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Tenebrio molitor-based edible lipids: Alternative extraction methods and evaluation of their biological properties

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O presente trabalho em nível de Doutorado foi avaliado e aprovado, em 28 de maio de 2024, pela banca examinadora composta pelos seguintes membros:

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Profª. Débora de Oliveira, Drª.
Orientadora

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Dedico aos meus pais, Cláudio e
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RESUMO

O inseto comestível *Tenebrio molitor* é uma promissora fonte alternativa de compostos bioativos. Sua composição nutricional inclui moléculas de alto valor agregado, principalmente proteínas e lipídios. Os lipídios do *T. molitor* são compostos majoritariamente por ácidos graxos saturados, poli-insaturados e monoinsaturados. Nesse sentido, o Capítulo 2 apresenta um artigo de revisão abordando o *T. molitor* como uma fonte promissora de compostos de interesse tecnológico, aplicada ao conceito de biorrefinaria No Capítulo 3 foram investigados métodos de extração ambientalmente favoráveis de lipídios a partir do *T. molitor*, a citar: fluido supercrítico (SC), banho de ultrassom (BU) e ponteira de ultrassom (PU); e comparados ao Soxhlet (SOX) (método controle). Esses métodos foram avaliados em diferentes solventes (hexano, éter de petróleo e etanol), tempos (5 min, 10 min, 15 min, 30 min, 74 min e 6 h) e temperaturas (temperatura ambiente, 45 °C e 70 °C). Os resultados foram promissores, visto que as extrações alternativas apresentaram rendimentos próximos aos valores obtidos pela extração com solvente orgânico (SOX). Além disso, os métodos de extração alternativos apresentaram maior produtividade (extração/tempo) em temperaturas mais amenas, como o método PU realizado em temperatura ambiente. De modo geral, os métodos alternativos de extração não afetaram as características fundamentais dos lipídios, tais como a composição. Assim, os ácidos majoritários foram os ácidos graxos oleico, linoleico e palmítico, respectivamente, caracterizados por GC-MS. Para avaliar a qualidade lipídica, foi realizada a determinação de ácidos graxos livres (AGL). Os lipídios de *T. molitor* extraídos por métodos alternativos apresentaram valores próximos aos obtidos pelo método convencional de SOX utilizando hexano como solvente. Portanto, os métodos de extração não interferiram na qualidade lipídica de ácidos graxos livres. A estabilidade oxidativa dos lipídios obtidos foi analisada, e a análise dos dados indicou que os lipídios extraídos apresentaram um tempo de indução (SC = 1,23 h, PU = 0,60 h, BU (hexano) = 0,27 h e BU (etanol) = 0,23 h) inferior ao SOX (1,64 h), porém, há significativa vantagem, em termo de qualidade e aplicação do lipídio obtido por meio dos métodos de extração alternativos. Foi avaliada a bioatividade dos lipídios extraídos através da análise da atividade antioxidante (DPPH e ABTS). Todos os óleos exibiram atividade antioxidante, os métodos de extração BU (DPPH = 3,56 μmol/g, ABTS = 3,86 μmol/g) e PU (DPPH = 2,07 μmol/g, ABTS = 3,32 μmol/g) demostraram uma atividade superior em relação ao método SOX (DPPH = 1,49 μmol/g, ABTS = 2,23 μmol/g e o método SC (DPPH = 0,17 μmol/g, ABTS = 0,28 μmol/g) menores valores em relação aos outros métodos. A atividade biológica dos óleos extraídos foi mensurada pelo ensaio de citotoxicidade e atividades anticancerígenas. Os óleos testados não apresentaram citotoxicidade nas células L929, as células permaneceram com atividade metabólica acima de 90% para ambos os métodos de extração com concentrações entre 10 e 1000 μg/mL. O óleo do método SOX induziu uma maior redução da viabilidade celular das células A549 e GL. O óleo do método SC também apresentou uma atividade anticancerígena, mas com uma menor redução quando comparado ao SOX. Outro ponto importante avaliado a partir dessas extrações foi a qualidade das proteínas recuperadas, medida através da solubilidade. Os métodos alternativos alteraram minimamente a estrutura proteica de *T. molitor*, em todos os métodos de extração as proteínas apresentaram aumento da solubilidade em pH alcalino, dados semelhantes ao comportamento do método controle SOX. Além disso, a temperatura de desnaturação da proteína foi determinada por DSC. Os métodos alternativos atingiram temperaturas de desnaturação inferiores (SC= 43,17 °C, BU=52,96 °C, PU = 59,19 °C) às temperaturas a partir da extração por SOX (66,86 °C). Portanto, lipídios bioativos do *T. molitor* podem ser obtidos por métodos de extração alternativos, sem interferência na qualidade da farinha desengordurada, principalmente das proteínas, contribuindo, desta forma, para a cadeia produtiva de insetos comestíveis.

Palavras-chave: *Tenebrio molitor*. Óleo. Extrações alternativas. Atividade bioativa. Atividade antitumoral.

RESUMO EXPANDIDO

Lipídios comestíveis à base de *Tenebrio molitor*: métodos alternativos de extração e avaliação de suas propriedades biológicas

INTRODUÇÃO

Insetos comestíveis, especialmente *Tenebrio molitor* L., são promissoras fontes de matéria-prima para a indústria alimentícia. Os insetos comestíveis apresentam grandes vantagens devido ao seu alto valor nutricional (JANTZEN et al., 2020), em especial proteínas, aminoácidos, lipídios, carboidratos e vitaminas (RUMBOS et al., 2020). Vale ressaltar que uma das vantagens do *T. molitor* é sua tolerância a diferentes condições ambientais, além de não necessitar de grande área para produção (SON et al., 2020b). Principalmente na fase larval, o *T. molitor* é considerado um alimento adequado para incorporação nas indústrias alimentícias e de rações para animais, com alto teor de proteínas e lipídios (GHOSH et al., 2017).

Os lipídios do *T. molitor* são, geralmente, considerados um subproduto da extração de proteínas e durante esse processo, e podem sofrer alterações estruturais (nutricionais) dependendo do método de extração utilizado, podendo alterar sabor, estabilidade e pureza (FERREIRA et al., 2022). O método de extração mais convencional é o Soxhlet, que utiliza solventes orgânicos em altas temperaturas (TZOMPA-SOSA et al., 2014), podendo conduzir à degradação de alguns compostos importantes, além da possível presença de resíduos de solventes presentes no óleo (DUBA; FIORI, 2015). Assim, métodos alternativos de extração são utilizados a fim de reduzir ou eliminar os efeitos desfavoráveis desse processo.

Alguns métodos alternativos de extração apresentam vantagens significativas sobre o SOX, por exemplo, a extração por fluido supercrítico, banho de ultrassom e ponteira de ultrassom, tecnologias em rápido desenvolvimento para melhorar a eficiência da extração, especialmente de compostos bioativos (WEN et al., 2018), que demandam menor tempo de extração, uso de solventes mais sustentáveis e temperaturas mais amenas.

Trabalhos presentes na literatura científica utilizaram a PU para obter extratos lipídicos de *T. molitor* e descreveram que o óleo apresentou efeito antioxidante com a inibição próxima a 80% do radical DPPH, assim como, lipídios de *T. molitor* extraído por SOX que apresentou efeitos anticancerígeno, inibindo o crescimento de células HepG2 e Caco-2, com valores de IC_{50} de 0,98% e 0,37%, respectivamente (HIERRO et al., 2020; WU et al., 2020a). Assim, como o objetivo de avaliar as propriedades bioativas dos lipídios de *T. molitor*, métodos alternativos de extração de lipídios, mais especificamente SC, BU e PU foram investigados, sendo avaliados o rendimento, estabilidade oxidativa e propriedades biológicas, incluindo atividades antioxidantes e efeitos anticancerígenos *in vitro* com células de linhagem celular de adenocarcinoma de pulmão humano e linhagem celular de glioma cerebral e então comparado com o método tradicional SOX.

OBJETIVOS

O objetivo geral deste trabalho foi investigar técnicas de extração alternativas dos lipídios do *T. molitor* e, em seguida, avaliar as atividades biológicas dos extratos obtidos.

Os objetivos específicos foram:

I. Avaliar métodos alternativos (fluido supercrítico, assistido por banho de ultrassom e assistido por sonda de ultrassom) de extração de óleo do *T. molitor* e comparar com o método tradicional (SOX) quanto ao perfil de ácidos graxos e estabilidade oxidativa dos óleos obtidos.

II. Avaliar o efeito do método de extração na atividade antioxidante do óleo por DPPH e ABTS.

III. Avaliar as atividades biológicas *in vitro* do óleo de *T. molitor* quanto à citotoxicidade e atividade antitumoral.

IV. Analisar a influência dos métodos alternativos em relação às proteínas desengorduradas obtidas após os processos de extração em relação ao teor de proteínas, solubilidade e sua estabilidade térmica, bem como a morfologia.

METODOLOGIA

Capítulo 2: Referente à revisão da literatura, onde parte deste capítulo foi publicado na revista "*Trends in Food Science & Technology*", como: "*Perspective on integrated biorefinery for biomass valorization from the edible insect Tenebrio molitor*" (https://doi.org/ 10.1016/j.tifs.2021.07.012). Os temas abordados englobam as principais informações sobre a composição nutricional do *Tenebrio molitor*, compostos bioativos presentes no inseto e alguns métodos não convencionais de extração de proteínas e lipídios. Por fim, apresenta-se o conceito de biorrefinaria aplicado ao *Tenebrio molitor* e uma breve revisão das principais técnicas de caracterização de materiais e potenciais aplicações.

Capítulo 3: Etapa experimental referente à extração dos lipídios, desde a obtenção das larvas do inseto *Tenebrio molitor*, congelamento (abate) e produção da farinha do inseto. Para a extração dos óleos, as farinhas de insetos foram submetidas a quatro métodos de extração: SOX, SC, BU e PU. O óleo foi extraído em aparelho Soxhlet por 6 h usando hexano ou éter de petróleo como solventes, imerso em manta de aquecimento a 60 - 70 °C. A extração por CO_2 supercrítico foi realizada em uma unidade de escala laboratorial, os experimentos foram realizados a uma taxa de fluxo de solvente constante de 0,8 kg CO_2/h. O tempo de extração foi fixado em 74 min de acordo com uma curva de extração cinética e as condições experimentais utilizadas foram 300 bar/45 °C, 300 bar/70 °C, 200 bar/45 °C e 200 bar/70 °C. Para extrações por BU, foi utilizado um sistema de banho de ultrassom (SSBu- 3,8 L, SolidSteel), operando a 40 kHz, e a extração foi realizada a 5, 15 e 30 min a uma temperatura de aproximadamente 45 °C, usando hexano, éter de petróleo ou etanol como solvente. As extrações PU foram realizadas etanol (99,95%), submetida a sonda ultrassônica por 5, 10 e 15 min em temperatura ambiente com amplitude de saída de sonicação de cerca de 20% em pulso contínuo por sonicação direta a 20 kHz. Foram calculados o rendimento de extração de óleo, análise da composição de ácidos graxos dos óleos extraídos por GC-MS, e para avaliar as propriedades do óleo foram realizadas as análises de determinação de ácidos graxos livres (FFA) por titulação e a estabilidade oxidativa do óleo, pelo método AOCS Cd 12b-92 (AOCS, 1997) em um equipamento Professional Biodiesel Rancimat 893 (Metrohm, Suíça). A atividade antioxidante dos lipídios foi medida pelo ensaio DPPḢ, solubilizado em etanol: hexano (70:30) e a atividade sequestrante de cátions do radical ABTS ($ABTS^{\bullet+}$). Os experimentos *in vitro*, foi estudada a citotoxicidade dos lipídios usando células L929 e analisada pelo ensaio MTS (CellTiter Aqueous One Solution; Promega). Para o estudo da atividade antitumoral, Linha celular de adenocarcinoma

de pulmão humano NSCLC (células A549) e linhagem celular de glioma cerebral (GL) foram usados como linhas celulares de câncer. A atividade antitumoral dos óleos foi analisada pelo ensaio MTS. Em paralelo, a caracterização da farinha desengordurada rica em proteínas foi realizada. Após extraído o óleo por diferentes métodos foram calculados o percentual de farinha desengordurada, o teor de nitrogênio total da farinha bruta e da farinha após os processos de extração foram analisados pelo protocolo de Kjeldahl segundo o método 928.08 (AOAC, 2000). O teor de proteína total foi calculado usando o fator de conversão de nitrogênio (N) de 6,25, convencional para alimentos e usando fatores de conversão especifica de insetos: 5,60 e 5,33. A caracterização da farinha desengordurada foi realizada através da análise de solubilidade e análise da estabilidade térmica das proteínas presentes na farinha, o perfil de desnaturação da farinha desengordurada (com proteínas mais concentradas) foi realizado de acordo com BAIGTS-ALLENDE et al. (2021) por meio de análise DSC (Jade-DSC Model, Perkin Elmer), para avaliar se algum método de extração de óleo causou alguma alteração nesta propriedade.

RESULTADOS E DISCUSSÃO

Os resultados referentes à etapa experimental serão apresentados a seguir. Em geral, as variações dos parâmetros analisados para cada método de extração apresentaram baixa correlação com o rendimento da extração de óleo. Os rendimentos de óleo das extrações de BU, comparando com o rendimento obtido pela extração convencional SOX usando éter de petróleo como solvente (38,84% ± 2,57), atingiram de ~ 49,88% a ~81% desse rendimento, valores consideráveis considerando as condições do processo. Na extração por SC obteve-se um melhor rendimento de óleo (31,66% ± 0,15) na extração a 300 bar e 70 °C. Em comparação com o método SOX que exigiu um tempo de extração maior (6 h), as extrações SC apresentam melhores resultados em termos de produtividade. O rendimento lipídico variou de 19,12% ± 0,007 (SC_200 bar_70 °C) a 38,83% ± 2,57 (SOX_E). Portanto, em relação aos rendimentos, os métodos considerados não convencionais apresentaram comportamento semelhante ao método SOX convencional, com destaque para a extração com sonda ultrassônica, que atingiu valores mais próximos do SOX (PU_E_Amb_15 min ≈ SOX_H_65 °C_6h).

A análise de CG-MS dos óleos extraídos identificou e quantificou os principais ácidos graxos de *T. molitor*, os mais abundantes foram: ácido oleico (C18:1) (39,97% a 41,35%), ácido linoleico (C 18:2) (24,20% a 25,06%) e ácido palmítico (C16:0) (14,97% a 15,55%). Esses resultados foram semelhantes aos reportados por Paul et al. (2017); Tzompa-Sosa, Yi, van Valenberg, van Boekel & Lakemont (2014), onde larvas do *T. molitor* foram extraídas com clorofórmio:metal e SOX, com rendimentos de 31,97% de conteúdo lipídico e 12,7 g/100 g de inseto fresco, respectivamente. Os métodos alternativos não modificaram a composição do óleo extraído em comparação com o método SOX, o que significa que a composição do óleo de *T. molitor* é estável e que a substituição de qualquer método de extração realizado não causará alterações em seus ácidos graxos. Os óleos de *T. molitor* extraídos por métodos não convencionais apresentaram 2,15% ± 0,23, 2,16% ± 0,18, 1,94% ± 0,03 e 2,19% ± 0,03 de FFA quando obtidos por extração com BU (hexano), BU (etanol), SC e PU, respectivamente. Os valores de FFA não mostraram diferenças relevantes entre os óleos extraídos com hexano e os métodos alternativos. As extrações alternativas apresentaram uma estabilidade oxidativa menor em comparação com extração SOX (1,64 h), com um período de indução de BU (hexano) = 0,27 h, BU (etanol) = 0,23 h, PU = 0,60 h e o método com o valor mais próximo ao SOX foi o SC = 1,23 h, demostrando uma vantagem em termo de qualidade e aplicação que esse método alternativo pode apresentar para o óleo.

Em geral, os extratos obtidos via extrações alternativas (BU e PU) demostraram uma atividade antioxidante igual ou superior em relação às obtidas a partir dos extratos referentes ao método SOX. Através do ensaio de DPPH os resultados foram de: SOX = 1,49 μmol/g, SC

= 0,17 µmol/g, BU (hexano) = 1,47 µmol/g, BU (etanol) = 3,56 µmol/g e PU = 2,07 µmol/g. Com base no ensaio da eliminação de cátions radicais ABTS, os resultados foram semelhantes ao ensaio anterior: SOX = 2,23µmol/g, SC = 0,28 µmol/g, BU (hexano) = 2,01 µmol/g, BU (etanol) = 3,86 µmol/g e PU = 3,32 µmol/g.

Em relação aos ensaios de citotoxicidade, segundo a ISO 10993/5 as células L929 permaneceram com a atividade metabólica acima 90% para o tratamento com concentrações de 10, 100, 250, 500, 750 e 1000 µg/mL dos óleos obtidos pelos dois métodos de extração, demonstrando sua propriedade não tóxica.

Em relação aos ensaios da atividade anticâncer, o óleo extraído por SOX apresentou uma redução dependente da célula testada e da concentração utilizada. Na concentração de 500, 750 e 1000 µg/mL apresentou uma redução de 10,56; 38,09 e 66,63%, respectivamente, na atividade metabólica da célula A549. A atividade metabólica da célula GL, na concentração de 500, 750 e 1.000 µg/mL apresentou uma redução de 45,36; 71,18 e 83,95%, respectivamente. O óleo extraído pelo método SC também apresentou uma atividade anticancerígena, mas com uma menor redução quando comparado ao SOX, a maior redução da viabilidade celular das células A549 foi de 23,48 % (500 µg/mL) e das células GL foi de 33,95% (750 µg/mL).

Os resultados obtidos na avaliação das propriedades bioativas do óleo revelaram aspectos promissores, os óleos não demonstraram citotoxicidade indicando um perfil de segurança relevante para possíveis aplicações. Além disso, a observação de atividade anticâncer nos óleos destaca seu potencial como agentes biologicamente ativos para possíveis aplicações terapêuticas. Em relação à caracterização da farinha desengordurada, o principal componente da farinha desengordurada obtida de *T. molitor* foram as proteínas. O teor de proteína atingiu a 71,8% (Pu_E_15 min). O teor de proteína bruta foi de 44,2 (g/100 g) ± 0,29, valor que está de acordo com os valores já obtidos em outros estudos com *T. molitor*. Após os processos de extração do óleo desse farelo, o teor de proteína bruta aumentou na faixa de 23,49 a 62,00%. O método de tratamento alterou minimamente a estrutura da proteína, em relação a análise de solubilidade. Como esperado, o pH teve um impacto significativo na solubilidade da proteína, um aumento na solubilidade foi observado à medida que o pH aumentou, indicando que a proteína de *T. molitor* pode ser solubilizada de forma mais efetiva em pH neutro ou alcalino. Temperaturas de desnaturação mais baixas foram encontradas por métodos não convencionais (SC= 43,17 °C, BU= 52,96 °C, PU= 59,19 °C) em relação ao SOX (66,86 °C). O método que mais impactou na temperatura de desnaturação foi o SC, isso pode estar relacionado à despressurização sofrida durante o processo de extração, que de alguma forma pode ter afetado esse resultado. No entanto, maiores valores de entalpia foram observados nos métodos PU (59,65 J/g), BU (46,25 J/g) e SC (25,55 J/g) em relação ao SOX (16,20 J/g). Segundo o estudo de BAIGTS-ALLENDE et al. (2021), provavelmente durante o aquecimento maiores ligações de hidrogênio foram quebradas ao longo do desdobramento das proteínas nos métodos não convencionais do que no método SOX.

