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"JÚLIO DE MESQUITA FILHO"

SCHOOL OF PHARMACEUTICAL SCIENCES

Bioscience and Biotechnology applied to Pharmacy Graduate Program

JOÃO FRANCISCO CABRAL DO NASCIMENTO

ENZYMATIC PRODUCTION OF DIETARY TAGS FROM PATAUÁ OIL:

investigation of intensification strategies in different reactors

Araraquara

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Araraquara, 23 de outubro de 2025.

I dedicate this work:

To my beloved parents, Amauri and Maria Adelaide.

To my dear sister, Ana Clara.

To my wonderful grandparents, Wilfrido and Annita.

To the cherished memory of Grandpa Rubens and Grandma Cida (*in memoriam*).

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STATEMENT OF RESPONSIBILITY

The opinions, hypotheses, and conclusions or recommendations expressed in this material are the sole responsibility of the author(s) and do not necessarily reflect the views of FAPESP or CAPES.

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Viver é melhor que sonhar

BELCHIOR, Antônio Carlos. Como nossos pais. 1976.

RESUMO

A crescente demanda por dietas mais saudáveis tem aumentado o interesse por lipídios funcionais, como os triacilgliceróis dietéticos (dTAGs), que rapidamente fornecem energia e podem contribuir para a prevenção de doenças como obesidade e distúrbios cardiovasculares. Neste estudo, o óleo de patauá (OP), um óleo amazônico nativo rico em ácidos graxos insaturados, foi utilizado como matéria-prima para reações de acidólise enzimática em meio livre de solvente com os ácidos butírico (C4:0) e cáprico (C10:0). Um delineamento do composto central rotacional (DCCR) foi aplicado para otimizar as reações de biotransformação de OP-C4 (incorporação de C4:0 nos TAGs de OP) e OP-C10 (incorporação de C10:0 nos TAGs de OP). Dois modelos polinomiais preditivos foram obtidos: OP-C4 (R^2 = 90,84%) e OP-C10 (R^2 = 99,21%). As condições ótimas foram identificadas como C4-MAX (50 °C, 11,7% de carga enzimática, razão molar OP:C4:0 de 1:6,5) e C10-MAX (66,8 °C, 10% de carga enzimática, razão molar OP:C10:0 de 1:6,5), com níveis máximos de incorporação de 20,76 ± 1,46 mol% (C4:0) e 48,87 ± 0,76 mol% (C10:0) após 24 h. O menor desempenho com C4:0 foi atribuído ao seu efeito desidratante no microambiente enzimático, por sua vez, o C10:0 aumentou a atividade catalítica, possivelmente por um efeito semelhante à bioimpressão no sítio ativo. A enzima utilizada (Lipura® Flex) demonstrou estabilidade operacional satisfatória, suportando cinco reações consecutivas (120 h) sem perda de produtividade. As tecnologias de intensificação de processos (ultrassom e micro-ondas) foram avaliadas separadamente. No sistema PO-C4, o ultrassom reduziu o tempo reacional de 24 h para 8 h, mantendo os níveis de incorporação (~20 mol%), enquanto as micro-ondas foram ineficazes. Em contraste, no sistema PO-C10, as micro-ondas reduziram o tempo de reação para 8 h, preservando a alta incorporação (~49 mol%), enquanto o ultrassom não apresentou efeito. Em ambos os casos, a produtividade obtida em 8 h foi equivalente à das reações convencionais em BSTR por 24 h, não apresentando diferença estatística significativa. Esses resultados ressaltam o potencial de aplicação industrial de tecnologias de intensificação na modificação de lipídios, combinando maior produtividade, redução do tempo. Por fim, destaca-se que não foi observada toxicidade para os dTAGs sintetizados no sistema BSTR de 24 h quando avaliados pelo ensaio de toxicidade em HET-CAM, apoiando seu potencial uso nas indústrias farmacêutica e, especialmente, cosmética.

Palavras-chave: acidólise; lipase; ultrassom; microondas; planejamento de experimentos.

