UNIVERSIDADE FEDERAL RURAL DO RIO DE JANEIRO
INSTITUTO DE TECNOLOGIA
DEPARTAMENTO DE TECNOLOGIA DE ALIMENTOS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

DISSERTAÇÃO

Efeito protetor da Pimenta Rosa (*Schinus terebinthifolia* Raddi) sobre a foto-oxidação lipídica em sistemas modelo contendo óleo de soja

LAURA MONTEIRO KELLER

2025

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RESUMO

KELLER, Laura Monteiro. **Efeito protetor da pimenta rosa (*Schinus terebinthifolia* Raddi) sobre a foto-oxidação lipídica em sistemas modelo contendo óleo de soja.** 139p. Dissertação (Mestrado em Ciência e Tecnologia de Alimentos). Instituto de Tecnologia. Departamento de Tecnologia de Alimentos, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, 2025.

O óleo de soja refinado é o segundo óleo vegetal mais consumido no mundo e, devido a sua composição em lipídios insaturados, é um produto de extrema suscetibilidade a degradação oxidativa, incluindo a foto-oxidação, causada pela ação das radiações ultravioleta e luminosa. A prática doméstica de condimentar óleos com temperos e especiarias, objetivando aromatizar e saborizar, pode ter um impacto positivo sobre a oxidação do óleo durante o armazenamento. A pimenta rosa (*Schinus terebinthifolia* Raddi; PR), é fruto da aroeira, nativa e abundante no Brasil, rica em compostos bioativos com potencial antioxidante. Esta pesquisa propôs a avaliação da estabilidade oxidativa do óleo de soja comercial refinado nas condições controle (0% PR) e condimentado com 2%, 4% e 6% de frutos *in natura* de PR, durante armazenamento por 15, 30, 45 e 60 dias sob exposição a luz e temperatura ambientes. Foi realizada a caracterização do extrato dos frutos em relação a fenólicos totais (7,40 ± 0,01 mg EAG/g), flavonoides totais (26,63 ± 1,59 mg EQ/g), capacidade antioxidante *in vitro* DPPH (87,43 ± 0,34 % de inibição) e FRAP (125,25 ± 1,76 μmol TE/g), caracterização de compostos bioativos por cromatografia líquida acoplada a espectrometria de massas de alta resolução (HPLC-QTOF-MS/MS) determinando compostos como ácido masticadienóico (*m/z* 453,3351), amentoflavona (*m/z* 537,0809), agatisflavona (*m/z* 537,0794) e ácido cítrico (*m/z* 191,0175). Para avaliação da oxidação lipídica dos óleos foram realizadas as análises de índice de peróxidos, formação de espécies reativas ao ácido tiobarbitúrico (TBARS), composição de ácidos graxos, teores dos principais fitosteróis (β-sitosterol, estigmasterol e campesterol) e seus respectivos óxidos. A exposição à luz durante o armazenamento levou ao aumento nos teores de peróxidos e TBARS. O perfil de ácidos graxos não apresentou diferenças significativas ($p>0,05$) ao longo de 60 dias. Os fitosteróis degradaram ao longo do tempo. A amostra controle no 60º apresentou redução de 57,8% do β-sitosterol, 54,5% do estigmasterol e 50,8% do campesterol. A amostra com 6% PR apresentou melhor proteção, com 16%, 33% e 36% de preservação do β-sitosterol, estigmasterol e campesterol, respectivamente. Dentre os óxidos analisados o 7-ceto e o 5,6 β-epóxido foram predominantemente formados nos três fitosteróis analisados. No último dia o total de óxidos formados foi de 52,88 μg/g na amostra controle, e as amostras adicionadas de PR apresentaram uma proteção de 23% (2% PR), 37% (4% PR) e 51% (6% PR) em relação ao total de óxidos formados. A adição da pimenta rosa foi eficaz em minimizar as reações de foto-oxidação do óleo de soja, demonstrando ser uma fonte natural antioxidante potencial de proteção do óleo durante o armazenamento.

Palavras-chave: *Schinus Terebhintifolia* Raddi, foto-oxidação, óleo de soja, estabilidade oxidativa.

ABSTRACT

KELLER, Laura Monteiro. **Protective effect of pink pepper (*Schinus terebinthifolia* Raddi) on lipid photooxidation in model systems containing soybean oil.** 139p. Dissertation (Master in Food Science and Technology). Institute of Technology, Food Technology Department, Federal Rural University of Rio de Janeiro, Seropédica, RJ, 2025.

Refined soybean oil is the second most consumed vegetable oil in the world and, due to its composition of unsaturated lipids, it is a product extremely susceptible to oxidative degradation, including photooxidation, caused by ultraviolet and light radiation. The domestic practice of seasoning oils with spices, aiming to aromatize and flavor, can have a positive impact on the oxidation of the oil during storage. Pink pepper (*Schinus terebinthifolia* Raddi; PP), is a fruit of the aroeira tree, native and abundant in Brazil, rich in bioactive compounds with antioxidant potential. This research proposed the evaluation of the oxidative stability of refined commercial soybean oil under control conditions (0% PP) and seasoned with 2%, 4% and 6% of fresh PP fruits, during storage for 15, 30, 45 and 60 days under exposure to light and room temperature. The characterization of the fruit extract was performed in relation to total phenolics (7.40 ± 0.01 mg EAG/g), total flavonoids (26.63 ± 1.59 mg EQ/g), in vitro antioxidant capacity DPPH (87.43 ± 0.34% inhibition) and FRAP (125.25 ± 1.76 μmol TE/g), characterization of bioactive compounds by liquid chromatography coupled to high-resolution mass spectrometry (HPLC-QTOF-MS/MS) determining some compounds such as masticadienoic acid (m/z 453.3351), amenthoflavone (m/z 537.0809), agathisflavone (m/z 537.0794) and citric acid (m/z 191.0175). To evaluate the lipid oxidation of the oils, analyses of peroxide index, formation of thiobarbituric acid reactive species (TBARS), fatty acid composition, levels of the main phytosterols (β-sitosterol, stigmasterol and campesterol) and their respective oxides were performed. Exposure to light during storage led to an increase in the levels of peroxides and TBARS. The fatty acids did not show significant differences ($p>0.05$) over 60 days. The phytosterols degraded over time. The control sample at 60 days showed a reduction of 57.8% of β-sitosterol, 54.5% of stigmasterol and 50.8% of campesterol. The sample with 6% PP showed better protection, with 16%, 33% and 36% preservation of β-sitosterol, stigmasterol and campesterol, respectively. Among the oxides analyzed, 7-keto and 5,6-β-epoxy were predominantly formed in the three phytosterols analyzed. On the last day, the total oxides formed was 52.88 μg/g in the control sample, and the samples added with PP showed a protection of 23% (2% PP), 37% (4% PP) and 51% (6% PP) in relation to the total oxides formed. The addition of pink pepper was effective in minimizing the photooxidation reactions of soybean oil, demonstrating that it is a potential natural antioxidant source for protecting the oil during storage.

Keywords: *Schinus Terebhintifolia* Raddi, photo-oxidation, soybean oil, oxidative stability.

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LISTA DE SIGLAS E ABREVIATURAS

AGMI Ácido graxo monoinsaturado

AGPI Ácido graxo poli-insaturado

AGS Ácido graxo saturado

BHA Butilidroxianisol/Butylhydroxyanisole

BHT Butilidroxitolueno/Butylhydroxytoluene

C16:0 Ácido palmítico

C18:0 Ácido esteárico

C18:1 ω9 cis Ácido oléico

C18:2 ω6 cis Ácido linoléico

C18:3 ω3 Ácido α-Linolênico

DPPH 2,2-difenill-1-picril-hidraliza

ESI Electrospray Ionization/ Ionização por Eletrospray

FRAP Ferric Reducing Antioxidant Power/Poder Antioxidante Redutor Férrico

GP Galato de propila

HPLC High Performance Liquid Chromatography

IP Índice de peróxidos

LC Liquid chromatography

LDL Low density lipoprotein

MS Mass Spectrometry

POC Produtos de oxidação do colesterol

POF Produtos de oxidação de fitosteróis

PR Pimenta rosa

QTOF Quadrupole time-of-flight

RTIQ Regulamento técnico de identidade e qualidade

SOT Schaal Oven Test

TBARS Thiobarbituric acid reactive substances

TBHQ Terc-butilidroquinona / Tert-butylhydroquinone

UPLC Ultra Performance Liquid Chromatography

USDA United States Department of Agriculture

UV Ultravioleta

ω3 / n3 Ômega 3

ω6 / n6 Ômega 6

ω9 / n9 Ômega 9

Sumário

INTRODUÇÃO GERAL

O Brasil lidera a produção mundial de grãos de soja, com safra estimada em 169,5 milhões de toneladas para 2024/25, aumento de 14,7% em relação à safra passada (CONAB, 2025), além disso, o Brasil é o terceiro maior produtor de óleo de soja (USDA, 2025). O óleo de soja é um óleo vegetal muito relevante em termos de produção, consequentemente muito consumido mundialmente, sendo a segunda maior produção global dentre os óleos vegetais. Em 2024 a produção do óleo de soja no mundo atingiu o volume de 65,86 milhões de toneladas, equivalendo a 715,9 milhões de litros. Estima-se que o consumo tenha sido em torno de 65,23 milhões de toneladas (Martini; Nerli; Malpiedi, 2022; USDA, 2025).

O óleo de soja é obtido de sementes de *Glycine max* pelos processos de extração e refino. Em sua composição, os ácidos graxos insaturados representam 85% do total de lipídios e, aproximadamente, 60% destes constituídos pelos ácidos graxos essenciais linoleico (35 – 60%) e linolênico (2,0 – 13%). Além disso, contêm 0,3 a 0,4% de fitosteróis, que são esteróis vegetais conhecidos pelo potencial hipocolesterolêmico, anti-inflamatório, antiaterogênico e anticancerígeno (Bai; Ma; Chen, 2021; Gasparetto; De Castilhos; Salau, 2022; Sun et al., 2022).

Devido a significativa quantidade de lipídios insaturados (ácidos graxos e fitosteróis) o óleo de soja é um produto propenso à degradação por oxidação lipídica (Zhao et al., 2019b). Em óleos vegetais, a oxidação causa mudanças sensoriais como sabor indesejável, odor de ranço e alterações na cor, além de mudanças no potencial nutricional pela perda de lipídios importantes, e formação de produtos de deletérios a saúde, que podem levar ao desenvolvimento de doenças cardiovasculares, neurológicas, cânceres e inflamações (Esposto et al., 2021; Grootveld et al., 2020; Martini; Nerli; Malpiedi, 2022).

Os principais mecanismos de oxidação lipídica em óleos vegetais são a auto-oxidação e a foto-oxidação. A oxidação ocorre entre as moléculas insaturadas e o oxigênio (O_2), na foto-oxidação é necessária a presença de radiação luminosa ou ultravioleta (UV) e moléculas fotossensibilizadoras ou cromóforas (Verduin, 2020). A auto-oxidação é catalisada pela ação de fatores como calor, enzimas, presença de íons metálicos e espécies reativas ao oxigênio, que atuam facilitando e acelerando as reações com o oxigênio (Wang et al., 2023). A perda de antioxidantes naturais como tocoferóis e carotenoides durante o refino do óleo de soja, torna-o ainda mais propenso a sofrer

oxidação (Martini; Nerli; Malpiedi, 2022). Além disso, as condições de armazenamento, temperatura e preparo podem acelerar sua degradação, tornando-o impróprio para consumo (Yang; Chu; Liu, 2005). Assim, estratégias industriais e domésticas são necessárias para mitigar a oxidação do óleo de soja.

Os antioxidantes sintéticos são amplamente utilizados pela indústria, em setores diversos, como cosméticos, farmacêutico, combustíveis e alimentos, a fim de aumentar a validade dos produtos de forma eficiente e com baixo custo. Entretanto, a ingestão a longo prazo de aditivos amplamente utilizados como o hidroxitolueno butilado (BHT) e o butil-hidroxianisol (BHA) apresentam efeitos deletérios à saúde e ao meio-ambiente (Liu; Mabury, 2020; Xu et al., 2021). No Brasil, os antioxidantes sintéticos permitidos pela legislação e os limites de adição são estabelecidos pela resolução nº 04, de 24 de novembro de 1988 do ministério da saúde (BRASIL, 2023; Kmiecik et al., 2011).

Limitar o uso dos antioxidantes sintéticos, no entanto, não é suficiente. A demanda crescente dos consumidores por produtos "*clean label*" (livres de aditivos sintéticos) e sustentáveis faz com que o consumo de produtos com aditivos sintéticos passe a ser evitado (Chauhan; Rao, 2024; Yang; Chu; Liu, 2005). Por isso, estratégias para a substituição desses aditivos têm sido estudadas. Em óleos vegetais, a adição de compostos naturais como extratos de folhas e frutos, especiarias *in natura* e subprodutos da agroindústria tem mostrado eficiência como potenciais antioxidantes (Abdo; Shaltout; Mansour, 2023; Arabsorkhi; Pourabdollah; Mashadi, 2023; Sharma; Chakkaravarthi; Bhattacharya, 2023; Umeda, 2021).

A pimenta rosa (*Schinus terebinthifolia* Raddi) é fruto da aroeira, árvore popularmente conhecida na América do Sul e presente em abundância em diversas regiões do Brasil (Ennigrou et al., 2017). Seus frutos, folhas e caules são ricos em compostos bioativos como terpenos e fenólicos, que lhes confere atividades antimicrobianas, antioxidantes e anti-inflamatórias. Seus frutos, utilizados pela medicina popular, também são vastamente empregados como condimentos na culinária (Ennigrou et al., 2017; Linden et al., 2020; Oliveira et al., 2020a). Os frutos e folhas da pimenta rosa foram aplicados em alimentos ricos em lipídios, a fim de avaliar o potencial dessas fontes em conferir estabilidade oxidativa, demonstrando capacidade de mitigar as reações de oxidação nesses alimentos (De Oliveira et al., 2022; Feriani et al., 2021; Feuereisen et al., 2017; Oliveira et al., 2020a; Vieira et al., 2023).

O objetivo do presente estudo foi avaliar o efeito protetor antioxidante dos frutos da pimenta rosa adicionados *in natura* a amostras de óleo comercial de soja refinado,

com exposição de luz natural, a fim de simular condições de luminosidade doméstica de armazenamento deste produto. Para isso o experimento foi conduzido de forma a ser reprodutível por indivíduos de forma simples e em cozinhas comuns.

OBJETIVOS

Objetivo geral

Estudar o efeito da adição de pimenta rosa (*Schinus terebinthifolia* Raddi) frente à oxidação lipídica em sistemas modelo contendo óleo de soja refinado, armazenado durante sessenta (60) dias, sob exposição de luz natural (12 h por dia) e temperatura ambiente (25 °C).

Objetivos específicos

- ✓ Caracterizar quimicamente o extrato da pimenta rosa (PR) quanto compostos fenólicos totais, flavonoides totais, compostos bioativos e capacidade antioxidante *in vitro* através dos métodos de DPPH e FRAP;
- ✓ Analisar as amostras de óleo de soja controle e óleo de soja com adição de pimenta rosa em três diferentes concentrações em 60 dias de armazenamento sob exposição de luz solar quanto ao índice de peróxidos, substâncias reativas ao ácido tiobarbitúrico (TBARS), ácidos graxos, fitosteróis (β-sitosterol, campesterol e estigmasterol) e óxidos de fitosterol.
- ✓ Analisar e correlacionar os dados de forma a concluir se a pimenta rosa pode ser adicionada ao óleo de soja como um antioxidante natural de forma a mitigar a foto-oxidação

REVISÃO DE LITERATURA

1 Óleo de soja

Os óleos são assim denominados pelo seu estado líquido a 25 °C (Fennema; Damodaran; Parkin, 2017; Wang et al., 2023). De acordo com o regulamento técnico de identidade e qualidade (RTIQ) dos óleos vegetais refinados (Instrução normativa nº 49, de 22 de dezembro de 2006), define-se óleo vegetal comestível como "produto alimentício constituído principalmente por triacilgliceróis de ácidos graxos, obtidos unicamente de matéria-prima vegetal, refinado mediante o emprego de processos tecnológicos adequados. Estes poderão conter pequenas quantidades de outros lipídios, tais como fosfolipídios, constituintes insaponificáveis e ácidos graxos livres, naturalmente presentes no óleo vegetal, além de ausência de turvação e substâncias em suspensão, como sujidades, parasitos e larvas.".

Em relação aos grãos, a soja é apontada como o mais produzido, importado e exportado no mundo. A produção mundial de grãos de soja atingiu 427.14 milhões de toneladas em 2024, e o Brasil foi o maior produtor e exportador dessa commoditie (USDA, 2025).

Em relação aos óleos vegetais, o óleo de soja é o segundo óleo mais produzido, importado e exportado no mundo, sendo o óleo de palma o primeiro. Em 2024, 65.86 milhões (M) de toneladas (t) de óleo de soja foram produzidas, 11.79M t importadas e 12.65M t exportadas. O Brasil é o terceiro maior produtor de óleo de soja do mundo e o segundo maior exportador. Já em relação ao consumo doméstico mundial, a USDA estimou 65.27M t, sendo também o segundo óleo mais consumido domesticamente, atrás do óleo de palma. Em relação ao consumo doméstico por país, o Brasil encontra-se em terceiro lugar, com 9,725M t consumidas em 2024 (USDA, 2025).

Como o segundo óleo vegetal mais consumido no mundo, grande parte da produção dos grãos de soja são destinados à produção de óleo comestível. No Brasil, o óleo de soja responde por aproximadamente 95% do consumo entre os óleos vegetais, utilizado nas cozinhas preferencialmente como base de frituras (Martini; Nerli; Malpiedi, 2022).

O óleo de soja é um produto extraído dos grãos de soja (*Glycine max L*), que possuem em sua composição aproximadamente 40% de proteínas, 20% de lipídios, 15% de carboidratos, 5% de cinzas. Além de compostos fenólicos variando de 0,29 a 0,51 % (Gasparetto; De Castilhos; Salau, 2022). Para a extração do óleo são realizadas as etapas de tratamento dos grãos (pré-limpeza, descascamento, condicionamento, trituração e

cozimento) e de extração química, com o uso de solventes orgânicos, e física, por prensagem. Após a extração, o óleo bruto é submetido aos processos de degomagem, neutralização, branqueamento e desodorização, transformando-o em óleo comestível. No óleo de soja os ácidos graxos representam aproximadamente 96% da sua composição, dentre eles 60% de poli-insaturados, 24% monoinsaturados e 16% saturados. Dentre eles, aproximadamente 54% de ácido linoleico (C18:2 ω-6), 23% ácido oleico (C18:1), 11% ácido palmítico (C16:0), 8% ácido linolênico (C18:3 ω-3) e 4% ácido esteárico (C18:0) (Sun et al., 2022).

Tabela 1: Composição dos principais ácidos graxos do óleo de soja (adaptado de Alves et al., 2019).

ÁCIDO GRAXO	%
Ácido palmítico (C16:0)	12,20
Ácido palmitoléico (C16:1)	0,01
Ácido esteárico (C18:0)	6,50
Ácido oleico (C18:1cis ω9)	29,10
Ácido linoleico (C18:2cis ω6)	44,10
Ácido α-linolênico (C18:3 ω3)	2,70
∑AGS	18,70
∑AGMI	29,11
∑AGPI	46,80
Total	**100,00**

Legenda: AGS: ácidos graxos saturados; AGMI: ácidos graxos monoinsaturados; AGPI: ácidos graxos poli-insaturados.

Nas matrizes vegetais predominam os ácidos graxos insaturados oleico, linoleico e α-linolênico, que desempenham diversas funções no organismo humano, como serem precursores de moléculas bioativas e servirem de estoque para constituintes da barreira extracelular (He et al., 2020). A ingestão de ácidos graxos insaturados, principalmente os ácidos das famílias ômega 3 (ω3) e ômega 6 (ω6), é associada a efeitos positivos para a saúde, como potencial anti-inflamatório, anticancerígeno e antioxidante, além de contribuir também para a redução do risco de desenvolvimento de doenças cardiovasculares (Liu et al., 2023; Messina; Shearer; Petersen, 2021; Ravaut et al., 2021; Tutor et al., 2024).

1.1. Fitosteróis no óleo de soja

Além de ácidos graxos, óleos vegetais também são fontes de fitosteróis. Os Fitosteróis (FS) são esteróis insaturados formados a partir do metabolismo secundário de vegetais. Presentes principalmente em óleos vegetais comestíveis, sementes, cereais, castanhas, legumes e frutas (Cabral; Klein, 2017; Rhazi et al., 2022), eles compõe a estrutura das membranas celulares e participam de seu funcionamento (Barkas et al., 2023). Mais de 250 diferentes moléculas de fitosteróis já foram identificados na literatura, e nos óleos vegetais estão presentes numa faixa de 0,03 a 1,1%. Os FS predominantes nos óleos vegetais são o β-sitosterol, campesterol e estigmasterol (Figura 1) (He et al., 2018; Kmiecik et al., 2011).

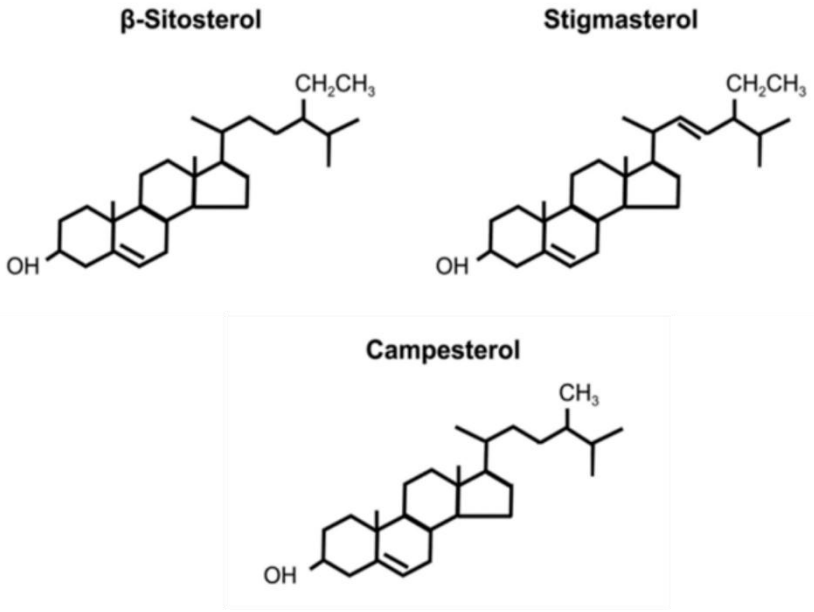

Figura 1: Estrutura plana dos principais fitosteróis encontrados em óleos vegetais (Matsuoka, 2022).

Os esteróis vegetais são biossinteticamente derivados do esqualeno e pertencem ao grupo dos triterpenos. Sua estrutura básica é conhecida como ciclopentanoperidrofenantreno, análoga à estrutura do colesterol, diferenciando-se apenas pela estrutura da cadeia lateral "R" (Figura 2). A maior parte dos fitosteróis possuem cadeias longas com 28 ou 29 átomos de carbono e com uma ou duas ligações duplas em sua estrutura (Furse et al., 2023; Poudel et al., 2022), além disso, os fitosteróis ocorrem principalmente como esterol livre ou esterificado com ácidos graxos ou com

monossacarídeos (Bai; Ma; Chen, 2021).

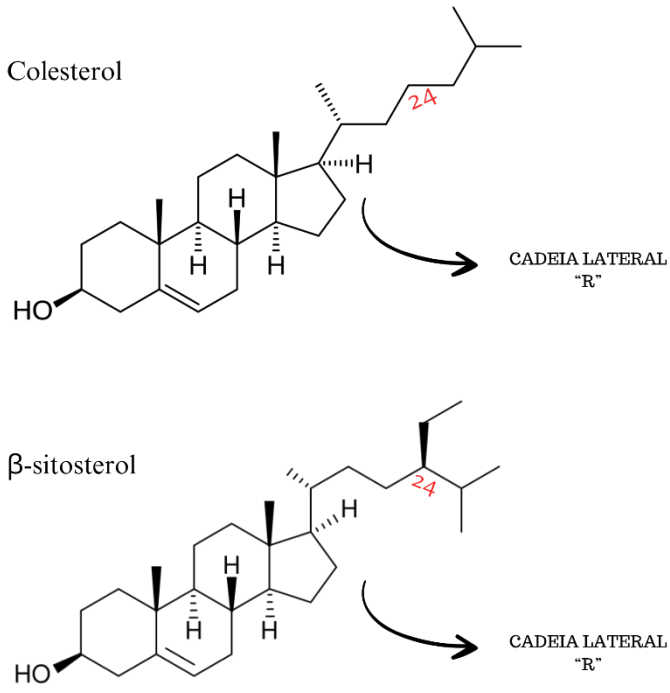

Figura 2: Comparação entre as estruturas químicas do colesterol e do fitosterol β-sitosterol (Autora, 2025).

No óleo de soja, a composição aproximada desses fitosteróis é de 124 a 173 mg/100g de β-sitosterol, 34 a 82 mg/100g de campesterol e 37 a 64 mg/100g de estigmasterol (Chang et al., 2020). O β-sitosterol aparece em maiores concentrações também em outros óleos vegetais como nos óleos de canola, milho e azeite de oliva (Kmiecik et al., 2011). A concentração de fitosteróis nos óleos depende de diversos fatores como genética das sementes, localização da plantação e condições de crescimento da planta, como temperatura (Vlahakis; Hazebroek, 2000)

De forma similar, o processo de refino afeta significativamente as concentrações de fitosteróis dos óleos vegetais. Verleyen et al. (2002) avaliaram a influência dos processos de refino em alguns óleos, incluindo o óleo de soja, que apresentou total de 326,6 mg/100g de fitosteróis antes dos processos de refino. Os fitosteróis foram quantificados após cada uma das etapas dos processos de refino físico (degomagem, branqueamento e desodorização) e químico (degomagem, neutralização, branqueamento e desodorização). Ao final dos dois tipos de refino os fitosteróis totais foram reduzidos, o processo físico resultou numa perda de aproximadamente 13%, enquanto o refino químico resultou em 34% de perda do total de fitosteróis. Além da perda dos esteróis, o

refino do óleo também causa a perda compostos antioxidantes naturalmente presentes nos óleos, como tocoferóis. No mesmo estudo os autores registraram uma perda de 36% do total de tocoferóis no processo de refino físico e 50% de perda no processo químico.

A Sociedade Europeia de Aterosclerose recomenda a ingestão diária de esteróis vegetais para a redução das concentrações séricas de colesterol LDL (*low-density lipoprotein*), de no mínimo 2 g/dia na refeição principal, por indivíduos em certas condições de saúde, como, pessoas com níveis elevados de colesterol e não qualificadas para farmacoterapia (Barkas et al., 2023; Plat et al., 2019). Além disso, a ingestão de FS também podem impactar positivamente em outras lipoproteínas e na homeostase da glicose (Barkas et al., 2023). O excesso de colesterol LDL na corrente sanguínea está relacionado ao desenvolvimento de doenças cardiovasculares (Guo et al., 2023). A capacidade de redução da absorção do colesterol pelos FS ocorre devido à similaridade entre as moléculas, que ocorre majoritariamente através da inibição da absorção do colesterol no lúmen intestinal, durante a digestão (Barkas et al., 2023). O grupo etila adicional presente no carbono 24 (Figura 2) é o que diferencia as moléculas do colesterol e do β-sitosterol (Marangoni; Poli, 2010).

Estudos farmacológicos têm demonstrado outros benefícios da ingestão de FS. Liu *et al*. (2024) reportaram o potencial cicatrizante do β-sitosterol frente a úlceras diabéticas em ratos, indicando a possível associação deste fitosterol com a redução de amputações em humanos. Jayaraman *et al.* (2023) demonstraram a atividade do β-sitosterol mediante as vias apoptóticas em células de câncer bucal, o sexto câncer mais diagnosticado no mundo. Em revisão de literatura, Khan et al. (2022) reuniran evidências acerca da eficácia do β-sitosterol contra os cânceres de mama, próstata, colo, pulmão e leucemia, destacando o potencial desse fitosterol frente múltiplas vias de sinalização celular, como ciclo celular, apoptose, angiogênese e metástase. Além disso, efeitos anti-inflamatórios, hepatoprotetores, antioxidantes, cardioprotetores e antidiabéticos também vêm sendo associados ao β-sitosterol.

O campesterol e o estigmasterol também são relacionados a benefícios à saúde. O campesterol é conhecido por seu potencial na regulação de citocinas, que sugerem benefícios terapêuticos no tratamento da artrite reumatoide (Nazir et al., 2023). O estigmasterol exibe uma ampla gama de propriedades farmacológicas, incluindo efeitos anticancerígenos, anti-inflamatórios, antidiabéticos, imunomoduladores e neuroprotetores, demonstrando também potencial no tratamento da doença inflamatória

intestinal, regulando as respostas imunológicas (Bakrim et al., 2022).

Apesar do valor nutricional atribuído ao óleo de soja, em razão do seu perfil de ácidos graxos e fitosteróis, a presença de insaturações nestes compostos os tornam altamente suscetíveis às reações de oxidação (Aktas et al., 2023; Qi et al., 2023), o que pode comprometer a qualidade sensorial e nutricional desse alimento. Além disso, os compostos bioativos presentes nos grãos de soja capazes de mitigar as reações de oxidação, como tocoferóis e carotenoides, são, em sua maior parte, removidos durante o processo de refino, principalmente durante os processos de desodorização e branqueamento (Jamoussi et al., 2022; Martini; Nerli; Malpiedi, 2022). Tais fatores destacam a importância de se investigar a ocorrência de processos oxidativos no óleo de soja, os quais podem ocorrer desde o seu processamento.

2 Oxidação lipídica

As reações de oxidação em lipídios são a maior causa de deterioração de alimentos. Elas ocorrem quando a matriz é exposta a fatores como oxigênio (O_2), radiação, enzimas, altas temperaturas e compostos como íons metálicos e clorofila. Essas reações ocorrem através da quebra de ligações insaturadas, e formação de diferentes produtos de oxidação. Este processo resulta no comprometimento da qualidade de alimentos, através da formação de sabores e odores indesejáveis, alterações na coloração, perda de propriedades funcionais e formação de produtos de oxidação tóxicos. Em alimentos, a oxidação lipídica ocorre principalmente pelas vias enzimática e não enzimática. Durante a vida útil dos produtos a oxidação ocorre pela via não enzimática, pelos mecanismos de auto-oxidação e foto-oxidação (Shahidi; Hossain, 2022; Wang et al., 2023), e, as reações enzimáticas, ocorrem geralmente em sistemas biológicos, com ação de enzimas como as lipoxigenases.

O processo de auto-oxidação lipídica (Figura 3) é o mais comum e ocorre como uma reação espontânea entre a insaturação da molécula de lipídio e o oxigênio atmosférico por reação em cadeia com radicais livres. Esse mecanismo ocorre em três etapas, que diferem entre si pelos compostos formados. A primeira etapa é a fase de indução ou iniciação, que ocorre quando um átomo de hidrogênio é removido do grupo metileno de um ácido graxo insaturado, levando a formação de um radical livre. Para que a reação ocorra, faz-se necessário a presença de oxigênio e de energia capaz de converter o oxigênio para seu estado singlete. Esta fase é marcada pelo baixo consumo de oxigênio,

baixas concentrações de peróxidos, aumento nas concentrações de radicais livres e ausência de alterações sensoriais (Lorenzo et al., 2018; Wang et al., 2023).

A segunda fase é a propagação, onde o radical livre que foi formado reage com o oxigênio atmosférico (O_2), gerando um radical peróxido. Esses radicais seguem reagindo com outros ácidos graxos insaturados, produzindo outro radical livre e hidroperóxidos, seguindo uma reação em cadeia de radicais livres. Os hidroperóxidos são moléculas instáveis, que se decompõem rapidamente gerando aldeídos, cetonas e álcoois, dentre estes estão os agentes indesejáveis de sabor e odor. Esta fase é caracterizada pelo alto consumo de oxigênio, aumento da concentração de peróxidos e o início das alterações sensoriais, com o aparecimento de odores característicos da rancidez (Lorenzo et al., 2018).

A terceira fase ou terminação, ocorre quando dois radicais livres reagem entre si, formando produtos estáveis. Esta fase é caracterizada pelo decréscimo da concentração de peróxidos, do consumo de oxigênio e por fortes alterações sensoriais, como sabor e cheiro característicos, alteração de viscosidade e cor (Eskin; Shahidi, 2015; Lorenzo et al., 2018).

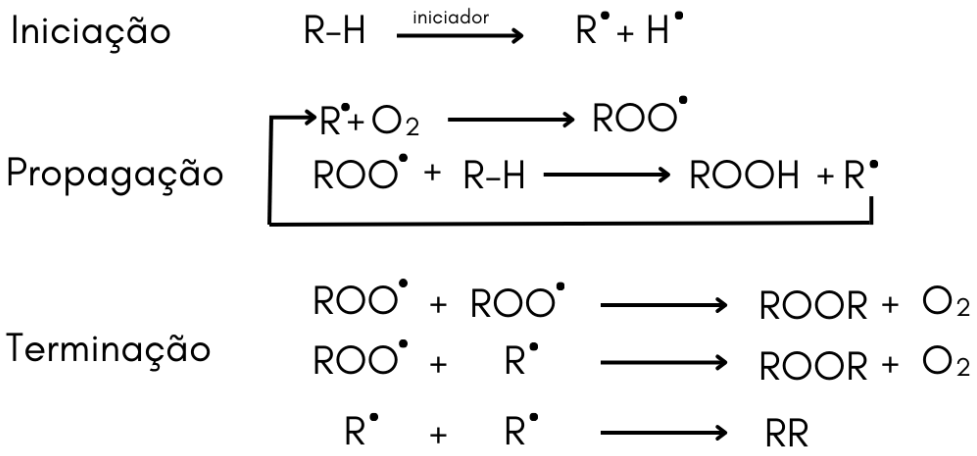

Figura 3: Mecanismo geral de oxidação lipídica (adaptado de: Wang et al., 2023).

2.1. Foto-oxidação

As radiações ultravioleta e luminosa podem induzir a degradação de alimentos através de reações fotoquímicas. Nesse tipo de reação, um fóton é absorvido por um elétron de um átomo ou de uma região de uma molécula fotossensível. A absorção da radiação luminosa torna-o excitado pelo aumento no nível energético, então, para que a reação do átomo excitado e a molécula aconteça, duas condições devem ser satisfeitas: a

energia do fóton deve ser suficientemente alta para que seja absorvida e também deve ser alta o suficiente para quebrar e formar ligações. Pode-se então classificar as reações fotoquímicas em dois tipos: diretas e fotossensibilizadas (Verduin, 2020).

A reação fotoquímica direta é aquela em que o fóton é absorvido diretamente pela molécula, levando a reação química direta, como acontece nas reações de ionização e isomerização. A reação fotossensibilizada é aquela em que deve haver a intermediação de composto fotossensível, que irá absorver a radiação luminosa e transferi-la através de uma reação química. O composto fotossensível é um cromóforo que possui ligações duplas conjugadas, e estão presentes em diversas matrizes alimentares como a clorofila, presentes nos vegetais, a mioglobina nos músculos animais e algumas vitaminas. A foto-oxidação é um tipo de reação fotossensibilizada, nesse caso, o composto fotossensível é excitado após absorver um fóton, tornando-se reativo e induzindo reações em cadeia com as moléculas suscetíveis na matriz e o oxigênio (Verduin, 2020).

As reações de foto-oxidação em alimentos podem ocorrer por diferentes mecanismos, a depender das características intrínsecas e extrínsecas do alimento. A foto-oxidação é comumente dividida em dois tipos de mecanismos, tipo I e tipo II. As reações do tipo I ocorrem majoritariamente em alimentos caracteristicamente hidrofílicos e em condições anaeróbicas, uma vez que o oxigênio apresenta baixa solubilidade em água. Nesse mecanismo o composto fotossensível, após absorver energia da luz, passa para um estado tripleto excitado (^{3}sen*), tornando-se reativo e interagindo diretamente com a fração molecular suscetível da matriz alimentar, através da transferência de elétrons e abstração de hidrogênio. Durante a transferência de elétrons, o composto fotossensibilizado em seu estado reativo reage com o oxigênio molecular (O_2) para formar o ânion superóxido (Lu; Zhao, 2017; Min; Boff, 2002; Verduin, 2020).

