UNIVERSIDADE FEDERAL DO ESTADO DO RIO DE JANEIRO - UNIRIO

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE – CCBS

PROGRAMA DE PÓS-GRADUAÇÃO EM ALIMENTOS E NUTRIÇÃO - PPGAN

Millena Cristina Barros Santos

Untargeted metabolomics for the characterization of wheat, rice and some of their coproducts applying conventional, deep eutectic solvents and *in situ* biotransformation

Rio de Janeiro

December, 2021

Millena Cristina Barros Santos

Untargeted metabolomics for the characterization of wheat, rice and some of their coproducts applying conventional, deep eutectic solvents and *in situ* biotransformation

Thesis defense presented to the jury in the PhD course of the Graduate Program in Food and Nutrition, Federal University of the State of Rio de Janeiro.

Supervisor: Prof. Dr. Mariana Simões Larraz Ferreira

Co-supervisor: Prof. Dr. Claire Bourlieu-Lacanal

Rio de Janeiro

December, 2021

S237

Santos, Millena Cristina Barros
Untargeted metabolomics for the characterization of wheat, rice and some of their coproducts applying conventional, deep eutectic solvents and in situ biotransformation / Millena Cristina Barros Santos. -- Rio de Janeiro, 2021.
275

Orientadora: Profa Dra Mariana Simões Larraz Ferreira.
Coorientadora: Dra Claire Bourlieu-Lacanal.
Tese (Doutorado) - Universidade Federal do Estado do Rio de Janeiro, Programa de Pós-Graduação em Alimentos e Nutrição, 2021.

1. Metabolomics based on LC-MS. 2. Phenolic compounds. 3. Conventional and NaDES extraction. 4. Wheat and rice. 5. Coproducts. I. Simões Larraz Ferreira, Profa Dra Mariana , orient. II. Bourlieu-Lacanal, Dra Claire, coorient. III. Título.

Millena Cristina Barros Santos

Untargeted metabolomics for the characterization of wheat, rice and some of their coproducts applying conventional, deep eutectic solvents and *in situ* biotransformation

PhD thesis presented to the Graduate Program in Food and Nutrition at the Federal University of the State of Rio de Janeiro as partial requirement for the title of PhD in Food and Nutrition.

Approved on: 17/12/2021.

JURY

President: Prof. Dr. Mariana Simões Larraz Ferreira
(PPGAN/UNIRIO) – Rio de Janeiro, Brazil

Prof. Dr. Elizabeth Ryan
Colorado State University - Colorado, United States

Dr Pierre Villeneuve
CIRAD - Centre de Coopération Internationale en Recherche Agronomique pour le Développement - Montpellier, France

Prof Dr Jesús Lozano-Sánchez
Department of Analytical Chemistry – University of Granada, Spain

Prof Dr Daniel Pelrone
UFRJ - Instituto de Química, Rio de Janeiro, Brazil

Dedico este trabalho aos meus pais e ao meu irmão.

Agradeço à Universidade Federal do Estado do Rio de Janeiro (UNIRIO).

Ao Programa de Pós-Graduação em Alimentos e Nutrição (PPGAN) por tudo que me foi oferecido durante esses exatos 4 anos e 9 meses, pela oportunidade de acesso à laboratórios com equipamentos de última geração e de participar de eventos científicos apresentando meu trabalho.

Agradeço à CAPES pela bolsa concedida para execução do Doutorado.

À FAPERJ pela bolsa concedida para execução do Doutorado Sanduíche.

Ao CNPq e FAPERJ por financiarem a pesquisa.

Aos membros da banca por terem aceitado o convite de avaliarem o meu trabalho (Profa. Dra Elizabeth Ryan, Dr. Pierre Villeneuve, Prof. Dr. Jesús Lozano-Sánchez e Prof. Dr. Daniel Perrone).

À empresa OR Melhoramento de Sementes Ltda., BioTrigo e EMBRAPA Trigo pelo fornecimento das amostras de grãos de trigo e farinhas refinadas utilizadas nesse estudo e ao Centro Francês de Arroz pelo fornecimento das amostras de farelo de arroz incluídas na coleção estudada.

À minha orientadora Profa. Dra. Mariana Simões Larraz Ferreira e coorientadora Dra Claire-Bourlieu-Lacanal, por serem duas pesquisadoras incríveis, literalmente mulheres-maravilhas que são uma inspiração para mim na vida e na ciência. Por dividirem comigo todo o conhecimento que elas possuem, muitas vezes repetidamente e com muita paciência. E por serem para mim muito mais que orientadoras, mas muitas vezes amigas e um cadinho de psicólogas. Por me incentivarem, acreditarem e buscarem o melhor de mim. Por me confiarem parcerias que foram importantíssimas para o meu desenvolvimento e crescimento pessoal e profissional. Por terem me proporcionado o inimaginável, o que nem em sonho eu pensei um dia viver.

À Profa. Dra. Mariana Simões Larraz Ferreira, por ter me aberto as portas há 8 anos quando eu buscava ainda entrar na pesquisa. Por ter me escolhido e confiado em mim e no meu trabalho, sem nem ao menos me conhecer. Por buscar e proporcionar o melhor para mim (e para todos os seus orientandos): melhores condições trabalhos, equipamentos, auxílios, participações em eventos científicos. Por pensar e se dedicar a nós em todos os momentos. Por estar sempre presente, ser uma excelente líder nos dando voz, segurança e tratando seus discentes de igual para igual. Por compartilhar de momentos como orientadora, amiga e psicóloga, dividindo momentos dentro e fora do laboratório, de alegria, de tristeza e de angústias. Por escrever meu nome com 2L desde o começo. Por me apresentar à Claire e por

me dar a oportunidade de conhecer outro país/cultura. Um abraço especial ao Pedro (amigo do Marcão) e ao Caetaninho.

À Dra. Claire Bourlieu-Lacanal, pour m'avoir reçue à Montpellier, pour m'avoir ouvert les portes de son laboratoire mais aussi de sa maison. Pour avoir été toujours présente et occupée de me fournir le meilleur séjour et les meilleurs enseignements. Pour m'avoir littéralement donnée une famille incroyable en France. Un câlin très spécial à tes parents, Hélène et Jean-Yves, à ton mari Olivier, à ta sœur Laure et Benjamin, et aux petits Coline, Emma, Sasha et Ambre qui m'ont rempli d'amour, de moments inoubliables et m'ont offert un stage qui a renforcé mon désir d'être maman.

Ao Prof. Luiz Cameron, diretor do Centro de Inovação em Espectrometria de Massas da UNIRIO, por me permitir acesso ao mesmo e por ter sido o responsável pelo meu primeiro contato com a Profa Mariana (lá em 2013).

À Profa. Dra. Édira Castello Branco de Andrade Gonçalves e a Profa, Dra. Maria Gabriela Koblitz, por estarem sempre presentes e trabalharem com excelência garantindo a qualidade oferecida aos discentes do PPGAN.

Ao Prof. Dr. Daniel Perrone, que foi membro das minhas bancas desde o início, com pontuações importantes, e críticas pontuais e construtivas que me desafiaram a buscar maior aprofundamento no conhecimento e evoluir como pesquisadora.

Ao Dr. Michael Murgu, Robinson Giraldes e Gustavo Almeida, da Waters Corporation, por todo ensinamento relacionado às análises ômicas, por toda disponibilidade em nos ajudar e tirar dúvidas com muita paciência.

Ao Team Glúten e às bioativas e bioativos mais queridos do Laboratório de Bioativos (Luciana Lima, Mariana Pimentel, Thais Alves, Carolina Thomaz, Talita Braga, Verônica Mayrinck, Diego Kalili Calandrini, Talita Pimenta, Oscar Lombo, Ingrid Maia, Sónia Rodrigues, Nathália Sales, Renata Marenda) que dividiram diariamente o trabalho, a bancada, as lutas, os cafés com canela, os bolos, risadas e inúmeros momentos que estão guardados para sempre no meu coração. Dividir esses anos com vocês foi incrível e me fizeram crescer muito. Vocês tornaram leve o que sozinha seria muito pesado de carregar.

Em especial ao meu trio metabolômico: Lhulhu e Carol. Vocês são o LC do meu MS.

Às moradoras do apto 110, em especial Thais, Larissa, Carol, Thamara e Juliana. Foram anos maravilhosos dividindo a vida e as lutas da pesquisa.

À l'équipe française pour m'avoir reçu à bras ouverts, pour avoir compris mon français débutant et pour avoir été important pour mon progrès en langue et en recherche. Pour toutes

les connaissances qui m'ont été transmises. Pour tous les moments partagés à l'intérieur et à l'extérieur du laboratoire. En particulier au Dr. Pierre Villeneuve, Dr. Erwann Durand, à Bruno Barea, à Nathalie Barouh et au Dr. Jérôme Lecompte.

Aux cher(e)s ami(e)s que j'ai rencontrées à Montpellier et qui ont partagé avec moi des moments inoubliables, en particulier Marcia Ortega et Julien, Amal Fenaghra, Mèlina Robert, Samantha Lamy et Thibaut, Guilherme Reis et Juliana, Oscar Laguna et Fanny, et Isidora Ilic.

Aos meus pais e ao meu irmão, que são minha base e foram primordiais na minha decisão de entrar na pesquisa, me dando todo o suporte que precisei (como sempre), por estarem sempre comigo, me apoiando, incentivando e me dando muito amor. Eu amo muito vocês!

Aos meus familiares e amigos que, além de entenderem minhas ausências, sempre me incentivaram e torceram pra que eu tivesse muito sucesso nesse percurso. Em especial, meus primos Thiago, Bruna, Maicon, Amanda, Iessa e Cláudia, minhas tias Rose, Leilah e Cláudia, minhas amigas Fabiana, Mayara, Leide, Renata e Rachel, e meus amigos Alan e Jeferson.

À Deus, por ter colocado pessoas maravilhosas em meu caminho, por ter me dado forças e guiado nos momentos em que achei que não seria possível.

À Ciência.

À Pesquisa.

Ao ensino público e de qualidade.

“Tão importante quanto à chegada é o caminho percorrido, pois o que vivemos no percurso nos faz chegar mais evoluídos.”

Plínio de Sá

Abstract

Wheat (*T. aestivum*) and rice (*O. sativa L.*) are the most important cereals in the world, being among the top three crops. The nutritional benefits of cereals are notably related not only to their dietary fibers but also to their phytochemicals. Cereal grains and their coproducts are rich in secondary metabolites showing a wide spectrum of bioactivities. The class of major interest is the phenolic compounds (PC) due for instance to their protective effect against the development of chronic diseases and great antioxidant capacity. PC composition is strongly affected by genetic and external factors throughout the grain growth until grain processing. PC can be found in soluble, soluble-conjugated or insoluble forms linked to cell wall components and are concentrated in the grain outer layers. These outer layers also enclosed important amount of bioactive polar lipids (PL) such as phospholipids that are the backbone of cell membranes. PC extraction should be considered as a pre-treatment allowing the development of dietary or pharmaceutical formulations. PC have a moderate to low solubility in apolar media that restricts their application in oil-based products. Improving their amphiphilic character by biotransformation (e.g., esterification with acyl chain) can be a strategy to preserve their functions and enhance their release. The choice of the solvent to extract PC is crucial to maximize yield, recovery, and subsequent application. Natural deep eutectic solvents (NaDES) appear as green alternative to conventional organic solvents due to their capacity to improve the solubility, stability of some amphiphilic bioactive compounds and to their good enzymatic compatibility. The chemical complexity of these extracts can be resolved by metabolomics techniques that allow a broad and reliable identification of compounds with different physicochemical properties. In this study, the comprehensive characterization of bioactive components, such as PC but also some lipids (including PL), extracted based on different strategies from several genotypes of wheat, rice and their coproducts, were performed by ultra-performance liquid chromatography coupled to mass spectrometry *en tandem* (UPLC-MS-MS). Wheat metabolomic analysis revealed different profiles of PC, with 237 PC identified during grain maturation and the highest diversity and abundance in immature grains. Despite of the milling process, refined wheat flours presented an interesting phenolic profile with 43 PC identified. Undoubtedly, extracts from aleurone and wheat bran enclose a distinct set of PC (globally 44 PC). Stabilized rice brans (RB) showed high PC contents, especially for pigmented rice (89 PC in red and black RB), together with an interesting amount of unsaturated fatty acid. Lipidomics revealed the presence of bioactive lipids, 163 lipids in RB (e.g., PL and oxylipins) in a large collection of RB and highlighted putative modulations in the lipid profile in human plasma (e.g., endocannabinoids) associated with RB consumption. Due to this interesting chemical profile, RB was selected for the one-pot enzymatic biotransformation, combining PC and lipids to obtain enriched potentially esterified or transesterified extracts with enhanced protection against lipid oxidation. We could evidence that NaDES can be used as reaction medium and remain in the formulation, unlike conventional solvents. Chromatographic changes after biotransformation suggests a structural rearrangement between PC and lipids and a pro-oxidant effect of the NaDES tested was evidenced. NaDES showed a specificity to extract some PC and proved to be a sustainable media for *in situ* biotransformation of the PC using a lipase.

Keywords: aleurone, bran, HPLC, LC-MS, NaDES, phenolic compounds, lipids

Resumo

O trigo (*T. aestivum*) e o arroz (*O. sativa L.*) são os cereais mais importantes do mundo, estando entre os três principais cultivos. Os benefícios nutricionais dos cereais estão relacionados não apenas com suas fibras alimentares, mas também com seus fitoquímicos. Os cereais e seus coprodutos são ricos em metabólitos secundários, que apresentam um amplo espectro de bioatividades. A classe de maior interesse são os compostos fenólicos (CF), devido, por exemplo, a seu efeito protetor quanto ao risco do desenvolvimento de doenças crônicas e excelente capacidade antioxidante. A composição dos CF é fortemente afetada por fatores genéticos e externos desde a maturação até o processamento dos grãos. Os CF podem ser encontrados nas formas solúveis, conjugadas ou insolúveis ligadas a componentes da parede celular e concentram-se nas camadas externas do grão. Estas camadas também apresentam importantes quantidades de lipídios polares (LP) bioativos, como os fosfolipídios presentes das membranas celulares. A extração de CF deve ser considerada como um pré-tratamento que permite o desenvolvimento de formulações alimentícias ou farmacêuticas. Os CF apresentam solubilidade moderada a baixa em meios apolares, o que restringe sua aplicação em produtos à base de óleo. Melhorar o caráter anfifílico dos CF por biotransformação (ex., esterificação com grupo acila) pode ser uma estratégia para preservar suas funções e melhorar sua liberação. A escolha do solvente é crucial para maximizar o rendimento, a recuperação e a subsequente aplicação dos CF. Os solventes eutéticos profundos naturais (do inglês, NaDES) aparecem como uma alternativa sustentável aos solventes orgânicos convencionais devido à sua capacidade de melhorar a solubilidade, estabilidade de alguns compostos bioativos anfifílicos e à sua boa compatibilidade enzimática. A complexidade química desses extratos pode ser resolvida por técnicas metabolômicas que permitem identificação ampla e confiável de compostos com diferentes propriedades físico-químicas. Neste estudo, a caracterização abrangente de componentes bioativos, como os CF, mas também lipídios (incluindo LP), extraídos com base em diferentes estratégias a partir de diversos genótipos de trigo, arroz e seus coprodutos, foi realizada por cromatografia líquida de ultra-performance acoplada à espectrometria de massa *em tandem* (UPLC-MS-MS). A metabolômica do trigo revelou diferentes perfis de CF, com 237 CF identificados ao longo da maturação do grão e com maior diversidade e abundância em grãos imaturos. Apesar do processo de moagem, as farinhas de trigo refinadas apresentaram um perfil fenólico interessante com 43 CF. Extratos obtidos da camada aleurona e de farelo de trigo representam uma fonte distinta de CF (44 CF). Os farelos de arroz (FA) estabilizados apresentaram alto conteúdo de CF, especialmente em arroz pigmentado (89 CF em arroz vermelho e negro), juntamente com um interessante perfil de ácidos graxos insaturados. A lipidômica do FA revelou a presença de lipídios bioativos, 163 lipídios (ex., LP e oxilipinas) e destacou modulações putativas no perfil lipídico no plasma humano (ex., endocanabinoides) associados ao consumo de FA. Devido ao interessante perfil químico do FA, ele foi selecionado para a biotransformação enzimática de um ponto combinando CF e lipídios (esterificados ou transesterificados) para obter extratos de FA potencialmente enriquecidos com maior proteção contra a oxidação lipídica. Foi possível provar que o NaDES pode ser usado como meio reacional e permanecer na formulação, ao contrário dos solventes convencionais. As mudanças cromatográficas após a biotransformação sugerem um rearranjo estrutural entre CF e lipídios, e um efeito pró-oxidante dos NaDES testados foi evidenciado. Os NaDES mostraram uma especificidade para extrair certos CF e provaram ser um meio sustentável para a biotransformação *in situ* de CF usando uma lipase.

Palavras-chave: aleurona, farelo, HPLC, LC-MS, NaDES, compostos fenólicos, lipídios.

List of figures

List of tables

Abbreviations

ANOVA - Analysis of variance

CALB - *Candida Antarctica* Lipase B

CID – Collision Induce Dissociation

DAG - Diacylglycerols

DES - Deep Eutectic Solvents

ESI – electrospray source ionization

FFA – Free Fatty Acids

GC-MS - Gas-chromatography Mass Spectrometry

HCA - Hierarchical Cluster Analysis

HILIC - hydrophilic interaction liquid chromatography

HPLC – High-Performance Liquid Chromatography

iCALB - Immobilized *Candida Antarctica* Lipase B

LC-MS – Liquid Chromatography Mass Spectrometer

m/z – mass/charge ratio

MAG - Monoacylglycerols

MUFA – Monounsaturated fatty acids

NaDES - Natural deep eutectic solvents

NEO-PUFA - Non-enzymatic oxidation of polyunsaturated fatty acids

OPLS - Orthogonal Partial Least Squares

OPLS-DA - Orthogonal Partial Least Squares-Discriminant Analysis

PC - Phenolic compounds

PCA - Principal Component Analysis

PL – Polar lipids

PLS – Partial Least Squares

PLS-DA – Partial Least Squares -Discriminant Analysis

PUFA – Polyunsaturated fatty acids

RB – Rice bran

SD - Standard Deviation

TAG – Triacylglycerols

TLC – Thin-Layer Chromatograph

UPLC-MS/MS - Ultra Performance Liquid Chromatograph coupled to tandem Mass Spectrometry

UPLC-MS^{E} - Ultra Performance Liquid Chromatograph coupled to Mass Spectrometry based on multiplex data independent acquisition

VIP - Variance Important Projection

Summary

General introduction, objectives, and strategy

This PhD thesis was carried out at the Graduate Program in Food and Nutrition (Programa de Pós-Graduação em Alimentos e Nutrição – PPGAN) within the line of research "Processing, quality, valorization of food, coproducts and residues" that seeks to meet the challenges of the agricultural and industrial sectors, considering the need for increasing the production of safe and healthy food; the more efficient use of natural resources, the production of biomaterials and biodegradable packaging, and the use of new raw materials, having Bioeconomy as a strand. This line of research has four research projects, and this thesis is part of the project "Metabolomics and proteomics of food" that aims to produce innovative results through the application of modern analytical techniques, such as omics tools, as well as the use of green chemistry and bioprocesses to reduce food waste and promote safe, healthy, and sustainable food.

It is worth mentioning that the objectives of this work contemplates 3 of the 17 Sustainable Development Goals instituted by the UN to protect the planet: #2 (zero hunger), #12 (responsible consumption and production) and, #17 (Partnerships for the goals) that emphasizes the importance of partnerships to promote development and integration of different countries, here represented by the study of a huge collection of food samples, in addition to the scientific exchange between Brazilian and international institutions.

This research was focused to improve knowledge about the chemical composition of wheat and rice, the most consumed cereals by humans and the staple food for nearly half the world population, and to promote the valorization of their coproducts. Comprehensive information about the metabolome of these two cereals and their coproducts will help the selection of varieties with an improved retention and diversity of bioactive compounds leading to the better valorization of wheat, rice and their coproducts for human food applications and also for non-food applications (e.g., pharmaceutical applications).

Cereals are considered as the main source of energy for humans due to their high polysaccharides content that must represent 50% of total energy intake in human diet. They are especially of great nutritional importance in developing countries where they represent the main staple food products, also contributing to the protein apport. These grains also present a richness of many health-promoting components, which have high complexity and diversity of biological effects (Serna-Saldivar, 2010b). The consumption of whole grains and their beneficial effects on human health were previously associated with the presence of fiber,

minerals, and vitamins present in the outer layers. More recent studies revealed indeed that they are also due to the phytochemicals, particularly to the phenolic compounds (PC).

Despite the large production and consumption of cereals in the world, malnutrition is still a reality. According to the 2020 Global Nutrition Report, one out of nine persons suffers from hunger while, paradoxically, one out of three is overweight or obese, in addition to the huge amount of non-communicable diseases related to diet present in many countries. In this challenging nutritional context, the main output of the present work is *to improve human nutrition by providing strategies to enrich our diet with bioactives present in cereals and especially abundant in their coproducts*. The prerequisite for achieving such output is to efficiently extract and comprehensively characterize the PC profiles present in the two cereals most consumed by humans: wheat and rice. In addition, a full characterization of lipids, an important nutrient in rice bran (RB) was conducted on a collection of RB.

The starting point of this thesis followed three hypotheses: i) wheat and rice PC contribute, through the consumption of whole grains or bran fractions, to rebalance human diet otherwise deficient in bioactive compounds; ii) omics tools are high throughput methods able to elucidate the diversified profile of PC and other bioactive compounds present in wheat and rice, iii) biological (cultivar, maturation state, different tissues/fractions) or chemical (extraction solvents, biotransformation) levers can be used to investigate and modulate the concentration of the bioactive compounds from wheat and rice.

Hence, the main objectives we want to reach in this thesis can be defined as follows:

(i) the exhaustive characterization, focusing on PC and lipids, by untargeted metabolomics approaches of rice and wheat, considering different stages of grain maturation, genetic diversity and the resulting coproducts obtained from the industrial milling; (ii) the evaluation of different solvents (conventional and deep eutectic) as extraction media for rice and wheat PC from coproducts, (iii) the development of enzymatically biotransformed PC rich extracts combining lipids and PC from pigmented RB, with potentially enhanced antioxidant activity.

The results obtained during this thesis are structured in seven scientific papers, three of them are already published in peer reviewed scientific journals, one is submitted, and the last three are in preparation for submission. In addition, a first chapter covers the literature review, and a final chapter presents an overall discussion of the current work, summarizes the main conclusions, and opens perspectives for future work. The present manuscript is organized in five major parts encompassing 9 chapters:

Chapter 1 is dedicated to the literature review focusing on the most significant cereals for promoting human health, the main coproducts generated by their industrial processing and the worldwide pressing need to support sustainable and nutritional food by the valorization of these coproducts. The review also emphasizes the beneficial effects of the PC, the major class of secondary metabolites present in cereals. In addition, updated techniques of extraction, analysis and promising applications of these PC are detailed. The possibility of extracting them applying green solvents, such as the Deep Euctectic Solvent (DES), with pros and cons of such green chemistry principles is discussed.

- Part I of this thesis is entitled "Phenolic profiling based on untargeted metabolomics in wheat" and consists in the comprehensive characterization of PC in wheat grains and flours by untargeted metabolomics. More precisely, this chapter followed PC biosynthesis during grain growth (immature grains), studied the effect of genotypes and flour processing on PC profile. Two chapters are presented in this part, each one consisting of a previously published article.
- Part II is entitled "Characterization and valorization of PC in rice and wheat coproducts" and the main goal was to characterize by classic chromatography and metabolomics tools the two cereal coproducts applying conventional and green solvents to effectively extract PC. Two chapters are presented in this part, one already published and the other in preparation for submission.
- Part III is entitled "Rice coproducts as an important source of a complex mixture of bioactive compounds" and is based on the results found and presented in the previous chapters. Indeed, a vast literature review was performed on this coproduct, and a gap in the knowledge was found in the elucidation of the lipid profile of RB from omics tools. In order to narrow this gap, we have gathered an important RB collection, across a global set of rice cultivars grown under different environmental and agricultural conditions to deeply study and advance the valorization of RB as a supplement for human nutrition. This collection was physico-chemically characterized, and its lipid profile was elucidated by applying cutting-edge lipidomics approach. In this part two articles are presented: both in preparation for submission.
- Part IV is entitled "Application and valorization of pigmented RB coproduct extracts in heterogeneous media". For this last step, all the characterizations previously performed were considered to select the ideal coproduct (here RB). The aim of this part was to valorize the rice coproduct developing a potentially enriched extract, using eutectic solvent as extractant and enzyme reaction medium promoting an *in vitro* biotransformation between the

main compounds present in the RB (PC and lipids). In this final part, one chapter is presented with recently submitted short communication.

- Part V is dedicated to a general discussion encompassing the main results found in this thesis, the concluding remarks, and some proposed prospects for future works.

This doctoral thesis was executed in the "Direct Doctorate" modality, being the first thesis developed in this mode in the PPGAN. This thesis was possible thanks to a research network established by PPGAN-UNIRIO that I would like to mention already at this stage, since their involvement was important for the success of the project. First of all, this thesis was carried out in the framework of the research group of CNPq “Proteomics and metabolomics of bioactive compounds - Omics sciences applied to organisms of economic and biotechnological interest” in the Laboratory of Bioactives and Center of Innovation in Mass Spectrometry (IMasS) of UNIRIO, where all metabolomics data acquisition and processing was performed. A part of this thesis was integrated within a network of French Institutions: The French National Institute for Food, Agriculture and Environment (INRAE), the “Institut Agro” and the Center for International Cooperation in Agricultural Research for Development (CIRAD) with 2 internships that represented 21 months spent abroad in the city of Montpellier, France. Two research groups specialized in cereal analysis (Grain(e)s, directed by V. Lullien-Pellerin) and in green chemistry and antioxidant activity (LiFFHe, directed by P. Villeneuve with co-supervising by E. Durand, N. Barouh and B. Barea), welcomed me in the best possible way, allowed me to have access to a vast knowledge and worked closely with me during these months.

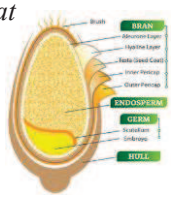
The non-omics analyses of wheat coproducts (bran and aleurone layer) and initial characterization of NaDES were performed at the laboratory in INRAE thanks to the collaboration with Melina Robert. On a collaborative point of view, the partnership with Dr. Elizabeth Ryan (USC, Fort Collins, Colorado) allowed to obtain a great exchange of knowledge and the possibility to treat lipidomics data from RB and human plasma. It was also made possible thanks to the collaboration with Dr. Thierry Durand and all his team (IBMM, Montpellier, France) to analyze key oxylipins (phytoprostanes and phytofurans) present in the RB collection, using targeted lipidomics.

Thesis design

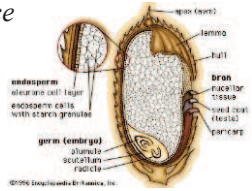
***Main output*: to improve human nutrition by providing strategies to enrich our diet with bioactives present in cereals and especially abundant in their coproducts**

2 main cereals consumed by human

Wheat



Rice



High amount of coproducts: gold vein for bioactives?

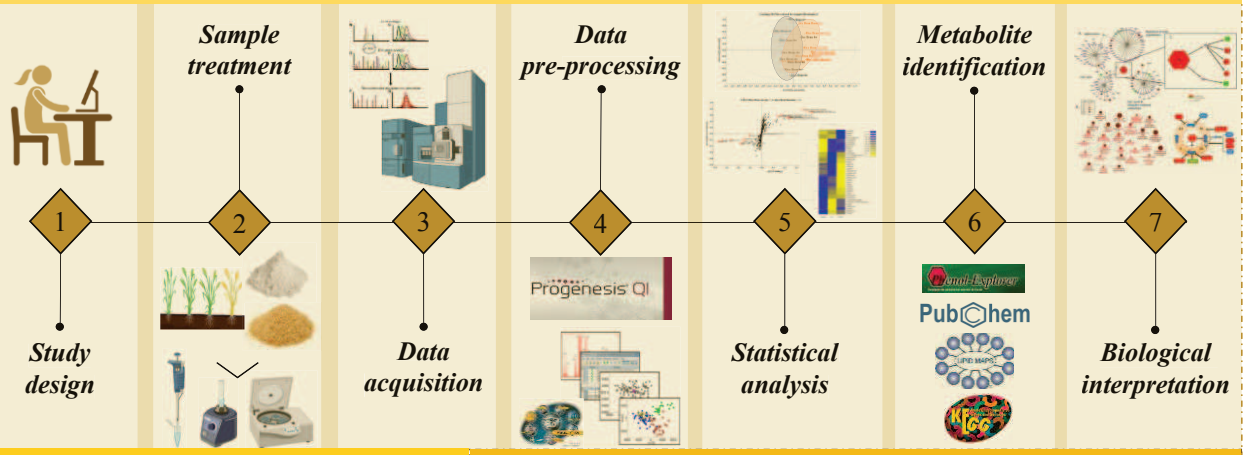
Wheat

Bran and aleurone

Rice

Brans (34) including colored samples

Untargeted metabolomics (LC-MS^E) as central high-throughput tool



Wheat grains: what is the impact on phenolic compounds (PC) profiles of:

- Genotypes (n=16)
- Maturation stages (n=4 stages)
- Milling process (n=14 flours)

Can these coproducts be used to extract bioactive PC and lipids?

- On wheat bran or aleurone using conventional solvents or NaDES
- On a rice bran collection (n=17) => richness of colored rice brans, comparison of CS vs NADES for PC extraction, characterization of bioactive lipids extracted using CS

Can we further enhance rice bran extracts functionalities by using NaDES and enzyme biotransformation in one-pot reaction?

Phase 1: Extraction — *1h*

S1: NaDES (ChCl:1,2-propanediol:water, 1:1:1) **pH ~ 5,42**

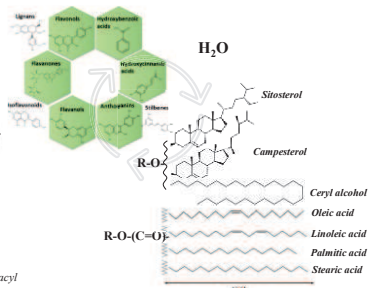
S2: NaDES (ChCl:xylitol_HCl, 1:1:_5%) **pH ~ 0,00**

+KOH, pH

25 mg rice bran, 1 mL NaDES, 40°C, 200 rpm

Phase 2: Biotransformation — *24h*

+ *iCALB* lipase



*10 mg of **iCALB** to 1 mL of NaDES, 50°C, 200 rpm*

Phase 3: Test of activities

Antioxidant assay

Biotransformed extracts NaDES formulation - Comparison of antioxidant efficiencies in emulsion

Chapter 1

Literature review

Chapter 1 – Literature review

1. Main cereal consumed by humans

Maize, wheat, and rice are the three most consumed cereals in the world (FAOSTAT, 2020). Maize, although being the first, is mostly used for animal feed, as can be seen in Figure 1, making wheat and rice the cereals mainly consumed by humans.

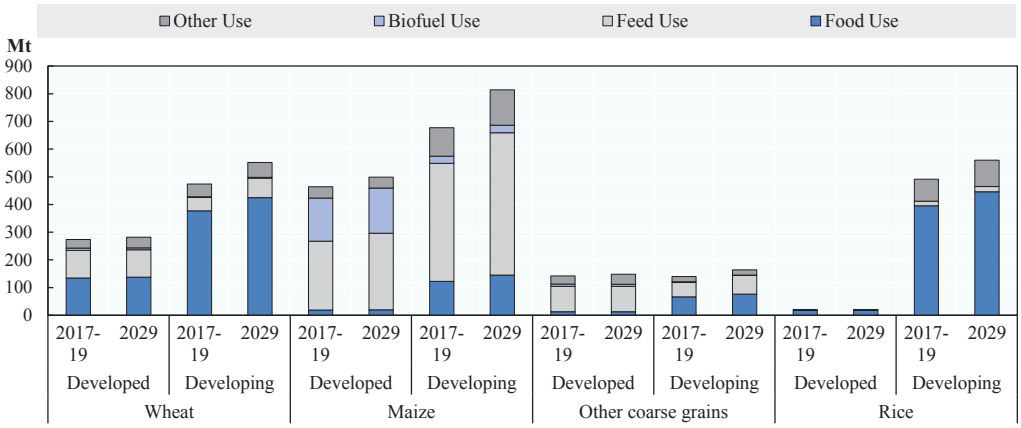


Figure 1 - Cereal use in developed and developing countries. Source: OECD/FAO (2020), "OECD-FAO Agricultural Outlook", OECD Agriculture statistics (database).

1.1. Wheat

Wheat (*Triticum spp.*) is a cereal of the family *Poaceae* (also known as *Gramineae*), belonging to the subfamily *Pooideae* and to the genus *Triticum*. The wheat species are classified according to their number of chromosomes and can be diploid, tetraploid or hexaploid. Due to the viscoelasticity of its storage proteins, wheat is considered as the most suitable raw material for pasta and bread making. Depending on the species and the technological suitability, wheat can be used to produce bread, pasta, cake, biscuit, and others, such as animal feed or in the non-food industry (Katyal et al., 2016; Kersting et al., 1994)

The wide range of applications makes wheat a food matrix of great economic and nutritional importance. *Triticum aestivum* is the second most produced cereal in the world, being on the top cereal crops in terms of volume production and world consumption. The wheat global production has been increasing over the years, reaching more than 700 million tonnes per year, projected to reach 839 million tonnes by 2029 (FAOSTAT, 2018; USDA, 2020). The five largest wheat consuming regions are the European Union, China, India, the Russian Federation, and the United States, accounting for 55% of global wheat use (FAOSTAT, 2020). France is the largest producer of wheat in Europe (37 million tonnes per

year) and this crop is cultivated throughout the country. On the other hand, Brazilian wheat consumption reaches more than 57 kg per capita, but the country produces only 6-7 million tonnes per year of common wheat (*T. aestivum*), from a cultivated area of 2.4 million hectares. Due to this scenario, Brazil imports every year around 50% of the amount need to supply the internal market, it means around 6.5 million tonnes (ABITRIGO, 2018) (CONAB,2021).

1.1.1. Structure and chemistry composition

Wheat grain is essentially composed of the germ, the outer layers (bran) and endosperm (Surget & Barron, 2005), presenting different structure and composition (Fig. 2).

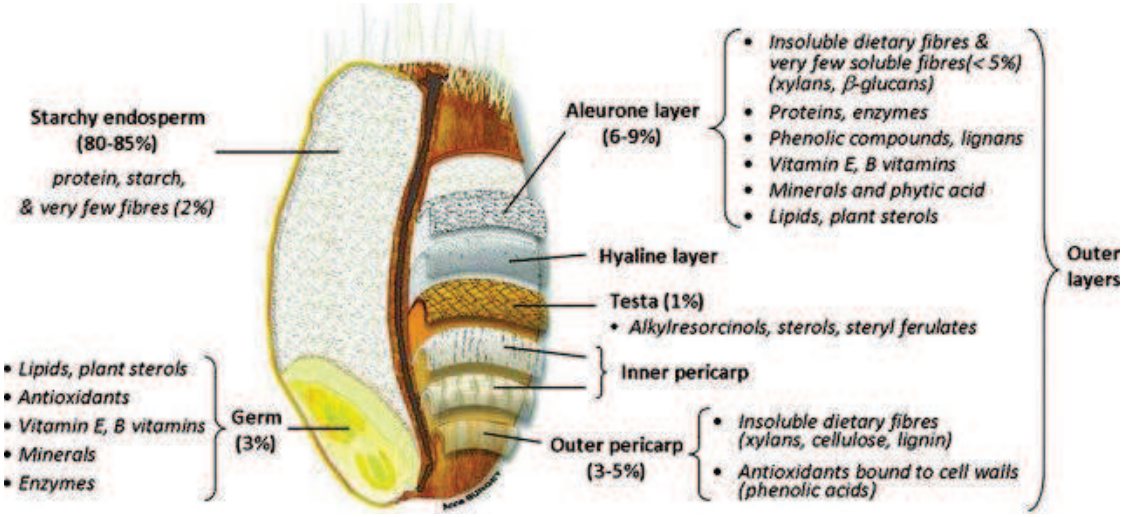


Figure 2 - Structures and composition of wheat grain tissues by Onipe (2015).

Wheat grain, after milling processes, can be subdivided into white flour, bran and germ. The flour, composed of the starchy endosperm and some parts of the aleurone layer, is the main product obtained. Another part of the aleurone is kept even in the refined flour (Fig. 3), which enriches the flour with its compounds. Aleurone layer (5-8% of the wheat grain weight) is a rich source of macro and micronutrients showing strong potential for food uses, even more because it is the most internal layer and is less exposed to pesticides and environmental factors when compared to the pericarp (Brouns et al., 2012; Hemery et al., 2010). The germ (3%) is essentially composed of lipids, proteins (albumins and globulins), vitamins, minerals, and soluble sugars.

The bran fraction (14%) is considered the coproduct of milling process; however, the use of wheat bran for human consumption has progressively increased over the years. In fact, many studies have shown that wheat grain has large nutraceutical properties associated with

its rich composition in bioactive compounds, mainly PC, carotenoids, alkylresorcinols and phytosterols (Serna-Saldivar, 2010b).

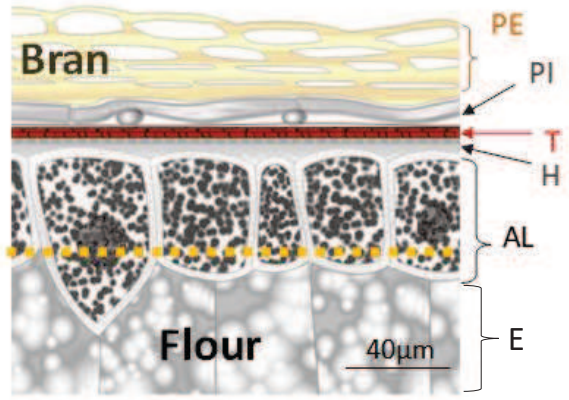


Figure 3 – Representation of cross-section of tissues of wheat grain. PE = pericarp extern, PI = pericarp , T = testa, H = hyaline, AL = aleurone, E = endosperm adapted from Surget and Barron (2005).

The content of bioactive compounds is differently distributed in the grain tissues and therefore there is a variation in the profile and concentration according to the percentage of bran in wheat flour (Hemery et al., 2007). The interest in the health benefits provided by cereals has led to an increased focus on the variation of the phytochemical composition among the different varieties and wheat species (Dinelli et al., 2009; Li, Shewry & Ward, 2008; Wang et al., 2013).

1.1.2. Development of the grain

It is widely accepted that the consumption of whole mature grains can bring health benefits. Moreover, it has been recently postulated that the consumption of immature grains, collected during the grain development before the harvest, also presents interest (Kim & Kim, 2017). The attractiveness of immature wheat grains has increased throughout the world due to the presence of nutrients and bioactive compounds in a higher extent than in mature grains, such as dietary fibers, fructo-oligosaccharides and PC (Özkaya et al., 2018).

The wheat grain development is divided into different stages of grain filling, which occurs after flowering (anthesis). These stages can be briefly described as milky, pasty and physiological maturity (Large, 1954; Zadoks et al., 1974) (Fig. 4). The stages of wheat grain filling are very important because they determine the final nutritional composition and quality of the grain and can be directly affected by some external factors, also impacting the accumulation of phytochemicals.

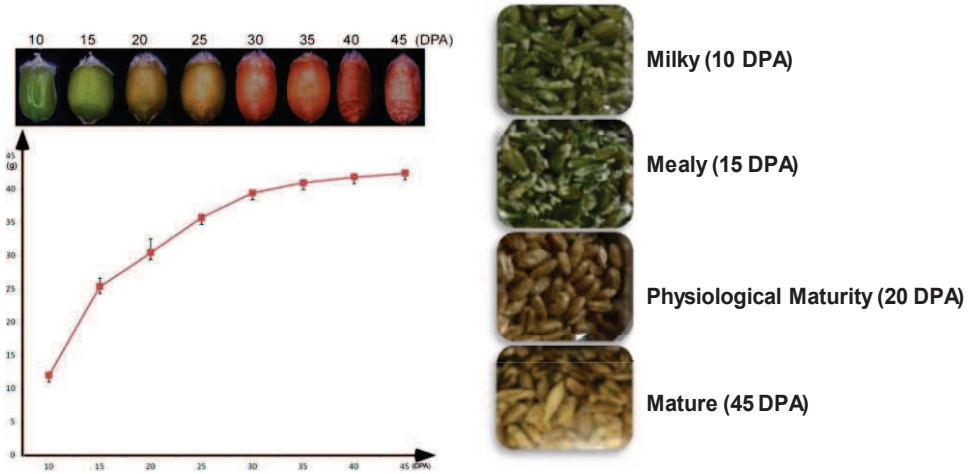


Figure 4 - Morphological characteristics and grain weight changes in the stages of wheat development stage. DPA: days post anthesis. Adapted from *Zhen et al.*, 2016 and *Santos et al., 2019*.

Immature wheat grains showed higher antioxidant and antiproliferative activities than mature grains, suggesting immature wheat as a potential grain source with antioxidant capacity and anticancer effects (Kim & Kim, 2016). Moreover, Kim and Kim (2017) showed the acceptability of products based on immature grains was improved when the consumer was aware of their greater antioxidant activity, despite some sensorial limitations such as green color and after taste. It is also important to consider the presence of antinutritional factors that impair the bioavailability of other compounds, such as phytate and even some polyphenols (Fardet, 2010; Nadeem et al., 2010).

However, there are still very few studies that have focused on the phenolic composition of the immature grains and even fewer that have evaluated the *in vivo* bioactive potential. Some studies have evaluated the association of enzymatic activity with PC content quantified by high performance liquid chromatography (HPLC) (McCallum & Walker, 1990, 1991). Zhen et al. (2016) showed for the first time the dynamic of primary compounds formation applying metabolomics tools during the wheat grain development. Santos et al. (2019) fully investigated, for the first time, the phenolic-profiling by metabolomic approaches of different wheat genotypes during the grain development and showed differences among cultivars and the different phases of grain development.

1.2. Rice

Rice (*Oryza sativa L.*) is a cereal belonging to the genus *Oryza* and is one of the most widely cultivated cereals around the world. The rice global production and consumption are expected to increase and the FAO's forecast of global rice use in 2029 is 582 million tonnes.

Asia is the major contributor for the world production and consumption with a per capita consumption of 78 kg/year. Africa, Latin American and Caribbean are also important consumers with a mean consumption per capita of 28 kg/year followed by North America (14 kg/year) and by Europe (5 kg/year), the lowest consumer (FAOSTAT, 2020).

According to Serna-Saldivar (2010a), rice can be divided in three major classes: long, medium and short. Rice is also classified by region, morphology, and chemical properties in three major groups: Japonica, Indica and Javanica. Beside this and considering the global market, FAOSTAT (2020) classifies as two main categories of traded rice: Indica and Japonica. There are also other classifications, more related to technological aspects, such as glutinous, black, red, aromatic and others.

1.2.1. Structure and chemical composition

Rice grain is essentially composed of the hull (or husk), bran, endosperm and germ and each tissue have a different structure (Fig. 5). The rice grain composition is similar to wheat due to the analogous grain structure, and it is also influenced by genetic and external factors (soil and environment). Rice germ is rich in protein, lipid, vitamins and minerals (Moongngarm et al., 2012). The endosperm consists of starch granules, proteins, vitamins, minerals, phytic acid and PC. Rice bran (RB) is a rich source of insoluble fiber, proteins and minerals and PC (Serna-Saldivar, 2010b).

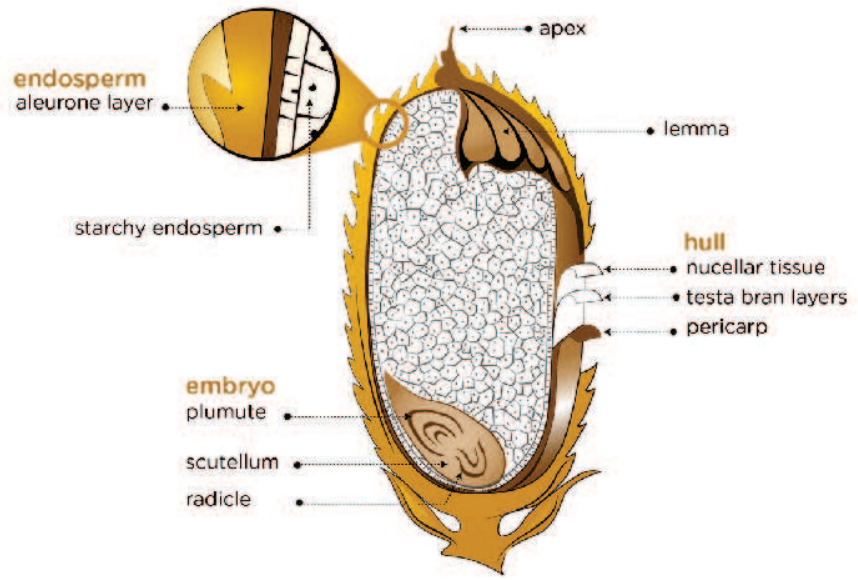


Figure 5 - Structure of the rice grain from (Shakri et al., 2021).

The RB health promoting properties are attributed mainly to its phytochemicals, including also γ-oryzanol, tocopherols, tocotrienols, anthocyanins and phytosterols. The presence of these compounds increased the research interest in pigmented rice varieties, especially in brown species (Mahanta and Saikia, 2016). Nowadays, the quality parameters for cooked rice grain are not only related to its sensory characteristics but also to the presence of bioactive compounds (Melini et al., 2019).

2. Coproducts of wheat and rice generation

Food industries generate large amounts of agri-food residues that bring negative impacts to the environment, besides economic problems. Due to this problem, sustainable strategies are becoming more and more important and necessary to allow the reuse and valorization of these residues/coproducts and guarantee the nutritional and food contribution of the entire population.

Cereal's coproducts present a rich composition and are considered good source of nutrients such as fibers, lipids, vitamins, and minerals and a large diversity of phytochemicals. Indeed, vegetal coproducts are rich in secondary metabolites which have a broad spectrum of bioactivities, showing a particular protective role against inflammatory, cardiovascular diseases and cancers. The composition of food coproducts is usually rich and variable (Serena & Knudsen, 2007), but the presence of PC is a constant feature among cereal coproducts. PC have been used as biomarkers of the peripheral tissues to monitor wheat grain fractionation and obtain nutritionally enriched fractions (Barron et al., 2007; Hemery et al., 2007).

The two cereals selected for this study are the second and third most cultivated in the word (wheat and rice, respectively). The habit of consumption of polished grains and refined flours led to an increase in the amount of agro-industrial residues and, consequently, the volume of coproducts generated (Brouns et al., 2012). Pruckler et al. [4] reported that wheat bran biomass can be roughly estimated to 150 million tons per year, and RB is estimated to 50 million tons per year according to (Cozzano Ferreira et al., 2019). Therefore, to improve the transformation of coproducts into novel products and functional ingredients became a priority.

The coproducts of wheat comprehends bran (the outer layers of the kernel including part of the aleurone layer), shorts (a mixture of bran, endosperm and germ) and feed flour (particles of endosperm with fine fragments of the outer layers) or red dog flour (a mixture of bran and low grade endosperm) and germ (wheat embryo) (Sarfaraz et al., 2017). Depending on the extraction rate applied during the wheat milling process, different types of flours can

be obtained (Hemery et al., 2007). The milling process tends to increase the enzymatic activity due to the raise of the surface area, resulting from the release of substrates and macromolecular disintegration (Silva et al., 2012) and, consequently, the nutritional value is altered. During this process, the enzymatic activity tends to increase due to the raise of the surface area, resulting from the release of substrates and macromolecular disintegration (Silva et al., 2012).

Brazilian legislation classifies wheat flour according to the extraction rate: whole wheat flour, special or high-quality wheat flour and common wheat flour. Hence, flours are classified in type 1, 2 or 3, according to the maximum ash contents of 0.8; 1.4 and 2.5%, respectively (MAPA, 2005). Whole wheat flour has a maximum extraction rate of 95% (Wang et al., 2013), thus maintaining all the layers composed by the grain.

The coproducts of rice consist of rice straw, ash, broken rice, the husk (awm and lemma) and the bran (pericarp, seed coast, aleurone and some part of germ) (Gopala Krishna et al., 2012) (Fig. 6). Rice husk and bran are the most important coproducts. RB can be generated with different ratios depending on the process (Moraes et al., 2014).

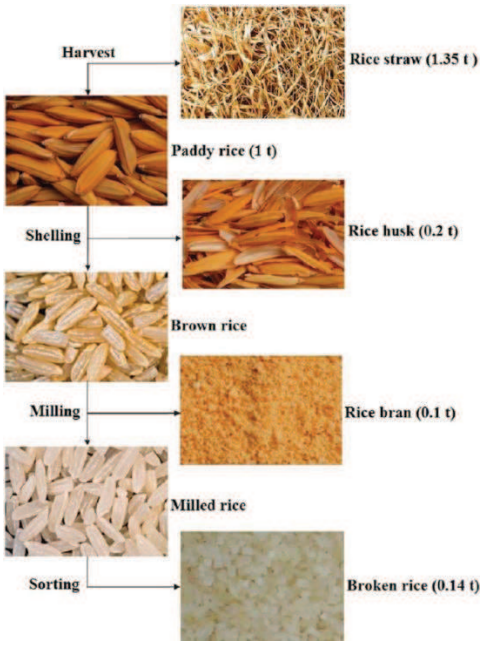


Figure 6 - Different coproducts during the rice process from Moraes et al (2014).

Wheat and rice coproducts, mainly the bran, found applications in the industry and are widely incorporated in the production of breakfast cereals, animal feeds, dietary foods, and vitamin complexes. In addition, rice can be an interesting alternative for consumers following a gluten-free diet. Indeed, the fact that RB - unlike wheat bran - contains a percentage of

germ, this coproduct has drawn the attention of researchers due to its lipid content and its possible applications. RB lipids have shown compelling impacts on cholesterol regulation (Wang et al., 2014) and immune functions (Castanho et al., 2019). These benefits can be associated to the presence of different lipids: phytosterols, tocopherols, tocotrienols, unsaturated fatty acids, minor polar lipids such as phospholipids, galactolipids and sphingolipids. RB oil production has been one strategy to extend use of RB from processing, and consumption is associated with direct effects on cholesterol metabolism (Sugano & Tsuji, 1997).

Another promise strategy to add value to this coproduct is the synthesis of functional biomolecules, with greater applicability to human health (e.g., through enriched extracts with better bioavailability), from PC and lipids. In Part 5 of this thesis, we addressed this strategy based on molecules synthesized from inserted lipid chains. However, to get positive results, it is necessary to elucidate the lipid composition present in this matrix. Indeed, there is a gap in the knowledge of the global lipid profile of RB that can be achieved by the study of the main portion of the metabolome, the lipidome, by using specific metabolomics approaches, specially called lipidomics.

3. Phenolic compounds – major class of secondary metabolites

PC represent the most diversified and abundant class of phytochemicals in plants, and they derived from phenylalanine, tyrosine and tryptophan residues (Fig. 7).

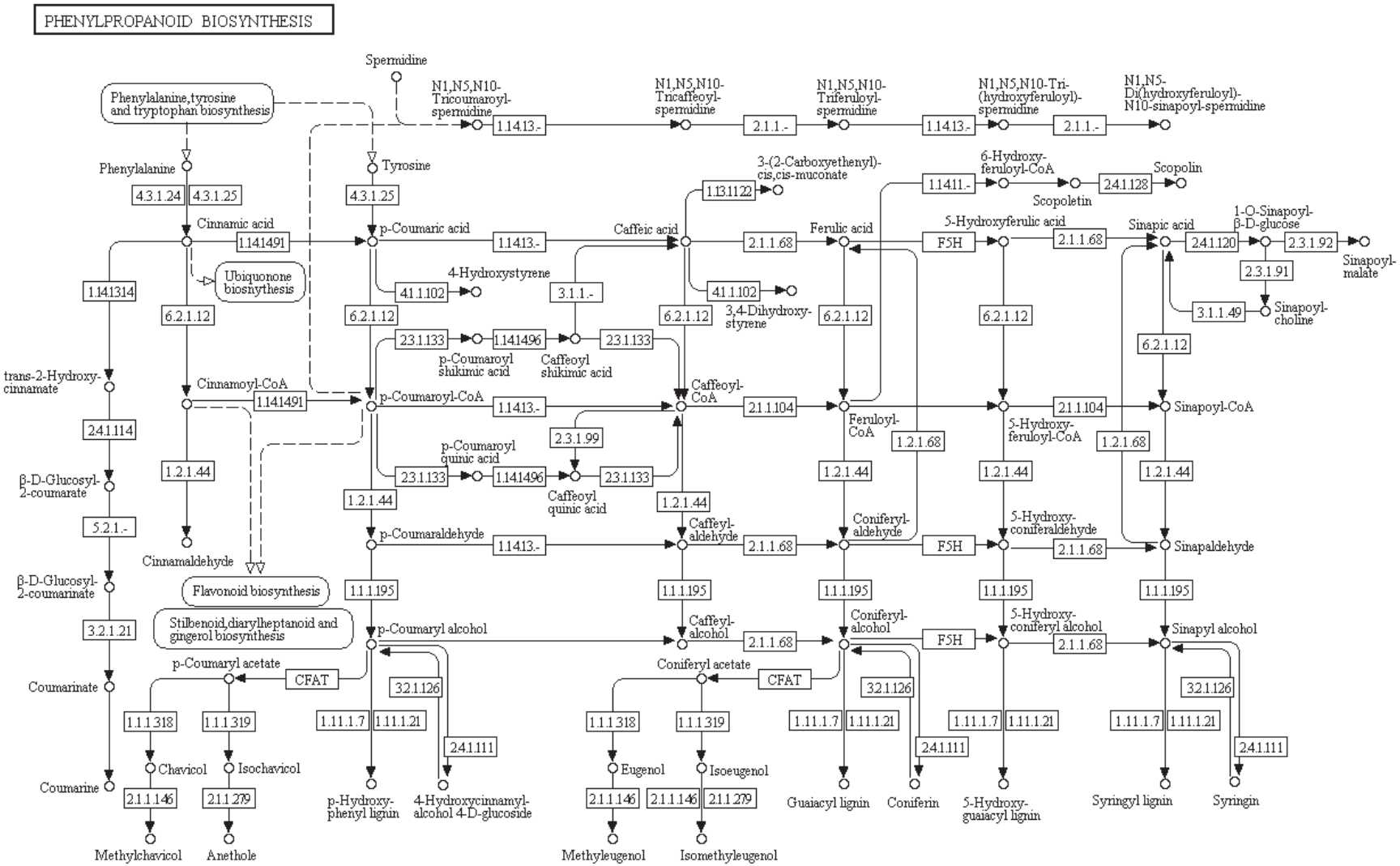


Figure 7 - Phenylpropanoid biosynthesis map (Source: *http://www.genome.jp/dbget-bin/www_bget?map00940*).

PC are secondary metabolites produced as a defense mechanism against predators and pathogens. They are essential for plant growth and reproduction, and contribute as well to its sensory and pigmentation characteristics (Balasundram et al., 2006). PC can be found in nature under several forms: soluble free, soluble conjugated (predominantly as *O*- or *C*-glycoside conjugates (Johnson et al., 2021) and insoluble, bound to matrix. In this insoluble/bound form, PC are esterified to the cell wall components or macronutrients (Irakli et al., 2012a). They can also polymerize to form polyphenols with a higher molecular weight (e.g., tannins), and according to their structure, can be classified as phenolic acids, flavonoids, stilbenes, lignans, and other polyphenols (figure 8). PC can then present a great diversity and complexity of chemical structures, and a vast presence of isomers, so a large gamma of polarity and bioactivities.

