxidant power of each compound is directly correlated with its molecular structure, like number and position of hydroxyl groups, degree of hydroxylation, distance between carbonyl and aromatic ring as well as the quantity of aromatic ring. Thus, characterization of phenolic compounds is very important in the studies of antioxidant capacity (Zhang & Tsao, 2016). It is important to highlight that the antioxidant activity can happen in different pathways: free radical scavenging, endogenous antioxidant enzymes activation, prevention of lipid peroxidation, prevention of DNA damage (Carocho, Morales, & Ferreira, 2018).

Concerning the necessity and tendencies of natural food or natural ways for human consumption, it is important to valorize natural process. Therefore, it is already seen in literature the positives consequences of germination, an efficient and economical process that causes dynamic and complex positive changes in bioactive compounds and nutritional quality as already registered by some researchers (Sritongtae et al., 2017; Chen et al., 2017; Mamilla & Mishra, 2017). Besides that, mustard grains were not studied yet with the objective of analyze the behavior of phenolic compounds as well as to their antioxidant properties during germination.

Thus, the aim of this study was to evaluate the mustard germination and its relation with phenolic compounds composition and antioxidant activities. The establishment of a correlation between the bioactive properties of the free and bound fractions present in the extracts was also proposed

## 2. Material and Methods

### 2.1 Reagents

The Folin and Ciocalteau's phenol reagent, sodium carbonate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), potassium persulphate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH); sodium nitrite, aluminum chloride, sodium hydroxide; vanillin, fluorescein, α,α′-Azodiisobutyramidine dihydrochloride (AAPH), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) and hydrated iron chloride were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were purchased in the grade commercially available.

### 2.2 Samples and germination conditions

Samples of two varieties of mustard grains namely black (*Brassica nigra*) and white (*Sinapsis alba*), were purchased in a local market from Piracicaba (Sao Paulo, Brazil). Mustard seeds were soaked for 12 hours with water (1g/100 mL). Subsequently, seeds were placed in a piece of paper filter that was curled up and placed in the adequate germination conditions (Table 1 and 2). After the specific time of germination, each assay was lyophilized, ground and subsequently defatted by using n-hexane. Defatted and dried seeds were stored at -18°C.

### 2.3 Extraction of soluble (free) and insoluble-bound phenolics

For soluble fraction from mustard (SFM), 1g of defatted sample was mixed with 30 mL of a solution of water/acetone (1:1, v/v) over 20 min under stirring (150 rpm) at 25°C. This procedure was repeated two more times and the combined extracts (SFM) were stored at -18°C for further analysis. The residue after removal of SFM was used for the extraction of insoluble fraction from mustard (IFM). For this, 1g of the residue was mixed with 15 mL of 2 mol/L NaOH for 4h under stirring. Hydrolyzed samples were acidified with 4.63 mL of HCl 6 mol/L and then extracted with ethyl acetate five times. Subsequently, the solvent was removed using a rotatory evaporator, followed by dissolving the resultant solids in water/acetone (1:1, v/v). Extracts (IFM) were also stored at -18°C for further analysis (Yeo & Shahidi, 2017).

### 2.4 Determination of total phenolic content

The total phenolics were estimated according to the method of Swain & Hillis (1959) with a modified version as described by de Camargo, Vieira, Regitano-D'Arce, Calori-

Domingues, & Canniatti-Brazaca, (2012). The Folin and Ciocalteau's phenol reagent (0.5 mL) was mixed with 0.5 mL of extracts in a tube, and 4 mL of distilled water. After 3 minutes of incubation, 1 mL of saturated sodium carbonate solution (0,3 g $mL^1$) was added to each tube. The reaction mixtures were allowed to stand for 2 hours at room temperature in the dark. The absorbance was read at 760 nm. The total amount of phenolic compounds was expressed as mg of gallic acid equivalents per gram dry weight of defatted sample (mg GAE $g^{-1}$).

### 2.5 Determination of total flavonoids content

Total flavonoid content was measured as described by Zhishen, Mengcheng, & Jianming (1999). Briefly, 0.5 mL of mustard extract (properly deluded) was dissolved in 2.5 mL of distilled water and then 0.15 mL of 5% $NaNO_2$ was also added. After standing for 5 min, the samples were allowed to react with 0.15 mL of 10% $AlCl_3$ and the mixture was left at room temperature for more 6 minutes in the dark. Then, 1 mL of 4% NaOH and 1.2 mL of water were added. The absorbance was read at 510 nm and total flavonoid content was expressed as mg catechin equivalents per gram of dry weight of defatted sample (mg CE $g^{-1}$).