CONCLUSÃO

Os lipídios obtidos do *T. molitor* foram abundantes em ácido oleico (2.524,94 a 3.073,29 mg/g), com destaque para o método de extração alternativo SC que apresentou um rendimento de 31,66 % e uma estabilidade oxidativa de 1,23 h a 110 °C, valores próximos ao método tradicional. Os lipídios extraídos revelaram propriedades notáveis, incluindo atividade antioxidante e anticancerígena. A observação da propriedade não tóxica em relação às células L929 destaca a segurança desses lipídios, reforçando a viabilidade de sua incorporação em produtos finais. Em relação das suas propriedades bioativas, atingiu uma redução de 83,95% da

viabilidade celular das células GL utilizando uma concentração de óleo de 1000 μg/mL. Em relação à farinha desengordurada, essa apresentou alto teor de proteínas, após os processos alcançou uma porcentagem de até 71,8% de proteínas. Além disso, observou-se que os métodos alternativos de extração de lipídios não impactaram a solubilidade das proteínas, sendo o pH o principal fator influenciador. Portanto, a aplicação do conceito de biorrefinaria ao *T. molitor* proporciona uma abordagem sustentável para a produção de alimentos, além de ser uma estratégia eficiente para a obtenção de produtos bioativos de boa qualidade, ajustando-se às demandas crescentes por inovações de saúde e responsabilidade ambiental.

Palavras-chave: *Tenebrio molitor*. Óleo. Extrações não convencionais. Atividade bioativa. Atividade antitumoral.

ABSTRACT

The edible insect *Tenebrio molitor* is a promising alternative source of bioactive compounds. Its nutritional composition includes molecules with high added value, mainly proteins and lipids. *T. molitor* lipids are mainly composed of saturated, polyunsaturated, and monounsaturated fatty acids. In this sense, Chapter 2 presents a review article addressing *T. molitor* as a promising source of compounds of technological interest, applied to the concept of biorefinery. In Chapter 3, environmentally favorable extraction methods of lipids from *T. molitor* were investigated: supercritical fluid (SC), ultrasound bath (BU) and ultrasound probe (PU); and compared to Soxhlet (SOX) (control method). These methods were evaluated in different solvents (hexane, petroleum ether and ethanol), times (5 min, 10 min, 15 min, 30 min, 74 min and 6 h) and temperatures (room temperature, 45 °C and 70 °C). The results were promising, since alternative extractions presented yields close to the values obtained by extraction with organic solvent (SOX). Furthermore, alternative extraction methods showed higher productivity (extraction/time) at milder temperatures, such as the PU method carried out at room temperature. In general, alternative extraction methods did not affect the fundamental characteristics of the lipids, such as composition. Thus, the majority acids were oleic, linoleic, and palmitic fatty acids, respectively, characterized by GC-MS. To evaluate lipid quality, free fatty acids (FFA) were determined. *T. molitor* lipids extracted by alternative methods presented values close to those obtained by the conventional SOX method using hexane as solvent. Therefore, the extraction methods did not interfere with the lipid quality of free fatty acids. The oxidative stability of the obtained lipids was analyzed, and data analysis indicated that the extracted lipids presented an induction time (SC = 1.23 h, PU = 0.60 h, BU (hexane) = 0.27 h and BU (ethanol) = 0.23 h) lower than SOX (1.64 h), however, there is a significant advantage, in terms of quality and application of the lipid obtained through alternative extraction methods. The bioactivity of the extracted lipids was evaluated through the analysis of antioxidant activity (DPPH and ABTS). All oils exhibited antioxidant activity, the BU (DPPH = 3.56 μmol/g, ABTS = 3.86 μmol/g) and PU (DPPH = 2.07 μmol/g, ABTS = 3.32 μmol/g) extraction methods demonstrated superior activity compared to the SOX method (DPPH = 1.49 μmol/g, ABTS = 2.23 μmol/g) and the SC method (DPPH = 0.17 μmol/g, ABTS = 0.28 μmol/g) lower values compared to other methods. The biological activity of the extracted oils was measured by cytotoxicity assay and anticancer activities. The tested oils did not show cytotoxicity in L929 cells, the cells remained with metabolic activity above 90% for both extraction methods with concentrations between 10 and 1000 μg/mL. SOX method oil induced a greater reduction in cell viability of A549 and GL cells. The oil from the SC method also showed anticancer activity, but with a smaller reduction when compared to SOX. Another important point assessed from these extractions was the quality of the recovered proteins, measured through solubility. The alternative methods minimally altered the protein structure of *T. molitor*, in all extraction methods the proteins showed increased solubility in alkaline pH, data similar to the behavior of the SOX control method. Furthermore, the protein denaturation temperature was determined by DSC. Alternative methods achieved lower denaturation temperatures (SC= 43.17 °C, BU=52.96 °C, PU = 59.19 °C) than the temperatures from SOX extraction (66.86 °C). Therefore, bioactive lipids from *T. molitor* can be obtained by alternative extraction methods, without interfering with the quality of the defatted meal, especially proteins, thus contributing to the production chain of edible insects.

Keywords: *Tenebrio molitor*. Oil. Alternative extractions. Bioactive activity. Antitumoral activity.

LIST OF FIGURES

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LIST OF ABBREVIATIONS

SOX	Soxhlet
SC	Supercritical fluid
BU	Ultrasound bath
PU	Ultrasound probe
HepG2	Hepatocellular Carcinoma cell line
Caco-2	Human epithelial cell line
CFU	Colony Forming Units
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2,2-azino-bis (3-ethylbenzothiazolin) -6-sulfonic acid
ACE	Angiotensin I-converting enzyme
IC50	Half Maximal Inhibitory Concentration
MTS	3-(4,5-Dimethylthiazol-2-Yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2h-Tetrazolium
FAME	Fatty acid methyl esters
DSC	Differential Scanning Calorimeters
GC/ MS	Gas chromatography-mass spectrometry

SUMMARY

CONCEPTUAL DIAGRAM

Tenebrio molitor-based edible lipids:
Alternative extraction methods and evaluation of their biological properties.

Why?

- *Tenebrio molitor* is an edible insect abundantly in nutrients.
- *T. molitor* is one of the most promising edible insects for large-scale production, it has a short development cycle.
- The valorization of *T. molitor* biomass through its conversion into valuable components, in which the extraction of oil (lipids) from insects does not compromise the protein fraction.
- The lipid fraction of *T. molitor* larvae demonstrates remarkable potential for several applications, being a promising source of bioactive compounds.

What has already been done?

- According to the Scopus database, the main focuses of *T. molitor* studies are associated with it as a food source and a substitute for traditional animal food.
- Previous studies showed that *T. molitor* oil, extracted by Soxhlet extraction, inhibited the growth of human hepatocellular carcinoma (HepG2) and colorectal adenocarcinoma (Caco-2) cells.

Study hypothesis.

- Alternative extraction methods, supercritical fluid, ultrasound bath and ultrasound probe, to obtain different molecules of high added value, simultaneously, from *T. molitor*.
- *T. molitor* oil has anti-cancer activity.

Experimental methods

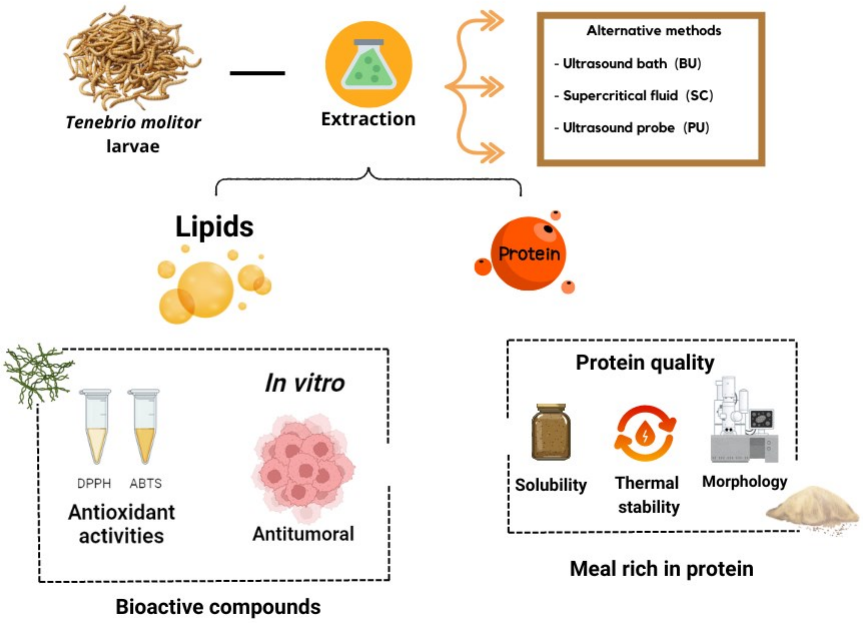
- Evaluate the yield of extractions using alternative methods and compare them with the traditional Soxhlet method.
- Analyze the influence of alternative methods on oils obtained after extraction: Identification of fatty acids, percentage of free fatty acids and the oxidative stability of the oils.
- Evaluate the effect of the unconventional extraction method determined on the antioxidant activity of the oil by DPPH and ABTS.

- Evaluate the in vitro biological activities of *T. molitor* oil regarding cytotoxicity and antitumor activity against the human lung adenocarcinoma cell line NSCLC (A549 cells) and brain glioma (GL) cell line.
- Analyze the influence of alternative methods on defatted proteins obtained after extraction processes: protein content, solubility and thermal stability, and morphology.

Answers

- Do the alternative methods present any differences in relation to the Soxhlet method, regarding the oil yield and its characterization?
- What is the effect of the alternative extraction method on the antioxidant activity of the oil by DPPH and ABTS?
- Does *T. molitor* oil show cytotoxicity?
- Does *T. molitor* oil inhibit the growth of cells from human lung adenocarcinoma cell line NSCLC (A549 cells) and brain glioma cell line (GL cells)?
- Do the oil extraction methods induce any damage to the proteins?

SCHEMATIC DIAGRAM OF THE EXPERIMENTAL ASSAYS



1 CHAPTER 1: INTRODUCTION

Edible insects, especially *Tenebrio molitor* L., are promising food source in the food industry. Edible insects present great advantages due to their high nutritional value (JANTZEN et al., 2020), in particular proteins, amino acids, lipids, carbohydrates, and vitamins (RUMBOS et al., 2020). It is worth noting that one of the advantages of *T. molitor* is its tolerance to different environmental conditions, in addition it does not require a large area for breeding (SON et al., 2020b). Mainly, at the larval stage, *T. molitor* is considered a suitable food, with a high content of proteins and lipids (GHOSH et al., 2017).

T. molitor lipids are usually a by-product of protein extraction and during this process, may suffer nutritional changes depending on the method used, which may alter flavor, stability, and purity (FERREIRA et al., 2022). The most conventional extraction method is Soxhlet, which uses organic solvents at high temperatures (TZOMPA-SOSA et al., 2014), making it possible to degrade some important compounds, in addition to the possible presence of solvent residues present in the oil (DUBA; FIORI, 2015). Thus, alternative extraction methods are used to reduce or eliminate these unfavorable effects in this process.

In this sense, some alternative extraction methods have significant advantages over Soxhlet, for instance CO_2 supercritical extraction and ultrasound-assisted extraction, a rapidly developing technology to improve extraction efficiency, especially of bioactive compounds (WEN et al., 2018), that demand shorter extraction time, use of cleaner solvents and milder temperatures.

In this sense, the work carried out by HIERRO et al. (2020), used the ultrasound method using ethanol as a solvent to obtain extracts from *T. molitor* that showed antioxidant activity and lipase inhibitory activity. To validate the hypothesis of lipids as a bioactive component, according to HIERRO et al. (2020) *T. molitor* oil, extracted by Soxhlet, showed anticancer effects in in vitro assays, inhibiting the growth of HepG2 and Caco-2 cells, with IC_{50} values of 0.98% and 0.37%, respectively.

To achieve the industrial applications of *T. molitor*-based lipids at industrial scale, it is necessary to investigate extraction methods and their yields, the oxidative stability of *T. molitor*-based lipids and their biological properties.

1.1 OBJECTIVES

1.1.1 General objective

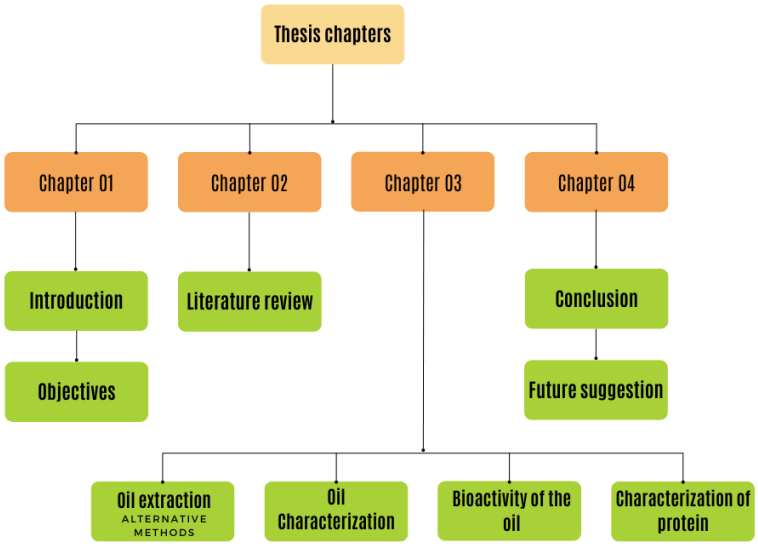
The aim of this thesis is to investigate alternative extraction techniques for *T. molitor-based lipids* and the biological activities of the different extracts.

1.1.2 Specific objectives

- Evaluate alternative methods (supercritical fluid, ultrasound bath-assisted and ultrasound probe assisted) of extracting oil from the *T. molitor* and compare with the traditional method (Soxhlet) regarding the fatty acid profile and oxidative stability of obtained oils.
- Evaluate the effect of unconventional extraction method determined on the antioxidant activity of the oil by DPPH and ABTS.
- Evaluate the *in vitro* biological activities of *T. molitor* oil regarding cytotoxicity and antitumoral activity.
- Analyze the influence of alternative methods on defatted proteins obtained after extraction processes: protein content, solubility, and thermal stability, as well as morphology.

1.2 THESIS OUTLINE

The structure of the thesis is presented below:



Chapter 02: Literature review

In this chapter, an approach is made to *Tenebrio molitor*: nutritional composition, reproduction, and food safety. An assessment is made on the biorefinery proposal: alternative and ecologically favorable extraction processes are suggested to extract the lipid part and simultaneously, protein recovery.

This chapter was published in Trends in Food Science & Technology Journal.

DA COSTA ROCHA, Ana Cláudia; DE ANDRADE, Cristiano José; DE OLIVEIRA, Débora. Perspective on integrated biorefinery for valorization of biomass from the edible insect Tenebrio molitor. **Trends in Food Science & Technology**, v. 116, p. 480-491, 2021.

Chapter 03: Extraction yield, fatty acid profile, properties, and bioactive evaluation of lipids from *Tenebrio molitor*: comparison among alternative extraction methods and Soxhlet

This chapter presents the main results obtained in the experimental stage of lipid extraction from *T. molitor*. The insect oil was extracted by different extraction methods: supercritical fluid, ultrasound bath and ultrasound probe. The yield, fatty acid composition, oxidative stability and percentage of free fatty acids were analyzed. The bioactivity of the extracted oil was also analyzed, such as antioxidant activity, cytotoxicity, and antitumor activity. At the same time, the characterization of defatted meal rich in proteins was carried out.

2 CHAPTER 2: LITERATURE REVIEW

This chapter presents a review of the available literature on the subjects covered in this work. Part of this chapter was published in Trends in Food Science & Technology, volume 116, October 2021, pages 480-491 as: "Perspective on integrated biorefinery for biomass valorization from the edible insect *Tenebrio molitor*" (https://doi.org/10.1016/j.tifs.2021.07.012).

Firstly, the main information on the nutritional composition of *T. molitor*, bioactive compounds present in the insect, and some non-conventional methods of protein and lipids extraction is presented. Finally, biorefinery concept applied to *T. molitor* and a brief review of the main material characterization techniques and potential applications are also discussed.

2.1 INTRODUCTION

The world population increased from 1 billion in 1800 to 7.7 billion today, and it is still growing (the current world population growth rate is 1.05 per year). Thus, the challenges correlated to food production, especially in underdeveloped countries, have been, proportionally, expanding. In this sense, the demand for protein ingredients is expected to enhance at a rate of 9.1% from 2020 to 2027 (PAM ISMAIL et al., 2020), and according to the FAO (Food and Agriculture Organization of The United Nations), world consumption of beef, pork, and chicken will increase 1.4% per year until 2024. Therefore, extensive research to find new mainly sustainable protein sources is needed.

Edible insects are an alternative to replace traditional and unsustainable food sources, mainly protein-based sources. According to (HUIS et al., 2013), the potential benefits of using insects are health, environmental, economic, and social sectors, increasing edible insect chain development, and consequently, global food security. According to the European regulation 2015/2283 (European Parliament and Council of the European Union) "…the basis of scientific and technological developments that have occurred since 1997, is appropriate to review, clarify and update the categories of food which constitute novel foods. Those categories should cover whole insects and their parts…". Therefore, it is inevitable that insect-based foods will be widely marketed. Currently in Brazil, no specific regulation authorizes the production and commercialization of insects for consumption. Companies that intend to use insects in food

manufacturing must request a safety assessment from the National Health Surveillance Agency (ANVISA) (MARQUES et al., 2021).

Biorefinery is an overall concept of a processing plant or strategy where biomass feedstocks are converted and extracted into a wide range spectrum of valuable products (TAKKELLAPATI; LI; GONZALEZ, 2018). Alternative technologies for efficient and environmentally friendly extraction are, ideally, associated with the biorefinery concept (DRAGONE et al., 2020).

In this sense, the insect biorefinery correlates the valorization of biomass by its conversion into chemical compounds that can be used for food ingredients, animal feed, biomaterials, biomolecules, and bioenergy (AZAGOH; HUBERT; MEZDOUR, 2015).

Many species of insects have been consumed, such as *Hermetia illucens*, *Bombyx mori*, *Acheta domesticus*, *Allomyrina dichotoma*, *Gryllus bimaculatus*, among others (DE CASTRO et al., 2018; IMATHIU, 2020). *T. molitor* is one of the most promising edible insects for production at a large scale (RUMBOS et al., 2020) since it has a short development cycle (adulthood in 10 weeks and a female can produce 160 eggs in her life cycle), high raw material conversion rate (GRAU; VILCINSKAS; JOOP, 2017; HONG; HAN; KIM, 2020), easy cultivation and does not demand a large area (≈ 3.6 m^2 per year for one kg of larvae) (SON et al., 2020a).