O mecanismo do tipo II (Figura 4) ocorre em condições aeróbicas, visto que o O_2 tem melhor solubilidade sob condições hidrofóbicas, portanto, ocorre comumente em alimentos lipídicos e emulsões, como o leite. Nesse mecanismo, o elétron do composto fotossensível (cromóforo) em seu estado fundamental (^{1}sen) absorve um fóton da radiação luminosa (*hv*), elevando a camada energética e tornando-se excitado, num estado singleto instável, que rapidamente é convertido em uma molécula excitada no estado tripleto (^{3}sen*) por cruzamento interssistema. Esse cromóforo no seu estado excitado transfere energia para molécula de oxigênio no seu estado fundamental ($^3O_2$), transformando-o em singleto ($^1O_2$), pelo mecanismo de aniquilação tripleto-tripleto. O composto fotossensibilizador retorna ao seu estado fundamental e pode recomeçar as

reações para gerar mais oxigênio singleto. Compostos fotossensibilizadores podem gerar de 10^3 a 10^5 moléculas de $^1O_2$ até se tornarem inativos (Min; Boff, 2002). O oxigênio singleto, rapidamente reage com as ligações π dos lipídios insaturados (Shankar et al., 2015; Yang et al., 2020), formando hidroperóxidos, compostos primários de oxidação que rapidamente se convertem em outros produtos, chamados produtos secundários.

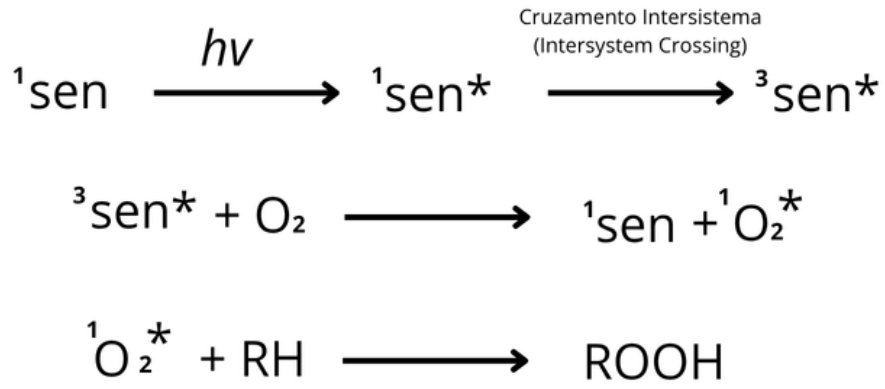

Figura 4: Representação esquemática da foto-oxidação do tipo II (Adaptado de: Min; Boff, 2002).

Os ácidos graxos podem ser oxidados pelo mecanismo de foto-oxidação. Chan et al. (1977) demonstraram através das reações de foto-oxidação a partir do linoleato de metila e oleato de metila, utilizando riboflavina e eritrosina como fotossensibilizadores. Os autores observaram que os ácidos graxos podem oxidar, tanto pelo mecanismo do tipo I (observado com a adição de riboflavina), quanto pelo tipo II (adição de eritrosina). Neste experimento a foto-oxidação predominou sobre a auto-oxidação em condições moderadas (25 ºC, 5000 lux), sendo os hidroperóxidos os principais produtos.

No mecanismo de foto-oxidação de fitosteróis em óleos vegetais, predomina a ocorrência do mecanismo tipo II, através da ativação pela energia da radiação luminosa visível e ultravioleta (200-700 nm). A presença da insaturação entre os carbonos C5 e C6 (Figura 5), torna o β-sitosterol altamente susceptível a reação com o oxigênio singleto, dando origem aos produtos primários 5α-hidroperóxido (5α-OOH), 6α- hidroperóxido e 6β- hidroperóxido, nesta etapa a taxa de geração dos produtos 5α é significativamente maior do que os derivados 6α e 6β. Os óxidos 7α- e 7 β-hidroperóxido são os produtos predominantes das reações do 5α-OOH, devido à instabilidade deste óxido (Yang et al., 2020). Esse mecanismo de foto-oxidação ocorre da mesma forma na molécula de colesterol, uma vez que as estruturas compartilham o mesmo núcleo e possuem entalpia

de dissociação similares. Logo, os produtos de oxidação de fitosteróis (POFs) são similares aos produtos de oxidação do colesterol (POCs), e podem apresentar efeitos deletérios análogos no organismo humano (Wang; Lu, 2018; Yang et al., 2020).

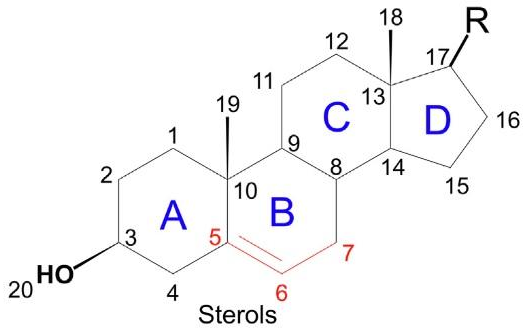

Figura 5: Estrutura plana geral numerada dos esteróis (Yang et al., 2020).

A degradação de fitosteróis via oxidação é evidenciada pela formação destes produtos da oxidação, onde são formados produtos primários (hidroperóxidos), que são decompostos em produtos secundários polares (cetonas, álcoois e epóxidos) e apolares (esteradienos e esteratrienos). Os produtos terciários podem ser dímeros, oligômeros ou polímeros. Alguns desses compostos são associados ao desenvolvimento de doenças como cânceres, doenças cardiovasculares, doenças neuro degenerativas e aterosclerose (Grootveld et al., 2020; Zhao et al., 2021).

A degradação do β-sitosterol em óleos vegetais foi avaliada sob exposição de luz solar natural, radiação UV artificial e irradiação durante 30 dias, com análise das amostras em intervalos de 10 dias. O conteúdo inicial de β-sitosterol no óleo de soja foi de 169 mg/100 gramas, além disso foram encontrados traços dos óxidos 7α-hidroxisitosterol e 7β-hidroxisitosterol no dia zero, justificados pela possível formação desses compostos durante os processos de extração e refino dos óleos. Nas amostras expostas a luz solar foi observado um rápido aumento nas concentrações de óxidos durante 20 dias de exposição, chegando a 1070 μg/g de óxidos no óleo de soja. Após os 20 dias foi observada a redução na concentração dos três principais óxidos (7α-hidroxi, 7β-hidroxi e 7ceto-hidroxi), que foi justificada pela possível degradação e formação de compostos secundários. Ao final dos 30 dias de observação, 49% do β-sitosterol havia sido degradado (Zhang et al*.,* 2006).

Zhao *et al.* (2019b) conduziram um estudo para avaliar a foto-oxidação dos

fitosteróis campesterol, estigmasterol, brassicasterol e β-sitosterol em três diferentes intensidades luminosas: 10.000, 20.000 e 30.000 lx, por 14 dias. Após 14 dias de exposição, a quantidade total de óxidos formados variou de 696,9 µg/g na amostra controle (avaliada no escuro) a 4.481,9 µg/g na amostra exposta a 30.000 lx. Os óxidos formados em maiores quantidades foram 7β-hidroxi, 7α-hidroxi, 5,6β-epóxi, 7-ceto, 5,6α-epóxi e 6β-hidroxi, respectivamente. A oxidação no carbono 7 é favorecida na oxidação de fitosteróis, quando o oxigênio simples reage com o hidrogênio alílico do carbono 7, originando posteriormente os produtos secundários 7-hidroxi e 7-ceto. Os resultados demonstraram degradação de aproximadamente 70% do total de FS ao final dos 14 dias nas amostras expostas a maior intensidade luminosa (30.000lux), constatando a correlação negativa entre intensidade da exposição a luz e total de FS.

A toxicidade e os efeitos deletérios dos óxidos de esteróis para o organismo humano já são conhecidos desde os anos 80. Ainda que cada óxido possua diferentes efeitos no organismo, a aterogenicidade e a citotoxicidade desses compostos já foi reconhecida pela ciência (Brown; Jessup, 1999). Atualmente, os efeitos desses compostos foram investigados. Yan e Cao (2024) avaliaram o comportamento do 7-cetositosterol na microbiota intestinal de camundongos e observaram que esse óxido aumentou a concentração de bactérias patogênicas e diminuiu a concentração de bactérias produtoras de ácidos graxos de cadeia curta, indicando o potencial do 7-cetositosterol em agravar a inflamação do cólon através da modulação da microbiota intestinal (Yan; Cao, 2024).

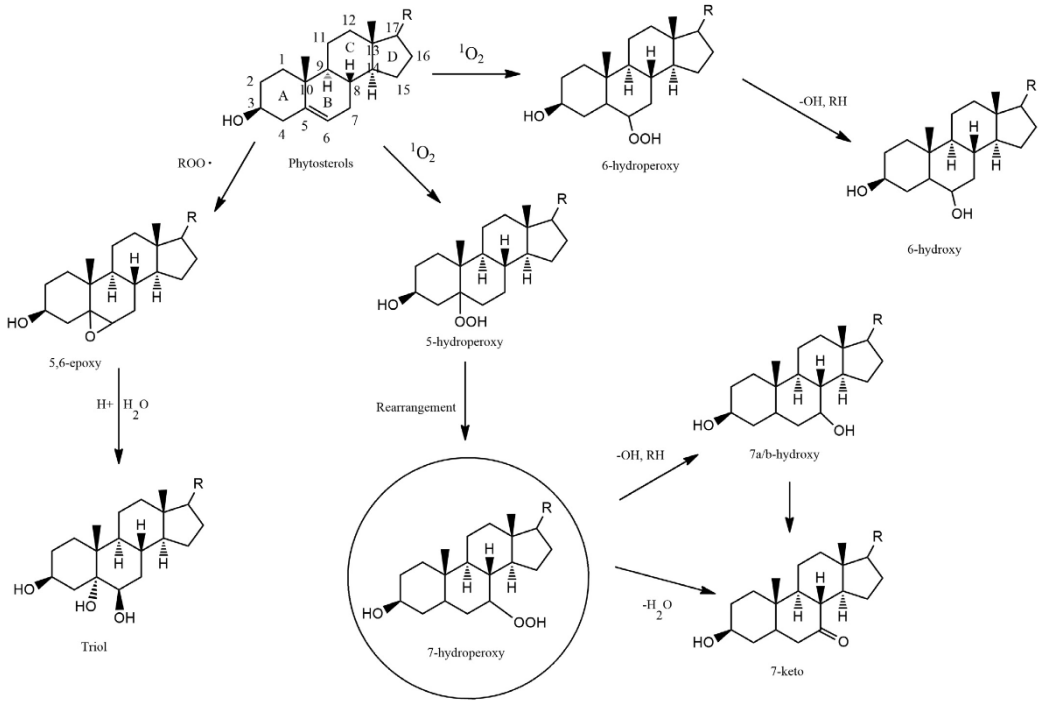

Figura 6: Principais produtos da foto-oxidação de fitosteróis (Zhao et al., 2019).

Yang *et al.* (2022) avaliaram o efeito da adição dos compostos fotossensibilizadores (CF) riboflavina e clorofila em bebida de soja sob diferentes comprimentos de onda (ultravioleta A – UVA, violeta, vermelho, azul, verde e amarelo – 365-665 nm) na foto-oxidação dos principais fitosteróis da soja, β-sitosterol e estigmasterol, durante 12 dias de exposição. Deve-se considerar maior oxidação produto quando comparado ao óleo, por tratar-se de emulsão óleo em água, possuindo regiões hidrofílicas, hidrofóbicas, anfifílicas e quimicamente ativas em regiões próximas. Os principais fitosteróis do leite β-sitosterol e estigmasterol apresentaram o mesmo padrão de degradação quando submetidos aos diferentes tipos de radiação, após 12 dias de exposição as radiações violeta e ultravioleta. A (UVA) foram as que induziram maior oxidação dos dois fitosteróis, reduzindo-os em 42% e 21% no estigmasterol, e 45% e 35% no β-sitosterol. Em relação aos produtos de oxidação dos fitosteróis, as maiores concentrações foram igualmente registradas nas amostras expostas as radiações violeta e UVA, com aproximadamente 30000 μg/mL e 27500 μg/mL ao final de 12 dias.

Em razão dos conhecidos efeitos dos compostos formados pela degradação de lipídios via oxidação, faz-se necessário a busca por estratégias para mitigar essas reações e aumentar a vida útil dos produtos. O óleo de soja é comercializado em garrafas PET

transparentes e, alguns fabricantes, adicionam na embalagem filtros de radiação UV (Ribeiro; Jorge, 2017; Yang et al., 2023), sendo, além disso adicionados antioxidantes sintéticos. Entretanto, a adição de antioxidantes sintéticos, representa uma preocupação aos consumidores, uma vez que seu consumo prolongado pode acarretar problemas a saúde e contaminação do meio ambiente (Schmidtkunz et al., 2020; Xu et al., 2021), dessa forma, vêm sendo observada a busca por estratégias para a diminuição ou substituição desses aditivos pelo emprego de fontes naturais.

3 Emprego de antioxidantes sintéticos e naturais em óleo de soja

Os antioxidantes são substâncias adicionadas aos alimentos para minimizar as reações de oxidação, através, principalmente, da captura ou da neutralização dos radicais livres, os quais são percursores dos processos oxidativos (Silva et al., 2022)

Os antioxidantes sintéticos são extensamente utilizados pela indústria de alimentos, cosmética e farmacêutica em razão do alto rendimento, estabilidade e baixos custos no emprego dos mesmos. Na indústria de óleos comestíveis os antioxidantes sintéticos mais utilizados são os fenólicos, principalmente o butilhidroxianisol (BHA), o butilhidroxitolueno (BHT), o galato de propila (GP) e o terc-butilhidroquinona (TBHQ) (Figura 6). Os compostos fenólicos possuem um anel fenol em sua estrutura e permitem a doação de um próton a um radical livre, interrompendo a propagação da reação de oxidação (Carocho; Morales; Ferreira, 2018; Xu et al., 2021).

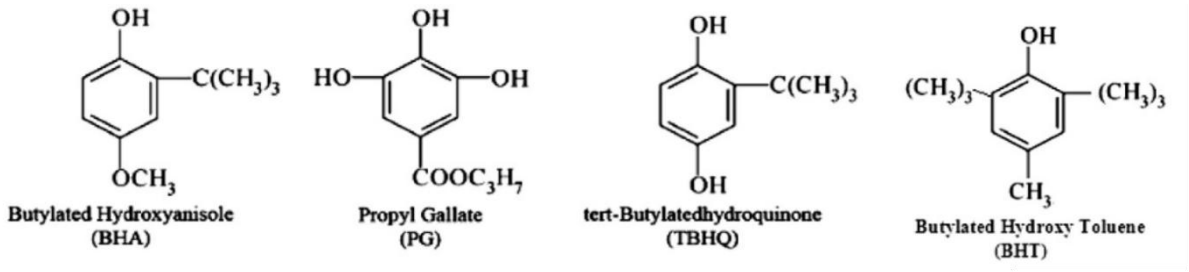

Figura 7: Estrutura química dos antioxidantes sintéticos BHA, TBHQ, PG e BHT (AGARWAL; KHURANA, 2013).

Apesar das vantagens do emprego dos aditivos sintéticos, estudos reportam riscos à saúde humana associados ao consumo a longo prazo de antioxidantes sintéticos, os quais podem ainda acumular no meio ambiente. Antioxidantes fenólicos sintéticos são encontrados como contaminantes em ambientes como águas de rios, suspensos no ar ou

depositados com poeiras, e em animais que habitam esses ambientes, portanto, podendo ser ingeridos pelo ser humano através de outras vias, além do alimento. No organismo, esses compostos podem comprometer a coagulação sanguínea e causar mutação e tumores (Esazadeh et al., 2024; Fries; Püttmann, 2002; Liu; Mabury, 2020; Wang et al., 2016).

Em razão desses males, a utilização de antioxidantes sintéticos é geralmente restrita. No Brasil, a RDC Nº 740/22 limita a utilização dos antioxidantes classificando os tipos de aditivos e quantidades por tipo de alimentos. Em óleos e gorduras vegetais refinados, a quantidade máxima que pode ser adicionada dos antioxidantes BHT, BHA, TBHQ e GP é de 200 mg/kg ou mg/L sozinhos, ou de forma combinada (BRASIL, 2024).

Em contrapartida, composto fenólicos e outros compostos com potencial antioxidante também são amplamente encontrados no reino vegetal, como em frutas, especiarias, ervas e até mesmo no óleo de soja. O grão de soja possui compostos naturais como tocoferóis, compostos fenólicos e carotenoides que são, em parte, perdidos durante o processamento do grão e produção do óleo. Por isso, faz-se necessária a adição de compostos antioxidantes ao óleo, que é comercializado em garrafas PET transparentes, e por isso é exposto a radiação durante sua vida útil (Kim et al., 2006; Lopes; Courrol, 2023). Tendo em vista os males causados pela ingestão dos compostos sintéticos, a busca por alternativas que contribuam para a manutenção da estabilidade oxidativa do óleo de soja compreende estratégias como a inserção de fontes naturais adicionais de compostos antioxidantes.

A adição de compostos naturais como substituintes aos sintéticos tem sido estudada principalmente a partir da segunda década do século XXI. Temperos, ervas, extratos, diferentes partes de vegetais e subprodutos já foram adicionados ao óleo de soja com o intuito de avaliar a estabilidade oxidativa tanto durante o armazenamento quanto durante o aquecimento para o preparo térmico de alimentos (Guo et al., 2016; Ribeiro; Jorge, 2017; Sharma et al., 2019).

Óleos condimentados, saborizados ou aromatizados são os óleos vegetais comestíveis adicionados de temperos e condimentos, os quais podem ser preparados por infusão ou maceração. Esta é uma prática doméstica culturalmente praticada no Brasil, que consiste em adicionar uma fonte vegetal ao óleo e deixa-la imergida por alguns dias ou durante seu uso. Normalmente, os óleos são retirados de sua embalagem original, que por vezes oferece proteção contra incidência de radiação e são colocados em outros recipientes, deixando o produto exposto a foto-oxidação. Visto que esses materiais

vegetais adicionados possuem compostos antioxidantes naturais capazes de minimizar os processos oxidativos, torna-se válido investigar estabilidade do produto em relação a oxidação lipídica durante o armazenamento com exposição a luz (Tinello; Lante, 2020; Yang; Chu; Liu, 2005)

A Tabela 2 apresenta estudos que avaliaram a estabilidade oxidativa de óleos de soja adicionados de fontes naturais de compostos antioxidantes em diferentes concentrações, combinações, temperaturas e tempos de armazenamento.

Quadro 1: Estudos acerca da adição de fontes naturais como antioxidantes em óleo de soja.

Fonte antoxidante	Concentrações utilizadas	Tratamento	Resultados	Referência
Óleo essencial de cominho (*Cuminum cyminum*), segurelha-anual (*Satureja hortensis*) e cardamomo (*Elettaria cardamom*)	Adicionados individualmente nas concentrações 0,2; 0,4 e 0,6 % (v/v)	Armazenamento a 20 °C, 30 °C e 40 °C /180 dias	Concentrações ótimas de 0,4% e 0,6% foram identificadas para reduzir acidez e peróxidos. Temperaturas 20 °C e 30 °C foram ideais para melhorar a estabilidade do óleo	DOLATI et al. (2016)
Óleo de semente de *Berberis integerrima* (OSB)	Adições de 2% e 5% em comparação ao controle (sem antioxidante) e amostra com TBHQ (200 ppm)	Armazenamento a 25 ºC/ 14 dias	O BSO aumentou significativamente a estabilidade do óleo de soja durante o armazenamento, efeito comparado ao antioxidante sintético TBHQ.	TAVAKOLI, SAHARI e BARZEGAR (2017)
Curcumina extraída do açafrão-da-terra (*Curcuma longa* L.)	120, 160 e 200 ppm, comparação com a-tocoferol e TBHQ.	Armazenamento sob exposição à luz, a 25 °C e 55°C/ 90 dias	A 200 ppm, a curcumina apresentou atividade antioxidante comparável ao α-tocoferol e ao TBHQ.	ESHGHI et al. (2014)
Pó de açafrão (*Curcuma longa*) e gengibre (*Zingiber officinale*)	Adição de 10% dos pós liofilizados de açafrão e gengibre, comparados com adição de 0,02% de BHT.	Armazenamento a 62 °C/ 28 dias)	Melhora da estabilidade oxidativa dos óleos adicionados de pó de açafrão. Propriedades antioxidantes atribuídas ao alto teor de fenólicos (6-gingerol e curcumina).	TINELLO e LANTE (2020)

Compostos fenólicos do extrato hidroalcóolico de pimenta malagueta (Capsicum frutescens)	100 mg/kg e 200 mg/kg (extrato), comparação com TBHQ e BHA, ambos a 100 e 200 mg/kg.	Armazenamento a 60 ºC/ 20 dias	Extrato a 200 mg reduziu efetivamente a formação de peróxidos e dienos conjugados após 20 dias de armazenamento, efeito comparável ao antioxidante TBHQ.	JORGE et al.(2018)
Nanoemulsão de extrato etanólico de casca de berinjela	Concentrações variando de 1.000 a 9.000 ppm, com adição de 8,9 e 10% de emulsificante de ésteres condensados de ácido ricinoleico de monolaurato de tetraglicerina (CR-310)	Armazenamento a 60 °C/ 30 dias	As nanoemulsões restringiram a oxidação e apresentaram efeitos comparáveis ao óleo de soja comercial com TBHQ, o teste em forno de Schaal indicou que as nanoemulsões podem controlar a oxidação do óleo de soja por 10 a 15 meses. A formulação a 8000 ppm e 10% de CR-310 apresentou a maior estabilidade oxidativa e liberação controlada de compostos polifenólicos.	SHARMA et al. (2023)
Pó liofilizado de extrato hidroalcóolico de casca de cebola roxa (*Allium cepa* L.)	200 mg/kg (extrato em compostos fenólicos); 100 mg/kg (extrato em compostos fenólicos) em combinação com TBHQ, palmitato de ascorbila (PA) e tocoferol	Armazenamento a 60 ºC/ 21 dias	TBHQ, PA e TBHQ+PA apresentaram maior estabilidade oxidativa. O pó liofilizado proporcionou proteção moderada durante o período inicial de armazenamento em comparação ao óleo de soja isoladamente.	UMEDA e JORGE (2021)
extrato hidroalcóolico de casca de café (*Coffea arabica* L.)	200 mg/kg (extrato em compostos fenólicos); 100 mg/kg (extrato em compostos fenólicos) em combinação com TBHQ e BHA	Armazenamento a 60 ºC/ 20 dias	O extrato de casca de café (EC) demonstrou um efeito sinérgico com o TBHQ, aumentando a estabilidade oxidativa do óleo de soja durante o armazenamento. A combinação de EC e TBHQ manteve a estabilidade oxidativa por 20 dias, reduzindo o ganho de massa. O EC retardou o início do ganho de massa em comparação com o BHA.	RIBEIRO e JORGE (2017)

Hidrolisados proteicos (HP) isolados obtidos de peixes de carpa cruciana (*Carassius carassius*) e de intestino de vaca e extrato de folha de oliveira (EFO) encapsulado com goma arábica e maltodextrina	200, 500 e 1000 mg/kg de pó hidrolisado proteico de peixe e de vaca. O extrato de folha de oliveira nas concentrações de 364,6 mg/kg e 1823 mg/kg. Foram analisados separadamente: extrato, extrato encapsulado com as gomas de forma individual e combinada	Armazenamento a 55 ºC/ 20 dias	O HP de peixe a 1000 mg/kg e o HP de intestino bovino a 500 e 1000 mg/kg apresentaram proteção oxidativa superior em comparação com o BHT a 100 e 200 mg/kg. O EFO demonstrou atividade antioxidante significativa, com o encapsulamento melhorando a estabilidade térmica dos compostos fenólicos, mas reduzindo a eficiência antioxidante.	TAGHVAEI et al. (2014)
Extrato de alecrim com 70% de ácido carnósico (*Rosmarinus officinalis* L.)	400 mg/kg de extrato em comparação com adição de 200 mg/kg da mistura de BHA com BHT a 50% cada.	Armazenamento a 62 ºC/ 24 dias	O extrato de alecrim aumentou significativamente os períodos de indução do óleo de soja em comparação aos antioxidantes sintéticos. A incorporação do extrato melhorou a capacidade antioxidante e o teor total de fenólicos nos óleos, diminuiu o índice de peróxidos e retardou a degradação de tocoferóis e ácidos graxos poli-insaturados.	YANG et al. (2016)
Extrato aquoso liofilizado de folhas de romã, goiaba e uva (1:1:1)	200, 400 e 800 ppm de extrato e 400 ppm de extrato combinado com 100 ppm de BHT	Armazenamento a 65 ºC/ 30 dias	A mistura do extrato aquoso liofilizado apresentou alto teor de fenólicos e flavonoides, sendo o ácido elágico e a naringenina os compostos mais abundantes. O extrato a 800 ppm demonstrou eficácia no retardamento da rancidez no óleo de soja, comparável ao BHT sintético. O extrato reduziu efetivamente a hidrólise, a polimerização e a oxidação de lipídios, resultando em menores valores de ácido, peróxido, p-anisidina, TBARS e produtos conjugados no óleo.	MANSOUR et al. (2022)

Extrato líquido (EL) de Cashew Nut (*Anacardium occidentale*) e as frações concentradas (C) e filtradas (F) do extrato líquido obtido por éter de petróleo.	Adição de 200 mg/kg de óleo das frações EL, C e F	Armazenamento a 30 ºC, 40 ºC, 50 °C e 60 ºC/ 5 dias.	O líquido da casca da castanha de caju contendo cardóis, cardanóis e ácidos anacárdicos foi menos eficaz do que o controle com antioxidante sintético. A fração filtrada, rica em cardóis e cardanóis pode ser usada como antioxidante natural no óleo de soja como alternativa a um antioxidante sintético.	GAITÁN-JIMÉNEZ et al. (2022)
Extrato metanólico de folhas de acácia-branca (*Moringa oleifera* Lam.)	0,02; 0,05; 0,08 e 0,1% de extrato; comparados adição de 0,02% de BHT, 0,02% de ácido ascórbico, 0,02% de ácido cítrico, 0,02% de α-tocoferol e três diferentes combinações de 0,1% do extrato com 0,02% de ácido ascórbico, ácido cítrico e α-tocoferol.	Armazenamento (60°C/ 12 dias) e Termo-oxidação da adição 0.1% (105 °C, 120 ºC, 150 °C e 180°C/24h)	O estudo demonstrou que o extrato das folhas reduziu significativamente a taxa de formação de produtos de oxidação primária no óleo durante o armazenamento, e seu efeito antioxidante foi aumentado com o aumento das concentrações. Além disso, o extrato exibiu efeitos antioxidantes sinérgicos quando combinado com ácido ascórbico, ácido cítrico ou α-tocoferol.	ZHAO et al. (2019a)

Estudos que avaliam estabilidade oxidativa de óleo sob armazenamento, geralmente utilizam os métodos Rancimat, método de oxigênio ativo ou teste de forno em escala, para avaliar o estado de oxidação da amostra (Antolovich et al., 2002). A maioria dos trabalhos conduzidos com óleo de soja utiliza teste de forno em escala, do inglês *"Schaal oven test"*, que consiste em manter o produto em estufa entre 60 °C a 63 °C durante horas ou dias. Utiliza-se essas condições com base no modelo de Arrhenius, que descreve que 24 horas de armazenamento de óleos a 60 °C em estufa, corresponde a 30 dias de armazenamento a temperatura ambiente (Antolovich et al., 2002; Pokhrel et al., 2024). No entanto, devido ao uso do forno, estes testes não consideram a exposição do óleo de soja a luz. Assim, como a literatura já evidencia o impacto da temperatura durante o armazenamento em termos de oxidação lipídica, é preciso investigar a influência também da incidência de luz, avaliando-se a foto-oxidação.

Tavakoli, Sahari e Barzegar (2017) avaliaram o efeito da adição de 2% e 5% do óleo de semente de *Berberis integérrima* em óleo de soja ao longo de 14 dias de armazenamento a temperatura ambiente e sem exposição de luz. Os autores avaliaram índice de peróxidos, substâncias reativas ao ácido tiobarbitúrico (TBARS), dienos e trienos conjugados e o teste de Rancimat. As amostras foram comparadas com amostras de óleo de soja sem adição de qualquer antioxidante e com adição do antioxidante sintético TBHQ. Os autores observaram que o efeito do óleo de semente de *Berberis integérrima* teve eficácia similar ao antioxidante sintético, e a maior adição obteve melhores resultados sobre a menor.

Alguns trabalhos avaliaram o efeito de diferentes temperaturas durante o armazenamento de óleos com antioxidantes naturais. Gaitán-Jiménez *et al.* (2022) prepararam extrato de líquido de casca de castanha de caju com éter de petróleo. Os autores avaliaram o extrato e duas frações dele obtidas, o concentrado e filtrado, cada um adicionado na concentração de 200 mg por quilograma de óleo de soja e armazenados em fornos nas temperaturas de 30, 40, 50 e 60 ºC, sem exposição a luz, comparando-os com amostras de óleo de soja sem antioxidante e com antioxidante sintético. A adição do filtrado obtido do extrato da casca da castanha, fração rica em bioativos, foi a amostra que obteve os resultados mais próximos aos antioxidantes sintéticos e maior eficácia em manter a estabilidade oxidativa em todas as temperaturas avaliadas.

Os óleos essenciais das ervas cominho, segurelha-anual e cardamomo foram adicionados ao óleo de soja em diferentes concentrações para avaliação da estabilidade oxidativa sob armazenamento em diferentes condições de temperatura. O óleo de

segurelha-anual foi o mais eficaz na redução da taxa de oxidação. Foi concluído que a concentração testada influenciou a oxidação, com as maiores concentrações de óleo essencial apresentando menores taxas de oxidação, além disso, quanto maior o tempo e a temperatura de armazenamento, menor a estabilidade oxidativa (Dolati et al., 2016).

Eshghi *et al.* (2014) avaliaram o efeito da adição de curcumina extraída do açafrão-da-terra na estabilidade oxidativa de óleos de soja durante o armazenamento sob diferentes temperaturas durante 90 dias. Nesse estudo os autores avaliaram também a influência da luz, comparando com amostras armazenadas no escuro. Tanto a luz como a temperatura impactaram negativamente na estabilidade oxidativa, entretanto a adição de 200 ppm de curcumina mostrou-se tão eficaz quanto os antioxidantes TBHQ a 120 ppm, e o α-tocopherol a 200 ppm.

Tinello e Lante (2020) simularam a aromatização doméstica de óleo de soja, adicionando 10% (p/p) dos pós liofilizados obtidos comercialmente de gengibre e cúrcuma e procedendo o teste de armazenamento "*Schaal oven test*" (SOT) durante 20 dias, sem exposição de luz. A estabilidade oxidativa foi melhorada pela adição dos pós comparados a amostras de óleo de soja sem antioxidantes e com 0,02% de BHT, dentre eles a cúrcuma apresentou melhor resposta, demonstrando potencial de substituição do BHT. De maneira similar o extrato da casca de café e da casca de berinjela foram avaliados através do SOT, durante 20 e 30 dias, respectivamente. Ambos os extratos agroindustriais apresentaram resultados satisfatórios e demonstraram ser possíveis substituintes aos antioxidantes sintéticos.

De maneira similar, Mansour et al. (2022) prepararam extrato aquoso de folhas secas de romã, goiaba e uva (1:1:1) e avaliaram a oxidação de óleo de soja sob armazenamento a 65 ºC, durante 30 dias, através de análises que avaliam o estágio de oxidação do produto, como índice de peróxidos (IP) e substâncias reativas ao ácido tiobarbitúrico (TBARS), os autores observaram que para ambas análises as amostras adicionadas de antioxidante sintético BHT, com 800 ppm do extrato e com a mistura de BHT (0.01%) com o extrato (0.04%), foram as que apresentaram os melhores resultados, com menores níveis de oxidação, com valores de IP entre aproximadamente 15 e 18 meq O_2/kg, e TBARS aproximadamente 0,75 mg MDA/kg de óleo.

Zhao et al. (2019a) determinaram a estabilidade oxidativa de óleo de soja adicionado de extrato metanólico de folhas de acácia-branca durante o armazenamento. As amostras de óleo de soja refinado foram adicionadas de 0,02%, 0,05%, 0,08% e 0,1% de extrato e o

armazenamento foi avaliado durante 12 dias a 60 ºC, os autores também adicionaram BHT (0,02%) como controle positivo, e óleo de soja refinado sem adição de antioxidantes como controle negativo. Após 12 dias de armazenamento, o controle negativo apresentou o maior valor de peróxidos (129,31 meq O_2/kg), enquanto as amostras com 0,1% de extrato e com BHT apresentaram os menores, 56,40 e 65,37 meq O_2/kg, respectivamente, demonstrando que o desempenho do extrato natural para oxidação primária é ainda melhor do que a adição máxima permitida do antioxidante sintético BHT. Para avaliar os produtos de oxidação secundária os autores utilizaram a análise do índice de *p*-anisidina, e similarmente ao IP o controle negativo apresentou maior oxidação (15,85) e a amostra com 0,1% de extrato menor valor (9,26), representando redução de 44% do índice após 12 dias de armazenamento, a amostra com BHT não apresentou diferença estatística, indicando similar eficácia.

O emprego de fontes naturais de compostos antioxidantes em óleo de soja tem apresentado resultados promissores, destacando esta prática como uma estratégia viável mesmo em ambiente doméstico. O efeito positivo dos compostos naturais sobre a estabilidade de alimentos já é conhecido, entretanto, ainda existem diversas fontes e condições de tratamento inexploradas. Tratando-se de fontes vegetais, é importante destacar que cada região do mundo possui uma flora característica, portanto, faz-se necessário o estudo de espécies abundantes e de fácil acesso no Brasil.

4 Pimenta rosa (*Schinus terebinthifolia* Raddi)

A pimenta rosa é o fruto da aroeira (*Schinus terebinthifolia* Raddi), uma planta nativa da América do Sul e amplamente distribuída por grande parte do território brasileiro, desde o Nordeste até o Sul do país, podendo também ser encontrada em partes da América Central, América do Norte, África, Europa e Ásia (Figura 8). Membro da família Anacardiaceae, a aroeira se apresenta como um arbusto com folhas pontiagudas estreitas, cresce cerca de 4 a 10 metros, e, quando frutifica, fica recoberta de cachos com frutos pequenos de cor vermelha e brilhante (Ennigrou et al., 2017). Durante a última década, a exploração do fruto esteve restrita à colheita manual de populações tradicionais. No entanto, a espécie tem ganhado visibilidade na culinária internacional, levando ao aumento considerável na demanda pela indústria de especiarias. A pimenta rosa apresenta sabor suave, levemente apimentado e é empregada em diversas preparações na forma de grãos inteiros ou moídos (De Souza; Arthur; Nogueira, 2012; Oliveira et al., 2020c).

Figura 10: Folhas e frutos da aroeira (elaborado pela autora, 2024).

Além do emprego culinário, a aroeira também é valorizada como planta medicinal, onde diversas partes da mesma são utilizadas no tratamento de infecções urinárias, feridas, reumatismo e inflamações (Rosas; Correa; Das Graças Henriques, 2019; Silva et al., 2023). Tais aplicações medicinais são decorrentes de importantes propriedades atribuídas à aroeira e seus frutos, como efeito antioxidante, cicatrizante, antitumoral, anti-inflamatório e atividade antimicrobiana, os quais são relacionados a diversidade de metabólitos secundários presentes nesta espécie (Cabral et al., 2021; Ennigrou et al., 2017; Oliveira et al., 2020a).