### 2.6 Determination of condensed tannins content

Condensed tannins content was determined according to the method of Broadhurst & Jones (1978). Each extract (30 µL) or standard solution was mixed (950 µL) with vanillin (4% with methanol). It was added concentrated HCL (405 µL) and the mixed solution was incubated at 30°C in the dark for 20 minutes. Absorbance was read at 500 nm. Catechin was used to make the standard curve (0.05–0.8 mg $mL^{-1}$) and the results were expressed at mg catechin equivalents per gram of dry weight of defatted sample (mg CE $g^{-1}$).

### 2.7 Determination of the antioxidant properties

#### 2.7.1 Determination of ABTS radical cation scavenging activity

The ABTS assay (Al-Duais et al., 2009) was performed using a microplate reader Spectra Max M3 (Molecular Devices, LLC, Sunnyvale, CA, USA). The ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6- sulphonic acid)] radical cation (generated by oxidation with potassium persulphate) was prepared in 75 mM potassium phosphate buffer saline solution (PBS) (pH 7.4). At the time of analysis, the working solution of ABTS radical cation (potassium persulphate (140 mM, 0.088 mL) and ABTS (7 mM, 10 mL) in PBS) was prepared by diluting its stock solution in PBS to reach an absorbance value of 0.70 ± 0.20 (734 nm).

Mustard extracts were diluted in PBS to reach a final concentration of 2.5 mg $mL^{-1}$. Aliquots of 20 µL of each extract were added to 220 µL of ABTS radical cation solution and

the absorbance was read at 734 nm after 6 min. The control assay was made with distilled water in place of the samples. A standard curve was prepared using different concentrations of Trolox (2.5-200 µM) and the results were expressed as µmol of Trolox equivalents per g of sample (µmol TE $g^{-1}$).

### 2.7.2 Determination of DPPH radical scavenging activity

DPPH radical scavenging activity was carried out according to the method described by Al-Duais, Müller, Böhm, & Jetschke, (2009) Briefly, 134 µL of 150 µM DPPH radical solution, which was freshly made in ethanol, was added to 66 µL of appropriately diluted extracts (2.5 mg $mL^{-1}$) or standards. After 45 min of incubation at room temperature in the dark, the absorbance was measured at 517 nm using a microplate reader Spectra Max M3 (Molecular Devices, LLC, Sunnyvale, CA, USA). Ethanol was used as blank. A standard curve was prepared with different concentrations of Trolox (20-140 µM) and the results were expressed as µmol of Trolox equivalents per g of sample (µmol TE $g^{-1}$).

### 2.7.3 Measurement of oxygen radical absorbance capacity (ORAC)

ORAC assay was conducted as described by (Chisté et al., 2011) and it was carried out using a microplate reader Spectra Max M3 (Molecular Devices, LLC, Sunnyvale, CA, USA). The reaction mixture consisted of 30 µL of appropriately diluted extract, blank, or Trolox were mixed with 60 µL of 0.4 µg $mL^{-1}$ fluorescein solution and 110 µL of AAPH (108 mg.$mL^{-1}$) added directly in the microplate that was then, incubated at 37°C for 120 min. All the solutions were prepared using phosphate buffer 75mM (pH 7.4). Fluorescence was determined and recorded every min for 120 min with an excitation and emission wavelengths of 485 and 538 nm, respectively. Antioxidant activity was calculated using the difference between the area under the fluorescence decay curve (AUC) of samples and blank. Different concentrations (12.5-400 µM) of Trolox were used to prepare the standard curve. The ORAC values were expressed as µmol of Trolox equivalents per gram of dry weight of defatted sample (µmol TE $g^{-1}$).

### 2.7.4 Measurement of ferric reducing/antioxidant power (FRAP) assay

FRAP assay was performed according to Benzie & Strain (1996) with some modifications proposed by Wiriyaphan, Chitsomboon, & Yongsawadigul (2012). FRAP reagent was prepared by mixing 2.5 mL of 10 mM TPTZ solution in 40 mM HCl; 25 mL of 0.3 M acetate buffer (pH 3.6), and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ solution. Aliquots of 100 µL of mustard extracts previously diluted, blank or Trolox (5-250 µM) was mixed with 1 mL of

fresh FRAP reagent. Then, the reaction mixture was incubated at 37 °C, for 15 min, in a water bath. Absorbance of each sample was monitored at 593 nm. Results were expressed as μmol of Trolox equivalents per gram of dry weight of defatted sample (μmol TE $g^{-1}$).