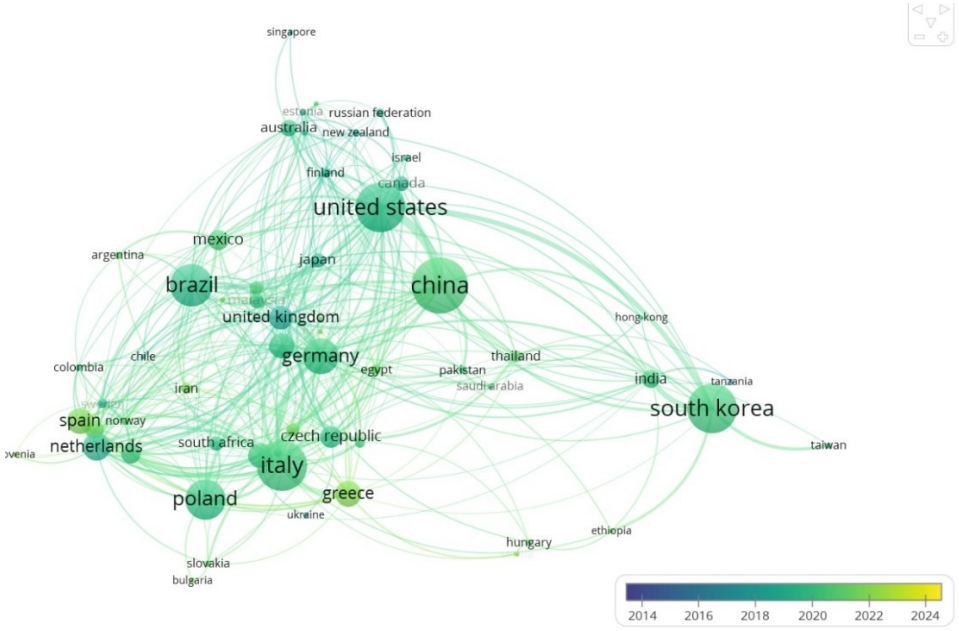
Regarding the breeding/reproduction of the insect *T. molitor*, Oonincx et al. (2010) reported that the amount of greenhouse gases generated is lower (1 mg NH_3/kg body mass/day) when compared to cattle (14-170 mg NH_3/kg body mass/day), and pigs (4,8-75 mg /kg body mass/day). In addition, the CO_2 production (greenhouse gas) by *T. molitor* is lower than cattle and swine, 22.35%, and 16.85%, respectively. *T. molitor* also demands lower space, the land use of production of one kg of fresh *T. molitor* larvae was 3.6 m^2 per year, whose 85% was required to cultivate feed grains, and 14% to produce carrots, 13.6 as less than compared to beef and 3.5 as less to pigs (DE VRIES; DE BOER, 2010; GRAU; VILCINSKAS; JOOP, 2017; OONINCX; DE BOER, 2012) and, water consumption of freshwater (3.5 times lower than that of beef) (MIGLIETTA et al., 2015). Thus, *T. molitor* trends to be more sustainable, when compared to traditional protein sources. In addition, according to the United Nations, in 2024, the world population will reach 8 billion people. Consequently, 1 out of 9 people will not have enough food, in particular proteins. In this sense, *T. molitor* is a very promising alternative. *Tenebrio molitor* can also convert organic wastes into nutritionally rich biomass. (RUSCHIONI et al., 2020) described the production of *T. molitor* larvae by using solid residues from the olive

oil industry. The feeding substrate for the *T. molitor* composed of 25% olive pomace and 75% wheat bran lead to the highest productivity (weight: 0.192 g; rate of survival: 78%; development time: 112 days) and resulted in a significant increase in moisture (21.96%), fiber (16.77% DM), and fat (6.18% DM). (ZHENG et al., 2013) cultivated *T. molitor* fed with decomposing vegetables (carrots, lettuce seeds, and leaves). About 4000 *T. molitor* larvae (0.017 g, individual weight) were fed 5000 g of decomposing vegetables (1.25 g/larva) for 9 weeks, yielding about 704.1 g of fresh insect larvae (0.176 g, individual weight) and 234.8 g of dry biomass.

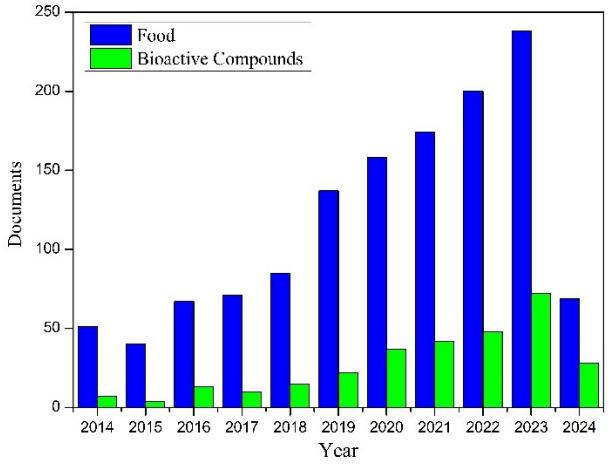
Concerning and trying to show the relevance of this subject in an organized way, a systematic search on the scientific database was performed to identify trends in research using *Tenebrio molitor*. The search using "*Tenebrio molitor*" OR "yellow mealworm" OR "mealworm" keywords in abstract and citation database Scopus (www.scopus.com) resulted in 1,405 documents from 2010 to September 15th, 2020. The countries most related to *Tenebrio molitor* featured in this search were the USA (13.02%), China (11.24%), and Brazil (10.23%). It is important to note that a relevant number of patents related to the insect *T. molitor* are available in the literature. Most of the patents are chinese (≈36.90%) and correlated to methods of producing edible food for animals based on insects. Second, the United States has a significant number of patents, with about 18.54% compared to patents on the insect worldwide.

Figure 1 (a) presents the compilation of this information, showing the number of documents and the number of patents published over the years.

Figure 1. (a) Countries that published the most about *Tenebrio molitor* and (b) comparative chart of document numbers on the main applications of *Tenebrio molitor* for food and as a source of bioactive compounds, published from 2014 to 2024 (January to April) according to Scopus database platform (www.scopus.com).



(a)



(b)

Source: Scopus database, VOSviwer

Another search, aiming to identify the insect-based application, was also carried out at the Scopus platform. The keywords were i) “bioactive compounds” OR “bioactivity” OR “bioactive peptides” OR “bioactive lipids” OR “antithrombotic” OR “inhibitory” OR “hydrolysis” OR “antioxidant” OR “hydrolysates” AND “*Tenebrio molitor*” OR “mealworm” OR “yellow mealworm"; and ii) “food” OR “animal feed” OR “meal” OR “diet” AND "*Tenebrio molitor*” OR “mealworm” OR “yellow mealworm”. The number of documents for each class (bioactive compound or food) is shown in Figure 1 (b).

There is a significant increase in publications related to *T. molitor*. Countries such as United States, China, Brazil, Germany, and South Korea have invested in studies and patents

related to the use of this edible insect. As shown in Figure 1, the main focuses of these studies are associated with *T. molitor* as a source of food and substitute of traditional animal feeds. This raw material is a valuable source of bioactive compounds. Nevertheless, these bioactive compounds have been poorly investigated. They need to be further explored for a valorization of this insect as biomass.

Traditionally, edible insects are used as food snacks or applied to food formulation. In this sense, insect-based products have remarkable biological properties related to unique molecules, for instance, bioactive peptides. (ZIELINSKA; BARANIAK; KARAS, 2017) demonstrated that the *T. molitor* has bioactive peptides with antioxidant activity, which was determined as free radical scavenging activity with an IC_{50} value of 5.3 µg/mL against $ABTS^{\bullet+}$, and anti-inflammatory activity, determined as inhibitory activity of lipoxygenase with an IC_{50} value of 1.57 µg/mL. Similarly, (HIERRO et al., 2020) obtained an inhibitory effect on pancreatic lipase (value around IC_{50} 0.4 mg/mL) from bioactive extracts of the mealworm, obtained by the extraction by pressurized liquid, using aqueous ethanol as a solvent. Whereas Chen, Jiang, Lu, Chen & Huang (2019) (CHEN et al., 2019) demonstrated that antithrombotic peptides were produced from *T. molitor* proteins by enzymatic treatment with commercial enzymes pepsin and trypsin. They obtained the two peptides SLVDAIGMGP and AGFAGDDAPR through separation and purification with potential antithrombotic activity. Besides that, the lipid fraction of *T. molitor* demonstrated the potential to produce natural chemotherapeutic anticancer agents (WU et al., 2020b). Thus, theoretically, it proves the potential of the biorefinery concept for *T. molitor* biomass.

The biorefinery concept applied to edible insects has already been reported (AZAGOH; HUBERT; MEZDOUR, 2015; GIROTTO; COSSU, 2019; RAJENDRAN et al., 2018). However, no data correlated to the biorefinery concept to *T. molitor* was found in the open literature. The extraction of different high added-value molecules, simultaneously, from this insect using green and efficient technologies - biorefinery concept - can make faster the implantation/implementation of this technology worldwide. Therefore, this review aimed to critically discuss the current state of the art and future trends on *T. molitor* as an alternative nutritional source, including proteins, fatty acids, bioactive peptides, minerals, vitamins, among others, and its correlation to biorefinery concept focused on promising mainly in unconventional extraction methods.

2.2 NUTRITIONAL COMPOSITION OF *TENEBRIO MOLITOR*

The mealworm (*T. molitor*) is a typical beetle in the family Tenebrionidae, order Coleoptera, typically found in tropical and temperate regions. It presents a complete metamorphosis divided into 4 phases: egg, larva, pupa, and adult. The life cycle can last up to two years (HILL, 2002).

The *T. molitor* insect is considered a suitable food (larval stage), with a high content of proteins and lipids, including mono and polyunsaturated fatty acids (EFSA, 2015; GHOSH et al., 2017). The larval phase lasts from 90 to 140 days, and the insect larvae length can vary between 12 and 32 mm with a light yellow-brown color (SELALEDI; MBAJIORGU; MABELEBELE, 2020). Some of the factors that influence the development of insects are temperature and humidity. The mealworm insects are ideally breeding at 28 °C, and high humidity (>70%) (JAJIC et al., 2019). According to Morales-Ramos et al. (2019), the optimized production of *T. molitor* biomass could be achieved by selecting specific characteristics, such as the body size. The selection of yellow mealworms with larger pupal size for 8 years showed better performance in the growth rate (8.825 mg/year), fertility (an increase of 213.28 eggs produced), and conversion efficiency (6.2%) of the food eaten when compared to ancestors.

Mealworms are nutritionally rich in proteins, fats, and micronutrients, such as minerals and vitamins. The composition of these nutrients can vary according to environmental factors, such as nutrition/diet and stage of development, and according to processing and production methods. Table 1 shows the average nutritional composition of mealworms.

Table 1. Nutritional composition of mealworms (*T. molitor*).

Crude protein, %	Crude fat %	Crude fiber %	Ash %	Nitrogen-free extract (NFE) %	Moisture %	Reference
53.22	34.54	6.26	4.04	1.94	-	(GHOSH et al., 2017)
46.44	32.7	4.58	2.86	8.09	5.33	(RAVZANAADII et al., 2012)
55.83	25.19	7.15	4.84	3.68	3.31	(JAJIC et al., 2019)
52.1	32.3	-	3.6	11.5	0.5	(SON et al., 2020a)

Source: Author

The protein content of *T. molitor* (13 - 22 g/100 fresh weight) is relatively similar to conventional sources such as beef (23 g/100 g fresh weight) and pork (~22 g/100 fresh weight) (GHOSH et al., 2017; REIG; ARISTOY; TOLDRÁ, 2013; WILLIAMS, 2007). The defatted

mealworm powder obtained by Son, Choi, Hwang, Nho & Kim (2020) (SON et al., 2020a) had 32.3% ± 1.0% of lipid content, which is significantly higher than soybean or meat. Mealworms are also rich in micronutrients, sources of iron, iodine, and magnesium, and high in zinc (NOWAK et al., 2016). The iron and zinc contents found in the insect by Ghosh et al. (2017) (GHOSH et al., 2017) (10.02 and 11.74 mg/100g of dry material, respectively) were higher than that found in chickens, pork, beef, and chicken eggs.

It is important to detail the impact of proteins, fatty acids, vitamins, and minerals on the intestinal microbiota. For instance, Kwon et al. (2020) (KWON et al., 2020) evaluated the prebiotic effect of exuviae of the mealworm in 20% of the diet fed to BALB/mice. Higher concentrations of lactic acid bacteria (4.5 CFU/mL) were found in the intestine of mice fed with mealworm exuviae for 8 weeks, mainly promoting the growth of *Bifidobacteriaceae* and *Lactobacillaceae*, indicating that feeding could positively influence the intestine environment of the mice, suggesting the potential of mealworms as a prebiotic for human intestinal health. De Carvalho et al. (2019) demonstrated by *in vitro* digestion model that *T. molitor* insect flour had a positive impact on the intestinal microbiota, promoting the growth of groups of *Bacteroidaceae* and *Prevotellaceae,* bacteria related to the proteolytic and saccharolytic activity that confer benefits to the host.

The mealworms (*T. molitor*) are excellent alternative sources of proteins and fatty acids, as shown in Table 1, comparable to traditional sources of vegetables and animals. They are not proved toxicity regarding the safety associated with ingesting mealworms and their derivates (CAPPELLI et al., 2020). Nevertheless, they can contaminate themselves by taking up toxic substances from their breeding environment, mainly by their diet. Therefore, they need food surveillance systems, with security in the production chains to guarantee product quality. An important issue is that, according to Csapó, Albert & Csapóné Kiss (2009) (CSAPÓ; ALBERT; CSAPÓNÉ KISS, 2009), insects can contain substantial amounts of D-amino acids that can lead to toxicological effects. In this sense, people allergic to crustaceans and mites can present hives, nausea, abdominal cramps, and vomiting after consuming mealworm. Broekman et al. (2016) (BROEKMAN et al., 2016) reported that 15 patients allergic to shrimp and shrimp/HDM were tested in a trial to assess allergenicity with yellow mealworm. As a result, all patients were identified as sensitive to mealworm ingestion. In particular, the risk could be associated with IgE binding to tropomyosin and arginine kinase, and sarcoplasmic calcium-binding protein, and myosin light chain (shellfish allergens).

2.2.1 Protein

Proteins are large chains of amino acids fundamentals for maintaining and developing physiological functions (SARMADI; ISMAIL, 2010). Protein quality is related to its digestibility and composition of essential amino acids, mainly the balance between essential and non-essential amino acids (RAVI et al., 2020).

The protein content in edible insects is usually obtained by determining the total nitrogen content multiplied by a conversion factor nitrogen (6.25) into the protein (MARIOTTI; TOMÉ; MIRAND, 2008). However, it is often overestimated due to the non-protein nitrogen content present. In this sense, Janssen, Vincken, Van Den Broek, Fogliano & Lakemond (2017) (JANSSEN et al., 2017) determined the specific nitrogen-to-protein conversion factor for *T. molitor* larvae. The authors described that the specific nitrogen-to-protein conversion factors were 4.76 and 5.60 for the whole larvae and soluble protein extracted from the insect, respectively. Similarly, Boulos, Tännler & Nyström (2020) (BOULOS; TÄNNLER; NYSTRÖM, 2020) found an average of 5.33 nitrogen-to-protein conversion factor for *T. molitor* obtained from three European commercial breeders.

According to Wu, Ding, Yin et al. (2020a) (WU et al., 2020c), the essential amino acids (124.86 ± 3.82 mg/g DW) of the mealworms (*T. molitor*) indicated that the quality of the protein was comparable to conventional sources and was rich in sulfur, and sulfur-rich amino acids have great antioxidant potential. Valine, leucine, and lysine were the most abundant essential amino acids, corresponding to 15.15, 17.7, and 12.6% respectively, of the total amino acids. Similarly, Li, Zhao & Liu (2013) (LI; ZHAO; LIU, 2013) proved that the yellow mealworm contains all the essential amino acids necessary for human nutrition. In addition, the mass fraction (essential amino acids/total amino acids) was ≈44.7%.

According to Panini et al. (2017) (PANINI et al., 2017), juvenile shrimp (*L. vannamei*) was not affected by the replacement of fish meal by mealworm meal. However, the digestibility data showed that methionine was the first limiting amino acid of the insect-based meal. On the other hand, Stone, Tanaka & Nickerson (2019) (STONE; TANAKA; NICKERSON, 2019) indicated that the limiting amino acid of *T. molitor* was lysine. In addition, mealworm extracts studied by Zhao, Vázquez-Gutiérrez, Johansson, Landberg & Langton (2016) (ZHAO et al., 2016) contained total sulfur amino acids (methionine + cysteine) lower that recommended level for humans. Considering this information, it is worth to mention that mealworms can be nutritionally supplemented to overcome amino acid content limitations.

Digestibility is also a relevant factor, since it determines the total amount of nutrients that will be absorbed. As reported by Gravel & Doyen (2020) (GRAVEL; DOYEN, 2020), insect proteins have good digestibility, often higher than vegetable proteins. According to Yoo et al. (2019) (YOO et al., 2019), the flour from the larvae of *T. molitor* in the pig diet exhibited an apparent ideal digestibility of lysine, histidine, and arginine greater than when fed fish meal. A negative effect of the D-amino acid configuration is the reduction of protein digestibility, consequently its bioavailability (CSAPÓ; ALBERT; CSAPÓNÉ KISS, 2009). Corrigan & Seinivasant (1966) (CORRIGAN; SEINIVASANT, 1966) evaluated insect species *Tetraopes tetrophthalmus, Labidomera clivicollis,* and *Popillia japonica*. They did not find D-serine, where for *Bombyx morie* D-serine was detected at early larval stages and then increased in the pupal stage. In *T. molitor*, the presence of D-amino acids was beneficial for the organism; the neuropeptide analog proctolin obtained by replacing native L-amino acids with its D-isomers was examined by cardioexcitatory testing in the heart of the yellow mealworm, *in vitro*. The simple replacement of L- by D-configuration at position 1 (arginine), and 3 (leucine), and 4 (proline) led to increased cardioexcitatory effects (KUCZER et al., 1996).

Yi et al. (2013) (YI et al., 2013) determined through the electrophoresis in sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) the distribution of the molecular weight of the *T. molitor* protein fractions. Bands lower than 14 kDa have been associated with antifreeze-type proteins (from 8.5 to 13 kDa), bands between 32 to 95 kDa may be related to enzymes, and other proteins (melanization inhibiting protein, β-glycosidase, trypsin-like proteinases) and molecular weights between 14 and 30 kDa could originate from *T. molitor* cuticle proteins.

Regarding the functional food properties, Zielińska, Karaś & Baraniak (2018) (ZIELIŃSKA; KARAŚ; BARANIAK, 2018) analyzed the solubility of *T. molitor* protein at different pH ranges. According to the results, the protein showed greater solubility at the pH extremes, 97% at pH 11 and 86% at pH 2, and less solubility was found around pH 5 for *T. molitor* (3%), data similar to legumes (kidney bean flour, for example) solubility. The good solubility of *T. molitor* protein in this pH range facilitates its use as a food additive in acidic and alkaline food ingredients.