Their biochemical activities depend on their chemical structures and on their partition in the system that has to be stabilized (Shahidi & Ambigaipalan, 2015), but they are typically classified as natural antioxidants. This antioxidant capacity is explained by their ability to transfer a hydrogen atom and stabilize molecules in an oxidation scenario. PC from diet can acts as effective health promoters with helpful features for both, food applications and human cells. Indeed, PC can decrease the oxidative stress, delay or inhibit disorders associated with metabolic processes of various diseases, such as atherosclerosis, cancer, hyperlipidemia, among other inflammatory and cardiovascular diseases (Luthria et al., 2015; Vermerris & Nicholson, 2006).

The beneficial character of PC is widely known and proven *in vitro*, but their action *in vivo* is influenced by several factors (e.g., some generated metabolites can present no bioactivity). Although studies about the bioaccessibility of PC are still limited, recently the absorption at different stages of the digestive tract and the potential of these compounds to modulate the intestinal microbiota was evaluated (Nignpense et al., 2021). Moreover, it is suggested that glycosylated molecules are more bioavailable because the glycoside has a protective effect, allowing the aglycone to be released after hydrolysis or other enzymatic actions, increasing the its biological activity (Johnson et al., 2021).

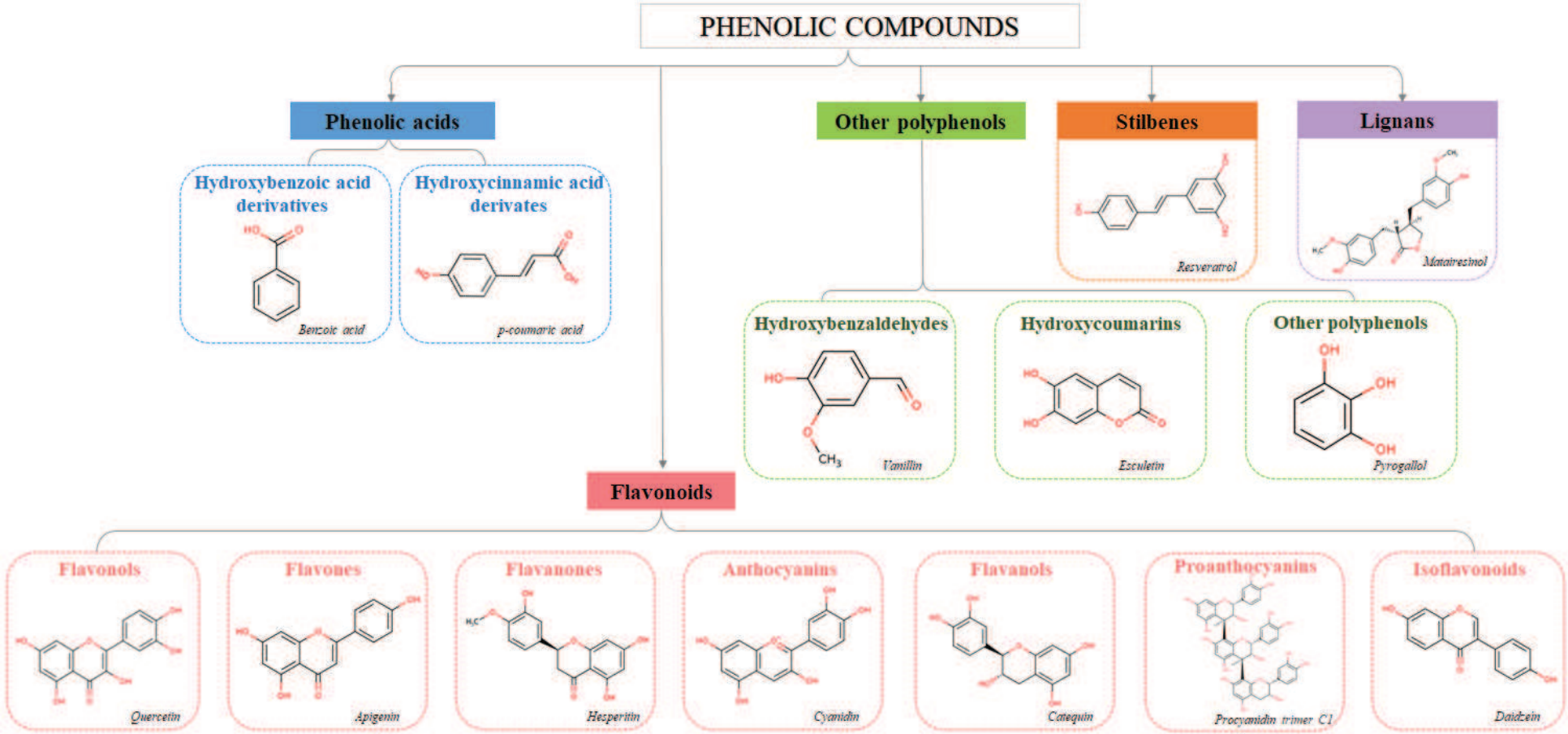


Figure 8 – Phenolic compounds classes.

3.1. Phenolic compounds of wheat and rice and strategies for their valorization

Wheat

The phenolic content is strongly subjected to the influence of wheat grain processes, especially to grain milling. Wang et al. (2013) studied five different extraction rates (milling yield), ranging from 60 to 100% and reported the concentration of compounds increased as the extraction rate increased, despite of the similar profile of PC in these samples. Indeed, PC are found in great concentration in the bran in both forms, free and bound, however the great majority of the wheat PC is under the bound form. Studies suggest that bound PC can exert a better antioxidant effect because they are bound to components of the cell wall, resist gastrointestinal digestion being better absorbed in the intestine, becoming more bioavailable in the body (Angelino et al., 2017; Pandey & Rizvi, 2009).

The main classes of PC found in wheat are phenolic acids, flavonoids, coumarins, lignans, tannins and stilbenes. The most commonly class found in wheat is phenolic acids, which are divided into two subgroups: i) hydroxybenzoic acid derivatives, including vanillic acid, gallic acid, *p*-hydroxybenzoic and syringic acids (the most abundant in the free form) (Wang et al., 2013; Zhang et al., 2012) and ii) hydroxycinnamic acid derivatives, which include ferulic acid, the most abundant compound reported in both free and bound form (Wang et al., 2013), *p*-coumaric acid, caffeic acid and sinapinic acid (Saulnier et al., 2007; Verma et al., 2009).

Beside this, specific phenolics were also identified in different varieties of wheat, such as fertaric acid, *p*-coumaroylquinic acid, kaempferide, epigallocatechin gallate and tricin (Sharma et al., 2016). This study also showed that PC may play a negative role in the final product due to the interactions with gluten proteins. The effects of growing conditions also impact on the production of wheat secondary metabolites (Shamloo et al., 2017). By 2019, the knowledge on the temporal changes of the phenolic profile of wheat during grain development was still limited (McCallum & Walker, 1991). Santos et al. (2019) revealed by UPLC-MS^E, the phenolic profile during different stages of grain development (milky, softy, physiological maturity and mature) showing progressively decrease of the number of PC along grain development and considering immature grains as a rich source of PC and highlighting the presence of a great number of isomers.

Rice

In rice, bound PC are also predominant and ferulic acid is the most abundant compound of this group (Zhang et al., 2015). The predominant classes are phenolic acids

(white rice), anthocyanins (black rice) and proanthocyanins (red rice) (Min et al., 2011; Zaupa et al., 2015). Pang et al. (2018) compared the total phenolic content and antioxidant capacity between white and pigmented rice (black and red) and found higher values in black followed by red rice. White rice had the lowest score for PC and antioxidant capacity. They showed that bound PC extracted for the three types of rice had the highest antioxidant capacity.

In fact, the PC composition are differentially distributed an varied according to the rice fraction (Ti et al., 2014). Phenolic acids such as *p*-coumaric, syringic, vanillic, caffeic, sinapic, *p*-hydroxybenzoic and protocatechuic acids have been reported in whole rice grains (Shao et al., 2014a; Zaupa et al., 2015). Anthocyanin group belongs to the subclass of flavonoids and are generally present under the free form. They have been found within the outer layers of pigmented black rice grains and cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside have already been identified (Zhang et al., 2010).

The influence of the rice grain maturation was reported by Shao et al. (2014b) in four stages of development after flowering in black, red and white rice grains. The black rice had the higher total bound PC in comparison with white and red rice, irrespective to the maturation stage. Anthocyanin levels in the black rice increased from the second week after flowering and the PC presents in red rice decreased by half after the third week post-flowering. These results have shown that the pigmented rice (black and red) have a great content of PC and potentially a high associated bioactivity.

Due to the phytochemical richness and diversity of these pigmented rice grains, studies applying particular extraction together with advanced metabolomic tools are important for the identification and the characterization of the PC present. Santos et al. (2021) characterized PC in black and red pigmented RB applying conventional organic solvents and NaDES. UPLC-MS^E results revealed not only a distinct profile, but also that acidic NaDES-based extracts exhibited unique PC and a greater antioxidant capacity.

4. Metabolomic approaches to identify phenolic compounds

The *omics* sciences search for the accurately characterization and quantification of biological molecules and play a central role in the integrated knowledge process of organisms, being among the most advanced approaches of chemical mapping.

The *omic* suffix means collectively considering all constituents and metabolomic refers, therefore, to the study of the set of metabolites from organisms or cells. Metabolomic plays a fundamental role in the understanding of biological systems, defining metabolic

phenotypes and has been applied for the investigation of secondary metabolites that have important biological effects, such as chemopreventive and antiproliferative activity on cancer cells (Graziani et al., 2018; Wang & Chen, 2013).

Metabolomics has become increasingly popular in recent years for many applications ranging from food analysis, clinical diagnostics, human health and even biotechnological investigation. Despite technological advances, metabolomic studies can still have limitations due to the difficulty in identifying all metabolites, a class of compounds with great chemical diversity.

Metabolomics can be defined as the use of advanced analytical platforms to characterize the composition of metabolites (typically compounds with molecular mass less than 1500 Da) of a particular system or organism (Bijttebier et al., 2016; Lacalle-Bergeron et al., 2021). To capture all metabolites present in an organism, different analytical platforms must be combined, due to their different chemical properties (Bijttebier et al., 2016). In the last decade, metabolomics mass spectrometry-based studies have been presented as an indispensable technique for the analysis of biomolecules, components of the primary and secondary metabolism of plants and animals, enabling the application of metabolomics techniques to the analysis of food and target bioactive compounds (Lacalle-Bergeron et al., 2021).

Metabolomic studies depend on the application of suitable analytical platforms to determine the multiple metabolites present in complex matrices. The application of these approaches promotes a comprehensive characterization of the target metabolites and increase chemical knowledge over their biotransformation. Therefore, metabolomics plays a key role in understanding cell systems, defining metabolic phenotypes and identifying the function of unknown genes. The untargeted metabolomic workflow (figure 9) demonstrates important steps for a quality analysis: 1) study design, 2) sample treatment, 3) data acquisition, 4) data pre-processing, 5) statistical analysis, 6) metabolite identification and 7) biological interpretation.

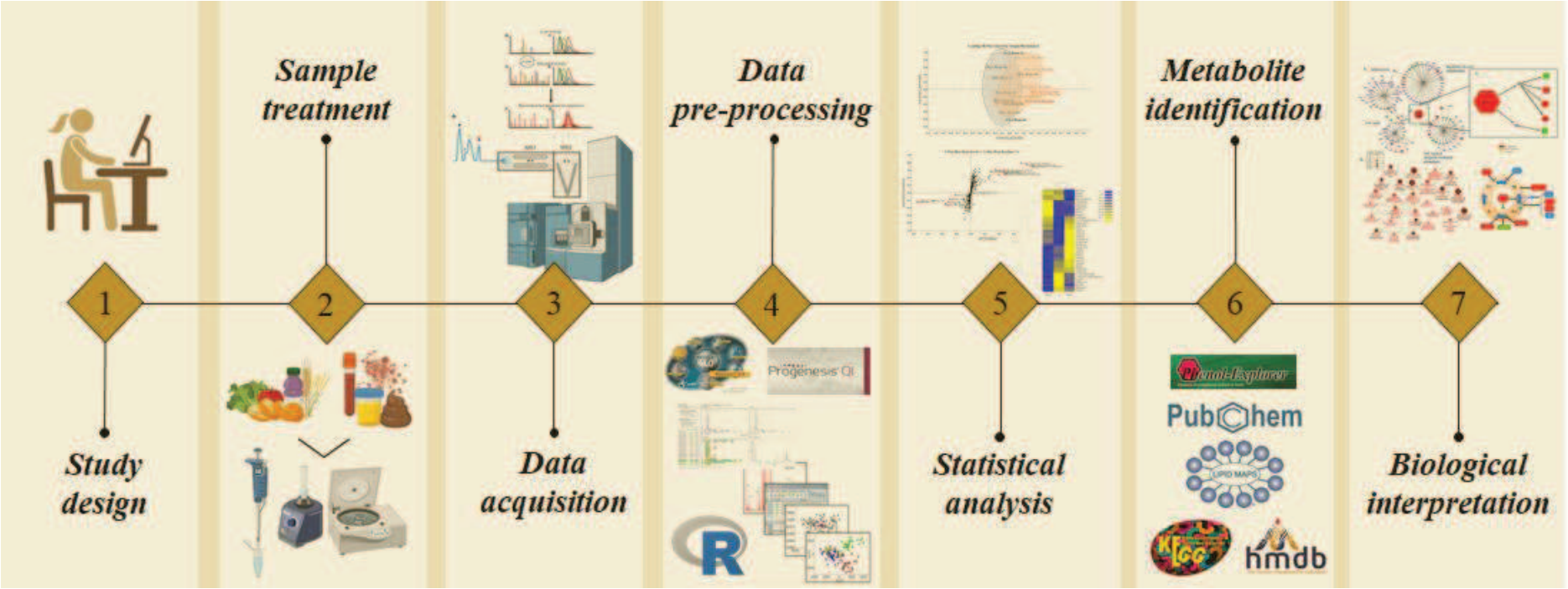


Figure 9 - Untargeted metabolomics workflow.

4.1. Extraction of phenolic compounds for mass spectrometry analysis

The complexity of the plant samples requires sequential extractions for the further recovery of these compounds, as well as a cleaning step to eliminate impurities. This step is fundamental for the analysis of aqueous based extracts by MS, since the presence of salt within the extracts can cause ions suppression, adducts formation, negatively affecting the identification of the compounds due to the presence of non-volatile residues (Álvarez-Sánchez et al., 2010). Sequential extraction based on chemical hydrolysis (alkaline and acidic) is required due to the presence of insoluble PC. This step highly increases the quantity of extracted compounds, since bound PC are in a higher proportion than free PC in the grains (Irakli et al., 2012a; Nicoletti et al., 2013; Vadivel et al., 2017; Zhang et al., 2012).

Organic solvents do not guarantee a good extraction of all classes of PC, mainly due to the wide difference in terms of molecular structure, polarity, and physico-chemical properties. The combination of organic solvents, binary or ternary mixtures, usually with water may significantly increase the efficiency of extraction. In addition, sometimes is necessary to assist extraction with other physical techniques to increase its efficiency (e.g., ultrasonic, microwaves, high pressure). Chromatography techniques such as solid phase extraction (SPE) are widely used to remove molecules that may cause interference in the analysis (Irakli et al., 2012a; Nicoletti et al., 2013; Vadivel et al., 2017).

In this challenging context, a new "class" of extraction media has been studied: the deep eutectic solvent (DES) (Radošević et al., 2016) or natural deep eutectic solvents (NaDES) depending of the components used (Chemat et al., 2019). NaDES can be obtained by combination of components in specific proportions capable of achieving a eutectic point (in which the melting point is as low as possible), where one of the components acts as receiver and other as hydrogen donor.

They are considered as a greener option than conventional solvents: they are low cost, biodegradable, non-flammable and have a relatively low toxicity. NaDES toxicity depends on its composition (type and molar ratio of components) (Mbous et al., 2017; Radošević et al., 2015; Wen et al., 2015). NaDES are considered a great extraction media since their components can form hydrogen bonds with the molecules to be extracted. Because of this H-bond network, the solubility of the molecule is increased (Dai et al., 2013; Morrison et al., 2009).

NaDES based on choline chloride, polyols, urea, organic acids and sugars emerged as green and cost-competitive solvents to extract bioactive compounds from plant co-products,

due to these distinguished properties which allow also the dissolution of low polar compounds possessing therapeutic activities (Jablonský et al., 2018). NaDES application is a promising strategy to the valorization of cereal/pseudocereal co-products, achieving until 95% of recovery of important phytochemicals (Huang et al., 2017b). Santos et al. (2021) underlined the great extraction capacity of NaDES, and most importantly, the extraction specificity of acidic NaDES for certain PC.

The NaDES extraction can be considered as a pretreatment of the cereal coproduct allowing further steps for the compound recovery. The use of NaDES to extract PC can be very useful due to its tuneability, meaning that selectivity of NaDES for a target compound can be tailored by varying its composition. An additional strategy can be used as a reaction medium in enzymatic reactions to the biotransformation/functionalization of the extract for example (Durand, Lecomte, Baréa, et al., 2013), developing ready-to-use extracts.

4.2. Untargeted metabolomics to identify phenolic compounds in wheat and rice – focus on liquid chromatography coupled to mass spectrometry

Among the most applied and efficient analytical techniques are the coupled techniques, such as the use of liquid chromatography coupled to mass spectrometry (LC-MS) (Fernández-Ochoa et al., 2021). In these studies, the amount of data generated is enormous and requires important computational analytical effort to deal with the systemic and comparative data. MS is an analytical technique used to identify and quantify chemical species in their ionized forms by measuring their mass/charge (*m/z*) ratios in the gas phase. This tool became increasingly useful and has improved the understanding of biological processes in general through the identification, the quantification and the mapping of important functional molecules.

The metabolomic analysis can be targeted or quantitative, where the analysis provides specific information of known compounds quantification based on a high sensitivity, thus allowing the absolute quantification, while untargeted metabolomics promotes a fully analysis of metabolites from complex biological systems, such as food matrices, with lower sensitivity but allowing a relative quantification (Gorrochategui et al., 2016), especially when high resolution data independent analysis is applied (Feuerstein et al., 2021).

The chemical complexity of extracts obtained by organic solvents or NaDES can be resolved by characterization using untargeted metabolomic approaches. These approaches are extremely powerful in terms of identification of bioactive molecules that, even when present

in low concentrations or combined with other compounds, may exert bioactivities of extreme relevance.

The number of isomers of PC makes difficult the identification and it is necessary to combine the optimized extraction with an instrument that allows reproducible, reliable and robust identification. Dinelli et al. (2011) reported the presence of 104 isomers of PC highlighting remarkable differences in the phytochemical fingerprints of old and modern wheat varieties. Modern methods such as UPLC-MS^E are able to identify PC with different physicochemical properties (Nascimento, Santos, Lima, et al., 2018). In MS^E methods, the fragmentation of ions, obtained by the application of high energy in the collision cell, occurs simultaneously to the analysis of the precursors (low energy) and allows the simultaneously detection of precursor ions and fragments (MS-MS), improving identification (Souza et al., 2017).

According to Sumner et al. (2007), four levels of identification is describe on Metabolomics Standards Initiative (MSI): 1) Identified compounds (with standards), 2) putatively annotated compounds (based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries), 3) putatively characterized compound classes (based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class) and 4) Unknown compounds.

Metabolomic have been successfully applied to characterize the large diversity of PC in food with the concept of "Foodomics". This concept employs the omics tools to understand and to map the chemical compounds and also to characterize contaminants in foods (Herrero et al., 2012). PC undergo external influences, genetic variability and even grain milling processes. This approach permits to distinguish species (Khakimov et al., 2014), genotypes (Kusano et al., 2015), crops and maturation effects (Shao et al., 2014b), biotic stress (Balmer et al., 2013) and identification of biomarkers (Graziani et al., 2018), for instance.

The recent study conducted by Santos et al. (2019b) showed that immature wheat grains presented a more complex composition of phenolics with higher abundance than mature grains. These results can explain the previous works that showed the antioxidant potential and important bioactivity presented by immature wheat such as antiproliferative effect against cancer cells.

Metabolomic have been positively applied to characterize lipid diversity with the concept of "Lipidomics". The lipidomics emerged to study the complete set of lipids, the

lipidome, in a given cell or organism as well as to provide a large-scale data set of pathways and networks of cellular lipids in biological systems (Holčapek et al., 2018).

5. Promise chemical strategy to increase the applicability and bioactivity of phenolic compounds

5.1. Biotransformation of phenolic compounds in new molecules – *Phenolipids*

PC have drawn scientist's attention due to their great potential and different types of application in health or food sectors, especially as natural antioxidants. However, many of these compounds have limited solubility in lipid systems and to improve their solubility in lipid systems could extend their range of application, especially in cosmetic or pharmaceutical formulations.

Lipophilization or hydrophobation is a process where one hydrophilic compound is esterified (acid and alcohol), transesterified (ester and alcohol) or interesterified (ester and ester) (Fig. 10). This modification can be carried out by biocatalysis (enzyme) or chemical catalysis (acid or basic) onto a PC and leads to the formation of a new biomolecule called *phenolipids*. The chemical strategies are faintly used due to the instability of these molecules at high temperatures and alkaline conditions. Concomitantly, the enzymatic alternative (using lipase) has advantages: milder reaction conditions, selectivity, reduction of by-products, less purification steps, allowing a more environmentally friendly processes in agreement with this new era of green chemistry (Durand et al., 2013; Kahveci et al., 2015; Villeneuve, 2007).

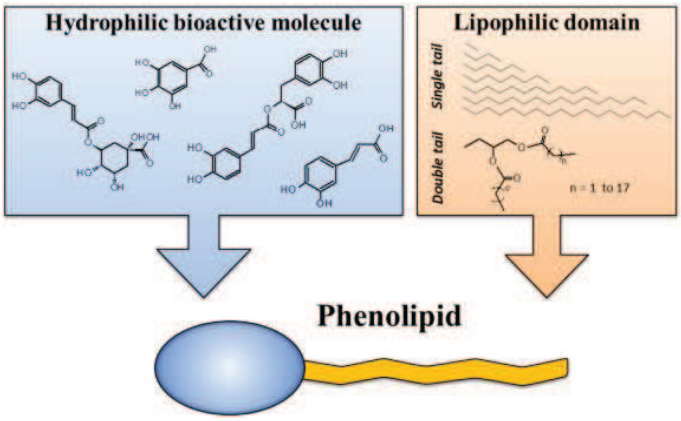


Figure 10 - Strategy for formation of phenolipids by Durand et al. (2017).

Changing the structure of PC may be a great strategy to change the polarity, modifying the hydrophilic/lipophilic character, and therefore producing new bioactive molecules with

better proprieties (antimicrobial action, retard of oxidative stress, enhanced bioavailability (Durand et al., 2017; Lopez Giraldo, 2009). However, the main challenge of the lipase-catalyzed lipophilization is optimizing the synthesis (in terms of yield and kinetics) with two substrates having different polarities. Therefore, it is essential to select a medium in which the two substrates will be soluble, at least partially, and where the activity/stability of the enzyme can be maintained at a satisfactory level.

Regarding the screening of antioxidant capacity, an important parameter is to find a method to quickly and efficiently evaluate the antioxidant potential in lipid dispersion (e.g. emulsion). The conjugated autoxidizable triene assay is an oil-in-water emulsion model system that may estimate the antioxidant activity (Laguerre et al., 2008). This method, using the tung oil as a direct marker of oxidation, allows for a simple, easy, reliable, fast and efficient assessment of antioxidant activities.

5.2. Biotransformation of molecules by lipophilization in alcoholysis reaction

Organic solvents are the most applied media for the functionalization reaction with a great efficacy even if is pure or in combination allowing a large solubilization of compounds with different polarities. The structure of the compound can also influence the reaction, as highlighted by Guyot et al. (1997) who showed that the presence of two OH groups in the chain of caffeic acid, analyzed in green coffee, makes the esterification unfeasible.

A model with different phenolic acids and fatty alcohols was studied and the esterification can be improved when utilize binary organic solvent, for example, hexane/2-butanone, but the ratio is very important and can change the enzyme activity and consequently the results (Karboune et al., 2005; Yang et al., 2012). The same occurs when pure solvents are applied. Depending on the polarity, the lipase reaction can be increased but the solubility of compounds (substrates) can be reduced in hydrophobic solvents, or the inverse for hydrophilic solvents.

Despite the positive results of esterification in organic media, these solvents have a considerable toxicity, inflammability and pollution. Thus, the research for new alternatives concerning a better media for this reaction has begun, such as the utilization of DES (Durand et al., 2012). In non-polar solvents, such as hexane, lipases generally maintain good activity, but the hydrophilic PC have very low solubility. Inversely, in polar organic media, the PC have good solubility but lipases often present limited activity. Thus, there is a technical dilemma in choosing an effective system in which a polar substrate (e.g., PC) and non-polar

(e.g., a long chain alcohol) will have both high solubility and where the enzymes maintain a good activity.

5.3. Biotransformation of extracts in NaDES by molecules assembly or structuration

Some features like toxicity and biodegradability of NaDES have been subject of studies. Recent data partially call into the question about their non-toxicity and biodegradability (Hayyan, Hashim, Al-Saadi, et al., 2013; Hayyan, Hashim, Hayyan, et al., 2013). NaDES was initially considered non-toxic, biodegradable and environmentally friendly, but it was based on the individual properties of each component of NaDES, as mentioned earlier.

The component choline chloride (ChCl), the most applied for NaDES formulation, is present in many foods, and essential for cell development. It has been classified as an essential nutrient by the USDA (United States Department of Agriculture) and as a food supplement by the FDA (Food Drug Administration). The recommended proportion of ChCl for the consumption is between 550 mg to 3.5 g per day and it has been considered non-genotoxic, non-toxic for reproduction and development, and is authorized in animal feed.

Durand et al. (2012) analyzed the advantages and limitations of NaDES as green solvents for biotransformation since these solvents are known to solubilize a large range of compounds with thermal stability. The main finding was that the compositions of NaDES interfere directly in the reaction. The fact of the NaDES acts as hydrogen donor causes a competition in the reaction and can form by-products; however, some NaDES increase the activity and selectivity showing great and promising results. The use of NaDES as functionalization media showed that the enzymes are in general active and the *Candida antarctica* lipase B exhibited the highest activities, principally in ChCl-based NaDES. Based on this, despite some studies showed negative points, NaDES application is postulated as potential alternative media for biotransformation/functionalization, considering its composition (Durand, Lecomte, & Villeneuve, 2013).

Part I

Phenolic profiling based on untargeted metabolomics in wheat

The two cereals investigated in this thesis have similar structures and characteristics. This allowed us to select one of the two for some analyses. In this first part, wheat was chosen due to its high production and consumption ahead of rice and also because it is considered the most suitable raw material for the manufacture of bread and pasta, especially due to its unique texture and technological quality of storage proteins.

Aim: Comprehensive characterization of phenolic compounds in different genotypes of Brazilian wheat grains and flours by untargeted metabolomics, to follow biosynthesis during grain growth (immature grains), to study genotype effects, and the effect of flour processing.

Based on the following publications:

- Chapter 2: Metabolomics approach for the characterization of phenolic compounds in different wheat genotype during grain development – Special volume Food Research International (Impact factor: 6.475)

- Chapter 3: Foodomics in wheat flour reveals phenolic profile of different genotypes and technological qualities. LWT – Food Science and Technology (Impact Factor: 4.952)

Main results:

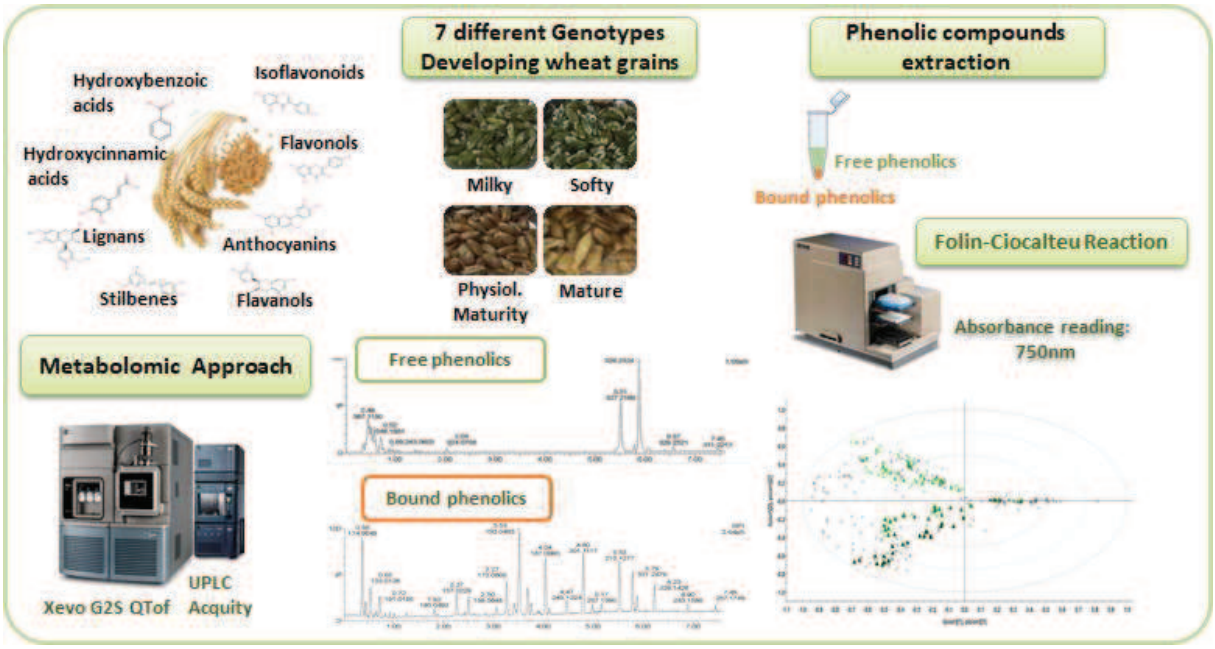
- Differentiated phenolic profile between immature and mature stages of wheat grain
- Immature grains showed higher relative abundance in PC than mature grains
- Brazilian refined flours present important PC in their composition even after industrial milling and thus representing an important source of PC due to its high consume

Chapter 2 – Metabolomic approach for characterization of phenolic compounds in different wheat genotypes during grain development

Millena Cristina Barros Santos[a]; Luciana Ribeiro da Silva Lima[a,b]; Fabiana Ramos Nascimento[a]; Talita Pimenta do Nascimento[a]; Luiz Claudio Cameron[c*]; Mariana Simões Larraz Ferreira[a-c*]

[a]Laboratory of Bioactives, Food and Nutrition Graduate Program, Federal University of State of Rio de Janeiro, UNIRIO; Av. Pasteur, 296, Urca, 22290-240 Rio de Janeiro, Brazil.
[b]Department of Food Science, Nutrition School, UNIRIO;
[c]Laboratory of Protein Biochemistry, Center of Innovation in Mass Spectrometry, UNIRIO.

Published on Food Research International (FRI), 2019. DOI: https://doi.org/10.1016/j.foodres.2018.08.034



Highlights

- Phenolic profile of 7 genotypes of immature and mature wheat was studied by UPLC-MS
- 237 phenolics including isomers were tentatively identified as free and bound forms
- Immature grains presented the highest abundance of phenolics than mature grains
- Phenolic profiles are different among genotypes and during the grain development
- Brazilian wheat showed a rich composition and abundance of phenolic compounds

Abstract

The phenolic-profiling of seven different wheat (*Triticum aestivum*) genotypes was investigated for the first time during different stages of grain development (milky, softy, physiological maturity and mature). Free and bound phenolic compounds were extracted separately and analyzed by UPLC-QTOF-MS^E. Total phenolic content significantly decreased, up to 50% depending on the genotype, towards the maturation of grain. The highest content (free and bound) was observed in the most immature grains, while the lowest level was found in mature grains (408.0 and 165.0 GAE mg/100 g, respectively). Globally, 237 phenolic compounds were identified, divided into 5 classes: flavonoids (85), phenolic acids (77), other polyphenols (51), lignans (16) and stilbenes (8). UPLC-MS results showed a progressively decrease of the number of phenolic identification (ID) all along grain development, milky (213), softy (192), physiological maturity (169) and mature (144). The proportion bound to free phenolic progressively increased, reaching the maximum at physiological maturity, indicating a possible enzymatic reactions and complexation during grain growth. Ferulic acid, diphyllin, 4-hydroxybenzoic acid, ferulic acid isomer, apigenin 7-*O*-apiosyl-glucoside isomer and myricetin isomer were the most abundant compounds. Chemometric tools showed a clear separation between immature and mature grain for all genotypes. Phenolic profile varied significantly among genotypes, this result can help the selection of varieties towards a higher retention of bioactive compounds. Noteworthy, immature wheat grains can be considered a rich source of phenolic compounds and as an attractive ingredient to incorporate to functional foods.

Keywords: UPLC-MS, *Triticum aestivum*, Bioactive compounds, Free phenolics, Bound phenolics, Folin-Ciocalteu.

1. Introduction

The interest of the health benefits provided by cereals led to an increased focus on the variation of the phytochemical composition among the different varieties and wheat species (Dinelli et al., 2009; Li et al., 2008; Wang et al., 2013). Phenolic compounds are the main class of health-promoting phytochemicals of cereal grains. Besides the genetic diversity, external factors, such as location and growing conditions can strongly impact on secondary metabolites, especially on phenolic composition and content (Rascio et al., 2016; Shamloo et al., 2017; Wang et al., 2013; Zhang et al., 2012).

Phenolics represent the most diversified class of secondary metabolites in plants, they are produced as a defense mechanism against predators and pathogens, being essential for plant growth and reproduction, as well as contributors to sensory and pigment characteristics (Balasundram et al., 2006). In grains, phenolics can be found as soluble free or conjugates esterified with sugars or other low molecular mass compounds, or insoluble, which are bound through covalent cross-linking to cell wall components (Irakli et al., 2012a; Sosulski et al., 1982). Typically, the nomenclature most used are free (soluble, conjugated or not) and bound (insoluble) phenolic compounds.

Phenolic compounds are differentially distributed in wheat grain tissues. Together with phenolic acids, polyphenols such as lignans can be preferentially found on the living-cells aleurone layer, while specific phenolic are present in bran fractions (Hemery et al., 2007). They have been used as biomarkers of peripheral tissues of wheat grain to monitor fractionation processes and obtain nutritionally enriched fractions (Barron et al., 2007; Hemery et al., 2007). Hence, the milling process at different extraction rates also strongly affects the distribution of phytochemical profiles and antioxidant activities, including phenolic acids (Wang et al., 2013). Wheat bran is particularly rich in phenolic acids covalently bound to cell wall polymers (Adom & Liu, 2002; Wang et al., 2013).

Bound phenolics represent the most abundant fraction in wheat grains and have been associated with the greatest antioxidant activity, when compared to free PC or flavonoids (Adom & Liu, 2002; Özkaya et al., 2018). Moreover, alkaline hydrolysis proved to be the most effective to release phenolic acids showing greater antioxidant activity than free or acid hydrolysable fractions (Kyung-Hee et al, 2006). Ferulic acid is the most abundant compound in bound form, followed by vanillic acid, syringic acid, caffeic acid, p-coumaric acid and sinapic acid, found in both forms (Verma et al., 2009; Wang et al., 2013; Zhang et al., 2012).

In addition to those major compounds, intermediates and products of the ferulic acid biosynthesis pathway, have been identified by modern separation techniques coupled to mass spectrometry (HPLC–MS, UPLC–MS, CE–MS), allowing the simultaneous characterization of metabolomic profile (Zhen et al., 2016) and reliable identification of multiple class of phenolic compounds (Di Loreto et al., 2017; Sharma et al., 2016). For instance, some inter-variety specific phenolics were identified, such as fertaric acid, *p*-coumaroylquinic acid, kaempferide, epigallocatechin gallate and tricin (Sharma et al., 2016). However, even if some studies shown the effects of growing conditions have a significantly impact on the production of wheat secondary metabolites (Shamloo et al., 2017), the temporal changes of the phenolic profile of wheat during grain development is limited (McCallum & Walker, 1991).

The grain development or maturation process is divided into different stages after flowering: milky, softy or dough stage, physiological maturity and then maturity (Large, 1954; Zadoks et al., 1974). During the wheat grain filling (i.e. before physiological maturity, when the final grain weight is defined), important changes occur such as dry mass accumulation (starch and protein), decrease in cellular redox potential, where protein sulfhydryl groups are oxidized into a polymeric structure, impacting in the final nutrient composition and grain quality (Ferreira et al., 2012). The content of phytochemicals presenting antioxidant activity also varied during grain maturation stages. Total phenolic content progressively decreased with the grain development, together with antioxidant activity, probably related to the seed growth and oxidative metabolism (Özkaya et al., 2018).

Indeed, the popularity of immature wheat grains is increasing throughout the world due to the presence of nutrients and bioactive compounds in a higher extent than mature grains, such as dietary fibers, fructooligosaccharides and phenolic compounds (Özkaya et al., 2018). Immature wheat grains showed higher antioxidant and antiproliferative activities than mature grains, suggesting immature wheat as a potential grain source with antioxidant capacity and anticancer effects (Kim & Kim, 2016). Moreover, Kim and Kim (2017) showed the acceptability of products based on immature grains was improved when the consumer was aware of their greater antioxidant activity, despite some sensorial limitations such as green color and after taste.

In this study, metabolomic tools were applied for the first time to follow changes in the phenolic-profiling of seven different genotypes of wheat (*Triticum aestivum*) during different stages of grain development. A modern non-targeted method based on a multiplexed MS-MS acquisition with simultaneous application of low and high collision energy (MS^E)

(Oliveira et al., 2018) was used to identify and relatively quantify secondary metabolites of wheat. The comparative multivariate data analysis showed specific phenolics belonging of immature and mature grains. Noteworthy, differences in phenolic profile and composition were found among genotypes and during the grain development. The data shown is the most comprehensive and detailed metabolomic-based profile of wheat phenolic compound to date.

2. Material and methods

2.1.Chemicals

MS-grade solvents acetonitrile and methanol, celite® 545 and the following reference standards were purchased from Sigma–Aldrich (St. Louis, MO, USA): vanillic acid, *p*-coumaric acid, catechin, caffeic acid, ellagic acid, *trans*-ferulic acid, kaempferol, myricetin, pyrogallol, flavonone, quercetin, gallic acid, epicatechin, 4-hydroxybenzyl alcohol, 4-hydroxy benzaldehyde, 4-hydroxybenzoic acid, 4-phenylacetic acid, synapinic acid, benzoic acid, quercetin 3 glycoside, 3,4-dihidroxy phenylacetic acid, epigallocatechin, epigatechin gallate, chlorogenic acid, 2,5-dihydroxy benzoic acid, 4-methoxycinnamic acid, 2-hydroxycinnamic acid, 3-hydroxy-4-methoxycinnamic acid, trans cinnamic acid, 3-methoxycinnamic acid and L-(-)-3-phenylacetic acid. Formic acid LC-MS eluent additive was purchased from Fluka (Switzerland). Ultra pure water was obtained through the Barnstead™ Smart2Pure™ (Thermo Fisher Scientific, USA) purification system. Other unmarked reagents were of analytical grade.

2.2.Plant material

Immature grains from seven genotypes (Campeiro, ORS25, ORS1401, ORS1402, Marfim, Jadeite and Ametista) of common wheat (*Triticum aestivum*) were kindly provided by OR Melhoramento de Sementes (Passo Fundo, Brazil). The grains were cultivated at the same location in Coxilha (Latitude S 28°09’33’’, Longitude W 52°18’23’’, South region, Brazil) during 2015/2016, with normal harvest and management of seeds. Wheat ears from different technological qualities were harvested during 4 stages of grain development, defined according to the moisture content of the grains: milky (60%), softy (45%), physiological maturity (33%) and mature (12%) (Ferreira et al., 2012; Large, 1954). Ten ears of each sample were stored at -80 °C and freeze-dried (Ls-3000, Terroni, Brazil). Whole grains are cryogenically ground for 2 min using a ball mill and the resulting powder was maintained at -80 °C until analysis.

2.3. Sequential extraction of free and bound phenolic compounds

Phenolic compounds from ground whole grains were extracted in triplicate according to the method reported by Dinelli et al. (2011) with modifications. Free phenolics were extracted from 70 mg of ground grains, manually macerated with 50 mg of celite and 80% ethanol. Samples were homogenized at 200 rpm and 25 °C for 10 min in a shaker (TE420, Tecnal, Brazil) and centrifuged (5.000 x *g*, 10 min, 25 °C) (Heraeus Megafuge 16R Centrifuge, ThermoFisher Scientific, Germany). The supernatants were reserved and the extraction was repeated. The two supernatants of each sample replicate were combined and dried in an evaporator centrifuge (Savant, SpeedVac Concentrator, ThermoFisher Scientific, USA).

The pellets resulting from extraction of free phenolics were submitted to alkaline hydrolysis assisted by ultrasound, followed by acid hydrolysis. The pellets were resuspended with 3.5 mL of 4M NaOH and then placed in an ultrasonic bath (42 kHz) for 90 min at 40 °C. The pH was adjusted to below 2.0 with concentrated HCl and the samples were centrifuged at 2.000 x *g* for 5 min. The supernatants were washed with 7 mL of ethyl acetate and then centrifuged (10.000 x *g,* 5 min, 10 °C). This step was repeated for 3 times and the final supernatants were combined and dried in a rotary evaporator (Laborota 4000 Heidolph, Germany) at 200 rpm coupled to an ultra-thermostatic bath with circulator (Q214M2, Quimis, Brazil) at 40 °C. At the end of extraction, all dried extracts were resuspended in 1.5 mL of 2% methanol, 5% acetonitrile and 93% MilliQ water and filtered with analytical hydrophilic filters with 13 mm diameter and 0.22 µm pores, transferred to total recovery vials and stored at -20 °C until analysis.

2.4. Determination of total phenolic contents

The total phenolic content in free and bound extracts was determined in triplicate according to the Folin–Ciocalteu method (Singleton et al., 1999) adapted to microplates. Absorbance readings were performed at 750 nm on a microplate reader (FlexStation III, Molecular Devices). The standard curve was performed with seven known concentrations of gallic acid (5-130 mg/L) in the reaction mixture. The absorbance of the reaction mixture with water instead of the extract or standard was subtracted from the absorbance of the reaction with the sample. Results were expressed as mg of gallic acid equivalents (Balmer et al.) per 100 g of grain (dry basis).

2.5. Determination of the profile of phenolic compounds by UPLC-MS^E

Standard compounds were individually prepared in aqueous methanol. An aliquot of each stock solution was mixed at a concentration of 10 ppm for each compound and diluted in the initial mobile phase. The mix solution of 31 standard compounds or extracts (2 µL) were injected in triplicate into the system UPLC Acquity (Waters Co., Milford, MA) coupled to the Xevo G2-S Q-Tof (Waters Co., Manchester, UK) equipped with an electrospray ionization source (Phonsatta et al.). The standard mix was used to optimize the chromatographic separation, MS detection and the reproducibility of the system.

The chromatographic separation was carried out using a UPLC HSS T3 C18 column (100 mm x 2.1 mm, 1.8 µm particle diameter) (Liu et al.) maintained at 30 °C. Flow of 0.6 $mL.min^{-1}$ of ultra-pure water containing 0.3% formic acid and 5 mM ammonium formate (mobile phase A) and acetonitrile containing 0.3% formic acid (mobile phase B) was used according to the gradient method: 0 min - 97% A; 6.78 min - 50% A; 7.36 min - 15% A; 8.51 min - 15% A; 9.09 min-97% A. The capillary and cone voltage were set at 2.0 kV and 30 V, respectively. The dessolvation gas (N_2) was set at 800 $L.h^{-1}$ and 500 °C, the cone gas was set at 50 L.h-1 and the source at 120 °C. Data were acquired using a multiplexed MS/MS acquisition with alternating low and high energy acquisition (MS^E), from *m/z* 50 to 1000, operating in negative ion mode ESI (-), on centroid mode. MS/MS experiments were performed with collision energy ranging from 30 to 55 eV using ultrapure argon (Ar) as a collision gas. All acquisitions were performed using leucine encephalin (Leu-Enk, *m/z* 554.2615, [M-H]-) for lock mass calibration and MassLynx 4.1 SCN 9.16 (Liu et al.) software was used to acquire the data.

2.6. UPLC-MS^E data processing

The data were processed by using the software Progenesis QI v.2.1 (NonLinear Dynamics, Waters Co) with the following conditions: all runs, automatic limits, centroid data, full width resolution at half maximum of 30.000 and ionization in negative mode. For the reliable identification of compounds, comparison was made with analytical standards and database, based on neutral mass isotope distribution, retention time and MS/MS fragments applying MetaScope using the customized database of polyphenols from PubChem (https://pubch em.ncbi.nlm.nih.gov/) and other metabolites from KEGG (http://www.genom e.jp/kegg/). For the non-targeted identifications, the parameters generated by the software were applied in the descending order of importance: precursor exact mass error and fragment mass error (<10 ppm); isotopic similarity (>80%); score >30, and highest fragmentation score. In addition, only compounds present in the three technical replicates (3/3) and presenting CV

<30% were considered as tentatively identified. The data were exported to the XLSTAT software (Addinsoft, France) and Metaboanalyst 3.0 web server (Xia & Wishart, 2016) for analysis of multivariate data (HCA, hierarchical cluster analysis and PCA, principal component analysis) generated from UPLC-MS.

2.7. Statistical analysis

Statistical analysis was performed to compare means of total content and relative abundance of PC applying one-way ANOVA (Tukey, $p< 0.05$) by using XLSTAT (Addinsoft, v. 2018.2.50452).

3. Results and discussion

3.1. Total phenolic content in wheat grains at different stages of maturation

In this work, the total phenolic content was determined in both, free and bound forms, during four different stages of grain development in seven different genotypes (Table 1). Total phenolic contents were represented by the sum of free and bound forms. The total phenolic content significantly decreased from immature (milky) to mature grains, around 25-45% of reduction, depending on the cultivar. Globally, this result was found for free and bound content for all the genotypes. Remarkably, there are two distinguished groups with a great division between immature (milky and softy) and mature (physiological maturation and mature) samples (Figure 1). It is possible to see a slightly decreased between the two most immature grains (milky and softy) and then a significantly decreased towards physiological maturity and mature. Özkaya et al. (2018) also observed a decrease around 50-60% in total phenolic contents during grain development for three different cultivars and suggest there is effect of dilution of the pool of phenolic compounds present in immature grains, caused by seed size growth. Taking all genotypes together, it is possible to observe a progressive increase in bound to free ratio from immature grains (1.5:1), milky and softy, reaching the maximum at physiological mature stage (2.5:1). Equivalent ratios were found for immature grains in previous studies (Özkaya et al., 2018; Kim & Kim, 2016). In other words, the concentration of bound phenolic was higher than free phenolic in all stages of grain maturation, representing 76.5% (milky) and 60% (mature). Dinelli et al., (2011) also showed that bound fraction contributed to the total phenolic content for 72%, for 22 genotypes of old and modern Italian common wheat.

In this study, the highest phenolic content of free and bound was observed in milky grains from ORS25 genotype (177.4 mg and 230.6 mg GAE/100 g, respectively), while the

lowest contents were observed in the genotype Jadeite at the physiological maturity stage for free PC (40.3 mg GAE/100 g) and for bound in mature grains (103.4 mg GAE/100 g) (Table 1). In the same way, the highest total content was also found in milky grains of ORS25 (408.0 mg GAE/100 g) and the lowest content in mature grains of Jadeite (165.0 mg GAE/100 g). Similar contents were reported in previous studies (Özkaya et al., 2018; Kim & Kim, 2016). Although, the phenolic content of free and bound significantly varied during grain development, in mature grains total phenolic content were similar between genotypes (CV=9%). However, the Folin methods do not measure only reduction capacity of phenolic compounds but also of other reducing power substances like ascorbic acid, aromatic amines and sugars (Box, 1983).

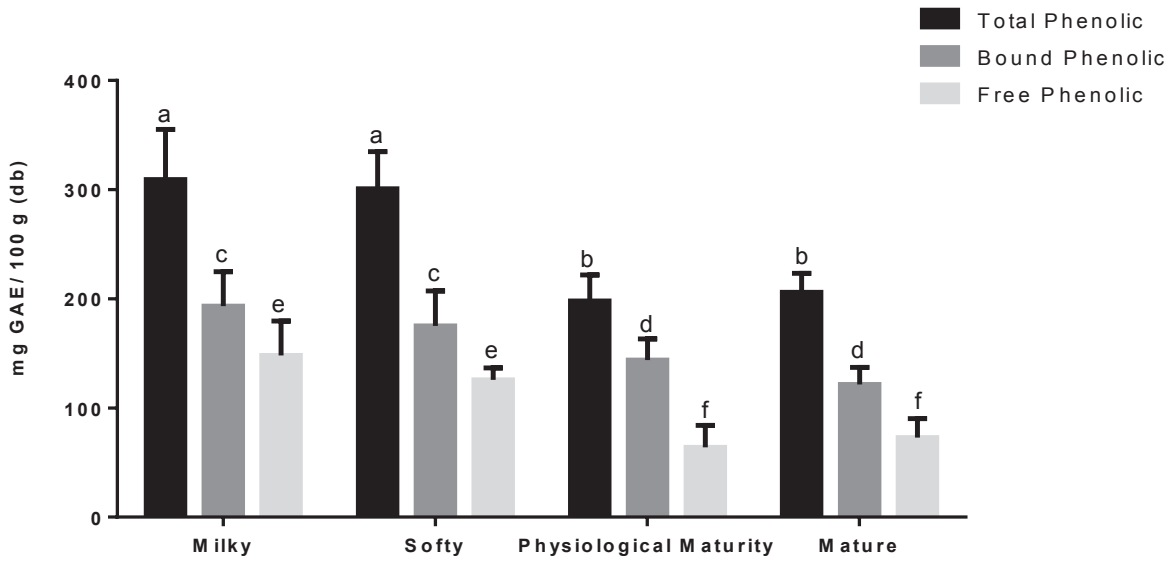


Figure 1. Total phenolic content in different genotypes of wheat grains at different stages of maturation. Data mean averages of triplicates of all genotypes samples. Different letters mean significant difference ($p< 0.05$) and bars represent standard deviation.

Table 1. Content of free, bound and total phenolic compounds in different genotypes of wheat grains at different stages of maturation.

Genotype	Free phenolic content (mg GAE/100 g)c				Bound phenolic content (mg GAE/100 g)				Total phenolic content (mg GAE/100 g)*			
	Milky	Softy	Phys. Maturity	Mature	Milky	Softy	Phys. Maturity	Mature	Milky	Softy	Phys. Maturity	Mature
Campeiro	83.9±12.7^{Bc}	142.2±2.7 ^{Aa}	99.5±1.6^{Ba}	58.6±0.3^{Cd}	218.2±1.6^{Ac}	155.9±2.7^{Bd}	136.2±7.6^{Bab}	121.5±18.9^{Ba}	302.1±11.2^{Ad}	303.2±7.3^{Ab}	235.7±9.0 ^{Ba}	179.8±19.1^{Ca}
Marfim	113.7±1.4^{Bc}	123.3±3.4^{Ad}	63,9±1.1^{Cd}	61.6±0.6^{Cd}	193.4±2.3^{Ad}	206.1±2.9^{Bab}	161.3±1.3^{Ca}	143.9±0.9^{Da}	307.1±3.7^{Bde}	331.4±4.3^{Aa}	225.5±2.3^{Ca}	206.1±0.6^{Da}
Ametista	154.0±2.1^{Aab}	128.7±1.8^{Bc}	51.6±0.6^{De}	73.2±1.1^{Cc}	224.9±0.4^{Ab}	208.7±15.4^{Aa}	146.4±14.8^{Ba}	134.2±1.1^{Ba}	378.8±1.7^{Ab}	330.0±35.9^{Ba}	198.0±14.2^{Cb}	207.4±2.2^{Ca}
Jadeite	114.2±0.6^{Ac}	105.3±2.1^{Be}	40.3±1.3^{Df}	51.6±5.5^{Ce}	166.6±0.8^{Af}	175.2±4.7^{Bcd}	127.8±3.0^{Cbc}	113.7±3.1^{Db}	279.8±0.6^{Af}	282.6±11.0^{Ab}	167.1±2.2^{Bd}	165.0±2.4^{Bb}
ORS1401	148.1±2.0^{Ab}	125.7±1.8^{Bcd}	41.3±1.3^{Df}	74.0±1.2^{Cc}	138.5±0.4^{Bg}	134.7±0.8^{Ce}	146.4±1.7^{Aab}	139.1±0.8^{Ba}	286.9±2.3^{Aef}	257.9±2.7^{Bc}	187.7±2.9^{Dc}	213.2±1.9^{Ca}
ORS1402	157.0±23.8^{Aab}	122.5±1.0^{Ad}	76.0±2.0^{Cc}	103.4±1.0^{Da}	181.0±1.2^{Ae}	132.8±1.3^{Be}	159.8±14.4^{Ba}	103.4±1.0^{Cb}	339.6±22.9^{Ac}	254.9±28.5^{Bc}	232.7±13.0^{Ca}	206.8±2.0^{Ca}
ORS25	177.4±0.5^{Aa}	134.1±1.7^{Bb}	83.0±1.2^{Db}	87.3±0.4^{Cb}	230.6±0.5^{Aa}	184.7±16.2^{Bbc}	111.2±0.8^{Cc}	116.9±0.8^{Cb}	408.0±1.0^{Aa}	336.7±18.0^{Ba}	193.5±0.5^{Cbc}	204.2±0.4^{Ca}

Uppercase letters indicated significant difference ($p< 0.05$) between columns for the same genotype, separately for each group (free, bound and total). Lowercase letters indicated significant difference ($p<0.05$) between lines or genotypes for the same maturation stage, separately for each group (free, bound and total). *Total content is the sum of free and bound phenolic content for each genotype; Data are means ± standard deviation (n = 3).

3.2. Identification of phenolic compounds and other secondary metabolites by UPLC-MS^E

The phenolic profiles of seven different genotypes were followed during different grain development phases by UPLC-MS^E method, providing the most comprehensive screening in wheat grains to date. Globally, a total of 237 phenolic compounds were tentatively identified (including isomers), among them 10 compounds were fully confirmed by reference standards: caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, cinammic acid that were present in both extracts (free and bound), while vanillin was identified only in free extract and the 4 hydroxybenzoic acid, gentisic acid, gallic acid and syringic acid only in bound extracts (Table 2). The complete table with all information of the detected compounds is summarized in data in brief (see Appendix 1 in Nascimento et al., submitted). A high number of phenolic compounds (50) were identified within same molecular formula and *m/z*, but distinct retention times (RT) and MS spectra, suggesting the occurrence of isomeric structures that significantly differ in their elution behavior. These isomeric compounds, such as ferulic acid and its derivatives, 3-feruloylquinic acid, feruloyl glucose and dehydrodiferulic acid were tentatively identified. These results showed the potential of the very sensible analytical instrumentation used, allowing the detection of several compounds. Previous works have also reported the presence of a high number of isomer forms of phenolics in wheat species (Dinelli et al., 2009, 2011). Other secondary metabolites were also identified (including isomeric forms), such as alkaloids (40), terpenoids (38) and polyketides (30) (see Table 2, Nascimento et al., submitted).

A total of 26 phenolic compounds were commonly identified in free and bound extracts. The number of phenolics identified in bound (180) were higher than in free (83) extracts, corroborating previous studies (Dinelli et al., 2009; Sharma et al., 2016; Wang et al., 2013). All wheat genotypes presented this same behavior. The phenolic acids (40%) were the most abundant class identified in free extract, while in bound were flavonoids (36%) and lignans and stilbenes were the minor classes in both extracts (Figure 2). The ferulic acid is typically the most abundant phenolic acid in grains (Angelino et al., 2017). In this study, ferulic acid was confidently identified in both extracts for all genotypes independently of the maturation stage and presented the highest relative abundance (Table 2). The most immature grains, milky stage of the cultivar (cv.) Campeiro presented the highest total ion abundance of ferulic acid. Moreover, 8 isomers of ferulic acids and 9 isoforms of dimeric ferulic acids were detected (see Table 1, Nascimento et al., submitted). 4 dehydrodimers of ferulic acid were

described in literature (Garcia-Conesa et al., 1997). In free extracts, only one diferulic acid isomer was identified, while in bound extract at least 8 isoforms were present in genotypes irrespective of stages of maturation studies (data not shown). Remarkably, this compound was associated with more effective antioxidant capacity than soluble ferulic acid (Andreasen et al., 2001).