### 2.8 Calculations and statistics

The results were statistically analyzed according to the Tukey test, using the software Minitab® 18 from Minitab Inc. (USA). The values were expressed as the arithmetic mean and were considered statistically different when the *p*-value ≤ 0.05.

## 3. Results and Discussion

Germination conditions were already studied by our research group, and results are not published yet. Besides, the best conditions for black and white mustard are presented in Tables 1 and 2 with all results. To choose one germination condition for each mustard variety, analyses of the content of some phenolic compounds and antioxidant activities *in vitro* were made for soluble (SFM) and insoluble fractions (IFM) of the extracts obtained from black and white mustard.

As one of the objectives was to choose one condition, the comparison was always made between non-germinated mustard and each pre-selected germination condition.

Total phenolic compounds of soluble fraction of black and white mustard increased in all germination conditions. Similar results were observed by Yeo & Shahidi (2015) that registered an increase in this fraction during lentil germination, what might indicate gradual synthesis of phenolic compounds during germination or liberation of phenolic compounds that are linked in macromolecules in cell wall, since insoluble fraction (IFM) showed a decrease in certain germination condition for both mustard, like 24 h, at 25°C with light and 48 h, at 30°C at light for white mustard and 48 h, at 25°C at alternated periods of light and darkness for the black one (Table 1 and 2).

For total flavonoids, the profile observed changed for each mustard variety and extracted fraction (Table 1 and 2). For white mustard, while soluble fraction increases, insoluble fraction decreases. An increase in total phenolic and flavonoid content was also observed in Foxtail millet after 48 hours of germination (Sharma, Saxena, & Riar, 2018) and brown rice germinated for 30 hours (Ti et al., 2014). This behavior may be explained by the release of insoluble-bound phenolics from cell wall matrix, decreasing insoluble fraction and increasing soluble fraction. Germination starts with grains imbibition, and hydrated cells are activated. These cells dispense hydrolytic enzymes that act hydrolyzing macromolecules such as protein and starch to smaller molecules, used for the growth and metabolism of the seeds, As a result, this can affect the content of phenolic compounds and their formation (Raven, et al., 2005). On

the other side, augment in flavonoids content may be explained by flavonoids' synthesis, known as flavonoid branch pathway, showed in Figure 1. For black mustard, total flavonoids profile did not present a clear variation as for white mustard. For soluble fraction, the assays 1 and 2 showed an increase in values of total flavonoids and assay 3 remained constant. For insoluble fraction, the flavonoid content did not change significantly ($p \leq 0.05$), except the assay 2 that had a small increase. The explanation for this behavior is specific for each condition, and follows ideas already said: release or synthesis of phenolic compounds and linkage with cell wall, what shows the complexity of this process (Shahidi & Yeo, 2016).

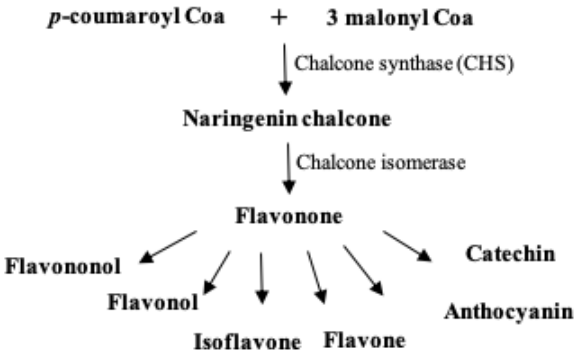


**Figure** 1. Flavonoid branch pathway. Based on (Shahidi & Yeo, 2016).

Condensed tannins are a class of flavonols, composed by monomers and procyanidins (Zhang & Tsao, 2016). For soluble fraction, condensed tannins increased for both mustards, independent of the germination condition, compared to the non-germinated grains, indicating an increase in flavonoids synthesis pathway or a release of tannins covalently linked in cell walls (IFM assays with decrease in values, as IFM 2 of white mustard). For IFM assays that showed an increase in values (IFM 1 and 3 for white mustard and IFM 3 for black mustard), it is possible to infer a formation of insoluble-bound phenolics in the cell wall, despite it has not yet been well stablished (Table 1 and 2). One of the evidences is that phenolics in the cytoplasmic can reach cell wall matrix and form covalent bounds with the cell wall substances such as hemicellulose, cellulose, structural proteins, arabinoxylans and pectin through ether, ester and C-C-bounds. The covalent bounds can enhance the rigid structure of the cell wall matrix, protect the cell of penetration fungi, a number of pathogens (Nicholson & Hammerschmidt, 1992) and UV damage (Jansen, 2001).