Gould & Wolf (2018) evaluated the emulsification capacity of the protein extracted from *T. molitor* larvae. Oil/water emulsions were prepared by mixing 80 g of aqueous protein solution at pH 7 with 20 g of sunflower oil. In response, 0.44% w/w based on the aqueous phase of the protein obtained from *T. molitor* was sufficient to obtain a limited process droplet size

spectrum. Under the same process conditions, it was needed 1.75% commercial whey protein to achieve the same situation. Regarding stability, the emulsion microstructure was analyzed and remained unchanged, except for flocculation after heating to 90 °C and at a pH close to the *T. molitor* isoelectric point. Lower concentration of *T. molitor* protein was needed to generate microstructure emulsions like whey protein, representing a promising alternative to protein-based emulsifiers for food formulations.

Therefore, the high protein content of *T. molitor*, which has all the essential amino acids, good digestibility, solubility, and emulsification capacity represents an interesting alternative source for growing consumption and new applications, either as a component of human food or feed or as nutraceutical applications.

2.2.2 Lipids

The lipid fraction of the mealworm insect is composed of saturated, polyunsaturated, and predominantly monosaturated fatty acids, the largest component being oleic acid (C18:1) (35.83 – 49.50 g/100g), then linoleic acid (C18:2) (21.82 – 30.23 g/100g) and palmitic acid (16:0) (16.72 – 21.33 g/100g) (Paul et al., 2017; Ravzanaadii, Kim, Choi, Hong & Kim, 2012; Tzompa-Sosa, Yi, van Valenberg, van Boekel & Lakemont, 2014).

Polyunsaturated acids of mealworms tend to contain high levels of omega 6 (≈ 27.2%). However, a diet enriched with 1% of flaxseed oil reduced the proportion of omega 6/omega 3 (n-6/n-3) from 21.7 to 6.3, close to the recommendation for human health (OONINCX et al., 2020), this ratio is commonly used as a parameter for the quality of these acids, the higher the proportion, the more it is associated with the development of diseases such as cancer and heart disease (PAUL et al., 2017). The n-6/n-3 ratio in the *T. molitor* oil (12.98%) in the (WU et al., 2020c) study was considerably lower than corn oil (64.15%) and olive oil (13.76%). The content of polyunsaturated fatty acids in the mealworm (19.8%) was also higher than pork (15.7%), lamb (2.3%), and beef (1.6%). In addition, the composition of fatty acids in mealworm oil is close to the characteristics of vegetable oil (Son et al., 2020). The most abundant acid, oleic acid, has positive effects on reducing insulin resistance (PALOMER et al., 2018) and has neuroprotective effects (Song et al., 2019).

As the proteins, the lipid fraction of *T. molitor* larvae shows a remarkable potential of applications. In general, extractions converge into just one compound, compromising the achievement and use of the other compounds. With an efficient fractionation of this biomass, it

is possible to guarantee enriched fractions of both proteins and lipids, generating thus a unique chain of by-products that can be used commercially, adding even more value to the insect.

2.2.3 Chitin/Chitosan

The insect exoskeleton is usually composed of chitin (poly (β- (1-4)) - *N* - acetyl -D- glucosamine) (ZHU et al., 2016). The traditional chitin chemical extraction process involves demineralization, deproteination, bleaching, and deacetylation steps operated by alkaline and acid treatment. Chitin is degraded mainly by two biological pathways: chitinase to produce oligomeric beta-*N*-acetylglucosamine and another possible degradation pathway is the deacetylation of chitin by which it is converted into chitosan. These two degradation pathways generally occur simultaneously (ZHANG et al., 2021). Chitosan has been widely applied in environmental and biomedical areas, and mainly by food industries, such as food packaging film, nanocapsules, and nanoparticles.

The content of chitin varies with insect species and its life cycle. According to Adámková et al. (2017) the chitin concentration of *T. molitor* biomass (giant larvae) - dry basis - was ≈6%, whereas the common larvae was ≈13%. Song et al. (2018) also investigated the production of chitin and chitosan from the exuvium and the whole body of *T. molitor*. As a result, the average yields of chitin were 18.01 and 4.92% for exuvium and whole body, respectively. The relative average chitosan yield (whole-body) was 3.65% and, the larval exuvium was 9.20% (based on dry weight).

Chitosan is often produced by deacetylation of chitin derived from crustacean residues. In this sense, *T. molitor* is also a promising alternative to obtaining this compound. For example, the solubility of chitosan prepared from mealworm was 97.4%, higher than that of shrimp (91.5%), demonstrating that the insect's chitosan was more soluble than the shrimp shell chitosan. The ash contents of the mealworm and shrimp shells were measured as 0.50 and 0.95%. The insect's chitosan had a lower ash content, which may be a key factor for the better solubility extracted from the insect (LUO et al., 2019).

Shin, Kim & Shin (2019) obtained chitosan by deacetylation of chitin. The chitin yields were 80, 78.33, and 83.33% in the *T. molitor* larvae, adults, and superworm, respectively. In addition, the author showed, for the first time, that chitosan from mealworm larvae (8% chitosan solution) had antimicrobial activity against pathogenic bacteria *Bacillus cereus*,

Listeria monocytogenes, Escherichia coli, and *Staphylococcus aureus*, with zones of fence inhibition 1–2 mm against the four bacteria.

2.3 BIOACTIVE COMPOUNDS

Bioactive compounds are molecules that interact with living tissue components producing a positive effect on human health (MARTIROSYAN; MILLER, 2018). The main classification of bioactive compounds are phenolic compounds, terpenes and terpenoids, and alkaloids (KHEZERLOU; JAFARI, 2020), and it has gained prominence for the identification, and characterization of these compounds, mainly in alternative sources, such as edible insects, which have promising levels of peptides and lipids (LUCAS et al., 2020).

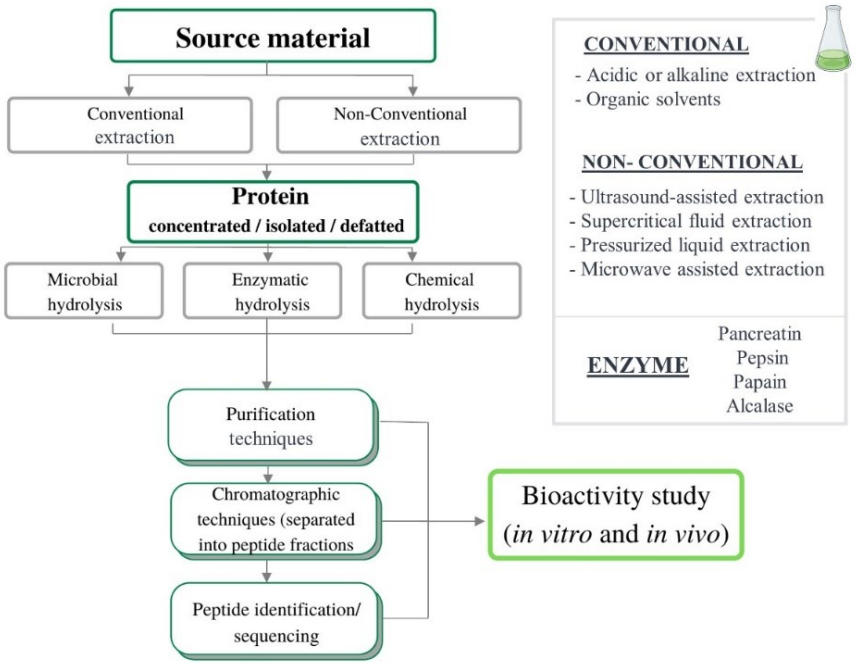
2.3.1 Bioactive peptides

Bioactive peptides have a wide range of biological functions, functional and nutraceutical ingredients, with antioxidant, antihypertensive, anti-inflammatory, antidiabetic characteristics and other activities that are strictly related to their amino acid sequence (DAI et al., 2013; SEO et al., 2017; ZIELINSKA; BARANIAK; KARAS, 2017). Bioactive peptides are inactive in the precursor structure. They can be released through enzymatic processes, microbial fermentation, or chemical hydrolysis (DALIRI; OH; LEE, 2017; OKOLIE et al., 2019; SÁNCHEZ; VÁZQUEZ, 2017). Figure 2 represents a flowchart based on the production of these bioactive peptides.

Enzymatic hydrolysis is the conventional method due to its high specificity and sustainability since it does not generate residues such as organic solvents and chemicals (NONGONIERMA; FITZGERALD, 2017; TU et al., 2018). Some factors influence the production of bioactive peptides by enzymatic hydrolysis, such as protein pre-treatment, pH, enzyme-substrate ratio, temperature, and processing time (NONGONIERMA; FITZGERALD, 2017; OKOLIE et al., 2019). In this sense, a commercial alcalase enzyme purchased from Novo (Seoul, Korea) was used for hydrolysis of the mealworm where new hepatoprotective peptides, Ala-Lys-Lys-His-Lys-Glu and Leu-Glu (CHO; LEE, 2020), and also a new (ACE) inhibitory peptide, Tyr-Ala-Asn (DAI et al., 2013). The hydrolysis of the mealworms with gastrointestinal enzymes (α-amylase, pepsin, pancreatin, and bile extract solution) showed high activity against $ABTS^{+}$ (2,2' -azinobis (3-ethylbenzothiazoline-6- sulfonic acid) (ZIELINSKA; BARANIAK; KARAS, 2017). Yu et al. (2017) evaluated five proteases for obtaining hydrolysates from the

mealworm: alcalase, bromelain, flavourzyme, neutrase, and papain. The degree of hydrolysis of the larvae treated with proteases of plant origin (bromelain and papain) was much lower when compared with the other microbial proteases (alcalase: 4,781.39 µg/mL; flavourzyme: 5,429.35 µg/mL; neutrase: 3,155.55 µg/mL; bromelain: 1,800 µg/mL and papain: 1,782.61 µg/mL).

Figure 2. Flowchart of production of bioactive peptides.



Source: Author

Table 2 presents some biofunctional peptides from protein hydrolysates of the mealworm (*T. molitor*).

Table 2. Bioactive effects from *Tenebrio molitor* biomass.

Bioactivity	Enzyme(s)	Enzyme Origin	Method	DH (%)	Reference
Antithrombotic	Pepsin (0.2 mg/mL pepsin solution (3000 U/mg), 37 °C, 2 h agitation, pH 2.0) and trypsin (0.2 mg/mL trypsin solution (250 U/mg), 37 °C, 5 h agitation, pH 7.5)	Commercial (MP Biomedicals, Santa Ana, CA, EUA)	Degreasing with isopropyl alcohol (in a ratio of 1:5 (w/v), 50 °C, 1 h).	21.5	(CHEN et al., 2019)
Antidiabetic	Flavorsome + Alcalase (Flavourzyme with 30 and 60 U/g protein; Alcalase with 12 and 72 mU/g protein, 55 °C, 8 h agitation)	Commercial (Novozymes, Bagsvaerd, Denmark)	Defatted using hexane;proteins extracted by sonication	-	(YOON et al., 2019)
Antioxidant	Alcalase (added to 1% (w / w), 55 °C ,100 rpm, 24 h)	Commercial (Novo, Seoul, Korea)	Dissolved in 10 mM sodium phosphate buffer (pH 7.0)	42.05	(YU et al., 2017)
Antioxidant	Gastrointestinal enzymes (α-amylase, pepsin, pancreatin, and bile extract solution) (α-amylase from hog pancreas (50 U/mg), pepsin from porcine gastric mucosa (250 U/mg), pancreatin from porcine pancreas, 4%, w/v in stimulated saliva solution, at 37 °C in darkness)	Commercial (Sigma-Marker, Sigma-Aldrich, St. Louis, MI, USA)	Thermal treatment: boiling, baking, and raw proteins extracted by basic extraction (NaOH)	-	(ZIELINSKA; BARANIAK; KARAS, 2017)
Antihypertensive	Trypsin (enzyme/substrate = 3%, pH 8, 50 °C)	Commercial (Nozoymes, Bagsvaerd, Denmark)	-	10	(PINO et al., 2020)
Antihypertensive	Gastrointestinal enzymes pepsin, trypsin, and α-chymotrypsin (To simulate stomach digestion: pH 2, pepsin enzyme/substrate 1:250 w/w, for 2 h 30 min at 37 °C. To simulate the digestive process that occurs in the small intestine: trypsin and α-chymotrypsin enzyme (1:1)/substrate 1:250 w/w) for 2,5 h at 37 °C, pH 6.5)	Commercial (Sigma-Aldrich, St Louis, USA)	Protein extracted with Tris/HCl buffer	-	(CITO et al., 2017a)
Antihypertensive	Alcalase (enzyme to protein ratio was 1:100, w/w) at 50 °C and pH 8.5)	Commercial (Xuemei Enzyme Preparations Science and Technology Co., Ltd., Wuxi, China)	Defatted using petroleum ether	20	(DAI et al., 2013)
Hepatoprotective	Alcalase (enzyme to mealworm ratio was 1:100, w/ v), at 100 rpm, 55 °C, 8 h)	Commercial (Novo, Seoul, Korea)	-	-	(CHO; LEE, 2020)

Antioxidants decrease the oxidation rate by inhibiting free radicals or complexing metals, which are responsible for damaging cells (inflammation). In the study by Zielińska et al. (2018), the peptide fraction of the mealworm (*T. molitor*) protein showed an enhanced chelating capacity of Fe^{2+} (EC_{50}: 2.21 μg/mL) than the standard EDTA (EC_{50}: 20.1 μg/mL) and ascorbic acid (EC_{50}: 10 μg/mL). Similarly, the antioxidant activity of the larvae hydrolysates was evaluated by the analysis of DPPH radical (2,2-diphenyl-1-picrylhydrazyl) (HIERRO et al., 2020). Alternatively, the $ABTS^{+}$ radical scavenging test (2,2-azino-bis (3-ethylbenzothiazolin) -6-sulfonic acid), a method based on the ability of antioxidants to capture the $ABTS^{+}$ cations, can be used. Flores et al. (2020) showed that approximately 100% of the radical $ABTS^{+}$ inhibition was reached by approximately 0.4 mg/mL of proteins extracted from the mealworms.

ACE plays an important role in the process of normalizing blood pressure. The conversion of angiotensin I into angiotensin II raises the blood pressure. Thus the ACE inhibition is an effective strategy to stabilize blood pressure (Cito et al., 2017). A study carried out in spontaneously hypertensive rats proved the antihypertensive, cardio, and neuroprotective effects of protein hydrolysates derived from the larval stage of the *T. molitor*. Dietary supplementation was safe, where no significant effect on the intake, physical characteristics, or weight gain of the animals was observed (PESSINA et al., 2020). The IC_{50} values for blood pressure reduction inhibiting ACE detected in samples of hydrolyzed proteins of mealworms by gastrointestinal proteases were considerably lower than the non-hydrolyzed samples (Cito et al., 2017b).

Additional bioactive activities of mealworm extracts were already investigated. Antithrombotic peptides (SLVDAIGMGP and AGFAGDDAPR) were produced from mealworm proteins after computational evaluations have been non-toxic and could inhibit thrombin activity through the mechanism of interaction with thrombin exosite 1 (CHEN et al., 2019). Antidiabetic peptides were studied by Yoon et al. (2019) through the inhibitory activity of α-glucosidase in the hydrolysates of *T. molitor* larvae, which showed increased effective inhibition when compared to non-hydrolyzed proteins. These results demonstrated the potential of mealworms (*T. molitor*) for their growing application, which can broader than animal feed, that is, be widely used in functional and nutraceutical products.

2.3.2 Bioactive lipids

Bioactive lipids are molecules that act on specific stimuli, affecting cell function, often signaling and regulating cell metabolism (HANNUN; OBEID, 2018).

Tocopherol is a powerful antioxidant compound, mainly against the harmful effects of reactive oxygen species (ROS), Son et al. (2020) performed the extraction of oil from the mealworm with ≈144.3 ± 3.0 mg of tocopherol/1,000 g of oil. In addition, γ-tocopherol was equivalent to ≈86% out of the total tocopherol types. Similarly, Jeon et al. (2016) reported that α-, γ- and δ tocopherol contents in insect larva oil were 5.74, 186.02, and 3.91 mg/kg, respectively. In addition, Li et al. (2011) carried out *in vivo* experiments with δ- and γ-tocopherol, at 0.17 or 0.3 (% g/g) in feeding mice. The authors concluded that both tocopherols decreased the tumor volume. In addition, both tocopherols reduced oxidative damage to DNA and the formation of nitrotyrosine. It is worth noting that, in general, α-tocopherol is the most active form.

Oleic and linoleic acids from the *T. molitor* larvae were analyzed as inhibitors of BACE1 (β-secretase) for the treatment of Alzheimer's disease. These free fatty acids exerted a considerable inhibitory activity against BACE1 (YOUN et al., 2014).

Wu et al. (2020) demonstrated the potential of oil from mealworms for the production of natural anticancer agents through the antiproliferative effects of the extracted oil on the growth of human hepatocellular carcinoma (HepG2) cells and colorectal adenocarcinoma ($CaCO^{-2}$), possibly the high levels of oleic and palmitic acids in the insect were responsible for this effect.

T. molitor represents a potential source of bioactive compounds. Its protein and lipid fractions need more robust data for the extraction and recovery of valuable components, in which the extraction of oil (lipids) from insects does not compromise the fraction of proteins and thus can be used both the bioactive peptides of this protein, as well as the lipids with bioactive properties of the extracted oil, ensuring quality and efficiency, to further expand the field of application of insect-based products.

2.4 NON-CONVENTIONAL METHODS OF PROTEIN AND LIPIDS EXTRACTION

The quality, content, safety, and yields of edible insect extracts are directly related to the processing/extraction stage. Conventional methods for obtaining the protein are acidic or

alkaline extractions, organic solvents, isoelectric precipitation, and the salting-in method. These conventional methods generally result in the degradation or transformation of the molecules of interest for any of the components (GRAVEL; DOYEN, 2020; OKOLIE et al., 2019). They are also more harmful to the environment due to the toxicity of some solvents and waste generation (KUMAR et al., 2017).

Ideally, the extraction methods of interest molecules from edible insects have to be aligned with the concept of biorefinery. Some alternative extraction methods have significant advantages over conventional processes, such as lower temperature, faster, eco-friendly solvents, among others (MELGAR-LALANNE; HERNÁNDEZ-ÁLVAREZ; SALINAS-CASTRO, 2019; SORITA; LEIMANN; FERREIRA, 2020). Among the unconventional extraction methods, ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), pressurized liquid extraction and microwave-assisted extraction have been described below for the recovery of valuable compounds from the edible insect.

2.4.1 Ultrasound-assisted extraction (UAE)

UAE is a quite interesting alternative to the conventional stirring extraction method. It is based on cavitation, which causes disruption of cellular structures, accelerating the reactions chemicals and possibly increase extraction yields. A greater transfer of heat and mass provided by ultrasound makes it one of the most promising technological processes (BOSILJKOV et al., 2016; CALDAS et al., 2018).

The selectivity potential of UAE proved that extracts of *T. molitor* by ethanol and water as solvent had a higher percentage of amino acids than water or ethanol UAE extraction (HIERRO et al., 2020). The insects were lyophilized and ground, and the extractions occurred for 15 minutes, at < 70 °C, with an ultrasonic probe (1/2 ″ in diameter) Branson SFX250 Digital Sonifier (Branson Ultrasonics, EUA), at a sonication output amplitude of 60% in continuous pulse by direct sonication at 20 kHz.