Table 2. Most abundant phenolic compounds and reference standard compounds identified in free (f) and bound (b) extracts.

Name of compound	Molecular formula	*m/z*	RT (min)	Score (%)	FS (%)	Fragment data	Error (ppm)	IS (%)	Class
Free compounds									
Diphyllin	$C_{21}H_{16}O_7$	379.0823	0.4	39.5	5.5	71.0120 (26.89); 85.0279 (6.34); 59.0116 (5.3); 267.0709 (3.82)	0.0	91.9	L
3-Feruloylquinic acid I	$C_{17}H_{20}O_9$	367.1044	0.4	43.9	26.6	129.0179 (75.02); 97.0280 (46.00); 75.0069 (33.60); 157.0324 (14.89); 187.0405 (13.51); 367.1041 (5.69); 205.0510 (5.67); 277.0722 (5.55); 191.0351 (4.22); 353.0884 (3.57); 147.0286 (3.54)	2.5	95.8	PA
1-*O*-Sinapoyl-beta-D-glucose	$C_{17}H_{22}O_{10}$	385.1146	0.4	42.2	22.2	367.1041 (13.61); 205.0510 (13.57); 277.0722 (13.29); 221.0455 (8.69); 207.0666 (8.60); 353.0884 (8.53); 263.0566 (5.23)	1.5	90.7	PA
Feruloyl glucose I	$C_{16}H_{20}O_9$	355.1029	0.4	42.0	21.2	157.0324 (14.89); 187.0405 (13.51); 205.0510 (5.67); 277.0722 (5.55); 191.0351 (4.22); 145.0303 (3.96); 221.0455 (3.63); 207.0666 (3.60); 203.0351 (3.00)	-1.5	90.5	PA
Chlorogenic acid	$C_{16}H_{18}O_9$	353.0886	0.4	43.8	26.6	129.0179 (75.02); 75.0069 (33.60); 187.0405 (13.51); 205.0510 (5.67); 277.0722 (5.55); 191.0351 (4.22); 221.0455 (3.63); 147.0286 (3.54); 203.0351 (3.00)	2.2	95.1	PA
Dalpanin	$C_{26}H_{30}O_{12}$	533.1714	0.5	49.6	62.5	101.0231(48.43); 113.0231 (39.61); 89.0229 (39.28); 341.1079 (37.40); 161.0443 (30.49); 71.0120 (26.89); 143.0337 (24.81); 119.0336 (13.83); 131.0337 (10.09); 87.0071 (6.45); 85.0279 (6.34); 125.0230 (6.27); 59.0116 (5.30)	9.2	95.5	F
Neohesperidin	$C_{28}H_{34}O_{15}$	609.1876	0.5	44.4	45.9	101.0231 (48.43); 113.0231 (39.61); 89.0229 (39.28); 161.0443 (30.49); 119.0336 (13.83); 323.0972 (11.35); 131.0337 (10.09); 85.0279 (6.34)	8.4	85.2	F
Dasytrichone	$C_{18}H_{16}O_4$	295.1002	0.9	35.3	nd	nd	8.9	86.1	F
Vanillin	$C_8H_8O_3$	151.0388	2.0	34.4	1.04	71.0118 (3.81); 68.9962 (3.52)	-8.5	80.4	OP
Caffeic acid	$C_9H_8O_4$	179.0337	2.7	36.4	nd	nd	-7.1	89.9	PA
Gentisic acid III	$C_7H_6O_4$	153.0180	2.8	47.2	46.8	109.0278 (81.56); 135.0071 (7.83)	-8.8	98.8	PA
Apigenin 7-*O*-apiosyl-glucoside I	$C_{26}H_{28}O_{14}$	563.1399	2.9	57.4	96.0	353.0652 (100); 383.0759 (45.51)	-1.3	92.4	F
Apigenin 7-*O*-apiosyl-glucoside II	$C_{26}H_{28}O_{14}$	563.1399	3.0	54.4	80.4	353.0653 (100); 383.0760 (64.84); 366.1179 (6.18); 325.0706 (1.64)	-1.3	93.1	F
***p*-Coumaric acid**	$C_9H_8O_3$	163.0387	3.3	56.2	91.7	119.0487 (100)	-8.5	99,0	PA
Ferulic acid	$C_{10}H_{10}O_4$	193.0492	3.5	47.2	47.6	134.0357 (100); 101.0231 (15.31); 113.0235 (6.01); 178.0256 (3.73)	-7.3	96.6	PA
Sinapic acid	$C_{11}H_{12}O_5$	223.0598	3.5	49.9	68.7	134.0357 (100); 113.0235 (6.01); 193.0494 (3.45)	-6.2	87.7	PA

Cinnamic Acid	$C_9H_8O_2$	147.0438	3.8	36.1	nd	nd	-8.9	90.3	PA
Zingerone II	$C_{11}H_{14}O_3$	193.0857	6.6	55.2	88.5	92.0259 (1.40); 177.0536 (1.75)	-6.9	95.4	PA
Elemicin II	$C_{12}H_{16}O_3$	207.1013	6.6	36.8	nd	nd	-6.7	91.8	OP
Bound compounds									
Gallic acid	$C_7H_6O_5$	169.0129	1.2	37.0	nd	nd	-8.2	94,0	PA
Gentisic acid II	$C_7H_6O_4$	153.018	1.6	55.0	86.0	152.0099 (11.31); 123.0076(1.94)	-9,0	99.1	PA
4-Hydroxybenzoic acid	$C_7H_6O_3$	137.0232	2.3	51.8	69.7	136.0150 (69.84); 108.0201 (21.09); 92.0252 (3.75)	-9,0	99.2	PA
Gentisic acid	$C_7H_6O_4$	153.0179	2.4	37.3	nd	nd	-9.3	96.7	PA
Myricetin I	$C_{15}H_{10}O_8$	317.0306	2.5	45.8	35.8	183.0283 (100); 111.0071 (68.64); 190.9972 (3.54)	1,0	94.2	F
Esculetin II	$C_9H_6O_4$	177.0179	2.6	54.1	84.7	133.0281 (72.68); 176.0098 (45.18); 104.0256 (21.88)	-7.8	94.3	OP
Caffeic acid	$C_9H_8O_4$	179.0336	2.7	54.8	83.8	135.0433 (100)	-7.5	98.7	PA
Syringic acid	$C_9H_{10}O_5$	197.0438	2.7	37.0	nd	nd	-8.9	94.9	PA
Gentisic acid IV	$C_7H_6O_4$	153.0179	3.2	40.1	11.0	107.0121 (34.25)	-9,0	99.3	PA
***p*-Coumaric acid**	$C_9H_8O_3$	163.0387	3.3	54.7	83.3	119.0487 (100)	-8.2	99.4	PA
Ferulic acid	$C_{10}H_{10}O_4$	193.0495	3.5	43.7	26.0	134.0359 (96.74); 178.0257 (21.96); 133.0280 (13.59); 149.0591 (8.42)	-5.6	99.1	PA
Sinapic acid	$C_{11}H_{12}O_5$	223.0598	3.5	40.1	14.3	193.0492 (22.01); 149.0228 (1.34)	-6.3	93.3	PA
Ferulic acid V	$C_{10}H_{10}O_4$	193.0494	3.7	49.9	57.4	134.0358 (100); 178.0255 (13.81); 133.0279 (8.14); 149.0581 (7.82)	-6.2	99.2	PA
5-8'-Benzofuran dehydrodiferulic acid I	$C_{20}H_{18}O_8$	385.0917	3.8	43.4	22.1	341.1016 (17.42); 326.0781 (9.22)	-3,0	98.5	PA
Diferulic IV	$C_{20}H_{18}O_8$	385.0917	4.5	43.3	22.8	341.1017 (18.41); 326.0774 (9.46); 325.0705 (4.72); 119.0492 (1.96)	-3.2	97.5	PA
5-8'-Benzofuran dehydrodiferulic acid II	$C_{20}H_{18}O_8$	385.0916	4.8	44.2	29.1	193.0493 (100); 134.0359 (31.62); 111.0799 (2.84)	-3.3	96,0	PA
Tetramethylscutellarein V	$C_{19}H_{18}O_6$	341.1019	4.9	42.4	18.9	147.0439 (15.36); 326.0781 (13.79); 159.0436 (9.55); 165.0546 (6.83); 195.0652 (4.38)	-3.5	97.2	F
Kaempferol	$C_{15}H_{10}O_6$	285.0392	5.2	36.8	nd	99.0069 (100)	-4.3	89.1	F
5-8'-Benzofuran dehydrodiferulic acid III	$C_{20}H_{18}O_8$	385.0916	5.6	39.7	7.63	109.0280 (67.27); 174.0307 (2.54); 159.0435 (2.08)	-3.3	94.8	PA
Zingerone II	$C_{11}H_{14}O_3$	193.0857	6.6	54.3	83.8	177.0540 (2.65)	-7,0	95.4	PA

m/z = mass/charge; RT = retention time; FS = fragmentation score; IS = isotope similarity; PA = phenolic acids; F = flavonoids; L = lignans; OP = other polyphenols. Bold represent reference standards and italic means reference compounds not identified in the list of the 15 most abundant compounds.

3.3. Evolution of phenolic profile during wheat grain development

From chromatograms, it is clear to see that bound extracts presented a higher number of peaks and hence a higher number of compounds identified than within free extracts (Supplementary Figure 1). The numbers of identified compounds progressively decreased with the grain development (Figure 2). For instance, in chromatograms of the cv. Jadeite (see Figure 1 in Nascimento et al., submitted), one can observe a clearly decrease in number and total ion count of peaks from immature stages (milky and softy) to mature. Noticeably, this result can be observed for FA (RT 3.5) especially concerning total ion abundance in bound extract along grain development.

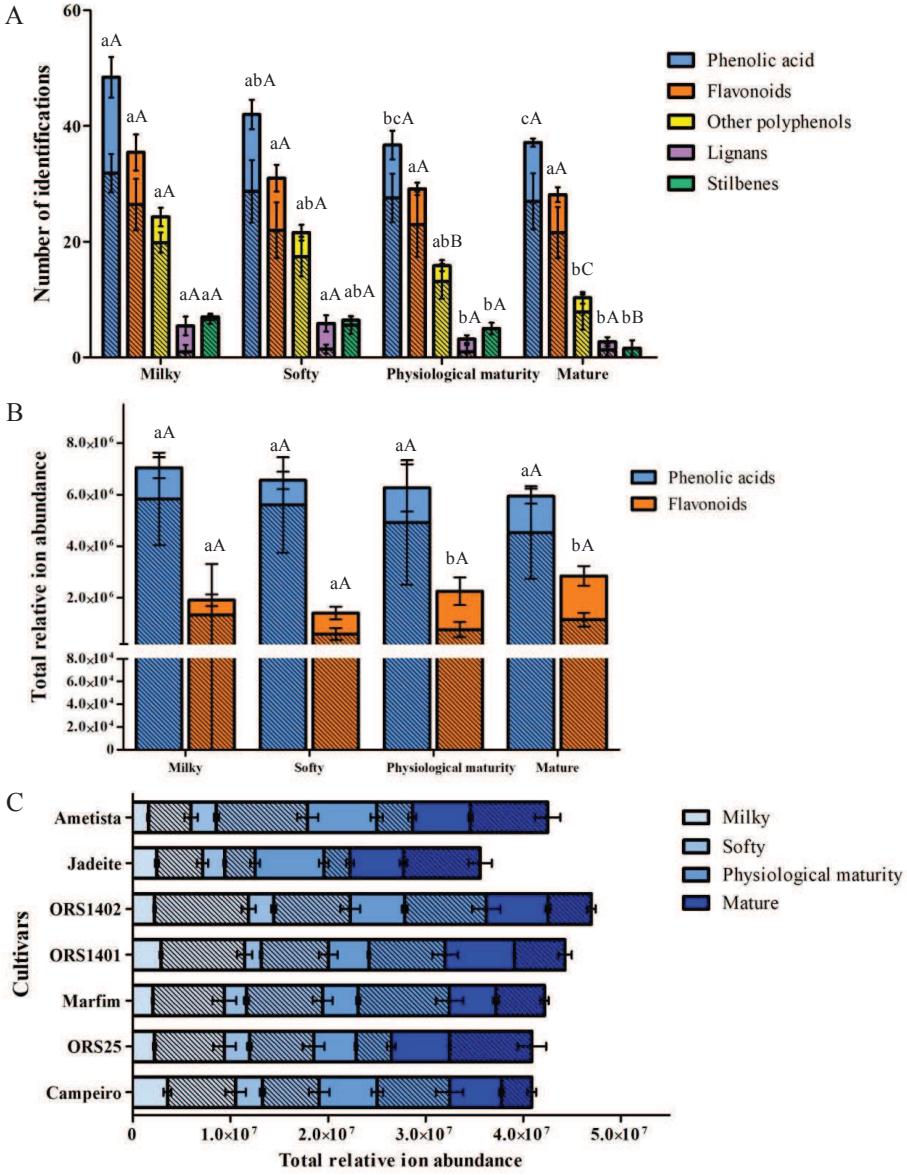


Figure 2. A. Number of phenolic compounds for each class and each stage of maturation. B. Total relative ion abundance of phenolic acids and flavonoids classes for free and bound extracts. C. Sum of the total relative ion abundance for each genotype during the grain wheat development. Data mean average of triplicates and bars represent standard deviation.

Globally, the number of phenolic compounds identifications decreased 32% towards the maturation of grain in both extracts and for all genotypes (Figure 2a). This result can be explained by the large presence of aminoacids in the initial stages of grain formation (Zhen et al., 2016), since these aminoacids (phenylalanine and tyrosine) are the precursors in the biosynthesis path of the phenolic compounds. The number of identification of lignans compounds also decresead along the grain maturation. The lignans were mainly identified in free extract in the most immature stages, it can be explained by the hydrolysis applied to release bound compounds that might be affected also molecular structure, causing interconversions between lignans (Durazzo et al., 2013). Other secondary metabolites identified also presented the same behavior during grain development, terpenoids and alkaloids showed a decrease of 77% and 76% in free extracts and 17% and 29% in bound extracts, respectively.

Although the number of identifications within each class (Figure 2a), decreased during the grain maturation (milky versus mature) in free and bound extracts, respectively for phenolic acids 39% and 9%, flavonoids 43% and 35%, other polyphenols 56% and 43%, when we correlate with total relative ion abundance, there is no significant difference (Figure 2b). Because total ion abundance for phenolic acids slightly decrease but flavonoid abundance rises, especially in free extract, during grain development. It can be suggested that flavonoids biosynthesis from cinnamoyl-CoA or p-coumaroyl-CoA occurs late in grain development. These results were also not correlated with spectrophometric results. It is known that Folin methods suffer influence of sugars, aminoacids or other reducing metabolites, thus the decrease found in Figure 1 can be related to these other metabolites that decrease during grain growth (Zhen et al., 2016). McCallum and Walker (1990) previously observed a higher activity of phenylalanine ammonia lyase (converts phenylalanine in cinnamic acid) in immature grains, without detection in mature grains. The reduction of this enzymatic activity suggests that with the maturation of grain the phenolic synthesis is decreased until stop.

The relative abundance of major phenolic compound, confidently identified based on standards, involved in the biosynthesis of ferulic acid was followed during grain maturation (Figure 3, for cv. Marfim). *P*-coumaric acid was the most abundant in free extract, probably due to the fact that this phenolic compound can be synthesized from two routes (tyrosine and phenylalanine), being considered as a key compound for the synthesis of all others phenolic compounds (phenylpropanoid biosynthesis map available in http://www.genome.jp/dbget-bin/www_bget?map00940). It is possible to see free phenolics decreasing during the grain

maturation, except for the cinammic acid, kept almost constant but showing the lowest abundance. From this result, it could be suggested a preferential route of ferulic acid synthesis from phenylalanine and cinnamic acid path. In bound extracts, the behavior of phenolic compound was more stable, showing bound or insoluble phenolic suffer minor alterations during grain development, corroborating the results showed by Silvestro et al. (2016). These authors showed that bound phenolics were mainly determined by genetic traits than environmental or year factors, while free phenolics were more susceptible to climatic conditions and location.

Genotypes also showed different abundance of phenolic compounds. It is possible to note the variation of total ion abundance in each stage of grain maturation (Figure 3c). The genotypes ORS25 and Ametista presented the highest total abundance in mature grains, which can have an impact in the composition of bioactive compounds in flour. The genetic diversity of phenolics was previously reported for Chinese and Italian, common and durum wheat cultivars (Dinelli et al., 2009; Laddomada et al., 2017; Zhang et al., 2012), but this is the first time that a comprehensive evaluation of genetic diversity of phenolic compounds is reported for Brazilian wheat.

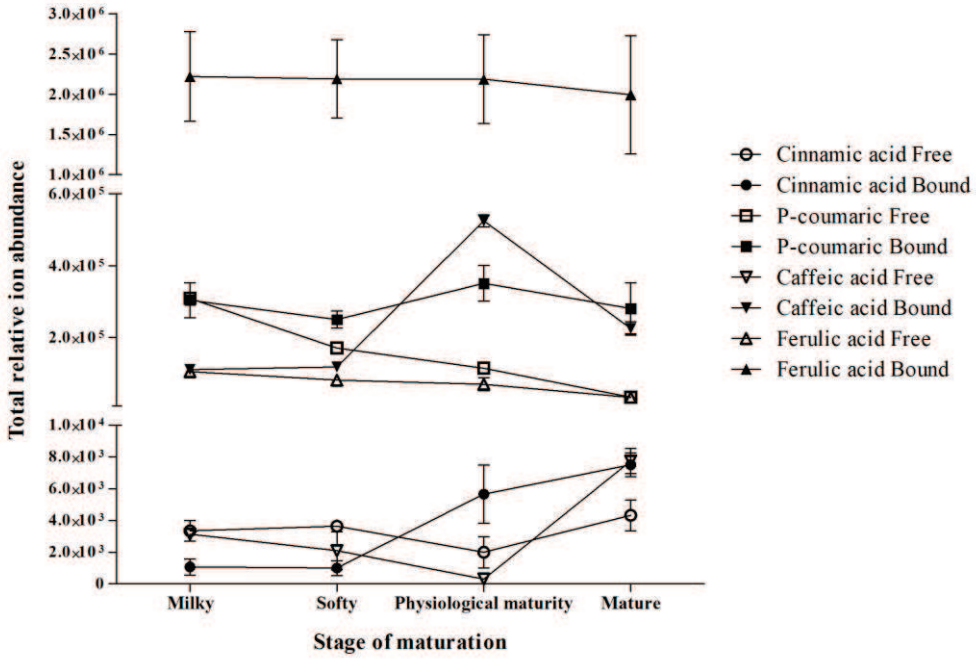


Figure 3. Monitoring of main phenolic compounds related to ferulic acid biosynthesis during the grain development.

3.4. Metabolomic tools for characterization of phenolic profile in different wheat genotypes during grain maturation

The phenolic profiles of seven wheat genotypes in different maturation stages were characterized by multivariate statistical methods (PCA and HCA) to recognize the distinct composition and behavior patterns of phenolic compounds in the different wheat genotypes during grain development. Moreover, the identification of discriminatory phenolics of immature and mature wheat grains may help to predict specific characteristics of a particular genotype and understand possible triggers for maturation stage changes (Phelix & Feltus, 2015; Steinfath et al., 2010). As a first step, PCA was applied to explore the variation in the dataset reduced and redistributed linearly as also randomly in scores (genotypes or maturation stage) and loadings (phenolic compounds). Cluster analysis was also carried out to investigate the degree of similarity or dissimilarity between the phenolic compounds relatively quantified in each sample.

Immature grains presented a differential phenolic profile than mature grains. The PCA biplot indicated a clear distinction between immature (milky and softy) and mature (physiological maturity and mature) stages in all genotypes, reaffirming the different patterns of the phenolic profile between them along grain maturation (Figure 4). The two main components (PC1 and PC2) explained in average 76% of the total variance observed in all genotypes evaluated. Discriminatory phenolic compounds were found mainly along PC2 (x-axis) and each genotype showed a specific composition able to explain changes in phenolic compounds during grain development (eigenvectors presented in Appendix 2, Nascimento et al., submitted).

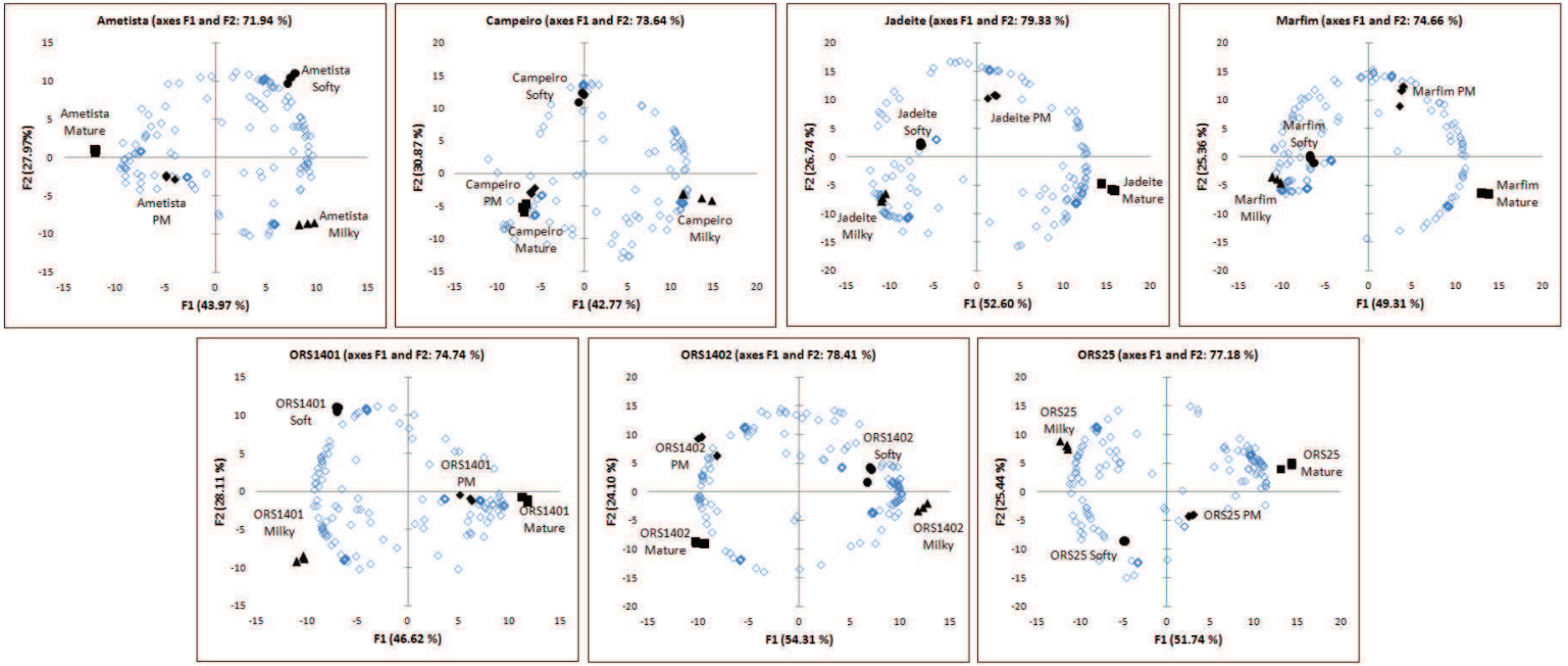


Figure 4. Phenolic profiling of immature (milky and softy stages) and mature (physiological maturity and mature stages) wheat genotypes. (a) Principal component analysis (PCA) of the identified phenolics in wheat genotypes.

In the cv. Ametista, variables from physiological maturity and mature stages were positively correlated, positioned on the same side of the plot but in different quadrants, showing these stages present differential composition of phenolics in this cultivar (Figure 4). On the other hand, the position of these variables in relation to softy and milky stages indicate negative correlation between them, and so they did not share common phenolic characteristics. Discriminatory phenolic compounds includes ferulic acid, 4-hydroxybenzoic acid, vanillic acid isomer I, gallic acid 3-*O*-gallate, juglone, 4-vinylguaiacol isomer I and gallic acid isomer I, predominantly found in bound form, except for free PC auriculoside isomer II, neohesperidin and sinapic acid (Figure 2 in Nascimento et al., submitted). Among phenolics identified, the flavonoid auriculoside isomer II was the main responsible for separation between maturation degrees in this cultivar. The HCA together with the correlation matrix (heatmap) highlights free and bound phenolics with relatively higher and lower abundance comparing genotypes and maturation stages. This same flavonoid was also differentially identified in wheat genotypes (Figure 5) and in greater abundance in mature samples of Ametista (Figure 2 in Nascimento et al., submitted).

In the cv. Campeiro, variables from physiological maturity and mature stages were positively correlated, almost superimposed whereas in opposite position are variables from milky stage (Figure 5). Variable corresponding to softy sample was isolated in the upper center quadrant, due to the unique phenolic composition in this specific stage. This result can be observed by HCA and heatmap, clearly pointing by a differential profile between softy and other samples (Figure 2, data in brief). Phenolics responsible by maximum variance in the PCA were found in the PC2, such as caffeic acid II and gardenin B II bound phenolics and glycitin I free phenolic. These compounds were equally important for separating maturation stages in this cultivar, but greater expression of them were found during softy stage as expected (Figure 5).

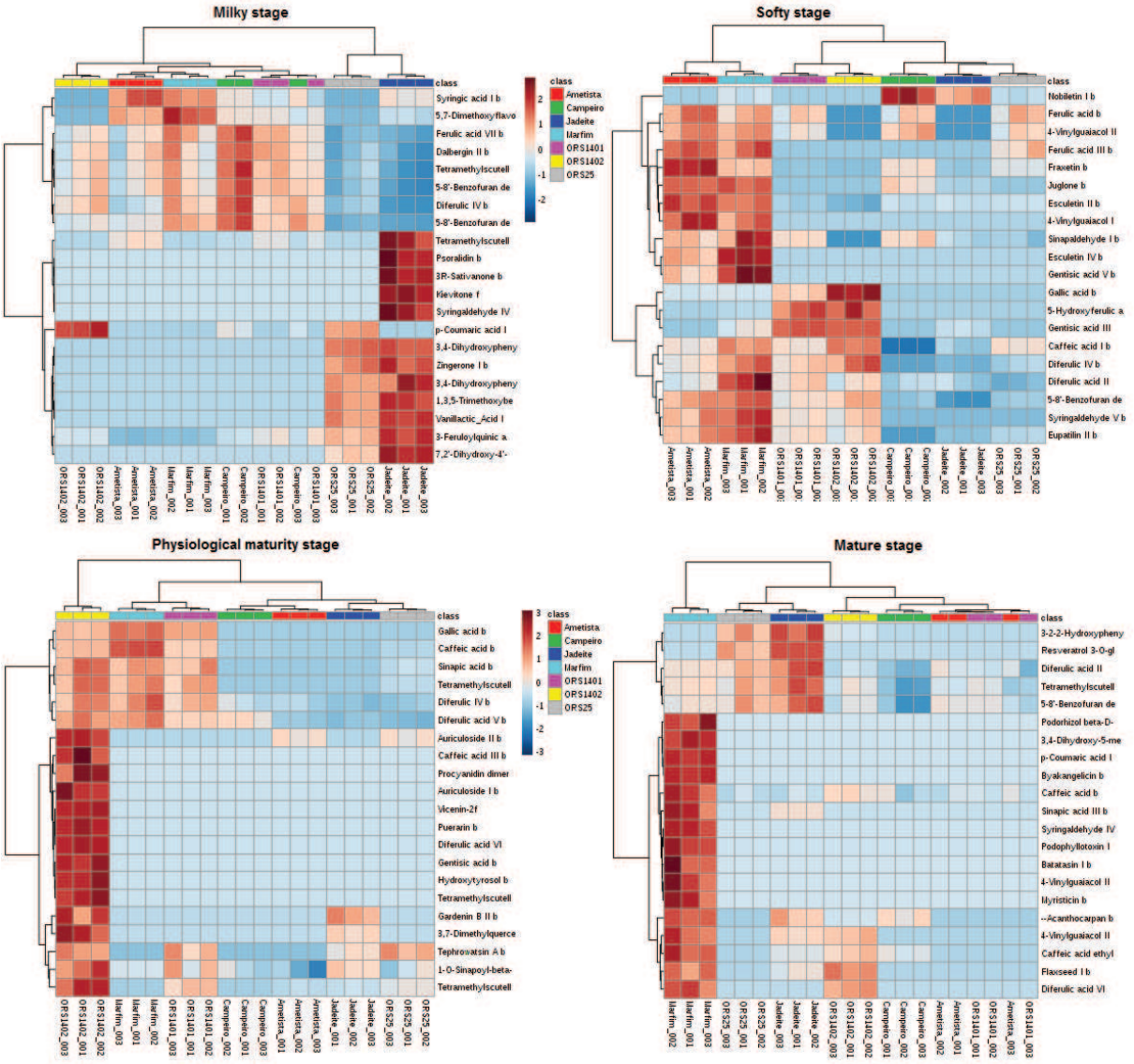


Figure 5. Hierarchical cluster analysis (HCA) and heatmap of the bound (b) and free (f) phenolics from wheat genotypes, which showed maximum variance (eigenvectors) along maturation stages.

In the cv. Jadeite, the variables from maturation stages were significantly distinguished (PC1 and PC2 80%) by the dissimilarity in the phenolic profile along maturation (Figure 4). The main discriminatory phenolic compounds were found along PC2, which includes the free forms of 3-feruloylquinic acid and neohesperidin; flaxseed II, esculetin II and leptodactylone II for bound phenolics. Among them, 3-feruloylquinic acid explained the strong separation between maturations stages, being identified in greater abundance in physiological maturity (Figure 2, data in brief). In the same way, for cv. Marfim, variables from softy and milky were positively correlated in the lower left quadrant, revealing a similar comparable phenolic profile (Figure 4). Variables from physiological maturity and mature stages were located in the opposite position, but with a low degree of similarity due to the distance in the plot. This result can also be observed in the HCA and

heatmap (Figure 2 in Nascimento et al., submitted). The *p*-coumaric acid 4-*O*-glucoside, coniferin, gentisic acid III, sinapic acid and 5-hydroxyferulic acid methyl ester III were the major discriminatory phenolics along maturation. They are predominantly bound phenolic, except for *p*-coumaric acid 4-*O*-glucoside and coniferin. Among them, one isomer of gentisic acid III was the most significant in the separation between maturation degrees in cv. Marfim (Figure 2 in Nascimento et al., submitted).

For the genotypes OR1401, OR1402 and OR25, milky and softy stages were also positively correlated (Figure 4). In opposite positions there were variables from physiological maturity and mature. However, each genotype showed a different composition of discriminatory compounds. Among them, *p*-coumaric acid I isomer was the most important compound for separating maturation stages in ORS1401, especially in physiological maturity. Previous works found this phenolic acid in low concentrations in different wheat genotypes (Liyana-Pathirana et al., 2006; Moore et al., 2005). The main discriminatory compound was the isomer of lignin, diferulic acid III, in the ORS1402 genotype, found in greater abundance in physiological maturity stage (Figure 2 in Nascimento et al., submitted). For ORS25, the flavanoid dalbergin I isomer was the main discriminatory compound between maturation and greater levels of them were found in mature stage (see Figure 2 in Nascimento et al., submitted).

Besides to evaluate each genotype in different phases of grain development, we also compared all genotypes together in each maturation stage. In this way, clear separation was also observed by the two main components (PC1 and PC2), which explained more than 46% of the total variance observed (see Figure 1 in Nascimento et al., submitted). Some phenolics discriminatory of each stage might be pointed as the main responsible for the separation between genotypes in each maturation degree (Figure 5). Among them the lignan isomer diferulic acid IV was the main compound explaining variability in milky stage, while 4-vinylguaiacol I isomer, a polyphenol, was the most important phenolic for separating genotypes in the softy stage. At physiological and mature stages, two genotypes presented a distinguished profile, ORS1402 and Marfim, respectively (Figure 5). In these stages, the flavonoid 3,7-dimethylquercetin II isomer and the hydroxycinnamic acid, caffeic acid ethyl ester II isomer were found among the most significant compounds in physiological and mature stages, respectively.

Although some recent studies showed that the relative distribution of free and bound phenolic acids did not significantly differ between wheat varieties (Shamloo et al., 2017),

others suggested that there is possible differences between biosynthetic pathway of secondary metabolites among durum wheat genotypes (Di Loreto et al., 2018). In this work, a comprehensive phenolic composition pattern were described and showed different profiles of phenolic compounds found either between wheat genotypes or during grain maturation phases.

4. Conclusion remarks

Immature wheat grains from all Brazilian genotypes here studied presented a more complex composition of phenolic compounds with higher abundance than mature grains. These results corroborate previous works showed the antioxidant potential and important bioactivity presented by immature wheat such as antiproliferative effect against cancer cells (Kim and Kim, 2016; 2017). Phenolic profile and composition were different among genotypes and during the different phases of grain development. Chemometric analysis (PCA and HCA) showed each genotype has discriminatory compounds specifics of maturation stage. Moreover, some genotypes showed a diversified composition and abundance of phenolics, such as Marfim and ORS1402, respectively, showing Brazilian wheat genotypes contains a rich phenolic composition and may be selected according to the profile of bioactive compounds. Selecting and breeding wheat genotypes that are high in bioactive compounds will improve agronomical traits of the wheat plants, enhance the keeping quality, stability, and safety of wheat products and improve the health beneficial properties associated with wheat consumption.

5. Acknowledgements and Funding

The authors are grateful to Prof. Dr. Luiz Carlos Gutkoski (UPF, Brazil) and OR Melhoramento de Sementes Ltda for providing and collecting samples; to Michael Murgu and Robinson Giraldes from Waters Corporation (Brazil) to their support on MS data processing and helpful technical assistance in mass spectrometry.

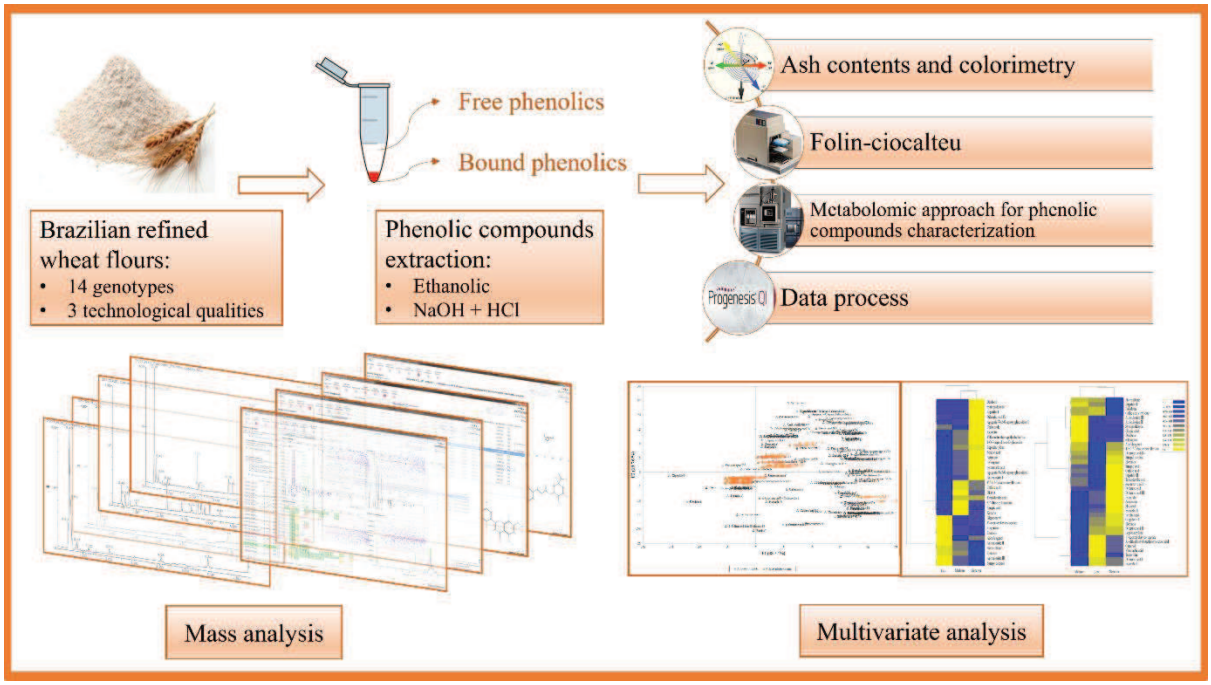
This work was financed by the Foundation for Research Support of State of Rio de Janeiro (FAPERJ) (26/111.484/2014; 26/203.312/2015), National Council for Scientific and Technological Development (CNPq) (448171/2014-7) and supported by Federal University of the State of Rio de Janeiro (UNIRIO), Brazilian Olympic Committee (COB), Coordination for Improvement of Personnel with Higher Education (CAPES), Funding Authority for Studies and Projects (FINEP), Sigma-Aldrich and Waters Corporation.

Chapter 3 – Foodomics in wheat flour reveals phenolic profile of different genotypes and technological qualities

Millena Cristina Barros Santos[a,b]; Luciana Ribeiro da Silva Lima[a,b]; Carolina Thomaz dos Santos D'Almeida[a,b]; Verônica Cristina Mayrinck Victorio[a,b]; Luiz Claudio Cameron[b]; Claire Bourlieu-Lacanal[c], Mariana Simões Larraz Ferreira[a,b*]

[a]Laboratory of Bioactives, Food and Nutrition Graduate Program (PPGAN), Federal University of the State of Rio de Janeiro, UNIRIO;
[b]Center of Innovation in Mass Spectrometry, Laboratory of Protein Biochemistry, UNIRIO.
[c]INRAE, UMR IATE, Institute Agro, Montpellier, France

Paper published on on LWT – Food Science and Technology, 2022. Doi: https://doi.org/10.1016/j.lwt.2021.112519



Highlights:

• First study to correlate phenolic profile and technological quality in Brazilian wheat flours.

• Metabolomics reveals the phenolic profile in different Brazilian genotypes in wheat flours.

• Phenolic compounds persist after the milling process in refined wheat flours.

• Fifteen phenolic compounds were detected in all genotypes studied.

• Multivariate analysis highlighted discriminating compounds among the technological qualities.

Abstract

Phenolic compounds (Martillanes et al.) strongly contribute to the beneficial health effects of wheat, but their interactions can affect the quality of end-use wheat products. Free and bound PC were comprehensively characterized in 14 wheat flours (*Triticum aestivum*) from different Brazilian genotypes and technological qualities by using a metabolomics approach (UPLC-ESI-QTOF-MS^{E}) combined with classical characterizations: colorimetry, ash, protein, starch and total phenolic content (TPC). Globally, 43 PC were identified: 33 (bound, 28 (free) and 15 in all flours, regardless of extract. Ferulic acid isomers were the most abundant PC, representing 25-50% of ion abundance depending on genotype. Campeiro, Sossego and Topázio genotypes showed a distinguished profile, with the highest total relative abundance of PC. TPC was significantly higher in flours with higher gluten strength (66.5-58.0 mg GAE/100 g flour). The ratio free-to-bound of PC averaged 1.15 between the flours of different technological qualities. Although PCA highlighted specific PC related to technological qualities, the genotype effect was very pronounced. This study correlates the phenolic profile and technological quality of wheat flours and provides the most recent data on the secondary metabolites profile, especially PC in refined flour, attesting to its significant nutritional importance due to its large consumption in refined forms.

Keywords: metabolomics, bound phenolics, end-use food, free phenolics, *Triticum aestivum*.

1. Introduction

Wheat (*Triticum spp*.) is the second most cultivated cereal in the world. In 2020, world wheat production reached 764 million tonnes, an increase of 4.9% over the last five years (USDA, 2020). In 2020, Brazil produced 6.23 million tonnes of common wheat from a cultivated area of 2.4 million hectares, but imported 6.6 million tonnes (CONAB, 2021) due to its high per capita consumption of 57 kg per year (ABITRIGO, 2018). Wheat is considered the most suitable raw material for bread and pasta due to its viscoelasticity and protein quality. Brazilian production and consumption are almost exclusively restricted to common wheat (*Triticum aestivum*) while durum wheat (*T. durum*) use remains limited; as a result the rheological properties define the technological quality of flour and thus the industrial end-use of wheat.

Furthermore, wheat research has intensified over the last years due to its bioactive compounds and wide range of reputed beneficial effects on human health. Epidemiological studies have shown that the consumption of whole wheat and grain-based products is associated with reducing chronic non-communicable diseases. The health benefits of cereals have fostered research on the phytochemical composition, mainly, the phenolic compounds (Martillanes et al.), of the different varieties and species of wheat (Dinelli et al., 2009; Fardet, 2010) .

Indeed, PC are ubiquitous compounds; they are secondary metabolites synthesized during plant development in response to stress conditions and are among the most abundant bioactive compounds on Earth (Saltveit, 2017). The largest proportion of PC is found in wheat's outer layers - aleurone, testa and pericarp - and exert a wide range of bioactivities, but their beneficial effects are generally attributed to their antioxidant activity (Shewry & Hey, 2015). PC can be found in three forms in wheat: soluble free, soluble conjugated (e.g., with mono and polysaccharides), and insoluble bound to cell wall components, such as arabinoxylan fibers, or macronutrients, such as proteins. The most abundant form found in wheat grains is the insoluble bound (77%), followed by the soluble conjugated (22%) and the soluble free form (<0.5 –1%) (Acosta-Estrada et al., 2014). Phenol-protein interactions can alter these molecules physicochemical properties, solubility, availability and digestibility (Ozdal et al., 2013). Due to these chemical interactions, PC can impact the quality of end-use products. Sharma et al. (2016) showed that wheat flours classified as "poor" had higher levels of PC and higher expression of enzymes related to their synthesis pathways when compared to "good" wheat flour.

Phenolic acids and flavonoids are the most important class and ferulic acid is the dominant phenolic acid in wheat (> 90%). However, agroclimatic parameters, such as location and growing conditions, and processing, such as wheat dry fractionation processes, strongly impact the PC profile (Hemery et al., 2007; Wang et al., 2013). This impact is evident in the milling process to obtain refined wheat flour; whole flours (100% extraction rate) presented the highest levels of PC (Wang et al., 2013). However, the consumption of refined white flour is six times higher than wholemeal flour. Despite this, information about refined flour's phenolic profile and content remains limited (Sharma et al., 2020; Shewry & Hey, 2015).

Due to the great diversity of PC and their isomers in cereals, especially in wheat grains, it is essential to employ advanced analytical techniques for reliable identification (Santos et al., 2019b). The metabolomic approach is useful since it combines different techniques, presenting high sensitivity, selectivity, and resolution. Similarly, the concept of "Foodomics" employs omics tools to understand and map the chemical compounds, as well as to characterize food contaminants (Herrero et al., 2012).

In this work, omics tools were applied to characterize the PC profile of wheat flour from different Brazilian genotypes classified into three different technological qualities: low, medium and superior. Thus, a total of 14 refined wheat flours were investigated, representing 12.5% of cultivars currently produced in Brazil, with different technological qualities and indicative end-use commercial classes (e.g., pasta, bread, or biscuit production). A modern non-targeted method based on a multiplexed MS-MS acquisition with simultaneous application of low (precursor ions) and high collision energy (MS^E) (fragment ions) was used to relatively identify and quantify the PC of wheat samples. Total phenolic, ash, protein, and starch contents and colorimetric properties were also determined, and some correlations were established.

2. Materials and methods

2.1. Chemicals

The following reference standards, as well as MS-grade acetonitrile and methanol, were purchased from Sigma-Aldrich (St. Louis, MO, USA): vanillic acid, *p*-coumaric acid, catechin, caffeic acid, ellagic acid, *trans*-ferulic acid, kaempferol, myricetin, pyrogallol, flavanone, quercetin, gallic acid, epicatechin, 4-hydroxybenzylalcohol, 4-

hydroxybenzaldehyde acid, 4-hydroxybenzoic acid, 4-hydroxybenzoic acid, 4-phenylacetic acid, sinapic acid, benzoic acid, quercetin-3-O-glucoside, 3,4-dihydroxy phenylacetic acid, epigallocatechin, epicatechingallate, chlorogenic acid, 2,5-dihydroxybenzoic acid, 4-methoxycinnamic acid, 2-hydroxycinnamic acid, 3-hydroxy-4-methoxycinnamic acid, *trans*-cinnamic acid, 3-methoxycinnamic acid, and L-(-)-3-phenylacetic acid. Formic acid was purchased from Fluka (Switzerland). Ultrapure water was obtained through the Barnstead™ Smart2Pure™ (Thermo Fisher Scientific, USA) purification system. Other unmarked reagents were of analytical grade.

2.2. Plant material

OR Melhoramento de Sementes and Biotrigo Genética (Passo Fundo, Brazil) kindly provided common winter wheat flours (*Triticum aestivum*) from 14 different Brazilian genotypes (Campeiro, ORS Vintecinco, ORS1401, ORS1402, Marfim, Jadeite 11, Ametista, ORS Vintesete, Topázio, Noble, Iguaçu, Sintonia, Sossego and Alpaca) and different technological qualities according to the gluten rheological properties (Supplementary table 1). All samples were cultivated in normal agronomical conditions at the same location in Coxilha (RS, Brazil) and were ground with the experimental grinder "Moinho Experimental VG 2000i" (Vitti Molinos, Santa Catarina, Brazil) at the classical flour extraction rate (~50 to 55%). The flours were kept at -20 °C for three months before use.

2.3. Moisture, ash, protein and starch contents and colorimetric parameters

The moisture, ash and protein content of wheat flours were determined according to the AACCI methods (44-15.02 and 08.01.01, respectively) (AACCI) through a micro-Kjeldahl with a conversion factor of 5.7 (AOAC, 1984). The total starch content was determined using the Megazyme assay (K-TSTA 07/11) (AOAC Method 996.11, AACCI Method 76-13). The colorimetric parameters were determined in triplicate by reflectance colorimeter (CM-5, Konica Minolta, Japan) using the CIELAB color model, hue angle and chroma (C^*) as parameters.

2.4.Extraction of free and bound phenolic compounds

PC from wheat flour was extracted in triplicate (Santos et al., 2019). An amount of 70 mg of sample and 50 mg of celite were weighed, manually macerated and then extracted with 80% ethanol. Samples were stirred (200 rpm, 10 min, 25 °C) and centrifuged (5000 x*g*, 10

min, 25 °C). The extraction was repeated twice, and the collected supernatants were combined. The extracts were dried in an evaporator centrifuge (Speed Vac Concentrator, Thermo Scientific, USA). The pellets were resuspended with NaOH (4M) and submerged in an ultrasonic bath (42 kHz, 90 min, 40 °C). After the alkaline hydrolysis, acid hydrolysis was performed with concentrated HCl (~ pH 2), and the samples were centrifuged (2000 x*g*, 5 min). The supernatant was washed three times with ethyl acetate (7 mL) and centrifuged between each step (10000 x*g*, 5 min, 10 °C). The combined extracts were dried in a rotary evaporator (200 rpm, 40 °C) (Laborota 4000 Heidolph) coupled to a chiller. All dried extracts were resuspended in 1.5 mL of 2% methanol, 5% acetonitrile and 93% ultrapure water and then filtered (13 mm, 0.22 µm), transferred to vials, and stored at -20 °C until analysis.

2.5. Folin–Ciocalteu reducing capacity

Total PC in the obtained extracts were estimated by measuring their capacity to reduce Folin-Ciocalteu reagent, it was estimated in triplicate in free and bound extracts of wheat flours based on the original method (Singleton et al., 1999) adapted to microplates. Absorbance was determined at 750 nm on a microplate reader (FlexStation III, Molecular Devices). The standard curve was carried out with gallic acid (5-130 mg/L) in the reaction mixture. Results were expressed as mg gallic acid equivalents per 100 g of sample (dry basis).

2.6. Determination of the phenolic compounds profile by UPLC-MS^E

For each analysis, a mixed solution of standard compounds (10 ppm) or extracts (2 µL) was injected in triplicate into the system UPLC Acquity (Waters, Milford, MA) coupled to the Xevo G2-S Q-TOF (Waters, Manchester, UK), which was equipped with an electrospray ionization source according to Santos et al. (2019). The separation was carried out using a UPLC HSS T3 C18 column (100 x 2.1 mm, 1.8 µm particle diameter) (Liu et al.) at 30 °C, with a flow of 0.6 mL/min of ultra-pure water and 5 mM ammonium formate (mobile phase A) and acetonitrile (mobile phase B), both containing 0.3% formic acid. The gradient method was applied as follows: 0 min - 97% A; 6.78 min - 50% A; 7.36 min - 15% A; 8.51 min - 15% A; and 9.09 min - 97% A. The capillary and cone voltage were set at 2.0 kV and 30 V, respectively. The desolvation gas (N_2) was set at 800 L/h and 500 °C, and the cone gas was set at 50 L/h and the source at 120 °C. Data was acquired through the centroid mode using a multiplexed MS/MS acquisition with alternating low and high-energy acquisition (MS^E), from *m/z* 50 to 1000, operating in negative ion mode ESI (-). MS/MS experiments were performed with a collision energy ramp (30 to 55 eV) and ultrapure argon

(Ar) as collision gas. All acquisitions were performed using leucine encephalin (Leu-Enk) for calibration and MassLynx 4.1 (Liu et al.) software for the data.

2.7. UPLC-MS^E data processing

The putative identifications followed the levels of identification according to Sumner et al. (2007).The software Progenesis QI was used under the following conditions to analyze the data set: all runs, automatic limits, centroid data, and resolution of 30000, and negative ion mode. For the identification, neutral mass isotope distribution, retention time, and MS/MS fragments from standards were used by applying MetaScope based on the comparison with polyphenols database from PubChem, Kegg and the online database Phenol Explorer as per Santos et al. (2019). The non-targeted identifications followed these parameters, in descending order of importance: comparison between the experimental and theoretical *m/z*; isotopic similarity (> 80); exact mass error (< 10 ppm); score > 30; highest fragmentation score; and all parameters generated by the software used. In addition, other factors such as comparison sample characteristics, retention time, literature data and chemical characteristics of the molecule were used as criteria for tentative identification of multiple or unknown compounds.

2.8.Statistical analysis

All analyses were performed in technical triplicate, and the results are reported as mean values ± standard deviation (SD). The XLSTAT software (Addinsoft, France) was used to perform the statistical analysis among samples (Tukey's test, $p<0.05$, and one-way ANOVA) and the heatmaps. The omics data was exported to the EZInfo software (Waters, USA) for multivariate analysis to Principal Components Analysis (PCA) and S-plot. The graphic representation of the results was created using the GraphPad Prism (5.0) software.

3. Results and discussion

3.1. Physico-chemical characterization of wheat flours

Ash content, usually, indicates the contamination of flour with bran particles during milling and thus provides an estimation of the degree of separation of bran and germ from endosperm during milling. The presence of bran darkens the color of the products (Katyal et al., 2016). The ash content averaged 0.77% and ranged from 0.54 to 1.24% across the 14 flours, in accordance with Brazilian legislative guideline for refined wheat flour (maximum value of ash: 1.4%) (BRASIL, 2005).

Although studies do not relate the ash content to the technological quality, but external factors such as soil and, weather conditions. Concerning the technological quality effect, this study showed a significant difference in the ash content (p<0.05) between the medium/superior flours and the low flour. The ash content ranged from 0.54 to 0.95% in low, 0.59 to 0.95% in medium, and 0.58 to 1.24% in superior wheat flours (Table 1). The ash content of low wheat flours (average 0.69%) was similar to the findings of the previous literature (0.66 ± 0.03) for refined wheat flour (Dhiraj & Prabhasankar, 2013). Conversely, medium and superior flours had an average ash content that was 12% and 20% higher than expected, respectively. Refined wheat flours with high ash content are associated with non-endosperm (e.g., bran) contamination during refining. However, all flour varieties were submitted to the same extraction rate (white wheat flour: 50%). In addition, contamination would also have impacted the protein and starch analyses. Nevertheless, they did not show a good correlation, and the starch content did not change between the three technological qualities. Therefore, this difference can be explained by the following two hypotheses: a) particular characteristics of the genotype, wheat class, and cultivar determine mineral/ash content variability (Czaja et al., 2020); and b) there is a correlation between flour technological quality and ash content, corroborating the findings of Yousaf et al. (2019).

In general, L*, a*, and b* coordinates of flours ranged from 85 to 91, 0.3 to 1.1, and 7.3 to 11.3, respectively (Table 1). The same reported trends for ash content were obtained for colorimetric results. A significant impact of technological quality on colorimetric parameters ($p< 0.05$) was also found. According to parameters, the superior flour is darker (L*) and redder (a*) compared to the others, while the b* axis is similar to medium flour, indicating that both have the same yellow color. In contrast, low flours are lighter, less red, and less yellow. As expected, a positive correlation between ash content with a* and b* ($r = 0.90$, $p = 3.5\text{-}6$ and $r = 0.84$, $p = 0.03$) was observed. At the same time, the brightness (L*) and hue angle were negatively correlated with ash content ($r=0.90$, $p= 2.37\text{-}4$ and $r=0.92$, $p= 2.47\text{-}4$, respectively). Previous studies have also shown a similar correlation between ash and CIELAB parameters (Katyal et al., 2016).

The increase of ash content in flour is attractive from a nutritional point of view; however, it negatively impacts the flour's technological characteristics (Carson & Edwards, 2009; Hemery et al., 2011). As observed in the colorimetric analyses, the high-ash flour was characterized by a darker color, resulting in the possible rejection of the final product (Bucsella et al., 2016). Furthermore, the ash content was also associated with greater activity

of proteolytic and amylolytic enzymes; in other words, dietary fiber and non-gluten proteins disintegrated, weakening the protein matrix during dough formation (Bucsella et al., 2016; Carson & Edwards, 2009). According to commercial classification (BRASIL, 2010), the superior quality is equivalent to improved wheat mixed with (low quality) basic flours for bakery products (e.g., bread, biscuits, and cakes). With this blend, the high ash content and colorimetric parameters of superior quality flours would not impact the final product.

Table 1 - Contents of ash, starch, protein, colorimetric parameters, and phenolic contents in the different wheat flours.