It was clearly evidenced that the content of phenolic compounds, tannins and flavonoids have changed during germination. The question was how it would affect the antioxidant

properties of the mustard extracts and how the composition changed. For antioxidant activities, FRAP, ABTS, DPPH ORAC methods were applied for all extracts. Because assays of free radical scavenging capacity of antioxidant compounds are classified in two categories – single-electron transfer assay (SET) and hydrogen atom transfer-based assays (HAT), it is highly recommended studies focus on the *in vitro* antioxidant activity for both mechanisms. The most accurate estimate of a plan sample's antioxidant activity is a combination of assays (Tan & Lim, 2015).

ABTS measures antioxidant capacity of donate hydrogen and DPPH is based on electron transference (Al-Duais, Müller, Böhm, & Jetschke, 2009). FRAP analyses antioxidant capacity of iron ion chelation (Benzie & Strain, 1996), and ORAC, a HAT methodology, measures inhibition ability against the peroxyl radical-induced oxidation (Chisté et al., 2011).

Extracts obtained from white and black germinated mustard in all conditions showed increases in antioxidant activity for soluble fraction, except for the sample from black mustard in DPPH assay. This augment in antioxidant activity during germination was already reported in germinated olive seeds (*Olea europaea* L) (Falcinelli et al., 2018); foxtail millet (*Setaria italica*) (Sharma, Saxena, & Riar, 2018b); brown rice (*Oryza sativa* L.) (Cho & Lim, 2018); chickpea sprouts (*Cicer arietinum* L.) (Hayta & İşçimen, 2017); broad beans (*Vicia faba* L.), lupine seeds (*Lupinus albus*), chickpea seeds (*Cicer arietinum* L.), lentil seeds (*Lens culinaris*), fenugreek seeds (*Trigonella foenum-graecum* L.) and common beans (*Phaseolus vulgaris*) (Saleh, Hassan, Mansour, Fahmy, & El-Bedawey, 2017).

The increase of antioxidant activity in soluble extracts, can be explained by the release and synthesis of phenolic compounds during germination, as said before. On the other size, insoluble fraction from mustard showed a different profile for each analysis and mustard variety. For white mustard, assay 3, for all antioxidant assays presented high values when compared with non-germinated mustard. For black mustard, it is observed a decrease in antioxidant activity for bound phenolics independent of the germination condition. This behavior may be explained by the fact that in general, IFM showed a decrease in phenolic compounds during germination for black mustard (Table 1 and 2).

It is clearly that germination causes an important and positive transformations in phenolic compounds composition and antioxidant properties of the mustard grains. According to our results, the best germination condition to maximize the content of phenolic compounds and antioxidant activities for white mustard is assay 3 (72 h of germination at 25°C in the dark) when soluble and insoluble fraction showed nearly 2-fold increase for most analyzes. For black mustard, assay 1 (48 h of germination at 25°C with alternation of light and dark periods) was chosen. At this condition, soluble fraction was nearly 1.5-fold higher than non-germinated

mustard for all analyses, except ABTS, that was 2.6-folds higher with germination. Besides, insoluble fraction at this condition, maintained the same value or decreased for all analyses done. 72h, 30°C, light/darkness, condition that presented the best values for insoluble fraction, was not the best for soluble fraction (Table 1 and 2).

Results showed that germinated mustard is a good option to improve the antioxidant properties of sprouts. It is also a cheap and easy way of mustard preparation that is mainly consumed as a sauce.

**Table 1.** Non-germinated and germinated conditions used to obtain the white mustard extracts from soluble and insoluble-bound fractions and the results for some phenolic compounds and antioxidant properties.