Ozuna et al. (2015) suggested that high-intensity ultrasound as a pre-treatment and during the process of protein hydrolysis could increase the efficiency of hydrolysates, since it changes the protein structures, such as hydrogen bonds and hydrophobic interactions. Exposing more hydrolysis sites accessible by the enzyme, changing the molecular properties, being able to generate new peptides.

Choi et al. (2017) applied sonication to defatted mealworm samples. The insect-based flour was dispersed in distilled water and 9.46 nM ascorbic acid, the suspension was sonicated

for 20 minutes, using a Sonics® Vibra-Cell ™ VCX 750 ultrasonic unit (Sonics & Materials Inc., USA), operating at 20 kHz with sonication output amplitude 75%, where aliquots were collected at various time intervals. After 15 minutes of sonication, there was a 28% increase in protein yield.

Based on the related studies results, ultrasound-assisted extraction represents a promising alternative technology to increase extraction efficiency, with the ability to replace organic solvents with solvents that do not have toxic effects, falling within the green extraction area.

2.4.2 Supercritical fluid extraction (SFE)

Supercritical fluids as solvents present enhanced transport properties than liquids, such as low viscosity and relatively high diffusivity. Thus, they can diffuse quickly through solid materials and obtain faster extraction rates. Carbon dioxide (CO_2) is the most used solvent due to its safety, greater availability, and low cost. Also, it allows supercritical operations with relatively low pressures and temperatures close to the environment (DA SILVA; ROCHA-SANTOS; DUARTE, 2016; HERRERO; CIFUENTES; IBAÑEZ, 2006). The supercritical CO_2 (SC - CO_2) extraction of the mealworm oil was performed by Purschke et al. (2017) using a 500 bar pilot-scale extraction unit equipped with a 2 L. The use of the kinetic extractor indicated that the increased pressure increased oil solubility; greater solubility was achieved at 400 bar. Maximum larval degreasing was achieved under 400/250 bar, 45 °C, and 105 min. The physical-chemical properties and the composition of the oil extracted with SC-CO_2 were compared with the oil obtained by extraction with a conventional hexane method.

Tang & Dai (2016) identified the immunomodulatory activity of extracts of mealworms (*T. molitor*) obtained through a supercritical CO_2 fluid system in the immune system of mice. The extraction occurred at 50 ºC and 30 MPa with a CO_2 feed of 8 kg/h. The extracts showed efficient results to the indices of a nonspecific cellular and humoral immune response. The tests confirmed the increase in the serum NO content, the activity of acid phosphatase and alkaline phosphatase with the application of the obtained extract, indicating the capacity to protect the biological immune system of the mice.

Laroche et al. (2019) investigated the effects of SC-CO_2 extraction and methods with conventional solvents hexane, petroleum ether, ethyl acetate, and ethanol on the extraction of mealworm lipids (*T. molitor*). The extraction with supercritical CO_2 was carried out under

conditions of 55 °C and 325 bar with a flow rate of 10 g/mL. As a result, the extraction yields of SC-CO_2 were similar to conventional methods (22%). Regarding the extracted lipid profile, SC-CO_2 extracted the highest proportion of *trans*-vaccenic acid and was more efficient for the extraction of C16:0 in relation to the extractions of Soxhlet with hexane or petroleum ether or ethyl acetate. In general, SFE is an excellent tool for obtaining nutritionally valuable compounds, including from *T. molitor*, applied in a process combination that can improve the use of biomass, mainly for obtaining purified extracts, without residual solvents and environmentally friendly contributing to the better use of the fractions obtained from this insect, being positive and promising for the food, cosmetic and pharmaceutical industries.

2.4.3 Pressurized liquid extraction (PLE)

PLE extraction involves the use of liquid solvents at high pressures and temperatures. The solvent is above its boiling point, changing the solubility, surface tension, and mass transfer properties, resulting in a better extraction process. One of the great advantages of this technique is reducing the extraction time (AZMIR et al., 2013).

Otero et al. (2020) studied the mealworm's fatty acid profile extracted with PLE and compared it with the initial insect. This type of extraction caused a relevant decrease in total polyunsaturated fatty acids (PUFA) and total saturated fatty acids (SFA). However, it led to the enrichment of monounsaturated fatty acids (MUFA) in the extracts due to increased oleic acid. Extractions were performed at 120 °C for 15 min.

Hierro et al. (2020) evaluated the inhibitory capacity of pancreatic lipase as a bioactive activity of the *T. molitor* extract obtained by the PLE method, using ethanol and aqueous ethanol as solvents. Extractions were carried out under conditions of 120 °C for 15 min and 100 bar, using N_2 as the compressor gas. As a result, the conditions of the PLE allowed a more effective concentration of pancreatic lipase inhibitory compounds, where the inhibitory activity presented a better value for the *T. molitor* of IC_{50} (0.15 mg/mL) using aqueous ethanol, being this IC_{50} comparable to natural plant extracts that are often used for this activity.

Thus, PLE represents a promising alternative to obtaining unique extracts from *T. molitor* and other edible insects, generating added value for the edible insect industry mainly by the production of bioactive ingredients.

2.4.4 Microwave-assisted extraction (MAE)

The mechanism of microwave-assisted extraction involves intracellular heating and the release of solutes from the matrix into the solvent. It is described as an efficient method, with low cost, faster heating, and smaller thermal gradients. It is a safe technique with good performance (AZMIR et al., 2013; OKOLIE et al., 2019). To the best of our knowledge, there is no data related to the extraction of mealworms assisted by microwave. However, microwave extraction was already investigated for other edible insects.

The positive effect of microwave-assisted enzymatic hydrolysis of cricket protein (*Gryllodes sigillatus*) was studied by Hall & Liceaga (2020). The sample exposed to microwave heating (MW-C) significantly improved the IC_{50} values for DPP-IV (0.31 mg/mL) and ACE (1.43 mg/mL). After adding the alcalase enzyme, the IC_{50} values for DPP-IV and ACE reached 0.27 and 0.096 mg/mL, respectively. The microwave (MarsExpress ™ CEM Co., Matthews, NC, USA) has been programmed to heat solutions to 55 °C and remains constant. Protein cleavage/reaction occurred faster under microwave heating, t = 10 min, compared to conventional hydrolysis, t = 20 min (room temperature).

Enzymatic processes, assisted by microwaves and ultrasound, have been explored to produce chitin, particularly more limited to shrimp residues (MOHAN et al., 2020). Future investigation is needed to address chitin extraction methods in *T. molitor*, which will contribute to increase the potential application of the insect-based chitosan, mainly by the food and pharmaceutical industries.

Despite the few studies using MAE for edible insects, it is a viable and economical method, which is worth applying and studying both for the extraction of compounds from *T. molitor* as in pre-treatment and during enzymatic hydrolysis processes.

2.5 BIOREFINERY CONCEPT APPLIED TO *TENEBRIO MOLITOR*

T. molitor has high nutritional value, a natural source of bioactive compounds. Thus, it is quite an interesting biomass. Appropriate eco-friendly fractionation processes of *T. molitor* that do not affect either quality or yield is a currently unexplored challenge. The insect biorefinery can generate products of substantial economic value. The production/reproduction of *T. molitor* is already considered environmentally sustainable. An insect biorefinery corresponds to an integration of technology that allows the valorization of insect biomass

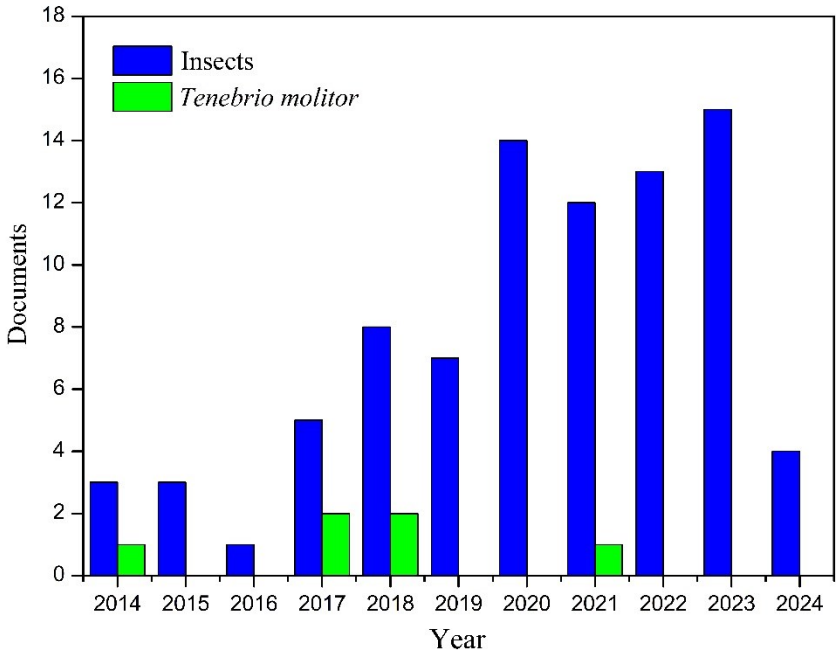
through its bioconversion into valuable ingredients with broad application, similar to green biorefineries (AZAGOH; HUBERT; MEZDOUR, 2015; RAJENDRAN et al., 2018). The biorefinery concept applied to insects challenges is mainly searching for innovative technologies from the collection of organic waste to final consumption, with products with high added value (RAVI et al., 2020).

Despite the relevance of the subject, the biorefinery concept applied to *T. molitor* was poorly investigated. Research on the Scopus database (www.scopus.com) was carried out on April 1th, 2024, to analyze the number of studies related to edible insects and mealworms (*T. molitor*). The search using the keywords "larvae" OR "insects" OR "edible insects" AND "combination of processes" OR "fractionation processes" OR "bio-fractionation" OR "biorefinery" outputted 97 documents for the period 2014 to 2024 (Figure 3). Regarding the type of documents, 59 (60.8%) are research articles, 21 (21.6%) are review articles, 8 (8.2%) book chapters, and 6 conference articles and notes (7.2%). Another more specific search was also performed using the keywords "mealworm" OR "yellow mealworm" OR "*Tenebrio molitor*" OR "larva *Tenebrio molitor*" OR "insect *Tenebrio molitor*" AND "combination of processes" OR " fractionation processes "OR" bio-fractionation "OR" biorefinery". As a result, only 6 documents were found between 2014 and 2024, 4 (66.7%) research articles, 1 (16.7%) book chapter and 1 (16.7%) review article. These articles mainly are addressed to the bioconversion of organic residues and the production of biofuels from these residues - often related to black soldier fly (*Hermetia illucens* L.). Therefore, the concept of biorefinery applied to *T. molitor* should be carefully and deeply investigated, considering the possibilities pointed out in this review article.

Figure 4 illustrates a biorefinery concept schematic for the insect *T. molitor*. The possible applications and valuation of new products since most of the studies found in the literature are related to food supplementation such as feed, partial replacement of foods, among

After being separated from the excrement and cleaned, the *T. molitor* biomass is pre-treated. This process consists of a preliminary heating step, such as bleaching, grinding process, and drying step, such as freeze-drying, to ensure this biomass safety and preservation.

Figure 3. According to the database platform Scopus, the number of publications related to the concept of biorefinery applied to the processing of edible insects in general and the concept of biorefinery applied specifically to *Tenebrio molitor*, published in the period from 2014 to 2024 (April 1, 2024). The data show the results for all document types: Article, review, book chapters, among others.



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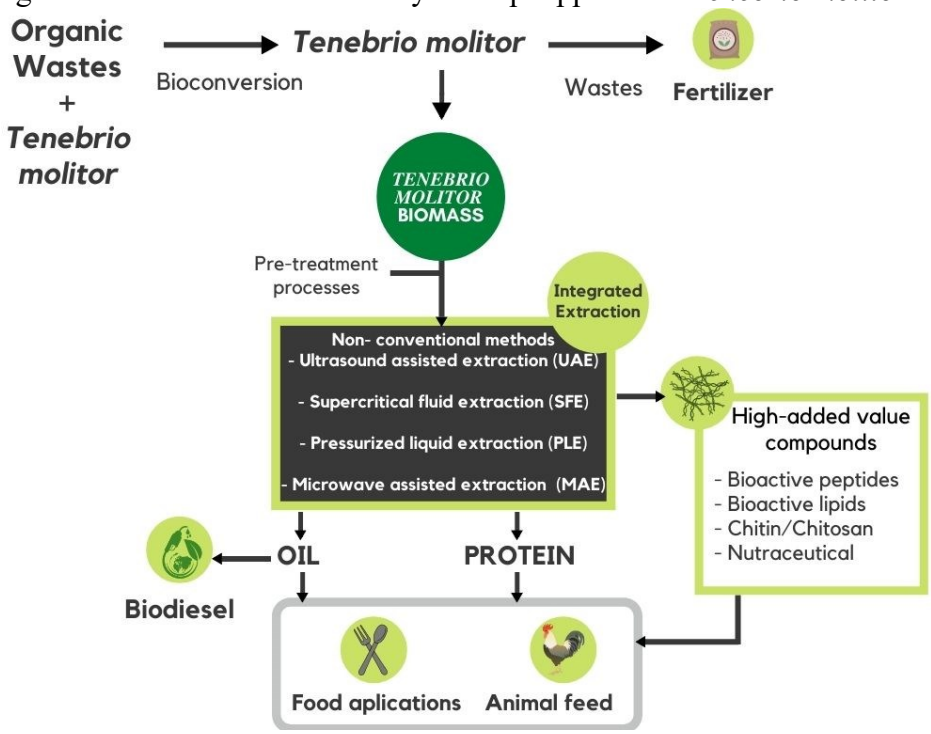
Then, the biomass is subjected to extraction processes. Process integration is proposed to recover valuable compounds, mainly protein and oil, which are the main components of this insect. Non-conventional extraction techniques can be combined to recover these components in a viable and more effective way. A possible combined process would be for the hexane solvent to be replaced by the SFE-CO_2 extraction in the oil extraction, mainly extracting the nonpolar fraction, such as oleic, linoleic, and palmitic acid, without compromising the protein obtained from solvent residues. Thus, UAE extraction using a mix of water and ethanol, where more polar extracts are obtained, rich in free amino acids (HIERRO et al., 2020). However, these integration processes must be fundamentally correlated to the economic viability data, mainly for developing countries (feasibility), since these technologies generally require high investment costs, compared to conventional methods. On the other hand, as already mentioned, eco-friendly unconventional extraction methods have advantages in terms of environmental regulations and a wider range of applications (human and animal feed) since there are no solvent residues. In addition, the integrated biorefinery tends to be more efficient (biomass fractionation). The quality of the products obtained can justify the high initial costs for

implanting these technologies in large scale. So, a full investigation on the technical feasibility of them are necessary to base further steps on scaling up, for instance.

Figure 4. Flowchart of biorefinery concept applied for *Tenebrio molitor*.



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As seen in Figure 4, several products can be obtained from the oil and the extracted protein. From the oil, the fraction rich in saturated fatty acids, such as palmitic acid, obtained mainly in the SFE-CO_2 extraction, can be applied in the production of biodiesel. Part of the extracted oil can be applied as food and feed components, mainly the fraction rich in essential fatty acids. As already discussed, this oil obtained from *T. molitor* also presented bioactive compounds, adding even more value to the extract.

The fraction of protein obtained after the extraction processes can be applied as a component of animal feed, one of its most excellent applications today. Nevertheless, valuable bioactive compounds can also be obtained from enzymatic hydrolysis. It suggests an integrated process of microwave-assisted enzymatic hydrolysis to enhance the bioactivity of the peptides obtained. As already mentioned, no study has been carried out with the *T. molitor*, but it has shown positive results with the *Gryllodes sigillatus* (Hall & Liceaga, 2020). The proteins obtained can also be applied in food formulation since they have all the essential amino acids. Also, Houben et al. (2020) indicated that the excrement of the mealworms has great potential as a partial or complete substitute for mineral fertilizer. A larvae diet that contained 66%

carbohydrates, basically celluloses and hemicelluloses, 6% fat, and 28% protein, can promote, from excrement, tolerance to abiotic stress in plants (POVEDA et al., 2019). It was the first study to report the ability of *T. molitor* feces to promote plant resistance to abiotic stress. As shown in Figure 4, the larvae can reuse putrescible foods with no added value and, from their digestion, obtain biomass rich in nutrients and excrement that has excellent potential for application advantage of everything that is generated. From biomass, high-added value molecules can be obtained, for instance, proteins composed of essential amino acids, lipids rich in fatty acids, peptides, polysaccharide chitin, among others.

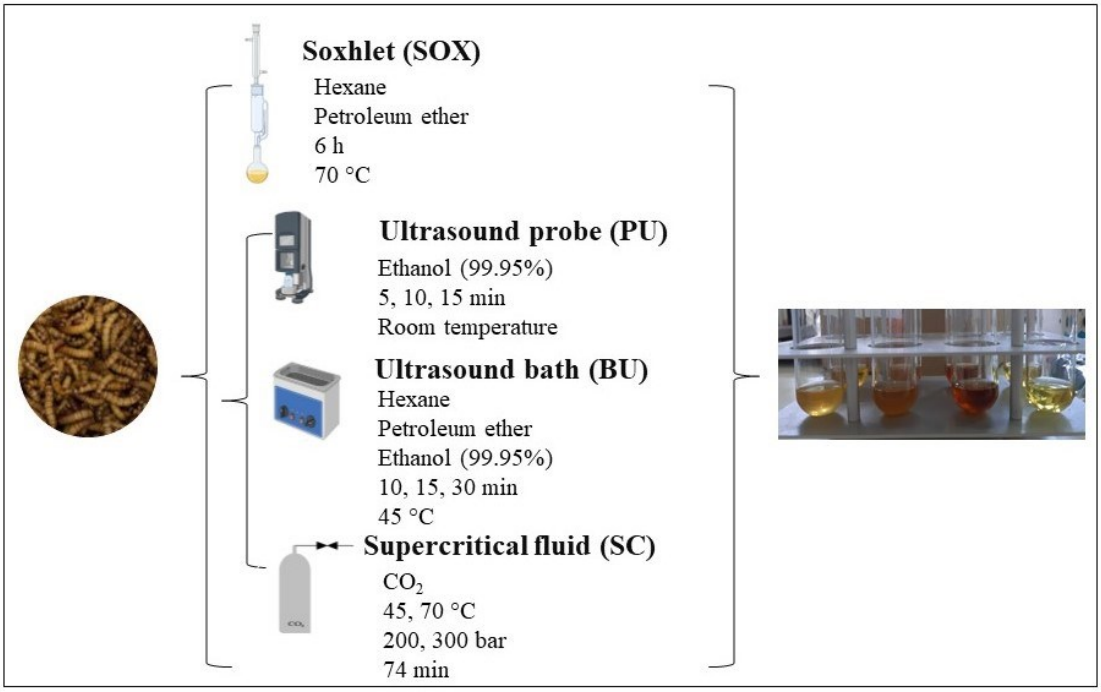
2.6 CONSIDERATIONS ABOUT THE STATE OF THE ART

From the exposed in this chapter, one can verify that the *T. molitor* biorefinery approach offers a large chain of sustainable products and relatively unconventional applications. Lipids extracted by unconventional extraction processes are attractive as a source of antithrombotic, antidiabetic, antioxidant, antihypertensive and hepatoprotective bioactive compounds, as reviewed in the literature. These processes can favor the implantation of insects as an industrial source of bioactive compounds, and it is necessary to scientifically investigate these possibilities. It's needed to analyze the feasibility of unconventional processes, as well as the effects they can cause on the product and, in addition, to explore processes that can make *T. molitor* lipids even better industrially.