A composição centesimal da pimenta rosa foi realizada por diferentes autores, com frutos de diferentes lugares do mundo. Vieira et al. (2023) reuniram diversos estudos que apresentam caracterização da pimenta rosa. A umidade apresentou valores de 9,55 a 39,00 g/ 100g, carboidratos apresentaram-se na faixa de 31,21 a 62,49 g/ 100g, proteínas entre 7,71 e 26,25 g/ 100g, lipídios de 5,11 a 25,15 g/ 100g, e cinzas de 0,19 a 14,24 g/ 100g. Essas quantificações referem-se a aproximadamente 12 estudos, o que justifica as variações, pelas diferentes condições de crescimento da planta. As fibras foram quantificadas apenas por Oliveira et al. (2020a), quantificadas em 17,90 g/ 100g.

Oliveira et al. (2020a) também avaliaram composição de ácidos graxos, compostos bioativos e capacidade antioxidante da pimenta rosa proveniente da Embrapa Agroindústria de Alimentos em Guaratiba, no Rio de Janeiro. Vinte ácidos graxos foram

identificados e quantificados, com destaque para os ácidos linoleico (36,3 g/100 g), oleico (18,71 g/100 g), palmítico (9,01 g/100 g) e palmitoléico (7,87 g/100 g). Em relação aos somatórios dos ácidos graxos, os poli-insaturados representam a maior concentração (37,01 g/ 100g), seguido dos monoinsaturados (29,32 g/ 100g), e dos saturados (26,53 g/ 100g).

As análises dos compostos bioativos fenólicos, flavonoides, antocianinas totais, carotenoides e o perfil de bioativos realizados por UHPLC-ESI-MS foram feitos a partir do extrato de metanol e água, na proporção 80:20. A caracterização de bioativos por cromatografia identificou 7 compostos, com destaque para os flavonoides tetrahidroamentoflavona, agatisflavona e hinokiflavona (Oliveira et al., 2020a). Dentre os metabólitos secundários com propriedades bioativas presentes na pimenta rosa, destacam-se os compostos fenólicos, os quais incluem principalmente ácidos fenólicos e flavonoides. Ácidos fenólicos como os ácidos gálico, vanílico e cumárico foram identificados, enquanto para os flavonoides a literatura reporta a presença de compostos como a quercetina, rutina, apigenina, naringina, amentoflavona e tetraidroamentoflavona (Linden et al., 2020; Menegali et al., 2020a; Oliveira et al., 2020a). Sabe-se, entretanto, que a composição química da pimenta rosa é determinada por fatores genéticos e ambientais, que causam variações significativas na composição dos frutos. Desta forma, os teores de compostos fitoquímicos podem ser afetados pelo tempo de maturação, genótipo, época da colheita, assim como a estocagem e as condições de processamento (Ennigrou et al., 2017; Vieira et al., 2023).

Variações no conteúdo de substâncias fenólicas totais durante diferentes estágios de maturação da pimenta rosa foram analisadas, com o objetivo de identificar possíveis alterações nas propriedades destes frutos. Teores mais elevados destes compostos foram observados no estágio mais avançado de maturação, sendo os menores teores no estágio intermediário. Já o conteúdo de flavonoides totais apresentou teores mais elevados no estágio intermediário. Os frutos intactos também foram comparados com o resíduo hidrodestilado, de forma que foram determinados conteúdos quase semelhantes de substâncias fenólicas e superiores ao de flavonoides totais no resíduo, demonstrando que o resíduo do processo de destilação pode representar uma excelente fonte de polifenóis (Ennigrou et al., 2017).

Além do conhecimento da composição química, pesquisas tem avaliado a efetividade da pimenta rosa como antioxidante natural em alimentos ricos em lipídios.

Devido à complexidade das matrizes alimentares, onde diferentes componentes podem interferir nas análises e resultados, antioxidantes naturais são aplicados primeiramente em sistemas modelos. Os sistemas modelos permitem avaliar e controlar parâmetros que possam influenciar no processo de oxidação e na efetividade do antioxidante (Barriuso et al., 2016).

Os estudos de Merlo *et al.* (2019) e Serrano-León *et al.* (2018) avaliaram incorporação do extrato de pimenta rosa em filmes de quitosana na avaliação da vida útil de filés de salmão e produtos reestruturados de frango, respectivamente. Ambos os estudos demonstraram a eficácia do extrato da pimenta rosa na mitigação da oxidação lipídica, além dos efeitos antimicrobianos. Para os produtos reestruturados de frango, os resultados obtidos com a adição de pimenta rosa foram tão satisfatórios quanto os obtidos com adição do antioxidante BHT.

Fagundes *et al.* (2020) utilizaram a pimenta rosa 10% (v/v) na condimentação de azeite de oliva, submetidas a maceração convencional, realizada sob as condições de abrigo de luz, a 20 ºC com agitação manual de 20 segundos/dia; e maceração em ultrassom, utilizando o equipamento de banho ultrassônico a 35 ºC por 37 minutos. Todas as amostras adicionadas apresentaram maior estabilidade oxidativa comparadas as amostras controle, entretanto, as amostras submetidas a maceração por ultrassom apresentaram maiores capacidades antioxidantes.

Menegalli *et al.* (2020) utilizaram extrato de pimenta rosa como antioxidante natural em hambúrgueres de frango e compararam com a adição do antioxidante sintético BHT. As amostras foram armazenadas durante 7 dias em embalagens com e sem vácuo a 2 °C e com incidência de luz branca (lâmpada fluorescente - 800 lúmens). A estabilidade oxidativa foi mensurada através da análise de substâncias reativas ao ácido tiobarbitúrico (TBARS), demonstrando que o tratamento com o extrato foi eficiente tanto quanto o BHT, com valores significantemente menores, até 79,4% de redução nas amostras com adição de pimenta rosa, quando comparadas as amostras controle. Para ambas as embalagens utilizadas o extrato mostrou-se eficaz em retardar as reações de oxidação.

Além do emprego de extratos, as adições do fruto integral também vêm sendo considerada. Em estudo realizado por Barreira *et al.* (2023), a pimenta rosa foi adicionada na sua forma integral no óleo de soja utilizado como líquido de cobertura durante o processamento de enlatamento de sardinhas (*Sardina pilchardus*) nas concentrações 0,25%, 0,50%, 0,75% e 1,0%, comparadas com amostra controle, sem adição de pimenta.

A oxidação ocasionada pelo processo, que submete o produto a 126 °C por 40 minutos, foi avaliada também pela quantificação de colesterol e óxidos de colesterol nas sardinhas e no óleo de soja antes e após o processamento. O colesterol das sardinhas sofreu menor degradação nas amostras adicionadas de 1,0%, que apresentaram 17% de proteção em relação ao controle. Foi observada também proteção da pimenta rosa em relação a formação de produtos de oxidação do colesterol, nas sardinhas controle foi observado aumento de 2,9 vezes do total de óxidos após o processamento, enquanto as amostras com 0,75% e 1,0% apresentaram aumento de 1,5 vezes, representando aproximadamente 48% de proteção promovida pela pimenta rosa. Já em relação ao óleo de soja, que não possui colesterol em sua composição, foi registrado 218,3 μg/g nas amostras controle após o enlatamento, indicando migração do colesterol e óxidos da sardinha para o líquido de cobertura. A amostra com 1,0% de adição de pimenta rosa foi a que apresentou menor quantidade de óxidos de colesterol (132,3 μg/g), corroborando com os resultados observados nas sardinhas, e demonstrando efeito protetor da pimenta rosa.

De Oliveira *et al.* (2020a) avaliaram o efeito da adição de 0,2% e 0,5% de pimenta rosa moída em sistema modelo contendo óleo de sardinha (*Sardinella brasiliensis*) aquecidos a 150 °C e 180 °C, comparando com amostras adicionadas do antioxidante sintético BHT e sem adição de antioxidante, através das análises de TBARS, perfil de ácidos graxos e colesterol e óxidos de colesterol. Na análise de TBARS os autores observaram que as adições de 0,5% de pimenta rosa apresentaram o mesmo efeito protetor do BHT quando o óleo foi aquecido a 180 °C. Na análise de perfil de ácidos graxos foi observada a redução da concentração de ácidos graxos, inclusive os saturados, em razão das altas temperaturas aplicadas. A adição de 0,5% de pimenta rosa foi eficaz em proteger os ácidos graxos quando comparadas a todas as outras amostras, mantendo 94% dos ácidos graxos poli-insaturados após o aquecimento a 180 °C. As amostras 0,5% e BHT protegeram o óleo da degradação do colesterol em 50% e 57%, respectivamente, a 180 °C. Já em relação a formação de óxidos de colesterol, a amostra BHT apresentou maior efetividade, ou seja, menor formação de produtos oxidados em 44%, no entanto, a amostra com 0,5% de aroeira também demonstrou efetividade com 23% de proteção da amostra, quando comparadas a amostra controle.

Da Silva et al. (2025) avaliaram a adição de pimenta rosa moída em salame de porco nas concentrações de 0,25%, 0,50%, 0,75% e 1,0%, além da amostra controle sem adição de pimenta. A oxidação foi avaliada após 28 dias de maturação dos salames através das

análises de TBARS, perfil de ácidos graxos, colesterol e óxidos de colesterol. A adição de 1,0% de pimenta rosa reduziu em 51% a formação de TBARS nos salames quando comparados ao controle. O mesmo foi observado em relação ao aumento do somatório de ácidos graxos poli-insaturados, 51% maior nas amostras 0,75% e 1,0% do que na amostra controle. O efeito de redução na oxidação promovido pela pimenta também foi observado na análise de produtos de oxidação do colesterol (POCs), que demonstraram uma redução no total de óxidos formados em totas as amostras, com maior eficácia na amostra 1,0%, que apresentou 82,6% de redução do total de POCs em comparação ao controle.

A literatura tem reportado o potencial da flora brasileira como aditivos naturais na conservação de alimentos. Entretanto, ainda são escassos os estudos referentes à utilização de plantas subutilizadas e não convencionais frequentemente encontradas em quintais domésticos, como a pimenta rosa.

Os estudos que utilizam fontes naturais como agentes antioxidantes em óleo de soja no geral avaliam a adição de extratos, forma não viável de aplicação em ambiente doméstico, que utiliza de forma frequente o óleo de soja. Os óleos adicionados de pimenta rosa podem ser uma opção acessível, com a finalidade de minimizar reações foto-oxidativas, possibilitando a utilização de um produto mais seguro, com sua qualidade e propriedades funcionais preservadas, principalmente provenientes do conteúdo de fitosteróis e ácidos graxos.

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CAPÍTULO I

Effect of pink pepper (*Schinus terebinthifolia* Raddi) fruits on lipid oxidative stability of refined soybean oil during natural light exposure

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ABSTRACT

Soybean oil is the second most consumed edible oil in the world and is highly prone to oxidative degradation due to its high content of unsaturated lipids. As a result, photo-oxidation can significantly compromise oil quality during storage, when the product is exposed to light and ultraviolet radiation. Therefore, the effect of incorporating pink pepper (*Schinus terebinthifolia* Raddi) into refined soybean oil at different concentrations (2%, 4%, 6%) was evaluated to assess its antioxidant potential during 60 days of storage under light exposure at room temperature. Lipid oxidation was mainly evidenced by the formation of secondary oxidation products during storage, monitored by reactive substances (TBARS) analysis. However, for samples containing 4% and 6% of the fruit, TBARS values decreased as pink pepper concentration increased. Additionally, a kinetic study confirmed a time-dependent increase in TBARS values, with a first-order kinetic model providing the best fit for TBARS formation. After 60 days of storage, β-sitosterol content in control samples was reduced by 57.8%, but its degradation was significantly minimized by adding pink pepper at 4% and 6% until day 45. These findings indicate the potential of pink pepper to act as an additive in refined soybean oil to mitigate nutritional losses due to β-sitosterol degradation and to limit oxidative damage, thereby preserving the product quality during storage under light exposure.

Keywords: vegetable oil, β-sitosterol, photo-oxidation, natural antioxidants, bioactive compounds.

1 INTRODUCTION

Soybean oil is one of the most relevant vegetable oils in terms of production, market, and consumption. As a versatile product, soybean oil plays a crucial role in culinary and cooking processes (Jia et al., 2024; Knowlton, 2022). Therefore, it has the second-largest global production among vegetable oils, reaching 65.86 million tons in 2024, with a corresponding consumption of 65.23 million tons (USDA, 2025). Regarding its composition, soybean oil contains essential fatty acids, phytosterols, tocopherols, and fat-soluble vitamins (Ghazani & Marangoni, 2016; Xiang et al., 2024). It is rich in unsaturated fatty acids (UFAs), with linoleic acid (C18:2ω6) as the major fatty acid, accounting for 47 to 52% (Abdo et al., 2023; Olagunju et al., 2022). Among the phytosterols, β-sitosterol is the predominant one (Ghazani & Marangoni, 2016; Zheng et al., 2024). Both classes of compounds play vital roles in the human body and contribute to beneficial effects associated with soybean consumption, like the reduced risk of developing cardiovascular diseases (Barkas et al., 2023; Messina et al., 2021; Pérez-Martínez et al., 2023). However, their unsaturated bonds make unsaturated fatty acids and β-sitosterol highly susceptible to oxidation (Kuksis & Pruzanski, 2017; Li et al., 2024).

Prooxidant factors induce oxidative processes in foods during storage and processing, including high temperatures, oxygen, and light (Jacobsen et al., 2021). Regarding soybean oil, it can undergo color changes and lipid oxidation may form volatile compounds that produce a rancid odor, causing great economic impact due to quality losses (Shahidi & Hossain, 2022; Zhang et al., 2023). The degradation of vitamins, phytosterols, and essential fatty acids may reduce its nutritional value (Li et al., 2024; Prabakaran et al., 2018; Tian et al., 2023). Furthermore, the oxidation of phytosterols and UFAs can lead to toxic products, affecting food safety (Grootveld et al., 2021; Yu et al., 2021).

Soybean oil is commonly stored in transparent plastic packaging, which may contain ultraviolet (UV) light-absorbing additives; however, without such filters, the oil remains exposed to light during storage (Coltro & Buratin, 2004; Lopes & Courrol, 2023). Photo-oxidation occurs when food is exposed to light radiation, oxygen, and chromophores via two mechanisms: a chromophore in its excited state reacts with unsaturated molecules (Type I) or a chromophore absorbs radiation and reacts with triplet molecular oxygen ($^3O_2$) to form singlet oxygen ($^1O_2$), which reacts with unsaturated compounds (type II) (Min & Boff, 2002; Verduin, 2020). Therefore, due to the presence of dissolved O_2 and chromophores like chlorophyll, soybean oil is highly susceptible to Type II photo-oxidation, which is more common under aerobic conditions in hydrophobic foods (Min & Boff, 2002; Yang et al., 2020).

The oil industry usually adds synthetic antioxidants to prevent oxidation and extend the shelf life of commercial oils. However, the long-term consumption of these additives may pose health risks, including carcinogenic, cytotoxic, oxidative-stress inducing, and endocrine-disrupting effects (Esazadeh et al., 2024; Ji et al., 2024). In this way, the replacement of synthetic antioxidants with natural alternatives, such as ginger and turmeric powders, pomegranate, orange, beetroot leaf, purslane, grape seed and eggplant peel extracts, has been investigated in soybean oil, showing promising results (Abdo et al., 2023; Freitas et al., 2017; Jorge et al., 2018; Sharma et al., 2023; Tinello & Lante, 2020). Additionally, this trend also attends to consumers' demand for food products with reduced levels of synthetic additives (Inguglia et al., 2023).

Pink pepper, a fruit from the Aroeira (*Schinus terebinthifolia* Raddi) tree, is native to South America and abundantly found in Brazil. In addition to being valued as a condiment worldwide, it is recognized for its rich composition in bioactive compounds (e.g. phenolic acids, flavonoids, anthocyanins, tannins), highlighting its potential as a food additive with antioxidant properties, antimicrobial effects, and enhanced nutritional value (Barreira et al., 2023; de Oliveira et al., 2020a; Vieira et al., 2023).

In this context, we hypothesized that pink pepper addition would reduce oxidative degradation and preserve β-sitosterol in vegetable oils. Therefore, pink pepper fruits were incorporated into commercial soybean oil at concentrations of 2, 4%, and 6% (w/v) to investigate their protective effect against the degradation of UFAs and β-sitosterol during storage under light exposure at room temperature. Additionally, the fruits were characterized for their *in vitro* antioxidant capacity and composition in bioactive compounds by HPLC-QTOF-MS/MS.

2 MATERIALS AND METHODS

2.1 Materials, chemicals, and solvents

Commercial refined soybean oil (ingredients: genetically modified soybean oil and the synthetic antioxidant citric acid) was acquired from a local market located in Seropédica, Rio de Janeiro, Brazil, in February 2024. Folin-Ciocalteu, 3-chloroacetic acid, sodium methoxide, thiobarbituric acid, quercetin, Trolox, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and β-sitosterol standard were obtained from Sigma-Aldrich (St. Luis, MO, USA). Undecanoic methyl ester and the standard mixture of fatty acid were purchased from Supelco TM 37 (FAME Mix 18919, Bellefonte, PA, USA). For HPLC analyses the performed with solvents of chromatographic

grade obtained as follows: 2-propanol (St. Louis, MO, USA), acetonitrile (Merck, Darmstadt, Germany), formic acid (Synth, São Paulo, Brazil), and n-hexane (Scharlau, Barcelona, Spain). All other chemicals were achieved from Vetec (São Paulo, Brazil).

2.2. Pink pepper (*Schinus terebinthifolia* Raddi)

Pink pepper (*Schinus terebinthifolia* Raddi) samples were collected from the Ademar Moreira Settlement agroecological cooperative, located in São Pedro da Aldeia, Rio de Janeiro, Brazil (latitude 22° 43' 18.343" S; longitude 42° 6' 52.049" W). The mature fruits were selected considering their visual quality aspects (round shape, bright pink color, firmness, and absence of injuries). Then, the fruits were dried in a ventilated oven (SOLAB, São Paulo, Brazil) (40 ºC/24 h) to remove moisture excess. The samples were immediately used in the experiment.

A methanolic extract (methanol/water, 80:20, v/v) was prepared for analyses as reported by de Oliveira et al. (2020a). Approximately 2 g of pink pepper were homogenized with the extracting solution and sonicated for 30 min (40 Hz) (Elmasonic P, Elma Schmidbauer GmbH, Singen, Germany). Subsequently, the mixture was centrifuged (NI 1813, Nova Instruments, São Paulo, Brazil) at 18,000 × *g* for 5 min at 25 °C. The supernatant was collected and transferred to a volumetric amber flask of 50 mL and the volume completed with the methanol/water solution.

2.2.1 Total content of phenolic compounds and flavonoids

The methanolic extract of pink pepper was evaluated by the Folin-Ciocalteu method according to Swain and Hillis (1959). The absorbance was measured at 725 nm using a spectrophotometer (WUv-m51, Weblabor, São Paulo, Brazil), and the results were presented as mg gallic acid equivalent (GAE)/g sample.

To quantify the flavonoids, the methanolic extract (250 µL) diluted in distilled water (1250 µL) was mixed with a 5% sodium nitrite solution (75 µL) and left to rest for 5 min. A 10% aluminum trichloride solution (150 µL) was added to the mixture. After 5 more min, 500 µL of 1 M sodium hydroxide solution and 775 µL of distilled water were added (de Oliveira et al., 2020a). The readings were performed at 510 nm and results were expressed in mg of quercetin equivalent (QE)/g sample.

2.2.2 *In vitro* antioxidant capacity

The *in vitro* antioxidant capacity was assessed using the DPPH and FRAP assays. The

DPPH radical scavenging test was performed by mixing the methanolic extract (100 µL) with 3.9 mL of a 0.06 mM methanolic DPPH solution. After incubation for 1 hour in the dark at 25°C, the absorbance was measured at 517 nm. The DPPH radical scavenging activity was calculated using Equation 1, where A_0 and A represent the absorbance of the control (DPPH) and the sample, respectively (de Oliveira et al., 2020a).

$$\% \text{ DPPH inhibition} = ((A_0 - A) / A_0) \times 100 \quad (1)$$

For the FRAP assay, the methanolic extract (90 µL) was diluted in distilled water (270 µL) and mixed with the 2.7 mL of the FRAP reagent, which was prepared by mixing 300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM hydrochloric acid, and 20 mM ferric chloride in proportions of 10:1:1 (v/v/v). After homogenization, the solution was heated in a water bath at 37 °C for 30 min (Thaipong et al., 2006). Then, it was cooled, and the absorbance was measured at 595 nm. The results were expressed as µmol Trolox equivalent (TE)/g sample.

2.2.3 Identification of bioactive compounds by HPLC-QTOF-MS/MS analyses

Bioactive compounds were identified by high-resolution chromatography analysis on an HPLC instrument (Agilent 1290 Infinity II LC) configured with an Agilent 6530 quadrupole time-of-flight (QTOF) mass spectrometer (Agilent, Santa Clara, CA, USA) equipped with a C18 column (50 mm × 2.1 mm × 1.8 µm, Zorbax extension), maintained at 40 ºC. The mobile phase was an aqueous solution containing 0.1% formic acid and 5 mM ammonium formate (A), and a methanol solution containing 0.1% formic acid and 5 mM ammonium formate (B), the flow rate was 0.35 mL/min, and the sample injection volume was 10 µL. The gradient elution program started up to 1 min with 35% B, then 35–80% B (1–20 min), 80–85% B (20–25 min), 85–90% B (25–30 min), 90–95% B (30–35 min), 95–100% B (35–38 min), and 100% B for 7 min, followed by a column equilibration for 5 min. The mass spectra were acquired in positive and negative modes using electrospray ionization (ESI) source with the following operating conditions: dry gas (N_2) at 12 L/min; dry temperature at 300 °C; nebulizer at 38 psi; capillary voltage at 3500 V; fragmentor voltage at 120 V. The analysis was carried out in the MS full scan mode monitored in the mass range of 100 to 800 *m/z*. Subsequently, an MS/MS experiment was performed and the parameters set as follows: MS spectrum acquisition rates of 4 spectra/s and collision energies of 10, 20, 40, and 60 V. All operations, data acquisition, and

analysis were performed in Mass-Hunter Acquisition software version 11.0.

2.3 Photo-oxidation: sample preparation and experimental conditions

The commercial refined soybean oil, acquired on a date close to the date of manufacture, was bottled in transparent glass jars without a specific protection from UV light. Aliquots of 200 mL were distributed to the recipients according to the following treatments: oil without the addition of pink pepper (control) and oil with pink pepper at 2, 4, and 6% w/v (2% PP, 4% PP, and 6% PP, respectively). Pink pepper was used in its whole form. The fruits were added to the glass jars containing the oil and manually homogenized by shaking the jars, which were kept closed. The pepper concentrations used were established based on preliminary tests that considered the visual aspect of the mixture, simulating home preparation.

Four samples (jars containing the oil) were prepared for each treatment, which were randomly placed on a laboratory bench in Seropédica, Rio de Janeiro (latitude 22° 46' 37.316" S; longitude 43° 41' 9.416" W). Therefore, the samples were exposed to ambient light at room temperature (25 °C ± 1 °C), simulating a domestic environment, for 60 days. The average daily light exposure was 12 hours, from February 27th to April 27th, 2024. Additionally, the samples were randomly repositioned every 3 days until the 60 days was completed.

The samples from each treatment were taken for analysis on days 0, 15, 30, 45, and 60, where day 0 corresponds to the initial oxidation status. The contents from four jars were filtered to separate pink pepper fruits, and the oil was transferred and homogenized in another recipient. Therefore, a single sample was obtained for each treatment, and appropriate aliquots were taken for analysis.

2.3.1 Lux measurement

The average daily light incidence was measured using a lux meter (digital lux meter Vonder, LDV2000). Measurements were taken at 20 randomly selected points on the experimental bench, at two different times of the day (10 a.m. and 4 p.m.), totalling 40 measurements per day. The daily light exposure duration was determined by recording the number of daylight hours throughout the experimental period. Based on these data, both the total light exposure time and the average daily light incidence were calculated. From February 27 to April 27, the average daily exposure was 11 hours, and 53 min and the lux average was 851 ± 82.

2.3.2 Soybean oil analyses

2.3.2.1 Peroxide value (PV)

The peroxide value was determined according to the method 965.33 (AOAC, 2005). Samples (5 g) were dissolved in an acetic acid-chloroform solution (3:2 v/v, 30 mL), and then a saturated iodide solution (0.5 mL) was added. After homogenization for 1 min, distilled water (30 mL) and starch indicator (1 mL) were added. The resultant solution was titrated with sodium thiosulfate (0.01 N) until the yellow color disappeared. Peroxide values were calculated using Equation 2 and the results were expressed as meq O_2/kg sample.

$$PV\ (meq\ O_2/kg\ sample) = (V - Vb) \times N \times 1000/\ m \qquad (2)$$

Where V and Vb are the volumes of sodium thiosulfate (mL) used during titration for the samples and the blank, respectively; N is the sodium thiosulfate normality, and m is the sample mass (g).

2.3.2.2 Thiobarbituric acid reactive substances (TBARS)

Samples (0.5 g) were mixed with 5 mL of a thiobarbituric acid solution prepared by mixing a chloric acetic acid solution (0.25 M) with a trichloroacetic acid solution (15%) and a thiobarbituric acid solution (0.375%). The mixtures were heated in a boiling water bath under constant magnetic stirring for 10 min. Subsequently, they were immediately cooled, sonicated (Elmasonic P, Elma Schmidbauer GmbH, Singen, Germany) for 30 min and centrifuged (NI 1813, Nova Instruments, São Paulo, Brazil) at 18,000 × *g* for 10 min. The absorbance of the supernatant was measured at 532 nm and results were expressed as mg of malondialdehyde (MDA)/kg sample (Buege & Aust, 1978).

2.3.2.3 Fatty acid composition

The oil samples were converted into methyl esters through transesterification with sodium methoxide, followed by phase separation and extraction with hexane, according to Zhu et al. (2011). Fatty acids were separated and identified by gas chromatography using a gas chromatograph (Shimadzu GC 2010, Tokyo, Japan) with a flame ionization detector and a SP®-2560 capillary column (100 m × 0.25 mm i.d., 0.20 μm film thickness) (Sigma-Aldrich, St. Luis, MO, USA), operating in the split mode (1:50). Chromatographic conditions were programed as described by de Oliveira et al. (2020a). Hydrogen was the carrier gas (1 mL/min),

and nitrogen was used as the make-up gas (30 mL/min). The identification of chromatographic peaks was performed by comparing the retention times with those of FAME standards, while fatty acids were quantified by internal standardization with undecanoic methyl ester (C11:0).

2.3.2.4 β-sitosterol analysis by HPLC-APCI-MS

β-sitosterol was obtained directly from soybean oil samples by cold saponification as described by Azadmard-Damirchi and Dutta (2009). High performance liquid chromatography was conducted for identification and quantification of β-sitosterol using a Prominence-Shimadzu equipment (Kyoto, Japan) with an LC-20AD pump, SPD-M20A detector, CTO-20A oven, SIL-10AF autosampler, CBM-20A controller, and the LabSolution software. Atmospheric Pressure Chemical Ionization (APCI) in the positive mode and selective ion monitoring (SIM) mode were applied, according to de Oliveira et al. (2020a). A CN Hyperchrome column (250 mm × 4,3mm × 5,0 μm) (Phenomenex, Colorado, USA) was used. The mobile phase was n-hexane:2-propanol (97:3, v/v), at a flow rate of 1 mL/min. Oven temperature was set at 32 ºC and the injection volume was 10 μL. β-sitosterol was determined by comparing the retention time of the peak in samples with that of the standard reference. Moreover, the *m/z* 395, which was previously observed for the standard, was also considered. β-sitosterol quantification was performed by external standardization using standard calibration curve (r=0.995).

2.4 Kinetic modeling for peroxide value and TBARS

The mathematical models were used to evaluate the rates of formation and decomposition of primary oxidation products (peroxides) and the formation of secondary oxidation products (TBARS) as a function of storage time and concentration (Van Boekel, 1996). The models (Equations 3, 4, and 5) were based on fundamental kinetic models. In addition, a quadratic model was tested (Equation 6) for the peroxide values. The model adequacy was verified using the coefficient of determination R^2 and the Shapiro-Wilk test to evaluate the normality of the residues. Models with R^2 values closest to 1.0 and residuals that followed a normal distribution (p_value_Shapiro_Wilk > 0.05) were considered the most appropriate, according to the following equations.

$$y = C_0 \pm kt \quad (3)$$

$$y = C_0 * e^{\pm kt} \quad (4)$$

$$y = {C_0}/{1 \pm C_0 * kt} \quad (5)$$

$$y = C_0 + b_1 * t + b_{11} * t^2 \quad (6)$$

Where y is the response, C_0 is the initial value of the compound of interest, b_1 is the slope or linear effect of concentrations of pink pepper, b_{11} is the quadratic effect of pink pepper concentrations; t is the time, and k is the reaction rate (mg. $g^{-1}.h^{-1}$).

2.5 Statistical analysis

A two-way ANOVA (analysis of variance) was applied to evaluate the effects of pink pepper addition and storage time. Then, Tukey's post-hoc mean comparison test was performed where differences were detected. Results were expressed as mean ± standard deviation (n = 3), and analyses were carried out using 5% of significance.

Principal Component Analysis (PCA) and Hierarchical Clustering of Principal Component analysis (HCPC) were used for multivariate statistics. PCA was performed on standardized data to evaluate variable and sample relationships. HCPC was applied using the PCA-derived coordinates, the distances were measured using a Euclidian distance, and clusters were formed by the Ward´s method. A heatmap was generated to visualize the influence of variables on the identified sample groups, with clustering based on the HCPC results. Finally, Pearson's correlation test was conducted to evaluate relationships among response variables like PV, TBARS, fatty acids, and β-sitosterol. The strength of correlations was interpreted according to the scale proposed by Teles et al. (2019).

3 RESULTS AND DISCUSSION

3.1 Pink pepper characterization

3.1.1 Phenolic compounds, flavonoids, and antioxidant capacity of pink pepper extract

The total phenolic content determined in the pink pepper extract was 7.40 ± 0.01 mg GAE/g (Table 1), which was lower than the one reported by de Oliveira et al. (2020a) (13.66 mg GAE/g). In contrast, closer levels were found by Costa et al. (2015) for fruit extract obtained by Soxhlet using ethanol (5.44 mg GAE/g) and by Sassi et al. (2020) for a methanolic extract (9.21 mg GAE/g).

Regarding total flavonoids, a content of 26.63 ± 1.59 mg QE/g was found in pink pepper extract (Table 1). Variable results can be found in the literature, from 1.88 mg QE/g in an

ethanolic extract (Pinto et al.,2020) to 10.33 mg QE/g in a methanolic extract (de Oliveira et al., 2020a). Sauthier et al. (2024) evaluated pink pepper fruits from different origins and found values ranging from 1.01 to 2.84 mg QE/g in methanolic extracts of freeze-dried fruits, while Tlili et al. (2018) determined contents of 39.05 and 37.21 mg QE/g for methanolic extracts of pink pepper fruits from Tunis and Gafsa, respectively.

Table 1: Total contents of phenolic compounds and flavonoids, and antioxidant capacity determined by DPPH and FRAP assays of pink pepper extract.

Method	**Result**
Total phenolic (mg GAE/g sample)	7.40 ± 0.01
Total flavonoids (mg QE/g sample)	26.63 ± 1.59
DPPH (% inhibition)	87.43 ± 0.34
FRAP (μmol TE/g)	125.25 ± 1.76

Results presented as mean ± standard deviation, n=3.

Phenolic compounds are the main antioxidant compounds found in pink pepper, primarily represented by phenolic acids and flavonoids (Martini et al., 2021; Vieira et al., 2023). However, the levels of those phytochemicals, which are secondary metabolites of plants, are influenced by several parameters, such as genotype, ripening state, origin, light exposure, climate, and others. Furthermore, other factors, such as extract preparation conditions, fruit pre-treatment, and temperature, can influence the quantification of these compounds (Ennigrou et al., 2017; Silva et al., 2024).

The *in vitro* antioxidant capacity was measured by DPPH and FRAP assays. Pink pepper extract showed an inhibition against DPPH of 87.43 ± 0.34 % (Table 1). A higher protection was reported for an ethanolic extract, with 95% of inhibition (Barreira et al., 2023), while 42.68% was found in a methanolic extract (de Oliveira et al., 2020a).

For FRAP assay, a value of 125.25 ± 1.76 μmol TE/g was determined (Table 1), which was higher than the one found by Barreira et al. (2023) (58.51 μmol TE/g) in an ethanolic extract and Martini et al. (2021) (30 μmol TE/g) for an extract prepared with a methanol, water, and formic acid solution. Other studies reported the antioxidant capacity of pink pepper fruit extracts by the FRAP assay; however, the different standards used for quantification make

comparison of values unfeasible. A review by Vieira et al. (2023) compiled data on the antioxidant capacity of pink pepper, including results from the DPPH assay, demonstrating that values can range from 1.3% to 95.6%. Studies have evaluated different pink pepper samples from around the world, using extracts prepared under different extraction conditions and methodologies which, although based on similar principles, may present procedural modifications. These factors may influence the results, leading to different findings. Therefore, comparing values across literature is challenging, which highlights the necessity of characterizing each extract individually.

3.1.2 Bioactive compounds from pink pepper extract determined by HPLC-QTOF-MS/MS

Ten compounds, including flavonoids, organic acids, phenolic compounds, and terpenes, were identified by HPLC-QTOF-MS/MS in the negative ion mode (Table 2). The identified compounds were amentoflavone ($[M-H]^-$ *m/z* 537.0809), agathisflavone ($[M-H]^-$ *m/z* 537.0794), tetrahydroagathisflavone ($[M-H]^-$ *m/z* 541.1112), citric acid ($[M-H]^-$ *m/z* 191.0175), gallic acid ($[M-H]^-$ *m/z* 169.0129), masticadienoic acid ($[M-H]^-$ *m/z* 453.3351), triterpene acids ($[M-H]^-$ *m/z* 471.3457 and 471.3567), methyl digallate ($[M-H]^-$ *m/z* 335.0404), methyl gallate ($[M-H]^-$ *m/z* 183.0288), and ethyl gallate ($[M-H]^-$ *m/z* 197.0456). The proposal identification was conducted by comparing retention times, molecular masses, fragmentation patterns, and molecular formulas with data reported in the literature.

Flavones were the main bioactive compounds determined in pink pepper, including amentoflavone, agathisflavone, and tetrahydroagathisflavone, in agreement with Gomes et al. (2020). These compounds belong to the biflavonoids group, a class of polyphenolic compounds composed of two flavonoid units linked together, which contributes to their complex chemical structure and biological activities, presenting antioxidant, anti-inflammatory, and antimicrobial properties (Andrade et al., 2018; Xiong et al., 2021).

Methyl gallate, methyl digallate, and ethyl gallate were also identified for Carneiro et al. (2024) and Gomes et al. (2020), who used UHPLC-QTOF-MS and ESI (-) FT-ICR MS methods, respectively. Methyl and ethyl gallate compounds are derived from gallic acid, a phenolic acid widely valued by its high anti-radical potential (Ceruks et al., 2007). As demonstrated by Farhoosh and Nyström (2018), gallic acid and methyl gallate were more effective than the synthetic antioxidant TBHQ in preventing the oxidation of sunflower oil at 120 °C, where a combined treatment with a 75:25 ratio of methyl gallate to gallic acid exhibited

the highest antioxidant potential.

The triterpene acid masticadienoic acid was also identified by Carneiro et al. (2024) by UHPLC-QTOF-MS, supporting both our findings and those reported by Gomes et al. (2020), who found phenolic compounds and triterpenes ($[M-H]^-$ *(m/z)* 471.34861) in residues from pink pepper tree processing. Terpene compounds can act as anti-inflammatory, antioxidant, antiangiogenic, and antimicrobial agents (Jain et al., 1995; Tang et al., 2020).

Citric acid, which was determined in pink pepper extract, is an organic acid with both antimicrobial and antioxidant activities (Fernández-Fernández et al., 2010), including metal ion chelating and sequestering properties (Mahdavian, 2021; Martínez et al., 2018). Therefore, due to these functional characteristics, synthetic citric acid is widely used as a preservative by the food industry (Martínez et al., 2018).