ABSTRACT

The growing demand for healthier diets has increased interest in functional lipids such as dietary triacylglycerols (dTAGs), which provide rapid energy and may contribute to the prevention of obesity and cardiovascular diseases. In this study, Patauá oil (PO), a native Amazonian oil rich in unsaturated fatty acids, was used as a raw material for solvent-free enzymatic acidolysis with butyric (C4:0) and capric (C10:0) acids. A central composite rotatable design (CCRD) was applied to optimize the reactions: the biotransformations of PO-C4 (incorporation of C4:0 into the TAGs of PO) and PO-C10 (incorporation of C10:0 into the TAGs of PO). Two polynomial predictive models were obtained: for PO-C4 (R^2 = 90.84%) and PO-C10 (R^2 = 99.21%). Optimal conditions were identified as C4-MAX (50 °C, 11.7% enzyme loading, PO:C4:0 molar ratio 1:6.5) and C10-MAX (66.8 °C, 10% enzyme loading, PO:C10:0 molar ratio 1:6.5), with maximum incorporation levels of 20.76 ± 1.46 mol% (C4:0) and 48.87 ± 0.76 mol% (C10:0) after 24 h. The reduced performance with C4:0 is attributed to its dehydrating effect on the enzymatic microenvironment, whereas C10:0 enhanced catalytic activity, likely through a bioimprinting-like effect. Lipura® Flex also demonstrated satisfactory operational stability, supporting five consecutive reactions (120 h) without loss of productivity. Process intensification technologies were evaluated separately. In the PO-C4 system, ultrasound reduced the reaction time from 24 h to 8 h while maintaining incorporation levels (~20 mol%), whereas microwaves were ineffective. Conversely, in the PO-C10 system, microwaves reduced the reaction time to 8 h while preserving high incorporation (~49 mol%), while ultrasound showed no effect. In both cases, productivity achieved in 8 h equaled that of conventional 24 h BSTR reactions. Importantly, no toxicity was observed for the dTAGs synthesized in the 24 h BSTR system when evaluated by the HET-CAM toxicity assay, supporting their potential use in pharmaceutical and, especially, cosmetic formulations.

Keywords: acidolysis; lipase; ultrasound; microwave; design of experiments.

LIST OF ILLUSTRATIONS

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

TAG – Triacylglycerol

dTAG – Dietary Triacylglycerol

SLS – Triacylglycerol containing short-chain fatty acids at the sn-1 and sn-3 positions, while having a long-chain fatty acid at the sn-2 position

MLM – Triacylglycerol containing medium-chain fatty acids at the sn-1 and sn-3 positions, while having a long-chain fatty acid at the sn-2 position

CALB – Lipase B from *Candida antarctica*

PO – Patauá oil

BSTR – Batch Stirred Tank Reactor

PIT – Process Intensification Technology

C4:4 – Butyric Acid

C10:0 – Capric Acid

CCRD – Central Composite Rotatable Design

PO-C4 – Patauá oil enriched with butyric acid

PO-C10 – Patauá oil enriched with capric acid

ANOVA – One-way Analysis of Variance

RSM – Response Surface Methodology

AOCS – American Oil Chemists' Society

C4-MAX – Maximized condition for butyric acid incorporation into the triacylglycerols of Patauá oil

C10-MAX – Maximized condition for capric acid incorporation into the triacylglycerols of Patauá oil

DF – Degree of Freedom

SS – Sum of Squares

MS – Mean Square

Fcal – Calculated value for the F-test applied

Ftab – Critical value for decision-making in the F-test

p – Significance level

HET-CAM – Hen's Egg Test on the Chorioallantoic Membrane

LIST OF SYMBOLS

® – Registered trademark

R^2 – Coefficient of determination

% – Percentage

g – Gram

µL – Microliter

mL – Milliliter

kg – Kilogram

v – Volume

min – Minute

µmol – Micromole

µm – Micrometer

mm – Millimeter

cm – Centimeter

rpm – Revolutions per minute

U – International unit for enzymes

°C – Degrees Celsius

$\boldsymbol{MW_{oil}}$ – Estimated average molecular weight of the oil

$\boldsymbol{fa_i}$ – Decimal percentage composition of each fatty acid identified in the oil composition

$\boldsymbol{MW_i}$ – Molecular weight of the respective fatty acid identified in the oil composition

$\boldsymbol{A_{ester}}$ – Esterification activity of the enzyme, expressed in U/g

$\propto_1 \ and \ \propto_2$ – Slope coefficients obtained from the linear regression for each reaction duplicate

$\boldsymbol{w_{enzyme}}$ – Mass of the enzyme weighed and used in the assay

$\boldsymbol{ID}$ – Incorporation degree (mol%) of medium- or short-chain fatty acids into the triacylglycerols of Patauá oil

$\boldsymbol{IFA}$ – Number of moles of medium- or short-chain fatty acids in the triacylglycerols of Patauá oil

$\boldsymbol{TM}$ – Total number of moles of fatty acids in the triacylglycerols of Patauá oil

x_1 – Temperature

x_2 – Enzyme loading

x_3 – Molar ratio

Y_{C4} – Incorporation degree of butyric acid into the TAGs of Patauá oil

Y_{C10} – Incorporation degree of capric acid into the TAGs of Patauá oil

FA_{sn-2} – Incorporation degree (mol%) of medium- or short-chain fatty acids specifically in the sn-2 position, calculated relative only to the sn-2 positions of TAGs from PO.