3 CHAPTER 3: EXTRACTION YIELD, FATTY ACID PROFILE, PROPERTIES AND BIOACTIVE EVALUATION OF LIPIDS FROM *TENEBRIO molitor*: COMPARISON AMONG ALTERNATIVE EXTRACTION METHODS AND SOXHLET

This chapter presents the main results obtained in the experimental stage of lipids. The objective was to extract *T. molitor* oil by different extraction methods, mainly by alternative methods (SC, BU, and PU), as represented in Figure 5. The results were compared with the conventional SOX extraction method and analyzed the yield, fatty acids composition, oxidative stability, and free fatty acids percentage. The bioactivity of the extracted oil was also analyzed, such as antioxidant activity, cytotoxicity, and antitumor activities. The results presented is chapter was submitted to publication in International Journal of Biological Macromolecules.

Figure 5. Representation of *T. molitor* lipid extraction by different extraction technologies, from conventional to alternative methods.



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3.1 INTRODUCTION

The extraction process plays a significative role on the development of new products from alternative raw materials, including a direct influence on the viability and efficiency of the final products obtained. The extraction process has been used for a long time and its innovation continues to be the target of researchers and especially industries, resulting in new, more innovative, economic, and environmentally correct methods (TAMBUN; ALEXANDER; GINTING, 2021).

Usually, the conventional extraction techniques used are time-consuming and operate at high temperatures, which limits the integrity of thermo-sensitive molecules. In addition, a large part of the process requires the use of high-purity solvents, which increases operating costs and also represents an environmental challenge (PÉRES et al., 2006). Traditionally, mainly in academic studies, SOX extraction is used in the extraction of lipids. It has been increasingly used for the extraction of bioactive compounds from natural raw materials, mainly as a comparative method of alternative extraction methods, which seek to improve the challenges of conventional extraction (AZMIR et al., 2013).

In the last decade, eco-friendly extraction methods have been investigated such as the ultrasound-assisted technique and supercritical fluid extraction. To increasingly validate these alternative methods, studies need to be deepened and analyzed.

Studies report that the oil extracted from *T. molitor* exhibits antioxidant properties, attributed to the presence of bioactive compounds in the oil, such as polyunsaturated fatty acids, tocopherols and carotenoids (ERRICO et al., 2022; GHARIBZAHEDI; ALTINTAS, 2023) which have demonstrated efficacy in neutralizing free radicals, protecting against oxidative stress. Investigations into the insect-based oil cytotoxicity reveal potential applications of the oil, suggesting a promising safety profile for human consumption. Regarding antitumor activity, *T. molitor* oil may have inhibitory effects on the growth of tumor cells, pointing to possible therapeutic applications in the fight against cancer (WU et al., 2020b). However, it is crucial to highlight that more research is needed to fully understand the scope of these properties and to validate the viability of *T. molitor* oil as an effective anti-tumor agent.

Therefore, some alternative methods were used and compared with Soxhlet to extract lipids from the insect *T. molitor*, the extracted oils were analyzed in terms of yields, and interference of the methods used on the properties of the extracted oil and investigated their

bioactive activities. Subsequently, the quality of the proteins present in the defatted meal was examined and analyzed for the interference of the methods used on their properties. Thus, the biorefinery approach for *T. molitor* can be established, to maximize the properties and potential of the insect chain.

3.2 MATERIAL AND METHODS

3.2.1 Raw materials and chemicals

The insect *Tenebrio molitor* larvae were purchased from a company specialized in edible insect production (Agrin Criação e Comércio de Insetos LTDA, São Paulo, Brazil).

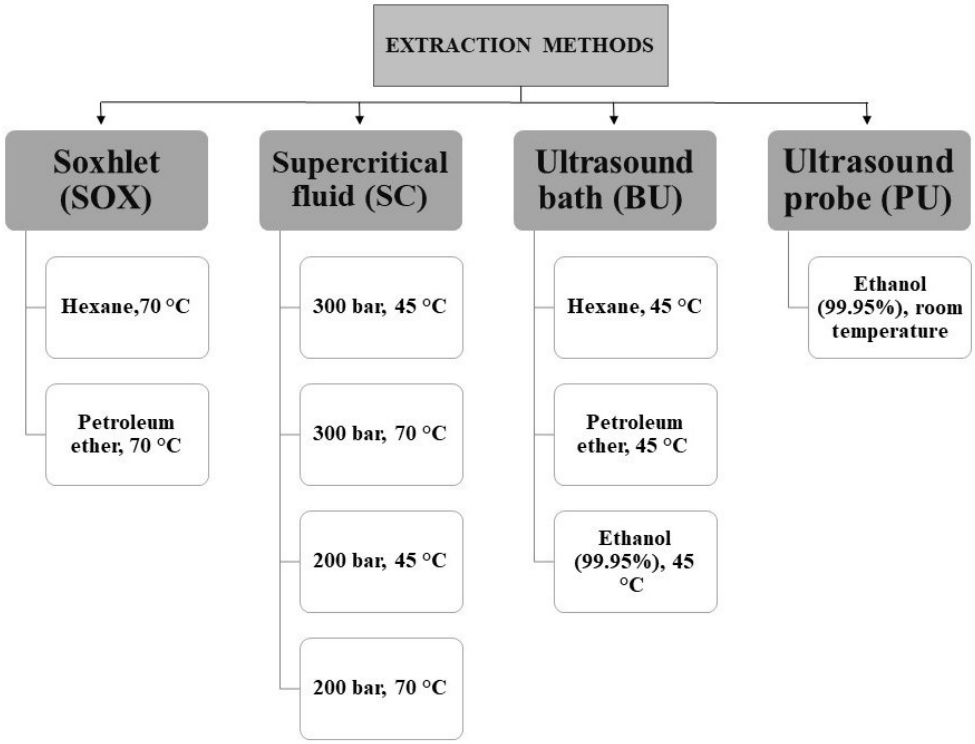
Hexane P.A. (Nuclear), absolute ethyl alcohol 99.8% P.A. (NEON), methyl alcohol P.A. (Dinâmica), bovine serum albumin protein (Sigma- Aldrich), petroleum ether P.A. (Dinâmica), carbon dioxide (99.9%, pure), potassium hydroxide P.A. (NEON), sulfuric acid P.A. ACS (NEON), Ethyl Ether P.A. (SYNTH), phenolphthalein P.A. (SYNTH), sodium hydroxide P.A. (Dinâmica), hydrochloric acid 37% P.A./ACS (NEON), copper (II) sulfate pentahydrate P.A. (Dinâmica), sodium carbonate P.A. (SYNTH), Folin Ciocalteu reagent (Sigma- Aldrich), methanol P.A. (Êxodo scientific), Isopropanol P.A (Êxodo scientific), CellTiter Aqueous One Solution (Promega).

3.2.2 Lipid extraction

Prior to extraction the live larvae were fasted for 24 h to clear their gastrointestinal tract of any residual food, after this period, mealworms were washed abundantly with tap water, then were kept on a colander for removing excess water and the remaining water was removed with a paper towel (SON et al., 2020a). The mealworms were freezed (slaughter), ground in a knife mill (EDB-5 Model, DeLeo), kept in sealed bags, and stored in a -80 °C freezer (HIERRO et al., 2020; TZOMPA-SOSA et al., 2014). Just before the extraction, the insects-based material was freeze-dried, and then kept in desiccator.

For extracting oils, the insect meals were submitted to four extraction methods (Figure 6): SOX, SC, BU and PU. For the SOX, BU and PU methods, the defatted insect meals and the lipid extracts after the solvent evaporation process were dried at 40 °C in an oven for 24 h. The defatted meal from all methods was kept in a desiccator at room temperature and the extracted oils were stored at -20 °C until further use, both protected from light.

Figure 6. Diagram of the extraction processes carried out to obtain the *T. molitor* oil in the present study.



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3.2.2.1 Soxhlet extraction

For all extractions using solvents, the mass/solvent ratio was determined: 3g of *T. molitor* meal/200 mL of solvent, according to MIN; ELLEFSON (2010), with some modifications. The oil was extracted in a SOX apparatus for 6 h using hexane or petroleum ether as solvents, immersed in a heating blanket at 60 - 70 °C. Then, the solvents were removed using a rotary evaporator (RV 10 basic, IKA) in a water bath at 40 °C for about 60 min until no solvent detection. After the rotary evaporation process, the oil spent 24h in a vacuum oven to remove residual hexane (ESCORSIM et al., 2018).

3.2.2.2 Supercritical fluid extraction

Supercritical CO_2 extraction was performed in a laboratory-scale unit, according to LAROCHE et al. (2019). 15 g of the mealworm were placed inside the extraction column and

the empty space was filled with cotton and glass beads. The experiments were performed at constant solvent flow rate 0.8 kg CO_2/h. The extraction time was set at 74 min according to one previous kinetic extraction curve carried out at 200 bar/45 °C and 1 kg CO_2/h. The experimental conditions used were 300 bar/45 °C, 300 bar/70 °C, 200 bar/45°C and 200 bar/45 °C. Aiming to evaluate the impact of pressure and temperature on the extraction yield, the extractions were carried out by varying the pressure to 200-300 °C and temperature to 45-70 °C. Samples were collected in amber flasks previously weighted on an analytical balance and stored in amber flasks at -20 °C prior to analysis (PURSCHKE et al., 2017).

3.2.2.3 Ultrasound bath assisted extraction

T. molitor meal sample (3 g) was placed in a screw cap flask and 200 mL of solvent (hexane, petroleum ether or ethanol) was added, based on the mass: solvent ratio of the Soxhlet method previously described. The flask was placed in an ultrasound bath system (SSBu- 3.8 L, SolidSteel), operating at 40 kHz, and the extraction was performed at 5, 15, and 30 min at approximately 45 °C. The temperature control of the water bath by ultrasound was performed. After the extraction step, the solid-liquid solutions were filtered on filter paper to recover the defatted meal. The solvents were removed using a rotary evaporator (RV 10 basic, IKA) in a water bath at 40 °C for about 60 min until no solvent detection (HIERRO et al., 2020).

3.2.2.4 Ultrasound probe assisted extraction

Extractions were carried out as described by Navarro del Hierro et al. (2020) with modifications, basically 3 g of insect sample was mixture with 200 mL the ethanol (99.95%), and submitted an ultrasonic probe (model DES500, Unique) for 5, 10, and 15 min at room temperature at a sonication output amplitude about of 20% in continuous pulse by direct sonication at 20 kHz. Then, the mixture was then filtered on filter paper in vacuum to recover the defatted meal. The ethanol was removed using a rotary evaporator (RV 10 basic, IKA) in a water bath at 60 °C for about 60 min until no solvent detection.

3.2.3 Oil characterization

3.2.3.1 Extraction yield

The oil extraction yield was calculated as the weight of the total material extracted in relation to the initial insect sample weight and expressed as a percentage. Regarding meal, the percentage of defatted meal was calculated with the final weight of the meal after the drying step in the oven in relation to the weight of the initial sample and expressed as a percentage (TESFAYE; TEFERA, 2017).

3.2.3.2 Identification of fatty acids by gas chromatography coupled to mass spectrometry

The extracted oils were characterized by gas chromatography coupled to mass spectrometry (CG-MS) after the stage of methylation of fatty acids to obtain their respective fatty acid methyl esters (FAME), according to O'FALLON et al. (2007). In this step, 40 µL of oil were added into a glass tube with 0.7 mL KOH (10M), and 5.3 mL methanol, after the agitation, was heated at 55 °C for 1.5 h in a shaker (TE-424, Refrigerated incubator with shake, TECNAL), shaking the tube every 10 min for 10 s in a vortex. Next, the tube was taken and cooled to room temperature in cold tap water, followed by the addition of 0.58 mL H_2SO_4 (12M). Then, the tube was heated again under the same conditions (55 °C for 1.5 h), and after, 3 mL of hexane were added to the tube, vortexed for 5 min, and centrifuged at 4000 rpm for 5 min. Then, FAMEs-containing supernatant was collected and analyzed by GC. The GC/MS analysis was performed on instrument Agilent GC 7890A coupled with MS detector Agilent 5975C. The column, an HP-5MS (Agilent) fused silica capillary column (30 m length x 250 µm i.d. x 0.25 µm film thickness composed of 5% phenyl-95% methylpolysiloxane) was connected to an EI source (Electron Impact Ionization) operating at 70 eV with a quadrupole mass analyzer. The mass scan ranged from 40 to 550 m/z. Helium was used as the carrier gas at a flow rate of 1.2 mL/ min. The injector (with a split ratio of 1:25) and interface temperatures were 300 ºC. The solvent delay was 3.0 minutes. The injection volume was 1.0 µL with autosampler Agilent GC Sampler 80 equipped with a 10 µL syringe. The oven temperature program consisted of ramping up from 60 ºC for 3 min, then 4 °C/min to 270 °C for 2 min and then 30 °C/min to 300 °C for 5 min. Total run time of 63,5 min. The compounds were identified by comparing their mass spectra with those from the National Institute of Standards and

Technology (NIST, 2011), following the same procedure as the samples (LUO et al., 2018 with modifications).

3.2.3.3 Determination of free fatty acids

The total titratable acidity was determined for all samples according to Vicentini-Polette et al. (2021), with modifications. Approximately 0.4 g of oil were used, dissolved in 5.0 mL of ether-alcohol solution (2:1), and added 50 µL of 1% phenolphthalein indicator in ethanol. Titration was performed using 0.1 M sodium hydroxide solution in distilled water until the appearance of pink color. The FFA content was calculated according to Equation 1.

$$\% FFA = \frac{(V*M*28.2)}{m} \quad (1)$$

Where % FFA represents the percentage of free fatty acids, V corresponds to the volume of solvent used, M is the Molarity of the NaOH solution, and m is the mass of the oil sample. The results were expressed in oleic acid content (% m/m), and all analyses were conducted in duplicate.

3.2.3.4 Oxidative stability

The evaluation of the oxidative stability index (OSI) followed the AOCS Cd 12b-92 method (AOCS, 1997) in a Professional Biodiesel Rancimat 893 equipment (Metrohm, Switzerland), according to TEIXEIRA et al. (2020). The analysis was performed using 2.5 g of oil, from each extraction a condition was selected for evaluation of the oxidative stability based on the yield and at similar times and temperatures. The apparatus was set to operate at 110 °C with 20 L/h of airflow. The OSI was expressed in hours in duplicate runs. Data were recorded using StabNet 1.1 Build 21 software.

3.2.4 Antioxidant activity

3.2.4.1 DPPH assay

The antioxidant activity of lipids is measured by the DPPḢ assay (BRAND-WILLIAMS; CUVELIER; BERSET, 1995). The oil was solubilized in ethanol: hexane (70:30), where 50 µL of this solution was added to microplates and mixed with 250 µL of a DPPḢ solution (125 µmol/L) in methanol. Samples were incubated at room temperature for 30 min in

the dark. The reading was carried out at 517 nm on the microplate reader. The remaining DPPḢ concentration of all samples was calculated from a DPPḢ calibration curve. Control samples are obtained using the same procedure but without the presence of oil.

3.2.4.2 ABTS radical scavenging activity assay

The ABTS radical cation scavenging activity ($ABTS^{\bullet+}$) was determined according to RE et al. (1999). The radical solution was prepared with ABTS and potassium persulfate, diluted in water until a final concentration of 2.45 mmol/L and left in the absence of light at room temperature for 16 h to allow radical development. Then, the solution was diluted with distilled water, around 4 mL of the ABTS radical to 250 mL of distilled water, to obtain an absorbance of 0.70 at 735 nm. 20 µL of the oil diluted in ethanol: hexane (70:30) was mixed with 280 µL of the diluted ABTS radical solution in a microplate, being kept in the absence of light for 30 min. Absorbance was measured at 734 nm. The scavenging effect was calculated from a standard calibration curve.

3.2.5 *In vitro* experiments

3.2.5.1 Preparation of fatty acids and cell culture

Initially, for the preparation of these concentrations, a mass of 5.12 mg of extracted oils was diluted in 1 mL of isopropanol P.A., resulting in a main solution (5,120 µg/mL) 10x more concentrated and, from there, were realized serial dilutions (1:1) using the same solvent in equivalent.

3.2.5.2 Cell Culture

The cells Mouse fibroblast cell line (L929), human lung adenocarcinoma NSCLC cell line (A549) and brain glioma cell line (GL) were purchased from SIGMA and were grown in DMEM (Dulbecco's modified Eagle's medium, Gibco©) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco ©) and antibiotic (penicillin and streptomycin) (Gibco©, USA). All cell lines were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

3.2.5.3 Cytotoxicity

L929 cells were plated in 96-well cell culture plates (10,000 cells per well). After 24 h, the media were replaced and the cells were treated with oils extracted by the SOX and SC methods at the following concentrations: 10, 50, 100, 250, 500, 750, and 1000 µg/mL and left for 24 h, incubated at 37 °C in a humidified cell culture with 5% CO_2.

After 24 h, the cytotoxicity of the oils was analyzed using the MTS assay (CellTiter Aqueous One Solution; Promega) according to the manufacturer instructions, 120 µl of MTS solution (0.2 µl MTS/ µL of medium) was added to each well. After incubation for 2 h at 37°C, 100 µL of the solution was collected for analysis. Cell viability was measured using a microplate spectrophotometer (Molecular Devices) at 490 nm. Cytotoxicity analysis was performed in individual experiments per ISO 10993/5.

3.2.5.4 Antitumoral activity

A549 and GL cells were used as cancer cell lines. Both cells followed the same study procedures. Cells were plated in 96-well cell culture plates (10,000 cells per well). After 24 h, the media were replaced and the cells were treated with oils at concentrations of 500, 750, and 1,000 µg/mL and left for 24 h, incubated at 37 °C in a humidified cell culture with 5% CO_2.

The antitumor activity of the oils was analyzed using the MTS assay (CellTiter Aqueous One Solution; Promega) as described in the topic above. Cell viability was measured using a microplate spectrophotometer (Molecular Devices) at 490 nm.

3.2.6 Characterization of protein-rich defatted meal

3.2.6.1 Protein content and solubility

The total nitrogen content of the crude meal and the meals after the extraction processes were analyzed using the Kjeldahl protocol according to method 928.08 (AOAC, 2000). Total protein content was calculated using the nitrogen conversion factor (N) of 6.25. Furthermore, they were calculated using the insect-specific conversion factors obtained by JANSSEN et al. (2017) and BOULOS; TÄNNLER; NYSTRÖM (2020), of 5.60 and 5.33, respectively.