| Assays | SFM | IFM |
|---|---|---|
| **Total phenolic compounds (mg GAE $g^{-1}$)** | | |
| Non-germinated | $23.05 \pm 0.31^{c}$ | $2.96 \pm 0.1^{b}$ |
| 1) 24h, 25°C, light | $29.56 \pm 2.1^{b}$ | $2.22 \pm 0.12^{c}$ |
| 2) 48h, 30°C, light | $31.06 \pm 0.23^{b}$ | $2.31 \pm 0.08^{c}$ |
| 3) 72h, 25°C, darkness | $34.32 \pm 0.76^{a}$ | $4.00 \pm 0.13^{a}$ |
| **Total flavonoids (mg CE $g^{-1}$)** | | |
| Non-germinated | $6.04 \pm 0.39^{d}$ | $0.65 \pm 0.05^{a}$ |
| 1) 24h, 25°C, light | $7.38 \pm 0.44^{c}$ | $0.30 \pm 0.014^{b}$ |
| 2) 48h, 30°C, light | $9.01 \pm 0.50^{b}$ | $0.30 \pm 0.024^{b}$ |
| 3) 72h, 25°C, darkness | $10.42 \pm 0.27^{a}$ | $0.58 \pm 0.008^{a}$ |
| **Condensed tannins (mg CE $g^{-1}$)** | | |
| Non-germinated | $1.16 \pm 0.02^{c}$ | $0.94 \pm 0.008^{c}$ |
| 1) 24h, 25°C, light | $2.14 \pm 0.004^{b}$ | $1.02 \pm 0.01^{b}$ |
| 2) 48h, 30°C, light | $2.73 \pm 0.02^{a}$ | $0.58 \pm 0.0^{d}$ |
| 3) 72h, 25°C, darkness | $2.08 \pm 0.09^{b}$ | $2.56 \pm 0.003^{a}$ |
| **FRAP (µmol TE g-$^{1}$)** | | |
| Non-germinated | $128.08 \pm 6.531^{b}$ | $21.00 \pm 0.55^{b}$ |
| 1) 24h, 25°C, light | $141.67 \pm 2.62^{b}$ | $12.82 \pm 0.074^{c}$ |
| 2) 48h, 30°C, light | $206.07 \pm 1.1^{a}$ | $11.19 \pm 0.27^{d}$ |
| 3) 72h, 25°C, darkness | $215.25 \pm 16.6^{a}$ | $24.29 \pm 0.53^{a}$ |
| **DPPH (µmol TE $g^{-1}$)** | | |
| Non-germinated | $49.92 \pm 0.67^{c}$ | $10.13 \pm 0.06^{c}$ |
| 1) 24h, 25°C, light | $95.71 \pm 2.93^{a}$ | $12.24 \pm 0.76^{b}$ |
| 2) 48h, 30°C, light | $89.58 \pm 2.03^{a}$ | $8.81 \pm 0.46^{d}$ |
| 3) 72h, 25°C, darkness | $71.16 \pm 5.13^{b}$ | $14.59 \pm 0.42^{a}$ |
| **ABTS (µmol TE $g^{-1}$)** | | |
| Non-germinated | $180.95 \pm 2.7^{c}$ | $26.72 \pm 1.38^{c}$ |
| 1) 24h, 25°C, light | $299.71 \pm 9.37^{b}$ | $34.93 \pm 1.46^{b}$ |
| 2) 48h, 30°C, light | $343.28 \pm 9.85^{a}$ | $23.35 \pm 0.77^{c}$ |
| 3) 72h, 25°C, darkness | $300.83 \pm 6.67^{b}$ | $58.44 \pm 1.23^{a}$ |
| **ORAC (µmol TE $g^{-1}$)** | | |
| Non-germinated | $823.31 \pm 2.1^{c}$ | $60.96 \pm 6.09^{b}$ |
| 1) 24h, 25°C, light | $995.92 \pm 16.29^{b}$ | $36.72 \pm 3.67^{c}$ |
| 2) 48h, 30°C, light | $1024.05 \pm 55.83^{b}$ | $33.51 \pm 2.91^{c}$ |
| 3) 72h, 25°C, darkness | $1191.43 \pm 46.62^{a}$ | $143.69 \pm 3.21^{a}$ |

Values in the same column with the same letters are not significantly different ($p \leq 0.05$)

**Table 2.** Non-germinated and germinated conditions used to obtain the black mustard extracts from soluble and insoluble-bound fractions and the results for some phenolic compounds and antioxidant properties.