Genotype	Ash (% db)	Protein (% db)	Starch (% db)	Colorimetric analysis					Folin-Ciocalteu (mg GAE/100 g db)				
				L*	a*	b*	C*	h_{ab}	Free (F)	Ratio (F/T)	Bound (B)	Ratio (B/T)	Total (T)
Alpaca	0.54±0.06^{e}	9.30±1.33^{f}	62.99±0.71^{e}	91.17±0.08^{a}	0.31±0.01^{j}	7.64±0.02^{h}	7.65±0.02^{bcd}	1.53±0.04^{a}	28.43±0.44^{g}	0.60	18.92±0.47^{i}	0.40	47.35±0.91^{h}
Ametista	0.79±0.11^{bcd}	14.89±0.02^{a}	69.24±1.80^{bcde}	85.48±0.02^{i}	1.02±0.00^{d}	9.99±0.02^{e}	10.04±0.02^{f}	1.47±0.01^{i}	30.93±0.30^{ef}	0.51	29.77±0.30^{de}	0.49	60.70±0.60^{e}
Campeiro	0.71±0.04^{cde}	10.93±0.01^{def}	70.45±0.73^{abcde}	89.95±0.09^{d}	0.55±0.01^{h}	7.78±0.08^{h}	7.80±0.08^{d}	1.50±0.02^{c}	31.83±0.72^{e}	0.44	40.47±1.47^{a}	0.56	72.29±2.19^{b}
Iguaçu	0.80±0.05^{bcd}	8.97±0.72^{f}	65.09±0.94^{cde}	86.93±0.01^{g}	0.88 ±0.01^{e}	11.28±0.03^{a}	11.31±0.03^{e}	1.49±0.04^{e}	29.60±0.15^{fg}	0.44	37.21±0.73^{b}	0.56	66.81±0.88^{cd}
Jadeite	0.78±0.03^{bcd}	13.65±0.51^{ab}	72.36±1.92^{ab}	85.80±0.03^{h}	1.05±0.00^{c}	10.19±0.02^{d}	10.24±0.02^{cd}	1.47±0.01^{i}	30.91±0.44^{ef}	0.53	27.03±1.65^{fg}	0.47	57.94±2.09^{f}
Marfim	0.66±0.02^{de}	15.60±0.19^{ab}	67.18±1.98^{bcde}	90.24±0.05^{c}	0.53±0.00^{h}	7.30±0.05^{i}	7.32±0.05^{g}	1.50±0.03^{d}	25.31±0.14^{h}	0.52	22.99±0.77^{h}	0.48	48.31±0.91^{h}
Noble	0.58±0.04^{e}	14.00±0.21^{ab}	67.85±0.90^{bcde}	89.92±0.17^{d}	0.59±0.02^{g}	8.09±0.16^{g}	8.12±0.16^{ab}	1.50±0.07^{d}	52.36±0.06^{a}	0.68	24.68±0.31^{gh}	0.32	77.04±0.91^{a}
ORS1401	0.59±0.09^{e}	13.50±0.00^{abc}	72.57±1.85^{abc}	87.32±0.02^{f}	0.76±0.00^{f}	10.59±0.04^{c}	10.61±0.04^{bcd}	1.50±0.01^{cd}	44.74±0.74^{b}	0.65	23.82±0.00^{h}	0.35	68.56±0.74^{g}
ORS1402	0.85±0.03^{bc}	13.65±0.21^{cde}	70.61±2.96^{abc}	87.60±0.02^{e}	0.89±0.01^{e}	9.42±0.02^{f}	9.46±0.02^{de}	1.48±0.03^{g}	30.04±0.33^{f}	0.51	28.64±0.90^{ef}	0.49	58.68±1.23^{b}
ORSVintecinco	0.95±0.02^{b}	13.62±0.50^{ab}	71.61±3.25^{abc}	89.94±0.05^{d}	0.60±0.00^{g}	7.42±0.01^{i}	7.45±0.01^{h}	1.49±0.00^{f}	24.39±0.42^{h}	0.52	22.75±0.44^{h}	0.48	47.14±0.86^{h}
ORSVintesete	0.56±0.02^{e}	13.52±0.38^{abc}	76.94±7.28^{a}	90.71±0.07^{b}	0.41±0.01^{i}	7.46±0.05^{i}	7.47±0.05^{i}	1.52±0.03^{b}	31.94±0.76^{e}	0.59	22.15±0.39^{cd}	0.41	54.09±1.15^{de}
Sintonia	1.24±0.10^{bcd}	10.90±0.39 ^{def}	67.73±1.04^{bcde}	87.63±0.05^{e}	0.87 ±0.00^{e}	11.08±0.03^{b}	11.11±0.03^{cd}	1.49±0.01^{e}	32.27 ±0.39^{e}	0.58	23.76±0.98^{h}	0.42	56.04±1.37^{fg}
Sossego	0.81±0.02^{b}	10.57±0.69^{ef}	64.24±0.40^{de}	85.25±0.06^{j}	1.08±0.00^{b}	11.17±0.07^{ab}	11.22±0.07^{a}	1.47±0.04^{h}	36.55±0.00^{c}	0.54	31.32±0.73^{d}	0.46	67.87±0.73^{c}
Topázio	0.95±0.02^{a}	12.79±0.34^{bcd}	70.71±0.76^{abcd}	85.14±0.02^{j}	1.12±0.01^{a}	10.07±0.03^{de}	10.13±0.03^{abc}	1.46±0.02^{j}	34.67±0.08^{d}	0.50	34.24±0.84^{c}	0.50	68.91±0.92^{c}
Low*	0.69±0.03^{B}	11.84±0.22^{B}	70.38±1.60^{A}	90.44±0.07^{A}	0.47±0.00^{C}	7.58±0.03^{B}	9.57±0.06^{B}	1.51±0.00^{A}	29.15±0.21^{C}	0.50	28.93±0.30^{B}	0.50	58.08±0.50^{C}
Medium*	0.77±0.02^{A}	12.19±0.10^{B}	68.72±1.50^{A}	87.61±0.02^{B}	0.83±0.00^{B}	9.91±0.03^{A}	10.92±0.08^{A}	1.49±0.00^{B}	33.78±0.09^{B}	0.56	26.16±0.43^{C}	0.44	59.89±0.38^{B}
Superior*	0.84±0.04^{A}	12.85±0.17^{A}	69.12±0.36^{A}	86.65±0.04^{C}	0.93±0.00^{A}	9.92±0.05^{A}	11.04±0.05^{A}	1.48±0.00^{C}	35.69±0.14^{A}	0.54	30.82±0.54^{A}	0.46	66.51±0.59^{A}

C*: croma, h_{ab}: hue angle. a-j subscribed letters indicated a significant difference (p<0.05) between the genotypes in the same columns and A-C indicates a significant difference (p<0.05) between the technological qualities. Low represents Alpaca, Campeiro, ORS25 and ORS27 varieties; Medium represents Marfim, ORS1401, ORS1402, Sintonia and Sossego varieties; and Superior represents Ametista, Jadeite, Iguaçu, Noble and Topázio varieties. Data are means ± SD (n = 3).

3.2. Total phenolic content in different cultivars of wheat flour

Although the Folin-Ciocalteu method may present interferences due to the reaction of other reducing molecules present in the extracts (Górnaś et al., 2016; Huang et al., 2005), in this work, the total phenolic content was estimated by measuring the reducing capacity of the obtained extracts, enriched in phenolic compounds, considering free and bound phenolics. Table 1 shows the TPC of the different wheat flours. The TPC averaged 62 mg GAE/100 g and ranged between 47 and 77 mg GAE/100 g for the 14 wheat flours. As expected, these values were lower than those previously found for the same cultivars in mature wheat whole grains (Santos et al., 2019). However, the TPC of the present study was greater than the values found for some wheat grains (53.1 mg GAE/100 g) and different winter wheat flours (11.3 - 37.1 mg GAE/100 g) (Alvarez-Jubete et al., 2010; Yu et al., 2004). The technological quality effect was again evidenced; the TPC of medium wheat flours was significantly higher (3%) than low flours, and superior wheat flours were significantly higher than low and medium flours (15% and 11%, respectively), corroborating the results found for ash content and colorimetric parameters. More generally in our data, TPC of flours showed a positive but not significant correlation with ash content ($r = 0.85$, $p = 0.43$), and with redness (a*) ($r = 0.81$, $p = 0.03$), while the brightness (L*) was inversely correlated to TPC ($r = 0.82$, $p = 2.92^{-7}$).

An analysis of each extract (Table 1) showed that free extract generally had 16% more phenolic content than bound extract ($p < 0.05$). Although, the majority of previous studies have reported that PC are mostly found in bound forms (60-75%) in wheat grains (*Triticum spp.)*, Liyana-Pathirana and Shahidi (2006) have shown that the presence of bran influences. These authors reported that the highest percentage of bound PC was found in bran (84%), while 59% was found in whole grain and only 49% in wheat white flour, corroborating our data. The superior sample had 22% and 6% more free PC than low and medium flours, respectively. Similarly, free PC were higher in superior flours, and medium showed the lowest value (7% and 18% less than low and superior samples, respectively).

The mean ratio of free-to-bound (F/B) PC in low flours was 1.0 to 1.3 in medium and 1.2 in superior flours, showing different profiles between the technological qualities. Although the bakery effect was not evaluated in our study, the literature has shown that this processing is able to change the F/B ratio. Bread, cookie and muffin production releases bound PC, increasing the free PC content and boosting its bioavailability (Abdel-Aal & Rabalski, 2013).

However, it is important to note that this effect appeared dependent on the baking recipe, heating conditions and PC class.

3.3. Identification of phenolic compounds by UPLC-MS^E

3.3.1. Comparison of different Brazilian genotypes

This work tentatively identified a total of 43 PC including isomers. Some compounds were fully confirmed by reference standards, such as: caffeic acid, ferulic acid, *p*-coumaric and sinapic acid, present in both extracts (free and bound). Overall, five classes of PC were found in this study: flavonoids (32%), phenolic acids (30%), other polyphenols (26%), stilbenes (7%) and lignans (5%).

Table 2 presents the complete table with all information about the putative compounds, including the experimental exact mass [M-H$^-$], the retention time, the isotopic similarity, the mass error of precursors, the fragmentation score and the fragments ions (MS/MS) generated. The MS/MS data that resulted from the specific fragmentation of precursor ions from the breaking of structural bonds in the collision cell is an important parameter for confirmation the identified PC in a non-targeted approach (Ncube et al., 2014).

Table 2. Putative identification of phenolic compounds in Brazilian refined wheat flour by UPLC-MS^E.

	Compound	Molecular formula	*m/z* [M-H$^-$]	RT (min)	Score (%)	FS (%)	Fragments	Mass Error (ppm)	IS (%)
	PHENOLIC ACIDS								
1	3.4-dihydroxy-5-methoxybenzoic acid	$C_8H_8O_5$	183.0281	0.94	36.6	ND	-	-6.88	90.65
2	Gallic acid ethyl ester	$C_9H_{10}O_5$	197.0442	1.31	38.4	13.2	101.0244 (61.76%); 125.0244 (36.51%); 143.0349 (1.47%)	-6.94	86.54
3	***Caffeic acid****	$C_9H_8O_4$	179.0332	2.67	56.4	96.3	135.0435 (100%)	-9.08	95.48
4	Diferulic acid I	$C_{20}H_{18}O_8$	385.0913	3.59	49.5	59.9	341.1030 (100%)	-3.60	91.87
5	Diferulic acid II	$C_{20}H_{18}O_8$	385.0913	3.75	36.9	ND	-	-3.50	89.10
6	Diferulic acid III*	$C_{20}H_{18}O_8$	385.0912	3.83	37.7	ND	-	-4.40	93.58
7	Diferulic acid IV*	$C_{20}H_{18}O_8$	385.0913	4.47	37.6	ND	-	-4.12	92.84
8	Diferulic acid V	$C_{20}H_{18}O_8$	385.0911	4.79	36.6	ND	-	-4.69	88.24
9	Ellagic acid	$C_{14}H_6O_8$	300.9971	3.50	51.8	67.7	299.9981 (31.31%)	-6.12	98.16
10	***Trans-ferulic acid****	**$C_{10}H_{10}O_4$**	**193.0491**	**3.53**	**53.4**	**77.6**	**134.0373 (100%); 178.0257 (11.52%); 133.0295 (6.47%); 149.0609 (3.14%);**	**-8.15**	**98.70**
11	Ferulic acid*	$C_{10}H_{10}O_4$	193.0488	3.69	55	87.4	134.0373 (100%); 178.0255 (4.29%); 149.0608 (1.64%)	-9.33	97.62
12	***p*-Coumaric acid I***	**$C_9H_8O_3$**	**163.0384**	**3.29**	**53.2**	**82.7**	**119.0490 (100%)**	**-9.95**	**94.00**
13	**Sinapic acid**	**$C_{11}H_{12}O_5$**	**223.0594**	**3.53**	**35.7**	**ND**	**-**	**-8.12**	**87.65**
	FLAVONOIDS								
14	3.7-Dimethylquercetin	$C_{17}H_{14}O_7$	329.0650	5.71	36	ND	-	-5.22	86.23
15	Apigenin 7-*O*-apiosyl-glucoside I*	$C_{26}H_{28}O_{14}$	563.1400	2.87	56.8	92.1	353.0667 (100%); 383.0772 (66.69%)	-1.17	93.48
16	Apigenin 7-*O*-apiosyl-glucoside II*	$C_{26}H_{28}O_{14}$	563.1399	3.01	56.1	87.2	353.0653 (19.03%); 383.0760 (14.10%)	-1.29	94.64
17	Auriculoside I	$C_{22}H_{26}O_{10}$	449.1426	1.15	36.2	0.11	71.0138 (9.76%)	-6.03	88.01
18	Auriculoside II	$C_{22}H_{26}O_{10}$	449.1492	6.31	35.5	ND	-	8.60	86.83
19	Carlinoside	$C_{26}H_{28}O_{15}$	579.1354	2.74	37	ND	-	-1.29	86.80
20	Daidzein	$C_{15}H_{10}O_4$	253.0495	4.73	35.8	ND	-	-4.41	84.19
21	Eupatorin I	$C_{18}H_{16}O_7$	343.0808	4.76	35.7	ND	-	-4.54	83.63
22	Eupatorin II	$C_{18}H_{16}O_7$	343.0817	5.31	35.7	ND	-	-1.81	80.88

23	Koparin	$C_{16}H_{12}O_6$	299.0551	5.76	35.8	ND	-	-3.39	82.87
24	Okanin	$C_{15}H_{12}O_6$	287.0561	2.03	40	16.6	101.0244 (19.31%); 113.2044 (9.29%); 125.0231 (3.51%); 99.0437 (5.11%); 261.0404 (1.99%)	-.019	83.77
25	Psoralidin	$C_{20}H_{16}O_5$	335.0900	1.01	35.8	ND	-	-7.36	87.21
26	Tectoridin	$C_{22}H_{22}O_{11}$	461.1059	3.21	35.5	ND	-	-6.62	85.17
27	Tetramethylscutellarein	$C_{19}H_{18}O_6$	341.1017	4.90	35.9	ND	-	-3.86	83.77
	OTHER POLYPHENOLS								
28	1-*O*-Sinapoyl-beta-D-glucose	$C_{17}H_{22}O_{10}$	385.1125	3.15	56.3	95.8	113.0244 (100%)	-3.91	90.39
29	1'-Acetoxychavicol acetate	$C_{13}H_{14}O_4$	233.0805	5.97	35.8	ND	-	-6.13	85.98
30	Acetyleugenol	$C_{12}H_{14}O_3$	205.0855	5.61	37.9	ND	-	-72.9	97.70
31	Catechol	$C_6H_6O_2$	109.0287	0.77	37	ND	-	-7.14	93.26
32	*Elemicin*	$C_{12}H_{16}O_3$	207.1011	5.55	35.7	ND	-	-7.67	87.10
33	*Esculetin I*	$C_9H_6O_4$	177.0177	2.62	42	25.9	133.0295 (19.45%)	-9.22	94.01
34	Esculetin II	$C_9H_6O_4$	177.0176	3.89	35.9	ND	-	-9.66	89.91
35	Leptodactylone	$C_{11}H_{10}O_5$	221.0435	4.32	36.7	ND	-	-9.05	93.46
36	*Sinapoyl alcohol**	$C_{11}H_{14}O_4$	209.0802	5.82	36.1	ND	-	-8.09	89.46
37	Syringaldehyde	$C_9H_{10}O_4$	181.0489	4.25	35.8	ND	-	-9.71	89.87
38	Vanillactic acid	$C_{10}H_{12}O_5$	211.0595	1.91	53.3	82.7	136.0166 (100%); 151.0400 (98.56%)	-8.14	92.66
	LIGNANS								
39	4'-Demethyldeoxypodophyllotoxin	$C_{21}H_{20}O_7$	383.1130	0.92	36.5	ND	-	-1.54	84.15
40	Flaxseed	$C_{26}H_{38}O_{12}$	541.2290	2.19	38.7	ND	-	0.07	93.41
	STILBENES								
41	Astringin	$C_{20}H_{22}O_9$	405.1152	0.95	38.6	13.2	180.0639 (20.27%)	-9.69	90.20
42	Pterostilbene	$C_{16}H_{16}O_3$	255.1044	6.17	36.1	ND	-	6.76	88.00
43	Resveratrol 3-*O*-glucoside	$C_{20}H_{22}O_8$	389.1220	3.48	35.8	ND	-	-5.72	85.51

m/z: mass/charge; RT: retention time; FS: fragmentation score; IS: isotope similarity. Bold: reference standards; italic: compounds identified in both extracts; *Phenolic compounds found in all samples studied.

Fifteen PC were identified in all 14 wheat flours, showing that despite genetic variability and the influence of external factors on phenotype, wheat contains a core content of PC. Some of these identified compounds, such as *p*-coumaric acid, caffeic acid and ferulic acid, stand out since they are present in the main synthetic pathways of other PC (phenylpropanoid biosynthesis map available at http://www.genome.jp/dbget-bin/www_bget?map00940). In our previous study, the phenolic profile was followed along with the development of wheat grains in seven genotypes of wheat (Campeiro, ORS Vintecinco, ORS1401, ORS1402, Marfim, Jadeite 11, Ametista); furthermore, a total of 100 PC were identified even in mature grains (used for flour production), including isomeric forms (Santos et al., 2019). In the current work, it was possible to compare the phenolic profile of the same wheat mature grains with the respective flours in these seven genotypes, that showed the presence of 26 PC. It means that a high number of PC remains present even after the grain milling process.

A high number of PC in refined wheat flours, including a high amount of ferulic acid and its isoforms, is of great importance and further reinforces the positive aspects attributed to wheat consumption, even if it is refined flour (Wieser et al., 2020). Indeed, these PC present in flours are likely to resist the baking process (hydration, fermentation, heat treatment) and can be found in the final products. The impact of the baking process on PC will differ depending on whether they are bound or free. High temperatures applied in the baking process can decrease the concentration of bound PC, probably due to broken bonds, but do not eliminate the PC. On the contrary, heat treatments can positively affect some matrices, increasing free PC and as a result, PC bioavailability (Lu et al., 2017). Lu et al. (2014) showed similar results, reporting that PC (initially higher in wholemeal flour than refined flour) remained present in the final product after the fermentation and baking process. The genotypes Ametista, Iguaçu, ORS1402, Sossego and Topázio showed the highest number of total PC (Figure 1A).

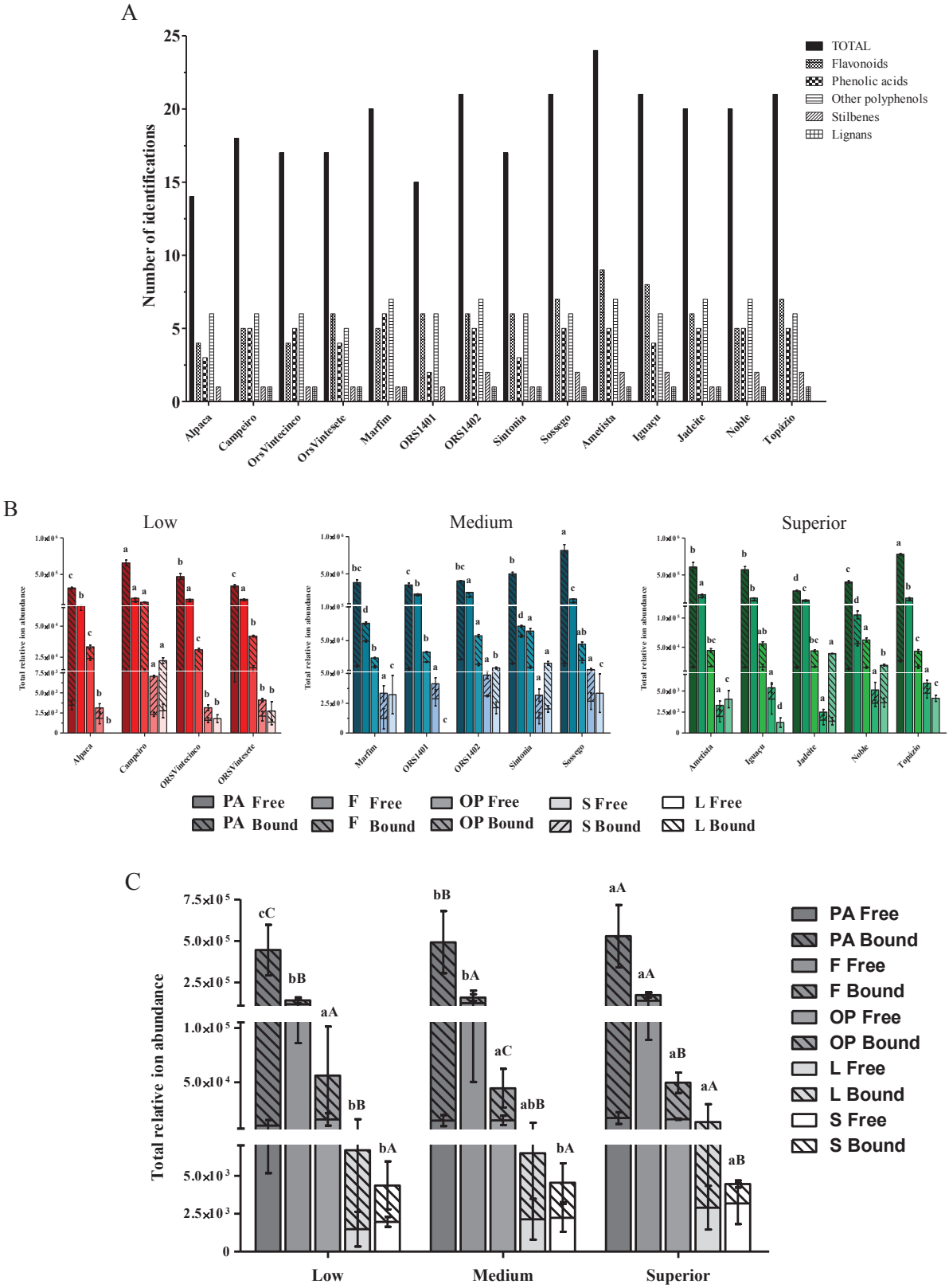


Figure 1. A. Number of identifications of phenolic classes for each wheat flour. B. Total relative ion abundance of phenolic classes for each wheat flour grouped by technological class (Low, Medium and Superior). C. Average of total relative ion abundance of phenolic classes for wheat flours by technological quality. OP: Other polyphenols; PA: phenolic acid; F: flavonoids; S stilbenes and L: lignans. Means ± SD (n=3). Different letters mean a significant difference (p<0.05) between free (lowercase) and bound (uppercase) extract samples, respectively, and within the same group (Fig. B) and comparing different technological classes (Fig. C).

Corroborating previous studies (Dinelli et al., 2009; Wang et al., 2013), the number of free PC (28) identified in this study was lower than the number of bound PC (33). This lower diversity of free PC was true for some classes of secondary metabolites identified in this study: phenolic acid, other polyphenols, lignans and stilbenes. Moreover, some compounds such as stilbenes, some lignans and flavonoids and some dimers of ferulic acid were identified only in bound extracts, reaffirming the importance of performing the hydrolysis step for the identification of compounds.

As expected, the isomers of ferulic acid were the most abundant PC in free and bound extracts. The ferulic acid is known to be the most abundant phenolic acid in cereals, especially in wheat, and is known for its potential health benefits (Luthria et al. 2015). The isomers of ferulic acid were responsible to 25-50% of ion abundance of phenolic compounds depending on genotype (Figure 2). The next most abundant compounds in free extracts included apigenin 7-O-apiosyl-glucoside (flavonoid), *p*-coumaric (phenolic acid) and diferulic acid (phenolic acid).

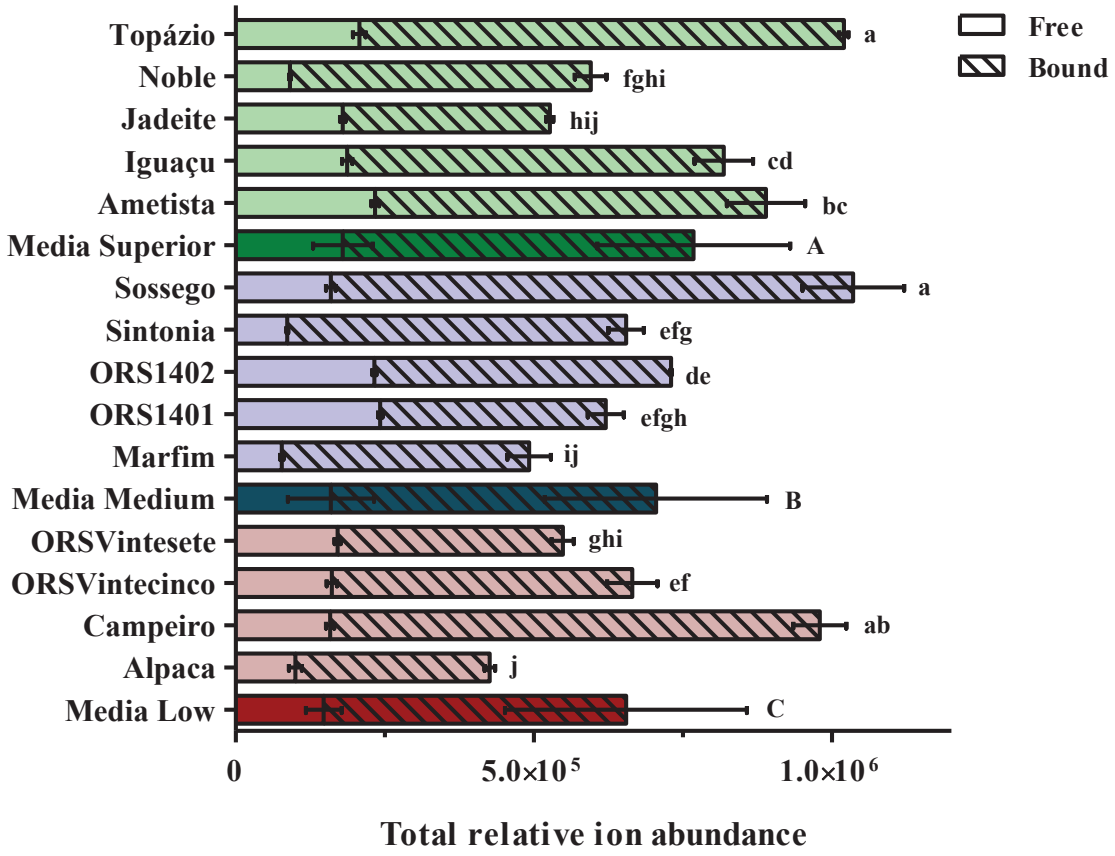


Figure 2. Total relative ion abundance of phenolic compounds in each genotype (free + bound). Means ± SD (n=3). Different lowercase letters mean a significant difference between samples. Uppercase letters mean a significant difference between technological qualities means (p<0.05).

In bound extracts, the most abundant compounds belong to the phenolic acids class and the most abundant ferulic acid isomers were followed by ellagic acid, *p*-coumaric and one isomer of diferulic acid. The presence of the carboxylic acid grouping allows these compounds to perform ester-like reactions. Besides this, five isoforms of diferulic acid were detected; among these, four isoforms were detected exclusively in bound extracts. Only the genotype Alpaca did these isoforms. The other genotypes presented all dimers in bound extract and at least one of the dimers in free extracts. These compounds have already been found in immature wheat grains (Santos et al. 2019) and present potential antioxidant activity able to inhibit the lipid peroxidation (Garcia-Conesa et al., 1999). The genotypes Campeiro, Sossego and Topázio showed the highest total relative abundance of PC (Figure 2). In this study the main classes were the flavonoids and phenolic acids corroborating the literature (Figure 1B) (Wang et al., 2013; Zhang et al., 2012).

The average of total ion abundance for each compound by genotype is displayed in the supplementary table 2. The difference between the two extracts can be evidenced by applying multivariate data, such as the principal components analysis (PCA), which allows for elucidating differences between the complex samples. In Figure 3, it is possible to observe the difference in the phenolic profiles found in the free extract and the bound extract.

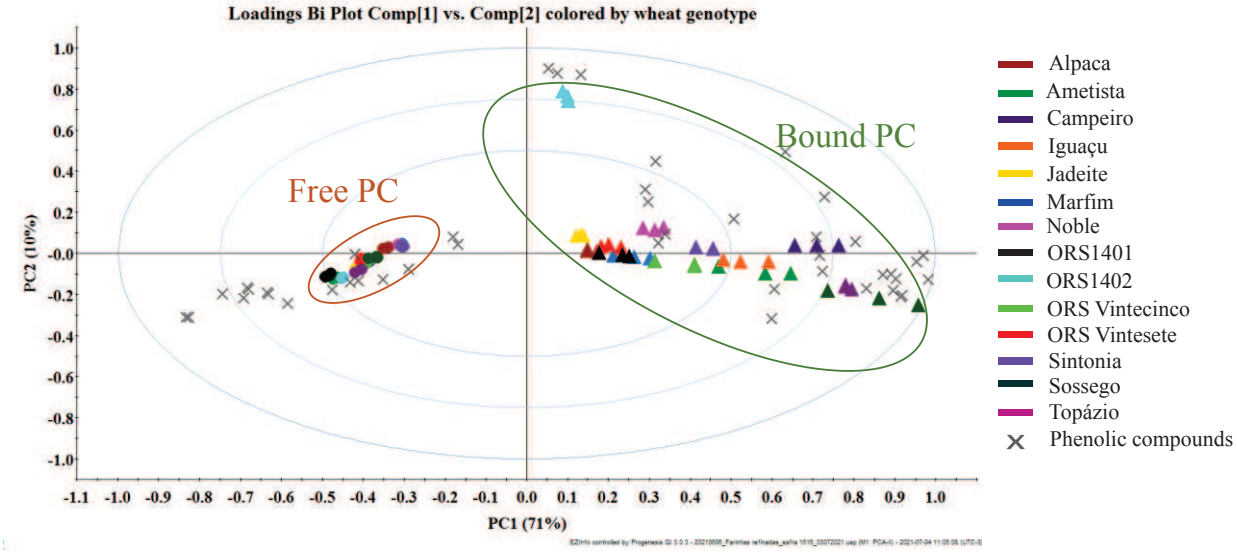


Figure 3 – Principal Components Analysis of all putative identified phenolic compounds in wheat flours of different genotypes. Dot: free phenolic compounds; Triangle: bound phenolic compounds; x means identified phenolic compounds.

Looking at each genotype's total relative ion abundance (figure 2), the abundance of free PC remained more constant than for bound PC. Previous works have suggested that bound PC are genotypically predetermined, while free PC are more likely influenced by external factors (Silvestro et al., 2016). However, our results have shown that genotypes with the same technological quality demonstrate variations that may be linked with phenotype conditions or individual genotype factors with highly variable bound PC. Thus, it is possible to conclude that external factors influence the relative abundance of PC in wheat flours but have limited influence on the global profile of PC which is mainly determined by the genotype.

3.3.2. Correlation between the differences in the phenolic profile and the technological quality of refined wheat flour

Concerning the technological quality effect (low *vs* medium *vs* superior) and the number of putative PC, superior wheat flours showed higher PC numbers. The number of identifications of phenolic acids and flavonoids were preponderant, as well as the total relative ion abundance. A significant impact of technological quality was observed in total relative abundance of PC ($p<0.05$) (Figure 2), where the wheat flour classified as superior presented the most abundance of PC followed by medium and low qualities, corroborating the Folin-Ciocalteu results. Contrary to this result, Indian varieties classified as poor showed higher levels of PC and higher expression of enzymes related to their synthesis pathways when compared to "good" wheat flour (Sharma et al., 2016).

Apart from proteomics studies of metabolic non-prolamin proteins (Victorio et al., 2018) and gluten proteins (Victorio et al., 2021) recently applied to Brazilian wheat flours, there are no studies that have investigated the phenolic profile. To the best of our knowledge, this is the first study to correlate the phenolic profile with the technological quality of Brazilian wheat flours. A multivariate analysis was applied to determine the possible differences between the technological qualities studied (low *vs*. medium *vs*. superior).

The covariance p[1] and correlation p [1] loadings from a two-class orthogonal partial least squares discriminant analysis (OPLS-DA) model are displayed in an S-plot format (Figure 4). Considering the total relative abundance and all compounds putatively identified in the free and bound extract, the upper right quadrant of the S-plot shows the elevation of PC, in a specific technological quality, while the lower-left quadrant presents a comparison of the elevation of PC according to technological quality: low versus medium (Figure 4A),

medium versus superior (Figure 4B) and low versus superior (Figure 4C). The further away from the *x*-axis the PC is, the greater the contribution to the variation between the technological qualities, while the further away from the *y*-axis, the greater the reliability of the analytical result – thus, the significance.

Each S-plot evaluated the discriminant PC by variable importance in projection (VIP). In Figure 4A, the abundance of a glycosylated flavonoids isomer (auriculoside isomer II) and ellagic acid differs from medium to low. Ferulic acid, esculetin I, and vanillactic acid were the responsible PC in comparisons of low to medium. Figure 4B shows the most discriminant PC when comparing medium to superior in the upper right quadrant (superior). While ellagic acid was the most discriminant in medium wheat flours, four PC are highlighted in superior: ferulic acid, diferulic III (phenolic acids), psoralidin (flavonoid) and the lignan flaxseed. Figure 4C shows the difference between low and superior; the most discriminant PC selected by VIP are present in superior wheat flours: *trans*-ferulic acid, *p*-coumaric acid, diferulic acid IV and auriculoside I, from phenolic acids and flavonoids classes.

The putative PC were illustrated in two heatmaps for a visual representation of abundance to compare the differences between technological qualities for each extract (Figure 5). The clusters were built from the quantitative correlations between compounds (on the left) and between wheat flours (at the top). The color gradient represents the variation in the abundance of these compounds, ranging from blue to yellow, where the darkest blue color represents the least abundant compounds and the darkest yellow the most abundant.

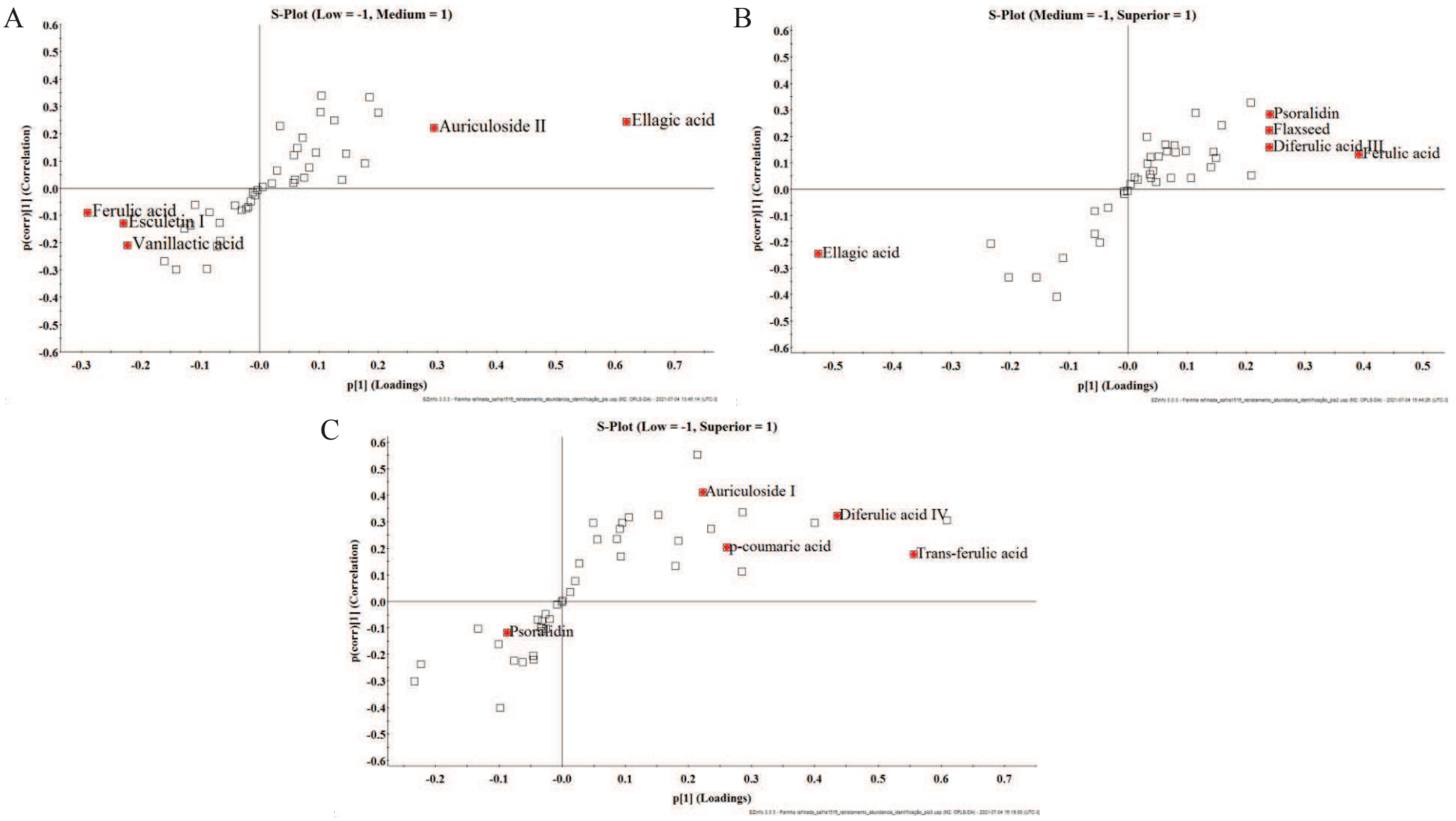


Figure 4 – S-plots comparing the wheat flours by pairs between technological quality. A) Low *vs* Medium; B) Medium *vs* Superior and C) Low *vs* Superior. Marked in red: the five discriminants phenolic compounds - VIP (Variance Important Projection).

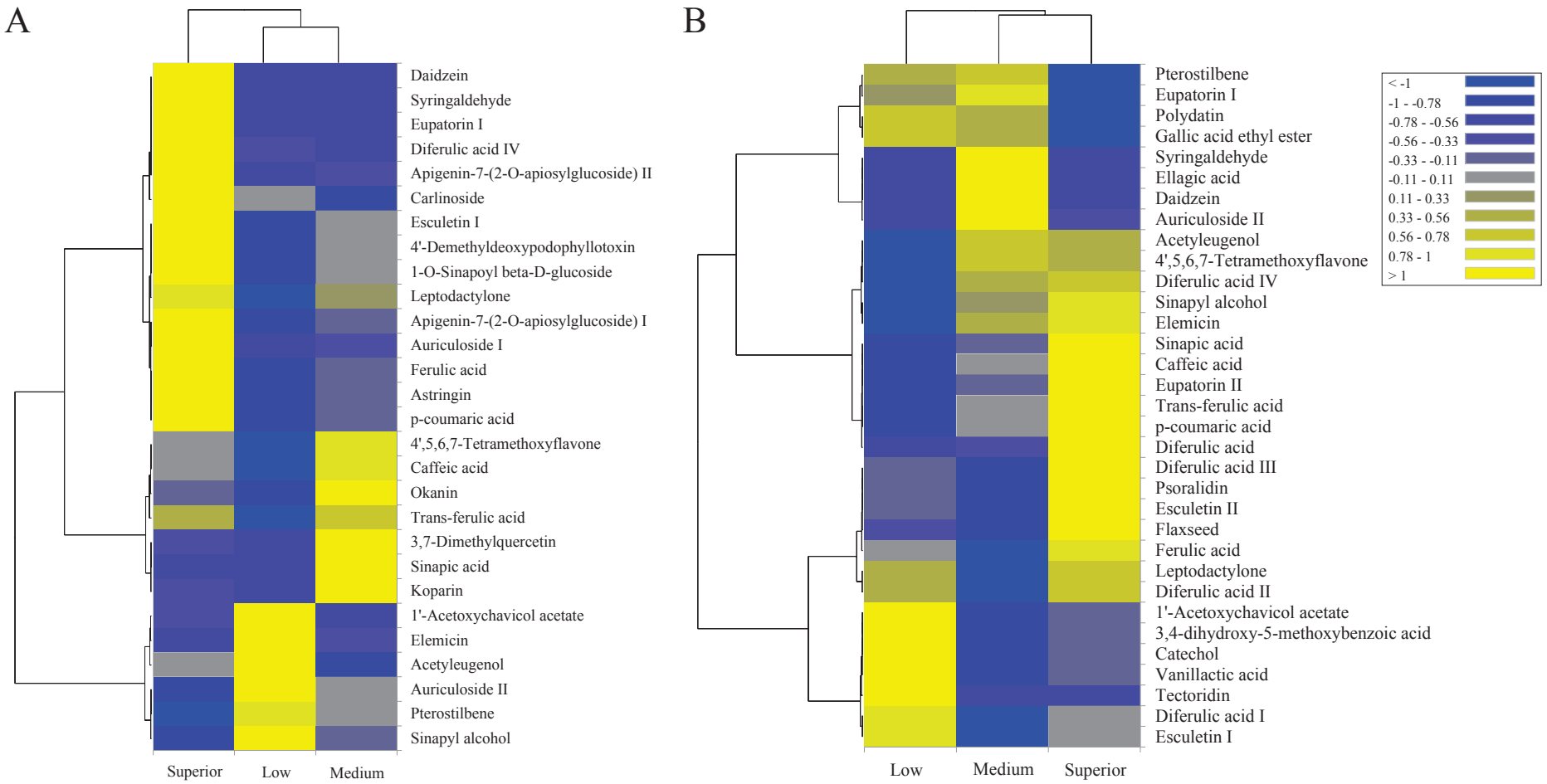


Figure 5. Heatmap of the putative phenolic compounds identified by UPLC-MS^E in different technological qualities of wheat flours: A: free extracts and B: bound extracts. Color legend generated from XLStat calculations based on the total ion abundances of each compound, which the extreme scores (-1 and +1) represent a scale meaning the maximum and minimum presence of a given compound in each variety.

The free PC are displayed in figure 5A. The differential compounds lead to the formation of two important clusters separating superior from the medium and low qualities. The low-quality flours showed a higher abundance of non-polar compounds, which mostly belonged to the class of other polyphenols. While in the superior flours, the presence of glycosylated and esterified PC is noteworthy. The clustering separation was different for the bound PC (Figure 5B) due to the phenolic profile. Low-quality flours were separated from the others due to the highest abundance of other polyphenols and stilbenes classes. The superior grouped with medium wheat flours and showed a higher abundance of dimers of ferulic acid and phenolic acids (*p*-coumaric, isomers of ferulic acid, and caffeic acid).

4. Conclusion

This research is the first study to correlate the phenolic profile with the technological quality of wheat flours. This study showed a characterization of PC and provides the most recent database of the phytochemical composition of common Brazilian wheat flours from different genotypes and technological qualities. The phenolic profile present in refined wheat flour is of significant nutritional importance for human health, considering the large consumption of this flour in the form of bread, cakes, pasta and cookies and the persistence of PC during the baking process. A metabolomics-based characterization could help build a database of wheat flour composition in secondary metabolites, useful for functional bakery products formulation/innovation or even for an improved understanding of the interactions between PC and cereal storage proteins. Despite a similar profile of phenolic compounds, the studied genotypes showed a difference in abundance. The superior technological quality presented highest levels of PC than medium and low. The multivariate analysis allowed to highlight discriminating compounds among the technological qualities. Such comprehensive approaches could help select a genotype and its expression as the wheat phenotype for a given application according to its bioactive compounds' profile and gluten composition.

5. Acknowledgements and funding

The authors are grateful to Prof. Dr. Luiz Carlos Gutkoski, OR Melhoramento de Sementes Ltda and Biotrigo Genética for providing and collecting samples; to Fabiana Ramos for her support in the Folin-Ciocalteu analysis; to Dr. Michael Murgu, Robinson Giraldes and Gustavo Almeida from the Waters Corporation (Brazil) for their support of the MS data processing and helpful technical assistance.

This work was financed by the Foundation for the Research Support of the State of Rio de Janeiro (FAPERJ) (26/010.100988/2018; 26/202/709-2018), the National Council for Scientific and Technological Development (CNPq) (427116/2018-0; 310343/2019-4), the Coordination for Improvement of Personnel with Higher Education (CAPES) (financial code 001) and supported by the Federal University of the State of Rio de Janeiro (UNIRIO).

Supplementary data

Supplementary Table 1. Determination of wheat quality of the flour samples.

Genotype	Technological classification	Gluten force, W (J)	Dough stability (min)	Water absorption (%)
Alpaca	Low	143	8.0	51.1
Campeiro	Low	115	6.7	49.2
ORSVintecinco	Low	138	7.3	53.6
ORSVintesete	Low	162	8.8	57.5
Marfim	Medium	311	17.7	55.8
ORS 1401	Medium	286	22.3	60.3
ORS 1402	Medium	206	10.5	58.8
Sintonia	Medium	308	20.0	61.5
Sossego	Medium	313	20.0	62.5
Ametista	Superior	437	20.9	62.2
Iguaçu	Superior	351	16.8	63.5
Jadeíte 11	Superior	418	14.9	61.5
Noble	Superior	378	23.7	64.1
Topázio	Superior*	Not available	Not available	Not available

*Not available, information provided by the suppliers.

Part II

Characterization and valorization of phenolic compounds of wheat and rice coproducts

In the part I, we showed that wheat grains have a great presence of PC but due to the industrial milling, many of these compounds end up being lost in the refined flour to be present within the coproducts, the outer layers where they are found in more abundant form.

Aim: In this context, the main goal of this part was to analyze by metabolomics tools only the coproducts generated applying conventional and green solvents to extract phenolic compounds. In part 2 we added the RB due to the large amount of rice coproducts generated like wheat.

Based on the following publications:

- Chapter 4: (submission process): Natural deep eutectic solvents as effective extraction media for polar bioactive compounds from wheat coproducts. Journal of Agricultural and Food Chemistry (Impact factor: 5.279)

- Chapter 5: (published) Metabolomics of Pigmented Rice Coproducts Applying Conventional or Deep Eutectic Extraction Solvents Reveal a Potential Antioxidant Source for Human Nutrition (2021) Metabolites (Impact factor: 4.932)

Main results:

- PC composition revealed by metabolomics add value and upgrade opportunities for food and pharmaceutical uses of these industrial co-products
- Higher extraction efficiency in terms of number of molecules using conventional solvents than NaDES in general is observed on wheat and rice coproducts
- NaDES enable extraction of "unique" PC and can define specific applications
- NaDES showed certain incompatibility with the applied metabolomics methods

Chapter 4 – Natural deep eutectic solvents as effective extraction media for phenolic compounds from wheat fractions

Millena C.B. Santos[1]; Melina Robert[2] ; Nathalie Barouh[3]; Erwann Durand[3]; Cecile Barron[1]; Frederic Carriere[4]; Valerie Micard[2]; Valerie Lullien[2]; Veronique Vie[5]; Pierre Villeneuve[3]; Mariana S. L. Ferreira[1] and Claire Bourlieu-Lacanal[2]*

[1]Food and Nutrition Postgraduate Program - PPGAN, Federal University of State of Rio de Janeiro, UNIRIO – Brazil
[2]INRAE, UMR IATE, Institute Agro, Montpellier, France
[3]Qualisud, Univ Montpellier, Avignon Université, CIRAD, Institut Agro, Université de La Réunion, Montpellier, France.
[4]UMR 7282 EIPL CNRS, Aix Marseille University,Marseille – France
[5]IPR Institute of Physics, Rennes University 1, Rennes -France

---

Paper to submit on Journal of Agriculture and Food Chemistry

Abstract

Aleurone and wheat bran are coproducts of wheat milling industry and constitute a sustainable and interesting matrix to extract phenolic compounds. Sustainable strategies are becoming more and more important and necessary to allow the reuse and valorization of these coproducts. Natural deep eutectic solvents (NaDES) are considered as cheap, easy to handle, biodegradable solvents showing strong capacity to solubilize phytochemicals. Seven formulations of NaDES were characterized and applied to extract phenolic compounds from wheat bran and aleurone layer and compared with classical organic solvents. The extracts were characterized by DPPH reducing power, HPLC-DAD-UV and UPLC-MS-MS. Different mixtures of conventional solvents showed similarities efficiencies to extract phenolic of wheat fractions. NaDES based on choline chloride:lactic acid (1:10) increased this amount 1.6 fold. Acidic NaDES extracts showed the best reducing power capacity and specificity to extract phenolic compounds revealed by metabolomic tools. This NaDES formulation also showed a strong tensioactive property (22 mN/m at 0.5% in TRIS buffer), which can contribute to its high extractive power. NaDES can be considered a good and "green" media to obtain a concentrate of phenolic compounds with high added-value from wheat coproducts.

Keywords: NaDES, phenolic compounds, wheat bran, aleurone, DPPH

1. Introduction

Whole wheat grains play an important role in the prevention of chronic diseases due to their nutritional composition and the presence of phytochemicals. The interest on the health benefits provided by cereals has led to a greatest focus on the variation of the phytochemical composition among the different varieties and species of wheat (Dinelli et al., 2009; Zhang et al., 2012). Besides this functional value, wheat grains have a great technological and economic importance being considered the most suitable raw material for the manufacture of bakery products, pasta and biscuits, explaining its elevated consumption.

Refined flour is the main wheat-based product consumed worldwide. Recently, we have shown using omics tools that refined flour has a diversified and abundant profile of phenolic compounds (Santos et al., 2022), but the contribution of the aleurone layer cannot be neglected (Brouns et al., 2022). Indeed, refined flour has an underwhelming bioactives composition compared to wholemeal due to the removal of germ and bran, in which most of the phytochemicals are located (Hemery et al., 2007; Sahidi, Danielski and Ikeda, 2021) (Fereidoon et al., 2021). Hence, the outermost fractions of wheat grains (*i.e.* bran and aleurone layer) are coproducts of wheat milling industry and constitute a sustainable and interesting matrix to extract bioactive compounds.

The phenolic compounds (PC) present numerous bioactivities, among them the most attractive is their their ability to limit oxidative stress in the body or in food applications. The classical methods of extraction of these compounds employs large quantities of organic solvents. Conventional (organic) solvents (e.g., ethanol, methanol, acetone, isopronanol) have been extensively used to extract these PC from different wheat genotypes and wheat cultivars (A. Di Loreto, 2018; Di Silvestro et al., 2017; Rascio et al., 2016), from wheat flours produced at different rates of milling (Wang et al., 2013), from different wheat layers or tissues (Barron et al., 2007; Hemery et al., 2007), using different technological qualities (Sharma et al., 2016), and different stages of grain maturation (Santos et al., 2018), showing that exist a great diversity of compounds with an elevated number of isomers (Nascimento, Santos, Luciana Ribeiro da Silva, et al., 2018).

In spite of the proven efficacy of organic solvents, an innovative “class” of substances has been lately proposed as green alternative to extract bioactive compounds (Radošević et al., 2016): the natural deep eutectic solvent (NaDES). NaDES are based on ionic liquids and are composed of an organic cation and an organic or inorganic anion, which allows a multitude of combinations. These solvents have environmentally desirable properties, such as

the fact that these solvents are non-volatile, not flammable, have good chemical stability, low cost, biodegradability, are easy to handle, and have relatively low toxicity (Mbous et al., 2017; Radošević et al., 2015; Wen et al., 2015). Due to all these properties, NaDES have become increasingly important in the food and non-food industries.

NaDES have been pointes out as a great extraction media, especially due to the hydrogen bonds that they can induce with the biomolecules to be extracted, increasing their solubility (Dai et al., 2013) and allowing the extraction of specific compounds as recently showed by Santos et al. (2021) based on omics tools. NaDES can also be used as reaction media allowing further steps such as the functionalization (Durand et al., 2013), representing a novel strategy to obtain enriched extracts with higher bioactive potential and feasible for application as natural additives.

The chemical complexity of these extracts requires the use of robust tools with high resolution and sensitivity such as liquid chromatography and mass spectrometry. In wheat, these compounds have been broadly characterized and estimated by diverse methodologies, from traditional quantitative methodologies (Borrás-Linares et al., 2015; McCallum & Walker, 1990, 1991) to high-resolution and sensitive techniques (Dinelli et al., 2011; Dinelli et al., 2009; Irakli et al., 2012a). In this work, PC from two wheat coproducts, aleurone layer and bran, were extracted by applying different NaDES formulations and classical organic solvents, for comparison purposes. All extracts were then characterized by HPLC-DAD-UV and UPLC-MS-MS.

2. Materials and methods

2.1. Chemicals

Choline chloride, lactic acid, 1,2-propanediol, glycerol, citric acid, betaine, perchloric acid titration, ethanol, n-propanol, acetone, methanol, water (CHROMASOLV® Plus grade HPLC), 2,2,1-diphenyl-1-picrylhydrazyl (DPPH), hexylresorcinol, ferulic, *p*-coumaric, sinapic and vanillic acids were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant material

Hard wheat bran and industrial aleurone layer were isolated from Bermude cultivar (*T. aestivum*) by industrial grinding and gently donated by Bühler (Uzwil, Switzerland).

2.2.1. Fractioning and granulometric characterization

Hard wheat bran was cryoground with a ball mill (Fritsch Pulverizer Mill, Germany) at different times (2, 5 and 10 min) to obtain different particle size populations. The aleurone layer and the different wheat bran particles were analyzed with a particle size analyzer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK) in dry and wet modes using ethanol as dispersing liquid.

2.3. Preparation of extraction solvents

2.3.1. Conventional solvents

Three mixtures of conventional solvents were prepared as follow: acetone/water (50:50 v/v), n-propanol/water (70:30 v/v) and ethanol/water (60:40 v/v).

2.3.2. Synthesis of natural deep eutectic solvents (NaDES)

Seven different formulations based on GRAS components and according a the previous study by Ruesgas-Ramón et al. (2017) were prepared. Briefly, NaDES were synthetized by mixing the different components at 60 °C under orbital shaking for 45 min at 400 rpm (Table 1). Some of NaDES formulations (2, 3, 4 and 5) were prepared in both anhydrous and hydrated version (2b, 3b, 4b and 5b). Moreover, NaDES 4b and 5b were left for additional 24 h shaking at the same conditions. The water activity (Aw, Aqualab, CX-2, Gemini, Netherland), the water content (Coulometer Karl Fisher GRS 2000, KF TITRATOR, Bioblock scientific, Germany) and the pH were determined in all NaDES formulations. The Aqualab was calibrated with saturated solutions of LiCl (A_w=0.11), NaCl (A_w=0.75).

Table 1 - Composition of different formulations of natural deep eutectic solvents (NaDES).

Name	Component 1	Component 2	Component 3	Molar ratio	Aw	KF	pH
NaDES 1	ChCl	1,2-propanediol	water	1:1:1	0.51 ± 0.03	7.20 ± 0.50	5.42
NaDES 2	ChCl	Glycerol	x	1:2	0.12 ± 0.01	0.20 ± 0.01	3.90
NaDES 2b	ChCl	Glycerol	water	1:2:2	0.13 ± 0.02	0.40 ± 0.01	3.95
NaDES 3	ChCl	Lactic acid	x	1:10	0.29 ± 0.00	6.30 ± 0.25	-0.77
NaDES 3b	ChCl	Lactic acid	water	1:10:6	0.41 ± 0.01	13.60 ± 0.25	-0.34
NaDES 4	ChCl	Citric acid	x	1:2	-	-	-
NaDES 4b	ChCl	Citric acid	water	1:2:3	0.36 ± 0.00	13.30 ± 0.38	-0.90
NaDES 5	Betaine	Citric acid	x	1:1	-	-	-
NaDES 5b	Betaine	Citric acid	water	1:1:2	0.38 ± 0.03	10.10 ± 1.27	2.36

ChCl = Choline chloride; A_W: water activity; water content determined by KF: Karl Fisher titration.

2.4. Phenolic extraction by conventional solvents and natural deep eutectic solvents

Samples (20 mg of aleurone and 100 mg of wheat bran) were placed in vials then the solvent was added in a ratio 1:20 (0.4 mL for aleurone and 2 mL for wheat bran). The vials were stirred for 25 min, 40 °C at 400 rpm, then the samples were left to cool to room temperature (~25 °C) and centrifuged for 5 min at 4000 rpm (CR412 centrifuge; Jouan, Winchester, VA). The supernatants were filtered with a cellulose filter (0.45 µm) (Minisart Legallais, Montferrier-sur-Lez, France), placed under nitrogen flow until to obtain a dried residue and stored at -4°C. Each extraction experiment was performed in triplicate.