Protein solubility was determined with BORREMANS et al. (2020) method with some modifications. 0.1 g of meal from each extraction process was mixed with 10 mL of distilled

water, and the pH of the mixture was adjusted to 5, 7, and 9 with 1 M NaOH or 1 M HCl. The solutions were stirred on a shaker (≈ 220 rpm) for 30 min and centrifuged (4000 SPEED r/min, 20 min, K14-0815C, KASVI). The protein concentration of the supernatant was assessed by the Lowry method using bovine serum albumin proteins as a standard.

The assay consisted of 100 µL of protein extracts (supernatants) added to 2 mL of a mixture of solution A, which consisted of 2% (m/v) of Na_2CO_3 in NaOH (0.1 M) with a solution B, consisting of 0.5% (w/v) of $CuSO_4.5H_2O$ and 1% (w/v) of sodium citrate (in a ratio of 50:1) and kept for 10 min at room temperature. Then, 0.2 mL of the reagent containing a mixture (1:1) of distilled water and Folin Cicoalteau reagent was added, vortexed, and left to stand for 30 min at room temperature. Absorption was analyzed at 750 nm using a UV/Vis spectrophotometer (Model 1105, BEL).

The procedure was performed in duplicate, and the protein solubility was calculated using Equation 2:

$$\text{Solubility (\%)} = \frac{[\text{ protein concentration in the supernatant }]}{\text{total protein concentration in the sample}} * 100 \quad (2)$$

3.2.6.2 Thermal stability

The denaturation profile of defatted meal was performed according to BAIGTS-ALLENDE et al. (2021) through DSC analysis (Jade-DSC Model, Perkin Elmer), mainly to assess whether any oil extraction method caused any change in this property. Meal samples were weighed and adjusted depending on the amount of crude protein per sample so that the amount of protein was the same in all samples: SOX: 4.24 mg, SC: 4.6 mg, PU: 4.23 mg and BU: 4.39 mg. The DSC equipment was set to a temperature schedule of 20 to 180 °C with a heating rate of 2 °C/min. The denaturation process was quantified by the thermal transition midpoint (Tm).

3.2.6.3 Morphology

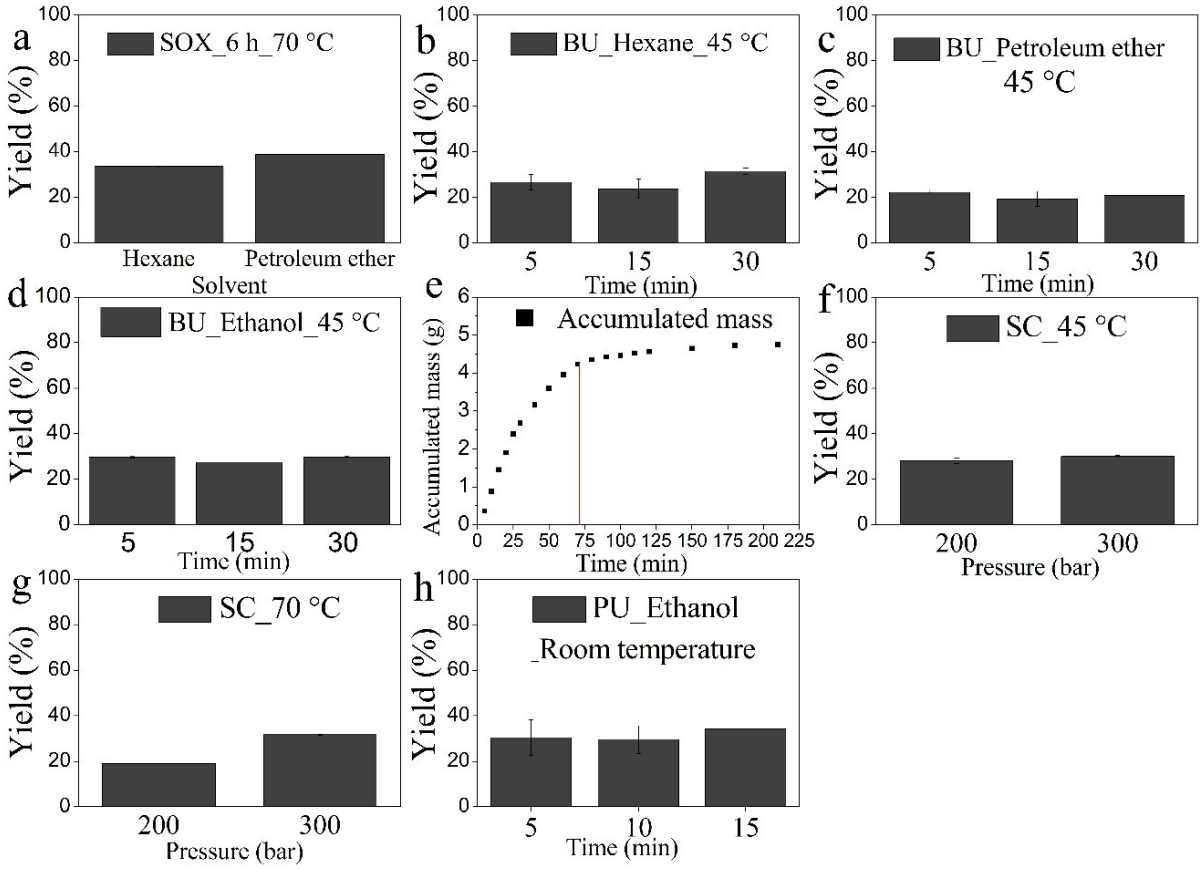
For morphological analysis, samples were lyophilized (Liotop, L110) using a critical point. The morphology of the samples was observed with a scanning electron microscope (model JSM 6390 LV, Jeol, Japan). Samples were analyzed at an accelerating voltage of 10 kV and micrographs at magnifications between 500 and 2500 ×.

3.3 RESULTS AND DISCUSSION

3.3.1 Extraction yield from different methods

The lipid extractions are usually affected by the extraction method. The influence of different extraction methods on the properties of *T. molitor* oil was investigated and compared with SOX conventional extraction technique (Figure 7). Also, it was evaluated the influence of solvent, extractions temperature, pressure and time as shown in Figure 6.

Figure 7. Yield in oil (% w/w of the insect meal) obtained from the different defatting methods. (a) conventional Soxhlet extraction (SOX), extraction with ultrasound bath (BU) using hexane (b), petroleum ether (c), and ethanol (d) as solvent at different times, (e) supercritical extraction (SC) kinetics to determine the best extraction time, (f) and (g) SC by varying the pressure and fixed temperature, (h) extraction with an ultrasound probe (PU) using ethanol as solvent at different times.



Source: Author

In general, the variations of the analyzed parameters show low influence on the oil yield. The oil yields of the BU extractions (Figure 7b,7c, 7d), comparing with the yield obtained

by the conventional extraction with SOX using petroleum ether as solvent (38.84% ± 2.57), reached from ~ 49.88% to ~81% of this yield, considerable values considering the process conditions. The extraction time is an important factor, as these yields were obtained in a time significantly shorter than SOX, with the longer time of 30 min. Regarding the BU method extractions using different solvents and time variations, a subtle variation in yield was observed among the solvents used (hexane, petroleum ether and ethanol): the highest yield reached 31.52% ± 1.4 using the hexane solvent, after the 30 min extraction. A parameter tested in this extraction was the variation of time, as mentioned above, the longer time tested showed better results, the value very similar to that of the SC extraction.

In relation to SC extraction, in the first moment, kinetics was performed at 200 bar, 45 °C with a flow rate of 1 kg CO_2/h to evaluate the optimal time to carry out the extraction of the oil from the meal of *T. molitor* by the method of supercritical extraction, it was observed (Figure 7e) that in 74 min was already had the maximum % of the accumulated mass. The percentage of oil obtained was considerably higher at higher pressure (300 bar), at both temperatures (45 and 70 °C), as can be seen in Figures 7f and 7g. Regarding the temperature of 70 °C, in Figure 7g it is observed the lowest oil yield value was obtained in all conditions, which was the process carried out at lower pressure (200 bar) and the highest oil yield value was obtained in all conditions that the extraction was carried out at 300 bar. In the combination of processes, a better oil yield (31.66 ± 0.155) was obtained in extraction at 300 bar and 70 °C, compared to the SOX method which required a longer extraction time (6 h), the extractions SC show better results. Based on oil yield, SC extraction, using a clean CO_2 solvent, is comparable and can replace conventional SOX extraction.

Despite a trend towards higher yields of PU extractions when compared to other unconventional methods (Figure 7h), the differences were not as significant as the ranges of yield variation are greater, staying in the range. One of the advantages of this method is the short extraction time (maximum 15 min) and being an extraction performed at room temperature. As with BU, the study parameter in this extraction was time. Despite little difference, a longer time (15 min) presented a better result.

In general, the yield values obtained in conventional and alternative extractions are very similar to the results obtained in other studies using *Tenebrio molitor* (Table 3).

Table 3. Yield values of lipids extracted from *T. molitor* found in the literature.

Oil Yield (% w/w)	Extraction Method	Reference
37.54	SOX/ Petroleum ether	Siow, Hao Sen, et al. (2021)
32.30	Shaker/ n-hexane	Son, Yang-Ju, et al. (2020)
24.06	High Hydrostatic Pressure/ 0,1 MPa, 40 °C	Ugur, Ahmet Erdem, et al. (2021)
22.10	SOX/ Hexane	Laroche, Myriam, et al. (2019)
25.50	Supercritical CO_2 / 325 bar, 55 °C	
38.84	SOX/ Petroleum ether	This study
31.52	BU/ Hexane/ 30 min	
31.66	SC / 300 bar, 70 °C	
34.24	PU/ Ethanol / 15 min	

Source: Author

The lipid yield ranged from 31.52% ± 1.4354 (BU_Hex_30 min) to 38.83% ± 2.57 (SOX_E). The alternatives methods showed similar behavior to the conventional method SOX).

Therefore, regarding performance, the methods showed few divergences, with the ultrasound probe being considered the best method. The extraction PU stands out, which reached values closer to the extraction with organic solvent (PU_E_Amb_15 min ≈ SOX_H_65 °C_6 h). Furthermore, other analyses were discussed below to evaluate the interference of unconventional methods on the extracted lipids.

3.3.2 Fatty acid composition of oils by GC–MS analysis

The analysis of the oils from different extraction methods by GC-MS identified the main fatty acids of *T. molitor*. The results are presented in Table 4. No extraction method altered or modified the fatty acids, all of them presented a very similar percentage and the same majority fatty acids. The most abundant were oleic acid (C18:1) (from 39.97% to 41.35%), linoleic acid (C18:2) (24.20% to 25.06) and palmitic acid (C16:0) (from 14.97% to 15.55%). These results were similar to those found in other studies on the lipid profile of the insect (PAUL et al., 2017; RAVZANAADII et al., 2012; TZOMPA-SOSA et al., 2014).

Table 4. Fatty acids (FAs) composition (%). The values expressed as percentages are data from duplicate experiments (mean ± standard deviation). SFA= saturated fatty acid, PUFA= polyunsaturated fatty acid, MUFA= Monounsaturated Fatty Acid.

Fatty acid	SOX (HEX)	SC (CO_2)	PU (ETH)	BU(HEX)	BU (ETH)
C 18:1	40.77 ± 0.31	41.35 ± 0.67	41.09 ± 0.48	40.52 ± 0.40	39.97 ± 0.64
C 18:2	24.91 ± 0.14	24.20 ± 0.11	24.59 ± 0.12	24.77 ± 0.06	25.06 ± 0.35
C 16:0	14.97 ± 0.18	15.15 ± 0.13	15.55 ± 0.11	14.97 ± 0.05	15.10 ± 0.10
C 14:0	6.75 ± 0.02	6.80 ± 0.02	6.66 ± 0.04	6.55 ± 0.02	6.69 ± 0.04
C 16:1	6.08 ± 0.76	6.89 ± 0.15	6.58 ± 0.08	6.96 ± 0.03	6.78 ± 0.15
C 18:0	2.25 ± 0.07	1.85 ± 0.04	2.22 ± 0.06	2.31 ± 0.02	2.40 ± 0.04
C 14:1	0.60 ± 0.05	0.56 ± 0.01	0.55 ± 0.02	0.57 ± 0.01	0.53 ± 0.04
C 12:0	0.43 ± 0.02	0.41 ± 0.02	0.38 ± 0.01	0.42 ± 0.01	0.41 ± 0.01
Others	3.22 ± 0.98	2.76 ± 0.60	2.34 ± 0.34	2.89 ± 0.28	3.04 ± 0.81
SFA	**24.41**	**24.22**	**24.83**	**24.27**	**24.60**
MUFA	**47.45**	**48.82**	**48.23**	**48.07**	**47.29**
PUFA	**24.91**	**24.20**	**24.59**	**24.78**	**25.06**
PUFA/ SFA	**1.02**	**0.99**	**0.99**	**1.02**	**1.01**

Source: Author

The ratio between PUFA/SFA acids ranged from 0.99 (SC and PU_ETH) to 1.02 (SOX_hex and BU_hex), this ratio is related to a healthy human diet, where the recommended ratio is close to 1 (PAUL et al., 2017). The lipids extracted in this study showed a ratio close to one, indicating nutritional potential.

It is worth noting that the rearing conditions can interfere in the insect fatty acid profile, which can justify that no extraction method showed significant changes since all were used the same batch of meal, with the same diet.

Therefore, no significant difference was found in relation to the composition of the oil extracted by the alternative methods and the SOX, which means that the composition of the *T. molitor* oil is highly stable and that the replacement of any extraction method performed will not cause changes in their fatty acids.

3.3.3 Oil characterization

For the characterization of the oil and defatted meal, a representative condition was selected for each extraction method used. These conditions were chosen based on common parameters between the extraction methods, such as the use of the same solvent, similar temperatures, or equivalent extraction time, which will allow analyzing, under comparable conditions, the possible differences between the extraction methods studied.

In the SOX extraction method, was selected the solvent hexane, the same one chosen for the BU extraction. In the BU method, in addition to hexane, the ethanol solvent was selected, with an extraction time of 15 minutes, to compare with the same parameters as the PU method, which uses the ethanol solvent for 15 minutes. For the SC method, the extraction carried out at 70 °C was selected, and the temperature reached by the SOX method during extraction. The determination of free fatty acids is a parameter that evaluates the quality of the oil. In general, higher values of FFAs have negative implications for oil quality (MAHBOUBIFAR et al., 2016; ZHU et al., 2019). Table 5 presents the FFA content of *T. molitor* oils extracted using different extraction methods. *T. molitor* oils extracted by alternatives methods showed % FFA of 2.19 ± 0.03, 2.15 ± 0.23, 1.94 ± 0.03 and 2.16 ± 0.18 when obtained by extraction with BU (solvent: hexane and ethanol), SC and PU, respectively.

Table 5. Free fatty acid (FFA) profile.

% FFA	Extraction Method	Reference
2.75±0.03	Soxhlet / hexane	
2.19±0.03	Ultrasonic bath/ hexane, 15 min	
2.15±0.23	Ultrasonic bath/ ethanol, 15 min	
1.94±0.03	Supercritical CO_2 / 300 bar, 70 °C	This study
2.16±0.18	Ultrasonic probe / Ethanol, 15 min	
3.92±0.2	Supercritical CO_2 / 325 bar, 55 °C	(PURSCHKE et al., 2017)

Source: Author

The FFA values showed no relevant differences between the oils extracted with hexane and the unconventional methods. In the literature, (PURSCHKE et al., 2017) found that no significant difference was found in the SOX and SC methods. The results found in this study were lower than the values reported in the literature, 7.58% ± 0.72 using hexane and 10.84% using petroleum ether as solvent in the SOX extraction of *T. molitor* oil (SETE DA CRUZ et al., 2022; SIOW et al., 2021).

The Rancimat method determines oxidative stability based on changes in conductivity by volatile acids produced along the accelerated oxidation of test oils. The induction period (IT) corresponds to the time that the oil starts its auto-oxidation, indicating its resistance to oxidation (CORDEIRO et al., 2013). The IT data of the samples (Table 6) demonstrated that extraction by the SOX method resulted in a longer induction period (1.64 h) compared to unconventional extractions, with value approached the observed in the work of SETE DA CRUZ et al. (2022) for extraction of *T. molitor* oil with Soxhlet.

Table 6. Oxidative stability of oils obtained by different extraction methods by the Rancimat method at an isothermal temperature of 110 °C.

	Soxhlet /hexane, 70 °C	**Supercritical/ CO_2**	**Ultrasonic bath/ hexane, 45 °C**	**Ultrasonic bath/ ethanol, 45 °C**	**Ultrasonic probe/ ethanol**
IT (hours)	1.64±0.056	1.23±0.134	0.60±0.099	0.27±0.085	0.23±0.014

Source: Author

The extracted oil has a higher degree of unsaturation (by the analysis of the fatty acid composition), usually edible oils with high amounts of polyunsaturated fatty acids and with a higher level of unsaturation are more susceptible to oxidation. In some cases, the antioxidants

present in the oil influence the stability of the oil more than the fatty acids. According to ALMOSELHY (2021), oxidative stability does not depend on a single parameter, it can be affected by the composition of fatty acids and a complex set of antioxidants.

Edible oils of peanut, corn, rapeseed oil and grape seed oil were characterized with TI values of 5.02; 4.85; 5.02, and 2.4 h respectively at 120 °C (MASZEWSKA et al., 2018). Compared to these edible oils, the studied oils showed a lower value. In this case, the fatty acid composition had a greater influence on lipid oxidation.

Among the unconventional methods, the induction time ranged from 0.23 to 1.23 h, with the highest oxidative stability being the oil extracted by the SC method. The oil extracted by the SC method achieved a little lower oil yield than SOX and still has greater stability and does not use a chemically toxic solvent.

Extraction methods that use ethanol as a solvent presented lower values when compared to SOX, a possible explanation would be the fact that ethanol is strongly polar, dissolving a large amount of polar lipids, making the polar lipid part easier to oxidize (LI; XIN; SUN, 2021).

Based on the results of the oil characterization, an oil obtained by an alternative extraction method was selected to continue the studies. The method selected was supercritical extraction, based on its results, being a green method and with potential.

3.3.4 Antioxidant activity

The potential antioxidant activity of the extracts was evaluated by testing the ability of the extracts to inhibit the DPPH· radical and elimination of ABTS radical cations ($ABTS^{\bullet+}$), the results are presented in Table 7.

Table 7. Antioxidant activity of oils extracted by different methods through DPPḢ and ABTS assay. Antioxidant capacity results were expressed in μmol of Trolox equivalent per gram of sample.