$ID_{sn-1\ or\ sn-3}$ – Incorporation degree (mol%) of medium- or short-chain fatty acids in the sn-1 or sn-3 position of TAGs from PO

ID_{sn-2} – Incorporation degree (mol%) of medium- or short-chain fatty acids in the sn-2 position of TAGs from PO, calculated relative to all fatty acid positions in the triacylglycerol molecule.

L – lysis score used in *HET-CAM* assay

H – hemorrhage score used in *HET-CAM* assay

C – coagulation score used in *HET-CAM* assay

TABLE OF CONTENTS

1 INTRODUCTION

The growing demand for sustainable and health-conscious food products has been driving advancements in research focused on alternative food sources that offer health benefits (Remonatto *et al.*, 2023). Among these, dietary triacylglycerols (dTAGs) stand out as a promising option, providing a balanced lipid source that may help prevent conditions such as obesity, diabetes, and cardiovascular diseases (Nestel *et al.*, 2021; Zhou *et al.*, 2022).

Dietary triacylglycerols are a special class of structured lipids that have incorporated medium- or short-chain fatty acids to positions of the triacylglycerol backbone (Miotti *et al.*, 2024; Tsuzuki, 2005). Their unique structure plays an important role in how they are metabolized: unlike conventional fats, dTAGs are broken down more efficiently in the body, which reduces fat accumulation and contributes to a lower caloric intake (Wang *et al.*, 2022).

The type of dTAG produced depends on the fatty acids used during synthesis. If short-chain fatty acids are incorporated in the sn-1,3 positions of the TAG backbone, the result is an SLS-type dTAG (short–long–short), characterized by short-chain fatty acids at the external positions and a long-chain fatty acid at the internal one, while the incorporation of medium-chain fatty acids leads to an MLM-type dTAG (medium–long–medium). These compounds can be produced through chemical or enzymatic processes.

Enzymatic synthesis has gained particular attention in this field, as it offers advantages over chemical methods such as fewer byproducts, lower energy consumption, and easier purification of the final product (Nascimento et al., 2024; Remonatto et al., 2022c). For dTAG enzymatic synthesis, lipases are applied as catalysts which can then operate through three different pathways: interesterification, esterification, or acidolysis (Utama *et al.*, 2019).

Among these, acidolysis is the most widely used, as it facilitates the acquisition of the desired products. Regarding this pathway, the substrates of the reaction are typically pure TAG or a vegetable oil (rich in TAGs) combined with the fatty acid intended to be incorporated (Bassan *et al.*, 2019; Costa *et al.*, 2018a; Fomuso; Akoh, 1997; Tsuzuki, 2005).

Because of this, one of the most important factors influencing the success of the enzymatic acidolysis reaction is the fatty acid composition of the vegetable oil. For

better results, the oil should be rich in long-chain TAGs, allowing for their strategic replacement with short- or medium-chain fatty acids (Atsakou *et al.*, 2023; Bassan *et al.*, 2019).

Oenocarpus bataua Mart. oil also known as Patauá oil (PO) has recently gained recognition for its diverse applications and economic potential in both local and global markets (Coradin; Camillo; Vieira, 2022). Patauá oil possess a remarkable composition, rich in oleic acid, essential fatty acids, antioxidants, and vitamins, making it an excellent candidate for the production of dTAGs (Almeida, 1952; Speranza; Ribeiro; Macedo, 2015).

Despite its potential, the enzymatic production of modified TAGs comes with challenges. The use of enzymes can be costly, and reaction times are often long to achieve the satisfactory level of fatty acid incorporation. To address these issues, process intensification techniques such as ultrasound (sonication) and microwave irradiation have been explored as ways to accelerate the reaction and improve overall efficiency (Carvalho de Melo *et al.*, 2023; Gogate, 2017; Khan; Rathod, 2018).