2.4.1. Bound extraction to quantify bound ferulic acid

Bound ferulic acid was extracted according to (Nardini et al., 2002) with some modifications. Samples (20 mg of aleurone and 50 mg of wheat bran) were placed in tubes and 1.5 mL methanol/water (2:1; v/v) then 13.5 mL NaOH 2N were added. The tubes were placed at 30 °C under orbital shaking for 30 min. The blanks were performed under the same conditions but using water instead of NaOH. Each experiment was performed in duplicate with the addition of EDTA (10 mM) and 1% of ascorbic acid in one of the two experiments. At the end of the incubation, the samples were acidified to pH 3 by adding HCl 4 N. An aliquot of 0.5 mL was taken and 300 mg of NaCl was added to increase the phase partitioning. Ethyl acetate was added to the solutions and this washing step was repeated 3 times (2 mL x 3), then the tubes were placed under orbital shaking. The tubes were then centrifuged for (room temperature, 5 min, 4000 rpm). The supernatants were dried under nitrogen flow and stored at -4°C. Each hydrolysis experiment was performed in duplicate.

2.5. HPLC-DAD analyses

The extracts obtained by conventional solvents and their hydrolysates were resuspended in a mixture of methanol:water (2:1; v/v) and filtered (0.45 µm), while the NaDES-based extracts were only diluted 5 times in MilliQ water before injection. The triplicates were injected into HPLC (LC-20AD, Shimadzu) equipped with a diode array and UV detectors (DAD SPO-M20A, Oven CTO-10ASvp, Shimadzu) at 280 nm. The separation was performed on a C18 grafted silica column (5 µm, 100 Å, 250 x 4.6 mm; Kinetex High purity, Phenomenex) with a flow rate 1 mL/min using a gradient method (mobile phase A: MeOH and B: water, both with 0.1% acetic acid): 0-5 min isocratic gradient to 10% B; 5-20 min: linear gradient to 100% B; 20-30 min isocratic gradient to 100% B; 30-35 min linear gradient to 10% B; 35-42 min: equilibration to 10% B. Standards (gallic acid, vanillic acid,

ferulic acid, coumaric acid, sinapic acid and hexylresorcinol) were injected in different concentrations and the quantification was performed by using a standard calibration curve of each phenolic.

2.6. UPLC-MS-MS analysis

Aleurone and wheat bran extracts obtained from classical organic solvents and from the two most efficient NaDES, based on HPLC results were characterized by omics tools. Analyses were performed by UPLC Acquity (Waters, Milford, USA) coupled to the XEVO G2-S Q-Tof (Waters, Manchester, UK) equipped with an electrospray ionization (Phonsatta et al.) source. Data were acquired in negative mode (ESI^-) using a multiplexed MS/MS acquisition (MS^E) with alternating low and high energy collision (30-55 v). Data generated were processed by using the software Progenesis QI (NonLinear Dynamics, Waters) and the PubChem and Phenol Explorer online databases according to (Santos et al., 2021).

2.7. DPPH assay

The antioxidant activity was determined by the DPPH method described by Brand-Williams et al. (1995) adapted to microplate assays (TECAN, Infinite M1000 PRO, Gröedig, Austria). Briefly, 20 µL of the ressuspended extracts and 180 µL of a methanolic solution of DPPH (final concentration in well 150 µM) were placed in the microplates (ANSI/SLAS (SBS) format (ThermoFischer, Courtaboeuf, France). The absorbance was immediately read at 515 nm. Trolox was used as control and blanks were made by replacing the samples with methanol. Blanks with each NaDES (or conventional solvents) were used in the same concentrations but without extract present to monitor possible activity of the NaDES itself. Each technical replicate was performed in 4 different wells and the average of these is then used. IC50 corresponds to the concentration of extracts (mg/mL) able to reduce 50% of the initial DPPH concentration. The assay was performed at 37 °C in triplicate for each sample.

2.8. Ellipsometry

The ellipsometric measurements were carried out with a conventional null ellipsometer using a He-Ne laser operating at wavelength λ=632.8 nm according with (Bourlieu et al., 2016). Before starting the experiment, the trough was cleaned with detergent to remove all traces of lipid, then with ethanol to remove the detergent and then rinsed with distilled water. Ellipsometric and tensiometric blank measurements were performed during 30

min on ultrapure water (Nanopure-UV). The surface pressure was measured with the Wilhelmy system from Nima technology (England) and then plotted as function of the molecular area, determined by dividing the number of molecules by the total surface (660 cm^2).

The two most promising NaDES, based on the HPLC results, have been characterized in terms of interface behavior. These characterizations were conducted in diluted aqueous media since these biophysical experiments require liquid/air interfaces. Several reactions were therefore carried out using the chosen NaDES in different concentrations (0.5% to 5%) and in the presence or absence of ferulic acid (0.5 mg/mL) in a buffered aqueous medium (pH 7, TRIS). The evolution of the reaction medium was followed over time by ellipsometry. For surfactant properties, the NaDES were mixed into "Langmuir films" based on mixtures of polar lipids: dioleoylphosphatidylcholine and dipalmitoylphosphatidylcholine (DOPC/DPPC at 18 mN/m, 30 mN/m) mimicking cell membranes.

2.9. Optical microscopy

The observation of the structures was performed on raw materials (wheat bran and aleurone layer) put in the presence of the different NaDES (1 mL) during 25 min and 15h. 20 mg of samples were put in a larger quantity of solvent than for the extractions to allow better dispersion and observation of the structures. For each raw material, a blank was made by replacing the extraction solvent by kerosene oil. Each condition was photographed in 3 copies by using an Olympus camera (20 x, luminosity 80 µs).

2.10. Statistical Analysis

The HPLC and DPPH data were submitted to one-way ANOVA (Tukey, $p < 0.05$) by using XLStat (v 2021.1.1).

3. Results and discussion

3.1. Physicochemical characterization of selected NaDES

Different NaDES were synthesized according to different molar ratios by simple agitation at 60 °C; some of formulations were anhydrous and others were hydrated. The hydrated formulations permitted to reduce the viscosity, increase the polarity and resulted in enhanced extraction power. Two formulations of NaDES (4 and 5, Table 1) despite

exceptionally long agitation time did not produce the eutectic mixture since crystals remained observable in the mixture. Thus, they were not further used.

The water content, pH and Aw were analyzed for the remaining seven NaDES (Table 1). It was expected that the more water present in the NaDES formulation, the more water was likely to be reactive. Indeed, it was noted that the higher the water content, the higher the Aw value, except for the NaDES 1, which seems to be characterized by a lower water sorption isotherm than the others. It is important to note that the set of elaborated NaDES have Aw incompatible with microbial development (Aw <0.6).

3.2. Granulometric profile of wheat fractions

The aleurone fraction and the different powders of wheat bran (resulting from different times of grinding) were characterized in terms of size distribution. The particle size analysis of the aleurone shows a monomodal distribution of the different particles. The median particle size (d_{50}) of this fraction was 162 ± 2.7 µm (Figure 1A) that seems to correspond to aggregates of three plant cells quite typical of an industrial aleurone (Antoine et al., 2004).

For wheat bran fraction, three grinding times were compared: 2, 5 and 10 min. The wheat bran fraction ground for 2 minutes also showed a monomodal distribution but with a shoulder (~ 20 µm) only in the dry. In both modes, dry and wet, the mode corresponded to particle size of around ~ 160 µm. For the other two grinding times (5 and 10 min), the shoulder was better defined and revealed a bimodal curve (with modes around 20 µm and 100 µm). The longer the grinding time, the more the powder tends towards a bimodal distribution with a higher volume corresponding to the smallest particles. These small particles are very important for the extraction of phenolic compounds: they contribute to increase the contact surface between particles and solvent (Rosa-Sibakov et al., 2015). In addition, a reduction in particle size can be accompanied by an increase in cell wall fractures, thus favoring the accessibility of their contents. However, long grinding time can also lead to local oxidation and degradation of PC.

Hence, although smaller fractions are considered better, the fractions milled for 2 min were used for further analyses taking into consideration: 1) average particle size of ~ 160 µm, i.e., similar to one found at 5 and 10 min, 2) the milling time can negatively impact the phenolic composition.

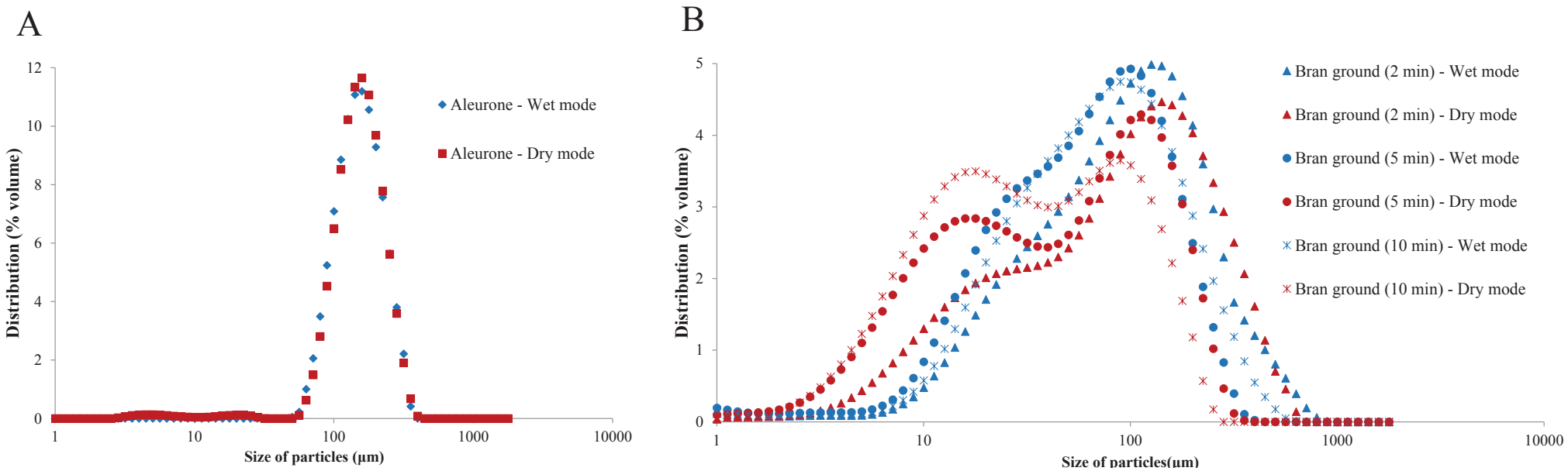


Figure 1 - Size distribution of particles in industrial aleurone layer (A) and cryoground wheat bran (B) by laser granulometry.

3.3. Free and bound extracts of phenolic compounds characterized by HPLC -DAD-UV

The quantification of the PC was based on external calibration curves built up with six standards of PC typically present in wheat fractions (gallic acid, vanillic acid, ferulic acid, coumaric acid, sinapic acid and hexylresorcinol) (Barron et al., 2011; Hemery et al., 2009).

3.3.1. Qualitative and quantitative comparison between conventional and natural deep eutectic solvents of free phenolics

Concerning the conventional solvents, similar results were observed for aleurone and wheat bran, considering the six phenolic acids analysed (Figure 2A). In aleurone extracts we could observe the highest efficiency for NaDES3, with values of total free PC extracted ranging from 4 to 62 mg/100 g of aleurone layer on all extracts and the lowest efficiency of the conventional mixtures. Wheat bran extracts contained from 2 to 10 mg/100 g free PC in all extracts and on this matrix conventional solvents were again less efficient than NaDES. But this time not only NaDES3 but NaDES1 showed better efficiency than conventional extracts. NaDES3, for instance, provided a total content of phenolic acids almost 5-fold higher than the best conventional solvents applied in wheat bran. Considering now, conventional solvents, most solvents presented for the considered six phenolic acids close extraction capacity.

Regarding more in details the six PC quantified in the different extracts, we can see that the conventional extracts applied to aleurone (Figure 2B), are dominated by vanillic, ferulic and coumaric/sinapic acids representing each around ~1 mg/100 g of aleurone (Figure 2B). Hexylresorcinol has been not extracted in enough quantity to be detected in aleurone applying ethanol or isopropanol mixtures but was extracted with acetone (~0.4 mg/100 g aleurone). In comparison, wheat bran conventional solvent extracts (Figure 2C) presented still a PC profile dominated by vanillic, ferulic and coumaric/sinapic acids but in higher amount than in aleurone conventional extracts. Highlighting ethanol and acetone mixtures as most efficient in extracting these compounds (notably ethanol presented amounts of 1.3 mg/100 g, 1.34 mg/100 g and 0.55 mg/100 g of vanillic, ferulic and coumaric/sinapic acids, respectively; and acetone presented amounts of 1.48mg/100 g, 1.42 mg/100 g and 0.63 mg/100 g of vanillic, ferulic and coumaric/sinapic acids, respectively).

Considering the quantification of typical phenolic acids and hexylresorcinol in the different NaDES extracts, results are displayed in Figure 2D and E. Results underlines a

differentiated profile in aleurone and wheat bran extracts. Again, we observe the best performance of NaDES3 and 3b, both formulated with lactic acid, this NaDES efficiency extracted vanillic acid (11.39 ± 1.11 mg/100 g 14.45 ± 2.11 mg/100 g, respectively) and coumaric/sinapic acid (20.29 ± 0.5 mg/100 g and 14.69 ± 0.43 mg/100 g) in aleurone layer (Figure 2D). Indeed, NaDES3 and NaDES3b solvents showed the highest efficiency for ferulic acid extraction, including comparison with conventional solvents. Amounts of 27.13 ± 3.63 mg/100 g and 19.06 ± 0.68 mg/100 g, respectively in aleurone layer *vs* 5.49 ± 1.22 mg/100 g and 3.69 ± 2.09 mg/100 g respectively in wheat bran (Figure 2E). The acidity of these NaDES have been pointed out to be responsible for hydrolysis of bound compounds, as previously reported by Santos et al. (2021) and Ruesgas-Ramón et al. (2017).

Considering now wheat fraction origin, we evidenced whatever the extract a higher content in free PC in aleurone than in wheat bran. This result was expected, as according to the literature, aleurone layer presents the highest content of phenolic acids (Brouns et al., 2012). In the present study, the differential performance of NaDES in solubilizing the PC may also have been enhanced by the smaller particle size of the ground aleurone, which led to a larger contact surface area with the solute.

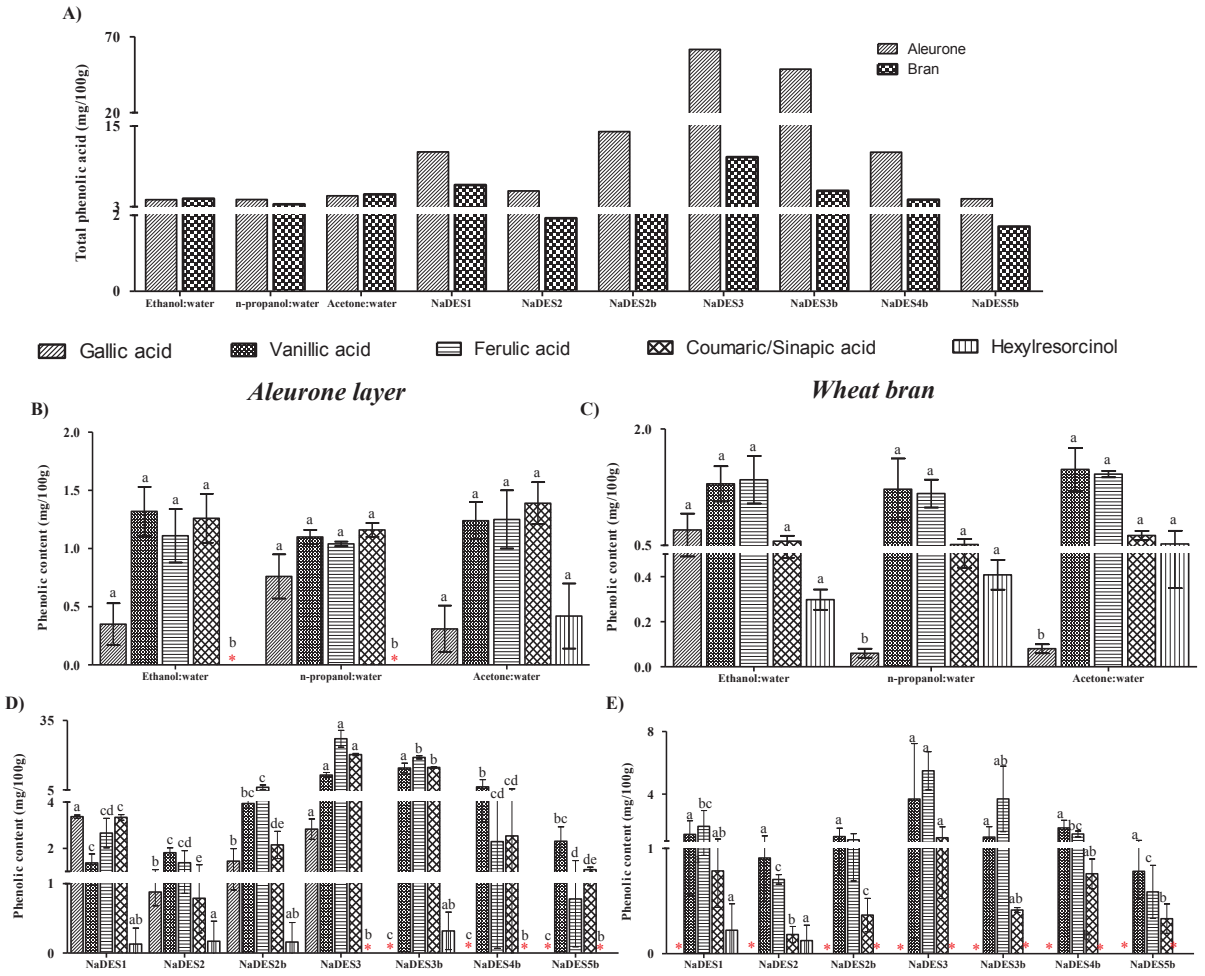
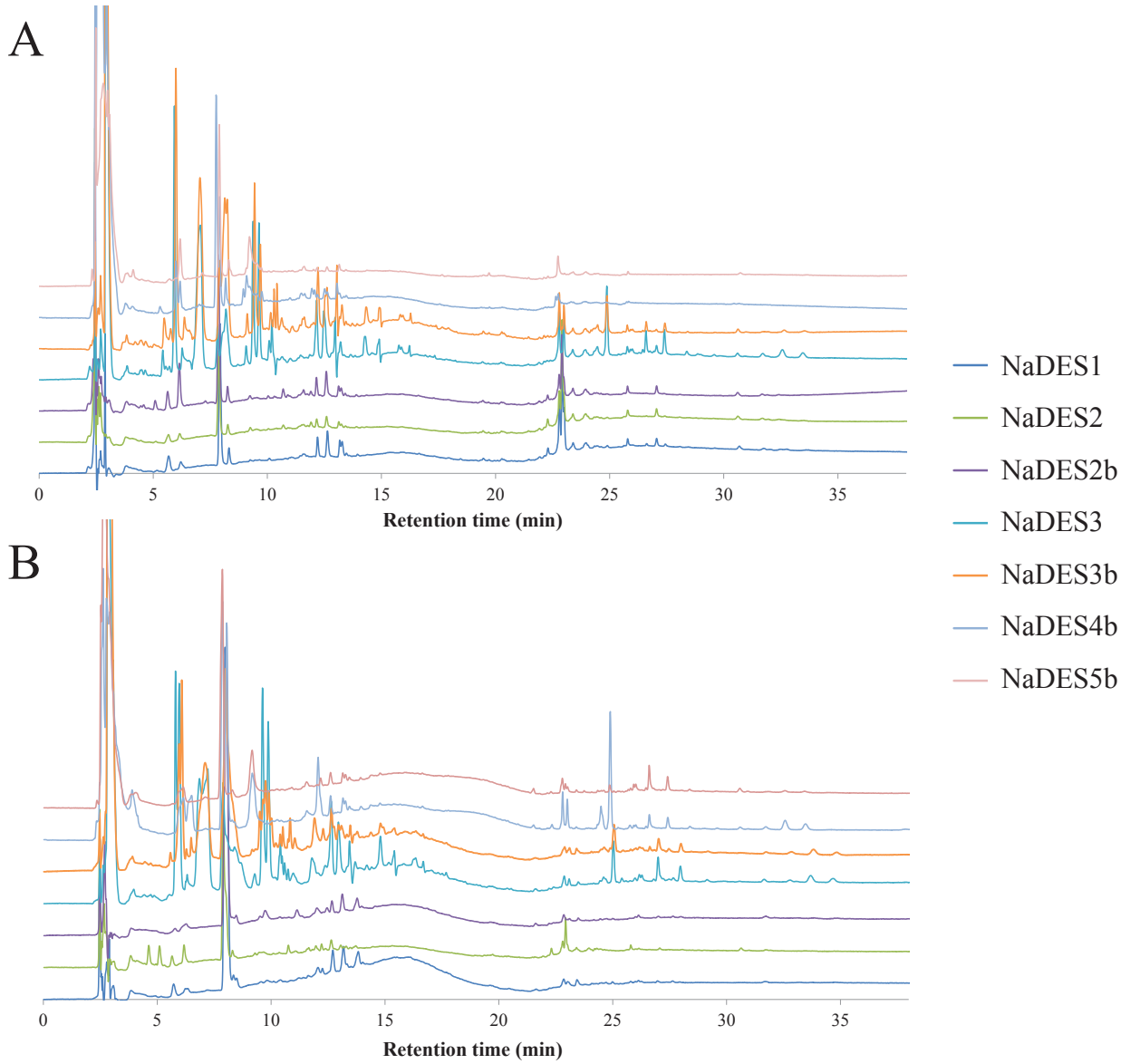


Figure 2 – Phenolic content of wheat fractions. A) Total amount of phenolics (mg/100g). Phenolic content extracted from wheat fractions using B and C) conventional solvents and, D and E) natural deep eutectic solvents. Lower case letters indicate comparison performed between different solvent efficiencies for each phenolic compounds ($p<0,05$).

Considering now the chromatography profile. Different profiles of chromatograms were obtained according to NaDES applied and the wheat fraction (Supplementary Figure 1). NaDES3 (ChCl:lactic acid) and NaDES3b (ChCl:lactic acid:water) presented the highest number of peaks (both 42) at 280 nm, and unique peaks in the retention time between 10-11 and 14-20 minutes. Consequently, NaDES3 was the NaDES that presented the highest diversity of PC. This fact can be related with the acidity of these NaDES (Table 1) able to hydrolyze the bound PC. The same results were found when this same formulation has been used by Santos et al (2021) to extract PC from pigmented rice bran.

Compounds eluting between 25-30 min seem to be better extracted on aleurone for NaDES 1, 2, 2b, 3 and 3b differently of NaDES 4b and 5b which allowed a better extraction of these compounds on wheat bran.

The NaDES 1, 2 and 2b showed the chromatograph profile with fewer peaks compared to the other NaDES for aleurone and wheat bran (Supplementary Figure 1).



Supplementary Figure 1 – UV-chromatogram of NaDES extracts: A) Aleurone layer and B) Wheat bran.

NaDES1 and NaDES3 have been showed the most effective extractors different PC and destined to be characterized by UPLC-MS^E.

3.3.2. Ferulic acid content in bound extracts

In wheat, as well as other cereals, the most part of PC are found in the bound form, as well, the most abundant phenolic in wheat in bound form is the ferulic acid (Saulnier et al., 2007; Verma et al., 2009). Hydrolyss has been performed to allow the quantification of the major phenolic acid, the ferulic acid in the two wheat fractions studied.

In this present study, aleurone layer presented a ferulic acid content of 800 ± 110 mg/100 g, 1.6-fold higher than wheat bran that presents 510 ± 40 mg/100 g). These results were coherent with literature (Mateo Anson et al., 2009; Rosa et al., 2013) which has already demonstrated that among wheat fractions, aleurone layer contains the highest content. Anson et al. (2009) also showed that aleurone present a content of ferulic acid of 1.2-fold more abundant than bran (680 mg/100 g of aleurone and 570 mg/100 g of wheat bran).

To prevent the degradation of PC during the hydrolyses process, a test with presence of EDTA and ascorbic acid were applied. However, the results have not shown difference for both, presenting a content of 780 ± 90 mg FA/100 g to aleurone and 530 ± 40 mg/100 g to wheat bran.

Hydrolyzed extracts allow to estimate the total content of ferulic acid in the samples. The results showed that bound PC represent almost the totality of ferulic acid (displayed in Figure 2). Indeed, free ferulic acid is present in very low amounts in wheat outermost layers, showing a content of approximately 1-20 to 1-6 mg/100 g of aleurone and wheat bran, respectively. Bound ferulic acid corresponded to 98% and 95%, respectively in these samples, and the ratio of bound to free ferulic acid has been very high for aleurone extracts and wheat bran extracts.

Comparing the hydrolyzed extracts with conventional free extracts, the amount of free ferulic acid showed a low extractible potential, varying between 0.23 to 0.69%. The fact that ferulic acid occurs mainly bound to the cell matrix or macronutrients reduces the effectiveness of simple organic solvent mixtures in extracting it when linked (Anson et al., 2009).

The NaDES extraction yield has been calculated considering the hydrolyzed extract as a total extract (free plus bound PC) obtained by conventional solvents. Although this yield has been high compared to the other solvents, the percentage of extraction compared to the ferulic acid yields after hydrolyses were low (NaDES3 ~0.34% and ~1.14% and NaDES3b ~0.17%

and 0.67% in aleurone and wheat bran, respectively). These results underline a higher efficiency when hydrolysis occurred under basic conditions in a conventional media.

3.4 Characterization of free extracts of wheat fractions obtained from conventional and natural deep eutectic solvents by UPLC-MS^E

Aleurone and wheat bran extracts of conventional solvents and the two most efficient NaDES (1 and 3) according to the HPLC results were characterized by metabolomics tools. From PubChem database, it was possible to identify globally, a total of 44 putative PC (Table 2). The identification of PC has been based on mass to charge (*m/z*), retention time, isotopic similarity, precursor mass error, the score, and the fragmentation score. The levels of each identification attempt followed the guidelines proposed by the Metabolomics Standards Initiative (Ncube et al.) as reported by Sumner et al. (2007).

Table 2 - Putative identification of phenolic compounds (PCs) in wheat fractions extracts by UPLC-MS^E.

	Putative identification	ID number (PubChem)	RT (min)	Molecular Formula	*m/z*	Mass Error (ppm)	Score	Fragmentation Score	Isotope Similarity (%)	Class	Samples
1	Isopimpinellin	68079	0.95	$C_{13}H_{10}O_5$	245.0458	1.19	36.9	0.0	86	OP	b, g
2	Apigenin -*O*-glucoside isomer I	622948	1.44	$C_{21}H_{24}O_9$	419.1348	0.00	38.2	0.0	91	F	g
3	Apigenin -*O*-glucoside isomer II	622948	1.48	$C_{21}H_{24}O_9$	419.1349	0.38	37.9	0.0	90	F	b
4	Dihydroxybenzoic acid isomer	1491	1.86	$C_7H_6O_4$	153.0179	-9.34	36.3	0.0	92	PA	b,c, d,e, f, h, i, j
5	Dihydrocaffeic acid	348154	2.06	$C_9H_{10}O_4$	181.0497	-5.25	36.8	0.0	90	PA	f, h, i, j
6	p-Coumaric acid 4-*O*-glucoside	9840292	2.10	$C_{15}H_{18}O_8$	325.0945	5.07	45.7	51.0	83	PA	h, i, j
7	3-Feruloylquinic acid	5317346	2.40	$C_{17}H_{20}O_9$	367.0998	-9.88	44.5	38.3	95	PA	c, h, i, j
8	Esculetin	5281416	2.51	$C_9H_6O_4$	177.0179	-8.29	36.1	0.0	90	OP	h, i, j
9	Gallic acid ethyl ester	152463	2.57	$C_9H_{10}O_5$	197.0440	-7.98	54.8	87.1	96	PA	b, g
10	Hydroxyphenylacetic acid isomer	11970	2.62	$C_8H_8O_3$	151.0390	-7.24	37.3	0.0	94	PA	c, d, e, i
11	**Caffeic acid**	689043	2.72	$C_9H_8O_4$	179.0337	-7.00	36.8	0.0	92	PA	b, c, d, e, f, g, h
12	Nortrachelogenin/ Isohydroxymatairesinol	394846	2.79	$C_{20}H_{22}O_7$	373.1300	1.93	38.2	0.0	93	L	b, g, i
13	Syringaresinol	100067	2.82	$C_{22}H_{26}O_8$	417.1567	2.95	37.5	0.0	91	L	b, g, i
14	Rosmarinic acid	5281792	2.93	$C_{18}H_{16}O_8$	359.0776	0.87	37.2	0.0	87	PA	b, g
15	Anethole / Estragole	637563	3.04	$C_{10}H_{12}O$	147.0801	-9.53	35.8	0.0	89	OP	h, i, j
16	Apigenin -*O*-apiosyl-glucoside isomer III	16211399	3.04	$C_{26}H_{28}O_{14}$	563.1399	-1.36	52.9	69.8	96	F	a, b, c, d, e, f, g, h, i, j
17	Vanillin	1183	3.14	$C_8H_8O_3$	151.0388	-8.03	36.4	0.0	91	PA	i
18	Feruloyl glucose isomer I	25245871	3.25	$C_{16}H_{20}O_9$	355.1020	-4.01	45.5	37.0	95	PA	h, i, j
19	Mellein	28516	3.32	$C_{10}H_{10}O_3$	177.0544	-7.47	52	79.4	89	OP	i, j
20	***p*-Coumaric acid**	322	3.35	$C_9H_8O_3$	163.0388	-7.96	36.2	0.0	90	PA	a, c, e, h, i, j
21	Feruloyl glucose isomer II	25245871	3.46	$C_{16}H_{20}O_9$	355.1020	-4.01	39	5.4	94	PA	h, i, j
22	Caffeic acid ethyl ester	5317238	3.51	$C_{11}H_{12}O_4$	207.0642	-9.95	36.2	0.0	92	PA	c, d, h, i, j
23	***Trans*-ferulic acid**	709	3.59	$C_{10}H_{10}O_4$	193.0498	-4.41	55.4	89.5	92	PA	a, c, d, e, h, i, j

24	Paeonol isomer I	11092	3.63	$C_9H_{10}O_3$	165.0543	-8.65	36.1	0.0	90	PA	b, i
25	Dihydro-*p*-coumaric acid	10394	3.78	$C_9H_{10}O_3$	165.0545	-7.35	37.3	4.5	90	PA	h, i
26	Ferulic acid	736186	3.81	$C_{10}H_{10}O_4$	193.0492	-7.34	37	0.0	93	PA	h, i, j
27	Cinnamic acid	444539	3.89	$C_9H_8O_2$	147.0438	-8.94	36.1	0.0	90	PA	c, d, e, h, i, j
28	Syringic acid	10742	3.93	$C_9H_{10}O_5$	197.0440	-7.60	36.2	0.0	90	PA	g
29	Scopoletin	5280460	4.05	$C_{10}H_8O_4$	191.0337	-6.80	36.2	0.0	89	OP	h, i, j
30	Carvacrol / Thymol	10364	4.08	$C_{10}H_{14}O$	149.0959	-8.83	53.4	87.1	89	OP	d, h, i, j
31	Sinapaldehyde	5280802	4.11	$C_{11}H_{12}O_4$	207.0648	-6.99	36	0.0	88	OP	h, i, j
32	Gardenin B	96539	4.25	$C_{19}H_{18}O_7$	357.0965	-4.09	35	0.0	80	F	h, i, j
33	Tetramethylscutellarein	96118	4.45	$C_{19}H_{18}O_6$	341.1019	-3.41	46.6	57.0	80	F	b, h, i, j
34	Nortrachelogenin	394846	4.62	$C_{20}H_{22}O_7$	373.1279	-3.71	52.2	76.1	89	L	d, f, g, h, i, j
35	Paeonol isomer II	11092	4.64	$C_9H_{10}O_3$	165.0547	-6.17	55.9	96.2	90	PA	i
36	Trihydroxyisoflavanone isomer	125100	4.72	$C_{15}H_{12}O_5$	271.0594	-6.75	35.3	0.0	84	F	g, h, i, j
37	Eupatorin / Cirsilineol	97214	4.82	$C_{18}H_{16}O_7$	343.0811	-3.67	35.9	0.0	84	F	h, i, j
38	Dihydroresveratrol	185914	5.00	$C_{14}H_{14}O_3$	229.0869	-0.45	36.9	0.0	85	S	b, g
39	Cyanidin	128861	5.10	$C_{15}H_{11}O_{6+}$	286.0465	-6.16	36.3	0.0	89	F	b, h, i, j
40	Violanone	24772948	5.16	$C_{17}H_{16}O_6$	315.0861	-4.08	36.8	6.6	82	F	h, i, j
41	Trihydroxyisoflavone isomer	5280443	5.68	$C_{15}H_{10}O_5$	269.0437	-6.76	35.3	0.0	84	F	f, h, i, j
42	Dimethylquercetin	5379096	5.76	$C_{17}H_{14}O_7$	329.0655	-3.66	38.5	1.8	95	F	a, c, d, h, i, j
43	Methoxyapigenin isomer I	5353911	5.81	$C_{16}H_{12}O_6$	299.0548	-4.52	36.5	0.0	88	F	a, d, e, g, h, i, j
44	Methoxyapigenin isomer II	5281628	6.40	$C_{16}H_{12}O_6$	299.0537	-8.06	34.8	0.0	83	F	h, i, j

m/z: ratio mass/charge; PA= phenolic acid; F: flavonoid; OP: other polyphenols; S: stilbene; L: lignans; a = Aleurone NaDES 1; b = Aleurone NaDES 3; c = Aleurone acetone:water; d = Aleurone ethanol:water; e = Aleurone n-propanol:water; f = wheat bran NaDES 1; g = wheat bran NaDES 3; h = wheat bran acetone:water; i = wheat bran ethanol:water; j= wheat bran n-propanol:water. NaDES1 (ChCl:1,2-propanediol) and NaDES3 (ChCl:lactic acid)

In this study, five classes of PC were evidenced: phenolic acids (43%), flavonoids (33%), other polyphenols (15%), lignans (7%) and stilbenes (2%). A higher diversity of PC has been identified in wheat bran than in aleurone layer extracts, for all solvents except for NaDES 3 (Figure 3). Simple phenolic acids such as ferulic acid isomers and *p*-coumaric acid were identified in aleurone and wheat bran extracts. The glycosylated form of ferulic and *p*-coumaric acid were also identified, as well as glycosylated flavonoids.

For wheat bran, among the conventional solvents, the ethanol/water mixture (60:40, v/v) proved to be the most efficient in extracting different PC, emphasizing flavonoids and other polyphenols. The ethanol mixture also stood out when compared to the two NaDES analyzed (NaDES 1 and NaDES 3). This result was also found when comparing the same solvents for the extraction of bioactive compounds in pigmented RB by Santos et al.(Santos et al., 2021). For aleurone layer, NaDES 3 was the solvent the most effective to extract different PC, while among the conventional solvents the ethanol/water presented a slightly better result.

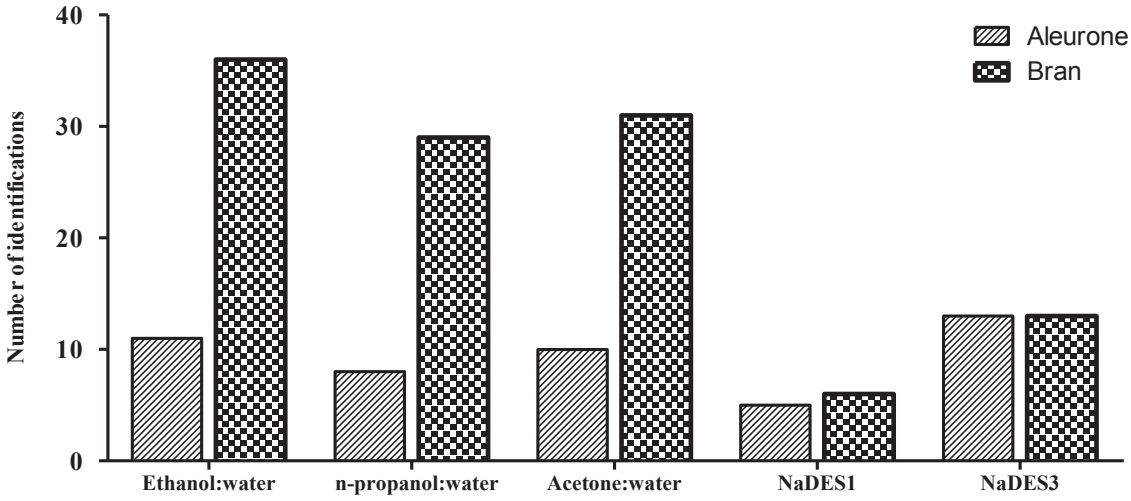


Figure 3 - Number of putative identifications in wheat fractions by solvent analyzed by UPLC-MS^E.

The total relative amount was calculated from the sum of abundances of PC identified in each extract (Figure 4). NaDES3 extract of aleurone appeared to be most efficient in extracting higher amounts than NaDES1 and conventional solvents. Wheat bran extracts showed the opposite, conventional solvents were most efficient in extracting higher amounts of PC than NaDES.

Compared to the absolute quantifications obtained in the HPLC analyses, the PC content evidenced from metabolomics allows us to visualize a positive correlation:

conventional solvents most efficient in extracting PC from wheat bran, while NaDES proved to be most efficient in extracting compounds from aleurone. This varying efficiency can be explained by the polarity of the solvent and the amount of water in the mixture. Fanali et al. (2020) recently showed that the efficiency of eutectic solvents in extracting PC from olive oil increased with higher water ratios.

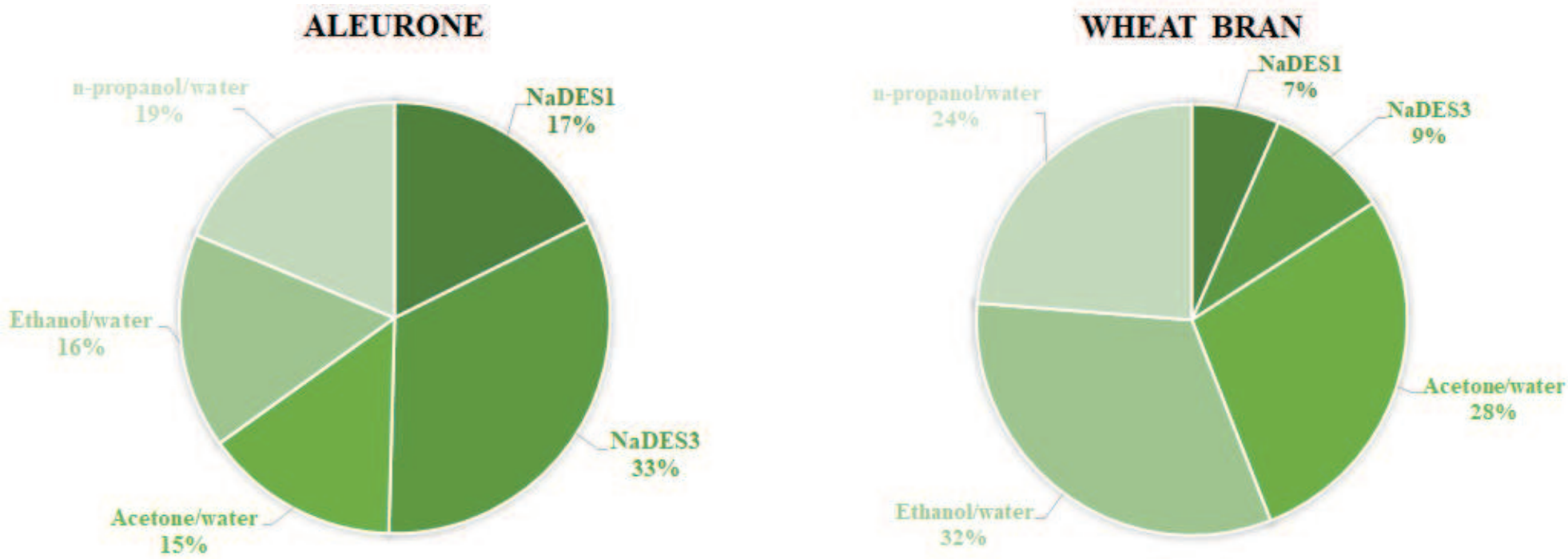


Figure 4 – Total relative abundance of phenolic compound for each extract to aleurone and wheat bran.

NaDES 3 presented a distinguished capacity of extract specific PC, showing 7 unique compounds (Table 2), probably due to its acid pH that may have favored the extraction of more specific PC according to the literature (Ruesgas-Ramón et al., 2017; Santos et al., 2021). Some of them already described in the literature for their well-known bioactivities: rosmarinic acid (*m/z* 359.0775 $[M–H]^-$), dihydroresveratrol (*m/z* 229.0871 $[M–H]^-$), two isomers of glycosylated apigenin (*m/z* 419.1348 $[M–H]^-$) and a ethyl ester of gallic acid (*m/z* 197.0440 $[M–H]^-$). Conversely, unique PC has been not present in NaDES 1 extracts.

3.5 Reducing power of the extracts assessed by DPPH radical scavenging assay

DPPH assay has been conducted to assess the reducing power of the wheat fractions extracts according to the different solvents. The IC_{50} was expressed in concentration of aleurone or wheat bran extract in mg/mL (Table 3). Among the conventional solvents, the ethanol mixture showed the highest reducing capacity in both aleurone and wheat bran fractions (IC_{50} 111.97 and 50.50 mg/mL, respectively), being in agreement with the literature (Abozed et al., 2014). For aleurone, the results of the reducing capacity also followed the phenolic acids quantification, where NaDES presented a much better performance than conventional solvents. Extracts obtained with NaDES3 and 3b required only half the concentration of the ethanolic extract to show the same reducing power (IC_{50} Ethanol:water = 111.97 mg/mL x NaDES3 = 50.10 mg/mL and NaDES3b = 53.07 mg/mL). For wheat bran extracts, obtained by conventional solvents, remained more efficient to scavenge DPPH than aleurone extract ($p<0.05$), corroborating the results found for the total PC determined by HPLC. NaDES extracts of wheat bran showed no difference in reducing power compared to aleurone extracts ($p<0.05$).

Control experiments have being also conducted using only the NaDES to determine their individual contribution to DPPH reduction (Santos et al., 2021). These control experiments pointed out that NaDES2 contributed less to the reducing power than the others NaDES (13%). The contribution of others NaDES remained responsible for 16% (NaDES 1), 20% (NaDES 2b), 33% (NaDES 3), 35% (NaDES 3b), 37% (NaDES4b) and 24% (NaDES5b) of the reducing capacity in these extracts.

Table 3 – IC_{50} values of aleurone and wheat bran extracts measured by DPPH assay with different extract solvents.

	IC_{50} (mg/mL)	
Solvents	**Aleurone**	**Wheat bran**
Acetone:water (50:50, v/v)	145.95 ± 40.28 ^{aA}	62.64 ± 15.91 ^{abA}
n-propanol:water (70:30, v/v)	122.89 ± 15.59 ^{abA}	68.90 ± 1.09 ^{abB}
Ethanol:water (60:40, v/v)	111.97 ± 8.58 ^{abcA}	50.50 ± 7.93 ^{bB}
NaDES1	123.81 ± 2.54 ^{abA}	95.31 ± 33.78 ^{abA}
NaDES2	112.76 ± 13.62 ^{abcA}	72.95 ± 13.14 ^{abA}
NaDES2b	66.76 ± 10.45 ^{cdA}	113.82 ± 11.57 ^{aA}
NaDES3	50.10 ± 7.38 ^{dA}	50.63 ± 5.52 ^{bA}
NaDES3b	53.07 ± 3.18 ^{dA}	53.75 ± 4.33 ^{bA}
NaDES4b	83.99 ± 26.05 ^{bcdA}	54.58 ± 4.74 ^{bA}
NaDES5b	90.99 ± 12.36 ^{bcdA}	70.53 ± 12.79 ^{abA}

a-d Lower case letters indicate comparison between solvents of the same sample (p<0,05).
A-B Upper case letters indicate comparison between samples with the same solvent (p<0,05).

3.6 Potential mechanism of action of the most effective NaDES3 (choline chloride: lactic acid 1:10) on specific wheat fractions

NaDES3 presented a distinguished extraction efficiency of PC and then was submitted to ellipsometry analyses. The values of ellipsometry angles are related to the refractive index of the medium studied. Different conditions were evaluated and illustrated in the Figure 5. As the percentage of NaDES3 increased, the refractive index increased slightly (Solution at 0.05% = 1.3347, 0.5% = 1.3352 and 5% = 1.3401). This same increase was observed when the components of NaDES3 were used separately. These results indicate a very discontinuous organization of NaDES at low concentrations that condense and organize much more homogeneously in thickness at 5%. Lactic acid seems to be the biggest contributor to the surfactant behavior of NaDES3; when used at 0.4% provoked an increase of 2.1° in delta angle and an increase of 12 mN/m in pressure.

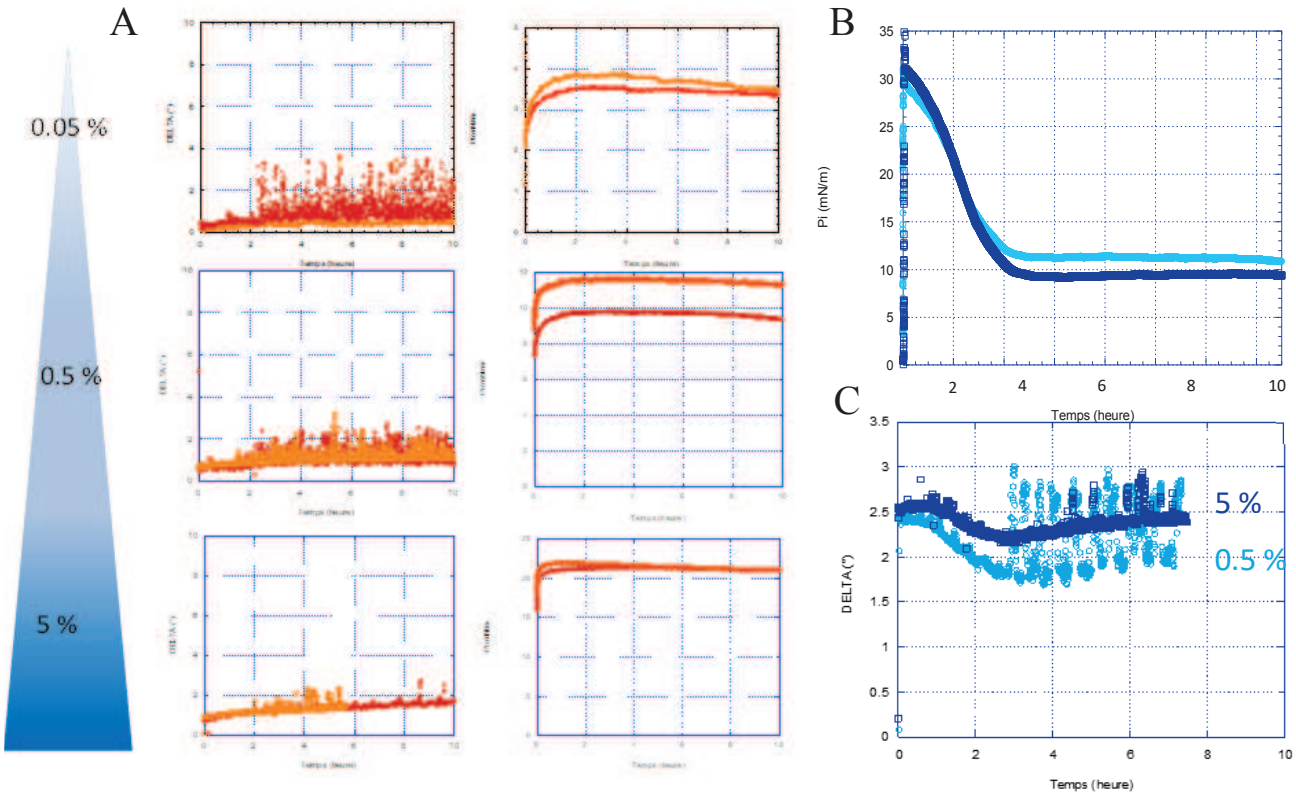


Figure 5 - Ellipsometric and surface tension at air/liquid interface in presence of increasing amounts of most effective DES (choline chloride: lactic acid 1:10).

When NaDES3 was placed in the aqueous phase, it very quickly causes an increase in the ellipsometric angle. The increase in angle was fast for NaDES3 after 30 min, the angle of NaDES3 goes from 0° to 1.5°; values close to equilibrium at 2.6° were reached in about 2 h (Figure 5A). This increase in ellipsometric angle reflects the rise of NaDES near the interface. This rise is also accompanied by an increase in surface pressure in the minutes following the addition of the NaDES. The equilibrium pressure reached by the NaDES3 was 20.8 mN/m. This surface tension indicates that NaDES adsorbs at the interface and changes the cohesion of the molecules at that interface. Similar pressures can be found when proteins adsorb to the interface.

A surfactant, or surface-active agent, are amphiphilic molecules (such as lipids) that modify the tension between two surfaces. When a surface-active molecule arrives at the surface, it decreases the surface tension and increases the surface pressure. NaDES3 thus presents a marked tensio-activity. The strong pressure increase (20.8 mN/m) for a small ellipsometric angle increase (2.6°C) indicates that a small thickness of molecules at the interface strongly modifies the cohesion of molecules at the liquid/air interface.

3.7 Effect of DES on the microstructures of wheat aleurone

Different images were taken after 25 min and 15 h of contact between the aleurone and the seven NaDES (Figure 6). Aleurone tissue structures showed different patterns according to the type of NaDES formulation applied. The cell walls were still discernible after 15 h of contact with the solvent. However, a careful observation allows to discern a discoloration in the center of the cells, at the level of the protein body for NaDES 2, 3, 3b and 4b (showed by arrows in Figure 5). In view of the profiles obtained for NaDES 3 and 3b, we could assume that the structures obtained with these formulations were more damaged than for the other NaDES, thus favoring the extraction of compounds. Besides to improve the bioacessibility, the structural modifications on wheat aleurone fraction can also lead to an altered metabolism of phenolic acids and other phytochemicals (Pekkinen et al., 2014).

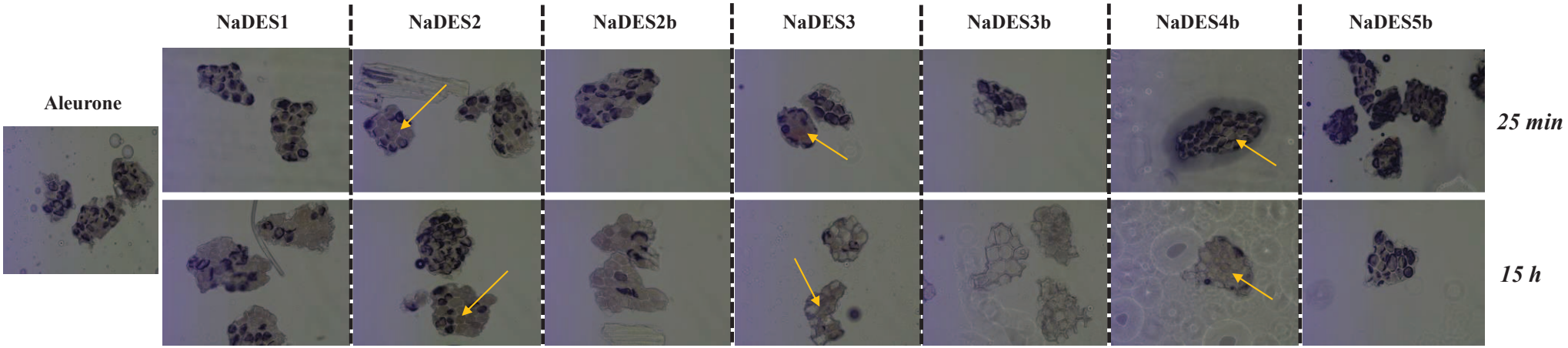


Figure 6 - Micrographs of dry aleurone residues after 25 min and 15 h of contact with the different NaDES (magnification 20×).

4. Conclusions

In the present study, complementary techniques were applied to characterize wheat fractions (aleurone and bran) extracts obtained either with conventional or deep eutectic solvents extraction procedures. Use of modern analytical tools allowed the quantification of the most typical phenolic acids and hexylresorcinol in aleurone and wheat bran as well as the description of the PC present in the different extracts. The quantification based on HPLC results underlined that, different solvents (with different polarities) allow the extraction of PC with different yields. Conventional solvents were less efficient in extracting the main phenolic acids from the two wheat coproducts but more efficient to extract a large number of PC. Specifically high extraction capacity of some of the NaDES (containing lactic acid) has been evidenced. Although conventional solvents have proven to be most efficient in extracting a greater diversity of PC by omics tools, NaDES3 (ChCl:lactic acid) allowed to extract specifics PC, probably in connection with its acidic character. Optical microscopy observations confirmed that NaDES3 affected the aleurone structures. In addition, a DPPH assay revealed that these wheat fractions extracted in NaDES presented an interesting reducing power although NaDES in themselves have specific pro-reducing or pro-oxidant activities. Globally, these DPPH assays opens the way to develop rich antioxidant wheat fractions formulas.

5. Acknowledgments

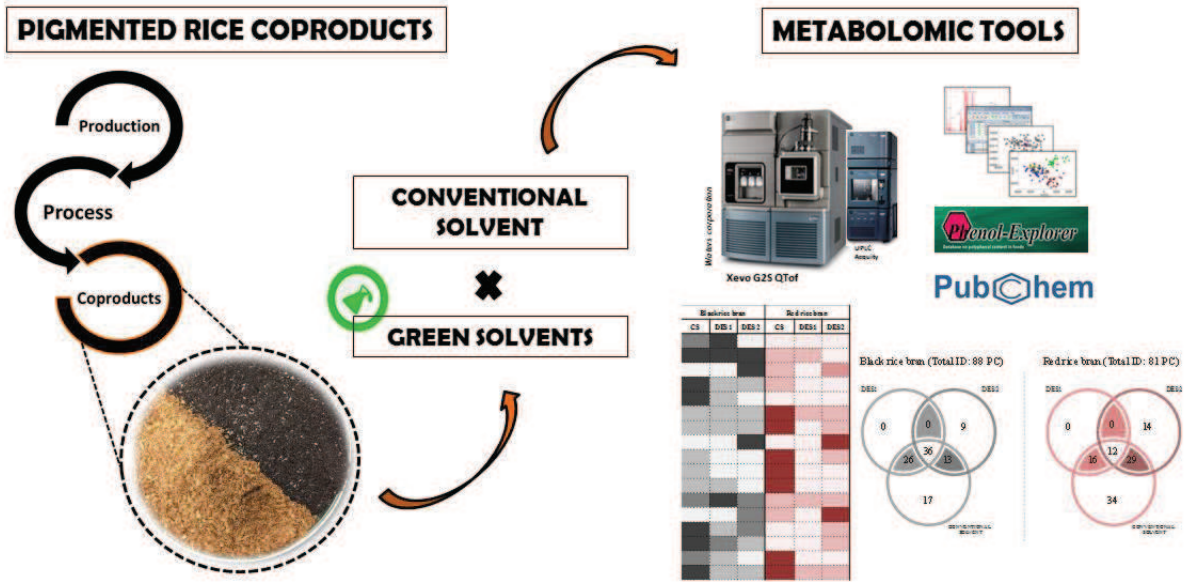
The authors are grateful to Bühler for providing wheat fractions. The authors thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, code 001), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, 26/010.100988/2018; 26/202/709–2018; 26/201.848/2019) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (310343/2019-4) for the financial support.