	Soxhlet (Hexane)	**Supercritical (CO_2)**	**Ultrasonic bath (Hexane)**	**Ultrasonic bath (Ethanol)**	**Ultrasonic probe (Ethanol)**
DPPH (μmol/g)	1.49 ± 0.02	0.17 ± 0.01	1.47 ± 0.04	3.56 ± 0.16	2.07 ± 0.06
ABTS (μmol/g)	2.23 ± 0.10	0.28 ± 0.04	2.01 ± 0.15	3.86 ± 0.33	3.32 ± 0.13

Source: Author

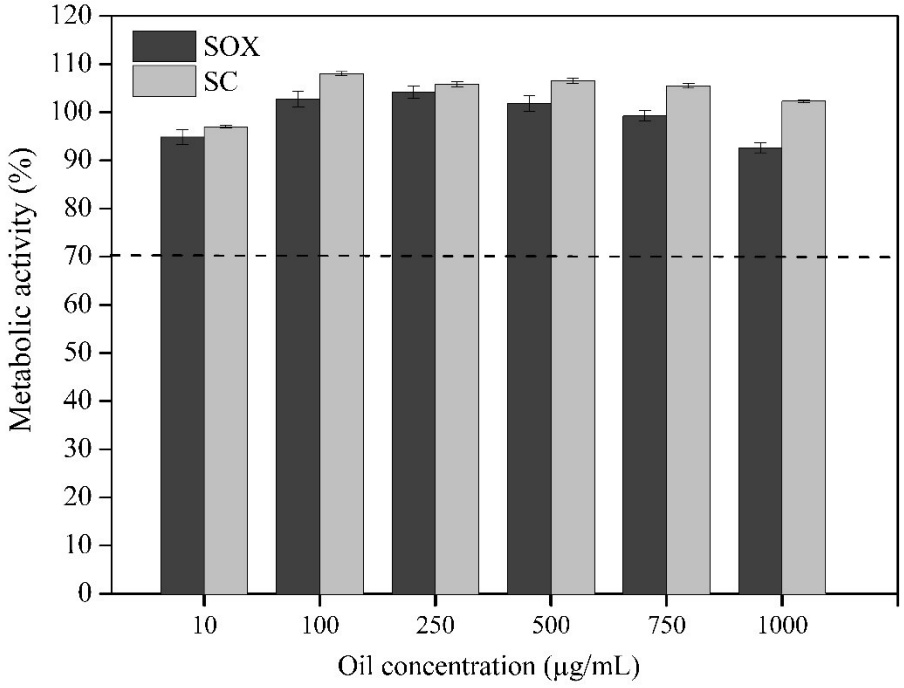
In general, extraction methods using an BU and PU demonstrated equal or greater activity compared to the SOX method. About the extraction method with ultrasonic bath, the extraction solvent affected the antioxidant values in which the oil obtained by ethanol was more efficient compared to hexane. The values of the extraction methods using hexane were very close (SOX and BU), being able to associate the solvent hexane with the less effective antioxidant potential. Extraction methods that used ethanol showed the highest antioxidant values of the oils.

The supercritical extraction method presented the lowest values of antioxidant activity about the other methods. The antioxidant action of the different classes of antioxidants that may be present in oils (tocopherols, phytosterols, polyphenols and flavonoids) may depend on the structure or concentration of each class present in the oil, which can associate with each other and influence the oxidative stability of the oil (GRAJZER et al., 2020). Therefore, it would be important to carry out some studies to more precisely identify these compounds that may be responsible for the antioxidant activity and oxidative stability of the oils studied.

3.3.5 Cytotoxicity

The *in vitro* cytotoxicity of the oils extracted by SOX and SC was evaluated with a murine fibroblast cell line (L929) using the MTS assay (Figure 8) to analyze whether the oils in the present study have any toxic effect that could cause any damage to the cells.

Figure 8. Metabolic activity of L929 cells after direct contact for 24 h with oil extracted from *T. molitor*.



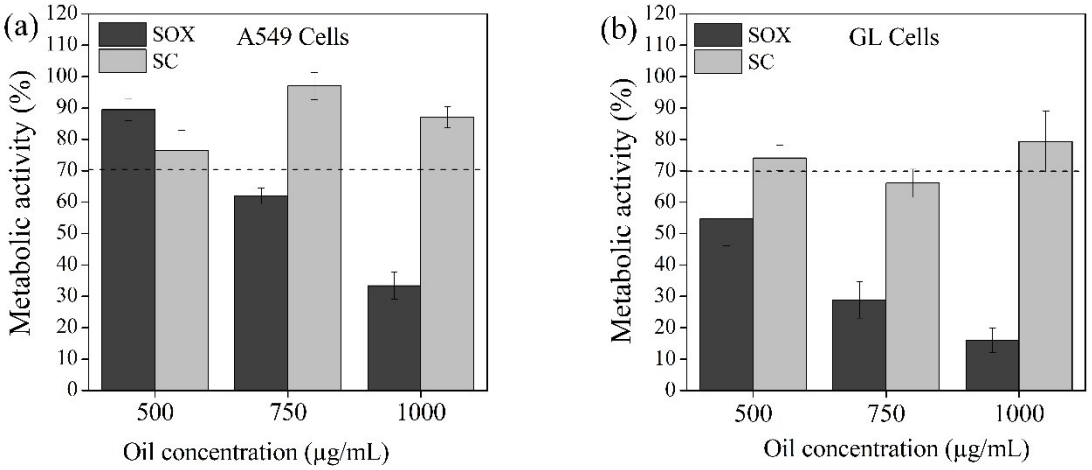
Source: Author

According to ISO 10993/5, the sample is non-toxic if cell viability is greater than 70%. The oils tested at various concentrations did not show cytotoxicity with a statistically significant difference. The cells remained with metabolic activity above 90% for treatment with concentrations of 10, 100, 250, 500, 750, and 1000 µg/mL of the oils obtained by the two extraction methods, demonstrating their non-toxic property.

3.3.6 Antitumoral activity

As observed in Figure 9 (a), the SOX method oil significantly induced a reduction in cell viability, this reduction was greater dependent on the concentration, but it was only from the concentration of 750 µg/mL that the oil began to affect metabolic activity of the A549 cell, with a cell viability of less than 70%. The effect observed in the oil from the SC method was a slight reduction in 24 h, in which, regardless of the concentration, cell viability was greater than 70%.

Figure 9. Percentage of cell viability of A549 (a) and GL (b) cells treated with oil extracted by the Soxhlet (SOX) and supercritical (SC) methods for 24 h. Cell viability was determined by the MTS cell viability assay.



Source: Author

The MTS assay found that the cell growth inhibition of GL cancer cells was greater when treated with the oil extracted from the SOX method than with the oil extracted from the SC method (Figure 9 b). Cell growth was impacted in a concentration-dependent manner over the 24h. The SOX method showed a cell viability of less than 70% at a concentration of 500 µg/mL and the SC method approached a 30% reduction in cell viability.

The anticancer effects of oils may be related to different mechanisms against cancer cells. Fatty acids have an impact on cell proliferation, high concentrations of certain fatty acids can lead to cell death by necrosis or apoptosis (ANDRADE et al., 2005; JÓZWIAK et al., 2020). Interestingly, based on the analysis of fatty acids demonstrated previously, there was no significant difference in the amount of fatty acids present in the oils from the extraction methods (SOX and SC), which could have led to this difference in cytotoxicity of the cells treated with these oils. Fatty acids are shown to exhibit selective cytotoxicity against study cancer cells while causing little or no damage to fibroblast cell line cells.

3.3.7 Protein content

The protein content of crude meals and defatted meals was determined by Kjeldahl analysis using a nitrogen-to-protein conversion factor of 6.25 normally used in food analysis. However, according to the literature, these values may be overestimated when calculated with

the traditional factor 6.25. With this, the protein content present in the insect flour was determined using two specific nitrogen conversion factors for insects, 5.60 and 5.33, found in the literature. The main component of the defatted meal obtained from *T. molitor* is proteins. The results of the protein content of raw meals and defatted meals are shown in Table 8.

Table 8. Comparison of protein content in raw and defatted meals by different methods (calculated on a dry weight basis) and different nitrogen conversion factors / SOX = Soxhlet, SC= Supercritical, BU = Ultrasound bath, PU= Ultrasound probe.

Process	Protein content (g/100 g)		
	F: 6.25	**F: 5.60**	**F: 5.33**
Raw Meal	44.20 ± 0.30	39.60 ± 0.18	37.70 ± 0.18
SOX	70.66 ± 1.55	63.31 ± 0.98	60.26 ± 0.94
SC	65.54 ± 3.36	58.73 ± 2.13	55.90 ± 2.02
BU	68.68 ± 1.62	61.54 ± 1.03	58.57 ± 0.98
PU	71.80 ± 1.92	64.34 ± 1.22	61.23 ± 1.16

Source: Author

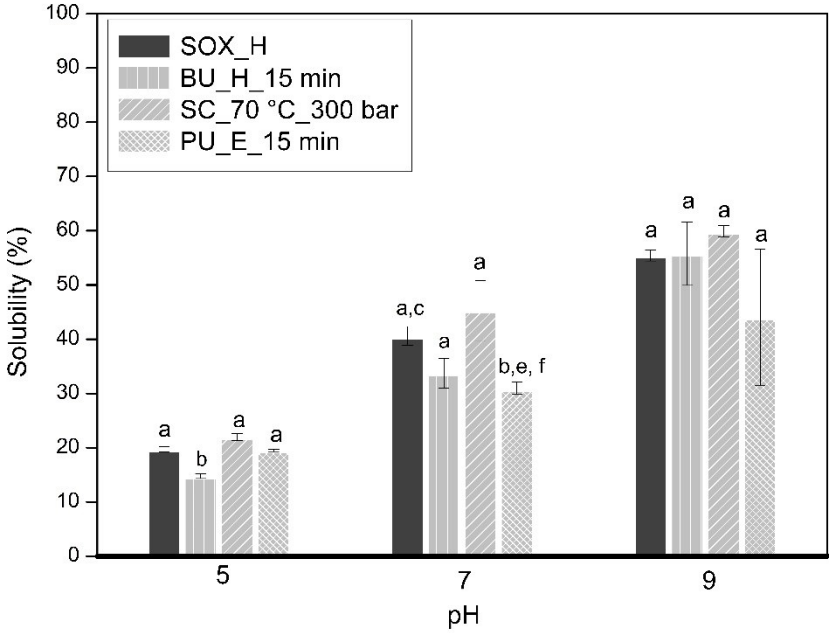
As can be observed, this study confirmed that the protein content in insects was significantly overestimated due to the use of factor 6.25. However, it is essential to carry out more studies to confirm and expand these findings, ensuring a more robust and precise understanding of the specific conversion factor of *T. molitor*.

Using the traditionally used conversion factor of 6.25, the crude protein content was 44.2 (g/100 g) ± 0.29, this value is in line with the values already obtained in other studies with *T. molitor* larvae, Jajic et al. (2019) obtained a protein content value of 55.83% and Ravzanaadii et al. (2012) obtained a content of 46.44%. About the protein content of defatted *T. molitor* larvae, this value reached 71.80% for samples defatted with the ultrasound tip. Yi et al. (2016) obtained a protein content value of 76.5% for *T. molitor* samples defatted with hexane by SOX. The protein contents of the meal defatted by these processes reported in this study, ranging from 65.54% (SC method) to 71.80% (PU method), were all higher than the protein content of the defatted soybean meal of 53.11 ± 0.95% reported by Rahman et al. (2021), for example, which indicates potential applications as a source of protein in the development of new foods. Comparing the values between the alternative methods, the values were in the same range of protein content, including the error values.

3.3.8 Solubility

Solubility is a functional property that depends on the physicochemical properties of proteins and influences their application characteristics (SAWADA et al., 2014; SIBT-E-ABBAS et al., 2020). The pH is one of the factors that interfere with the solubility of proteins, the isoelectric point of food proteins, including edible insects, are in the range of 3 to 6, that is, a greater tendency to obtain a lower solubility at pH close to these values (GKINALI et al., 2022). Figure 10 shows the protein solubility values of defatted meals from all extraction processes in terms of pH conditions (5, 7 and 9).

Figure 10. Protein solubility at pH 5, 7 and 9 of defatted *T. molitor* meal.



Source: Author

The treatment method minimally altered the protein structure. As expected, pH had a significant impact on protein solubility, an increase in solubility was observed as pH increased, indicating that *T. molitor* protein can be better solubilized at neutral to alkaline values of pH. This result was consistent with results reported in the literature, (GRAVEL et al., 2021), observed the same behavior for *T. molitor* proteins defatted with hexane using SOX, which was justified by the authors that the degreasing process can cause a change in the isoelectric point value, which can also be applied in this study.

All alternative extraction methods showed good solubility results and mainly that there was little protein denaturation during the degreasing processes. With an emphasis on the SC extraction, which showed good solubility results even superior to SOX, which may be related to the extraction temperature (70 °C), as the protein solubility is increased at higher temperatures (BUSSLER et al., 2016), which justifies the lower solubility values of the PU extraction, as it was performed at room temperature.

3.3.9 Thermal stability

The DSC analysis method has been used to detect heat-induced protein denaturation or protein structure unfolding, where the denaturation temperature (Td) indicates the thermostability of the protein (BRISHTI et al., 2021). For peak identification, the *Pyris software* was used. During the heating of the samples enthalpic peaks were observed, the results are shown in Table 9.

Table 9. Effect of different extraction methods on the thermal properties of proteins present in the defatted meal of the insect *T. molitor* obtained through the analysis of differential scanning calorimetry (DSC), where Td: denaturation temperature.

Extraction method	Td (°C)	ΔH (J/g)
Soxhlet	66.86	16.820
Ultrasound bath	52.96	46.250
Ultrasound probe	59.19	59.649
Supercritical fluid	43.17	25.554

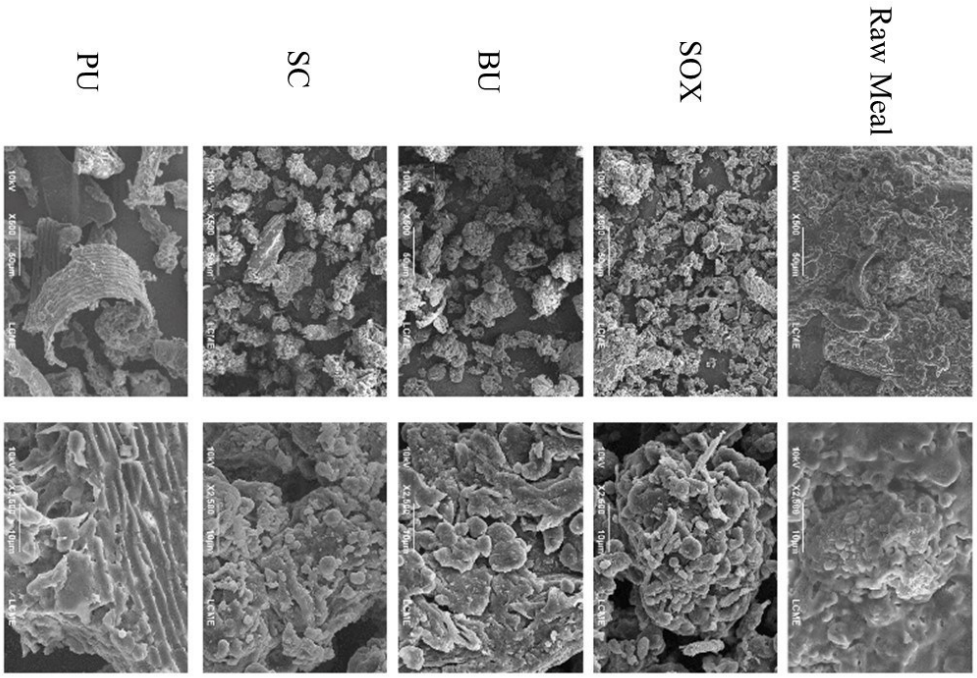
Source: Author

Lower denaturation temperatures were found by unconventional methods (SC= 43.17 °C, BU= 52.96 °C, PU= 59.19 °C) compared to SOX (66.86 °C). The method that most impacted the denaturation temperature was SC, this may be related to the depressurization suffered during the extraction process, which somehow may have affected this result. However, higher enthalpy values were observed in the PU (59.65 J/g), BU (46.25 J/g) and SC (25.55 J/g) methods in relation to the SOX (16.2 J/g). According to the study by BAIGTS-ALLENDE et al. (2021), probably during heating greater hydrogen bonds were broken along the unfolding of proteins in the unconventional methods than in the SOX method.

3.3.10 Morphology (Scanning Electron Microscopy)

To further investigate the impact of defatting methods on the protein structure of *T. molitor* meal, the surface morphology of raw meal and protein concentrates are shown in Figure 11.

Figure 11. Scanning electron micrographs from *T. molitor* meal, where SOX: soxhlet; BU: ultrasonic bath; H: hexane; SC: supercritical; PU: ultrasound tip and E: ethanol. Magnification of 500 and 2500 ×.



Source: Author

The raw meal showed a denser and globular surface, while the meals from the SOX, SC, BU methods showed a similar surface in relation to the formed globules but less dense. The images revealed differences only in the PU process sample. In the PU method, irregularly shaped particles were formed, exhibiting large particles with surface morphology similar to a scaly plaque.

3.4 CONCLUSION

The oils obtained from *T. molitor* are rich in oleic acid (41.35% to 39.97%), with emphasis on the alternative extraction method SC, which presented a yield of 31.66% and oxidative stability of 1. 23 h at 110 °C, values close to the traditional method. The extracted lipids revealed remarkable properties, including antioxidant and anticancer activity. The

observation of the non-toxic property concerning L929 cells highlights the safety of these lipids, reinforcing the feasibility of their incorporation into final products. In relation to its bioactive properties, it achieved an 83.95% reduction in the cell viability of GL cells using an oil concentration of 1000 μg/mL. Thus, the present study reinforces the feasibility and advantages of alternative extraction methods, not only preserving the characteristics of the oil but also enhancing beneficial properties, such as antioxidant and anticancer activity. As for defatted meal, it can be concluded that it has a high protein content, after the processes it reached a percentage of up to 71.8% proteins. Furthermore, it was observed that alternative methods had no impact on protein solubility, with pH being the main influencing factor.

4 CHAPTER 4: CONCLUSION AND PERSPECTIVES

4.1 CONCLUSION

The results obtained when analyzing the influences of each method on the characteristics of the oil highlight that the alternative methods did not significantly change the composition of the oil. Considerably, the percentages of free fatty acids remained consistent and similar between the methods (2.19% (BU_hexane), 2.15% (BU_ethanol), 1.94% (SC), 2.16% (PU)), as well as the amount of major fatty acids in the oils (oleic acid (41.35% to 39.97%), linoleic acid (24.20% to 25.06%) and palmitic acid (14.97% to 15.55%)). The extracted lipids demonstrated antioxidant properties, and it is also important to highlight that these lipids also showed non-toxic properties to L929 cells, indicating a safety profile for future biological applications of the oils. They also revealed anticancer activity, reducing 83.95% of cell viability of GL cells and 66.63% of A549 cells using an oil concentration of 1000 µg/mL extracted by SOX, highlighting a promising potential for therapeutic applications. In this way, integrating the biorefinery concept with *T. molitor* provides a sustainable approach to food production. It is highlighted as an efficient strategy for obtaining good quality bioactive products, adjusting to the growing demands for innovations in health and environmental responsibility.

4.2 PERSPECTIVES

- Evaluate the bioactive compounds present in the oil.
- Evaluate the mechanisms by which bioactive compounds exert their biological effects, investigating their interactions with specific cells and biomolecules.
- Evaluate *T. molitor* oil in the cell migration assay (wound healing).
- Evaluate the effects of oils on lipid accumulation in 3T3-L1 preadipocytes (Anti Adipogenic Activity).
- Evaluate the bioactive compounds present in *T. molitor*-based peptides.

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