Particularly, applying ultrasound generates a phenomenon called cavitation. In its effects, cavitation releases energy and makes it available at conversion sites or enzyme active sites, resulting in higher substrate conversion rates (Khan; Beg; Waghmare, 2021). Additionally, it reduces mass transfer limitations in the system, particularly in biocatalysis involving immobilized enzymes (Zhao et al., 2018; Remonatto et al., 2023; Utama et al., 2019; Zhang et al., 2020).

The application of microwaves in the synthesis process can intensify the reaction through two types of effects: thermal and non-thermal. The thermal effects of microwave irradiation increase the kinetic energy of the substrates, which enhances the frequency and efficiency of collisions between enzyme and substrate, thereby promoting product formation (Aprile *et al.*, 2023; Venturi *et al.*, 2023). In addition, microwave heating is directly absorbed by the reaction substrates, making it faster and more uniform than conventional methods, and providing energy for biocatalysis more efficiently (Carvalho de Melo *et al.*, 2023; Venturi *et al.*, 2023).

The non-thermal effects of microwave application are related to the principles of dipolar rotation induced by the alternating electromagnetic field. That is, studies suggest that dipolar rotation may influence the molecular orientation of substrates as well as the flexibility of the biocatalyst, favoring catalytic states and conformations that

are more active and interactive with the substrates in the biocatalytic mechanism of action (Khan; Rathod, 2018).

In general, the enzymatic production of dTAGs is already well established for various substrates and commercially available enzymes. In the literature, the main justification for studies involving these molecules is based on their nutritional functionality (Jadhav; Annapure, 2021; Wang *et al.*, 2022). For example, when consumed in foods, dTAGs tend not to accumulate in adipose tissue, provide fewer calories, and are also associated with the regulation of cholesterol levels (Osborn; Akoh, 2002; Souza-Gonçalves *et al.*, 2023). However, despite the recognized potential of structured lipids as bioactive molecules, their applications beyond the nutritional context have been little explored.

Lipids are also known to play an important role in the cosmetic industry, where they act as emollients, moisturizers, antioxidants, and perform other functional roles in formulations (Abdalla; Aroua; Gew, 2024). Nevertheless, in the case of dTAGs, little has been documented regarding possible applications of these modified lipids (Miotti *et al.*, 2024). This opens opportunities to investigate their behavior and effects in biological systems, starting with laboratory assays, whether *in vitro* or *in ovo*, that may provide promising insights into the use of dTAGs in cosmetic or pharmaceutical formulations.

The present study proposes the production of dietary triacylglycerols (dTAGs) via enzymatic acidolysis in order to enrich Patauá oil with butyric and capric acids. In this context, the aim was not only to investigate the synthesis of dTAGs from Patauá oil, but also to evaluate this process under different reaction conditions, seeking optimized parameters for the incorporation of the target fatty acids. Furthermore, the study assessed the potential of process intensification strategies, such as ultrasound and microwave irradiation, to enhance the performance of Lipura® Flex by reducing the time required for dTAG production. In addition, the biological potential of the synthesized dTAGs was examined through Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM) toxicity assay.

6 CONCLUSIONS

Based on the results obtained up to the synthesis stage, patauá oil proved to be a suitable substrate for the enzymatic production of dietary triacylglycerols (dTAGs), due to its high content of oleic (80.74%) and palmitic acids (10.00%).

The experimental design (CCRD) enabled the development and validation of significant polynomial models for the incorporation of butyric (R^2 = 90,84%) and capric (R^2 = 99,21%) acids, identifying optimal conditions for synthesis catalyzed by Lipura® Flex, with high efficiency and operational stability over up to five consecutive cycles (a total of 120 hours under reaction). The evaluated process intensification technologies were effective, reducing the reaction time from 24 h to 8 h, depending on the substrate, without compromising yield, demonstrating the feasibility of applying such strategies to accelerate the production of these biomolecules.

The obtained dTAGs (20.76 ± 1.46 mol% incorporation of C4:0 and 48.87 ± 0.76 mol% of C10:0 within 24 h in a BSTR system) showed no toxicity in *in ovo* assays, indicating potential for use in pharmaceutical and cosmetic formulations, where they can act as lipid carriers, emollients, or bioactive components in controlled-release systems. However, further studies are required to investigate their bioactive properties, stability, and performance in complex formulations.

The application of process intensification technologies, such as ultrasound and microwave irradiation, represents a promising approach for large-scale production of these compounds, shortening reaction times while maintaining high efficiency. Nevertheless, complementary evaluations of operational cost, energy consumption, and environmental impact are essential for the establishment and optimization of the synthesis process within a green chemistry framework.

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