Chapter 5 – Metabolomics of Pigmented Rice Coproducts Applying Conventional or Deep Eutectic Extraction Solvents Reveal a Potential Antioxidant Source for Human Nutrition

Millena Cristina Barros Santos[1,2], Nathalie Barouh[3,4], Erwann Durand[3,4], Bruno Baréa[3,4], Mélina Robert[3,4], Valérie Micard[4], Valérie Lullien-Pellerin[4], Pierre Villeneuve[3,4], Luiz Claudio Cameron[2], Elizabeth P. Ryan[5], Mariana Simões Larraz Ferreira[1,2]* and Claire Bourlieu-Lacanal[3,4]*

[1]LabBio, Laboratory of Bioactives, Food and Nutrition Graduate Program, PPGAN, Federal University of State of Rio de Janeiro, UNIRIO, 22290-240 Rio de Janeiro, Brazil
[2]IMasS-LBP, Center of Innovation in MS-Laboratory of Protein Biochemistry, UNIRIO, 22290-240 Rio de Janeiro, Brazil
[3]CIRAD, UMR IATE, 34398 Montpellier, France
[4]IATE, Univ Montpellier, INRAE, Institut Agro, 34000 Montpellier, France
[5]Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO 80523, USA

Paper published on Metabolites, 2021. DOI: https://doi.org/10.3390/metabo11020110



Abstract

Ric bran (RB) corresponds to the outer layers of whole grain rice and contains several phenolic compounds (PCs) that make it an interesting functional food ingredient. PC richness is enhanced in pigmented RB varieties and requires effective ways of extraction of these compounds. Therefore, we investigated conventional and deep eutectic solvents (DES) extraction methods to recover a wide array of PCs from red and black RB. The RB were extracted with ethanol/water (60:40, *v/v*) and two DES (choline chloride/1.2-propanediol/water, 1:1:1 and choline chloride/lactic acid, 1:10, mole ratios), based on Generally Recognized as Safe (Górnaś et al.) components. Besides the quantification of the most typical phenolic acids of cereals, nontargeted metabolomic approaches were applied to PCs profiling in the extracts. Globally, metabolomics revealed 89 PCs belonging to flavonoids (52%), phenolic acids (33%), other polyphenols (8%), lignans (6%) and stilbenes (1%) classes. All extracts, whatever the solvents, were highly concentrated in the main phenolic acids found in cereals (37–66 mg/100 g in black RB extracts vs. 6–20 mg/100 g in red RB extracts). However, the PC profile was highly dependent on the extraction solvent and specific PCs were extracted using the acidic DES. The PC-enriched DES extracts demonstrated interesting DPPH scavenging activity, which makes them candidates for novel antioxidant formulations.

Keywords: phenolic compounds; pigmented RB; green solvent; metabolomics; UPLC-MS^E; antioxidant

1. Introduction

Health-promoting properties of rice bran (RB) (*Oryza sativa* L.) have supported its application in food products for human consumption over the last decade. RB has been tested in the formulation of functional foods for human studies involving children and adults (Borresen & Ryan, 2014; Li et al., 2018a). Indeed, RB nutritional profile is well diversified in essential and nonessential nutrients, and contains lysine-rich proteins (Khir & Pan, 2019), lipids (Tong & Bao, 2019), fibers and phytochemicals (Zarei et al., 2018). Additionally, RB consumption can positively modulate intestinal microbiota, contribute to the production of novel primary and secondary metabolites, to the regulation of intestinal immunity for the protection against bacterial infection with Salmonella (Goodyear et al., 2015; Nealon et al., 2017) and to colon cancer prevention (Zarei et al., 2019).

RB bioactivity is enhanced in pigmented rice varieties (Zaupa et al., 2015), very likely because of the higher levels and diversity of phenolic compounds (PCs). These pigmented rice gather usual phenolic acids found in nonpigmented varieties such as *p*-coumaric, syringic, vanillic, caffeic, sinapic, *p*-hydroxybenzoic, isoferulic and protocatechuic acids (Pang et al., 2018; Shao et al., 2014a; Zaupa et al., 2015; Zhang et al., 2015), but owe their color to flavonoids. For instance, anthocyanins such as cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside and proanthocyanidins have already been identified in pigmented RB (Deng et al., 2013; Mbanjo et al., 2020; Zhang et al., 2010). These pigmented-rice flavonoids and phenolic acids play an essential role against oxidative stress and inflammation. Such protective role was demonstrated in mouse (Chakuton et al., 2012; Petroni et al., 2017) or human cell assays (Junmarkho & Hansakul, 2019). This antioxidant activity is reported to be even enhanced in black rice grains (Pang et al., 2018). Among the wide range of bioactivity for pigmented RB potentially linked to its high PCs load, antiaging properties have been evidenced by Sunthonkun et al. (2019). These authors have shown increased viability of yeast *Saccharomyces cerevisiae* when exposed to medium enriched in pigmented RB extracts. In addition, Khammanit et al. (2017) provided evidence for the antiproliferative properties of pigmented RB on HEK-293 cells. These antiproliferative effects were mediated by a reduction in ROS production, as well as an enhancement of antioxidant enzymes production.

Considering the high bioactivity of pigmented RB, it is an important and relevant scientific challenge to better examine their complex chemical PCs composition. Due to the wide range of PCs' polarity, effective extraction is a key issue to properly elucidate the components of the pigmented RB extracts. This is generally handled by combining mixtures of organic solvents (e.g., methanol, isopropanol, chloroform, acetone) with water, but their

efficiency is sometimes limited due to the low diversity of compounds extracted (Peanparkdee et al., 2019). In addition, physically-assisted solvent extraction techniques have also been proposed to increase bioactive compounds extraction efficiency (Ghasemzadeh et al., 2015).

In this context, the application of a novel class of green solvents, called deep eutectic solvents (DES), has been proposed as a promising strategy to improve the extraction efficiency of bioactive compounds from coproducts (Ruesgas-Ramón et al., 2017). DES show similar physical properties to the well-known ionic liquids (e.g., low vapor pressure, chemical and thermal stability, no flammability, high conductivity, high solubilizing capacity and low volatility) but with lower toxicity and better beneficial cost, accessibility and sustainability. What makes DES interesting is their unpredictable and strong solubilization capacity that was intensively used to provide enriched extracts with high phytochemical concentration and/or specificity (Ruesgas-Ramón et al., 2017). For instance, Huang et al. (2017b) successfully extracted the low soluble rutin reaching 95% recovery from buckwheat hull. In addition, DES can stabilize and protect molecules from degradation, mostly due to the supramolecular network of tightly interconnected molecules (Dai et al., 2016; Dai et al., 2014). Depending on the DES composition, they may offer all characteristics to design atom economy, efficient, low cost and sustainable development for ready-to-use formulation, fully compatible with food and feed applications (Ruesgas-Ramón et al., 2017).

To resolve the complexity of DES extracts, "foodomics" can be applied since it represents a high-throughput approach able to elucidate the food chemical complexity by using chromatography as a separation method coupled with high-resolution mass spectrometry. Foodomics ensures simultaneously the coverage of diverse chemical specimens (*e.g.,* amino acids, lipids, carbohydrates, phytochemicals) (Cifuentes, 2009; Herrero et al., 2012). These tools are used to identify and quantify chemical species in their ionized forms by measuring their mass/charge (*m/z*). In this study, we have investigated the potential of DES to extract PCs from pigmented RB in comparison to conventional ethanol/water solvent.

The chemometrics tools were applied for the chemical data generate characterization of the obtained extracts. Typical phenolic acids of cereals were also quantified with a high-performance liquid chromatography (HPLC) fitted with a diode-array detector (DAD).

The DPPH (2,2-diphenyl-1-picrylhydrazyl) test is based on the antioxidant activity of a hydrogen donor that will allow the reduction of DPPH (Brand-Williams et al., 1995). Indeed, antioxidant activity is defined as the ability of an organism to protect itself against free radicals. This assay was performed to assess the capacity of extracts to stabilize radicals.

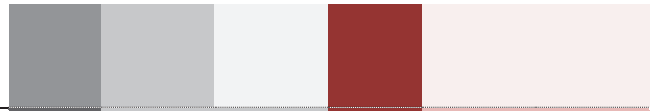
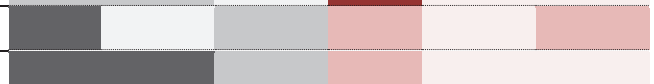
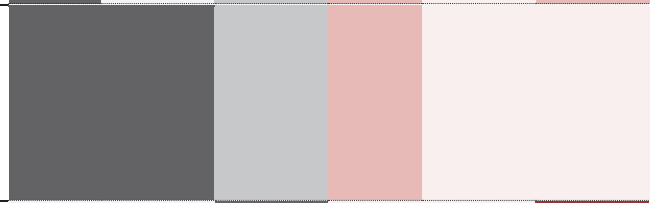
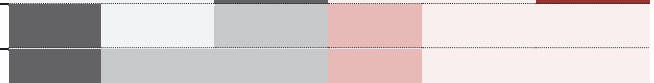
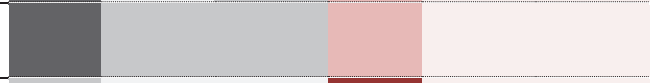
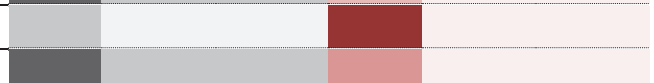
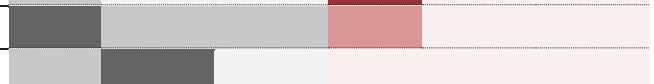
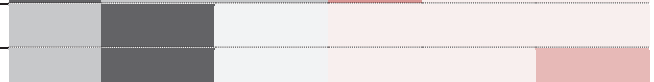
2. Results and Discussion

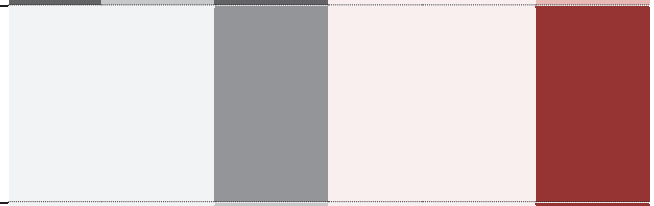
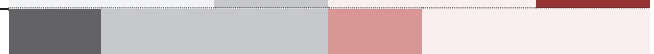
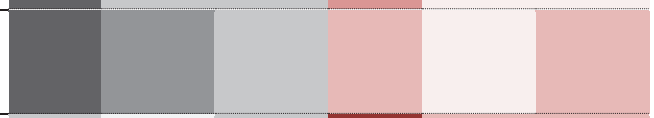
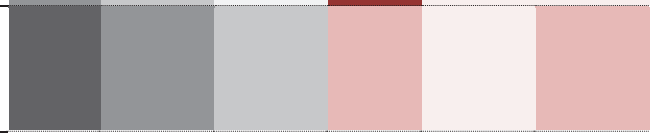
2.1. UPLC-MS^E Analyses to Unveil the Chemical Complexity and Diversity of PCs in RB Extracts

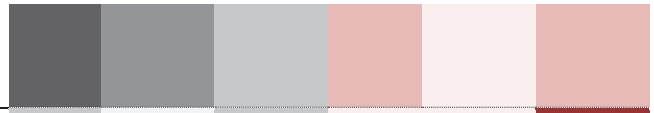
Considering the molecular complexity found in RB extracts, a putative identification of PCs and comparison between conventional and DES extracts with advanced UPLC-MS-MS tools was performed. Globally, a total of 89 PCs were tentatively identified in both extracts, including all extraction conditions. The putative PCs were identified following the recommendations of level 1 and 2 according to *Sumner et al. (2007)*,*(Schymanski et al., 2014)* considering mass to charge (*m/z*), retention time, isotopic similarity, precursor mass error, as well as the score and the fragmentation score for each identification attempt. These putative compounds are listed in the order of their retention time in Table 1. When all the parameters for the identification were equal, it was not possible to distinguish the compound and a multiple identification was proposed. In addition, compounds with the same m/z but with different retention times, were identified as isomers and were listed in the order of their retention time. In this work, 35 isomers of PCs were identified and 20 multiple identifications occurred.

Table 1. Putative identification of phenolic compounds (PCs) in pigmented rice bran (RB) extracts by UPLC-MS^E.

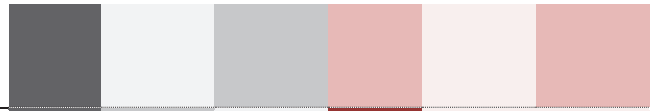
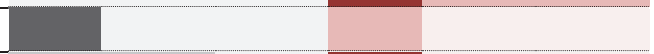
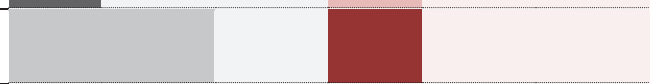
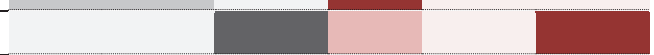
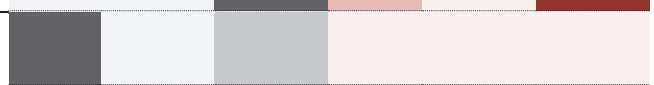
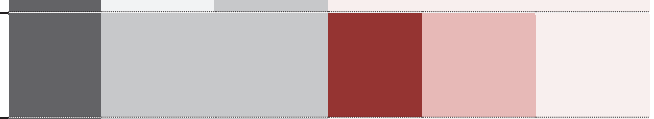
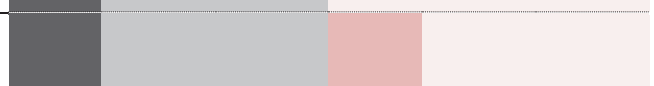
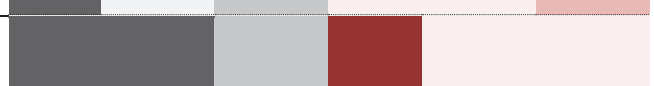
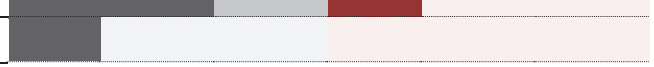
	Putative Compound	$[M-H]^-$	RT (min)	Molecular Formula	Score	FS	Fragments /Intensity	ME	IS	Black RB			Red RB		
										CS	DES1	DES2	CS	DES1	DES2
1	Gallic acid *	169.0131	1.26	$C_7H_6O_5$	37.3	0	Nd	−6.89	94.40						
2	Dihydroxybenzoic acid isomer I *	153.0181	1.41	$C_7H_6O_4$	37.1	0	Nd	−8.25	94.68						
3	4-Hydroxymandelic acid/Vanillic acid isomer I	167.0334	1.46	$C_8H_8O_4$	36.1	0	Nd	−9.59	90.85						
4	Dihydroxybenzoic acid isomer II	153.0180	1.48	$C_7H_6O_4$	37.1	0	Nd	−8.46	94.67						
5	Caffeoylquinic acid isomer I	353.0863	1.54	$C_{16}H_{18}O_9$	35.5	0	Nd	−4.19	82.23						
6	4-Hydroxymandelic acid/Vanillic acid isomer II	167.0337	1.64	$C_8H_8O_4$	37.8	0	Nd	−7.85	97.57						
7	Dihydroxybenzoic acid isomer III	153.0179	1.64	$C_7H_6O_4$	36.7	0	Nd	−9.25	93.70						
8	Apigenin 7-O-glucoside	419.1351	1.73	$C_{21}H_{24}O_9$	36.2	0	Nd	0.77	81.93						
9	Isorhamnetin/Rhamnetin/Nepetin	315.0498	1.74	$C_{16}H_{12}O_7$	37.7	0	Nd	−3.95	93.23						
10	Irilone	297.0392	1.75	$C_{16}H_{10}O_6$	35.6	0	Nd	−4.20	82.89						
11	Dihydro-p-coumaric acid/Methoxyphenylacetic acid	165.0545	1.81	$C_9H_{10}O_3$	36.3	0	Nd	−7.41	90.07						
12	Dihydroxybenzoic acid isomer IV	153.0180	1.85	$C_7H_6O_4$	38	0	Nd	−8.46	99.40						
13	Eriodictyol isomer I	287.0567	1.90	$C_{15}H_{12}O_6$	38.6	11.7	165.0543 (19%)	2.05	83.77						
14	Esculetin	177.0180	1.91	$C_9H_6O_4$	37.4	0	Nd	−7.54	95.53						
15	Quercetin 3-O-glucoside isomer I	463.0870	1.94	$C_{21}H_{20}O_{12}$	37.4	0	Nd	−2.69	90.16						

16	Syringic acid/Gallic acid ethyl ester/3,4-Dihydroxyphenyllactic acid	197.0444	2.09	$C_9H_{10}O_5$	36.5	0	Nd	−6.00	89.58	
17	Homovanillic acid/Dihydrocaffeic acid	181.0494	2.13	$C_9H_{10}O_4$	37.6	0	Nd	−6.85	95.91	
18	Myricetin	317.0290	2.16	$C_{15}H_{10}O_8$	38.9	5.13	124.0146 (17%), 123.0076 (11%)	−4.04	94.33	
19	Coumaroylquinic acid isomer I	337.0916	2.27	$C_{16}H_{18}O_8$	43.2	20.7	119.0488 (100%), 191.0549 (5%), 20111.0434 (2%)	−3.70	99.55	
20	4-Hydroxymandelic acid/Vanillic acid isomer III	167.0335	2.29	$C_8H_8O_4$	36.2	0	Nd	−9.01	90.85	
21	Cyanidin 3-*O*-beta-D-sambubioside	580.1488	2.38	$C_{26}H_{29}O_{15}+$	36.2	0.631	115.0401 (2%)	9.41	90.56	
22	Methylgallic acid	183.0289	2.42	$C_8H_8O_5$	38.6	0	Nd	−5.28	98.86	
23	Feruloylquinic acid isomer I	367.1023	2.45	$C_{17}H_{20}O_9$	57.3	91	134.0359 (100%), 193.0494 (29%), 200.0442 (25%), 117.0333 (14%), 123.0436 (7%), 155.0335 (2%)	−3.18	99.18	
24	Dihydroresveratrol	229.0878	2.54	$C_{14}H_{14}O_3$	36.2	0	Nd	3.43	85.26	
25	Scopoletin	191.0332	2.54	$C_{10}H_8O_4$	37	0	Nd	−9.04	95.14	
23	4-Hydroxymandelic acid/Vanillic acid isomer IV	167.0337	2.56	$C_8H_8O_4$	36.5	0	Nd	−7.53	90.85	
27	4'-O-Methylepigallocatechin	319.0809	2.58	$C_{16}H_{16}O_7$	35.5	0	Nd	−4.54	82.64	
28	Feruloyl glucose	355.1016	2.58	$C_{16}H_{20}O_9$	40.7	15.9	177.0545 (41%)	−5.08	93.62	
29	Bergapten/Xanthoxin	215.0335	2.60	$C_{12}H_8O_4$	44.9	36.8	191.0333 (100%)	−6.78	95.46	
30	Psoralen	185.0233	2.60	$C_{11}H_6O_3$	36.2	0	Nd	−6.24	88.21	
31	**(+)-Catechin ***	289.0705	2.67	$C_{15}H_{14}O_6$	35.7	0	Nd	−4.38	83.74	

32	Kaempferide	298.0465	2.69	$C_{16}H_{11}O_6^-$	39.4	20.9	175.0388 (76%), 134.0360 (27%), 193.0127 (13%), 117.0330 (1%)	−6.01	82.88	
33	**Caffeic acid ***	179.0335	2.72	$C_9H_8O_4$	38	0	Nd	−7.97	98.69	
34	Trihydroxyisoflavone	269.0443	2.73	$C_{15}H_{10}O_5$	38.6	0	Nd	−4.63	98.27	
35	Isorhamnetin 3-O-glucoside/Isorhamnetin 3-O-galactoside	477.1021	2.75	$C_{22}H_{22}O_{12}$	40.8	16.2	429.0818 (1%), 59.0113 (1%)	−3.68	92.19	
36	Hydroxymatairesinol isomer I	373.1303	2.77	$C_{20}H_{22}O_7$	43.2	28.9	205.0494 (100%),223.0601 (62%), 179.0700 (12%), 221.0805 (6%), 181.0491 (4%), 193.0854 (1%), 105.0331 (1%)	2.67	90.02	
37	Syringaresinol isomer I	417.1560	2.80	$C_{22}H_{26}O_8$	37.7	0	Nd	1.17	89.93	
38	Dicaffeoylquinic acid	515.1221	2.83	$C_{25}H_{24}O_{12}$	38.4	5.72	307.0909 (3%)	4.94	92.15	
39	Coumaroylquinic acid isomer II	337.0917	2.84	$C_{16}H_{18}O_8$	45.2	32.1	245.0803 (50%), 119.0486 (15%), 93.0327 (13%), 243.0651 (11%)	−3.56	98.01	
40	**(-)-Epicatechin**	289.0700	2.84	$C_{15}H_{14}O_6$	46.9	48.8	257.0438 (100%), 243.0651 (11%)	−6.23	92.53	
41	4-Hydroxymandelic acid/Vanillic acid isomer V	167.0337	2.90	$C_8H_8O_4$	51.6	75.8	151.0385 (100%), 123.0437 (14%), 135.0435 (4%), 105.0332 (1%)	−7.79	90.85	
42	3,4-Dihydroxyphenyllactic acid	197.0441	2.98	$C_9H_{10}O_5$	44.2	34.6	134.0357 (90%)	−7.18	94.67	
43	Feruloylquinic acid isomer II	367.1023	2.99	$C_{17}H_{20}O_9$	42.7	17.5	134.0357 (100%), 173.0443 (76%), 191.0546 (31%), 117.0330 (10%),	−3.03	99.41	

							111.0436 (10%), 155.0332 (7%), 75.0065 (5%)			
44	Rosmarinic acid	359.0775	3.02	$C_{18}H_{16}O_8$	35.9	0	Nd	0.85	80.69	
45	Quercetin 3-O-rutinoside/Kaempferol 3-O-sophoroside/Quercetin 3-O-rhamnosyl-galactoside/Kaempferol 3,7-O-diglucoside isomer I	609.1446	3.21	$C_{27}H_{30}O_{16}$	38	0	Nd	−2.44	92.86	
46	Tetrahydroxyisoflavone isomer I	285.0391	3.27	$C_{15}H_{10}O_6$	47.1	52.9	151.0386 (100%)	−4.70	88.27	
47	Eriodictyol 7-O-glucoside	449.1075	3.30	$C_{21}H_{22}O_{11}$	45.7	34.3	103.0386 (100%), 181.0498 (22%), 122.0359 (16%), 311.0760 (10%), 99.0075 (7%)	−3.24	97.82	
48	Dihydroxybenzoic acid isomer V	153.0181	3.31	$C_7H_6O_4$	38	0	Nd	−8.22	99.29	
49	Quercetin 3-O-rutinoside/Kaempferol 3-O-sophoroside/Quercetin 3-O-rhamnosyl-galactoside/Kaempferol 3,7-O-diglucoside isomer II	609.1454	3.38	$C_{27}H_{30}O_{16}$	42.7	16.9	300.0264 (8%)	−1.16	98.00	
50	Luteolin 7-O-rutinoside/Kaempferol 3-O-rutinoside/Chrysoeriol 7-O-apiosyl-glucoside	593.1495	3.39	$C_{27}H_{30}O_{15}$	37.1	1.66	103.0387 (7%), 175.0600 (2%)	−2.81	87.33	
51	Didymin/Poncirin	593.1884	3.40	$C_{28}H_{34}O_{14}$	36.7	2.47	103.0387 (100%), 175.0600 (23%)	1.41	82.63	
52	Salvianolic acid D	237.0395	3.44	$C_{11}H_{10}O_6$	38.2	0	Nd	−3.88	95.39	
53	***p*-coumaric acid ***	163.0389	3.47	$C_9H_8O_3$	40.8	12.3	163.0388 (28%),	−6.97	99.39	

							119.0488 (21%)			
54	Phloridzin	435.1277	3.48	$C_{21}H_{24}O_{10}$	37.2	7.68	103.0387 (100%)	−4.47	83.45	
55	Schisandrin B	399.1835	3.51	$C_{23}H_{28}O_6$	37.3	0	Nd	5.39	92.86	
56	Tectoridin	461.1080	3.54	$C_{22}H_{22}O_{11}$	41.8	16.4	341.0654 (4%), 146.0341 (1%)	−2.00	95.23	
57	Glycitin	445.1129	3.59	$C_{22}H_{22}O_{10}$	44.3	29.7	326.0777 (29%), 283.0593 (22%), 379.0769 (16%)	−2.51	94.59	
58	Isorhamnetin 3-O-rutinoside	461.1067	3.63	$C_{22}H_{22}O_{11}$	50.6	69.8	324.0255 (100%), 279.0288 (29%), 99.0070 (7%), 73.0274 (5%)	−4.82	88.73	
59	**Ferulic acid ***	**193.0495**	**3.64**	$C_{10}H_{10}O_4$	40	10.6	137.0590 (13%)	−5.68	96.05	
60	Paeoniflorin	479.1549	3.66	$C_{23}H_{28}O_{11}$	38.1	0	Nd	−2.09	92.84	
61	Tetrahydroxyisoflavone isomer II	285.0392	3.73	$C_{15}H_{10}O_6$	39.8	5.06	117.0331 (9%), 105.0330 (4%), 132.0206 (3%)	−4.38	99.00	
62	Violanone	315.0860	3.75	$C_{17}H_{16}O_6$	43.2	23.3	165.0543 (32%)	−4.45	98.07	
63	3,7-Dimethylquercetin/Jaceosidin/Tricin isomer I	329.0654	3.80	$C_{17}H_{14}O_7$	37.4	0	Nd	−3.97	91.62	
64	Diosmin	607.1656	3.81	$C_{28}H_{32}O_{15}$	38.6	1.83	89.0229 (100%)	−2.04	93.78	
65	Tetrahydroxyisoflavone isomer III	285.0393	3.88	$C_{15}H_{10}O_6$	38.4	0.795	123.0074 (1%)	−4.03	95.99	
66	Schisantherin A	535.2012	3.90	$C_{30}H_{32}O_9$	34.9	0.218	191.0701 (1%)	7.14	82.26	
67	Gardenin B	357.0968	3.91	$C_{19}H_{18}O_7$	47.4	51.3	209.0445 (100%), 315.0861 (45%), 239.0552 (32%), 327.0860 (22%), 345.0952 (17%),	−3.24	89.72	

							251.0550 (13%), 177.0547 (14%), 181.0491 (12%)			
68	Hesperidin	609.1820	3.93	$C_{28}H_{34}O_{15}$	36	0.274	161.0596 (4%)	−0.78	80.56	
69	Syringaldehyde	181.0495	4.29	$C_9H_{10}O_4$	38.4	0	Nd	−6.18	99.23	
70	Eriodictyol isomer II	287.0549	4.34	$C_{15}H_{12}O_6$	38.1	3.22	147.0071 (16%), 119.0123 (12%), 123.0075 (2%)	−4.19	92.29	
71	3,7-Dimethylquercetin/Jaceosidin/Tricin isomer II	329.0655	4.38	$C_{17}H_{14}O_7$	38.3	5.77	122.0355 (3%), 146.0350 (3%)	−3.70	90.18	
72	Nobiletin	401.1229	4.52	$C_{21}H_{22}O_8$	37.4	0	Nd	−3.14	90.76	
73	Hydroxymatairesinol isomer II	373.1281	4.62	$C_{20}H_{22}O_7$	38.1	2.26	146.0343 (10%)	−3.23	91.85	
74	Syringaresinol isomer II	417.1560	4.74	$C_{22}H_{26}O_8$	36.6	0	Nd	1.32	84.73	
75	Isorhamnetin isomer I	315.0495	4.79	$C_{16}H_{12}O_7$	49.4	59.6	175.3038 (100%), 160.0152 (20%)	−4.75	92.69	
76	Tetrahydroxyisoflavone isomer V	285.0393	5.01	$C_{15}H_{10}O_6$	42.7	21.6	133.0280 (100%), 132.0207 (9%), 179.0343 (4%)	−4.21	96.95	
77	3,7-Dimethylquercetin/Jaceosidin/Tricin isomer II	329.0648	5.04	$C_{17}H_{14}O_7$	40	10.8	121.0281 (35%), 139.0387 (4%), 147.0434 (3%), 119.0121 (2%)	−5.78	96.01	
78	**Quercetin**	**301.0342**	**5.06**	$C_{15}H_{10}O_7$	49.6	53.2	151.0023 (100%), 121.0181 (35%), 178.9973 (19%)	−3.80	99.43	
79	Dihydroquercetin *	303.0499	5.16	$C_{15}H_{12}O_7$	38.2	0	Nd	−3.67	95.13	
80	Trihydroxyisoflavanone isomer II	271.0595	5.38	$C_{15}H_{12}O_5$	37.4	0	Nd	−6.09	94.02	
81	Isorhamnetin isomer II	315.0498	5.56	$C_{16}H_{12}O_7$	40.1	6.44	117.0334 (13%)	−3.73	98.49	

82	6-Hydroxyluteolin/Morin	301.0338	5.56	$C_{15}H_{10}O_7$	37.5	0	Nd	−5.33	93.63	
83	Urolithin A	227.0337	5.56	$C_{13}H_8O_4$	39.8	19	183.0435 (100%), 182.0360 (24%), 167.0486 (7%)	−5.42	86.04	
84	Trihydroxyisoflavanone isomer III	271.0598	5.64	$C_{15}H_{12}O_5$	40	9.09	119.0487 (100%)	−4.98	96.67	
85	Hesperetin/Homoeriodictyol	301.0704	5.73	$C_{16}H_{14}O_6$	42.8	21.1	193.0492 (23%), 134.0358 (19%), 164.0097 (7%), 149.0591 (6%)	−4.41	97.88	
86	Dihydroxykaempferol	317.0290	5.75	$C_{15}H_{10}O_8$	42	23.2	271.0234 (100%), 107.0124 (11%)	−4.10	91.65	
87	Hispidulin	299.0545	5.80	$C_{16}H_{12}O_6$	37.6	0	Nd	−5.51	94.43	
88	Paeonol	165.0545	6.71	$C_9H_{10}O_3$	36.4	0	Nd	−7.14	90.07	
89	Rosmanol	345.1688	6.72	$C_{20}H_{26}O_5$	37.4	0	Nd	−5.74	93.66	
Number of identifications										**# 79** **# 49** **# 58** **# 67** **# 16** **# 43**

By relative ion abundance of each PC							
Black RB		**not present**		**< 50%**		**50 % < abundance <75 %**	**> 75%**
Red RB							

RT: retention time; FS: fragmentation score; ME: mass error; IS: isotope similarity; Nd: Not detected. Bold: reference standards; CS: conventional solvent. * identified by Zarei et al. (2018).

The PCs identified in this study belonged to different chemical classes that were listed by decreasing number of occurrences in the extracts (Figure 1). Regardless of the RB types and conditions of extraction, the flavonoids were the most representative class of PCs with 52% of occurrences, followed by the phenolic acids (33%), other polyphenols (8%), lignans (6%) and stilbenes (1%).

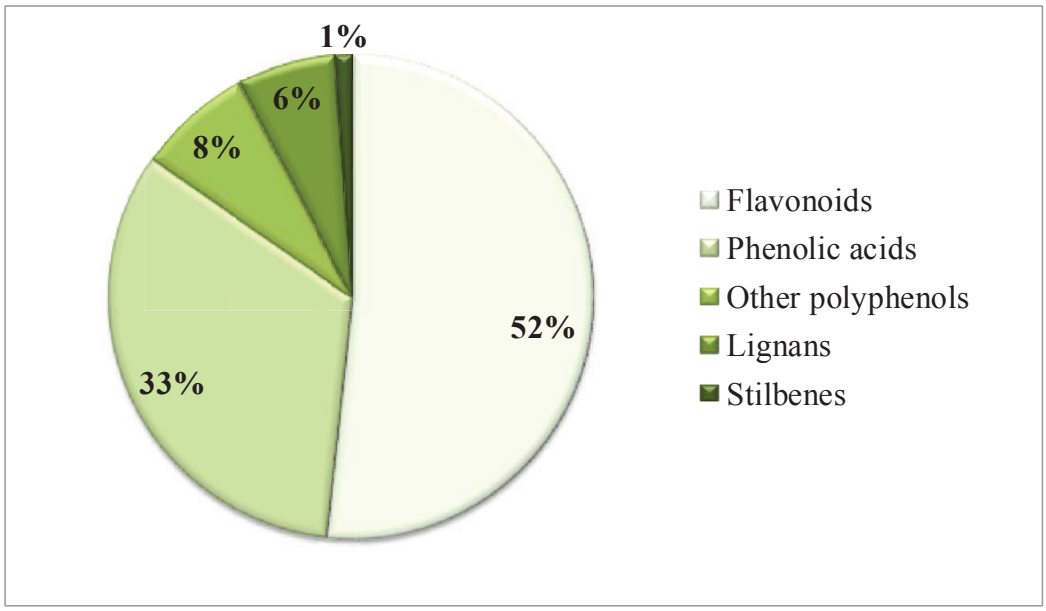


Figure 1. Percentage of number of tentatively identifications by class of phenolic compounds in all types of extracts whatever the RB.

As expected for pigmented rice, the flavonoids were the most abundant compounds present in the extracts, however only one anthocyanin (cyanidin 3-O-beta-D-sambubioside) was unambiguously identified. Considering the influence of the type of RB, a higher number of PCs was identified in the black rice RB (88) than in the red rice RB (81), which is in agreement with the literature (Shao et al., 2014a). Our results were in line with Pereira-Caro et al. (2013) who applied HPLC-PDA-MS^2 to identify and quantify compounds in pigmented rice Camargue grains (black rice cultivar Artemide and red rice cultivar Tam Tam). They registered the presence of 34 PCs (34 present in black rice vs. 20 in red rice). In addition, these authors also pointed out that black rice was 14 times richer in PCs than red rice. Among the identified PCs, the most abundant class were flavonoids (subclasses: anthocyanins, flavones, flavonols, and flavan-3-ols) corresponding to 60% of the total. More recently, a metabolomic study with 17 RB cultivars originated from 11 countries identified 23 PCs (Zarei et al., 2018). Among them, seven were also identified in the present study and are highlighted in Table 1. The presence of chlorogenic acids corroborates the results found by Pang et al. (2018) who identified two isomers of feruloylquinic acids. However, they did not detect the

presence of caffeoylquinic acid and coumaroylquinic acid. In the present work, we could identify the presence of two isomers of feruloylquinic acids (*m/z* 367.1023 $[M–H]^-$) and two isomers of coumaroylquinic acid (*m/z* 337.0916 $[M–H]^-$) among the discriminant PCs. In addition, one isomer of caffeoylquinic acid (*m/z* 353.0863 $[M–H]^-$) and one isomer of dicaffeoylquinic acid (*m/z* 515.1221 $[M–H]^-$) were also identified (Table 1).

Although the presence of various anthocyanins has not been evidenced, PCs closely related to anthocyanins metabolism (Chen & Hrazdina, 1981) have been identified such as dihydroquercetin, quercetin (the fourth most abundant PC identified in this study) and myricetin.

2.2. Focus on the Tentative Identification of Phenolic Compounds by Solvent

To better visualize common and unique PCs within the different extracts, PCs were displayed under the form of a Venn diagram (Figure 2). When looking at PCs in common in the three types of extracts either for black RB or for red RB, 36 common PCs out of 88 were identified in black RB versus 12 only out of 81 in red RB. This limited common pool of PCs underlines the specificity of extraction of each type of extract.

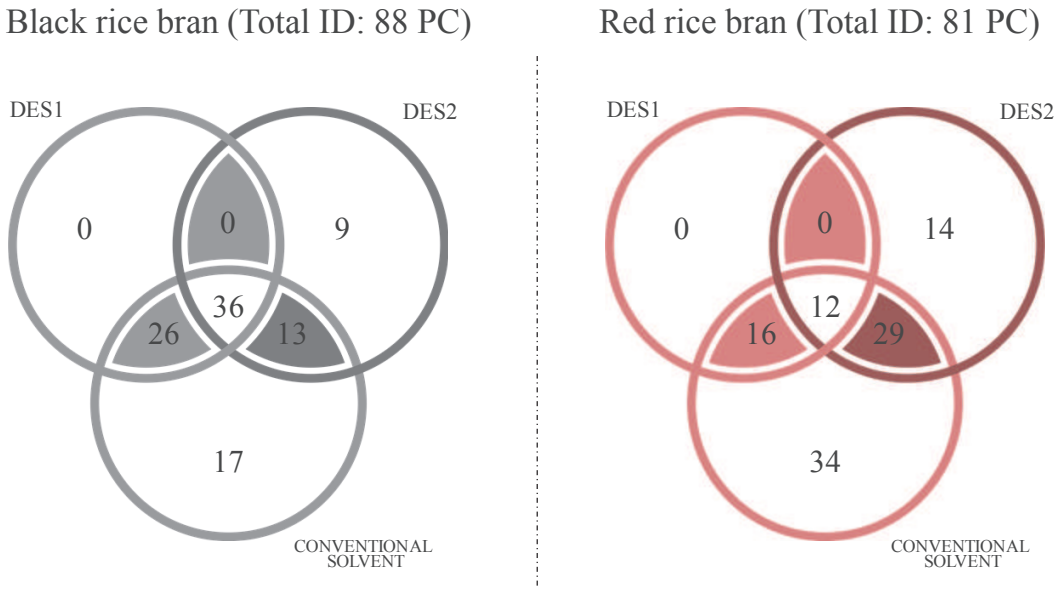


Figure 2. Venn diagrams of identified phenolic compounds by pigmented RB in the different extracts.

Focusing on unique compounds this time, the Venn diagram indicated some pools of unique compounds for conventional and DES2 (choline chloride/lactic acid, 1:10) extracts only. Unique PCs extracted with conventional solvents and identified by MS will not be discussed further since they have already been evidenced in the literature (Pereira-Caro et al.,

2013; Shao et al., 2014a; Zarei et al., 2018). Considering DES2 solvent, it allowed the extraction of additional and unique compounds as follows, nine unique PCs were obtained from black RB and 14 from red RB. The acidity of the DES2 is probably effective in triggering bound phenolic acid hydrolysis and their release in the extract, as already pointed out by Ruesgas-Ramón et al. [23]. Loypimai et al. (2017) compared different solvents and showed that acidified solvents (with added HCl) led to better yields of PCs extraction. In our study, we also observe that DES2 is able to extract glycosylated or ester cyclic molecules. In comparison, no unique PC was identified in DES1 extracts which had a pool of PCs in common with conventional extracts (respectively 26 for the Black RB and 16 for red RB).

If we try to identify these unique PCs in DES2, some of these PCs had already been described in the literature for their bioactivities. For instance, dihydroresveratrol (*m/z* 229.0871 $[M–H]^-$), rosmarinic acid (*m/z* 359.0775 $[M–H]^-$), and the paeonol (*m/z* 165.0545 $[M–H]^-$) are known to be biomarkers of inflammation pathways, protecting the nervous and cardiovascular system (Gakh et al., 2010; Luo et al., 2020), as well as inducer of apoptosis and proliferative inhibitor in liver and kidney (Bixi et al., 2016). Syringaresinol (*m/z* 417.1560 $[M–H]^-$) is a lignin compound that also plays a key role in inhibiting the proliferation of cancer cells (Park et al., 2008), but it can also play an important biotechnological role.

Indeed, Janvier et al. (2017) has shown that it can be an interesting substitute to the synthetic bisphenol A (BPA) compound in the polymer production. Eriodictyol (*m/z* 287.0567 $[M–H]^-$), being one of the precursors of anthocyanins (Zha & Koffas, 2017), is a flavonoid of a great importance. It is one of the main PCs present in citrus fruits, and exhibited antioxidant, antimicrobial, anti-inflammatory and antidiabetic activity (Li et al., 2016; Lv et al., 2019). It is used to mask bitter taste in beverages and in pharmaceutical industries (Rajan et al., 2018). Therefore, we have evidenced that DES2 is a good medium to extract specific RB PCs known for their biological activities. However, this specificity will very likely be dependent on the matrix (Ruesgas-Ramón et al., 2017).

To conclude the comparison of extraction potentials between conventional and DES solvents, although the conventional solvent extracted a higher number of PCs compared to DES, the possibility to extract different and unique PCs in DES2 offers an interesting alternative for both, the biomass characterization and the valorization of biomass-derived bioactive molecules.

2.3. Multivariate Analysis from the Measured Relative Abundance of Phenolic Compounds

The application of the multiplexed UPLC-MS^E method has enabled to quantify the relative abundance of identified compounds from the total ion counting. In Table 1, the relative abundance was summarized by color considering the percentage of each identified compound calculated from the total relative abundance (abundance higher than 75%, between 75% and 50% and lower than 50%).

Two isomers of dihydroxybenzoic acids were the most abundant and prevalent compounds in this study. According to the literature, one may suppose that these isomers were protocatechuic and gentisic acids (Pang et al., 2018; Shao et al., 2014b). Indeed, Zarei et al. (2018) evidenced that protocatechuic acid was the most abundant PCs of the pigmented RB varieties and was even present in larger quantities in black rice.

To analyze the interrelations between the samples, the data were submitted to multivariate analysis by principal components analysis (Figure 3). The relative ion abundance of each putative identified PC was considered as a variable and the score of each sample was calculated (Figure 3a). The principal component 1 (PC1) accounted for 56% and the PC2 for 25% of the total variation in the dataset. It is possible to distinguish black and red RB and to highlight the differences in the PCs profile between the extraction conditions. Indeed, the red RB extraction profiles obtained with the two DES stayed close when projected on PC1 and PC2, while PC1 axis helped separating the black RB from the red RB extracts. It was marked by a high concentration in flavonoids or phenolic acids derived from hydroxybenzoic acids and chlorogenic acids. Conversely, extracts with a low score on PC1 gathered the RB extracts and were marked with lignans and phenolic acids derived from hydroxycinnamic and hydroxyphenylacetic acids. PC2 resumed less variability but separated conventional extracts, on the superior quadrant presenting high scores, from DES extracts (inferior quadrant).

The covariance p[1] and correlation p(Nicoletti et al.)[1] loadings from a two-class Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) model (conventional solvent vs. DES) are displayed in a S-plot format (Figure 3b), where the variables (squares) are represented by the putative PCs. The upper right quadrant of the S-plot showed the PCs, which were elevated in DES extracts, while the lower left quadrant presented the PCs which were elevated in conventional solvent extracts. The measured intensities and factor of changes were based on the average of the measured values for each PCs in the group. To ease the reading of such an S-plot, we must specify: the further away from the *x*-axis the compound is, the greater the contribution to the variation between the groups, while the further away from the *y*-axis, the greater the reliability of the analytical result, thus the significance. The two

most important PCs that explained differences between DES and conventional extracts were the coumaric acid (−0.44; −0.95) and the syringaldehyde (−0.26; −1).

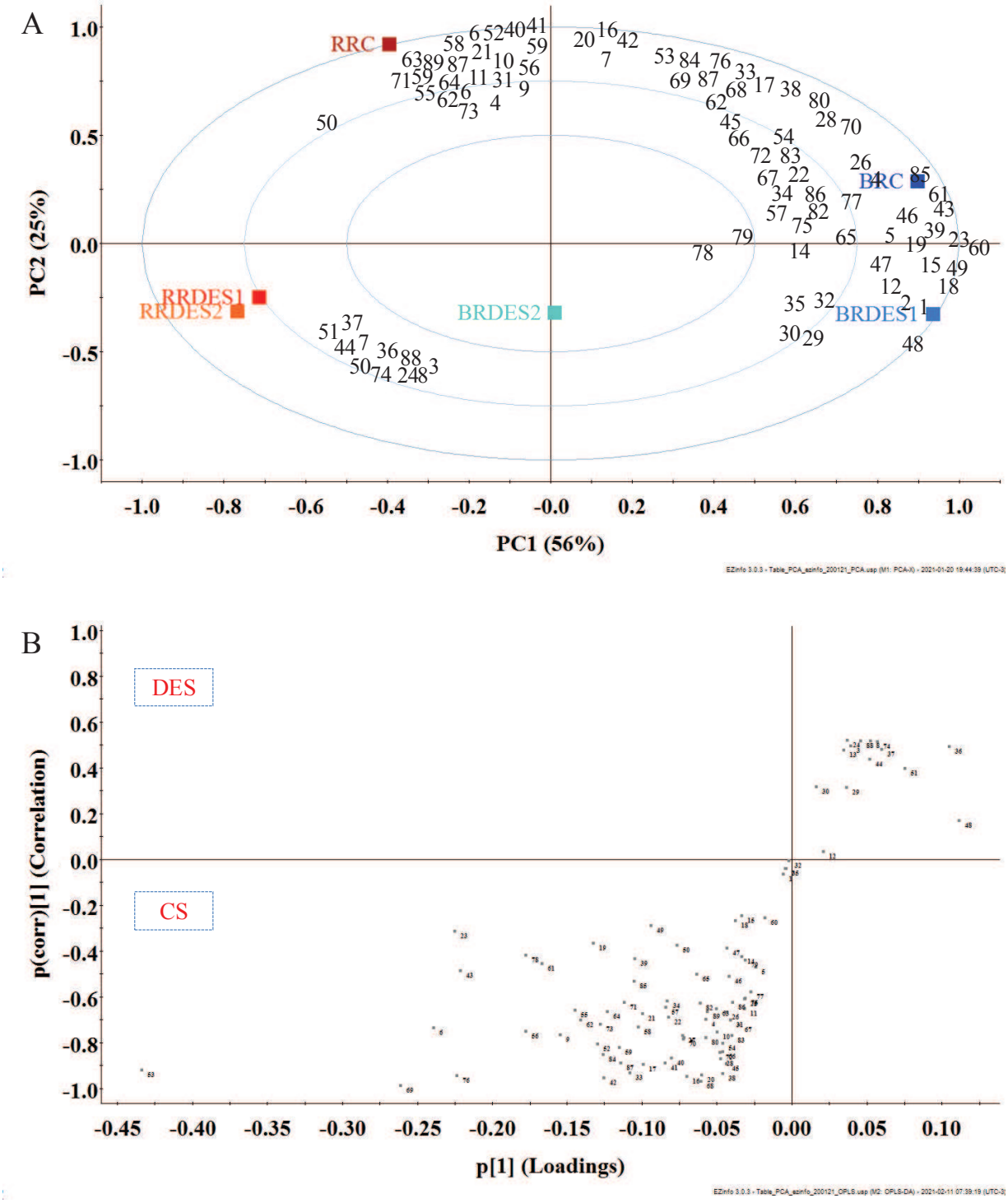


Figure 3. (**A**) Principal components analysis of PCs of pigmented RB extracted with conventional and deep eutectic solvents (DES) methods; (**B**) S-plots from Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) modeling of conventional solvent (Bucsella et al.) extracts versus DES extracts. The loadings are

represented by the respective numbers to each putative phenolic compound described in Table 2. Abbreviations: red rice conventional (RRC), red rice DES1 (RRDES1), red rice DES2 (RRDES2), black rice conventional (BRC), black rice DES1 (BRDES1), black rice DES2 (BRDES2).

2.4. Further Characterization of Extracts

2.4.1. Quantitative Examination of the Typical Phenolic Acids Found in Cereals

The quantification of five typical phenolic acids (gallic, vanillic, *p*-coumaric, sinapic and ferulic acids) in the different extracts of black and red RB was carried out and is displayed in Figure 4. These phenolic acids were selected because they had already been found in the pigmented RB (Irakli et al., 2012b; Shao et al., 2014a; Sumczynski et al., 2016; Zaupa et al., 2015). The quantification profile of those phenolic acids was different and dependent of the extraction conditions. In this work, conventional solvent and DES1 resulted in extracts with higher content of these five phenolic acids in comparison with DES2. This study points out that some DES solvents can have high extraction capacity leading to broad profile of extracted PCs and can thus be good substitutes for conventional solvents while other DES formulations can lead to narrower and more specific PC extraction profiles.

Moreover, such quantification of phenolic acids allowed strongly differentiating red and black RB extracts (Figure 4A). Indeed, whatever the solvent used for extraction, red RB extracts were less concentrated in phenolic acids than the black RB extracts. These results were coherent with literature which has already demonstrated that among colored rice, black rice outer layers contains more phenolics than red rice (Irakli et al., 2012b). Black RB extracts contained sixfold more phenolic acids than red RB, with a total ranging between 37–66 mg/100 g in black RB extracts vs. 6–20 mg/100 g in red RB extracts. These contents were in agreement with other authors in literature (Sumczynski et al., 2016). Among these compounds (Figure 4B), the ferulic acid was the most abundant in most cases (26–34 mg/100 g of black RB vs. 2–8 mg/100 g of red RB). Ferulic acid was indeed reported to be the main phenolic acid in cereals, especially in the bound fraction (Zhang et al., 2015). The second most concentrated phenolic acid quantified was vanillic acid in red RB, and *p*-coumaric/sinapic acid in black RB. These concentrations differed depending on the combination of solvent type (conventional solvent vs. DES1 vs. DES2) and of the type of matrix. Our results showed lower levels in vanillic acid in red RB compared with black RB in conventional extracts. Such result contrasted from the ones of Shao et al. (2014b) who reported that vanillic acid was found only in black rice. Gallic acid was the least concentrated phenolic acid among the five quantified compounds, being more concentrated in black RB than in red RB, in agreement with Shao et al. (2014a).

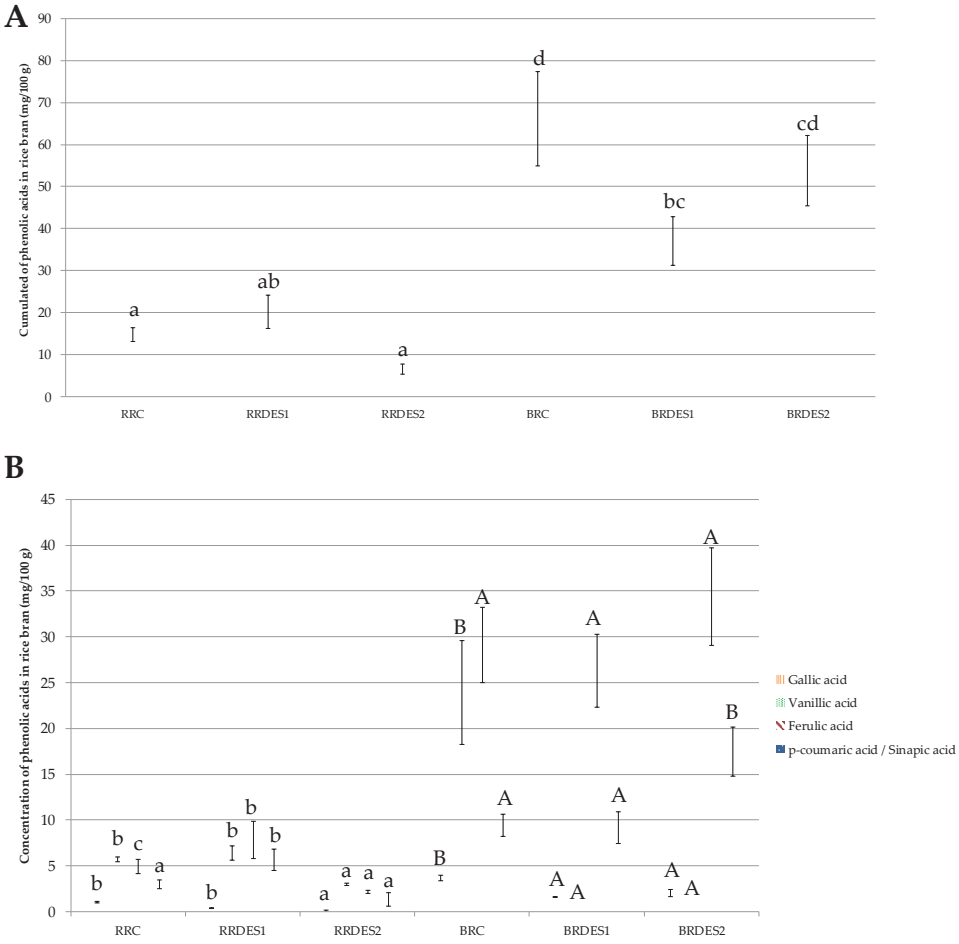


Figure 4. Concentration (mg/100 g) of RB extracts in five typical phenolic acids found in cereals. (**A**) Cumulated concentration of the five phenolic acids quantified in this study. Lowercase letters indicate significant differences in cumulated concentration of the amount of the five phenolic acids between extract ($p < 0.05$). (**B**) Quantification of gallic, vanillic, ferulic and *p*-coumaric/sinapic acids in RB extracts. Lowercase letters indicate significant differences between red RB extracts for a given phenolic acid. Uppercase letters indicate significant differences between black RB extracts for a given phenolic acid ($p < 0.05$). Abbreviations: red rice conventional (RRC), red rice DES1 (RRDES1), red rice DES2 (RRDES2), black rice conventional (BRC), black rice DES1 (BRDES1), black rice DES2 (BRDES2).

2.4.2. Scavenging Ability of RB Extracts Assessed by the DPPH Radical Assay

DPPH assay was conducted to assess the reducing power of the RB extracts by different solvents. The EC_{50} was expressed as equivalent of RB extract in mg/mL.

EC_{50} of 0.26 and 0.18 mg/mL were obtained for black RB DES1 and DES2 extracts respectively ($p < 0.01$). In comparison, higher EC_{50} of 0.46 and 0.36 mg/mL were obtained for red RB DES1 and DES2 extracts, respectively ($p < 0.01$).

Two conclusions can be drawn from these results:

(1) Whatever the DES, black RB extracts have higher capacity to reduce the DPPH radical, i.e., lower EC_{50}, in comparison with red RB extracts ($p < 0.01$). Such higher reducing capacity is coherent with black RB highest load in PCs (Goufo & Trindade, 2017). We must

precise that at concentrations of black RB extracts of 0.50 mg/mL, total reduction of DPPH was already reached and very likely explained by the high PC content of the extract. A review conducted by Goufo and Trindade (2014) has similarly shown that the EC_{50} of pigmented RB was 16 times lower than of nonpigmented RB extracts. Other studies have shown that the highest antioxidant capacity of pigmented rice compared to nonpigmented rice (Chakuton et al., 2012) was due to the presence of proanthocyanidins and anthocyanins. Such compounds would be abundant in red rice (Sabir et al., 2017) but even more in black rice (Chen et al., 2012; Min et al., 2011).

(2) Whatever the type of RB, DES1 RB extracts showed the best results in comparison to DES2 RB extracts. Control experiments were also conducted using DES only to determine their individual contribution to DPPH reduction. These control experiments pointed out that DES solvent on their own were responsible for 16% and 37%, DES1 and DES2 respectively, of the reducing capacity in these extracts.

3. Materials and Methods

3.1. Chemicals

Choline chloride (≥99%), lactic acid (~90%), 1.2-propanediol (≥99%), ethanol (reagent), methanol (CHROMASOLV® ≥ 99.9%), water (CHROMASOLV® Plus grade HPLC), 2,2,1-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) gallic, ferulic, *p*-coumaric, sinapic and vanillic acids were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Pigmented RBs

Red (*cv. TamTam*) and black (*cv. Artemide*) rice grains cultivated in Camargue were provided by the "Centre Français du Riz" (Arles, France). Grains (5 kg) were subject to dry abrasion using a DMS 500 huller (Electra, Poudenas, France) to obtain a fine bran fraction corresponding to between 4–7% of the grain mass. Acronyms were used to identify the analyzed RB extracts throughout the document: red rice conventional (RRC), red rice DES1 (RRDES1), red rice DES2 (RRDES2), black rice conventional (BRC), black rice DES1 (BRDES1), black rice DES2 (BRDES2).