| Assays | SFM | IFM |
|---|---|---|
| **Total phenolic compounds (mg GAE $g^{-1}$)** | | |
| Non-germinated | $13.21 \pm 0.47^{c}$ | $1.76 \pm 0.07^{a}$ |
| 1) 48h, 25°C, light/darkness | $19.00 \pm 0.92^{a}$ | $1.35 \pm 0.03^{b}$ |
| 2) 72h, 25°C, light | $16.92 \pm 0.08^{b}$ | $1.59 \pm 0.08^{a}$ |
| 3) 72h, 30°C, light/darkness | $19.11 \pm 0.98^{a}$ | $1.60 \pm 0.04^{a}$ |
| **Total flavonoids (mg CE $g^{-1}$)** | | |
| Non-germinated | $8.97 \pm 0.24^{b}$ | $0.51 \pm 0.002^{b}$ |
| 1) 48h, 25°C, light/darkness | $10.63 \pm 0.42^{a}$ | $0.50 \pm 0.02^{b}$ |
| 2) 72h, 25°C, light | $10.44 \pm 0.48^{a}$ | $0.61 \pm 0.02^{a}$ |
| 3) 72h, 30°C, light/darkness | $9.47 \pm 0.27^{b}$ | $0.54 \pm 0.05^{ab}$ |
| **Condensed tannins (mg CE $g^{-1}$)** | | |
| Non-germinated | $1.75 \pm 0.12^{c}$ | $0.28 \pm 0.004^{b}$ |
| 1) 48h, 25°C, light/darkness | $2.72 \pm 0.16^{a}$ | $0.27 \pm 0.02^{b}$ |
| 2) 72h, 25°C, light | $2.14 \pm 0.07^{b}$ | $0.54 \pm 0.02^{b}$ |
| 3) 72h, 30°C, light/darkness | $2.52 \pm 0.13^{a}$ | $1.33 \pm 0.26^{a}$ |
| **FRAP (µmol TE $g^{-1}$)** | | |
| Non-germinated | $115.92 \pm 2.7^{c}$ | $21.57 \pm 0.38^{b}$ |
| 1) 48h, 25°C, light/darkness | $149.69 \pm 2.49^{b}$ | $17.56 \pm 0.60^{c}$ |
| 2) 72h, 25°C, light | $156.98 \pm 3.55^{ab}$ | $16.74 \pm 0.15^{c}$ |
| 3) 72h, 30°C, light/darkness | $164.91 \pm 4.12^{a}$ | $23.64 \pm 0.41^{a}$ |
| **DPPH (µmol TE $g^{-1}$)** | | |
| Non-germinated | $60.59 \pm 0.61^{a}$ | $9.84 \pm 0.065^{a}$ |
| 1) 48h, 25°C, light/darkness | $62.54 \pm 0.65^{a}$ | $8.14 \pm 0.37^{b}$ |
| 2) 72h, 25°C, light | $62.58 \pm 1.28^{a}$ | $8.49 \pm 0.22^{b}$ |
| 3) 72h, 30°C, light/darkness | $43.34 \pm 2.86^{b}$ | $9.33 \pm 0.17^{a}$ |
| **ABTS (µmol TE $g^{-1}$)** | | |
| Non-germinated | $67.82 \pm 3.27^{c}$ | $17.03 \pm 1.01^{a}$ |
| 1) 48h, 25°C, light/darkness | $176.34 \pm 2.92^{a}$ | $14.63 \pm 1.31^{ab}$ |
| 2) 72h, 25°C, light | $161.64 \pm 14.33^{a}$ | $13.25 \pm 0.85^{b}$ |
| 3) 72h, 30°C, light/darkness | $134.21 \pm 8.16^{b}$ | $13.22 \pm 0.67^{b}$ |
| **ORAC (µmol TE $g^{-1}$)** | | |
| Non-germinated | $239.18 \pm 3.83^{c}$ | $37.76 \pm 2.59^{a}$ |
| 1) 48h, 25°C, light/darkness | $339.26 \pm 15.33^{b}$ | $31.69 \pm 0.64^{b}$ |
| 2) 72h, 25°C, light | $364.59 \pm 19.20^{b}$ | $31.87 \pm 0.51^{b}$ |
| 3) 72h, 30°C, light/darkness | $430.32 \pm 13.33^{a}$ | $30.92 \pm 0.39^{b}$ |

Values in the same column with the same letters are not significantly different ($p \leq 0.05$)

## 4. Conclusion

The increase in phenolic compounds content induced by germination resulted in the increase in antioxidant properties of white and black mustard mainly for the free fraction. Both free and bound fraction were synthesized and degraded during this process depending on germination condition, that was essential to maximize phenolic compounds and antioxidant properties of sprouts. In this sense, for white mustard, 72 h of germination at 25°C in the dark and for black mustard, 48 h of germination at 25°C with alternation of light and dark periods were selected as the most appropriate conditions of germination.

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**Discussão**

Considerando a diversidade de composição de cada grão presente na natureza, é necessário escolher o solvente adequado para extração de compostos bioativos, determinar as condições de processo que valorizem o teor dos compostos funcionais nele presentes, assim como identificar os compostos responsáveis pela bioatividade. Tendo em vista estes quesitos, os resultados encontrados no presente trabalho visaram responder e esclarecer os pontos acima citados, para grãos de mostarda branca (*Sinapsis alba*) e preta (*Brassica nigra*).