As presented above, pink pepper contains valuable active compounds that highlight its health-promoting benefits, primarily by combating oxidative stress (Feriani et al., 2021). Phenolic compounds, such as the ones identified herein, have one or more phenolic rings in their structure, acting as free radical scavengers by donating electrons or hydrogen atoms, thereby neutralizing reactive oxygen species (Vieira et al., 2023). In addition, these properties also indicate the use of pink pepper as a natural source of phenolics to extend the shelf life of food products by reducing oxidative processes (Ordoñez-Cano et al., 2024; Ullah et al., 2022). Menegali et al. (2020) incorporated pink pepper extract into chicken burgers packaged using different materials to assess their oxidative stability during 7 days of storage at 2 °C. Compared to a formulation containing the synthetic antioxidant butylated hydroxytoluene (BHT), the extract demonstrated equivalent effectiveness in delaying lipid oxidation. These findings indicate pink pepper as a promising natural alternative for enhancing the oxidative stability of food products during storage, supporting our research.

Table 1: Proposed identification of bioactive compounds of pink pepper extract in the negative ion mode by HPLC-QTOF-MS/MS.

RT (min)	Mass (average)	Ion [M-H] (*m/z*)	Fragments	Formula	Identification	References
0.540	192.0254	191.0175	158.8464; 148.8972; 102.9471	$C_6H_8O_7$	Citric acid	Fernández-Fernández et al. (2010); *metlin*, 2025
0.574	336.0470	335.0391	183.0302; 124.0165	$C_{15}H_{12}O_9$	Methyl digallate	Carneiro et al. (2024); Gomes et al. (2020)
0.674	332.0736	331.0657	183.0302; 169.0141; 124.0164	$C_{13}H_{16}O_{10}$	Glucogallin	Feriani et al. (2021)
0.941	170.0208	169.0129	125.0242	$C_7H_6O_3$-	Gallic acid	Carneiro et al. (2024); Feriani et al. (2021); *metlin (*2025)
2.891	184.0367	183.0288	168.0059; 124.0163	$C_8H_8O_5$	Methyl gallate	Carneiro et al. (2024); Gomes et al. (2020)
5.600	198.0535	197.0456	169.0134; 124.0168	$C_9H_{10}O_5$	Ethyl gallate	Carneiro et al. (2024); Gomes et al. (2020)
6.575	336.0483	335.0404	183.0306; 124.0168	$C_{15}H_{11}O_9$	Methyl digallate	Carneiro et al. (2024); Gomes et al. (2020)
8.517	538.0888	537.0809	479.1933; 375.0508; 297.0393; 169.0149	$C_{30}H_{18}O_{10}$	Amentoflavone	Gomes et al. (2020)
8.717	538.0873	537.0794	481.2089; 443.0415; 375.0501	$C_{30}H_{18}O_{10}$	Agathisflavone	Gomes et al. (2020)
8.792	542.1191	541.1112	480.3060; 255.2322; 389.1075	$C_{30}H_{22}O_{10}$	Tetrahydroagathisflavone	Gomes et al. (2020)
13.759	454.3430	453.3351	407.2925; 267.2693; 112.9850 isomer	$C_{30}H_{45}O_3$	Masticadienoic acid	Carneiro et al. (2024); Gomes et al. (2020)
14.465	472.3536	471.3457	399.3478; 311.2026; 149.0965 isomer	$C_{30}H_{48}O_4$	Triterpene acid	Gomes et al. (2020)

3.2 Effects of photo-oxidation

3.2.1 Peroxide values

The initial peroxide values of the samples on day 0 varied from 0.93 ± 0.10 (6% PP) to 1.46 ± 0.01 meq O2/kg oil (control), with no statistically significant differences among the different treatments ($p > 0.05$) (Table 3). After 15 days of storage, values increased significantly for all samples ($p \leq 0.05$), reaching their maximum value on day 45, when values ranged from 5.34 ± 0.60 (6% PP) to 7.24 ± 0.80 meq O_2/kg oil (control). At all evaluated time points, the control samples presented the highest peroxide values, followed by 2% PP, 4% PP, and 6% PP samples, respectively. Despite this, after the 45th day, the peroxide values showed a decrease, ranging from 4.30 ± 0.25 (6% PP) to 6.20 ± 0.32 (control) meq O_2/kg oil ($p \leq 0.05$). Peroxides are primary oxidation products characterized by one or more oxygen-oxygen (O-O) bonds, which are inherently unstable and prone to cleavage under mild conditions (Sanchez & Myers, 2000). Therefore, the observed decline in peroxide values after 45 days of storage suggests the degradation of these compounds over time.

Regarding the addition of pink pepper, its protective effect against lipid oxidation was demonstrated by the lower peroxide values determined in samples containing the natural antioxidant compared to the control during storage (Table 3). On day 45, which presented the highest peroxide levels, samples containing 4% and 6% pink pepper exhibited values that were 1.3 and 1.4 times lower, respectively, compared to the control ($p \leq 0.05$). The treatment with 2% PP did not differ significantly from the control ($p > 0.05$) on day 45. At the end of the storage period (day 60), the 2% PP treatment remained statistically indistinguishable from the control ($p > 0.05$). In contrast, 4% PP and 6% PP presented significantly lower peroxide values, 4.77 ± 0.17 and 4.30 ± 0.25 meq O_2/kg oil, respectively, compared to the control.

A comparison of the different concentrations of pink pepper revealed a similar protective effect for the 4% and 6% treatments on day 45 ($p > 0.05$), presenting percentages of peroxide inhibition as follows: 19% (4% PP) and 26% (6% PP). However, the addition of 2% PP did not provide significant protection against lipid oxidation, as the peroxide level was comparable to that of the control ($p > 0.05$). Overall, these findings suggest a concentration-dependent effect, with higher pink pepper concentrations resulting in greater antioxidant efficacy in inhibiting the formation of primary oxidation products. This behavior was also observed in previous studies carried out with different food matrices using pink pepper (Barreira et al., 2023; de Oliveira et al., 2020b; Martini et al., 2021; Oliveira et al., 2020). In vegetable oils, Fagundes et al. (2020) showed that adding

10% of ground pink pepper to olive oil using different maceration conditions (conventional maceration for 7 and 15 days, and ultrasound-assisted maceration) increased the oxidative stability. In control samples, the peroxide value was 15.82 meq O_2/kg. In contrast, incorporating 10% PP with the conventional maceration for 7 (CM7) and 15 days (CM15) resulted in 8.40 and 8.66 meq O_2/kg, respectively, while samples treated with ultrasound assisted maceration (UM) resulted in 7.14 meq O_2/kg, resulting in reductions in peroxide formation of 53% (CM7), 55% (CM15), and 45% (UM).

The incidence of light also plays a role in increasing peroxide values. Zhao et al. (2019) investigated a mixture of phytosterols dissolved in triolein, subjected to varying light intensities and stored in the dark at 25 °C for 14 days. The study observed an overall increase in peroxide levels across all samples during storage, but samples kept in the dark exhibited significantly lower peroxide values (12 meq O_2/kg of oil) compared to the samples exposed to the highest light intensity (32 meq O_2/kg of oil) by day 14. Moreover, the peroxide value of soybean oils doubled during the first 15 days of exposure to sunlight (8 hours per day), with no significant changes observed between day 15 and day 60 (Solefack et al., 2024).

To better understand the formation of peroxides during storage, a kinetic study was conducted. Among the tested models, the quadratic model, which corresponds to model 3 in bold (Supplementary material Table 1), best fitted the peroxide formation data in soybean oil samples. It exhibited the highest coefficient of determination (R^2) for all samples, while presenting the lowest Root Mean Squared Error (RMSE). Figure 1 presents both the empirical data (dots) and the theoretical values (dotted line) predicted by the models. The observed quadratic behavior indicates that during the storage time the peroxide value increased until a certain point due to the formation of primary oxidation compounds, followed by a decrease, suggesting their subsequent decomposition and conversion into secondary oxidation products.

Other studies have also identified the quadratic model as the most suitable for describing degradative processes in foods, such as the formation of oxidized diacylglycerols and triacylglycerols in vegetable oils during frying (Tarmizi et al., 2019). As theoretically expected, the peroxide level tends to increase initially and subsequently decrease. On the other hand, some studies on peroxide formation during soybean oil storage have reported a continuous increase in these compounds even after 60 days of storage (Abdo et al., 2023; Gaitán-Jiménez et al., 2022; Mansour et al., 2022; Solefack et al., 2024). However, comparisons among studies are challenging due to variations in experimental conditions (oil type, storage duration and temperature, and light exposure).

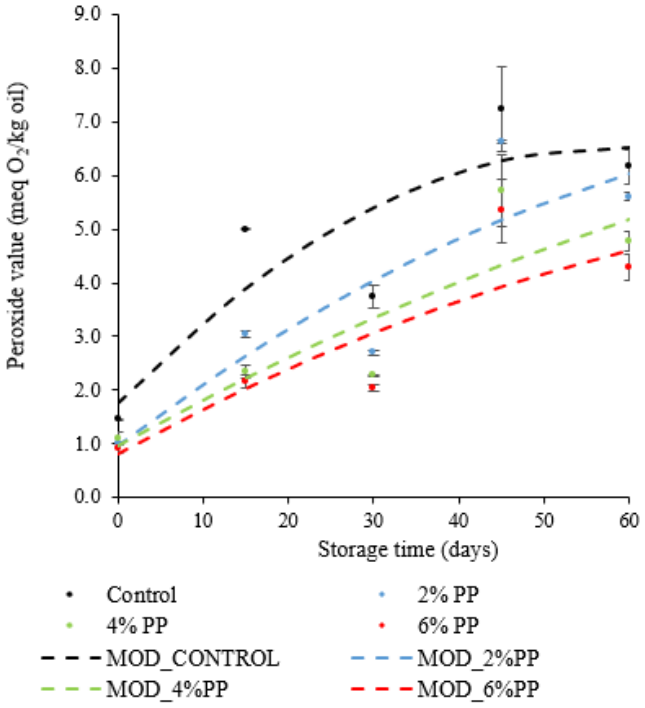

Figure 1: Dots and dashed lines represent experimental results and mathematical models, respectively, for control (black), 2% PP (blue), 4% PP (green), and 6% PP (red).

3.2.2 Formation of thiobarbituric acid reactive substances (TBARS)

The TBARS values determined on day 0 ranged from 0.69 ± 0.03 to 0.76 ± 0.05 mg MDA/kg oil, which were similar regardless of the treatment considered ($p>0.05$) (Table 3). From day 15 onwards, these values increased in all samples, where the greatest impact of storage on TBARS values was achieved on day 60. For the control samples, the following values were determined: 0.76 ± 0.05 mg MDA/kg oil (day 0), 1.03 ± 0.10 mg MDA/kg oil (day 15), 1.67 ± 0.07 mg MDA/kg oil (day 30), 1.79 ± 0.01 mg MDA/kg oil (day 45), and 2.31 ± 0.04 mg MDA/kg oil (day 60). However, the antioxidant potential of pink pepper was evidenced by the lower TBARS values in samples containing the fruit compared to the control ($p\leq0.05$). On day 60, treatments with pink pepper presented 2.25 ± 0.08 mg MDA/kg oil (2% PP), 2.04 ± 0.30 mg MDA/kg oil (4% PP), and 1.61 ± 0.01 mg MDA/kg oil (6% PP), corresponding to reductions in TBARS formation of 2.6%, 11.5%, and 30.2%, respectively, relative to the control. However, no statistically significant difference was observed between the 2% PP treatment and the control ($p>0.05$). For samples containing 4% and 6% PP, the higher the pink pepper

concentration, the lower the TBARS value. This protective effect was also observed on days 15, 30, and 45, during which samples supplemented with pink pepper consistently showed lower TBARS values.

The efficacy of pink pepper in inhibiting MDA formation has been demonstrated in various food matrices. The ethanolic extract of pink pepper (90 mg GAE/ kg meat) had an antioxidant efficiency comparable to that of BHT in chicken burgers stored under white light incidence, as evidenced by significantly lower TBARS values relative to the control (Menegali et al., 2020). Similarly, de Oliveira et al. (2020b) found that the addition of pink pepper (0.5%) to sardine oils heated to 150 °C and 180 °C resulted in a 26.73% reduction in MDA formation at 180 °C, a value statistically similar to that observed for BHT-treated samples (27.59% inhibition ($p>0.05$). Furthermore, the incorporation of an aqueous pink pepper extract (1.0%) into pork sausages stored for 420 days under freeze-storage (-18 °C) was more efficient than the application of 0.025% sodium erythorbate (Oliveira et al., 2020). Regarding soybean oils, the literature has highlighted the role of natural antioxidants in maintaining oxidative stability, as assessed by TBARS, including pomegranate, guava, and grape mixture extract (Mansour et al., 2022) and sumac fruit (*Rhus coriaria*) extract (Rahmati et al., 2022).

For the control sample, the zero-order kinetic model (model 1) provided the best fit, presenting the highest R^2 value (0.97) and the lowest RMSE (0.10) (Figure 2 and Supplementary material Table 2). In this case, TBARS formation in soybean oil was linearly dependent on storage time. In contrast, for the samples containing pink pepper, the second-order kinetic model yielded the best fit, with the highest R^2 values (2% PP: 0.99, 4% PP: 0.93, and 6% PP6: 0.87), and the lowest RMSE (2% PP: 0.05, 4% PP: 0.13, and 6% PP: 0.13). Among these treatments, the sample with 6% PP exhibited the lowest TBARS values, indicating enhanced oxidative stability attributed to the presence of pink pepper.

A first-order kinetic model better fitted the formation of TBARS in pork sausages stored at temperatures ranging from 5 to 35 °C (Wenjiao et al., 2014). Similarly, in minced raw beef meat containing plant extracts, TBARS values increased significantly with both storage time and temperature. Among the kinetic models evaluated, the Arrhenius model provided slightly better accuracy in predicting TBARS changes, describing the temperature dependency of lipid oxidation and offering a reliable framework for understanding the influence of thermal conditions on oxidation rates in meat products. Additionally, clove, allspice, and bay leaf were identified as the most effective antioxidant extracts in that context (Kaczmarek & Muzolf-Panek, 2021). In the

present study, the kinetic analysis suggests that the antioxidant efficacy of pink pepper increases with higher concentrations; however, its protective effect tends to diminish over prolonged storage periods.

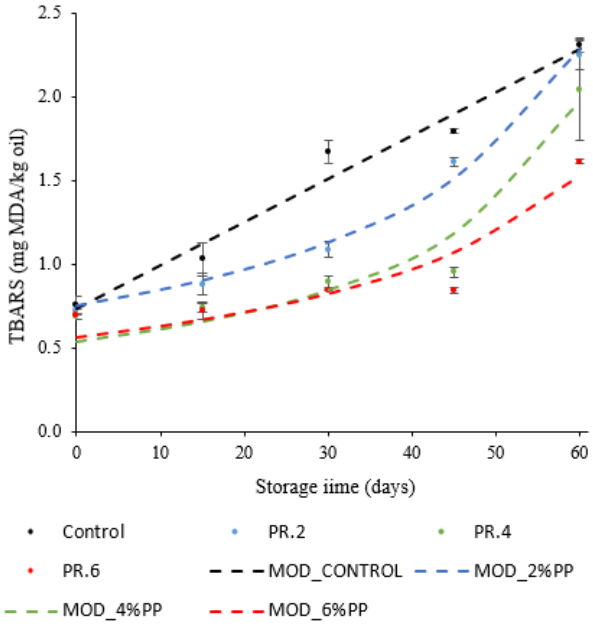

Figure 2: Dots and dashed lines represent experimental results and mathematical models, respectively, for control (black), 2% PP (blue), 4% PP (green), and 6% PP (red).

Table 2: Peroxide value (meq O2/kg oil), TBARS (mg MDA/kg), and β-sitosterol (mg/100g) in soybean oil samples. content (mg/100 g of oil) of soybean oil samples (control, 2% PP, 4% PP, and 6% PP).

Time (days)	Treatment	PV (meq O_2/kg oil)	TBARS (mg MDA/kg)	β-sitosterol (mg/100g)
0	Control	1.46 ± 0.01 $^{A;e}$	0.76 ± 0.05 $^{A;d}$	146.59 ± 5.88 $^{A;a}$
	2% PP	1.01 ± 0.06 $^{A;d}$	0.72 ± 0.03 $^{A;d}$	144.06 ± 5.41 $^{A;a}$
	4% PP	1.11 ± 0.12 $^{A;d}$	0.70 ± 0.02 $^{A;b}$	144.22 ± 9.04 $^{A;a}$
	6% PP	0.93 ± 0.10 $^{A;d}$	0.69 ± 0.03 $^{A;d}$	146.48 ± 5.58 $^{A;a}$
15	Control	5.00 ± 0.01 $^{A;c}$	1.03 ± 0.10 $^{A;c}$	120.88 ± 6.84 $^{C;b}$
	2% PP	3.05 ± 0.11 $^{B;c}$	0.88 ± 0.06 $^{AB;d}$	126.41 ± 8.55 $^{BC;b}$
	4% PP	2.35 ± 0.12 $^{C;c}$	0.74 ± 0.02 $^{B;cd}$	133.77 ± 5.81 $^{AB;a}$
	6% PP	2.16 ± 0.12 $^{C;c}$	0.72 ± 0.05 $^{B;b}$	138.72 ± 2.89 $^{A;a}$
30	Control	3.75 ± 0.21 $^{A;d}$	1.67 ± 0.07 $^{A;b}$	100.88 ± 7.27 $^{B;c}$
	2% PP	2.70 ± 0.22 $^{B;c}$	1.09 ± 0.05 $^{B;c}$	106.91 ± 6.92 $^{AB;c}$
	4% PP	2.29 ± 0.00 $^{B;c}$	0.89 ± 0.03 $^{C;bc}$	109.17 ± 1.37 $^{AB;b}$
	6% PP	2.04 ± 0.06 $^{B;c}$	0.85 ± 0.01 $^{C;b}$	113.4 ± 4.45 $^{A;b}$
45	Control	7.24 ± 0.80 $^{A;a}$	1.79 ± 0.01 $^{A;b}$	91.09 ± 3.09 $^{B;c}$
	2% PP	6.62 ± 0.44 $^{A;a}$	1.61 ± 0.10 $^{A;b}$	92.71 ± 2.66 $^{B;d}$
	4% PP	5.71 ± 0.67 $^{B;a}$	0.95 ± 0.03 $^{B;b}$	109.48 ± 7.61 $^{A;b}$
	6% PP	5.34 ± 0.60 $^{B;a}$	0.84 ± 0.02 $^{B;b}$	113.14 ± 5.76 $^{A;b}$
60	Control	6.20 ± 0.32 $^{A;b}$	2.31 ± 0.04 $^{A;a}$	61.80 ± 2.84 $^{A;d}$
	2% PP	5.61 ± 0.21 $^{A;b}$	2.25 ± 0.08 $^{A;a}$	64.35 ± 0.46 $^{A;e}$
	4% PP	4.77 ± 0.17 $^{B;b}$	2.04 ± 0.30 $^{B;a}$	67.75 ± 6.39 $^{A;c}$
	6% PP	4.30 ± 0.25 $^{B;b}$	1.61 ± 0.01 $^{C;a}$	73.87 ± 2.78 $^{A;c}$

Different capital letters indicate significant differences among treatments during the same storage time, and different lower letters indicate significant differences ($p \leq 0.05$) for the same treatment during storage time.

3.2.3. Fatty acids

The main fatty acids determined in the control soybean oil samples on day 0 were C18:2 ω6 cis (50.01 ± 0.52 g/100 g oil), C18:1 ω9 cis (21.74 ± 0.24 g/100 g oil), C18:3 ω3 (4.95 ± 0.04 g/100 g oil), and C16:0 (10.04 ± 0.02 g/100 g oil) (Table 4). The addition of pink pepper did not result in significant alterations in the fatty acid profile ($p>0.05$), which was similar to those reported for refined soybean oils by Ayyildiz et al. (2015) and Olagunju et al. (2022).

Additionally, storage time and exposure to natural light did not influence the fatty acid concentration of the samples, regardless of treatment. These findings are consistent with those by Pignitter et al. (2014), who investigated if fluorescent light accelerates lipid oxidation compared to dark storage in soybean oil under household conditions. While photo-oxidation was confirmed by an increase in peroxide values during storage under light exposure at 22 °C and 32 °C for 56 days, the concentrations of fatty acids were not affected.

Similarly, Mansour et al. (2022) evaluated the fatty acid content of soybean oil under accelerated storage (65 °C) for 0, 6, and 30 days. According to the Arrhenius model, 6 days at 65 °C is equivalent to approximately 180 days of storage at room temperature. The authors observed no significant changes in the saturated and unsaturated fatty acid content of refined soybean oil without added antioxidants after 6 days of storage. However, after 30 days (equivalent to approximately 30 months at room temperature), a 2.2-fold increase in total saturated fatty acids and a 1.3-fold decrease in total unsaturated fatty acids were recorded.

Table 3: Fatty acid composition (g/100 g oil) of soybean oil samples (control, 2% PP, 4% PP, and 6% PP).

Fatty acids	Day 0				Day 15			
	Control	2 % PP	4% PP	6% PP	Control	2 % PP	4% PP	6% PP
C16:0	10.04 ± 0.02 $^{A;a}$	10.02 ± 0.08 $^{A;a}$	9.94 ± 0.64 $^{A;a}$	9.88 ± 0.02 $^{A;a}$	9.94 ± 0.05 $^{A;ab}$	9.82 ± 0.06 $^{A;a}$	9.80 ± 0.11 $^{A;a}$	9.77 ± 0.03 $^{A;ab}$
C18:0	3.73 ± 0.04 $^{A;a}$	3.72 ± 0.03 $^{A;a}$	3.66 ± 0.09 $^{A;b}$	3.22 ± 0.18 $^{B;b}$	3.98 ± 0.08 $^{A;b}$	3.86 ± 0.06 $^{A;a}$	3.78 ± 0.06 $^{A;a}$	3.76 ± 0.05 $^{A;a}$
C18:1ω9 cis	21.74 ± 0.24 $^{A;a}$	21.65 ± 0.15 $^{A;a}$	21.58 ± 0.21 $^{A;ab}$	21.44 ± 0.10 $^{A;b}$	21.98 ± 0.17 $^{A;a}$	21.75 ± 0.23 $^{A;ab}$	21.72 ± 0.20 $^{A;a}$	21.62 ± 0.08 $^{A;a}$
C18:2ω6 cis	50.01 ± 0.52 $^{A;a}$	49.47 ± 0.22 $^{A;a}$	49.00 ± 0.13 $^{AB;a}$	46.64 ± 5.30 $^{B;a}$	49.49 ± 0.25 $^{A;a}$	49.39 ± 0.17 $^{A;a}$	49.34 ± 0.14 $^{A;a}$	49.35 ± 0.05 $^{A;a}$
C20:0	0.34 ± 0.01 $^{A;a}$	0.35 ± 0.01 $^{A;b}$	0.34 ± 0.05 $^{A;ab}$	0.33 ± 0.02 $^{A;b}$	0.41 ± 0.01 $^{A;a}$	0.38 ± 0.01 $^{AB;a}$	0.36 ± 0.03 $^{B;ab}$	0.34 ± 0.04 $^{B;a}$
C18:3ω6	0.23 ± 0.03 $^{A;a}$	0.25 ± 0.01 $^{B;a}$	0.24 ± 0.00 $^{B;a}$	0.22 ± 0.00 $^{B;b}$	0.26 ± 0.00 $^{A;a}$	0.25 ± 0.02 $^{AB;a}$	0.24 ± 0.02 $^{AB;a}$	0.23 ± 0.02 $^{B;b}$
C20:1ω9	0.44 ± 0.03 $^{A;a}$	0.42 ± 0.03 $^{AB;a}$	0.38 ± 0.00 $^{AB;a}$	0.36 ± 0.02 $^{B;b}$	0.46 ± 0.02 $^{A;a}$	0.41 ± 0.05 $^{A;a}$	0.39 ± 0.07 $^{A;a}$	0.38 ± 0.06 $^{A;a}$
C18:3ω3	4.95 ± 0.04 $^{A;a}$	4.94 ± 0.07 $^{A;a}$	4.10 ± 0.03 $^{A;ab}$	4.89 ± 0.11 $^{A;a}$	4.86 ± 0.04 $^{A;a}$	4.85 ± 0.10 $^{A;a}$	4.80 ± 0.02 $^{A;a}$	4.74 ± 0.09 $^{A;b}$
C20:3ω6	0.42 ± 0.00 $^{A;b}$	0.39 ± 0.00 $^{AB;bc}$	0.38 ± 0.02 $^{B;b}$	0.36 ± 0.02 $^{B;c}$	0.47 ± 0.01 $^{A;a}$	0.46 ± 0.02 $^{AB;a}$	0.44 ± 0.01 $^{BC;a}$	0.42 ± 0.02 $^{C;a}$
C24:0	0.22 ± 0.00 $^{A;a}$	0.18 ± 0.00 $^{B;ab}$	0.17 ± 0.01 $^{B;a}$	0.17 ± 0.01 $^{B;a}$	0.22 ± 0.00 $^{A;a}$	0.20 ± 0.01 $^{B;a}$	0.19 ± 0.00 $^{B;a}$	0.18 ± 0.02 $^{B;a}$
TFA	95.57 ± 0.25 $^{A;a}$	95.40 ± 0.38 $^{A;a}$	95.25 ± 0.15 $^{A;a}$	94.99 ± 0.01 $^{A;a}$	95.75 ± 0.21 $^{A;a}$	95.46 ± 0.26 $^{A;a}$	95.45 ± 0.29 $^{A;a}$	95.32 ± 0.18 $^{A;a}$
SFA	15.92 ± 3.05 $^{A;ab}$	14.28 ± 0.06 $^{B;a}$	14.26 ± 0.06 $^{B;a}$	13.66 ± 0.72 $^{B;a}$	14.38 ± 0.12 $^{A;bc}$	14.26 ± 0.05 $^{A;a}$	14.24 ± 0.03 $^{A;a}$	14.11 ± 0.06 $^{A;a}$
MUFA	22.10 ± 0.23 $^{A;a}$	22.07 ± 0.08 $^{A;ab}$	22.03 ± 0.15 $^{A;a}$	21.85 ± 0.08 $^{A;b}$	22.37 ± 0.22 $^{A;a}$	22.18 ± 0.22 $^{A;a}$	22.14 ± 0.22 $^{A;ab}$	22.03 ± 0.10 $^{A;a}$
UFA	55.48 ± 0.46 $^{A;a}$	54.96 ± 0.22 $^{AB;a}$	54.56 ± 0.19 $^{AB;a}$	53.40 ± 3.24 $^{B;a}$	55.01 ± 0.34 $^{A;a}$	54.91 ± 0.18 $^{A;a}$	54.87 ± 0.09 $^{A;a}$	54.82 ± 0.20 $^{A;a}$
ω3	4.94 ± 0.04 $^{A;a}$	4.94 ± 0.07 $^{A;a}$	4.90 ± 0.03 $^{A;ab}$	4.89 ± 0.11 $^{A;a}$	4.86 ± 0.04 $^{A;a}$	4.85 ± 0.10 $^{A;a}$	4.80 ± 0.02 $^{A;a}$	4.74 ± 0.09 $^{A;b}$
ω6	50.59 ± 0.49 $^{A;a}$	50.02 ± 0.18 $^{AB;a}$	49.62 ± 0.13 $^{AB;a}$	47.49 ± 4.99 $^{B;a}$	50.16 ± 0.23 $^{A;a}$	50.08 ± 0.13 $^{A;a}$	50.07 ± 0.07 $^{A;a}$	50.05 ± 0.14 $^{A;a}$

Table 4 (continued)

Fatty acids	Day 30				Day 45			
	Control	2 % PP	4% PP	6% PP	Control	2 % PP	4% PP	6% PP
C16:0	9.90 ± 0.04 A;ab	9.79 ± 0.10 A;a	9.90 ± 0.10 A;a	9.81 ± 0.07 A;a	10.02 ± 0.17 A;ab	9.87 ± 0.08 A;a	9.98 ± 0.19 A;a	9.84 ± 0.06 A;a
C18:0	3.78 ± 0.05 A;a	3.74 ± 0.09 A;a	3.76 ± 0.04 A;b	3.75 ± 0.03 A;a	3.68 ± 0.02 A;a	3.64 ± 0.00 A;a	3.66 ± 0.01 A;b	3.63 ± 0.05 A;a
C18:1ω9 cis	22.10 ± 0.66 A;a	21.57 ± 0.11 A;a	21.94 ± 0.42 A;ab	21.74 ± 0.15 A;a	22.31 ± 0.18 A;ab	21.77 ± 0.53 A;ab	22.18 ± 0.14 A;a	21.74 ± 0.63 A;a
C18:2ω6 cis	49.516 ± 0.071 A;a	48.82 ± 0.43 A;a	49.34 ± 0.10 A;a	49.32 ± 0.34 A;a	49.50 ± 0.43 A;a	49.12 ± 0.17 A;a	49.34 ± 0.50 A;a	48.85 ± 0.39 A;a
C20:0	0.37 ± 0.01 A;ab	0.35 ± 0.02 A;ab	0.37 ± 0.01 A;ab	0.35 ± 0.02 A;a	0.35 ± 0.01 A;ab	0.34 ± 0.02 A;a	0.35 ± 0.01 A;b	0.31 ± 0.03 A;b
C18:3ω6	0.26 ± 0.00 A;a	0.24 ± 0.01 A;a	0.25 ± 0.01 A;a	0.24 ± 0.02 A;ab	0.26 ± 0.01 A;a	0.26 ± 0.01 A;a	0.26 ± 0.01 A;a	0.24 ± 0.03 A;ab
C20:1ω9	0.45 ± 0.01 A;a	0.41 ± 0.06 A;a	0.45 ± 0.01 A;a	0.42 ± 0.04 A;ab	0.45 ± 0.01 A;a	0.43 ± 0.01 A;a	0.44 ± 0.01 A;a	0.40 ± 0.05 A;ab
C18:3ω3	4.96 ± 0.03 A;a	4.87 ± 0.06 A;ab	4.96 ± 0.05 A;a	4.88 ± 0.09 A;a	4.99 ± 0.04 A;a	4.96 ± 0.06 A;a	4.98 ± 0.01 A;a	4.95 ± 0.16 A;a
C20:3ω6	0.42 ± 0.01 A;b	0.42 ± 0.00 A;b	0.42 ± 0.01 A;ab	0.42 ± 0.02 A;a	0.40 ± 0.02 A;b	0.38 ± 0.00 A;c	0.40 ± 0.02 A;b	0.38 ± 0.01 A;b
C24:0	0.19 ± 0.00 A;a	0.18 ± 0.01 A;a	0.19 ± 0.02 A;b	0.19 ± 0.00 A;ab	0.19 ± 0.02 A;a	0.17 ± 0.00 A;a	0.18 ± 0.01 A;b	0.16 ± 0.00 A;b
TFA	95.60 ± 0.14 A;a	95.19 ± 0.27 A;a	95.58 ± 0.06 A;a	95.30 ± 0.21 A;a	95.67 ± 0.19 A;a	95.39 ± 0.49 A;a	95.55 ± 0.33 A;a	95.38 ± 0.32 A;a
SFA	14.23 ± 0.05 A;a	14.06 ± 0.19 A;a	14.19 ± 0.13 A;a	14.13 ± 0.12 A;c	14.17 ± 0.22 A;a	14.02 ± 0.06 A;c	14.15 ± 0.18 A;a	14.01 ± 0.10 A;a
MUFA	22.55 ± 0.65 A;a	22.00 ± 0.08 A;a	22.38 ± 0.42 A;ab	22.15 ± 0.11 A;a	22.76 ± 0.16 A;ab	22.22 ± 0.53 A;a	22.61 ± 0.15 A;a	22.14 ± 0.65 A;a
UFA	55.05 ± 0.07 A;a	54.36 ± 0.50 A;a	54.96 ± 0.36 A;a	54.96 ± 0.15 A;a	55.09 ± 0.47 A;a	54.73 ± 0.16 A;a	54.99 ± 0.54 A;a	54.46 ± 0.44 A;a
ω3	4.96 ± 0.03 A;a	4.87 ± 0.06 A;ab	4.96 ± 0.05 A;a	4.88 ± 0.09 A;a	4.99 ± 0.04 A;a	4.96 ± 0.06 A;a	4.98 ± 0.01 A;a	4.95 ± 0.16 A;a
ω6	50.17 ± 0.05 A;a	49.49 ± 0.44 A;a	50.03 ± 0.34 A;a	50.00 ± 0.10 A;a	50.14 ± 0.40 A;a	49.75 ± 0.15 A;a	50.00 ± 0.52 A;a	49.49 ± 0.38 A;a

Table 4 (continued)

Fatty acids	Day 60			
	Control	2 % PP	4% PP	6% PP
C16:0	9.61 ± 0.07 $^{AB;b}$	9.63 ± 0.25 $^{AB;a}$	9.82 ± 0.10 $^{A;a}$	9.36 ± 0.19 $^{B;b}$
C18:0	3.76 ± 0.08 $^{A;a}$	3.80 ± 0.01 $^{A;a}$	3.83 ± 0.55 $^{A;a}$	3.70 ± 0.02 $A^{;a}$
C18:1ω9 cis	21.73 ± 0.69 $^{A;a}$	22.19 ± 0.55 $^{A;a}$	22.78 ± 1.57 $^{A;a}$	20.62 ± 0.13 $^{B;b}$
C18:2ω6 cis	48.55 ± 0.91 $^{A;a}$	49.26 ± 0.29 $^{A;a}$	49.30 ± 0.70 $^{A;a}$	48.35 ± 0.42 $^{A;a}$
C20:0	0.35 ± 0.01 $^{A;b}$	0.36 ± 0.01 $^{A;a}$	0.38 ± 0.00 $^{A;a}$	0.35 ± 0.02 $^{A;ab}$
C18:3ω6	0.25 ± 0.00 $^{A;a}$	0.25 ± 0.01 $^{A;a}$	0.26 ± 0.01 $^{A;a}$	0.25 ± 0.02 $^{A;ab}$
C20:1ω9	0.44 ± 0.02 $^{A;a}$	0.44 ± 0.01 $^{A;a}$	0.45 ± 0.01 $^{A;a}$	0.42 ± 0.06 $^{A;ab}$
C18:3ω3	4.85 ± 0.14 $^{AB;a}$	4.91 ± 0.06 $^{A;a}$	4.93 ± 0.09 $^{A;a}$	4.67 ± 0.26 $^{B;b}$
C20:3ω6	0.42 ± 0.02 $^{A;ab}$	0.42 ± 0.01 $^{A;b}$	0.44 ± 0.02 $^{A;a}$	0.41 ± 0.03 $^{A;b}$
C24:0	0.18 ± 0.02 $^{A;a}$	0.19 ± 0.01 $^{A;a}$	0.19 ± 0.01 $^{A;a}$	0.18 ± 0.00 $^{A;b}$
TFA	95.32 ± 0.28 $^{A;a}$	95.46 ± 0.52 $^{A;a}$	95.73 ± 0.16 $^{A;a}$	95.25 ± 0.32 $^{A;a}$
SFA	13.98 ± 0.05 $^{A;a}$	14.11 ± 0.17 $^{A;a}$	14.22 ± 0.39 $^{A;a}$	13.88 ± 0.24 $^{A;a}$
MUFA	22.19 ± 0.69 $^{A;a}$	22.60 ± 0.52 $^{A;a}$	23.22 ± 1.57 $^{A;a}$	21.06 ± 0.14 $^{B;b}$
UFA	54.07 ± 1.06 $^{A;a}$	54.84 ± 0.27 $^{A;a}$	54.93 ± 0.81 $^{A;a}$	53.69 ± 0.18 $^{A;a}$
ω3	4.85 ± 0.14 $^{AB;a}$	4.91 ± 0.06 $^{A;a}$	4.93 ± 0.09 $^{A;a}$	4.67 ± 0.26 $^{B;b}$
ω6	49.22 ± 0.91 $^{A;a}$	49.93 ± 0.30 $^{A;a}$	50.00 ± 0.72 $^{A;a}$	49.02 ± 0.44 $^{A;a}$

Results are expressed as mean ± standard deviation (n=3). Different capital letters in the same line indicate significant differences among treatments during the same storage time. Different lower letters in the same column indicate significant differences for the same treatment during storage time ($p \leq 0.05$).