3.3. Preparation of Deep Eutectic Solvents

Two choline-chloride based DES in combination with lactic acid or 1.2-propanediol were selected based on GRAS components and according to the previous study from

Ruesgas-Ramón et al. (2017). DES were prepared by heating (60 °C), agitating (400 rpm) (IKA KS 4000 I control, Staufen, Germany) and mixing the components at the corresponding mole ratios (Table 1) in a closed bottle for 45 min until a clear liquid is formed (Ruesgas-Ramón et al., 2017). The water content (Coulometer Karl Fisher GRS 2000, KF TITRATOR, Bioblock scientific, France), water activity (a_w) (Aqualab, Decagon Devices Inc., Pullman, WA, USA) and pH (EcoScan pH 5 Palmtop pH-meter, Legallais, Montferrier-sur-Lez, France) were determined (Table 2)

Table 2. Composition of DES and measurement results A_w, Karl Fisher water content and pH.

Name	# 1	# 2	# 3	Molar Ratio	aw	Water Content	pH
DES1	Choline chloride	1.2-propanediol	Water	1:1:1	0.51 ± 0.03	7.2 ± 0.5	5.42
DES2	Choline chloride	Lactic acid	-	1:10	0.29 ± 0.00	6.3 ± 0.25	--

Abbreviations. a_W = water activity, # = component, --: negative values linked to very acidic and specific medium.

3.4. Extraction of Phenolic Compounds from RBs

One hundred mg of pigmented RB were extracted in triplicate with 2 mL of DES1 or DES2 or conventional solvent (ethanol/water, 60:40 *v/v*) in a closed amber glass flask in an orbital agitation (400 rpm, 40 °C, 25 min) (Cimarec Thermo Scientific Poly 15, Legallais, Montferrier-sur-Lez, France). The samples were cooled to room temperature (~20 °C) and centrifuged for 5 min at 4000 rpm (CR412 centrifuge; Jouan, Winchester, VA, USA). The supernatant was filtered with a cellulose filter (0.45 µm) (Minisart Legallais, Montferrier-sur-Lez, France), dried under nitrogen stream and then dried extracts were stored at −20 °C.

3.5. HPLC-DAD Characterization

The obtained extracts were analyzed in triplicate in a HPLC (LC-20AD with oven: CTO-10ASvp and detector DAD SPO-M20, Shimadzu, Noisiel, France) at 280 nm. Conventional solvent extracts were dissolved in a MeOH/water (2:1, *v/v*) and filtered on 0.45 µm cellulose filter (Minisart Legallais, Montferrier-sur-Lez, France). Those obtained by DES were diluted five times in water and then injected. The separation was carried out on a C18 column (Kinetex High purity, 5 µm, 100 A, 250 × 4.6 mm, Thermo Electron, Burlington, MA, USA) with the mobile phase A (MeOH, 0.1% acetic acid) and B (water, 0.1% acetic acid), flow rate of 1 mL/min and gradient method: 0–5 min: isocratic at 10% of B; 5–20 min:

linear gradient up to 100% B; 20–30 min isocratic at 100% B; 30–35 min linear gradient up to 10% B; 35–42 min: equilibration at 10% B. Quantification of phenolic acids was performed by using a standard calibration curve made with different concentrations (0.01–3 mg/mL) of pure ferulic, *p*-coumaric, sinapic, vanillic and gallic acids.

3.6. Metabolomic Analysis and Data Processing

Analyses were performed by UPLC Acquity (Waters, Milford, CT, USA) coupled to the Xevo G2-S Q-Tof (Waters, Manchester, UK) equipped with an electrospray ionization source and acquired using a multiplexed MS/MS acquisition with alternating low and high energy acquisition (MS^E). Data were processed by Progenesis QI (NonLinear Dynamics, Waters) with the PubChem and Phenol Explorer online database according to Santos et al. (2019a).

3.7. DPPH Assessment of the Reducing Power of Extracts

The DPPH scavenging antioxidant activity was estimated according to the traditional method (Brand-Williams et al., 1995) but adapted to microplate assay (TECAN, Infinite M1000 PRO, Gröedig, Austria). Briefly, 20 µL of the samples and 180 µL of a methanolic solution of DPPH (final concentration in well 150 µM) were added on microplates (ThermoFischer, Courtaboeuf, France) and the absorbance was immediately read at 515 nm, every 5 min for the first 20 min, and then every 20 min for 1 h. Blanks with DES or ethanol/water 60:40 *v/v* were carried out to evaluate and to subtract the reducing activity of solvents. EC_{50} corresponds to the concentration of RB extracts (mg/mL) able to reduce 50% of the initial DPPH. Assay was performed at 37 °C in triplicate for each sample.

3.8. Statistics Analysis

EZInfo v. 3.0.3 (Umetrics, Sweden) was used for the analysis of metabolomics multivariate data. Principal Component Analysis (PCA) using pareto-scaling (van den Berg et al., 2006) and S-plot by Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) were generated from UPLC-MS^E data. Matrices of data gathered all compounds' abundances for each type of solvent and the type of pigmented rice. The HPLC and DPPH data were submitted to one-way ANOVA (Tukey, $p < 0.05$) by using R statistics (v 4.0.2).

4. Conclusions

In the present study, complementary techniques were applied to characterize pigmented RB extracts obtained either with conventional or DES extraction procedures. Use of modern analytical tools allowed the quantification of the most typical phenolic acids in rice as well as the description of the PC present in the different extracts. This approach underlined the high extraction capacity of DES, and most importantly, the extraction specificity of acidic DES for certain PCs. Whatever the solvent (conventional or DES), black RB remained the most enriched in PCs, and the source of RB with the highest potential for applications in human nutrition. To the best of our knowledge, this work was the first omics approach to characterize PCs in the pigmented RB DES extracts. In addition, a DPPH assay revealed that these pigmented RB extracts in DES presented an interesting reducing power, which opens the way to conceive new-pigmented RB formulations with these innovative liquid mixtures. This approach of extraction and omic characterization of extracts should be repeated on other pigmented rice varieties including purple, black-purple, orange or brown variants, known for their high load in flavonoids, anthocyanin and proanthocyanidin (Mbanjo et al., 2020). Therefore, more extensive works are currently under investigation to develop dietary or pharmaceutical formulations to improve the health benefits associated with the presence of these bioactive compounds.

Author Contributions: Conceptualization, M.C.B.S., N.B., V.L.-P., M.S.L.F. and C.B.-L.; data curation, M.C.B.S., N.B. and M.R.; formal analysis, M.C.B.S., B.B. and M.R.; funding acquisition, L.C.C., M.S.L.F. and C.B.-L.; investigation, M.C.B.S., N.B., B.B. and M.R.; methodology, M.C.B.S., B.B. and M.R.; project administration, M.S.L.F. and C.B.-L.; resources, V.M., V.L.-P., P.V., L.C.C., M.S.L.F. and C.B.L.; supervision, E.D., P.V., M.S.L.F. and C.B.-L.; validation, M.C.B.S., E.D., B.B., V.M., V.L.-P., P.V., L.C.C., E.P.R., M.S.L.F. and C.B.-L.; visualization, M.C.B.S., N.B., M.S.L.F. and C.B.-L.; writing-original draft, M.C.B.S.; writing—review and editing, M.C.B.S., N.B., E.D., V.M., V.L.-P., P.V., E.P.R., M.S.L.F. and C.B.-L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financed by the Foundation for Research Support of State of Rio de Janeiro (FAPERJ) (26/010.100.988/2018; 26/202.709/2018; 26/201.848/2019), National Council for Scientific and Technological Development (CNPq) (427.116/2018; 310.34/2019-4) and supported by UNIRIO, Coordination for Improvement of Personnel with Higher

Education (CAPES) (financial code 001). This work was also financed by a cofunding from TRANSFORM INRAE (FRANCE) department.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors are grateful to the Centre Français du Riz (Arles, France) for providing rice grains and to G. Cros (IBMM, UMR CNRS 5247, Faculté de Pharmacie Montpellier) for its gift of collected and collecting samples. The authors are also grateful to L. Ollier (now in UMR QualiSud, CIRAD, Montpellier) for her help in preparation of bran samples. The authors thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, code 001), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ, 26/010.100988/2018; 26/202/709–2018; 26/201.848/2019) and National Council for Scientific Technological Development (CNPq) (310343/2019-4) for the financial support.

Conflicts of Interest: The authors declare no conflict of interest.

Part III

Rice coproducts as an important source of a complex mixture of bioactive compounds

From the results obtained in part II, RB showed an interesting phenolic profile compared to the wheat coproducts. One of the main final outputs of this thesis is to link PC with lipids to produce enriched extracts with higher antioxidant capacity and better applicability in different food formulations, especially those that are more hydrophobic. The RB presents a higher lipid content than wheat bran. This point was considered to select the RB as food matrix for the following experiments. So, a vast literature review was performed on this coproduct, and a gap was found in the elucidation of the lipid profile from omics tools. In order to increase representativeness and elucidate the composition of rice coproducts, the number of samples was substantially increased, accompanied by the need to characterize the lipid profile of these samples as mentioned above.

Aim: RB presents exciting opportunities as a novel food ingredient with beneficial effect on human health due to its richness in bioactive compounds. These bioactive compounds gather PC but also minor lipids that will be explored in the present chapter. However, RB is also a challenging food matrix due to its susceptibility to lipid peroxidation. In this part, we propose cutting-edge lipidomics approach across a global set of rice cultivars grown under different environmental and agricultural conditions to deeply study and advance the valorization of RB as a supplement for human nutrition.

Based on the following publications:

- Chapter 6 – Comprehensive chemical characterization of a RB collection gathering different cultivar, grown and collected under different environmental and agricultural conditions. To be submitted to the Journal of the American Oil Chemists' Society (Impact factor: 1.720)

- Chapter 7 – (In preparation for submission) RB lipidome identifies novel phospholipids, glycolipids and oxylipins and roles in lipid metabolism of hypercholesterolemic children. To

be submitted to Molecular Nutrition and Food Research, in December 2021 (impact factor: 5.28)

Main results:

- RB present an important and rich source of lipids from different classes
- Lipidomics highlights the presence of bioactive polar lipids and oxylipins
- Clinical trial emphasizes the presence of putative lipid modulations associated with RB consumption
- Red RB stood out for the highest abundance of PC and for preserved lipids (TAG) after heat-treatment and could be selected for biotransformation strategy

Chapter 6 – Comprehensive chemical characterization of rice bran collection gathering different cultivar, grown and collected under different environmental and agricultural conditions

Millena Cristina Barros Santos[1], Nathalie Barouh[2], Valérie Lullien[3], Elizabeth P Ryan[4], Mariana Simões Larraz Ferreira[1] and Claire Bourlieu-Lacanal[3]

[1]Laboratory of Bioactives, Food and Nutrition Graduate Program, Federal University of State of Rio de Janeiro (UNIRIO), Rio de Janeiro, Brazil
[2]CIRAD, UMR Qualisud, F-34398 Montpellier, France. Qualisud, Univ Montpellier, Avignon Université, CIRAD, Institut Agro, Université de La Réunion, Montpellier, France.
[3]UMR IATE 1208 INRAE/CIRAD/SUPAGRO/UM, Montpellier, France
[4]Department of Environmental and Radiological Health Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, 80523, USA

To be submitted to the Journal of the American Oil Chemists' Society

Abstract

Rice is a staple food consumed globally and its processing results in a 60 million of tons of rice bran (RB) per year worldwide. RB is typically wasted or sold as feed. However, RB presents a potential opportunity as a novel food ingredient with human health benefits related to its bioactive lipids. The aim of this study was to perform a physical-chemical characterization across a global set of rice cultivars grown under different environmental and agricultural conditions. Such characterization can help in the valorization of RB as a supplement for human nutrition. Physico-chemical analyses were performed such as particle size, moisture content and water activity. Thin layer, gas and liquid chromatography were applied for the elucidation of the lipid classes present, fatty acid profile and the content of phenolic compounds, respectively. The physical analyses of the size distribution of particles indicated heterogeneous distribution representative of variable milling process. Some RB presented water activity higher than 0.6 (France cultivars) that may lead to microbial deterioration and can be explained by post-harvest processing and storage of RB. All RB samples presented as a rich source of lipids including bioactive classes (polar lipids, phytosterols), although high amounts of free fatty acids were also observed (especially in France and Cambodia cultivars), associated do lipolysis. Total lipid profile was shown to be influenced by genotype, production location and post-harvest conditions. Fatty acid composition showed a high source of unsaturated fatty acids (oleic acid, C18:1n9 and linoleic acid, C18:2n6). Red RB (Madagascar) showed up to 50 times higher phenolic compound content than non-pigmented RB. This study thus corroborates the fact that RB is a potential source of bioactive compounds, highlighting lipid classes that play an important role in human metabolism (polar lipids). More robust analysis such as omics tools should be applied for a global characterization of the lipids present in RB.

Keywords: rice coproduct, lipids classes, phenolic compounds.

1. Introduction

Rice (*Oryza sativa L.*) is one of the most produced and consumed cereals in the world. Asia is the main contributor to world production and consumption, with a per capita consumption of 78 kg/year. Africa, Latin America (where Brazil is the largest producer) and the Caribbean are also important consumers with a per capita consumption of 28 kg/year followed by North America and Europe with 14 kg/year and 5 kg/year, respectively (FAOSTAT, 2020).

The health-promoting properties due to brown rice consumption are attributed mainly to its phytochemicals, also including γ-oryzanol, tocopherols, tocotrienols, anthocyanins, and phytosterols present in the outermost layers of the grain. The presence of these compounds has increased not only research interest in pigmented rice varieties (black and red), but also in brown species (Mahanta and Saikia, 2016). At present, the quality parameters of cooked rice grains are not only related to its sensory characteristics, but also to the presence of bioactive compounds (Melini et al., 2019).

The consumption of polished rice grains increases the amount of agro-industrial waste and, consequently, the volume of generated coproducts (e.g., bran). Therefore, to value these coproducts and transform them into new products and functional ingredients has become a priority. This can generate different applications for the industry with the aim of increasingly targeting human consumption. *In vivo* studies have shown that rice bran (RB) consumption holds great promise as a functional food acting to prevent Salmonella colonization and to regulate intestinal immunity (Goodyear et al., 2015).

The composition of food coproducts is generally rich in bioactive compounds (vitamins, minerals, phenolic compounds and lipids), but variable. Phenolic compounds exert a wide range of bioactivities, generally attributed to their high antioxidant activity, and participate in metabolic processes that can reduce the risk or inhibit the development of chronic non-communicable diseases, many of which have an immune background. Bioactivity depends on the structure, particularly the number and position of hydroxyl groups and the type of substitutions on the aromatic rings (Heim et al., 2002).

Like phenolic compounds, lipids also participate in various mechanisms of action and can have a positive impact on human health as well as in biotechnological processes (Rodrigues et al., 2017). Its amphiphilic character also allows its application in emulsifying and stabilizing media (Vázquez et al., 2019).

In this study was performed a physico-chemical characterization of RB focusing on for the chemical part lipids and phenolic compounds.

2. Material and methods

2.1. Reagents

Solvents (ethanol, chloroform, and methanol) and reagents (potassium hydroxide, phenolphatlein, methylate sodium, hydrochloric methanol, potassium iodide, thiodene, and sodium thiosulfate) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Fatty acid methyl esters (FAMEs) external calibration standards (Mixture ME 100) were obtained from Larodan (Sweden). The lipids standards: triacylglycerols, free fatty acids, diacylglycerols, monoacylglycerols, fatty alcohol, phytosterol, monogalactosyldiacylglycerols, digalactosyldiacylglycerols, phosphatidylcholine and phosphatidylserine, and gallic acid were obtained from Sigma-Aldrich (St. Louis, MI, USA). Gallic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Rice bran samples

Heat-stabilized RB (110 °C for 6 min) from 15 RB cultivars and 1 germ from different geographical locations and genetic backgrounds including. The cultivars are described on Table 1. Among them, 3 belong to two seasons (Jasmin Dry, Jasmin Wet and Non-Jasmin). Lowland and Highland (Madagascar) are pigmented (red) RB, while the others are non-pigmented RB.

Table 2 - Cultivars of RB from different geographical locations.

Country	Cultivar
Cambodja	Jasmin Dry Season I
	Jasmin Dry Season II
	Jasmin Wet Season I
	Jasmin Wet Season II
	Non-Jasmin I
	Non-Jasmin II
Egypt	Egyptian
France	Arelate
	Gageran
	Gines
	Manobi
	Opale
	Paco
	Rousty
	F. No-stab Germ
	F. No-stab RB
	F. Stab Germ
	F. Stab RB
Madagascar	Highland
	Lowland

2.3. Granulometry

The RB particles were analyzed with a particle size analyzer Mastersizer 2000 (Malvern Instruments, Worcestshire, Great Britain) in dry mode.

2.4. Moisture contents and water activity

The moisture content was determined in triplicate by drying 2 g of the samples at 105 °C in Memmert heat chamber (model 100 Schwabach, Germany) until constant weight. Samples and their dehydrated residues were precisely weighted on a Mettler AE166 balance (Viroflay, France). Water activity (a_w) was determined at 25 °C on approximately 1 g with a water activity meter 4TE AQUALAB (Hopkins, USA).

2.5. Lipid content

Total lipids were extracted from 2 g of RB by the Folch method (Folch et al., 1957) using chloroform/methanol (2:1, v/v). Extracted total lipids were stored at −20 °C until further analysis. The organic layer was filtered and collected. Solvent was evaporated with a rotavapor at 40 °C until constant weight and residual lipids were precisely weighted (Laborota 400-efficient, Heidolph, Schawabach, Germany).

2.6. Lipid profile by TLC

Organic phases from Folch extraction method were spotted on silica gel HPTLC plates (Merck, Saint-Quentin Fallavier, France) using a ATS4 (Camag, Muttenz, Switzerland). Lipid standards (TAG: triacylglycerol, FFA: free fatty acid, DAG: diacylglycerol, MAG: monoacylglycerol, MGDG: monogalactosyldiacylglycerol, DGDG: digalactosyldiacylglycerol, PC: phosphatidylcholine and PS: phosphatidylserine) were also spotted on each plate for calibration. The elution was performed in double migration using an automatic developing chamber ADC2 (Camag, Muttenz, Switzerland) as follows: 40 mm with $CHCl_3$/MeOH/water (19/4/0.5, v/v/v) and 80 mm with hexane/diethyl ether/formic acid (14/6/0.2, v/v/v). After elution, plates were dried at room temperature, dipped in the $CuSO_4$/H_3PO_4 reagent using a Chromatogram Immersion Device 3 (Camag, Muttenz, Switzerland), dried again at room temperature, and placed in an oven at 180 °C for 10 min. Absorbances of revealed spots and bands were measured at 500 nm using a Camag TLC Scanner 3 and analyzed by VISIONCAT software (Camag, Muttenz, Switzerland). Spots were qualitatively evaluated in comparison with lipid standards.

2.7. Fatty acid profile by GC

In 25 mL round bottom flask, RB samples (~300 mg) with about 5% internal standard (C17:0) were added to 3 mL sodium methylate solution with phenolphthalein. Reaction medium was refluxed for 10 min. 3 mL chlorohydric methanol were added to phenolphthalein discoloration and the mixture was refluxed again for 10 min and then cooled to ambient temperature. 6 mL hexane and 10 mL water were added, and the organic phase was recovered and analyzed by gas chromatography (Zhang et al.). A Focus GC (Thermo Electron Corporation, Massachusetts, USA) was used and equipped with a split injector (ratio of 1/20), a CP-Sil 88 Varian capillary column (50 m×0.25 mm with 0.2 µm film thickness; Chrompack, Mid-delburg, Netherlands), and helium 1 mL/min as the carrier gas was used. Fatty acids methyl esters (FAME) were analyzed by flame ionization detector and ChromCard software data system (version 2005, Thermo Fisher Scientific, Massachusetts, USA). The column

temperature started from 150 °C, then reached 150 to 225 °C with a rise of 5 °C/min and was kept at 225 °C for 10 min. The injector and detector temperatures were 250 and 270°C, respectively. FAME were identified using as external standards a mixture of methyl esters (Mix37, Sigma-aldrich,). Each sample was methylated in triplicate.

2.8. Statistical analysis

Statistical analysis will be performed to compare means of total content and relative abundance of PC applying one-way ANOVA (Tukey, $p< 0.05$) by using XLSTAT (Addinsoft, v. 2018.2.50452).

3. Results and discussion

3.1. Particle size and distribution a collection of heat-stabilized rice bran

The samples were submitted to particle size distribution analysis by sieving (Figure 1) and laser light granulometry (Figure 2).

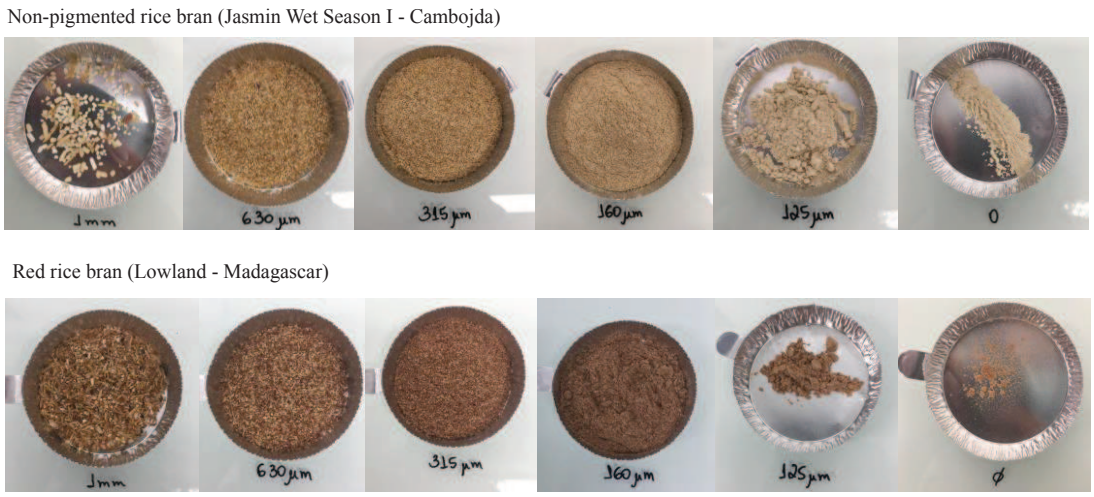


Figure 1 - Typical particle size distribution of two varieties of RB analyzed by sieving.

Particle size analysis showed a distribution of heterogeneous particles representative of variable milling processes, as can be observed in Figure 2. This Figure showed two important results: a) particles above 1000 µm were not found in Cambodja RB, which can be associated with the milling process and the mechanical strength of the grain; b) a higher amount (35 to 45%) of particles between 315 and 160 µm were observed. Due to the heterogeneity of the overall particle size, all samples were milled for further analysis.

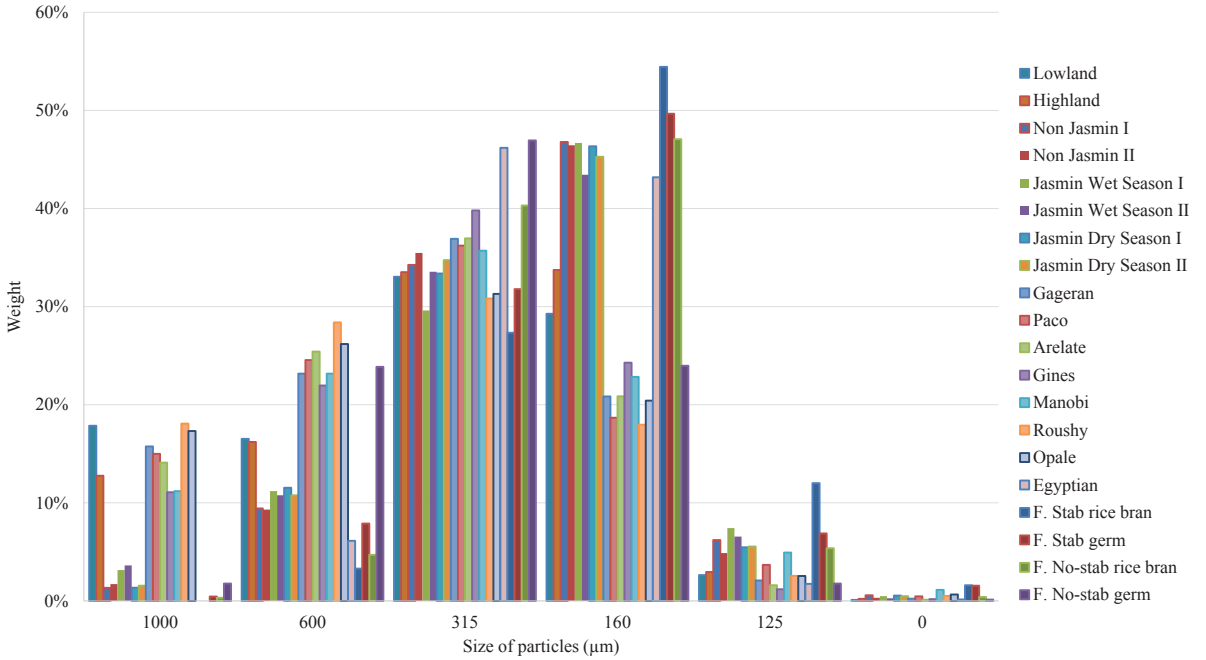


Figure 2 - Particle distribution of RB obtained by laser light granulometry.

3.2. Water activity and moisture content - parameters indicative of rice bran conservation and possibility of microorganism proliferation

The results of water activity (a_w) (Table 2) indicate that some samples presented a high value, such as the samples from France that presented an average of 0.63 ± 0.08. The samples from the other regions showed values of 0.48 ± 0.07, 0.49 ± 0.02, and 0.52 ± 0.00 for Cambodja, Madagascar, and Egypt, respectively. The samples that did not go through the stabilization process (F. No-stab Germ and F. No-stab Rice bran) stood out by presenting high values for water activity ($p<0.05$). Cambodja, Madagascar, and Egypt RB water activities are in line with data found in the literature (Moongngarm et al., 2012). The value higher than 0.6 is associated with a higher enzymatic activity and hight amount of free fatty acids, showing that the heat stabilization performed after the milling process of the rice grain was not sufficient for the deactivation of endogenous enzymes. This result reaffirms the importance of conducting heat-treatment as soon as possible in the post-harvest process.

.

Table 2 - RB variety collection, water activity and moisture content.

Country	Cultivar	Aw	Moisture (%)
Cambodja	Jasmin Dry Season I	0.540 ± 0.034 ^{fg}	8.18 ± 0.23 ^{fg}
	Jasmin Dry Season II	0.596 ± 0.020 ^{de}	8.78 ± 0.07 ^{f}
	Jasmin Wet Season I	0.445 ± 0.013 ^{h}	7.38 ± 0.15 ^{ij}
	Jasmin Wet Season II	0.421 ± 0.008 ^{hi}	7.21 ± 0.04 ^{j}
	Non-Jasmin I	0.445 ± 0.009 ^{h}	8.13 ± 0.11 ^{g}
	Non-Jasmin II	0.416 ± 0.003 ^{hi}	8.01 ± 0.06 ^{ghi}
Egypt	Egyptian	0.518 ± 0.019 ^{g}	8.12 ± 0.06 ^{gh}
France	Arelate	0.672 ± 0.003 ^{ab}	12.23 ± 0.33 ^{a}
	Gageran	0.635 ± 0.020 ^{bcd}	11.49 ± 0.16 ^{bc}
	Gines	0.564 ± 0.015 ^{ef}	10.56 ± 0.04 ^{de}
	Manobi	0.625 ± 0.002 ^{cd}	10.02 ± 0.15 ^{e}
	Opale	0.677 ± 0.012 ^{ab}	12.14 ± 0.42 ^{a}
	Paco	0.627 ± 0.021 ^{cd}	11.18 ± 0.09 ^{bcd}
	Rousty	0.642 ± 0.010 ^{bc}	11.73 ± 0.32 ^{ab}
	F. No-stab Germ	0.692 ± 0.001 ^{a}	11.04 ± 0.05 ^{cd}
	F. No-stab RB	0.69 ± 0.003 ^{a}	10.92 ± 0.40 ^{cd}
	F. Stab Germ	0.523 ± 0.001 ^{fg}	7.49 ± 0.12 ^{hij}
	F. Stab RB	0.652 ± 0.006 ^{abc}	9.92 ± 0.10 ^{e}
Madagascar	Highland	0.436 ± 0.013 ^{hi}	7.18 ± 0.08 ^{j}
	Lowland	0.401 ± 0.001 ^{i}	6.87 ± 0.13 j

Letters indicate significant differences between the RB genotypes for a given water activity or moisture content (%) (one-way ANOVA, $p< 0.05$)

The moisture content is an important parameter in controlling and marketing rice. This data is applied to correct the composition data, thus permitting the comparison among rice samples with different ash. The RB collection in this study presents a moisture content ranging between 6-12%. These results are in agreement with the literature, (Moongngarm et al., 2012) showing a moisture content 9-10% for RB and 6-8% for germ. In comparison, (Melini et al., 2019) reported 11.8% and 13.5% in pigmented RB.

3.3. Lipid analysis

3.3.1. Total lipid content and profile characterization

The total lipid content was performed, and the results are illustrated in Figure 3. Our RB data present a lipid content that ranging between 11.48 to 17.26 g/100 g of RB. As expected, the fraction of rice germs analyzed showed a higher content of lipids (23.02 ± 1.38 g/100g db), whether stabilized or not.

The variability of total lipid contents is remarkable on the global collection. If we analyze only the samples from France genotypes, we can say that Gines (15.02 g/100 g of RB) and Arelate (14.80 g/100g of RB) show no statistical difference between them, but Paco (13.56 g/100 g of RB), Opale (12.04 g/100 g of RB) and Manobi (11.48 g/100 g of RB) are statistically different.

Our results corroborate the values described in the literature, where the lipid content in RB can vary between 15 and 20% (Moongngarm et al., 2012). However, we can evidence a variable content between the cultivars (p<0.05) that may suffer an influence not only of the genotype but also, of the culture conditions.

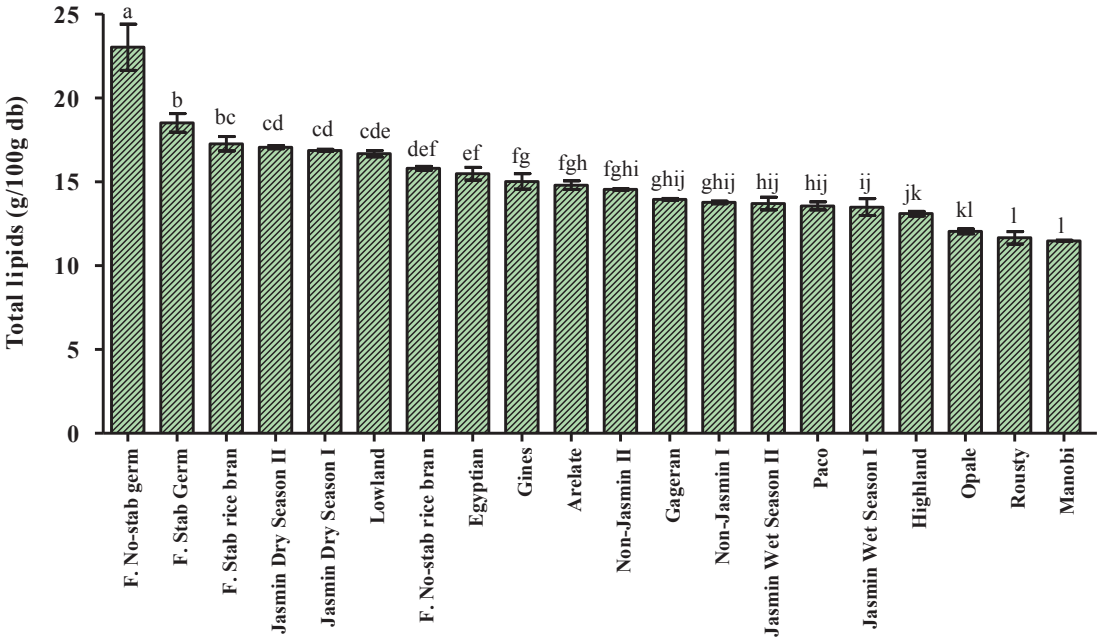


Figure 3 - Total lipids content (g/100 g of RB db) for each cultivar. Different lowercase letters mean a significant difference (one-way ANOVA, Tukey, p<0.05) between the cultivars.

The lipid extracts were submitted to a characterization by TLC. All samples were deposited in duplicate on the plate. In Figure 4, we can see that RB present different classes of lipids. We could identify classes such as triacylglycerols (TAG), diacylglycerols (DAG) and monoacylglicerols (MAG), free fatty acids (FFA), fatty alcohols, phytosterols and, also minor, but important polar lipids such as the galactolipid digalactosyldiacylglycerol (DGDG) and the phospholipids phostatidilcholine (Martillanes et al.), and phostatidylserine (Oliveira et al.). The polar lipids are related to important bioactivities (Liu et al., 2013).

Some RB varieties presented (e.g., Camargue, France) are lipolyzed. It is possible to observe a difference in the content of TAG, as well as in the content of FFA, evidencing a possible enzymatic activity from endogenous enzymes present in RB (lipase and peroxidase) (Brunschwiler et al., 2013; Liu et al., 2019). These activities would be responsible for lipid oxidation and hydrolysis of TAG, thus increasing the content of free fatty acids. It is also possible to evidence that the thermal stabilization performed was not enough to inactivate these endogenous enzymes. Those RB from Madagascar and Egypt have preserved triglycerides and a variation in polar lipid profile. The Cambodja samples showed no qualitative differences between the seasons (I and II), with similar gray scale for all classes.

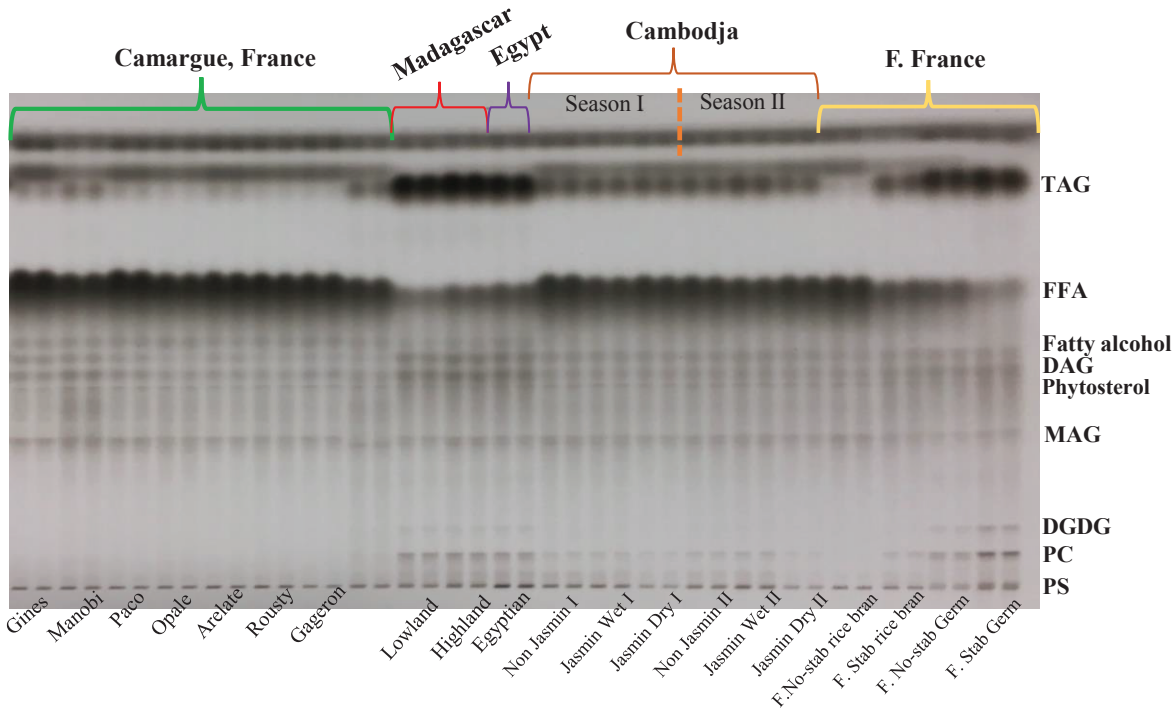


Figure 4 - Representative plate with all RB and germ varieties analyzed from thin-layer chromatography (TLC). TAG: triacylglycerol. FFA: free fatty acids. DAG: diacylglycerol, MAG: monoacylglycerol, MGDG: monogalactosyldiacylglycerol, PE: phostatidylethanolamine, DGDG: digalactosyldiacylglycerol, PC: phostatidilcholine, and PS: phostatidylserine

3.3.2. Fatty acid profile by gas chromatography reveals an important presence of polyunsaturated fatty acids

The fatty acid profile was performed by gas chromatography and the chromatogram is illustrated in Figure 5. We can highlight as major peaks the oleic (C18:1n9), linoleic

(C18:2n6) and palmitic (C16:0) acids, representing the most abundant fatty acids in RB, corroborating with literature (Gopala Krishna et al., 2012).

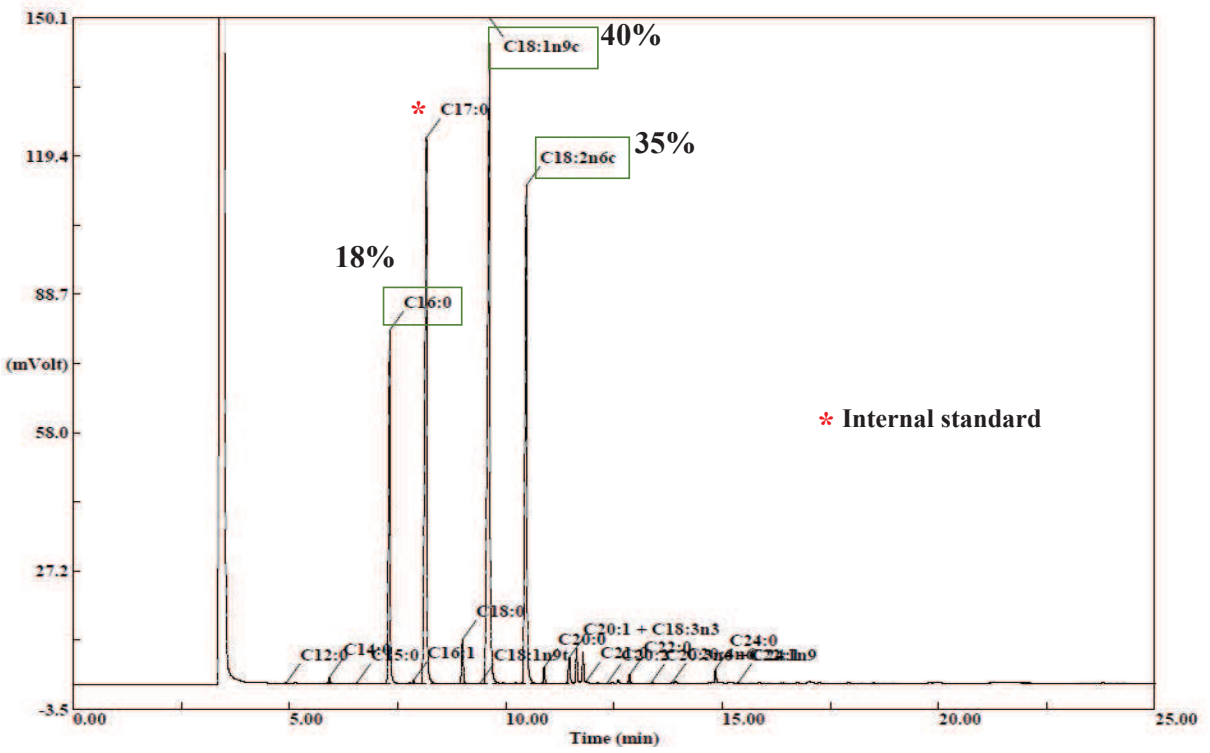


Figure 5 - Typical gas chromatogram of the RB fatty acid composition.

The global fatty acid profile as well as its quantification is described in Table 3. Among them, we can highlight the presence of long-chain fatty acids (C13 to C21) and very long-chain (C22 or more).

In Table 4, the content of each fatty acid classification (saturated, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA)) is described. Globally, the studied RB have a high content of MUFA and PUFA, ranged between 4.16 to 9.18 g/100g of RB and 4.14 to 10.02 g/100g of RB, respectively, with low saturated fatty acid content.

The non-stabilized RB sample showed higher content of saturated fatty acids, MUFA and PUFA compared to the other sample ($p<0.05$).

These major RB fatty acid profiles merit emphasis. Indeed unsaturated fatty acids can modulate diet-associated plasma lipid profile, and are associated with direct positive effects on cholesterol metabolism (Akkarach Bumrungpert et al., 2019). These compounds are mostly carried by triacylglycerols (85% of the lipid class in RB), as well as phospholipids, glycolipids, and sphingolipids, even if their concentrations are lower. Cuenoud et al. (2020)

showed that MAG can act as protectors against digestive actions, carrying omega 3 fatty acids and then modify their bioavailability.

Table 3 - Profile and content (g/100g of RB) of fatty acids present in RB.

Country	Cultivar	C14:0	C16:0	C16:1	C18:0	C18:1n9c	C18:2n6c	C20:0	C20:1+C18:3n3	C20:2	C22:0	C20:4n6	C24:0	C24:1
Cambodja	Non-Jasmin I	0.04 ± 0.00	2.76 ± 0.03	0.03 ± 0.00	0.35 ± 0.00	6.15 ± 0.05	4.30 ± 0.03	0.15 ± 0.00	0.21 ± 0.00	-	0.08 ± 0.00	0.01 ± 0.00	0.15 ± 0.00	-
	Non-Jasmin II	0.05 ± 0.00	2.94 ± 0.04	0.03 ± 0.00	0.38 ± 0.00	6.54 ± 0.08	4.55 ± 0.04	0.15 ± 0.00	0.22 ± 0.00	-	0.08 ± 0.00	0.01 ± 0.00	0.16 ± 0.00	-
	Jasmin Wet Season I	0.04 ± 0.00	2.74 ± 0.09	0.02 ± 0.00	0.34 ± 0.01	6.06 ± 0.17	4.06 ± 0.20	0.14 ± 0.00	0.20 ± 0.01	-	0.07 ± 0.00	-	0.14 ± 0.03	-
	Jasmin Wet Season II	0.04 ± 0.01	2.95 ± 0.16	0.03 ± 0.00	0.37 ± 0.02	6.56 ± 0.38	4.55 ± 0.26	0.15 ± 0.01	0.23 ± 0.01	-	0.09 ± 0.01	-	0.17 ± 0.02	-
	Jasmin Dry Season I	0.06 ± 0.00	3.93 ± 0.08	0.04 ± 0.00	0.48 ± 0.01	8.84 ± 0.16	6.00 ± 0.21	0.20 ± 0.00	0.29 ± 0.02	-	0.10 ± 0.00	-	0.20 ± 0.01	-
	Jasmin Dry Season II	0.06 ± 0.00	3.75 ± 0.22	0.04 ± 0.00	0.46 ± 0.02	8.39 ± 0.52	5.67 ± 0.35	0.20 ± 0.01	0.28 ± 0.02	-	0.10 ± 0.01	0.01 ± 0.00	0.20 ± 0.01	-
Egypt	Egyptian	0.04 ± 0.00	2.60 ± 0.03	0.02 ± 0.00	0.27 ± 0.01	6.79 ± 0.08	5.58 ± 0.06	0.11 ± 0.00	0.29 ± 0.00	-	0.10 ± 0.00	0.01 ± 0.00	0.23 ± 0.01	-
France	Gines	0.03 ± 0.00	2.37 ± 0.01	0.02 ± 0.00	0.28 ± 0.01	6.070 ± 0.22	5.52 ± 0.41	0.12 ± 0.01	0.25 ± 0.03	-	0.08 ± 0.02	0.01 ± 0.00	0.16 ± 0.06	-
	Manobi	0.02 ± 0.00	1.85 ± 0.05	0.02 ± 0.00	0.16 ± 0.00	4.36 ± 0.10	3.96 ± 0.10	0.07 ± 0.00	0.18 ± 0.01	0.01 ± 0.00	0.07 ± 0.00	0.01 ± 0.00	0.15 ± 0.01	-
	Paco	0.03 ± 0.00	2.21 ± 0.03	0.02 ± 0.00	0.23 ± 0.00	4.96 ± 0.06	5.00 ± 0.12	0.10 ± 0.00	0.23 ± 0.01	-	0.08 ± 0.00	0.01 ± 0.00	0.21 ± 0.00	-
	Opale	0.03 ± 0.00	1.84 ± 0.02	0.02 ± 0.00	0.17 ± 0.00	3.94 ± 0.03	3.93 ± 0.04	0.08 ± 0.00	0.20 ± 0.00	-	0.07 ± 0.00	0.01 ± 0.00	0.17 ± 0.01	-
	Arelate	0.02 ± 0.00	2.30 ± 0.03	0.02 ± 0.00	0.24 ± 0.00	5.64 ± 0.08	5.52 ± 0.08	0.12 ± 0.00	0.26 ± 0.01	-	0.09 ± 0.00	0.01 ± 0.00	0.21 ± 0.00	-
	Rousty	0.02 ± 0.00	1.89 ± 0.07	0.01 ± 0.00	0.20 ± 0.01	4.82 ± 0.17	4.28 ± 0.16	0.11 ± 0.00	0.23 ± 0.01	-	0.08 ± 0.00	0.01 ± 0.00	0.20 ± 0.01	-
	Gageran	0.02 ± 0.00	2.28 ± 0.05	0.02 ± 0.00	0.20 ± 0.01	5.36 ± 0.16	4.87 ± 0.21	0.06 ± 0.05	0.26 ± 0.02	-	0.09 ± 0.01	0.01 ± 0.00	0.22 ± 0.02	-
	F. No-stab RB	0.05 ± 0.00	2.67 ± 0.03	0.02 ± 0.00	0.29 ± 0.00	6.39 ± 0.11	5.86 ± 0.12	0.13 ± 0.00	0.33 ± 0.01	-	0.12 ± 0.00	-	0.28 ± 0.02	0.01 ± 0.00
	F. Stab RB	0.07 ± 0.01	3.29 ± 0.56	0.02 ± 0.00	0.37 ± 0.05	7.19 ± 1.21	7.21 ± 1.26	0.16 ± 0.02	0.40 ± 0.07	-	0.11 ± 0.02	0.01 ± 0.00	0.26 ± 0.04	0.01 ± 0.00
	F. No-Stab Germ	0.04 ± 0.00	4.54 ± 0.09	0.03 ± 0.00	0.54 ± 0.01	7.79 ± 0.17	9.59 ± 0.20	0.16 ± 0.00	0.42 ± 0.01	0.01 ± 0.00	0.07 ± 0.00	0.01 ± 0.00	0.12 ± 0.01	0.01 ± 0.00
	F. Stab Germ	0.04 ± 0.00	4.06 ± 0.37	0.02 ± 0.00	0.48 ± 0.04	6.61 ± 0.61	7.98 ± 0.71	0.14 ± 0.01	0.34 ± 0.03	-	0.06 ± 0.01	0.01 ± 0.00	0.13 ± 0.01	0.01 ± 0.00
Madagascar	Lowland	0.04 ± 0.00	3.01 ± 0.03	0.02 ± 0.00	0.39 ± 0.01	7.22 ± 0.08	5.75 ± 0.06	0.14 ± 0.00	0.32 ± 0.00	-	0.07 ± 0.00	0.01 ± 0.00	0.14 ± 0.00	-
	Highland	0.03 ± 0.00	2.01 ± 0.02	0.01 ± 0.00	0.29 ± 0.00	6.34 ± 0.07	4.31 ± 0.49	0.10 ± 0.01	0.24 ± 0.05	-	0.06 ± 0.00	0.01 ± 0.00	0.10 ± 0.00	-

Table 4 - Total content (g/100g of RB) of each fatty acid classification.

Country	Cultivar	Saturated	MUFA	PUFA
Cambodja	Non Jasmin I	3.53 ± 0.04 ^{efC}	6.39 ± 0.05 ^{efgA}	4.52 ± 0.03 ^{fB}
	Non Jasmin II	3.76 ± 0.04 ^{deC}	6.79 ± 0.08 ^{defgA}	4.78 ± 0.04 ^{defB}
	Jasmin Wet Season I	3.47 ± 0.13 ^{efgC}	6.29 ± 0.17 ^{efghA}	4.27 ± 0.21 ^{fB}
	Jasmin Wet Season II	3.76 ± 0.22 ^{deC}	6.82 ± 0.39 ^{defA}	4.79 ± 0.27 ^{defB}
	Jasmin Dry Season I	4.98 ± 0.11 ^{abC}	9.18 ± 0.17 ^{aA}	6.31 ± 0.22 ^{cB}
	Jasmin Dry Season II	4.77 ± 0.28 ^{bcC}	8.70 ± 0.54 ^{abA}	5.96 ± 0.37 ^{cdB}
Egypt	Egyptian	3.35 ± 0.04 ^{efgC}	7.10 ± 0.09 ^{cdeA}	5.88 ± 0.06 ^{cdB}
France	Gines	3.04 ± 0.11 ^{fghB}	6.34 ± 0.25 ^{efghA}	5.78 ± 0.44 ^{cdeA}
	Manobi	2.32 ± 0.06 ^{jC}	4.56 ± 0.11 ^{jkA}	4.16 ± 0.11 ^{fB}
	Paco	2.87 ± 0.03 ^{ghijB}	5.21 ± 0.07 ^{hijkA}	5.24 ± 0.12 ^{cdefA}
	Opale	2.35 ± 0.03 ^{ijB}	4.16 ± 0.03 ^{kA}	4.14 ± 0.04 ^{fA}
	Arelate	2.99 ± 0.03 ^{fghiB}	5.93 ± 0.08 ^{fghiA}	5.80 ± 0.09 ^{cdeA}
	Rousty	2.51 ± 0.09 ^{hijC}	5.06 ± 0.18 ^{ijkA}	4.52 ± 0.17 ^{fB}
	Gageran	2.88 ± 0.03 ^{fghijC}	5.64 ± 0.18 ^{ghijA}	5.14 ± 0.23 ^{cdefB}
	F. No-stab RB	3.52 ± 0.03 ^{aC}	6.75 ± 0.12 ^{abcA}	6.20 ± 0.13 ^{aB}
	F. Stab RB	4.25 ± 0.70 ^{efB}	7.62 ± 1.29 ^{defgA}	7.61 ± 1.33 ^{cA}
	F. No-Stab Germ	5.48 ± 0.11 ^{abC}	8.25 ± 0.16 ^{defB}	10.02 ± 0.20 ^{bA}
	F. Stab Germ	4.91 ± 0.45 ^{cdB}	6.98 ± 0.64 ^{bcdA}	8.33 ± 0.74 ^{bA}
Madagascar	Lowland	3.78 ± 0.04 ^{deC}	7.56 ± 0.09 ^{bcdA}	6.08 ± 0.07 ^{cB}
	Highland	2.59 ± 0.02 ^{hijC}	6.60 ± 0.11 ^{defgA}	4.56 ± 0.54 ^{efB}

Lowercase letters indicate significant differences between the RB genotypes for a given fatty acid classification. Uppercase letters indicate significant differences between the fatty acid classification for a given genotype (one-way ANOVA, Tukey $p<0.05$).

3.4. Phenolic content by HPLC-DAD

The quantification of phenolic content was determined by a calibration curve of gallic acid. Different amount of the phenolic compounds that were reliably quantified was show in Figure 7. In this study, the pigmented RB had a content up to 50 times higher than the non-pigmented bran samples ($p<0.05$). The content of phenolic compounds in the red RB samples from Madagascar is remarkable. Lowland and Highland varieties present 1723.2 ± 98.31 and 1144.95 ± 181.55 mg eq GA/100 g of RB, respectively. In a review by Goufo and Trindade (2014), phenolic contents in red RB can range from 408 to 2241.4 mg eq GA/100 g in RB. Santos et al. (2021) characterized from metabolomics tools red RB and highlighted the presence of different species, majority flavonoids, also, using green solvents as an alternative which may enable promising and sustainable applications. Furthermore, pigmented RB stands out for its high phenolic content, its phenolic diversity with different class of compounds, as well as its effective antioxidant activity, allowing applications of these RB and/or extracts for health promotion purposes (Borresen & Ryan, 2014; Park et al., 2017) or for the food, pharmaceutical and cosmetics industries (Friedman, 2013; Min et al., 2011).

The phenolics content of the non-pigmented varieties ranges from 8 to 45 mg eq GA/100 g. In Figure 6, it can be seen that the content, although with significant differences, allows the samples to be grouped by region. The samples from France presented an average of 32 mg eq GA/100 g of RB while samples from Cambodja presented an average 11.9 mg eq GA/100 g of RB. It is well established in the literature that the phenolics content/profile is highly sensitive to external factors. These results lead us to hypothesize that the phenol content is directly linked to the planting location/environmental and cultivation conditions. But post-harvest and processing factors of these samples should be considered. Heat treatment may be a factor that decreased the phenolic content of the RB from Cambodja (Zhang et al., 2015). The RB from France had water activity and lipid activity values that recall the inefficacy of the heat treatment, leading to the hypothesis that the heat treatment did not have an as great impact as on the Cambodjan RB.

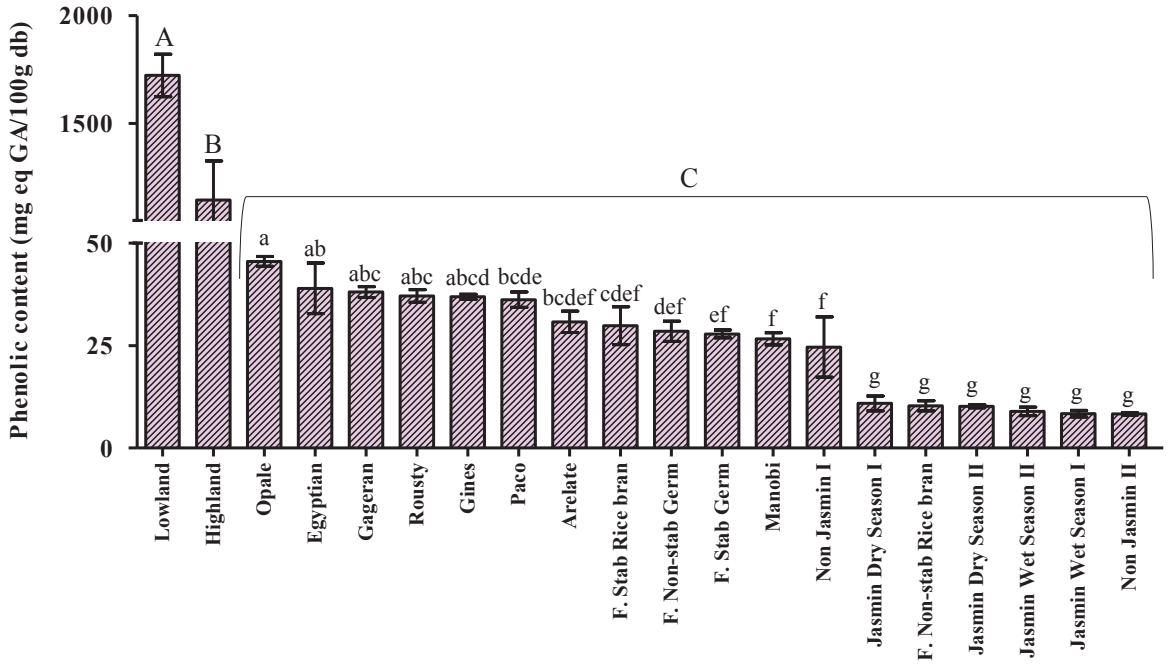


Figure 6 - Total content of phenolic compounds in mg eq GA/100 g of RB dry basis. Uppercase letters indicate significant differences between the phenolic content considering all samples and Lowercase letters indicate significant differences between the phenolic content excluding the two pigmented RB (lowland and highland from Madagascar) (one-way ANOVA, Tukey $p<0.05$).