A combinação binária de água com acetona, em proporções iguais, foi a melhor associação de solventes para extrair compostos fenólicos com propriedades antioxidantes dos grãos de mostarda. Nessa condição, os extratos de mostarda branca apresentaram 19,39 mg GAE $g^{-1}$, 109,25 e 61,25 μmol TE $g^{-1}$ para os métodos de ABTS e DPPH, respectivamente. Assim, a extração de compostos antioxidantes com água e acetona resultou em aumentos de 23 vezes para o teor de compostos fenólicos totais, 48 vezes para atividade antioxidante medida pelo método ABTS e 25 vezes para redução de radicais DPPH quando comparados com os extratos obtidos com acetona pura. Para as amostras de mostarda preta, o teor de compostos fenólicos totais foi 12,16 mg GAE $g^{-1}$, enquanto as atividades antioxidantes medidas pelos métodos ABTS e DPPH foram 36,22 e 26,24 μmol TE $g^{-1}$, respectivamente. Ainda, o extrato obtido com essa combinação de solventes apresentou valores de fenólicos totais e atividade antioxidante para ABTS e DPPH, 19, 31 e 27 vezes maiores, respectivamente, do que o produzido com acetona pura. A adição de água à solventes orgânicos, além de reduzir o impacto ao meio ambiente, aumenta a polaridade dos solventes orgânicos, extraindo uma maior quantidade e variedade de compostos fenólicos. O potencial antioxidante dos grãos de mostarda abordado nos resultados deste trabalho ainda não foi explorado literatura e, a melhor combinação de solventes foi elucidada pela primeira vez nesse trabalho colaborando para pesquisas futuras com esses grãos. Como complemento de resultados, nessa condição de extração, identificou-se a presença de ácido sinápico por cromatografia, que pode ser um dos compostos fenólicos responsáveis pela atividade antioxidante encontrada.

Adicionalmente, além dos aspectos relacionados à recuperação de compostos bioativos de matrizes vegetais, a aplicação de processos que levem à modificação do perfil destes compostos em termos qualitativos e quantitativos também merece destaque. Assim, a germinação de grãos e sementes ganhou espaço na literatura nos últimos anos. Este processo é conhecido por agregar valor nutricional, funcional e sensorial aos grãos germinados. Sabe-se também, que as condições ambientais em que é feita a germinação influenciam diretamente na composição final dos brotos, e consequentemente na bioatividade dos mesmos. Tendo em vista essa tendência nas pesquisas científicas, o trabalho desenvolvido nessa dissertação também

contemplou o estudo de diferentes condições ambientais e seus efeitos no teor de compostos fenólicos e propriedades antioxidantes no extrato solúvel (acetona:água) de grãos de mostarda preta e branca. Após os experimentos, foram escolhidas 3 condições para cada espécie, baseadas nas análises realizadas (teor de compostos fenólicos totais, DPPH e ABTS). Para mostarda branca, as seguintes condições foram definidas como mais adequadas: 1) 24 horas de germinação a 25°C na presença de luz durante todo processo, 2) 48 horas a 30°C na presença de luz e 3) 72 horas a 25°C no escuro. Nas condições supracitadas, os extratos apresentaram aumentos de 43%, 148% e 27% para atividade antioxidante mensurada pelos métodos ABTS e DPPH e para teor de compostos fenólicos totais, respectivamente, quando comparados com os obtidos a partir da mostarda não germinada. Na mesma comparação, para mostarda preta, as 3 condições selecionadas apresentaram aumentos de 15%, 105% e 82% para ABTS, DPPH e compostos fenólicos totais, respectivamente, a saber: 1) 48 horas de germinação a 25°C com períodos alternados de luz e escuro durante o processo, 2) 72 horas a 25°C na presença de luz e 3) 72 horas de germinação a 30°C alternando luz e escuro.