3.2.4. β-sitosterol

In the control samples on day 0, the β-sitosterol content of refined soybean oil was 146.59 ± 5.88 mg/100 g oil (Table 3), which was comparable to the values observed in samples containing pink pepper (from 144.06 ± 5.41 to 146.48 ± 5.58 mg/100 g oil). Variable values can be found for commercial soybean oils in the literature: 166.03 mg/100 g oil (Yang et al., 2019), 293.3 mg/100 g oil (Jorge et al., 2018), and 123.9 mg/100 g oil (Verleyen et al., 2002), as β-sitosterol level is likely influenced by factors such as soybean genotype and environmental growing conditions (Vlahakis & Hazebroek, 2000).

After 15 days of storage, lower contents of β-sitosterol were found, indicating its degradation over time. However, samples from the treatment with 2% PP (126.41 ± 8.55 mg/100 g), 4% PP (133.77 ± 5.81 mg/100 g), and 6% PP (138.72 ± 2.89 mg/100 g) maintained significantly higher β-sitosterol levels compared to the control (120.88 ± 6.84 mg/100 g) ($p \leq 0.05$), which reflected in lower percentages of β-sitosterol degradation. A similar trend was observed on day 30, with the control sample exhibiting a more pronounced decline in β-sitosterol content (100.88 ± 7.27 mg/100 g). In contrast, the treatments with pink pepper preserved β-sitosterol more effectively, corresponding to estimated protection rates of 7% (2% PP), 9% (4% PP), and 13% (6% PP) (Table 3).

Statistically significant differences were observed on day 45 when comparing the results from the 4% PP and 6% PP samples with the control and 2% PP samples ($p \leq 0.05$). Therefore, the degradation percentages were as follows: 37.9% (control), 35.6% (2% PP), 24.1% (4% PP), and 24.8% (6% PP) (Figure 3). At the end of the storage period (day 60), no statistically significant differences were detected between the evaluated samples, despite the numerical differences. This suggests the effectiveness of adding pink pepper up to day 45. The lowest β-sitosterol degradation was observed in the 6% PP samples, which presented a content of 73.87 ± 2.78 mg/100 g (49.6% degradation), while control samples had 61.80 ± 2.84 mg/100 g (57.8% degradation).

Phytosterols are sensitive to light and may undergo degradation depending on light intensity (Lu & Zhao, 2017; Zhao et al., 2019), resulting in the formation of products potentially toxic to humans. Zhang et al. (2006) reported a 49% reduction in β-sitosterol content in soybean oil exposed to natural sunlight through a glass window at room temperature over a 30-day period. Submitting a β-sitosterol standard dissolved in dimethyl sulfoxide to an incidence of 30,000 lx for 12 days led to a degradation of 43% (Yang et al., 2020). In addition to intensity, light frequency also has a direct influence in phytosterol degradation. As demonstrated by Yang et al. (2022), frequencies between 365–410 nm induce greater degradation of β-sitosterol

compared to those in the 465–665 nm range, which can be explained by their higher potential to form singlet oxygen, increasing the oxidation rate.

β-sitosterol is the predominant phytosterol found in soybean oil. Its intake is recommended by current dietary guidelines due to its ability to reduce the levels of low-density lipoprotein cholesterol, which is associated with an increased risk of developing coronary heart disease (Barkas et al., 2023). Furthermore, β-sitosterol presents anti-inflammatory properties that may benefit individuals with conditions such as arthritis. Its immune-modulating effects may enhance overall immune system function, while its role in managing benign prostatic hyperplasia contributes to improved urinary flow and prostate health. Moreover, additional biological activities attributed to β-sitosterol include wound-healing effects and potential anti-cancer properties (Chen et al., 2023; Khan et al., 2022; Liu et al., 2024).

Therefore, identifying strategies to mitigate β-sitosterol degradation, while preserving its functional properties and preventing the formation of oxidized compounds, is essential, primarily through the use of natural alternatives such as pink pepper. Natural antioxidants, like grape seed, purslane, green tea, and rosemary extract, have previously been shown to minimize β-sitosterol degradation under thermal conditions (Freitas et al., 2017; Jorge et al., 2018; Kmiecik et al., 2015). However, to the best of our knowledge, this is the first study to address the impact of incorporating a natural source of antioxidants into soybean oil, aiming at investigating its potential to reduce β-sitosterol degradation during storage under natural light exposure

In the present study, the bioactive composition of pink pepper was determined using HPLC-QTOF-MS/MS analysis, which confirmed the presence of phenolic compounds with well-known established antioxidant properties. For instance, agathisflavone was identified as a notable compound due to its structural features. It contains four hydroxyl groups, two of which actively donate hydrogen atoms to neutralize free radicals. The remaining hydroxyl groups, although not directly involved in hydrogen donation, contribute to antioxidant activity by stabilizing free radicals through resonance effects (Andrade et al., 2018).

In addition to phenolic compounds, terpenes were also identified in pink pepper. These compounds can act through free radical scavenging mechanisms or as indirect antioxidants by enhancing the antioxidant status in both enzymatic and non-enzymatic reactions (Gonzalez-Burgos & Gomez-Serranillos, 2012). Moreover, given their nonpolar nature, terpenes may significantly contribute to the oxidative stability of oil matrices, which are themselves predominantly nonpolar.

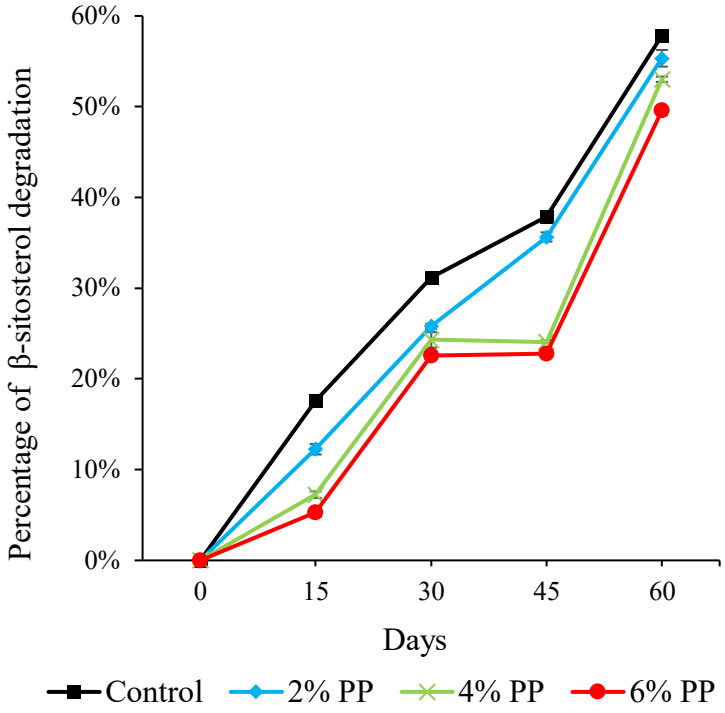

Figure 3: Degradation percentage of β-sitosterol in control (black), 2% PP (blue), 4% PP (green), and 6% PP samples (red).

3.2.5. Principal component analysis

Principal component analysis (PCA) was applied to build linear combinations with the original variables to reduce multidimensional data. As shown in Figure 4a, the first two principal components (Dim 1 and Dim 2) explain 74% of the total variability (41.2% and 33.1%, respectively), which is considered an adequate representation of the dataset's variance.

The heatmap (Figure 4b) corroborates the trends identified through PCA, indicating that the 4% PP and 6% PP treatments were associated with higher levels of PUFAs, ω3, and ω6 fatty acids. For β-sitosterol, its degradation was clearly influenced by storage time, with higher levels observed in samples analyzed on days 0 and 15, followed by a progressive decline over time. The correlogram (Figure 4c) shows that peroxide and TBARS values had a strong positive correlation with the time of storage (0.81 and 0.82, respectively), which may be attributed to the increased formation of oxidation products with prolonged storage. PUFAs exhibited a very strong positive correlation with ω6 fatty acids (0.94), consistent with the high proportion of ω6 in the fatty acid profile of soybean oil. In contrast, β-sitosterol content showed strong negative correlations with both storage time (–0.95) and TBARS values (–0.93).

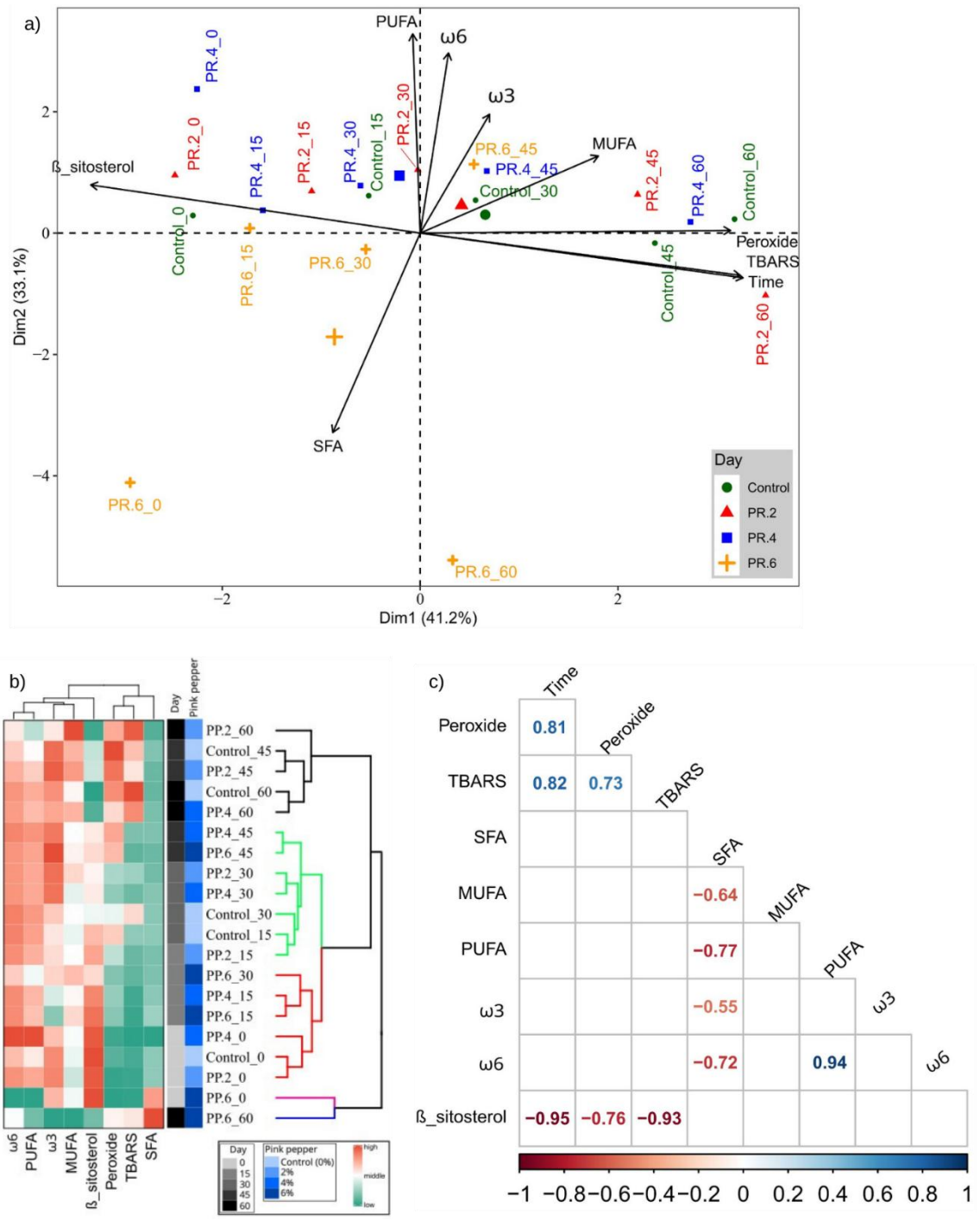

Figure 4: Multivariate analyses. PCA score plot (Dim1xDim2) and biplot for treatments and variables (a). Hierarchical cluster analysis (Heatmap) (b). Correlogram, where negative or positive correlations are represented by red or blue numbers, respectively (c).

4. CONCLUSION

This study demonstrated the negative impact of light exposure on lipid oxidation and β-sitosterol degradation in refined soybean oil. Both PV and TBARS increased over time in oil samples; however, lower values were determined in samples containing 4% PP and 6% PP, indicating the protective effect of pink pepper. HPLC-QTOF-MS/MS analysis identified several bioactive compounds in pink pepper, mainly biflavonoids, phenolic acids, and terpenes, which support its role as a natural antioxidant. The fatty acid levels did not present significant differences among samples during storage, while the degradation of β-sitosterol was evidenced by the lower contents found from day 15 onwards. However, samples treated with 4% PP and 6% PP maintained higher sterol levels until day 45. This highlights the need for further research to better understand the stability and effectiveness of pink pepper's antioxidant action during extended storage. Despite it, promising results were obtained, supporting the use of pink pepper as a natural preservative against lipid oxidation and β-sitosterol degradation in soybean refined oil, contributing to the maintenance of the nutritional quality of such applied and, consequently, consumed product.

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SUPPLEMENTARY MATERIAL (CAPÍTULO I)

Table 1: Mathematical models for peroxide values of soybean oil samples.

Treatment	Model (n)	C_0	k	β11	R^2	Shapiro	RMSE
Control	0	23.927	0.0776	-	0.6748	0.0133	1.1427
	1	29.087	0.0147	-	0.6085	0.1479	1.2578
	2	32.755	-0.0027	-	0.5507	0.4933	1.3570
	3	**17.496**	**0.1633**	**-0.0014**	**0.7469**	**0.4151**	**1.0081**
2% PP	0	1.2448	0.0851	-	0.7848	0.7381	0.9453
	1	1.9039	0.0204	-	0.7219	0.0689	1.0811
	2	2.3737	-0.0044	-	0.6480	0.2935	1.2347
	3	**0.9812**	**0.1202**	**-6,00E-04**	**0.7965**	**0.9978**	**0.9193**
4% PP	0	1.1068	0.0713	-	0.7785	0.6660	0.8068
	1	1.6291	0.0203	-	0.7276	0.0682	0.8991
	2	2.0296	-0.0051	-	0.6558	0.0547	1.0264
	3	**0.9796**	**0.0883**	**-3,00E-04**	**0.7823**	**0.7832**	**0.7998**
6% PP	0	0.9715	0.0661	-	0.7530	0.6108	0.8032
	1	1.4786	0.0204	-	0.6964	0.0658	0.8950
	2	1.8532	-0.0056	-	0.6234	0.0600	1.0119
	3	**0.8114**	**0.0875**	**-4,00E-04**	**0.7598**	**0.7814**	**0.7919**

$C_{0:}$ intercept; $b_{11:}$ quadratic effect of pink pepper concentrations; k: reaction rate (mg. $g^{-1}.h^{-1}$). The models highlighted in bold best fitted the experiments.

Table 2: Mathematical models for TBARS values of soybean oil samples.

Treatment	Model (n)	C_o	k	R^2	Shapiro	RMSE
Control	**0**	**0.7392**	**0.0258**	**0.9698**	**0.4213**	**0.0965**
	1	0.8643	0.0167	0.9472	0.0221	0.1282
	2	0.9740	-0.0101	0.9054	0.6954	0.1739
PP 2%	0	0.5527	0.0252	0.9231	0.4068	0.1545
	1	0.6349	0.0208	0.9884	0.8223	0.0610
	2	**0.7516**	**-0.0149**	**0.9911**	**0.0049**	**0.0538**
PP 4%	0	0.4779	0.0195	0.6857	0.9687	0.2801
	1	0.4734	0.0227	0.8275	0.2801	0.2110
	2	**0.5405**	**-0.0224**	**0.9345**	**0.0672**	**0.1340**
PP 6%	0	0.5566	0.0129	0,6583	0.9717	0.1979
	1	0.5474	0.0161	0,7636	0.4432	0.1659
	2	**0.5668**	**-0.0185**	**0,8678**	**0,12985**	**0,1268**

$C_{0:}$ intercept; $b_{11:}$ quadratic effect of pink pepper concentrations; k: reaction rate (mg. $g^{-1}.h^{-1}$). The models highlighted in bold best fitted the experiments.

CAPÍTULO II

Employment of pink pepper (*Schinus terebinthifolia* Raddi*)* as a strategy to mitigate phytosterol oxidation in soybean oil during light storage

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ABSTRACT

Soybean oil is rich in phytosterols, unsaturated lipids with potential benefits to human health, highlighting its hypocholesterolemic effects. However, due to its unsaturated composition soybean oil is susceptible to oxidative reactions, including photooxidation, which occurs when the product is exposed to visible and ultraviolet radiation, forming toxic phytosterols oxidation products (POCs). Therefore, the effect of adding pink pepper (*Schinus terebinthifolia* Raddi) to refined soybean oil at 2%, 4%, and 6% w/v was evaluated to assess its antioxidant potential against phytosterol oxides formation during 60 days of storage under natural light exposure at room temperature. For this purpose, the quantification of the main phytosterols, β-sitosterol, stigmasterol and campesterol, and their respective oxides (5,6α- and 5,6β-epoxy, 7-keto, 7α- and 7β-hydroxy) was performed by High Performance Liquid Chromatography with Mass Spectrometry, using Atmospheric Pressure Chemical Ionization (HPCL-APCI-MS). Structural identification was supported by High-Performance Liquid Chromatography coupled with Quadrupole Time-of-Flight Mass Spectrometry (HPLC-QTOF-MS/MS). Method validation was carried out by evaluating the linearity, precision, recovery, and the limits of detection and quantification using commercial standards of β-sitosterol, campesterol, stigmasterol, and 7-ketocholesterol, demonstrating the robustness of the analytical procedure. In addition, phytosterol oxides were prepared in the laboratory through thermo-oxidation of phytosterol standards, and their structures were confirmed by high-resolution chromatography. After 60 days, the three phytosterols have undergone degradation, with a 56% reduction in total phytosterol content and the formation of 52.88 μg/g of oxides in the control sample. Although all samples suffered some degree of phytosterol degradation and formation of oxides, those with pink pepper showed significantly reduced oxidation, demonstrating greater preservation of phytosterols and lower production of oxides. The sample with 6% pink pepper demonstrated 28% greater retention of total phytosterols and a 51% reduction in oxide formation compared to the control. The results indicate the potential of pink pepper as a natural antioxidant in refined soybean oil, capable of mitigating nutritional losses by protecting phytosterols from degradation. As a result, it contributes to maintaining the overall quality of the product during storage under light exposure.

Keywords: Methodology validation, phytosterol oxidation products, phytosterol photooxidation

1. INTRODUCTION

Soybean oil, the second most consumed vegetable oil in the world (USDA, 2025), is rich in phytosterols, presenting β-sitosterol as the predominant one. Phytosterols are secondary plant metabolites structurally similar to cholesterol, playing an important role in human health as they reduce cholesterol absorption in the intestine (Wang et al., 2024). In addition to their hypocholesterolemic effect, they have anti-inflammatory, hepatoprotective, cardioprotective, and antidiabetic properties (Khan et al., 2022), and also act as anticancer and immunomodulatory agents (Bakrim et al., 2022; Nazir et al., 2023).

However, phytosterol molecules have the same core structure as cholesterol, characterized by a four-ring steroid nucleus with a methyl or an ethyl group at carbon 24. The main phytosterols have a double bond between carbons 5 and 6 in the ring system, and an additional double bond at carbon 22 in the side chain. These double bonds make phytosterols particularly susceptible to oxidation, which leads to the formation of phytosterol oxidation products (POPs) (Furse et al., 2023; Poudel et al., 2022).

Studies have demonstrated that the ingestion of phytosterol oxidized products have deleterious effects similar to those of cholesterol oxides, as they may cause damage to the human body, including reduced cell viability, induction of apoptosis, cytotoxicity, and hepatic lipid disorders (Feng et al., 2022; Wang et al., 2021). Among these compounds, 7-ketosterol, one of the main oxides formed during oxidation, triggers inflammatory responses and alters immune-related proteins, which may cause negative changes in nutrient absorption (Laparra et al., 2015; Yan; Cao, 2024). Due to the adverse effects of these oxidation products, their identification and quantification are considered highly important for public health.

Oxidative reactions in oils are caused by factors such as light, oxygen, temperature, and pro-oxidant compounds, leading to changes in the product and the formation of various oxidation products, including phytosterol oxides. The main mechanisms of oil oxidation during shelf life are autooxidation and photooxidation. Photooxidation occurs in the presence of light, oxygen, and a photosensitizing compound. In soybean oil, chlorophyll acts as an important photosensitizer, also known as a chromophore.

Photosensitizer molecules absorb energy from light or ultraviolet radiation, entering an excited state. In this excited state, the photosensitizer transfers energy to triplet oxygen, enabling it to react directly with the double bonds present in phytosterols; this reaction mechanism is called Type II photooxidation (Min & Boff, 2002). The Type II mechanism generally occurs in lipid matrices, and the dissolved oxygen in vegetable oils facilitates this reaction (Choe & Min, 2006). The attack of singlet oxygen on the double bond located between

carbons 5 and 6 of the steroid nucleus generates several oxidation products, mainly 7-keto, 5,6-epoxy, and 7-hydroxy derivatives (Poudel et al., 2022; Scholz et al., 2015).

Due to the susceptibility of vegetable oils to oxidation, the industry commonly adds synthetic antioxidants to mitigate these reactions and extend shelf life. In vegetable oils, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ) are the most widely used synthetic antioxidants due to their high efficacy and low cost. Consequently, they are also found in various other industrialized products (Ham et al., 2019). However, studies have shown that the ingestion of these compounds and their metabolites can be harmful to human health, as they can bioaccumulate in the body and persist in the environment, contributing to soil and water contamination (Li et al., 2019; Wang et al., 2016; Xu et al., 2021).

To avoid the use of synthetic compounds, plant materials have been widely applied and evaluated as potential antioxidant substitutes in vegetable oils (Abdo, Shaltout & Mansour, 2023; Tinello & Lante, 2020; Umeda & Jorge, 2021). Pink pepper is the fruit of the aroeira tree (*Schinus terebinthifolia*), native to South America and abundant in several regions of Brazil. The fruits are pink or reddish when ripe and have a pleasant aroma and flavor. Therefore, they are popularly used to season foods. Pink pepper is rich in bioactive compounds such as phenolic acids and flavonoids, (Vieira et al., 2023), exhibiting antioxidant, anti-inflammatory, and antimicrobial properties (Feriani et al., 2021; Gomes et al., 2020).

The POPs face several challenges: the lack of commercial standards for POPs, the large number of POPs with very similar structures, and their presence only in trace amounts in food samples (Gachumi et al., 2021). Therefore, the isolation, separation, characterization, and quantification of these compounds are highly dependent on the analytical methods employed (Menéndez-Carreño, Knol & Janssen, 2016). Given these challenges, it is essential to develop methodologies that enable efficient separation, identification, and quantification of phytosterol oxides.

Considering the antioxidant potential demonstrated by pink pepper in various food systems (Barreira et al., 2023; Bittencourt Fagundes et al., 2020; Menegali et al., 2020b), the aim of this study was to evaluate the effect of adding pink pepper (2%, 4%, and 6% w/v) to commercial refined soybean oil and to investigate its protective effects against phytosterol oxidation under natural light exposure. The samples were stored for 60 days at room temperature (25°C ± 1°C) under typical domestic conditions. Additionally, a methodology for the identification and quantification of phytosterols and their oxides using HPLC-APCI-MS was developed and validated, with structural confirmation by HPLC-QTOF-MS.

MATERIALS AND METHODS

2.1. Chemicals and reagents

The standards β-sitosterol, stigmasterol, campesterol, 7-ketocholesterol (7-Keto), 7β-hydroxycholesterol (7β-OH), 5,6α-epoxycholesterol (5,6α-Ep), and 5,6β-epoxycholesterol (5,6β-Ep) were purchased from Sigma-Aldrich (St. Luis, MO, USA). The purities of these standards ranged from 95 to 99%. HPLC grade solvents (hexane, chloroform, ethyl acetate, and 2-propanol) were purchased from Sigma-Aldrich Brazil (São Paulo, SP, Brazil). All other analytical grade reagents were obtained from Merck Brazil (Rio de Janeiro, RJ, Brazil). Commercially refined soybean oils were obtained from a local market (Seropédica, Rio de Janeiro, Brazil), in February 2024, close to the date of manufacture. All other chemicals were achieved from Vetec (São Paulo, Brazil).

2.2. Samples

2.2.1. Pink pepper (*Schinus terebinthifolia* Raddi) samples

Pink pepper samples were collected from the Ademar Moreira Settlement agroecological cooperative, located in São Pedro da Aldeia, Rio de Janeiro, Brazil (latitude 22° 43' 18.343" S; longitude 42° 6' 52.049" W). After selection considering their visual quality aspects, the fruits were dried in a ventilated oven (SOLAB, São Paulo, Brazil) (40 ºC/24 h) to remove moisture excess. The amount required for the experiment was stored at -18 °C, in the absence of light.

2.2.2. Oil samples preparation and experimental conditions

The commercial refined soybean oil was bottled in transparent glass jars. Aliquots of 200 mL were distributed to the recipients according to the following treatments: oil without the addition of pink pepper (control) and oil with pink pepper at 2, 4, and 6% w/v (2% PP, 4% PP, and 6% PP, respectively). Pink pepper was used in its whole form. The fruits were added to the jars containing the oil and manually homogenized by shaking the jars. The pepper concentrations used were established based on preliminary tests that considered the visual aspect of the mixture, simulating home preparation. Four samples (jars containing the oil) were prepared for each treatment, which were randomly placed on a laboratory bench in Seropédica, Rio de Janeiro (latitude 22° 46' 37.316" S; longitude 43° 41' 9.416" W). Therefore, the samples were exposed to ambient light at room temperature (25°C ± 1 °C) and randomly repositioned every 3 days, simulating a domestic environment, for 60 days. The average daily light exposure was 12 hours, from February 27th to April 27th, 2024.

The samples from each treatment were taken for analysis on days 0, 15, 30, 45, and 60. The contents from four jars were filtered to separate pink pepper fruits, and the oil was transferred and homogenized in another recipient. Therefore, a single sample was obtained for each treatment, and appropriate aliquots were taken for analyses.

2.2.3. Preparation of sterol oxidation products

Since there is no commercial standard for phytosterol oxides, the oxides were produced by thermo-oxidation of standards, according to Conchillo et al. (2005), with modifications. 1 mL of each standard (β-sitosterol, campesterol and stigmasterol) was placed in open glass petri dishes 5 cm in diameter, in a static oven at 180°C for 2 hours. In order to reduce steps number and analytical costs, we choose not to use the extracts purification step, and the sterols are directly analyzed in HPLC-APCI-MS and HPLC-QTOF-MS/MS.

2.3 Pink pepper analysis

A methanolic extract (methanol/water, 80:20, v/v) of pink pepper was prepared for analyses, as reported by Oliveira et al. (2020a).

2.3.1. Bioactive compounds from pink pepper extract determined by HPLC-QTOF-MS/MS

Bioactive compounds were identified by high-resolution chromatography analysis on an HPLC instrument (Agilent 1290 Infinity II LC) configured with an Agilent 6530 quadrupole time-of-flight (QTOF) mass spectrometer (Agilent, Santa Clara, CA, USA) equipped with a C18 column (50 mm × 2.1 mm × 1.8 µm, Zorbax extension), maintained at 40 ºC. The mobile phase was an aqueous solution containing 0.1% formic acid and 5.0 mM ammonium formate (A), and a methanol solution containing 0.1% formic acid and 5 mM ammonium formate (B), the flow rate was 0.35 mL/min, and the sample injection volume was 10 µL. The gradient elution program started up to 1 min with 35% B, then 35–80% B (1–20 min), 80–85% B (20–25 min), 85–90% B (25–30 min), 90–95% B (30–35 min), 95–100% B (35–38 min), and 100% B for 7 min, followed by a column equilibration for 5 min. The mass spectra were acquired in positive and negative modes using electrospray ionization (ESI) source with the following operating conditions: dry gas (N_2) at 12 L/min; dry temperature at 300°C; nebulizer at 38 psi; capillary voltage at 3500 V; fragmentor voltage at 120 V. The analysis was carried out in the MS full scan mode monitored in the mass range of 100 to 800 *m/z*. Subsequently, an MS/MS experiment was performed and the parameters set as follows: MS spectrum acquisition rates of

4 spectra/s and collision energies of 10, 20, 40, and 60 V. All operations, data acquisition, and analysis were performed in Mass-Hunter Acquisition software version 11.0.

2.4. Phytosterols and phytosterol oxides analyses

2.4.1 Cold saponification

Phytosterols were extracted by direct cold saponification, according to Saldanha et al. (2006), with slightly modifications. 150 µg of oil sample with 10 mL of a 1M ethanolic solution of KOH, were submitted to continuous agitation at room temperature (23–25 ◦C) for 22 h in the dark. Then, the unsaponifiable matter was extracted 4 times with hexane (10 mL), and then hexane was distilled off at 35°C. The unsaponifiable fraction containing the sterols were dissolved with 1 mL de hexane, filtered (0.45 µM, PVDF, Millipore) and stored in glass vials at -20 ºC until chromatographic analyses.

2.4.2. High performance liquid chromatography with atmospheric pressure chemical ionization (HPLC-APCI-MS)

Prominence-Shimadzu equipment (Kyoto, Japan) with an LC-20AD pump, SPD-M20A detector, CTO-20A oven, SIL-10AF autosampler, CBM-20A controller and LabSolution software, with Atmospheric Pressure Chemical Ionization (APCI) in the positive ion mode and selective ion monitoring (SIM) mode was applied, according to de Oliveira et al. (2020), The full scan mode of MS was monitored in the mass range of 100 to 800 m/z. The column used to identify phytosterols and oxides were CN Hyperchrome (250 mm × 4,3mm × 5,0 µm) (Phenomenex, Colorado, USA), the total time was 20 minutes with an isocratic elution with 97% hexane and 3% 2-propanol, and flow speed 1 mL. min^{-1}. Oven temperature was 32ºC and injection volume of 10 µL. Compounds were determined by comparing the retention times of peaks in the samples with cholesterol oxides reference standards, quantification was performed by external standardization using calibration curves of b-sitosterol, campesterol, stigmasterol, and 7-ketocholesterol oxide standards.

2.4.3. High-performance liquid chromatography with quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS/MS) analysis

High-resolution chromatography analysis was performed on an HPLC-QTOF-MS/MS instrument (Agilent 1290 Infinity II LC) configured with an Agilent 6530 quadrupole time-of-flight (QTOF) mass spectrometer (Agilent, Santa Clara, CA, USA) equipped with a column CN Hyperchrome (250 mm × 4,3mm × 5,0 µm) (Phenomenex, Colorado, USA). Samples (10 µL)

were tested at 40 °C with a flow rate of 0.35 ml.mL -1. The mobile phase was a n-hexane: propanol solution (97:3). The wavelength at the PDA was 254 and 360 nm. MS experiments were performed with an electron spray ionization (ESI) source in positive or negative mode with the following operating conditions: gas flow rate (N_2) at 12 $L.min^{-1}$; gas temperature at 300°C; nebulizer at 38 psi; capillary voltage at 3500 V; fragmentor voltage at 120 V. The full scan mode of MS was monitored in the mass range of 100 to 800 m/z. Subsequently, an MS/MS experiment was performed and the parameters set as follows: MS spectrum acquisition rates of 4 spectra/s and inclusion energies of 10, 20, 40 and 60 V. All operations, data acquisition and analysis were performed in Mass-hunter Acquisition software.

2.5. Method validation

Linearity, limit of detection and quantification, precision (within and between-day), and recovery, was the criteria according to the International Conference on Harmonization guidance for the Validation of Analytic Methods (2005). Standards β-sitosterol, stigmasterol, campesterol, 7-ketocholesterol (7-Keto) were used for validation purposes. 7-ketocholesterol was used as the standard for analytical method development, since phytosterol oxides are not commercially available (Menéndez-Carreño; Knol; Janssen, 2016).

Limit of detection (LOD) and limit of quantification (LOQ) were determined using the standard of the response method, with Equations 1 and 2.

$$LOD = (3.3\sigma)/S \quad (1)$$

$$LOQ = (10\sigma)/S \quad (2)$$

where σ is the standard deviation of the blank (control samples) and S is the slope of the calibration curve.

Standard deviation of the blank was calculated by analyzing the background (noise) of soybean oil samples. Extraction recoveries (%) were determined by comparing the absolute response of the analytes spiked in control samples before and after the extraction procedure, using eighteen determinations (three concentrations/six replicates each) of the compound. Method precision (within and between-day) was determined as the relative standard deviation (%RSD) with three replicates and ten concentrations, on the same analyst, for three days.

2.6. Statistical analysis

All tests were done in triplicate and data are expressed as mean ± standard deviation. ANOVA ($p < 0.05$) and Tukey's posteriori test were used to verify significant differences

among the results ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Pink pepper

3.1.1. Bioactive compounds from pink pepper extract

The main bioactive compounds found in pink pepper extract are shown in Table 1: methyl digallate (*m/z* 335.0391), tetrahydroamentoflavone (*m/z* 541.1112), triterpene acid (*m/z* 469.3323), and masticadienoic acid (*m/z* 453.3351). These identifications are in agreement with the literature. A study carried out by Silva et al. (2024) determined methyl digallate as the main component of pink pepper leaves, where masticadienoic acid was also detected. Tetrahydroamentoflavone, the triterpene and masticadienoic acid were previously identified in pink pepper fruits (Barreira et al., 2023; Gomes et al., 2020).

Table 1: Main bioactive compounds of pink pepper extract analyzed by HPLC-QTOF-MS/MS.

RT (min)	Ion [M-H] (*m/z*)	Formula	Identification	References
0.574	335.0391	$C_{15}H_{12}O_9$	Methyl digallate	Carneiro et al. (2024) Gomes et al. (2020)
8.82	541.1112	$C_{30}H_{22}O_{10}$	Tetrahydroamentoflavone	Gomes et al. (2020)
11.71	469.3323	$C_{30}H_{45}O_4$	Triterpene acid	Barreira et al. (2023) Gomes et al. (2020)
14.51	453.3351	$C_{30}H_{45}O_3$	Masticadienoic acid	Carneiro et al. (2024) Gomes et al. (2020

RT = retention time.

These compounds determined in pink pepper have potential health benefits, including antioxidant, antimicrobial, and anti-inflammatory properties (Gomes et al., 2020; Tang et al., 2020; Xiong et al., 2021). However, as pink pepper fruits were incorporated into the oil, it would be necessary to study the migration of these bioactive compounds to it under the conditions studied.

As previous study by Barreira et al. (2023) showed the antioxidant potential of pink pepper fruits when they were added to the liquid medium (soybean oil) of canned sardines (*Sardina pilchardus*). Therefore, the authors investigated the migration of bioactive compounds from pink pepper to the liquid medium, revealing that among the compounds identified in the fruit only masticadienoic acid was also found in the soybean oil, indicating its migration.

3.2. HPLC-APCI-MS and HPLC-QTOF-MS/MS analysis to separation and identification of phytosterols and phytosterol oxidation products (POPs)

3.2.1 Separation and identification by HPLC-APCI-MS

High performance liquid chromatography (HPLC) with atmospheric pressure chemical ionization (APCI-MS) was used to separate and identify the phytosterols and oxides (Figure 1). The chromatographic conditions for compound separation were based on the method described by Saldanha et al. (2006), as described in Sections 2.4.2 and 2.4.3. Each phytosterol was individually injected, and cholesterol oxide standards were used to determine the elution order. For compound identification, a full scan mode (200–800 m/z) was initially employed. The same sample was then injected three times in Selected Ion Monitoring (SIM) mode to identify each phytosterol and its corresponding oxide individually, as detailed in Table 2.

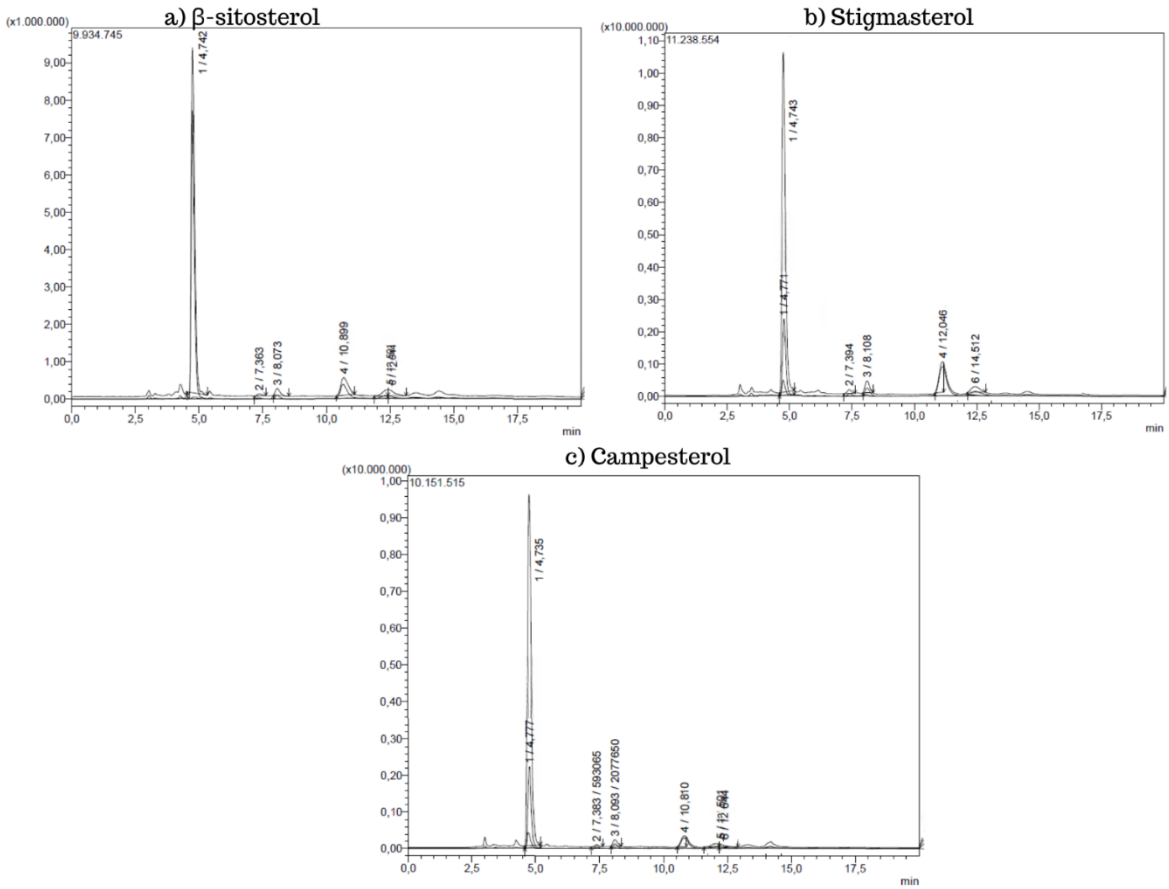

Figure 1: HPLC-APCI-MS chromatograms of thermo-oxidized standards β-sitosterol (a), stigmasterol (b), and campesterol (c).

Data on the fragmentation of phytosterols and phytosterol oxides were obtained by comparison with commercial standards and fragmentation patterns reported in the literature (Calaminici et al., 2024; Grün & Besseau, 2016; Menéndez-Carreño, Knol & Janssen, 2016).

A total of 15 POPs were obtained and identified from the thermo-oxidation of phytosterol standards. The spectrometric fragmentation data (*m/z*), retention times, and elution order of the main phytosterols and the synthesized POPs are summarized in Table 2.

The development of the preparative method was based on the study by Saldanha et al. (2006), who demonstrated that normal-phase HPLC using n-hexane/2-propanol (97:3, v/v) was effective in separating cholesterol oxides within a 30-minute run time. Due to the molecular and geometric similarities between cholesterol and phytosterol, particularly in terms of analyte polarity, the same chromatographic column and parameters were applicable for the analysis of phytosterols and their oxides. Each phytosterol and its respective oxides were identified based on their distinct ion masses, primarily through the formation of $[M+H–H_2O]^+$ ions, which result from in-source dehydration during ionization (Grün & Besseau, 2016; Saldanha et al., 2006), as shown in Table 2.

By performing a 20-minute run for each of the analyzed phytosterols, it was possible to identify the main compounds based on their mass and the respective oxides by analyzing retention times and fragment percentages, and comparing them with values reported in the literature. The phytosterols β-sitosterol (397 $[M+H-H_2O]^+$), campesterol (383 $[M+H-H_2O]^+$), and stigmasterol (395 $[M+H-H_2O]^+$) were eluted at similar retention times, approximately 4.3 minutes.

The respective oxides were identified based on their mass and fragmentation patterns. The first oxide to elute after the phytosterols was 5,6α-epoxide, appearing between 6.8 and 7.0 minutes. It shares the same molecular mass as 5,6β-epoxide, but the two were distinguished by differences in ion distribution and retention time. The 7-keto oxides eluted between 10.7 and 11.1 minutes and were easily detected due to their characteristic $[M+H]^+$ ion, which shows 100% relative intensity. The 7α- and 7β-hydroxy oxides exhibited similar retention times; however, they were identified based on their ion distribution patterns, in comparison with literature data. Both eluted around 12 minutes, with an approximate difference of 0.1 minute between the oxides, with 7β- eluting earlier than 7α.

Table 2: Retention time and mass spectral ions (m/z) of phytosterols and phytosterol oxides by HPLC-APCI-MS and HPLC-QTOF-MS/MS methods.

			HPLC-APCI-MS íon (quantitative)			HPLC-QTOF-MS/MS ions (qualitative)
Compound	**TR (min)**	**ÍON**	**M+H**	**M+H-H_2O**	**M+H-2H_2O**	
Campesterol	4.393	400	-	**383**	-	383.3747; 399.3630
5,6α-epoxycampesterol	6.980	-	-	**399**	381	399.3624; 381.3524; 417.3733
5,6β- epoxycampesterol	7.863	-	-	**399**	381	399.3615; 381.3534; 355.3015
7-ketocampesterol	11.047	-	**415**	397	379*	415.3573; 427.3564; 409.3510
7β- hydroxycampesterol	12.697	-	-	399	**381**; 255*	381.3506; 399.3613; 411.3697
7α-hydroxycampesterol	12.714	-	-	399	**381**; 255*	399.3624; 381.3523; 391.2832; 369.3523
Stigmasterol	4.393	412	413	**395**	-	395.3656; 409.3477; 393.3506
5,6α-epoxystigmasterol	6.987	428	429	**411**	393>	411.3651; 409.3495; 412.3685
5,6β- epoxystigmasterol	7.375	428	429	**411**	393	411.3672; 409.3505; 407.3341
7-ketostigmasterol	10.866	426	**427**	409	391	427.388; 409.3484
7β- hydroxystigmasterol	12.064	428	429	**411**	393>	411.3675; 409.3503; 391.2901
7α- hydroxystigmasterol	12.114	428	429	**411**	393	411.3670; 409.3495; 391.2881
B-sitosterol	4.403	414	415	**397**	-	397.3935; 413.3795
5,6α-epoxysitosterol	6.887	-	-	**413**	395	413.3792; 429.3734; 391.2783
5,6β-epoxysitosterol	7.604	-	-	**413**	395	413.3883; 445.3689; 427.3585
7-ketositosterol	10.721	428	**429**	411	393	429.3806; 411.3649
7β- hydroxysitosterol	12.405	-	-	413	**395**	395.3683; 413.3768; 429.3737
7α-hydroxysitosterol	12.555	-	-	413	**395**	395.3690; 413.3788; 429.3727

3.2.2. Structures confirmation by HPLC-QTOF-MS/MS

High-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS/MS) was used to confirm the identity of each thermo-oxidized standard. In Figure 2, peaks 1a, 1b, and 1c correspond to the phytosterols β-sitosterol, stigmasterol, and campesterol, respectively. Peak 2 represents the 5,6α-epoxide, eluting between approximately 6.9 and 7.1 minutes, while peak 3 corresponds to the 5,6β-epoxide, eluting between 7.6 and 7.9 minutes. Peak 4 represents 7-keto, detected between 10.6 and 11.0 minutes. Peaks 5 and 6 correspond to the 7α- and 7β-hydroxy oxides, respectively, both eluting around 12 minutes, with some variations among the different phytosterols. The mass spectra for each peak are provided in the supplementary material.

The retention times obtained by HPLC-QTOF-MS/MS were identical to those observed in HPLC-APCI-MS, as both methods employed the same chromatographic column. The structures of phytosterols and their respective oxides were confirmed by mass spectrometry and ion fragmentation, as presented in the sixth column of Table 2.

High-resolution chromatography allows the identification of ions and their fragments with accuracy up to four decimal places, due to the high sensitivity of the method, even when analytes were present in trace amounts. It is an analytical technique used to isolate various compounds by combining the efficient ion fragmentation capability of quadrupole technology with the rapid analysis and high mass resolution of time-of-flight spectrometry (Ahmed et al., 2023). The same structures found by HPLC-APCI-MS were confirmed by HPLC-QTOF-MS/MS, at the same retention times and ion mass.

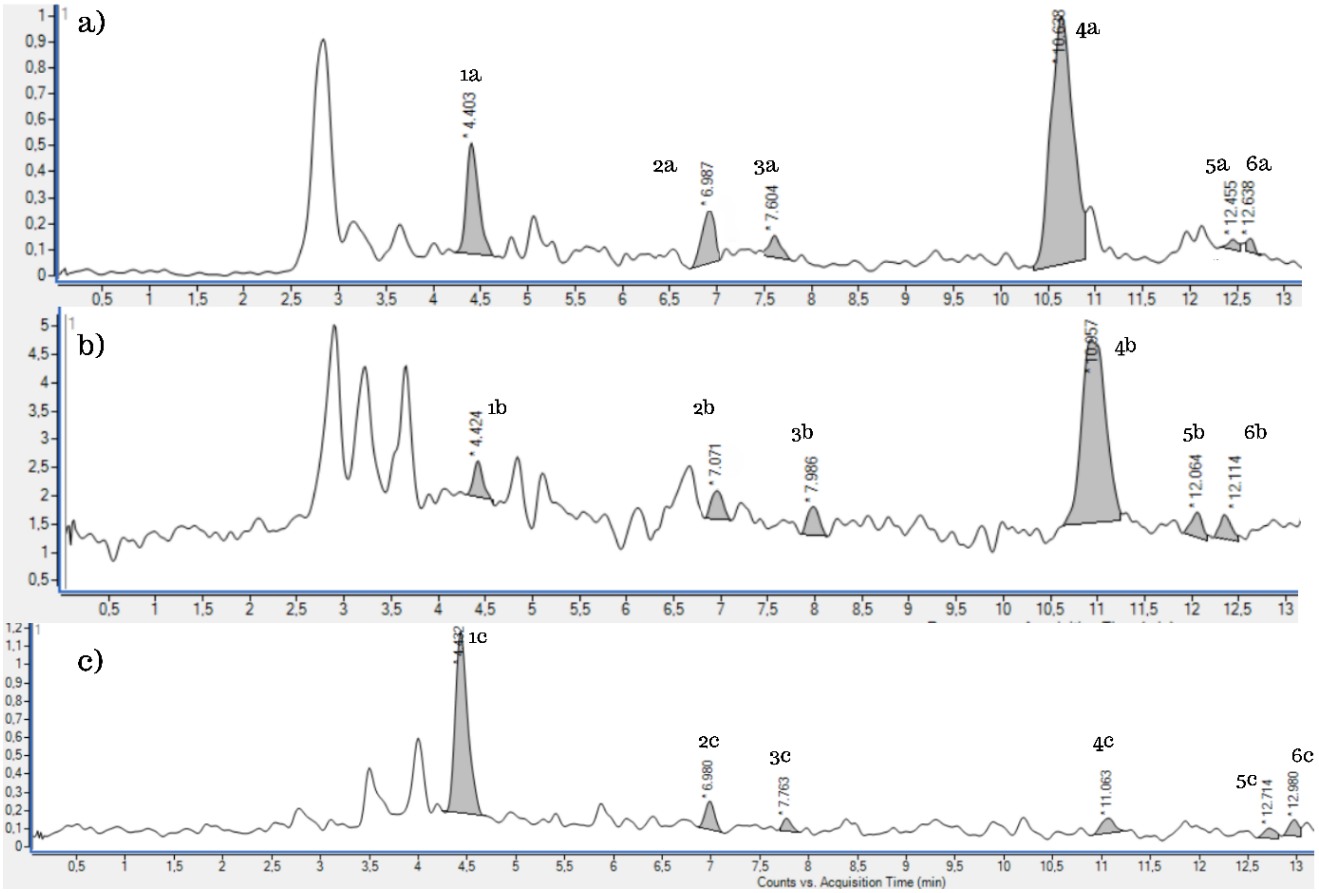

Figure 2: Chromatograms of the thermo-oxidized standards using HPLC-QTOF-MS/MS. β-sitosterol (a), stigmasterol (b), and campesterol (c) peaks.

3.3. Method validation

3.3.1. Linearity, limits of detection and quantification

Linearity was assessed through calibration curves for β-sitosterol, stigmasterol, campesterol, and 7-ketocholesterol ($R^2 > 0.99$), based on triplicate measurements at eight different concentrations (Table 2). All analytes exhibited high linearity, with R^2 values of 0.999. The concentration ranges used were 0.002–0.200 mg/mL for β-sitosterol, 0.001–0.120 mg/mL for campesterol, 0.001–0.120 mg/mL for stigmasterol, and 0.001–0.100 mg/mL for 7-ketocholesterol. Angular coefficients were also evaluated, with variability maintained below 10%, meeting the acceptance criteria. The experimental limits of detection (LOD) and quantification (LOQ) are presented in Table 3. Similarly, Calaminici et al. (2024) reported high linearity and angular coefficients during the validation of an HPLC-HRMS method for phytosterol oxide quantification. The LOD and LOQ values obtained in this study indicate higher sensitivity for HPLC-APCI-MS when compared to values reported in the literature for HPLC-APPI-MS and GC-MS methods (Grün & Besseau, 2016; Menéndez-Carreño, Knol &

Janssen, 2016).

Table 3: Linearity, limit of detection (LOD), and limit of quantification (LOQ) of phytosterols and 7-ketocholesterol standards.

Standards	Linearity		LOD (mg/kg)	LOQ (mg/kg)
	Linear equation	R^2		
β-sitosterol	y = 5E+08x + 129736	0.9999	1.67E-05	5.55E-05
Stigmasterol	y = 3E+08x + 87331	0.9973	1.54232E-05	5.14105E-05
Campesterol	y = 5E+08x + 5E+06	0.9924	1.98681E-05	6.62269E-05
7-ketocholesterol	y = 6E+08x + 3E+06	0.9921	6.31774E-06	2.10591E-05

3.3.2. Recovery and precision

Recovery of phytosterols and their oxidation products was determined by adding representative amounts of the standards to the soybean oil sample (Table 4), along with analysis of a blank sample to calculate the recovery for each addition. The recovery percentages ranged from 94.2% to 102.0%. The recovery was performed with three levels of addition of each standard to the refined soybean oil sample, in triplicate, at concentrations ranging from 0.075 to 0.350 mg, while 7-ketocholesterol was added at concentrations between 0.050 and 0.200 mg.

Menéndez-Carreño et al. (2016) reported similar recovery values during the validation of a method for quantifying phytosterols and phytosterol oxides in various food matrices using gas chromatography coupled with mass spectrometry (GC-MS). The authors conducted recovery experiments in vegetable, meat, fish, egg, and bakery product matrices, using three different addition levels for each phytosterol, ranging from 1.2 to 100 g/kg for campesterol, stigmasterol, and β-sitosterol. Recovery values ranged from 90.3% to 110.5% for these three phytosterols. In addition, cholesterol oxides were also evaluated, with 7-ketocholesterol added at concentrations of 2, 20, and 200 mg/kg, resulting in recoveries of 85.6%, 72.6%, and 73.2%, respectively.

Table 4: Recovery of phytosterols and 7-ketocholesterol from refined soybean sample.

Phytosterols and oxide	Spiked amount (µg)	Recovery (%)
β-sitosterol	75	95.5
	200	102.0
	350	96.3
Stigmasterol	75	98.6
	200	101.4
	350	95.4
Campesterol	75	95.5
	200	94.3
	350	94.2
7-Ketocholesterol	50	95.1
	100	95.8
	200	98.5

Concentration values and precision, expressed as relative standard deviation (RSD) for both between-day and within-day analyses, representing intermediate precision and repeatability, respectively, are presented in Table 5. The results ranged from 4.1% to 9.0%, Menéndez-Carreño et al. (2016) also evaluated concentration, between-day and within-day in their validation experiment. For concentration, the authors found different values due to the different matrix evaluated. However, for precision, the values found (less than 10%), an acceptable value for applying the evaluated method.

Table 5: Concentration, between- and within-day precision of phytosterols and 7-ketocholesterol in refined soybean oil sample.

Sterol	Concentration (mg/100 g oil)	Between-day RSD (%)	Within-day RSD (%)
β-sitosterol	146.6	6.1	9.0
Stigmasterol	59.16	5.1	7.6
Campesterol	28.28	4.1	9.3
Oxide	**Concentration (µg/100 g oil)**	**Between-day RSD (%)**	**Within-day RSD (%)**
7-Ketocholesterol	1.65	4.4	6.3

Thus, the validated methodology for the detection of phytosterols and their respective oxides by HPLC-APCI-MS proved to be robust, as demonstrated by the satisfactory results for linearity, recovery, precision, and detection limits. These parameters confirm the method's reliability for the identification and quantification of phytosterols and their oxidation products.

3.3. Soybean oil analyzes

3.4.1. Phytosterols

The quantification of phytosterols and their respective oxides was performed by HPLC-APCI-MS, based on the identification of mass spectra previously evaluated through high-resolution chromatography, which provided both ions profiles and retention times. Identification was also confirmed by retention time using the same chromatographic column. Three different methods were created in SIM mode, one for each major phytosterol group and their respective oxides. Each sample was injected in triplicate for each method, totaling 9 injections per sample.

β-sitosterol, stigmasterol, and, campesterol in soybean oil were analyzed by HPLC-APCI-MS throughout the 60 days of storage (Table 6). On day zero, β-sitosterol concentrations ranged from 144.06 ± 5.4 to 146.47 ± 5.58 ($p > 0.05$). Similarly, stigmasterol and campesterol showed no significant differences between samples, with concentrations of 59.16 ± 1.05 and 28.28 ± 1.13 mg/100 g of oil, respectively.

Different values have been reported in the literature, reflecting variability due to soybean variety, geographic origin, cultivation practices, post-harvest treatment, and oil processing methods (Bai, Ma & Chen, 2021). In raw soybean oil, β-sitosterol levels have been reported to range from 125–236 mg/100, stigmasterol from 47 to 77 mg/100 g, and campesterol from 62 to 131 mg/100 g, depending on the soybean genotype (Vlahakis & Hazebroek, 2000). In refined oils, differences are also observed based on the refining process. Chemical refining, commonly used in the food industry, results in lower phytosterol content compared to physical refining. For chemically refined soybean oil, Verleyen et al. (2002) reported 123.9 mg/100 g of β-sitosterol, 35.5 mg/100 g of stigmasterol, and 41.1 mg/100 g of campesterol. Conversely, Jorge et al. (2018) observed higher values in refined soybean oil, with 293.3 mg/100 g of β-sitosterol, 13.8 mg/100 g of stigmasterol, and 14.8 mg/100 g of campesterol. These variations highlight the influence of multiple factors on phytosterol composition in soybean oils.

All phytosterols showed a progressive decreased throughout the storage period. After 60 days, β-sitosterol concentrations ranged from 61.79 ± 2.84 (control) and 73.87 ± 2.78 mg/100 g (6% PP). The degradation of β-sitosterol reached 57.9% in the control, while the 6% PP sample showed a lower degradation percentage of 49.5% (Fig. 3a). For stigmasterol, the degradation percentages after 60 days were 54.5% (control), 52.8% (2% PP), 47.4% (4% PP), and 32.1% (6% PP), with statistically significant differences observed between the control and 6% PP samples (Fig. 3b). Likewise, campesterol showed a reduction in its content. The control

sample exhibited a 50.8% reduction, whereas the 6% PP sample showed a significantly lower degradation of 24.2% by the end of the storage period (Fig. 3c).

Kmiecik et al. (2021) evaluated the degradation of phytosterols and the antioxidant efficiency of ethanolic extracts of green tea (0.1%) and rosemary (0.02%) in refined rapeseed oil, comparing them to BHT and a control sample without antioxidants. The study was conducted under accelerated heating conditions using a Rancimat at 180 °C for 4 hours. The β-sitosterol degradation observed was 4% in the green tea extract sample and 3% in the rosemary extract sample, compared to 9% in the control. Campesterol degradation reached 6% and 4% in the green tea and rosemary treatments, respectively, whereas the control sample exhibited a 14.7% reduction. Notably, stigmasterol, which was initially quantified at 4 mg/100 g in unheated oil, showed no degradation in any of the samples during the heating process.

Yang et al. (2020) investigated the photooxidation mechanisms of phytosterol standards dissolved in dimethyl sulfoxide under LED light exposure (30,000 lux) at 25 ± 1 °C for 12 days in a controlled incubator. After the exposure period, β-sitosterol levels had decreased by approximately 50%, while stigmasterol and campesterol showed about 55% degradation relative to their initial concentrations. Similarly, Zhao et al. (2019) studied a standard phytosterol mixture dissolved in triolein exposed to LED lamp illumination at varying intensities (10,000, 20,000, and 30,000 lux) for 14 days at 25 °C, simulating commercial lighting conditions. In this study, phytosterol degradation occurred in all light-exposed samples, with the most intense illumination (30,000 lux) causing approximately 65% degradation of total phytosterols. In contrast, the control sample stored in the dark exhibited only 15% degradation. The same study also evaluated the influence of chlorophyll as a photosensitizer added to the phytosterol-triolein mixture at concentrations of 0.025%, 0.05%, and 0.1% under 20,000 lux exposure. The sample containing 0.1% chlorophyll showed the highest degradation rate (65%), while the sample without chlorophyll still showed significant degradation (55%), confirming the substantial impact of light exposure on phytosterol photooxidation, even in the absence of added sensitizers.

β-sitosterol is the predominant phytosterol in soybean oil. After 60 days of storage, even under mild conditions, its content decreased by nearly 60%, representing a significant loss of this bioactive compound. Phytosterols, particularly β-sitosterol, are known for their biological activities, notably the ability to reduce intestinal cholesterol absorption due to their structural similarity to cholesterol (Barkas et al., 2023), thereby contributing to a lower risk of developing cardiovascular diseases (Gylling et al., 2014). Additionally, β-sitosterol has shown potential in

inhibiting the progression of various cancers, including prostate, breast, and stomach cancers (Khan et al., 2022). Campesterol has been associated with cytokine regulation, suggesting therapeutic benefits in managing rheumatoid arthritis (Nazir et al., 2023). Stigmasterol, in turn, possesses a broad spectrum of pharmacological effects, including anticancer, anti-inflammatory, antidiabetic, immunomodulatory, and neuroprotective properties. It has also demonstrated potential in the treatment of inflammatory bowel disease through modulation of immune responses (Bakrim et al., 2022).

The results obtained demonstrate that refined soybean oil undergoes photo-oxidation when exposed to natural light, as evidenced by the degradation observed in all samples and the consequent significant loss of phytosterols. This degradation compromises part of the oil's biological functionality.

However, the addition of pink pepper to the oil exhibited a protective effect on phytosterol molecules. After 60 days of storage, the sample containing 6% pink pepper showed a preservation of 20% of β-sitosterol, 49% of stigmasterol, and 56% of campesterol compared to the control. This protective effect is attributed to the bioactive compounds present in the fruit, highlighting masticadienoic acid, as it is possibly useful as an antioxidant in soybean oil, as observed by Barreira et al. (2023). However, further studies are needed to confirm the compounds capable of migrating to soybean oil under the specific conditions studied. The other bioactive compounds also present in pink pepper, such as flavonoids, are capable of acting as antioxidants (De Oliveira et al., 2020). These molecules act mainly by neutralizing free radicals and chelating pro-oxidant metals (Kumar; Pandey, 2013), inhibiting oxidative processes and protecting sterol structures. The pronounced impact of light on soybean oil and the substantial degradation of its phytosterols underscore the importance of adopting strategies to prevent photo-oxidation. These may include the use of packaging with light- and UV-blocking properties or the incorporation of natural antioxidants, which offer sustainable and safe alternatives for enhancing product stability and nutritional value.

Table 6: Campesterol, stigmasterol and β-sitosterol contents (mg/100 g) in soybean oil samples during 60 days of storage.

Time (days)	Treatment	β-sitosterol	Stigmasterol	Campesterol
0	Control	146.60 ± 5.43 $^{A;a}$	59.16 ± 1.25 $^{A;a}$	28.28 ± 1.13 $^{A;a}$
	2% PP	144.06 ± 9.05 $^{A;a}$	59.11 ± 4.75 $^{A;a}$	27.33 ± 1.19 $^{A;a}$
	4% PP	144.22 ± 9.04 $^{A;a}$	59.02 ± 1.79 $^{A;a}$	27.15 ± 0.74 $^{A;a}$
	6% PP	146.47 ± 5.58 $^{A;a}$	59.09 ± 1.24 $^{A;a}$	28.57 ± 1.19 $^{A;a}$
15	Control	120.88 ± 6.84 $^{C;b}$	47.39 ± 0.96 $^{B;b}$	23.27 ± 0.33 $^{C;b}$
	2% PP	126.41 ± 8.55 $^{BC;b}$	47.38 ± 2.29 $^{B;b}$	23.22 ± 1.22 $^{C;b}$
	4% PP	133.77 ± 5.81 $^{AB;a}$	50.31 ± 0.65 $^{AB;b}$	25.46 ± 0.81 $^{B;ab}$
	6% PP	138.72 ± 2.89 $^{A;a}$	53.73 ± 1.04 $^{A;b}$	27.47 ± 0.48 $^{A;a}$
30	Control	100.84 ± 7.27 $^{B;c}$	39.55 ± 2.56 $^{;c}$	20.96 ± 0.71 $^{B;c}$
	2% PP	106.92 ± 6.92 $^{AB;c}$	42.81 ± 0.69 $^{BC;b}$	20.44 ± 0.51 $^{B;c}$
	4% PP	109.17 ± 1.37 $^{AB;b}$	45.47 ± 2.32 $^{AB;b}$	23.70 ± 0.21 $^{A;b}$
	6% PP	113.4 ± 4.45 $^{A;b}$	49.01 ± 3.31 $^{A;bc}$	25.34 ± 0.24 $^{A;b}$
45	Control	91.09 ± 3.09 $^{B;c}$	34.37 ± 1,87 $^{B;c}$	16.72 ± 0.68 $^{C;d}$
	2% PP	92.71 ± 2.67 $^{B;d}$	34.59 ± 0,98 $^{B;d}$	17.80 ± 0.29 $^{C;d}$
	4% PP	109.48 ± 7.61 $^{A;b}$	35.34 ± 0.52 $^{B;c}$	20.93 ± 1.3 $^{B;c}$
	6% PP	113.13 ± 5.76 $^{A;b}$	44.56 ± 1,56 $^{A;cd}$	23.12 ± 1.51 $^{A;c}$
60	Control	61.79 ± 2.84 $^{A;d}$	26.90 ± 1.55 $^{B;e}$	13.92 ± 0.64 $^{C;e}$
	2% PP	64.35 ± 0.46 $^{A;e}$	27.9 ± 1.25 $^{B;d}$	14.51 ± 0.57 $^{C;e}$
	4% PP	67.75 ± 6.39 $^{A;c}$	31.05 ± 0.48 $^{B;c}$	18.31 ± 0.78 $^{B;d}$
	6% PP	73.87 ± 2.78 $^{A;c}$	40.13 ± 3.97 $^{A;d}$	21.66 ± 0.56 $^{A;c}$

Results are expressed as mean ± standard deviation (n=3). Different capital letters in the same line indicate significant differences among treatments during the same storage time. Different lower letters in the same column indicate significant differences for the same treatment during storage time ($p \leq 0.05$).

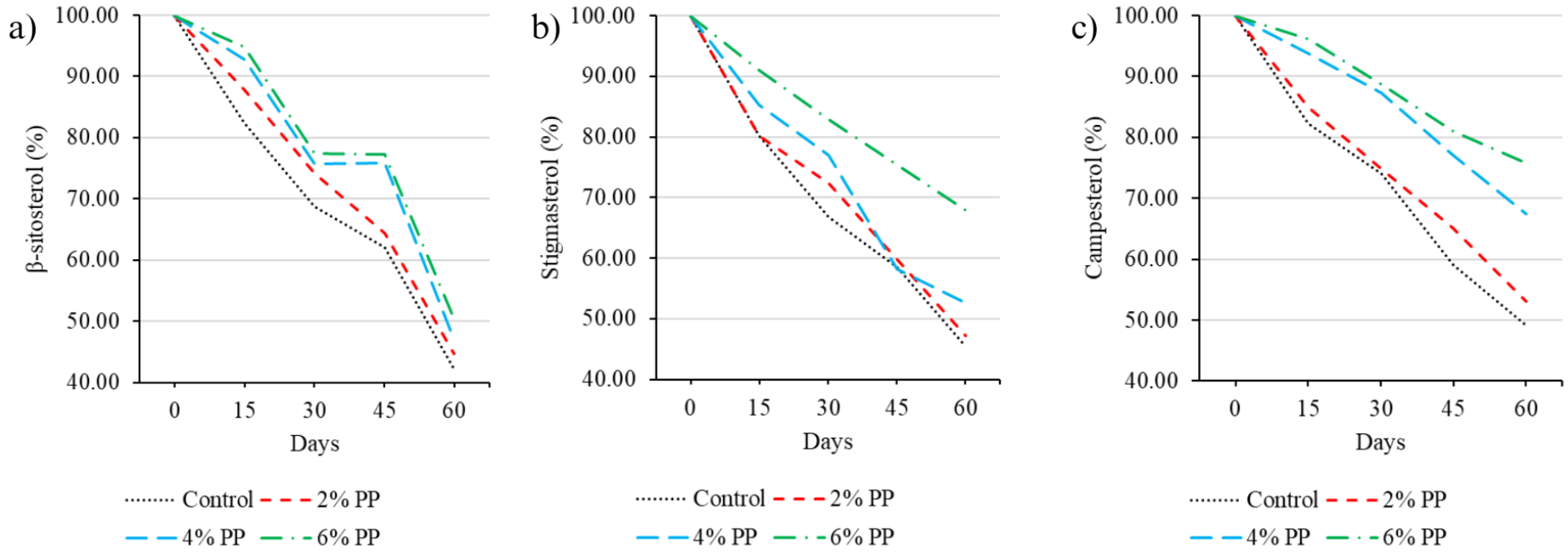

Figure 3: Degradation percentage of β-sitosterol (a), stigmasterol (b), and campesterol (c) in refined soybean samples during 60 days of storage.

3.3.2. Phytosterol oxides

Five oxidation products of the phytosterols β-sitosterol, stigmasterol, and campesterol were identified and quantified: 5,6α-epoxy, 5,6β-epoxy, 7-keto, 7α-hydroxy, and 7β-hydroxy (Table 7). On day zero, the total POPs were 6.61 μg/g in the control sample, 5.98 μg/g in the 2% PP sample, 5.68 μg/g in the 4% PP sample, and 5.22 μg/g in the 6% PP sample. For the oxides derived from β-sitosterol, all five studied oxides were detected, with the highest values found for 5,6β-epoxysitosterol (from 1.20 to 1.64 μg/g) and 7-ketositosterol (from 1.29 to 1.65 μg/g), with no statistically significant differences among the samples. For stigmasterol, the oxides quantified on day zero were 5,6β-epoxy (0.28 μg/g) and 7-keto (0.69 μg/g). For campesterol, 5,6β-epoxy (0.98 μg/g), 7-keto (0.01 μg/g), and 7β-hydroxy (0.15 μg/g) were detected in the control samples. The presence of oxides in soybean oil at day zero indicates that oxidation had already occurred during processing or prior storage.

By day 15, all phytosterol oxides showed an increase, including the formation of 0.14 μg/g of 7α-hydroxycampesterol in the control samples. Statistically significant differences in oxide formation were observed among the samples for all compounds except 5,6β-epoxycampesterol, which ranged from 1.06 to 1.17 μg/g. The total oxide concentration in the control sample doubled by day 15, whereas samples with 2%, 4%, and 6% PP exhibited smaller increases of 1.8, 1.5, and 1.4 times, respectively. A similar trend of moderated oxide formation was observed in the pink pepper-treated samples at day 30. Between days 15 and 30, 7-ketositosterol experienced the largest increase, rising by 40% in the control, 38% in the 2% PP sample, 8% in the 4% PP sample, and 12% in the 6% PP sample. By day 30, total oxide levels indicated protective effects from pink pepper addition, with reductions of 17% (2% PP), 35% (4% PP), and 48% (6% PP) relative to the control.

On day 45, the control sample exhibited the formation of 0.18 μg/g of 5,6α-epoxystigmasterol and 0.74 μg/g of 7β-hydroxystigmasterol. In contrast, samples with pink pepper addition showed the presence of 0.60 μg/g of 7β-hydroxystigmasterol, with no statistically significant differences among the different PP concentrations ($p > 0.05$). Regarding the total phytosterol oxidation products, the control sample experienced a 2.1-fold increase between days 30 and 45. Meanwhile, the samples containing pink pepper displayed a more moderate increase, corresponding to protection levels of 26% for 2% PP, 59% for 4% PP, and 65% for 6% PP, indicating a dose-dependent antioxidant effect of pink pepper in mitigating phytosterol oxidation.

On day 60, except for 7α-hydroxystigmasterol, which had newly formed, the total sum of

phytosterol oxides in the control sample reached 52.88 μg/g. In contrast, the samples with pink pepper addition showed significantly lower total oxide concentrations: 40.61 μg/g for 2% PP, 33.08 μg/g for 4% PP, and 25.89 μg/g for 6% PP. These correspond to protective effects of 23%, 37%, and 51% respectively, compared to the control. The predominant oxides across all three phytosterols were 7-keto and 5,6β-epoxy forms. Notably, oxides derived from β-sitosterol were found in the highest concentrations, consistent with its initial content being about 2.5 times that of stigmasterol and 5.2 times that of campesterol.

Regarding phytosterol oxidation products in vegetable oils, Poudel et al. (2022) analyzed campesterol and β-sitosterol oxides in canola oil and reported higher concentrations of 7-keto derivatives among the oxides studied, with 0.68 μg/g of 7-ketocampesterol and 0.79 μg/g of 7-ketositosterol. They also quantified 0.25 μg/g of 5,6-epoxycampesterol and 0.39 μg/g of 5,6-epoxysitosterol. Similarly, Cercaci et al. (2007) investigated the formation of β-sitosterol, stigmasterol, and campesterol oxides during bulk corn oil storage at 55°C for 40 days. They found that 7-keto was the predominant oxide formed, with a significant increase observed after 30 days, reaching approximately 20 μg/g of 7-ketositosterol by day 40.

Refined soybean oil was exposed to natural sunlight at room temperature for 30 days, and the oxidation products of β-sitosterol were analyzed. On day zero, only 7α- and 7β-hydroxysitosterol oxides were detected. By day 30, all evaluated oxides were quantified, with the highest concentrations found for 5,6β-epoxysitosterol (207 μg/g), 7-ketositosterol (192 μg/g), and 7β-hydroxysitosterol (180 μg/g) (Zhang et al., 2006).