Com base nas mudanças positivas observadas durante a germinação para as condições definidas como mais adequadas para aumento do teor de compostos fenólicos e propriedades antioxidantes, as frações solúvel e insolúvel das mesmas foram analisadas. O aumento do conteúdo de compostos fenólicos induzido pela germinação também resultou em aumento das propriedades antioxidantes das mostardas preta e branca, principalmente para a fração livre, solúvel. Notou-se que ambas as frações, solúvel e insolúvel, foram degradadas e sintetizadas durante a germinação em função da condição aplicada, o que foi essencial para maximizar o teor de compostos fenólicos e propriedades antioxidantes dos brotos. Para os extratos de mostarda branca, germinar durante 72 horas na temperatura de 25°C no escuro foi a condição escolhida com os maiores valores para ambas as frações. Para a fração solúvel o aumento foi de 1,5 vezes, em média, para todas as análises realizadas (teor de fenólicos totais, flavonoides totais, taninos condensados e atividades antioxidantes medidas pelos métodos ABTS, DPPH, FRAP e ORAC) quando comparadas com a fração solúvel da mostarda branca não germinada. A fração insolúvel desta mesma condição apresentou valores para as mesmas análises que variaram de 0,89 a 2,35 vezes maiores em comparação com a fração insolúvel do grão não germinado. Na mesma linha de análise dos resultados, a condição de germinação mais apropriada para mostarda preta foi incubação por 48 horas a 25°C com períodos alternados de luz e escuro. Nesta condição, os extratos da fração solúvel apresentaram valores de compostos fenólicos e atividade antioxidante até 2 vezes maiores que os valores apresentados pelo extrato da fração solúvel de mostarda preta não germinada. A fração insolúvel extraída desse grão apresentou comportamento contrário, com diminuição ou manutenção das respostas avaliadas

(teor de fenólicos totais, flavonoides totais, taninos condensados e atividades antioxidantes medidas pelos métodos ABTS, DPPH, FRAP e ORAC) durante a germinação.

Os resultados obtidos ao longo do presente trabalho mostraram o quão eficiente pode ser o processo de germinação na melhoria das propriedades bioativas de um grão quando realizado de maneira otimizada e adequada. As variações positivas durante a germinação nas duas frações em que os compostos fenólicos podem estar presentes são resultado de um processo complexo e contínuo em que todas transformações ocorrem ao mesmo tempo. De maneira simplificada, sabe-se que os fenólicos livres, presentes no endosperma do grão, podem ser transformados em fenólicos ligados à parede celular, que, em crescimento, precisa de compostos estruturais. Os fenólicos se ligam a macromoléculas e participam assim, da nova estrutura da planta que deve surgir. O contrário também acontece. Os compostos que estão ligados à parede celular, por ação enzimática, ativada pela absorção de água, são liberados para a fração solúvel, que muitas vezes apresenta aumento do teor de compostos fenólicos e/ou atividade antioxidante. Os brotos de mostarda são resultado de um processo simples, barato e caseiro que pode ser repetido facilmente e que apresenta valor agregado, principalmente por ser mais uma opção de consumo da mostarda.

## Conclusão geral

A germinação de grãos de mostarda preta e branca apresentou resultados positivos em relação às propriedades antioxidantes dos mesmos. Observou-se que germinar mostarda branca por 72 horas a 25°C no escuro e mostarda preta por 48 horas a 25°C com períodos alternados de luz e escuro são alternativas de consumo com maior valor funcional agregado. Os teores de compostos fenólicos totais, flavonoides totais, taninos condensados e atividade antioxidante por diversos métodos apresentaram aumentos significativos nos extratos obtidos a partir de grãos germinados quando comparados com os não germinados. Além disso, identificou-se a presença de ácido sinápico nos extratos produzidos com os grãos não germinados na condição estudada e considerada a mais adequada para extração: acetona:água, em iguais proporções. Por ser um processo fácil, barato e simples é uma alternativa para consumo dos grãos.

**Perspectivas futuras**

A utilização de mostarda germinada como fonte natural de compostos fenólicos com propriedades antioxidantes mostrou-se uma alternativa interessante para uma alimentação saudável e de fácil preparo, duas grandes tendências mundiais de alimentação. No entanto, mais estudos são necessários para a determinação do efeito real destes compostos em sistemas mais complexos. Sendo assim, algumas tendências propostas são descritas:

- Identificação dos compostos fenólicos presentes nos extratos solúveis e insolúveis dos grãos de mostarda branca e preta germinados e não germinados por UPLC-MS;

- Aplicação dos extratos em culturas de células para avaliação do potencial antioxidante *in vitro* desses compostos;

- Simulação de digestão *in vitro* para determinação de bioacessibilidade dos compostos antioxidantes presentes nos grãos;

- Aplicação *in vivo* dos extratos de mostarda para avaliação dos seus efeitos no organismo;

- Comparação dos resultados obtidos para os extratos deste estudo com extratos produzidos a partir de molhos de mostarda (a forma mais consumida desse grão);

- Avaliação de outras bioatividades dos extratos de grãos germinados, como atividades anticarcinogênica, anti-hipertensiva e anti-inflamatória.

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