Zhao et al. (2019) investigated the photooxidation of a mixture containing the four main phytosterols (β-sitosterol, campesterol, stigmasterol, and brassicasterol) added to triolein and stored under three different light intensities from a white LED lamp (10,000 lx, 20,000 lx, and 30,000 lx) for 14 days. A similar oxidation pattern was observed for all phytosterols evaluated. After 14 days, the total amount of oxides formed ranged from 696.9 μg/g in the control sample (kept in the dark) to 4481.9 μg/g in the sample exposed to 30,000 lx. The oxide formation pattern differed from that seen in the present study, with higher concentrations of 7β-hydroxy, 7α-hydroxy, 5,6β-epoxy, 7-keto, 5,6α-epoxy, and 6β-hydroxy derivatives, respectively. Oxidation at carbon 7 is favored during phytosterol oxidation, as molecular oxygen reacts with the allylic hydrogen at this position, subsequently forming secondary oxidation products such as 7-hydroxy and 7-keto derivatives.

The same authors also studied the effect of chlorophyll as a photosensitizing agent on the photooxidation of a phytosterol mixture in triolein, exposing the samples to 20,000 lx of white LED light for 14 days. They observed that photooxidation accelerated in a chlorophyll

concentration-dependent manner. At the end of the experiment, the predominant oxides were 7β-hydroxy, 5,6β-epoxy, 7α-hydroxy, 7-keto, 5,6α-epoxy, and 6β-hydroxy, a profile that differed from their previous study under varying light intensities without chlorophyll. The total oxide concentration ranged from 2966.2 ug/g (0% chlorophyll) to 3766.3 ug/g (0.1% chlorophyll). These differences in oxide formation can be explained by the different matrices and radiation types, as natural light exposure includes a spectrum of radiation types, including ultraviolet, which may influence oxidation pathways differently compared to LED light.

The formation of 5,6-epoxy products is generally initiated by free radicals from oxidation, which attack the double bond of carbons 5 and 6. In both cases, the formation of β products is favored due to the steric effect of the hydroxyl group on carbon 3 (Zhao et al., 2019), which explains the greater formation of 5,6β-epoxy compared to 5,6α products.

Exposure to light led to significant oxidation of the samples and substantial degradation of phytosterols, suggesting not only oxidation but also the possible formation of other degradation products. The highest formation of oxides was observed in the control sample, followed by the samples containing 2%, 4%, and 6% PP, respectively, across all types of oxides and time points evaluated. Figure 4 illustrates the progression of total oxide concentrations over the storage period. Although all samples showed an increase in oxidation products, those containing 4% and 6% pink pepper exhibited a less pronounced rise in total oxides, indicating the antioxidant and protective effects of pink pepper against photo-oxidation.

Due to the geometric similarity between cholesterol and phytosterols, the oxidation processes and resulting products are also similar, as is the potential for harmful effects in humans (Zhao et al., 2019b). Oxidation of phytosterols not only compromises their biological function, particularly their ability to act as hypocholesterolemic agents, but also leads to the formation of degradation products. These oxidation products have been associated with adverse health effects, including the development of cancer, cardiovascular diseases, neurodegenerative disorders, and atherosclerosis (Grootveld et al., 2020; Zhao et al., 2021).

Regarding the protective effect of pink pepper, its addition at concentrations of 0.25%, 0.5%, 0.75%, and 1.0% was evaluated in canned sardines with respect to the formation of cholesterol oxides after undergoing commercial sterilization (126 °C for 40 minutes). Pink pepper at the highest concentrations (0.75% and 1.0%) proved effective in mitigating cholesterol oxidation, reducing the formation of oxides by 47% in sardine fillets and by 39% in the soybean oil used as the packing medium (Barreira et al., 2023). Similarly, Oliveira et al. (2020) assessed cholesterol oxide formation in sardines (*Sardinella brasiliensis*) with the addition of 0.2% and 0.5% ground pink pepper, cooked in an oven at 150 °C and 180 °C for 7 minutes. A protective

effect was observed, with a 23.22% reduction in cholesterol oxidation products in the samples containing 0.5% pink pepper at 180 °C.

Kmiecik et al. (2009) evaluated the effect of ethanolic extracts of green tea (0.1%) and rosemary (0.02%) added to rapeseed oil heated at 180 °C for 4 hours, using both a Rancimat® and an Oxidograph®. These natural antioxidants were compared to the synthetic antioxidant BHT (0.02%). The study demonstrated a protective effect of 46% for green tea extract and 35% for rosemary, compared to 16% for BHT, indicating the superior effectiveness of these natural sources in mitigating the formation of oxidation products of phytosterols such as β-sitosterol, campesterol, avenasterol, brassicasterol, and stigmasterol.

Studies with natural sources have shown promise as potential alternatives to synthetic antioxidants, which have been associated with adverse health effects when consumed long-term, including interference with blood clotting, mutagenic potential, and tumor development (Esazadeh et al., 2024; Liu & Mabury, 2020). As in previous studies, pink pepper has also demonstrated effectiveness in reducing the formation of oxidation products (Da Silva et al., 2025; Martini et al., 2021; Menegali et al., 2020b), represents a practical strategy to enhance the oxidative stability and quality of soybean oil during storage. This antioxidant effect of pink pepper is attributed to its rich composition of bioactive compounds, mainly the phenolic constituents, phenolic acids and flavonoids. Among the flavonoids, glycosylated forms and the biflavonoids are predominat (Vieira et al., 2023). These bioactive act as antioxidants, primarily by donating hydrogen atoms or electrons to free radicals, thus interrupting oxidative chain reactions (Zeb, 2021).

Table 7: Contents of phytosterol oxides (μg/g) in refined soybean samples during 60 days of storage.

Phytosterol oxides (μg/g)	Control	2% PP	4% PP	6% PP
		DAY 0		
5.6α-epoxysitosterol	$0.83 \pm 0.05^{A;d}$	$0.52 \pm 0.04^{B;b}$	$0.42 \pm 0.02^{BC;d}$	$0.35 \pm 0.01^{C;d}$
5.6β-epoxysitosterol	$1.55 \pm 0.09^{A;d}$	$1.64 \pm 0.09^{A;d}$	$1.31 \pm 0.03^{A;d}$	$1.2 \pm 0.09^{A;d}$
7-ketositosterol	$1.65 \pm 0.14^{A;e}$	$1.29 \pm 0.01^{A;d}$	$1.48 \pm 0.01^{A;b}$	$1.33 \pm 0.08^{A;b}$
7α-hidroxisitosterol	$0.05 \pm 0^{A;e}$	$0.05 \pm 0^{A;e}$	$0.05 \pm 0^{A;c}$	$0.05 \pm 0^{A;c}$
7β-hydroxysitosterol	$0.42 \pm 0.02^{A;e}$	$0.42 \pm 0.03^{A;c}$	$0.38 \pm 0.01^{A;c}$	$0.33 \pm 0.01^{A;e}$
5.6α-epoxystigmasterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5.6β-epoxystigmasterol	$0.28 \pm 0.01^{A;e}$	$0.27 \pm 0.04^{A;d}$	$0.28 \pm 0.01^{A;e}$	$0.27 \pm 0.01^{A;e}$
7-ketostigmasterol	$0.69 \pm 0.06^{A;d}$	$0.68 \pm 0.05^{A;e}$	$0.66 \pm 0.04^{A;b}$	$0.65 \pm 0.02^{A;d}$
7α-hydroxystigmasterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7β-hydroxystigmasterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5.6α-epoxycampesterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5.6β-epoxycampesterol	$0.98 \pm 0.07^{A;d}$	$0.97 \pm 0.05^{A;d}$	$0.93 \pm 0.04^{A;c}$	$0.92 \pm 0.03^{A;b}$
7-ketocampesterol	$0.01 \pm 0^{A;c}$	$0.01 \pm 0^{A;d}$	$0.01 \pm 0^{A;c}$	$0.01 \pm 0^{A;d}$
7α-hydroxycampesterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7β-hydroxycampesterol	$0.15 \pm 0.01^{A;d}$	$0.14 \pm 0.01^{A;c}$	$0.14 \pm 0^{A;c}$	$0.14 \pm 0.01^{A;c}$
Total oxides	**6.61**	**5.98**	**5.68**	**5.22**
		DAY 15		
5.6α-epoxysitosterol	$1.15 \pm 0.07^{A;c}$	$0.93 \pm 0.01^{B;c}$	$0.7 \pm 0.06^{C;c}$	$0.67 \pm 0.01^{C;b}$
5.6β-epoxysitosterol	$3.17 \pm 0.15^{A;c}$	$2.28 \pm 0.05^{AB;cd}$	$2.1 \pm 0.23^{B;cd}$	$1.83 \pm 0.05^{B;cd}$
7-ketositosterol	$3.58 \pm 0.25^{A;d}$	$2.72 \pm 0.03^{AB;c}$	$1.67 \pm 0.09^{B;b}$	$1.58 \pm 0.06^{B;b}$
7α-hydroxysitosterol	$0.25 \pm 0.02^{A;d}$	$0.23 \pm 0^{AB;d}$	$0.13 \pm 0.01^{B;c}$	$0.12 \pm 0.01^{B;c}$
7β-hydroxysitosterol	$0.65 \pm 0.02^{A;d}$	$0.65 \pm 0.03^{A;d}$	$0.54 \pm 0.03^{AB;c}$	$0.46 \pm 0.03^{B;c}$
5.6α-epoxystigmasterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5.6β-epoxystigmasterol	$0.76 \pm 0.03^{A;d}$	$0.75 \pm 0.01^{A;d}$	$0.45 \pm 0.01^{B;d}$	$0.31 \pm 0.01^{C;cd}$
7-ketostigmasterol	$1.25 \pm 0.05^{A;d}$	$0.83 \pm 0.04^{B;d}$	$0.79 \pm 0.01^{B;cd}$	$0.72 \pm 0.01^{B;b}$
7α-hydroxystigmasterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7β-hydroxystigmasterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5.6α-epoxycampesterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5.6β-epoxycampesterol	$1.17 \pm 0.03^{A;c}$	$1.14 \pm 0.08^{A;c}$	$1.13 \pm 0.08^{A;b}$	$1.06 \pm 0.05^{A;ab}$
7-ketocampesterol	$1.69 \pm 0.11^{A;b}$	$1.38 \pm 0.04^{AB;b}$	$0.95 \pm 0.03^{BC;c}$	$0.6 \pm 0.03^{C;cd}$
7α-hydroxycampesterol	$0.14 \pm 0.01^{A;c}$	0 ± 0	0 ± 0	0 ± 0
7β-hydroxycampesterol	$0.18 \pm 0.01^{A;c}$	$0.17 \pm 0.01^{A;b}$	$0.16 \pm 0.01^{A;b}$	$0.13 \pm 0.01^{B;bc}$
Total oxides	**13.99**	**11.08**	**8.62**	**7.48**
		DAY 30		
5.6α-epoxysitosterol	$1.4 \pm 0.12^{A;b}$	$1.33 \pm 0.07^{A;a}$	$1.32 \pm 0.11^{A;b}$	$1.02 \pm 0.07^{B;bc}$

5.6β-epoxysitosterol	$3.13 \pm 0.27^{A;c}$	$2.98 \pm 0.03^{A;bc}$	$2.97 \pm 0.06^{A;bc}$	$2.29 \pm 1.82^{A;bc}$
7-ketositosterol	$5.02 \pm 0.29^{A;c}$	$3.76 \pm 0.08^{A;c}$	$1.80 \pm 0.05^{B;b}$	$1.77 \pm 0.09^{B;b}$
7α-hydroxysitosterol	$1.15 \pm 0.09^{A;c}$	$1.05 \pm 0.01^{AB;c}$	$0.91 \pm 0.04B^{C;b}$	$0.81 \pm 0.05^{C;b}$
7β-hydroxysitosterol	$0.8 \pm 0.05^{A;c}$	$0.65 \pm 0.05^{B;c}$	$0.50 \pm 0.03^{C;b}$	$0.43 \pm 0.02^{C;b}$
5.6α-epoxystigmasterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5.6β-epoxystigmasterol	$1.01 \pm 0.01^{A;c}$	$0.89 \pm 0.03^{B;c}$	$0.59 \pm 0.04^{C;c}$	$0.39 \pm 0.02^{D;c}$
7-ketostigmasterol	$1.48 \pm 0.02^{A;c}$	$1.04 \pm 0.04^{B;c}$	$0.87 \pm 0.02^{C;c}$	$0.80 \pm 0.01^{C;b}$
7α-hydroxystigmasterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7β-hydroxystigmasterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5.6α-epoxycampesterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5.6β-epoxycampesterol	$1.55 \pm 0.15^{A;b}$	$1.1 \pm 0.01^{B;cd}$	$1.09 \pm 0.01^{B;bc}$	$1.04 \pm 0.05^{B;ab}$
7-ketocampesterol	$1.8 \pm 0.14^{A;b}$	$1.57 \pm 0.02^{A;b}$	$1.21 \pm 0.03^{AB;bc}$	$0.71 \pm 0.02^{B;c}$
7α-hydroxycampesterol	$0.19 \pm 0.01^{A;c}$	$0.18 \pm 0.01^{A;b}$	$0.16 \pm 0^{B;b}$	$0.14 \pm 0^{C;ab}$
7β-hydroxycampesterol	$0.40 \pm 0.03^{A;b}$	$0.25 \pm 0.01^{B;c}$	$0.20 \pm 0.01^{B;b}$	0 ± 0
Total oxides	**17.93**	**14.8**	**11.62**	**9.4**
		DAY 45		
5.6α-epoxysitosterol	$1.55 \pm 0.1^{A;a}$	$1.49 \pm 0.03^{A;b}$	$1.30 \pm 0.04^{B;a}$	$1.18 \pm 0.03^{B;b}$
5.6β-epoxysitosterol	$4.35 \pm 0.16^{A;b}$	$3.47 \pm 0.36^{AB;b}$	$3.28 \pm 0.05^{B;b}$	$3.08 \pm 0.27^{B;b}$
7-ketositosterol	$16.84 \pm 2.02^{A;b}$	$11.36 \pm 0.71^{B;b}$	$2.52 \pm 0.26^{C;b}$	$2.16 \pm 0.15^{C;b}$
7α-hydroxysitosterol	$1.17 \pm 0.02^{A;b}$	$0.99 \pm 0.04^{B;b}$	$0.52 \pm 0^{C;b}$	$0.44 \pm 0.01^{C;b}$
7β-hydroxysitosterol	$2.30 \pm 0.06^{A;b}$	$2.27 \pm 0.07^{A;b}$	$1.04 \pm 0.01^{B;b}$	$0.87 \pm 0.05^{C;b}$
5.6α-epoxystigmasterol	$0.18 \pm 0.01^{A;b}$	0 ± 0	0 ± 0	0 ± 0
5.6β-epoxystigmasterol	$1.27 \pm 0.03^{A;b}$	$1.16 \pm 0.05^{B;b}$	$0.95 \pm 0.03^{C;b}$	$0.81 \pm 0.06^{D;b}$
7-ketostigmasterol	$2.38 \pm 0.23^{A;b}$	$1.35 \pm 0.06^{B;b}$	$1.08 \pm 0.02^{C;b}$	$0.84 \pm 0.02^{D;b}$
7α-hydroxystigmasterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7β-hydroxystigmasterol	$0.74 \pm 0.03^{A;b}$	$0.62 \pm 0.04^{B;b}$	$0.61 \pm 0.01^{B;b}$	$0.60 \pm 0^{B;b}$
5.6α-epoxycampesterol	$0.2 \pm 0^{A;b}$	$0.13 \pm 0.01^{B;b}$	$0.09 \pm 0^{C;b}$	0 ± 0
5.6β-epoxycampesterol	$2.28 \pm 0.10^{A;a}$	$1.64 \pm 0.09^{B;b}$	$1.43 \pm 0.09^{C;a}$	$1.11 \pm 0.04^{D;a}$
7-ketocampesterol	$3.37 \pm 0.27^{A;a}$	$2.94 \pm 0.1^{A;a}$	$1.81 \pm 0.01^{B;ab}$	$1.45 \pm 0.11^{B;b}$
7α-hydroxycampesterol	$0.23 \pm 0.01^{A;b}$	$0.23 \pm 0.01^{A;a}$	$0.20 \pm 0.01^{B;a}$	$0.14 \pm 0^{C;ab}$
7β-hydroxycampesterol	$0.43 \pm 0.02^{A;b}$	$0.38 \pm 0.02^{A;b}$	$0.27 \pm 0^{A;b}$	$0.15 \pm 0.01^{A;b}$
Total oxides	**37.29**	**27.72**	**15.29**	**12.95**
		DAY 60		
5.6 α-epoxysitosterol	$1.74 \pm 0.13^{A;a}$	$1.68 \pm 0.13^{A;a}$	$1.44 \pm 0.07^{B;a}$	$1.20 \pm 0.03^{C;bc}$
5.6β-epoxysitosterol	$5.61 \pm 0.11^{A;a}$	$4.94 \pm 0.3^{AB;a}$	$4.70 \pm 0.15^{AB;a}$	$4.27 \pm 0.2^{B;a}$
7-ketositosterol	$25.79 \pm 1.08^{A;a}$	$17.01 \pm 0.17^{B;a}$	$13.11 \pm 0.8^{C;a}$	$8.14 \pm 0.54^{D;a}$
7α-hydroxysitosterol	$2.69 \pm 0.12^{A;a}$	$2.40 \pm 0.12^{B;a}$	$1.42 \pm 0.11^{C;a}$	$1.20 \pm 0.07^{D;a}$

7β-hydroxysitosterol	$3.76 \pm 0.2^{A;a}$	$3.41 \pm 0.09^{B;a}$	$3.12 \pm 0.05^{C;a}$	$3.05 \pm 0.15^{C;a}$
5.6 α-epoxystigmasterol	$0.32 \pm 0.02^{A;a}$	$0.19 \pm 0.02^{B;a}$	0 ± 0	0 ± 0
5.6β-epoxystigmasterol	$1.96 \pm 0.12^{A;a}$	$1.76 \pm 0.08^{B;a}$	$1.59 \pm 0.09^{C;a}$	$1.12 \pm 0.05^{D;a}$
7-ketostigmasterol	$3.62 \pm 0.18^{A;a}$	$2.16 \pm 0.08^{B;a}$	$2.04 \pm 0.08^{B;a}$	$1.60 \pm 0.04^{C;a}$
7α-hydroxystigmasterol	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7β-hydroxystigmasterol	$0.97 \pm 0.04^{A;a}$	$0.90 \pm 0.03^{A;a}$	$0.73 \pm 0.04^{B;a}$	$0.8 \pm 0.03^{B;a}$
5.6 α-epoxycampesterol	$0.34 \pm 0.02^{A;a}$	$0.21 \pm 0.01^{B;a}$	$0.22 \pm 0.01^{B;a}$	$0.21 \pm 0.01^{B;a}$
5.6β-epoxycampesterol	$2.27 \pm 0.1^{A;a}$	$1.83 \pm 0.12^{B;a}$	$1.44 \pm 0.07^{C;a}$	$1.19 \pm 0.03^{D;a}$
7-ketocampesterol	$3.53 \pm 0.22^{A;a}$	$3.29 \pm 0.16^{A;a}$	$2.31 \pm 0.19^{B;a}$	$2.23 \pm 1.15^{B;a}$
7α-hydroxycampesterol	$0.28 \pm 0.01^{A;a}$	$0.24 \pm 0.01^{B;a}$	$0.21 \pm 0.02^{C;a}$	$0.15 \pm 0.01^{D;a}$
7β-hydroxycampesterol	$0.54 \pm 0.03^{A;a}$	$0.53 \pm 0.04^{A;a}$	$0.51 \pm 0.06^{A;a}$	$0.49 \pm 0.03^{A;a}$
Total oxides	**52.88**	**40.61**	**33.08**	**25.89**

Results are expressed as mean ± standard deviation (n=3). Different capital letters in the same line indicate significant differences among treatments during the same storage time. Different lower letters in the same column indicate significant differences for the same treatment during storage time ($p \leq 0.05$).

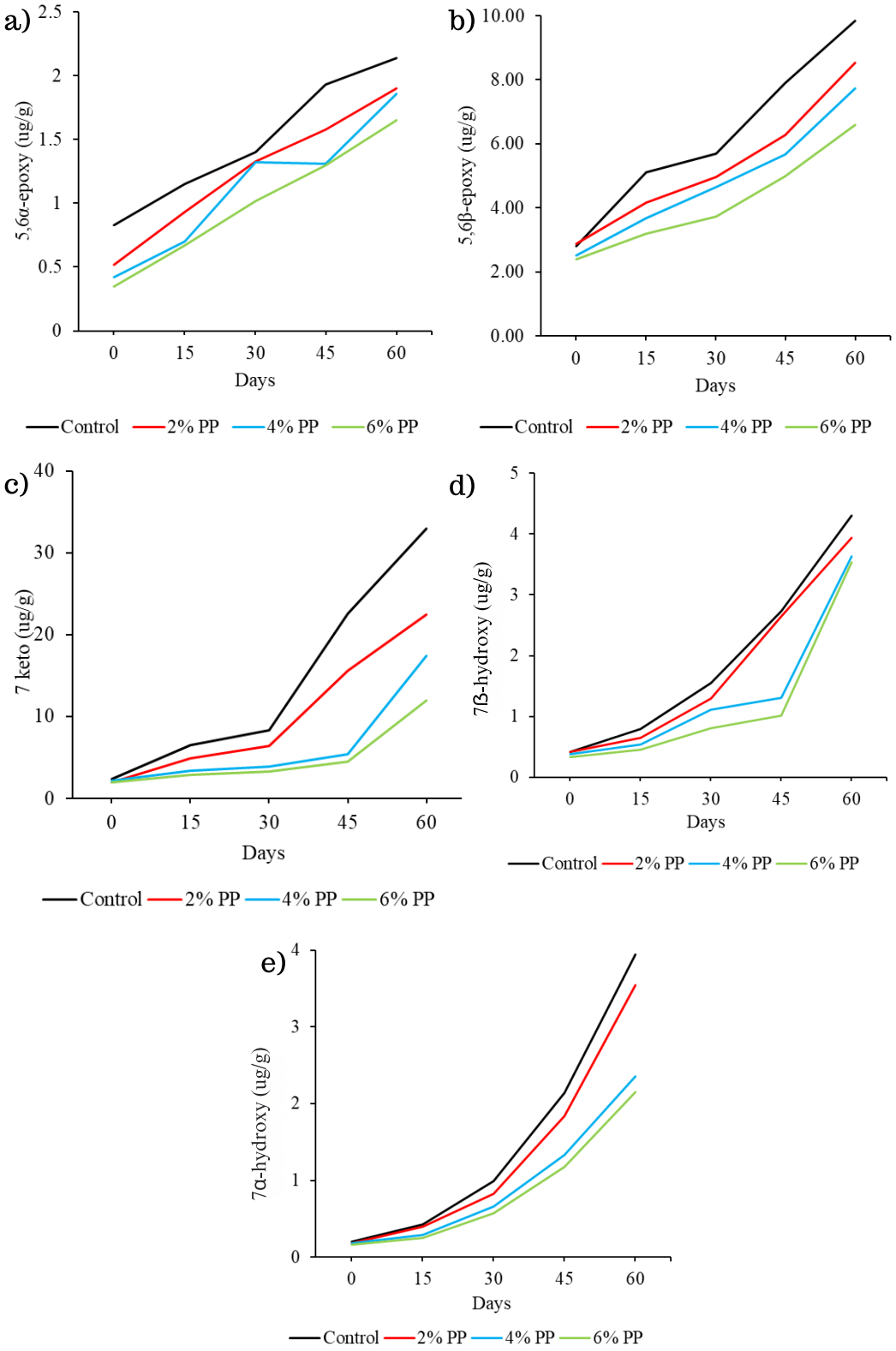

Figure 4: Sum of 5,6α-epoxy (a), 5,6β-epoxy (b), 7-keto (c), 7α-hydroxy (d), and 7β-hydroxy (e) oxides in refined soybean samples during 60 days of storage.

4. CONCLUSION

The methodology developed for quantifying the main phytosterols and their oxidation products in soybean oil samples using HPLC-APCI-MS proved to be robust and effective, meeting all validation parameters. Additionally, HPLC-QTOF-MS was essential for the identification of phytosterols and their thermo-oxidized oxides, especially given the absence of commercial standards for these compounds. As a result, it was possible to successfully identify and quantify the oxidation products of the three phytosterols studied.

All samples underwent oxidation with exposure to light. The control sample showed the greatest degradation of phytosterols and the highest formation of oxides, while samples containing pepper demonstrated reduced phytosterol degradation and lower levels of oxides, demonstrating the antioxidant capacity of pink pepper, mainly when it was added at 6%. Thus, these findings indicate that pink pepper was an effective natural antioxidant capable of protecting soybean oil against photo-oxidation during storage.

Future studies on the migration of pepper bioactive compounds at room temperature and under light exposure should be conducted to determine which bioactive compounds are primarily responsible for the antioxidant activity of soybean oil. We also suggest studying the protection of pink pepper in soybean oil after storage and heating to determine whether it provides protection at higher temperatures.

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Conflicts of Interest

The authors declare no conflict of interest.

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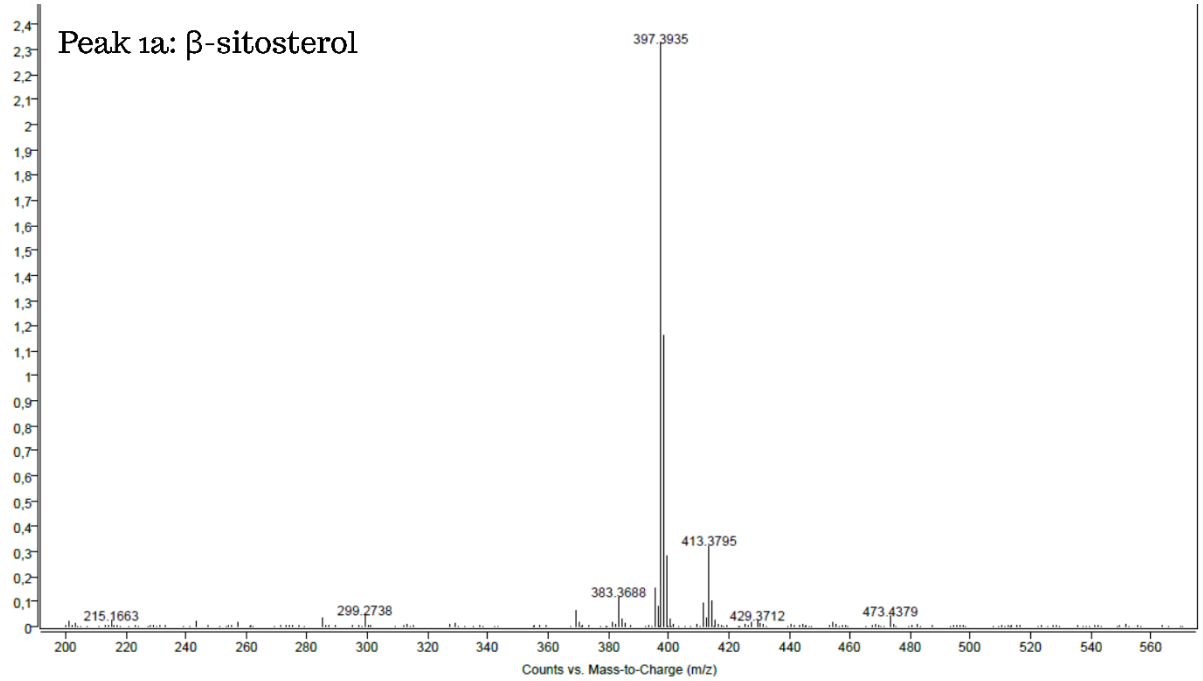
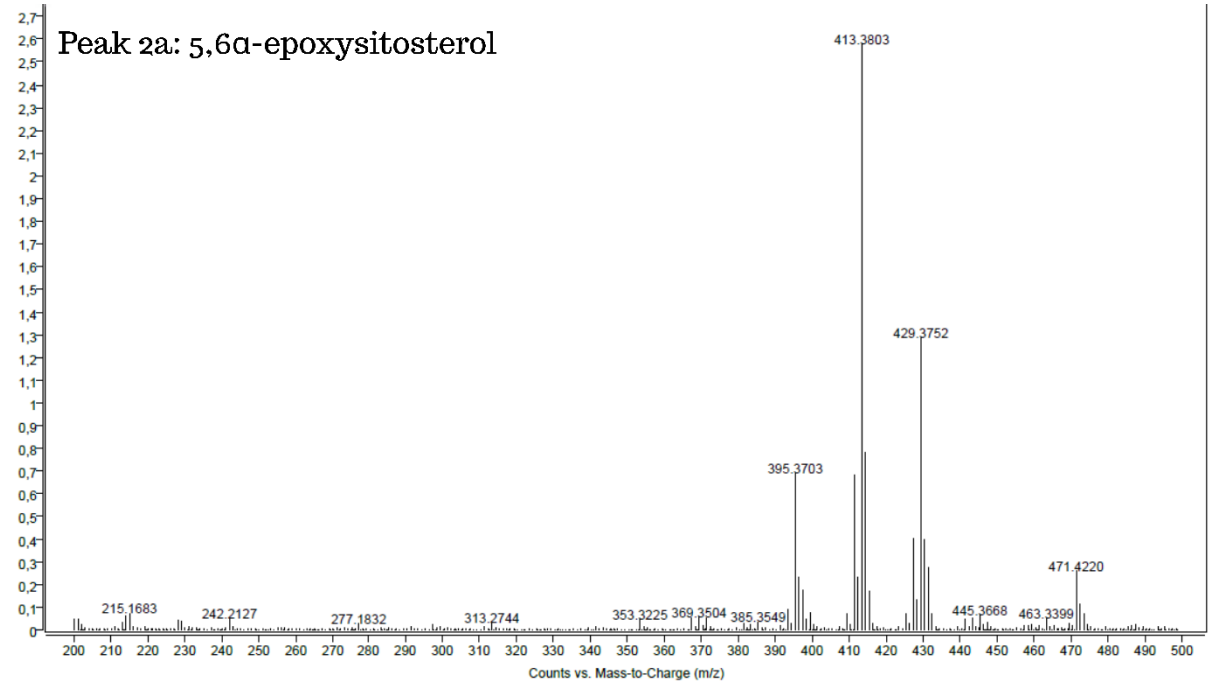
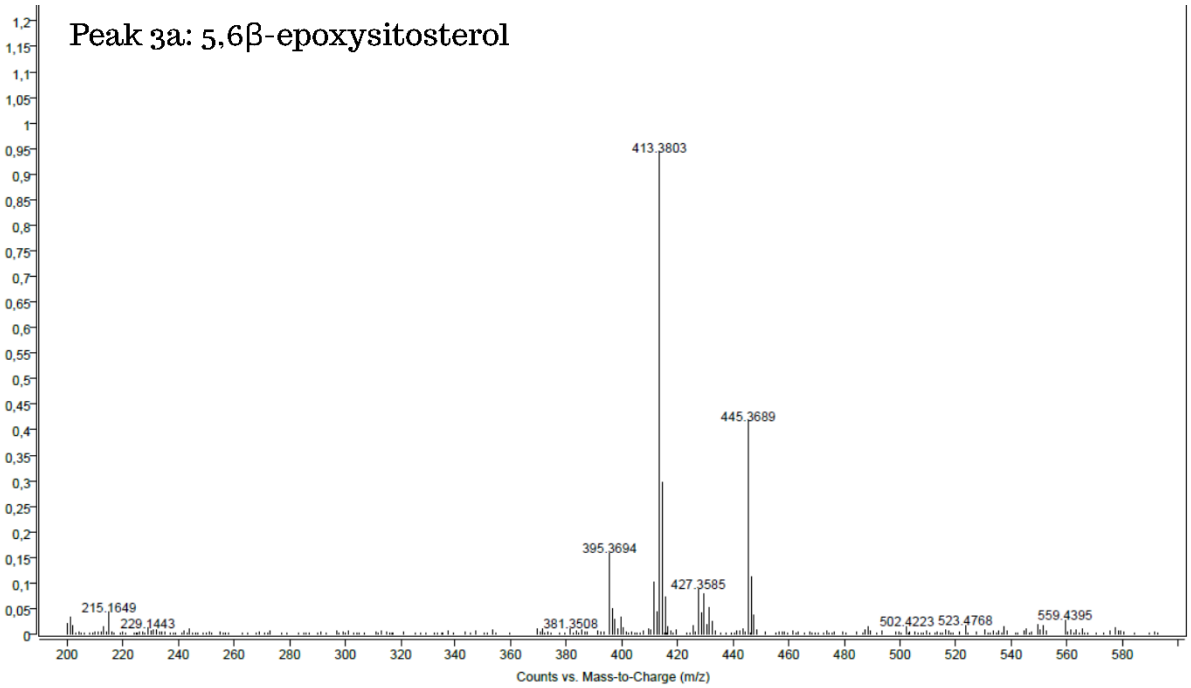
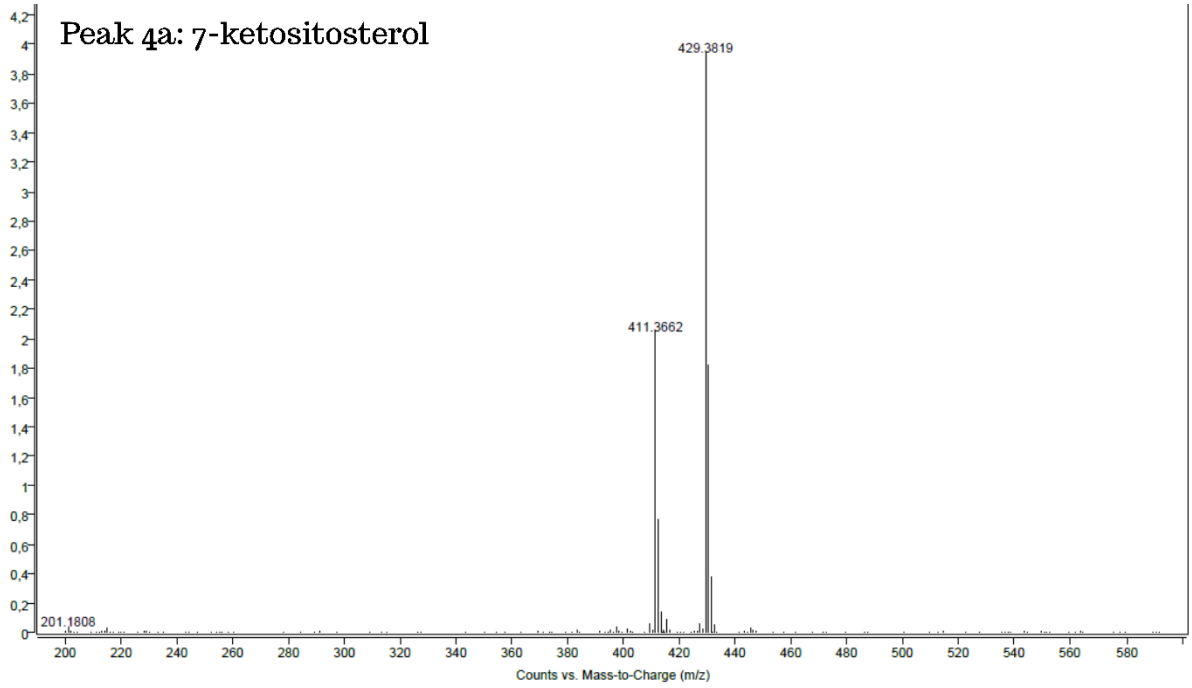
SUPPLEMENTARY MATERIAL (CAPÍTULO II)






Peak 3a: 5,6β-epoxysitosterol
413.3803
445.3689
395.3694
427.3585
215.1649
229.1443
381.3508
502.4223
523.4768
559.4395
Counts vs. Mass-to-Charge (m/z)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429.3819
411.3662
201.1808
Counts vs. Mass-to-Charge (m/z)

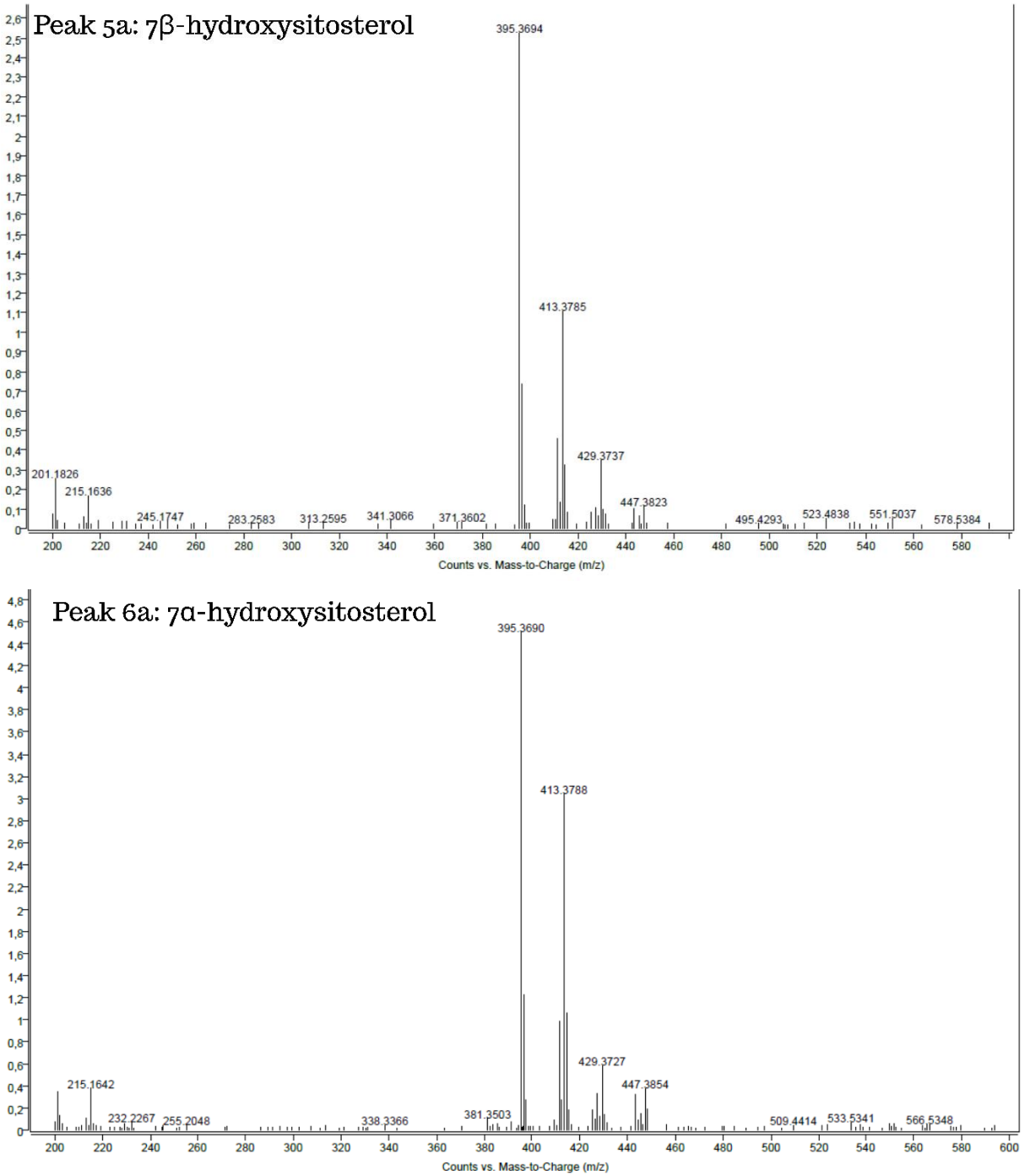
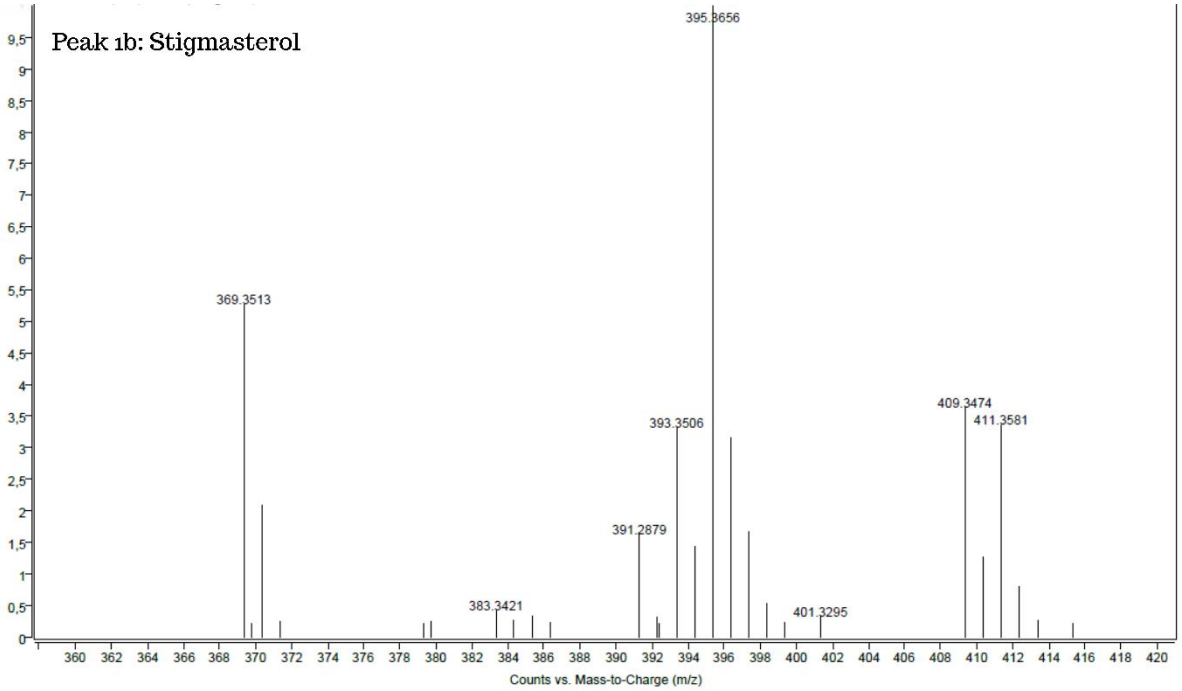
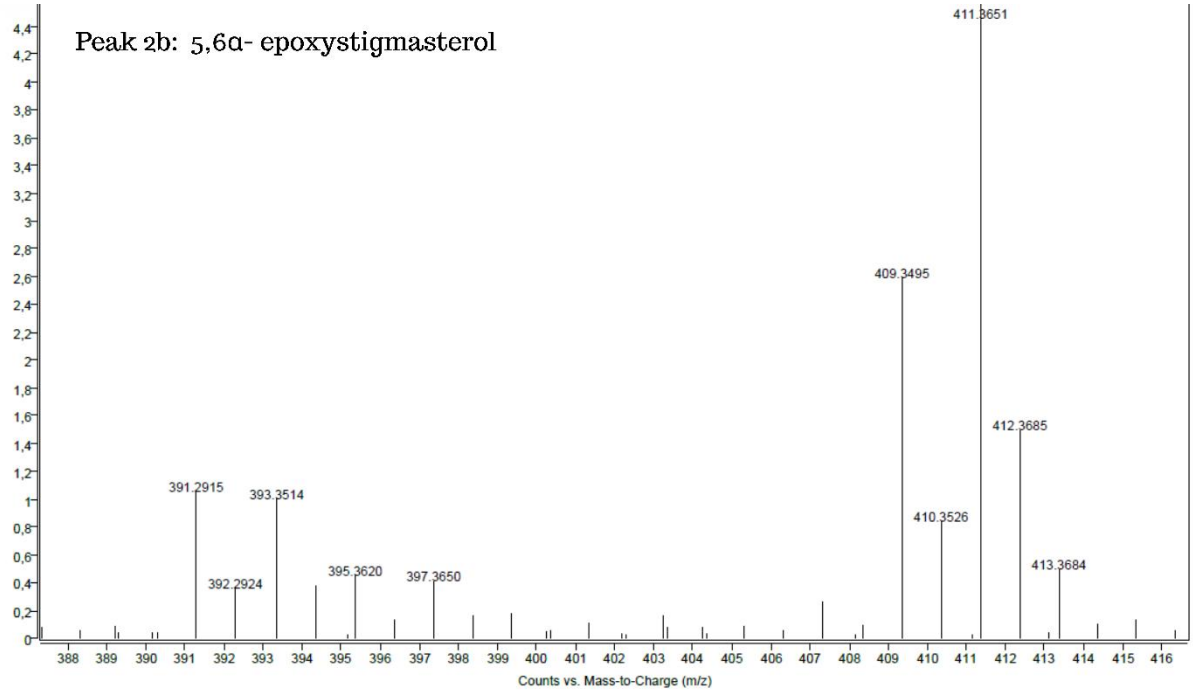
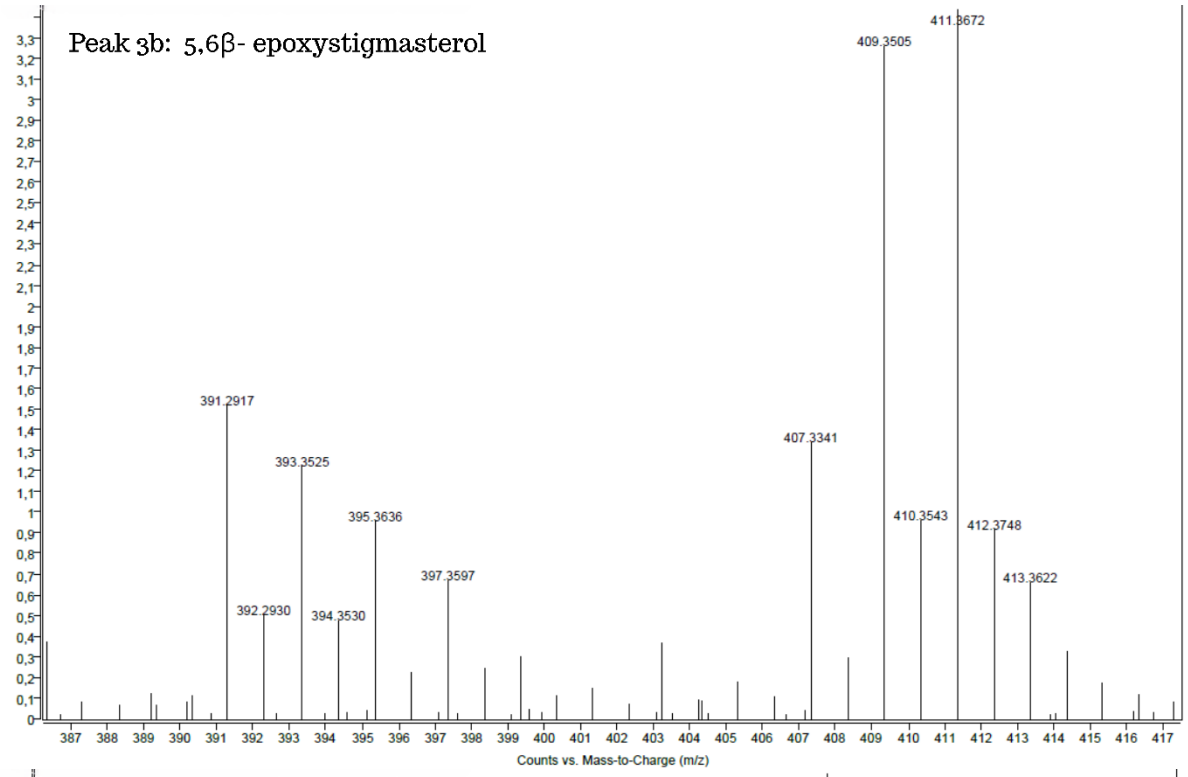
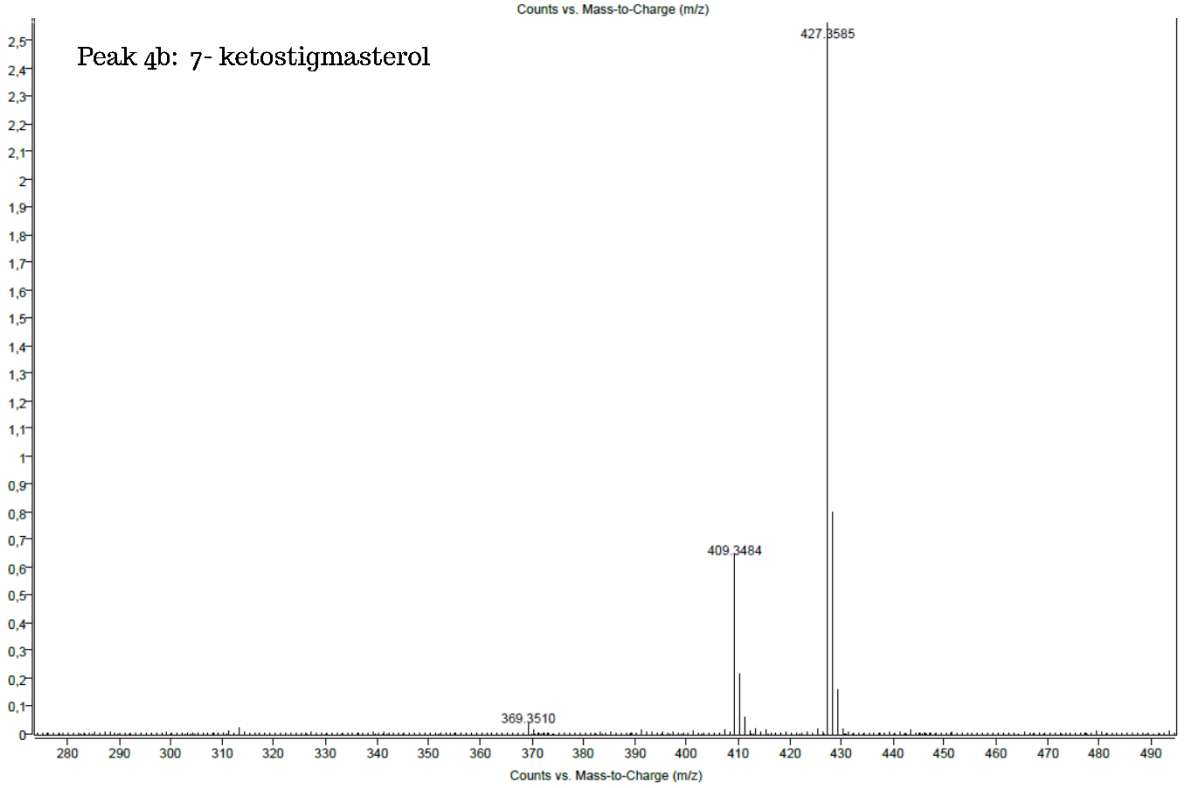


Figure 1: Peaks 1a to 6a. Mass spectrum of each peak of β-sitosterol standard thermo-oxidized referring to image 3a of chapter 2.


Peak 1b: Stigmasterol
395.3656
369.3513
409.3474
411.3581
393.3506
391.2879
383.3421
401.3295
Counts vs. Mass-to-Charge (m/z)


Peak 2b: 5,6α- epoxystigmasterol
411.3651
409.3495
412.3685
391.2915
393.3514
410.3526
413.3684
392.2924
395.3620
397.3650
Counts vs. Mass-to-Charge (m/z)


Peak 3b: 5,6β- epoxystigmasterol
411.3672
409.3505
391.2917
407.3341
393.3525
395.3636
410.3543
412.3748
397.3597
413.3622
392.2930
394.3530
Counts vs. Mass-to-Charge (m/z)


Peak 4b: 7- ketostigmasterol
427.3585
409.3484
369.3510
Counts vs. Mass-to-Charge (m/z)

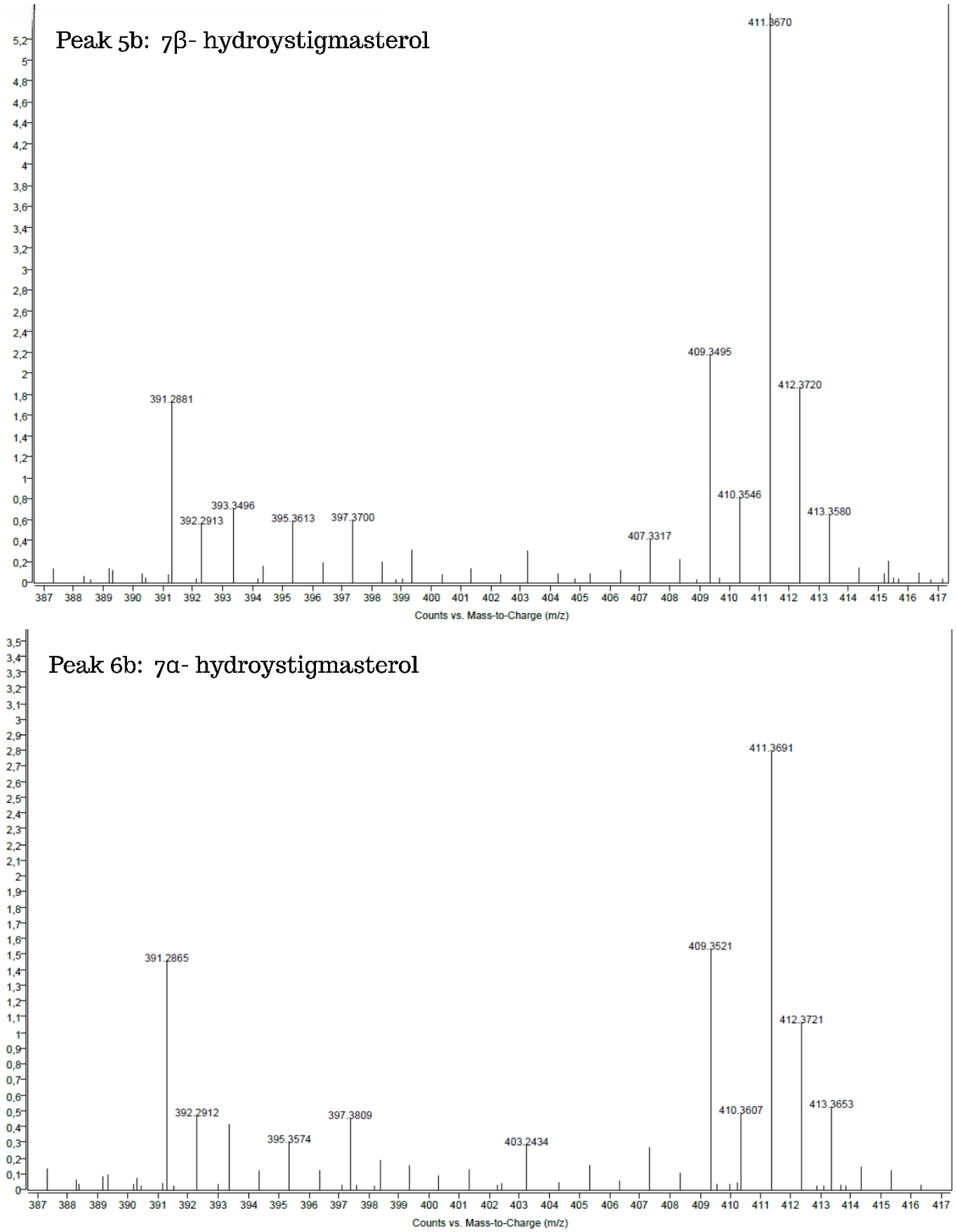
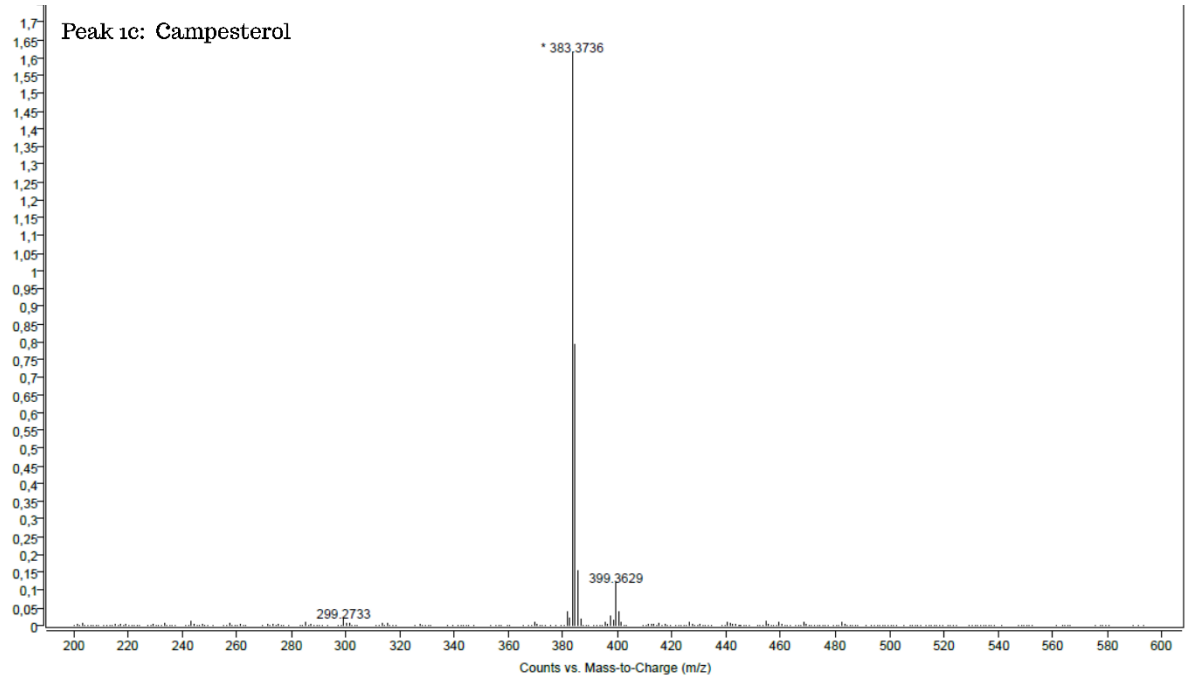
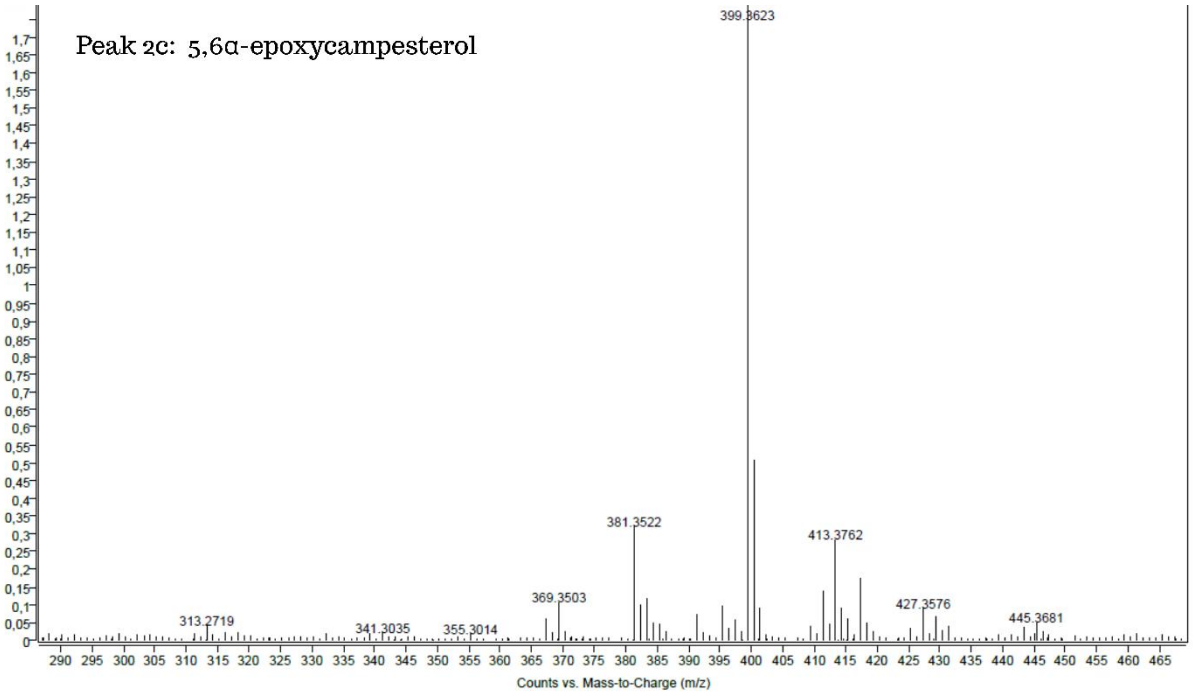
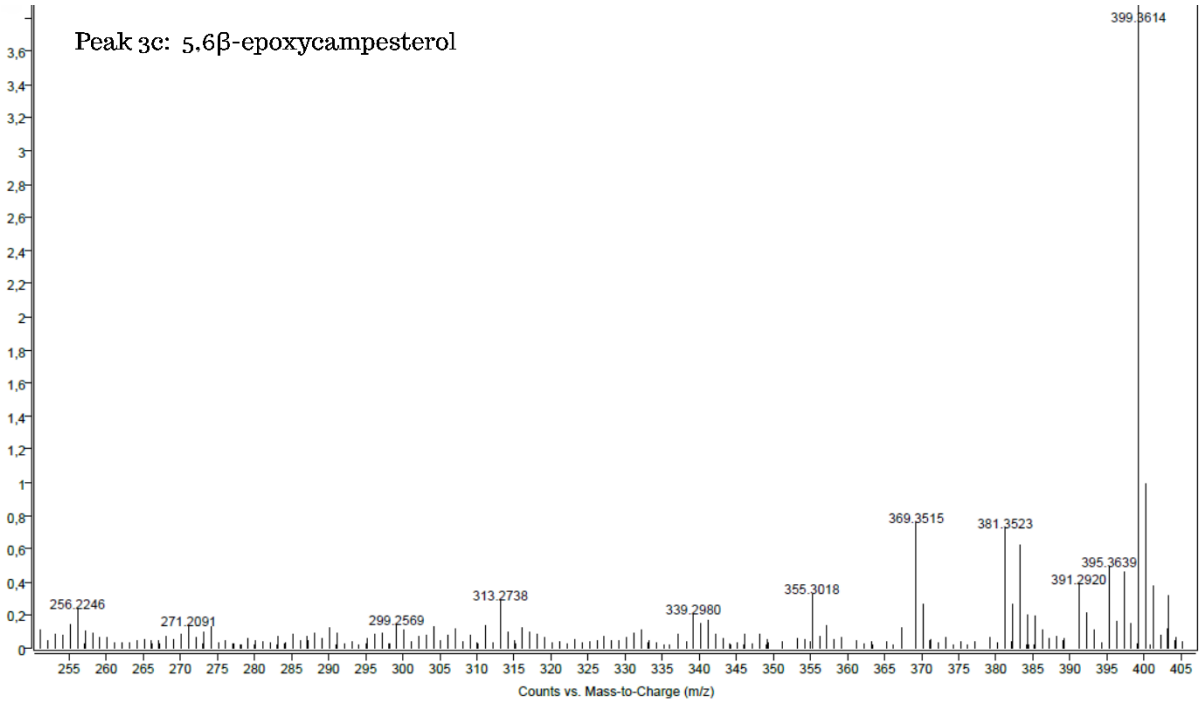
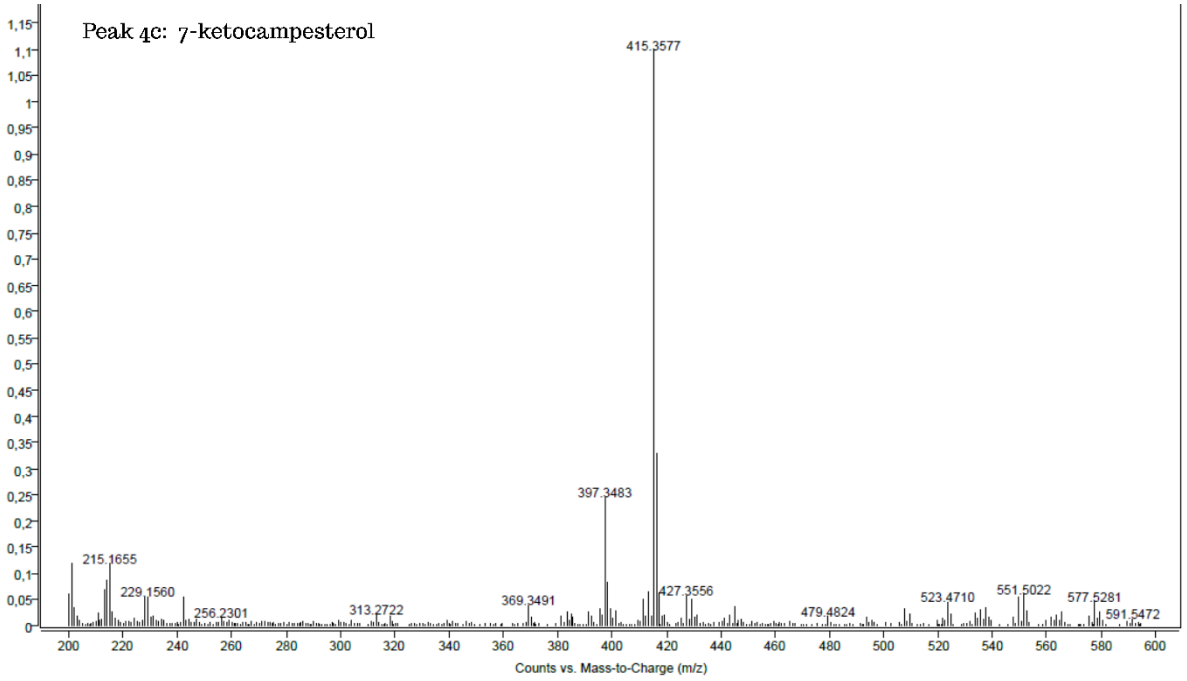


Figure 2: Peaks 1a to 6b. Mass spectrum of each peak of stigmasterol standard thermo-oxidized referring to image 3b of chapter 2.


Peak 1c: Campesterol
* 383,3736
399,3629
299,2733
Counts vs. Mass-to-Charge (m/z)


Peak 2c: 5,6α-epoxycampesterol
399,3623
381,3522
413,3762
369,3503
427,3576
313,2719
341,3035
355,3014
445,3681
Counts vs. Mass-to-Charge (m/z)


Peak 3c: 5,6β-epoxycampesterol
399.3614
369.3515
381.3523
395.3639
391.2920
355.3018
313.2738
339.2980
256.2246
271.2091
299.2569
Counts vs. Mass-to-Charge (m/z)


Peak 4c: 7-ketocampesterol
415.3577
397.3483
215.1655
229.1560
256.2301
313.2722
369.3491
427.3556
479.4824
523.4710
551.5022
577.5281
591.5472
Counts vs. Mass-to-Charge (m/z)

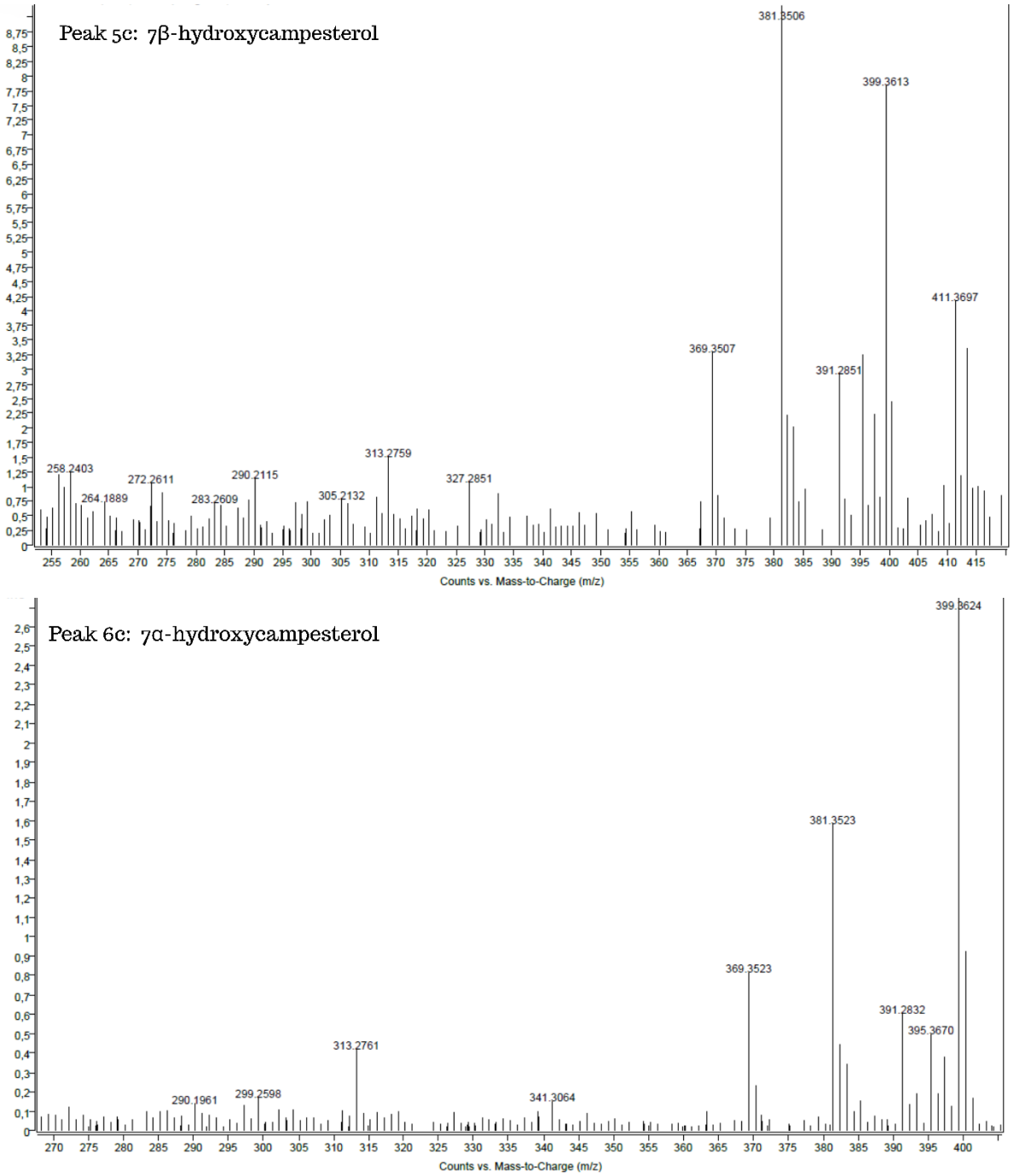


Figure 3: Peaks 1a to 6c. Mass spectrum of each peak of campesterol standard thermo-oxidized referring to image 3b of chapter 2.

CONSIDERAÇÕES FINAIS

A partir dos resultados obtidos foi possível concluir que a luz natural incidindo no óleo de soja refinado durante armazenamento teve impacto negativo na qualidade nutricional do produto, através da degradação de fitosteróis importantes para saúde humana e formação de produtos de oxidação de fitosteróis potencialmente tóxicos.

A pimenta rosa (*Schinus terebinthifolia* Raddi) é uma fonte de diversos compostos bioativos, com destaque para flavonoides, terpenos e o ácido masticadienoico, capazes de promover proteção antioxidante quando adicionada no óleo de soja. A cromatografia líquida de alta eficiência (HPLC-QTOF-MS/MS) do extrato da pimenta estudada detectou 12 compostos bioativos com potencial antioxidante, com 4 destes apresentando maiores concentrações, corroborando com estudos anteriores de que a pimenta rosa é uma fonte natural com significativo poder conservante.

A exposição do óleo de soja a luz natural, ainda que em temperatura branda, é capaz de degradar severamente os principais fitosteróis presentes no óleo. A pimenta rosa é capaz de promover proteção ao óleo, de forma diretamente dependente da concentração adicionada. A adição de 6% de pimenta ao óleo foi capaz de proteger de forma significativa o produto, demonstrando-se uma estratégia acessível, tanto para ambientes domésticos como comerciais, como forma de mitigar as perdas sofridas e preservar o valor nutricional e a segurança do produto quando há incidência de luz durante o armazenamento.

Além disso, sugere-se estudos futuros para avaliação da migração dos compostos bioativos da pimenta rosa para o óleo de soja nas condições de luz e temperaturas estudadas, para determinação dos compostos responsáveis pela ação antioxidante nas referidas condições. Também se sugere a avaliação da eficácia antioxidante do óleo após armazenamento submetido a termo-oxidação, em condições geralmente utilizadas para o óleo de soja.