3.5. Unsupervised multivariate analysis

The principal components analysis was performed on the encompassing our entire data matrix to allow us to make a global comparison in a more visual way (Figure 7).

Globally, the first 2 components explain 85% of the data. The variables were grouped as: water activity and moisture; lipid compounds; and phenolic compounds. Analyzing the observations, component 1 (F1) explains 56.08% by grouping the RB according to the variables: Lowland and Highland with higher phenolic content (pigmented RB), samples from France with higher water activity and ash content, and some Cambodjan varieties with higher lipid content. Component 2 (F2) explains 29.23% and separates the samples, in general, according to their countries, grouping only France samples, Egypt with Camboja and only Madagascar samples.

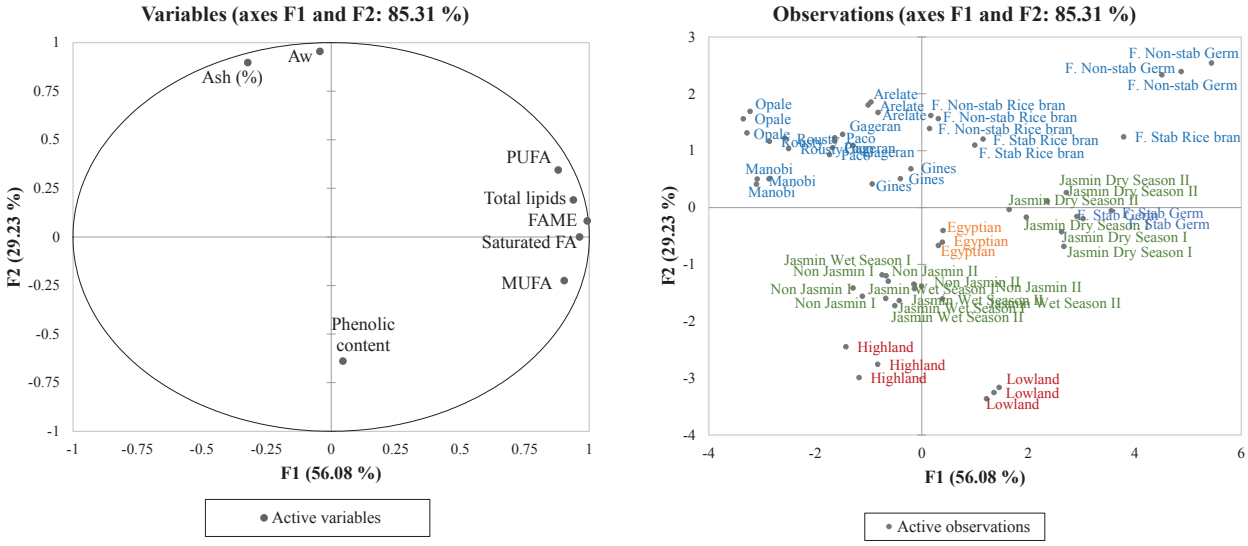


Figure 7 - Principal component analysis correlating all data present in the studied RB. Different colors represent the country of each RB. Red: Madagascar, green: Cambodja, orange: Egypt and blue: France.

4. Conclusions

The characterization of RB allows us to conclude the impact that some post-harvest processes can have on the quality of RB and its components. Some samples presented a high value of aw which may be associated with the post-harvest processing and storage of these bran. All RB samples presented as rich source of lipids (including bioactive classes, with a high amount of unsaturated fatty acids) and phenolic compounds. Total lipid and phenolic content were shown to be influenced by genotype, production location and postharvest condition, but even so, they presented contents that allow the valorization of this coproduct and its application in several sectors. RB has been shown to be a potential source of bioactive compounds, highlighting lipid classes that play an important role in human metabolism (polar lipids). More robust analysis such as omics tools (e.g., lipidomics) should be applied for a global characterization of the lipids present in RB.

Author contributions

Conceptualization, M.C.B.S., N.B., M.S.L.F., C.B.-L.; data curation, M.C.B.S., N.B., C.B.-L.; formal analysis, M.C.B.S., N.B.; funding acquisition, M.S.L.F. and C.B.-L.; investigation, M.C.B.S., N.B.; methodology, M.C.B.S.; project administration, M.S.L.F., C.B.-L.; resources, C.B.L., M.S.L.F.; supervision, N.B., M.S.L.F., C.B.-L.; validation, M.C.B.S., N.B., C.B.-L., M.S.L.F.; visualization, M.C.B.S., N.B., C.B.-L., M.S.L.F.; writing—original draft, M.C.B.S.;

writing—review and editing, M.C.B.S., N.B., M.S.L.F., C.B-L., All authors have read and agreed to the published version of the manuscript.

Acknowledgments: The authors are grateful to Dr Youna Hemery (Institute of Research for Development (IRD); UMR 204 Food and Nutrition Research in the Global South (NUTRIPASS), IRD/University of Montpellier/SupAgro, Montpellier, France) for providing the red RB for analysis.

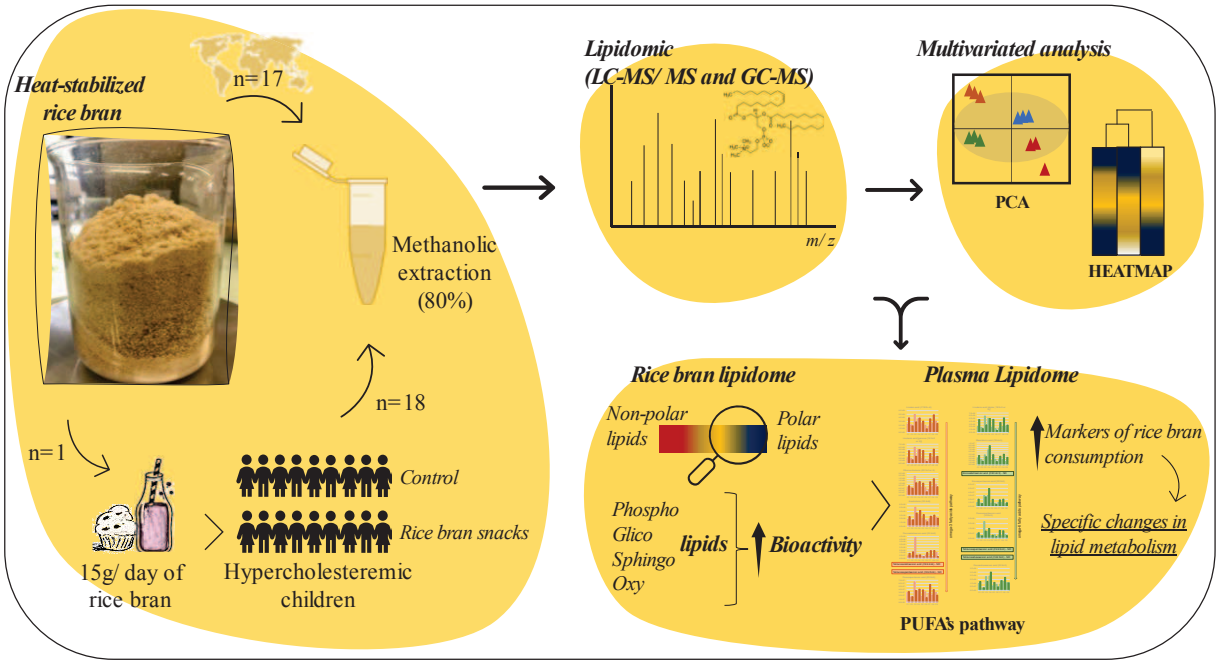
Funding: This research was financed by the Foundation for Research Support of State of Rio de Janeiro (FAPERJ) (26/010.100.988/2018; 26/202.709/2018; 26/201.848/2019), National Council for Scientific and Technological Development (CNPq) (427.116/2018; 310.34/2019-4) and supported by UNIRIO, Coordination for Improvement of Personnel with Higher Education (CAPES) (financial code 001).

Chapter 7 – Rice bran lipidome identifies novel phospholipids, glycolipids and oxylipins and roles in lipid metabolism of hypercholesterolemic children

Millena Cristina Barros Santos[1], Nathalie Barouh[2], Valérie Lullien[3], Valérie Micard[3], Pierre Villeneuve[2], Bingqing Zhou[4], Camille Oger[4], Claire Vigor[4], Thierry Durand[4], Mariana Simões Larraz Ferreira[1], Claire Bourlieu-Lacanal[3], Elizabeth P Ryan[5*]

(1) Laboratory of Bioactives, Food and Nutrition Graduate Program, Federal University of State of Rio de Janeiro (UNIRIO), Rio de Janeiro, Brazil.
(2) CIRAD, UMR Qualisud, F-34398 Montpellier, France.Qualisud, Univ Montpellier, Avignon Université, CIRAD, Institut Agro, Université de La Réunion, Montpellier, France.
(3) UMR IATE 1208 INRAE/CIRAD/SUPAGRO/UM, Montpellier, France
(4) Institut des Biomolecules Max Mousseron (IBMM), UMR 5247, CNRS, University of Montpellier, ENSCM, 34090 Montpellier, France
(5) Department of Environmental and Radiological Health Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, 80523, USA

Submission in progress to Molecular Nutrition and Food Research



Abstract

Rice bran (RB) is a major agricultural coproduct of rice milling and showed human health benefit following feeding trials in children and adults. Health benefits of dietary RB include, but are not limited to antimicrobial and anti-inflammatory functions, as well as mitigation of chronic metabolic disease. RB lipids linked to health promotion are gamma-oryzanol, other phytosterols and fatty acids. Lipidomic analysis such as ultra-performance liquid chromatography coupled with high-resolution tandem mass spectrometry (UPLC-MS/MS) and with an emphasis on targeted and untargeted, identified novel bioactive lipids of 17 genetically diverse varieties from 6 geographical locations has revealed novel polar lipids (methanolic extracts) and oxylipins. This diversified and comprehensive focus on novel RB lipids supports this emerging superfood as an important source of phytoprostanes and phytofurans. The Calrose-USA variety was selected for an in vivo study whereby RB supplementation at 15g/day for 28 days was completed in hypercholesteremic children (NCT01911390). Changes in plasma lipids from different subclasses were interrogated for linkages to RB consumption. Findings from this study have important implications for RB food product stabilization of bioactive lipids as well as programs that seek to advance RB as a functional food that regulates lipid metabolism in people at high risk for cardiometabolic disease conditions.

Keywords: bioactive lipids metabolism, lipidomics, polar lipids, phytoprostanes, phytofurans, RB lipids.

1. Introduction

Rice bran (RB) is a coproduct of industrial milling for white rice. RB is a rich source of nutrients, prebiotics and presents unique ratios of vitamins, minerals and phytochemicals. Dietary intake of RB has shown health beneficial properties for chronic disease prevention in animals and humans (both children and adults) (Henderson et al., 2012; Sheflin et al., 2017).

Targeted identification of RB lipids has primarily included gamma-oryzanol, tocopherols, tocotrienols, anthocyanins and phytosterols, all of which are already well discussed in the literature (Friedman, 2013; Moongngarm et al., 2012). Gamma-oryzanol and phytosterols have compelling impacts on cholesterol regulation (Wang et al., 2014) and immune functions (Castanho et al., 2019). RB is also a source of free fatty acids (e.g. palmitic acid, oleic acid, linoleic acid) that can increase antioxidant capacity in hyperlipidemia (Park et al., 2017). Extracts of RB are able to decrease peroxide values in food products susceptible to oxidation and to extend food shelf life. Lipid extracts of RB may be a sustainable alternative as a food additive to improve food quality and safety (Martillanes et al., 2020).

RB can become spoiled and rancid due to lipid peroxidation, a condition that can be delayed with proper stabilization, including the inactivation of endogenous lipases (Brunschwiler et al., 2013; Irakli et al., 2018; Liu et al., 2019). Heat-treatment to prevent lipid oxidation (Brown et al., 2017) represents one type of stabilization technology for expanding shelf-life and utility of RB for human consumption. *In vivo* studies show that heat-stabilized RB consumption leads to the modulation of microbiota composition (Sheflin et al., 2015), including, but not limited to the prevention of Salmonella colonization, the regulation of intestinal immunity and colorectal cancer protection (Goodyear et al., 2015; Sheflin et al., 2017; Yang et al., 2015).

The RB lipidome warrants molecular food and nutrition science attention for novel bioactives as was shown recently for antimicrobial actions (Kumar et al., 2012; Sheflin et al., 2017) and via an integration of untargeted and targeted metabolic profiling. Oxylipins, phospholipids, sphingolipids, and galactolipids are gaining research attention due to their potential bioactivity and nutritional importance (Shaheen et al.). Polar lipids are biological membrane constituents (Bourlieu et al., 2021) whereas oxylipins are products of enzymatic oxidation. Hydroxyoctadecadienoic acid (HODE), hydroxyeicosatetranoic acid (HETE) (Chehab et al., 2007) or non-enzymatic oxidation of polyunsaturated fatty acids (NEO-PUFA) (phytoprostanes - PhytoPs and phytofurans - PhytoFs) (Galano et al., 2017; Galano et al., 2018) can inhibit the growth of cancer cells and are also classified for anti-inflammatory

actions (Gabbs et al., 2015; Shearer & Walker, 2018). The depth and breadth of chemical diversity in the RB lipidome was investigated herein using 17 rice genotypes and for capacity to modulate human plasma metabolites after RB consumption. Given recent studies suggesting that a diet enriched in stabilized RB could modulate lipid metabolism (Borresen et al., 2017; Li et al., 2018b) we tested the hypothesis that the RB lipidome modulates the plasma lipidome of children after the daily intake of RB from the Calrose-United States variety. This study co-identified novel RB lipid classes in the food and the lipid metabolites occurrence in plasma after an intervention trial in hypercholesteremic children.

2. Experimental section

2.1. Rice bran collection

Heat-stabilized RB (110 °C for 6 min) from 17 rice genotypes from different geographical locations and genetic backgrounds including Cambodia (Cambodia), India (Njavara and Chennula), Kenya (SM1 and LM1), Mali (Khao Gaew, Shwetasoke, Gambiaka and DM16), Nepal (Nepal), Nicaragua (Nicaragua), and the United States (Calrose, Jasmine 85, SHZ, IAC600, LHT and RBT300) were used for untargeted and targeted lipidome analysis in the present study.

2.2. Polar lipids and enzymatic oxylipins of rice bran extraction

To focus on polar lipids and oxylipins, a methanolic extraction (80% aqueous methanol) was used to extract specifically these compounds, as previously described Zarei et al. (2017).

2.3. Total lipid profile determination by gas chromatography

In 25 mL round bottom flask, RB samples (~100 mg) with about 10% internal standard (C17:0) were added to 3 mL sodium methylate solution with phenolphthalein. Reaction medium was refluxed for 10 min. 3 mL chlorohydric methanol were added to phenolphthalein discoloration and the mixture was refluxed again for 10 min and then cooled to ambient temperature. 6 mL hexane and 10 mL water were added, and the organic phase was recovered and analyzed by gas chromatography (Zhang et al.). A Focus GC (Thermo Electron Corporation, Massachusetts, USA) was used and equipped with a split injector (ratio of 1/20), a CP-Sil 88 Varian capillary column (50 m×0.25 mm with 0.2 µm film thickness; Chrompack, Mid-delburg, Netherlands), and helium 1 mL/min as the carrier gas was used. Fatty acids methyl esters (FAME) were analyzed by flame ionization detector and ChromCard software

data system (version 2005, Thermo Fisher Scientific, Massachusetts, USA). The column temperature started from 150 °C, then reached 150 to 225 °C with a rise of 5 °C/min and was kept at 225 °C for 10 min. FAME were identified using as external standards a mixture of methyl esters (Mix37, Sigma-aldrich, Sweden). The injector and detector temperatures were 250 and 270°C, respectively. Each sample was methylated in triplicate.

2.4. Non-enzymatic oxylipins analysis by UPLC-MS-MS

The NEO-PUFA (PhytoPs and PhytoFs) present in RB were extracted following the methodology described by Pinciroli et al. (2017) with minor modifications. Briefly, 200 mg of crushed samples were placed into grinding matrix tubes (Lysing matrix D, MP Biochemicals, Illkirch, France) and added with 25 µL of BHT (1% in MeOH, w/v), 1 mL of MeOH and 4 µL of Internal Standard (IS, 1 µg/mL). The mixture was ground in the FastPrep-24 for 30 seconds, 6.5 m/s, (1 cycle) at room temperature, then transferred to 15mL centrifuge tube, and completed with 1 mL MeOH, and 1.5 mL of phosphate buffer (pH 2) saturated in NaCl. The set is placed in an IKA KS 4000 control shaker for 30 min (100 rpm-45sec / 90°-15sec), at room temperature. The samples extracts were centrifuged at 1500 rpm during 5 min. and supernatants were separated and added by 4 mL of cold chloroform. These solutions were vortexed for 30 seconds and then centrifuged at 2000 rpm for 5 min at cold temperature. Subsequently, aqueous phases were eliminated, and organic phases were isolated in a pyrex tube to be concentrated under a nitrogen flow at 40 °C. Hydrolysis of samples was performed by dissolving the dry extract into 950 µL of KOH (1 M in H_2O), followed by incubation into an shaker at 40 °C for 30 min and at 100 rpm. At the end of the incubation period, 1.0 mL of formic acid solution (40 mM) was added to the mixture and then stirred for 30 s. Solid phase extraction (SPE) was achieved by using Oasis mixed-mode ion-exchange sorbent cartridges previously, conditioned with 2 mL of methanol and equilibrated with 2 mL of 20 mM formic acid at pH 4.6. After loading the SPE column with samples, the cartridges were successively washed with: 2 mL of NH3 at 2 % (v/v), 2 mL of MeOH:formic acid 20 mM (30:70, v/v), 2 mL of hexane, and finally 2 mL of hexane:ethyl acetate (70:30, v/v). The column was dried for 1 to 2 minutes, then targeted metabolites were eluted with 2x1 mL of hexane:ethanol:acetic acid mixture (70:29.4:0.6, v/v/v) and then dried with N_2 (40°C, 1 h). The dry extracts were reconstituted with 100 µL of H_2O/ACN (83:17, v/v). Reconstituted samples were transferred in filtering eppendorf 0.45 µm and centrifuged at 10000 rpm for 1 min.

Chromatographic separation of PhytoPs and PhytoFs was performed using a micro liquid chromatography coupled with a QTRAP-MS/MS 5500 (Sciex Applied Biosystems), using the analytical column HALO C18 (0.5 x 100 mm, 2.7 μm, Eksigent Technologies, CA, USA). The column temperatures were 40 °C. The mobile phases consisted of LC-MS grade water/(ACN/MeOH 80:20) added with 0,1% formic acid. The injection volume and flow rate were 5μL and 0.03 mL min−1 upon the following gradient (min / %B): 0/17; 1.6/17; 2.85/21; 7.3/25; 8.8/28.5; 11/33.3; 15/40; 16.5/95; 18.9/95; 19/17; 21/17. The spectrometric analysis was conducted in Multiple Reaction Monitoring mode (MRM) operated in negative mode. Electrospray ionization (ESI) in negative mode was used as ionization source. Nitrogen (N_2) was used as curtain gas and the voltage was kept at −4.5 kV. Data acquisition and processing were performed using MultiQuant 3.0 software (Sciex Applied Biosystems). The quantification of PhytoPs and PhytoFs detected in RB was performed by constructing calibration curves of the ratio between analyte to IS area under the curve.

2.5. Clinical study

Plasma metabolome was completed from a previously described clinical trial (NCT01911390) Borresen et al. (2017) . Briefly, 18 hypercholesteremic children aged 8-13 years old were evaluated before and after 4 weeks of consumption using prepared snacks (low-fat, low-sodium, high-fiber and heart-healthy): containing 15g/day of RB: either 2 (114g) banana muffins with hazelnut or 1 (247g) banana, strawberry, and pineapple smoothie once a day for 4 weeks. Two study intervention groups were included herein a) 9 control participants that received snacks prepared without RB and b) 9 RB intervention participants that received 2 snacks per day prepared with 7.5g RB for a total daily dose of 15g/day. Blood (4 mL) was collected in EDTA tubes at the beginning of the study and after 4 weeks of daily consumption of the snacks. Blood was processed for plasma metabolite profiling by storing immediately on ice and final storage at -80°C prior to metabolite extraction.

2.6. Rice bran and plasma analysis

Lipids of RB (~100 mg) were extracted with 80% methanol by Metabolon, Inc. (Durham, NC, USA) and plasma was prepared using the automated MicroLab STAR® system (Hamilton Company, USA). To remove protein of plasma and recover small molecules, the sample was precipitated with methanol and vigorously shaken for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation Borresen et al. (2017). Untargeted

lipidomics of all RB extract samples were analyzed by UPLC-MS/MS and gas-chromatography mass spectrometry (GC-MS) in the positive and negative ionization mode platforms. The plasma extract was analyzed by reverse phase UPLC-MS/MS with positive and negative ion mode electrospray ionization and hydrophilic interaction liquid chromatography (HILIC) with negative ion mode electrospray ionization. The data were processed into Metabolon's Library Information Management System, according Li et al. (2018b). Briefly, raw data were obtained and peak-identified by Metabolon. The identified lipids were confirmed by comparison to an internal library of over 3.300 entries of purified standards or recurrent unknown entities maintained by Metabolon, based on the retention time/index, *m/z*, and chromatographic data. Metabolites were quantified in terms of relative ion abundance.

2.7. Statistical analysis

EZInfo v. 3.0.3 (Umetrics, Sweden) was used for the analysis of metabolomics multivariate data. Principal Component Analysis (PCA) using pareto-scaling (van den Berg et al., 2006) and S-plot by Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) were generated from UPLC-MS-MS data. The fatty acid profile data were submitted to one-way ANOVA (Tukey, $p < 0.05$) by using XLStat (v 2021.1.1).

3. Results and discussion

3.1. Rice bran lipidome

To deeply characterize novel lipids present in RB, we applied untargeted lipidomics to the methanolic extracts of 17 rice varieties, and identified 163 distinct lipids which substantially expanded upon the 20 classical compounds routinely quantified from RB oil fractions (Friedman, 2013). Supplementary Table 1 shows the complete metabolites list for the identified lipids. The RB lipidome encompassed nine distinct chemical classes and each of the nine groups differed by number and relative abundance (Figure 1) across rice varieties. In total, 118 lipids were determined as the core of the lipidome by definition of positive detection across all 17 varieties examined.

In a first glance, first focusing on number of identifications which reflects the diversity of compounds in the methanolic extract, we can say that the majority of these core lipids (72% of total number) did not differ in type by variety or growing conditions. There were 45 lipids that did vary by type among the RB collection and were therefore characterized to be

outside of the core lipidome composition. The 28% (45 lipids) associated with genotypic and environmental diversity merit attention for breeding programs. While the function and bioactivity of many RB lipids have been studied (Friedman, 2013), this lipidome, although focusing on semi and polar lipids, revealed novel and minor chemical lipid sub-groups that may demonstrate additive or synergistic activities when RB is consumed as a whole food ingredient.

Phospholipids comprised 34% of the lipidome in this analysis (diversity of structures), and represented the largest class within the lipid profile, which was followed by 19% for glycerolipids and free fatty acids. The lipids that make up 1-7% of the lipidome included galactolipids, oxylipins, vitamin E isoforms, sterols, fatty acid esters, amino fatty acids and terpenoids (Figure 1A).

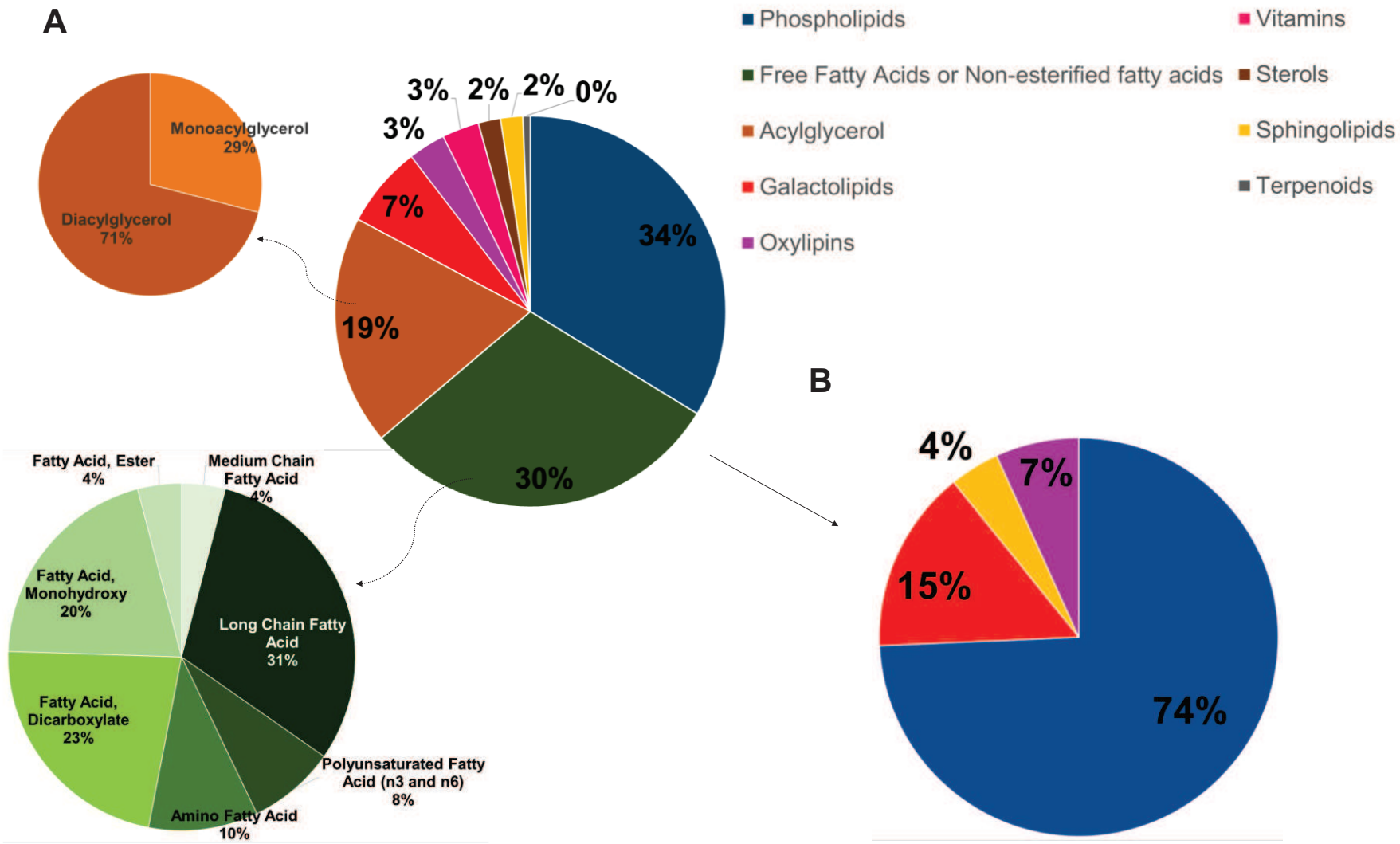


Figure 1 - RB lipidome comprises nine sub-classes, here represented by percentage of total number of identifications. This percentage of each sub-class for A) All identified chemical groups and sub-groups; B) selected polar lipids (phospholipids, galactolipids and sphingolipids) and oxylipins.

Phospholipids represent the greatest abundance in RB and a diverse spectrum of compounds, such high abundance and diversity could be explained as being the backbone of membrane cells and organelles. Liu et al. (2013) have previously shown phospholipids in rice whole grains as a major lipid class (~10-50% of total lipids) with important biological functions but did not separate the RB contribution to these findings. Among the subclasses of phospholipids, the phosphatidylcholines (PC) represent (35%) followed by the phosphatidylethanolamine (PE) (22%), and then phosphatidic acid and phosphatidylinositol (PI) are 16%, with the lowest amounts coming from phosphatidylglycerol (PG) (9%) and phosphatidylserine (Oliveira et al.) (2%). In a recent review, Robert et al. (Robert et al., 2020) reported similar classes in vegetable lecithin and more precisely a profile dominated by PC but with variable amount in PI in soy, sunflower or rapeseed lecithins. A recent study analyzing the phospholipidome in olive oil shows a higher presence/diversity of PG, followed by PA and PI (Antonelli et al., 2020). The glycerophospholipids present in our RB extracts are also found in the plasmatic membrane reported in mammalian cells and similar classes were found for example in cows milk coproducts such as butter serum in a prior study Gassi et al. (2016). These results thus emphasize the nutritional interest of RB coproducts since phospholipids cover a wide range of bioactivities that gather cholesterol-lowering properties, antiviral effects, memory improvement effect or improvement of cognitive function…) (Kullenberg et al., 2012; Liu et al., 2013).

The lower level presence of galactolipids (7%) and sphingolipids (2%), remain noteworthy molecules as these lipids are present in both plant and animal cell membranes. The galactolipids in plants can act like a storage depot for essential fatty acids even at low concentrations (Sahaka et al., 2020).

The diversity of compounds in a given class is not always reflected in their abundance in the samples. In untargeted analysis, the relative quantification can be calculated from the total relative ion abundance. Although polar lipids (phospholipids, galactolipids and sphingolipids) represented 40% of RB lipidome in terms of diversity, and oxylipins represent 3% (Figure 2A), the relative abundance of oxylipins is notably five times higher than the polar lipids, as shown in Figure 2B. The USA - RBT300 is a commercially available source of RB and has a particularly distinct profile with an inversion in the oxylipin/polar lipid relative abundance ratios that may be a result of differences in milling.

Phospholipids may also play a role in PUFA oxidation products and increasing oxylipins abundance. These oxilipins can be formed both enzymatically and non-

enzymatically when the sn-2 fatty acids of phospholipids are oxidized by radicals. So, depending on the fatty acid present in sn-2, the oxilipin generate will change (e.g., if is a arachidonic acid: HETEs will be formed, whereas if is a linoleic acid, HODEs wiil be formed) (Subbanagounder et al., 2000).

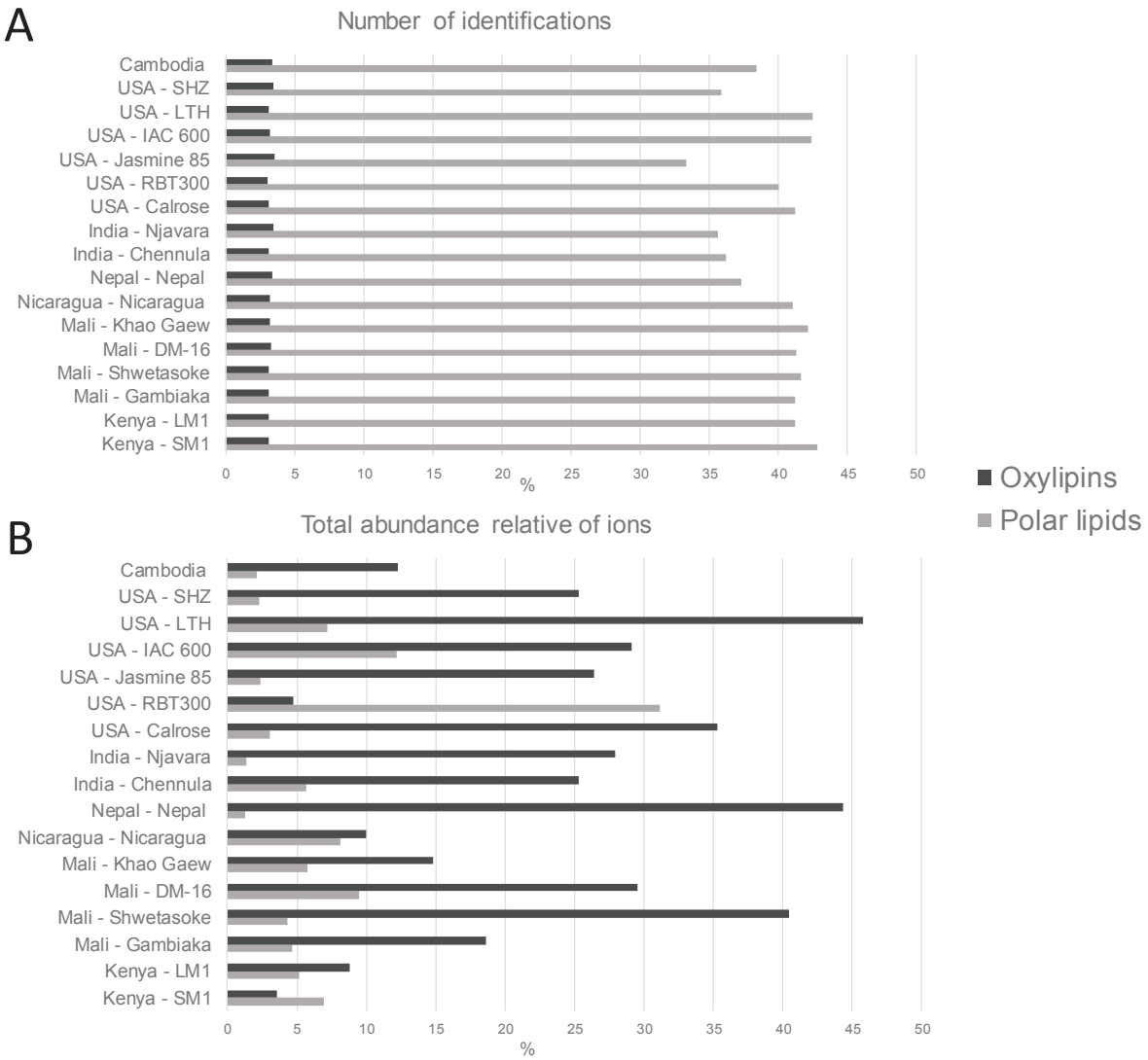


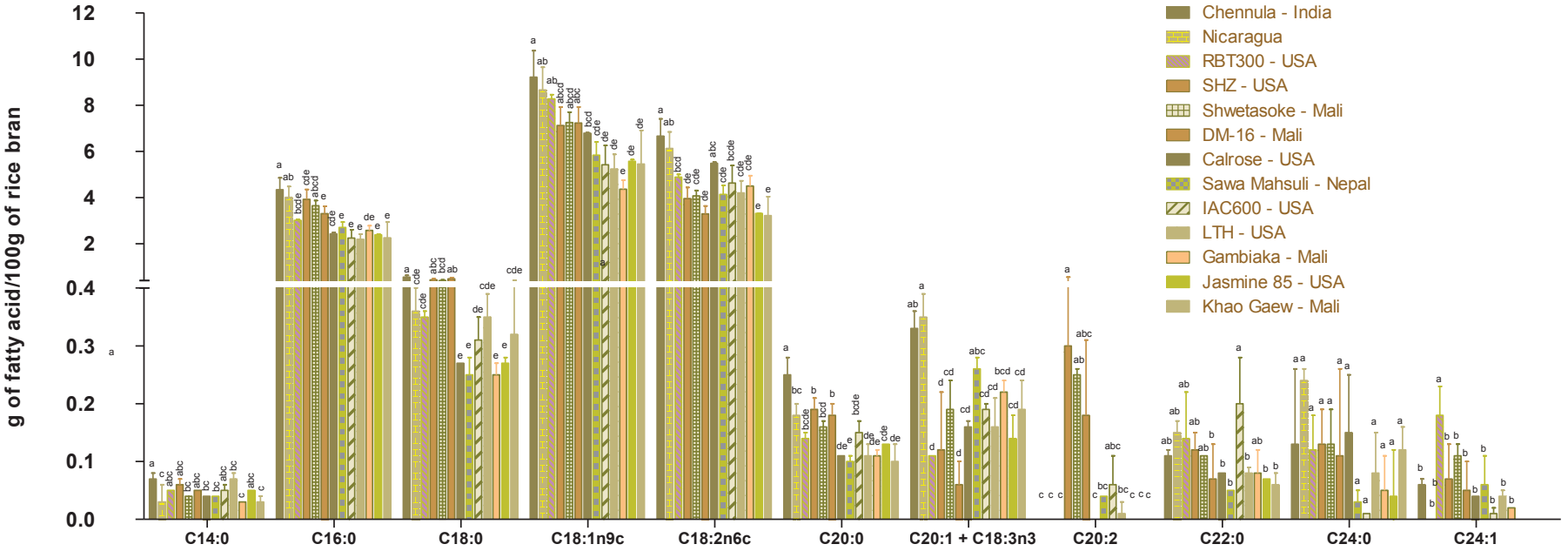
Figure 2 - Percentage of polar lipids (Wang et al.) and oxylipins (grey) in the RB lipidome, considering the number or abundance total like 100%. A: Percentage of number of diversified molecules of lipids; B: Total relative abundance of ions.

3.1.2. Rice bran fatty acid quantification across varieties

3.1.2.1. Fatty acid profile carried out by gas-chromatography

RB contains a similar fatty acid profile for all varieties (Figure 3A and B) and with prominent levels of oleic acid (~40%), linoleic acid (~35%) and stearic acid (~18%). These data are consistent with previous reports in the scientific literature (Friedman, 2013) indicating good conservation of FA whatever the genotype.

A)


g of fatty acid/100g of rice bran
12
10
8
6
4
2
0.4
0.3
0.2
0.1
0.0
C14:0
C16:0
C18:0
C18:1n9c
C18:2n6c
C20:0
C20:1 + C18:3n3
C20:2
C22:0
C24:0
C24:1
Chennula - India
Nicaragua
RBT300 - USA
SHZ - USA
Shwetasoke - Mali
DM-16 - Mali
Calrose - USA
Sawa Mahsuli - Nepal
IAC600 - USA
LTH - USA
Gambiaka - Mali
Jasmine 85 - USA
Khao Gaew - Mali

B)

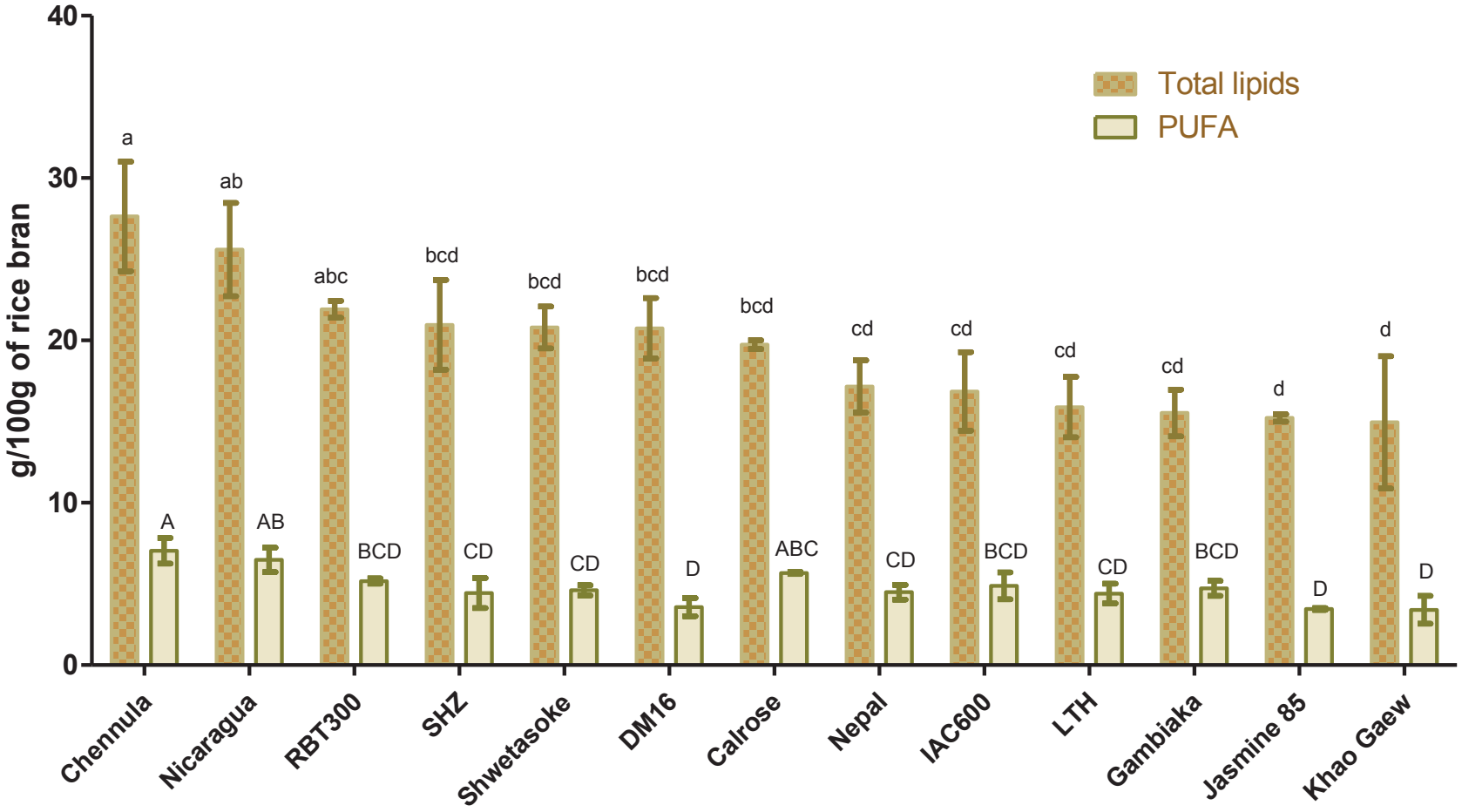


Figure 3 – Varietal differences and similarities in RB fatty acid profiles A) Polyunsaturated fatty acids and total lipids in RB (g of fatty acid/100 g of RB).B) RB Fatty acids in 13 RB samples (g of fatty acid/100 g of RB). Different lowercase letters mean a significant difference (one-way ANOVA, $p<0.05$) between the cultivars for each fatty acid.

Despite conservation of FA profile, total lipid does vary with variety and genotype ranging from 27.64 g/100 g of RB in Chennula to 14.95 g/100 g of RB in Khao Gaew. The variability of total lipid contents is remarkable if we analyze only the samples from Mali genotypes where Shwetasoke (20.79 g/100 g of RB) and DM16 (20.74 g/100g of RB) show no statistical difference between us, but Gambiaka (15.53 g/100 g of RB) and Khao Gaew (14.95 g/100 g of RB) are statistically different. These results confirm the importance contribution of lipid content on RB. Detailed attention to the environmental conditions of cultivation, agronomic practices and post-harvest processing are warranted to facilitate a strong understanding of the potential for additional modifiers of RB lipid composition. The PUFA contents in RB ranged between 3.41 to 7.05 g/100 g of RB (figure 3B). The Chennula genotype (India) had the highest content of PUFA, followed by Nicaragua (Nicaragua), Calrose and RBT300 (USA) with 6.49 g/100 g, 5.68 g/100 g and 5.18 g/100 g of RB, respectively. Levels indicated for linolenic acid, a fatty acid that is subject to enzymatic and non-enzymatic oxidation, is of special interest since its oxidation products have shown important bioactivities. Such interest supports our next focus developed in following section and on targeting oxylipins.

3.1.2.2. Non-enzymatic oxylipins analysis: emphasis in phytoprostanes and phytofurans

For the specific analysis of the NEO-PUFA, the RB that had the highest abundance of PUFA were selected. These included the India genotypes Njavara and Chennula, and the Mali genotypes Shwetasoke and DM16, one genotype from Nepal, one from Nicaragua, and the following from United States (Calrose, Jasmine 85, SHZ, LHT, IAC600 and RBT300). Studies highlight these NEO-PUFA for not only being considered novel markers in plants, but also for presenting relevant biological activities to humans (Leung et al., 2021).

The total amount of PhytoPs and PhytoFs were previously described in white grain flour, brown grain flour and RB (Pinciroli, Domínguez-Perles, Abellan, et al., 2018; Pinciroli et al., 2017; Pinciroli, Domínguez-Perles, Garbi, et al., 2018). Figure 4 shows the concentrations detected in the varieties tested herein in decreasing order of mass. This study demonstrates higher contents of total PhytoPs when compared to prior reports, and ranging between 316.98 ng/g to 2937.63 ng/g of RB. PhytoFs ranged between 117.05 ng/g to 1695.05 ng/g of RB. Among the genotypes, Shwetasoke from Mali showed the highest levels, and was significantly different from other varieties (p<0.05) with respect to total PhytoPs and PhytoFs (2937.63 ng/g of RB and 1695.05 ng/g of RB, respectively). The study conducted by Pinciroli

et al.(Pinciroli et al., 2017) with whole rice cultivars from Argentina indicated the highest content in RB as 118.00 ng/g and 27.74 ng/g for PhytoPs and PhytoFs, respectively.

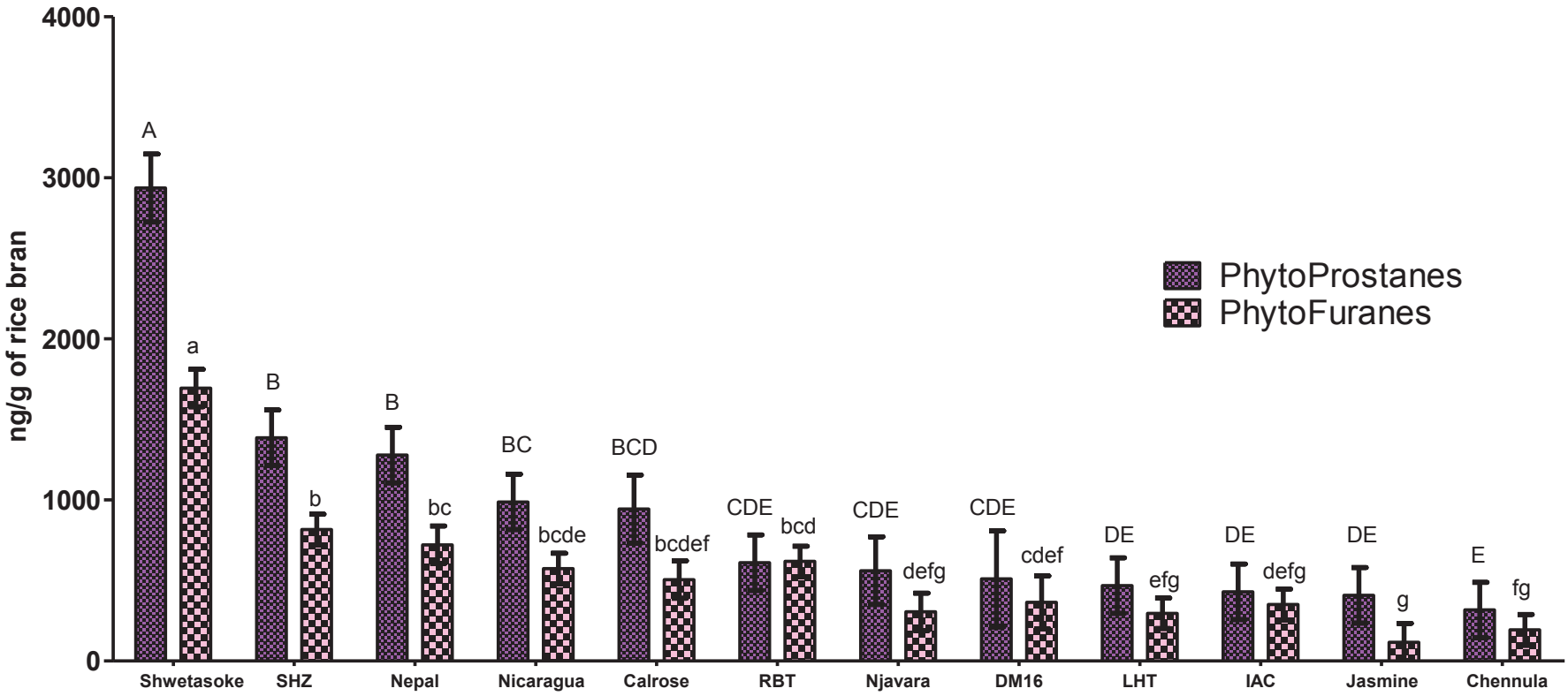


Figure 4 – Total content of PhytoPs and Phytofs in each genotype. Uppercase letters are significatly different at $p<0.05$ according to Fisher test between the PhytoPs content in each RB genotypes and lowercase letters are significatly different at $p<0.05$ according to Fisher test between the PhytoFs content in each RB genotype.

There were seven compounds of PhytoPs identified herein, namely *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-B_1-PhytoP, *ent*-9-L_1-PhytoP and 16(*RS*)-16-A_1-PhytoP,and three compounds of PhytoFs: *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF. All genotypes of RB in this study presented all PhytoPs and PhytoFs. The amount of each compound is displayed in Table 1 (with the exception of two PhytoFs (*ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF) due to coelution with other compounds that may influence the final content). The main PhytoP found in RB was 16(*RS*)-16-A_1-PhytoP (ranging between 60.16 ng/g of RB in Chennula from India to 1184.04 ng/g of RB in Shwetasoke from Mali) and *ent*-16-B_1-PhytoP (ranging from 53.62 ng/g of RB in Chennula from India to 657.82 ng/g of RB in Shwetasoke from Mali), while the main PhytoF found in RB was *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF (ranging between 72.32 ng/g of RB in Jasmine 85 from USA to 687.63 ng/g of RB in Shwetasoke from Mali). To our knowledge, this study is the first report on the detection of 16(*RS*)-16-A_1-PhytoP in RB.

Base on literature and on impact of temperature and storage conservation on PhytoF and PhytoP formation, we anticipate that there could be an influence of the country of origin on these lipids. Indeed the country of origin very likely influence postharvest conditions, milling process and storage time after milling in the rice industry (Pinciroli, Domínguez-Perles, Garbi, et al., 2018). However, the wide spectrum range of different PhytoP and PhytoF contents among the varieties did not allow for correlations by country of origin. In addition, we can say that quantification of PhytoPs and PhytoFs soon after the milling process would be ideal for accurate comparisons but is impossible considering our sampling scheme in various countries.

Table 3 - Phytoprostane and Phytofurane concentrations in RB for each genotype (ng/g of RB).

Genotype	Phytoprostanes							Phytofurans
	ent-16-F_{1t}-PhytoP	*ent-16-epi-16-F_{1t}-PhytoP*	*9-F1t-PhytoP*	*9-epi-9-F_{1t}-PhytoP*	*ent-16-B_1-PhytoP*	*ent-9-L_1-PhytoP*	*16(RS)-16-A_1-PhytoP*	*ent-16(RS)-9-epi-ST-Δ^{14}-10-PhytoF*
Chennula	39.96±4.36^{bcd}	29.56±2.44^{bcd}	54.86±8.58^{cd}	30.88±2.68^{cd}	53.67±4.49^{b}	47.96±5.83^{b}	60.14±4.02^{c}	76.00±9.48^{f}
Njavara	59.12±15.84^{abcd}	44.90±4.68^{abc}	89.63±9.37^{cd}	49.45±5.49^{bcd}	98.43±9.92^{b}	91.28±3.88^{b}	119.19±9.06^{c}	156.46±20.86^{def}
DM16	59.81±0.00^{abcd}	38.17±0.00^{bcd}	83.04±0.00^{cd}	36.63±0.00^{bcd}	93.21±0.00^{b}	86.46±0.00^{b}	112.57±0.00^{c}	199.88±0.00^{cdef}
Shwetasoke	89.65±42.49^{a}	67.96±29.64^{a}	250.55±98.36^{a}	94.29±14.53^{a}	657.82±266.00^{a}	593.31±292.32^{a}	1184.04±483.25^{a}	687.63±289.85^{a}
Nepal	86.47±23.09^{ab}	45.97±3.50^{abc}	200.67±47.14^{ab}	74.31±22.85^{ab}	253.58±58.23^{b}	227.39±57.62^{b}	386.00±92.69^{bc}	420.59±11.93^{b}
Nicaragua	95.50±6.29^{a}	69.65±2.03^{a}	111.33±13.92^{bcd}	71.06±7.13^{ab}	196.07±47.14^{b}	167.89±31.39^{b}	276.61±53.92^{bc}	385.19±52.46^{b}
Calrose	48.78±7.57^{abcd}	27.27±5.64^{bcd}	75.87±17.15^{cd}	45.60±8.56^{bcd}	208.19±1.87^{b}	212.22±6.49^{b}	326.26±6.19^{bc}	319.09±79.20^{bcd}
IAC 600	28.10±4.59^{cd}	20.09±4.11^{cd}	57.77±11.97^{cd}	23.40±5.51^{cd}	80.49±10.44^{b}	69.13±5.51^{b}	150.59±18.14^{c}	240.66±62.20^{cde}
Jasmine 85	24.33±4.67^{d}	16.31±3.64^{d}	31.00±2.69^{d}	17.06±1.43^{d}	99.95±31.75^{b}	92.25±29.30^{b}	127.41±35.16^{c}	72.32±4.72^{f}
LHT	54.20±5.72^{abcd}	49.61±5.06^{ab}	94.46±14.75^{bcd}	53.61±4.19^{bc}	65.00±8.31^{b}	65.22±5.84^{b}	85.93±7.30^{c}	148.33±26.13^{ef}
RBT 300	68.48±7.66^{abcd}	50.75±5.49^{ab}	102.19±7.91^{bcd}	49.99±5.58^{bcd}	110.54±11.51^{b}	89.18±8.68^{b}	139.13±12.56^{c}	246.90±14.35^{cde}
SHZ	75.80±23.26^{abc}	39.37±4.11^{bcd}	144.34±28.24^{abc}	80.16±8.22^{ab}	224.28±52.18^{b}	218.51±38.11^{b}	611.07±78.59^{b}	361.36±36.92^{bc}

Letters indicate significant differences between the RB genotypes for a given Phytoprostane or Phytofurane (one-way ANOVA, $p<0.001$)

3.1.3. Multivariate analysis of rice bran lipidomes

The multivariate analysis of partial least squares (PLS) confirmed the differentiation of the global lipid profiles of each variety (Figure 5). In the Figure 5A, across all cultivars and all identified lipids, the PLS component 1 (PC1) explains 80% of the total variation in the dataset, and PC2 6%. The PLS also shows the separation of the groups from the model. These results showed that the lipid profile is not specific to regions (or countries). Given the separation of genotypes grown in the same country, we conclude that the lipid profiles cannot be considered specific to each region, and that we should emphasize greater genotypic influence.

The PLS analysis highlighted lipids responsible for differentiation of RBs by cultivars by integrating the relative total abundance, including presence or absence of each metabolite. Figure 5 shows the 10 discriminant lipids by VIP (Variable importance in projection) (in red color) are fatty acids, glycerolipids, oxylipins and a polar lipid. These included oleic acid (18:1n9c), linoleic acid (18:2n6c), palmitic acid (16:0), linoleoyl-linoleoyl-glycerol (18:2/18:2) [1], oleoyl-linoleoyl-glycerol (18:1/18:2) [2], oleoyl-oleoyl-glycerol (18:1/18:1) [1] and palmitoyl-linoleoyl-glycerol (16:0/18:2) [2], two oxylipins: 13-HODE + 9-HODE and 9,10-epoxystearate and one polar lipid, the phospholipid glycerophosphorylcholine (PC).

Focusing on the polar lipids and oxylipins in Figure 5B, the PLS component 1 (PC1) accounted for 62% of the total variation in the dataset, and PC2 for 18%. We can note that the configuration of the groups has been changed in comparison with complete data analysis (all lipids in Figure 5A). Nepal RB was the variety that shows the most difference between the two PCA projection. The VIP compounds, in this case, indicated 3 oxylipins (13-HODE+9-HODE, 9,10-epoxystearate and 13-HOTrE - hydroxyoctadecatrienoic acid) and 7 phospholipids. To note, all these phospholipids have a PC polar head and with MUFA or PUFA acyls moieties: 1,2-dilinoleoyl-PC (18:2/18:2), 1-oleoyl-2-linoleoyl-PC (18:1/18:2), 1,2-dioleoyl-PC (18:1/18:1), 1-palmitoyl-2-oleoyl-PC (16:0/18:1), 1-oleoyl-PC (18:1), 1-palmitoyl-2-linoleoyl-PC (16:0/18:2)).

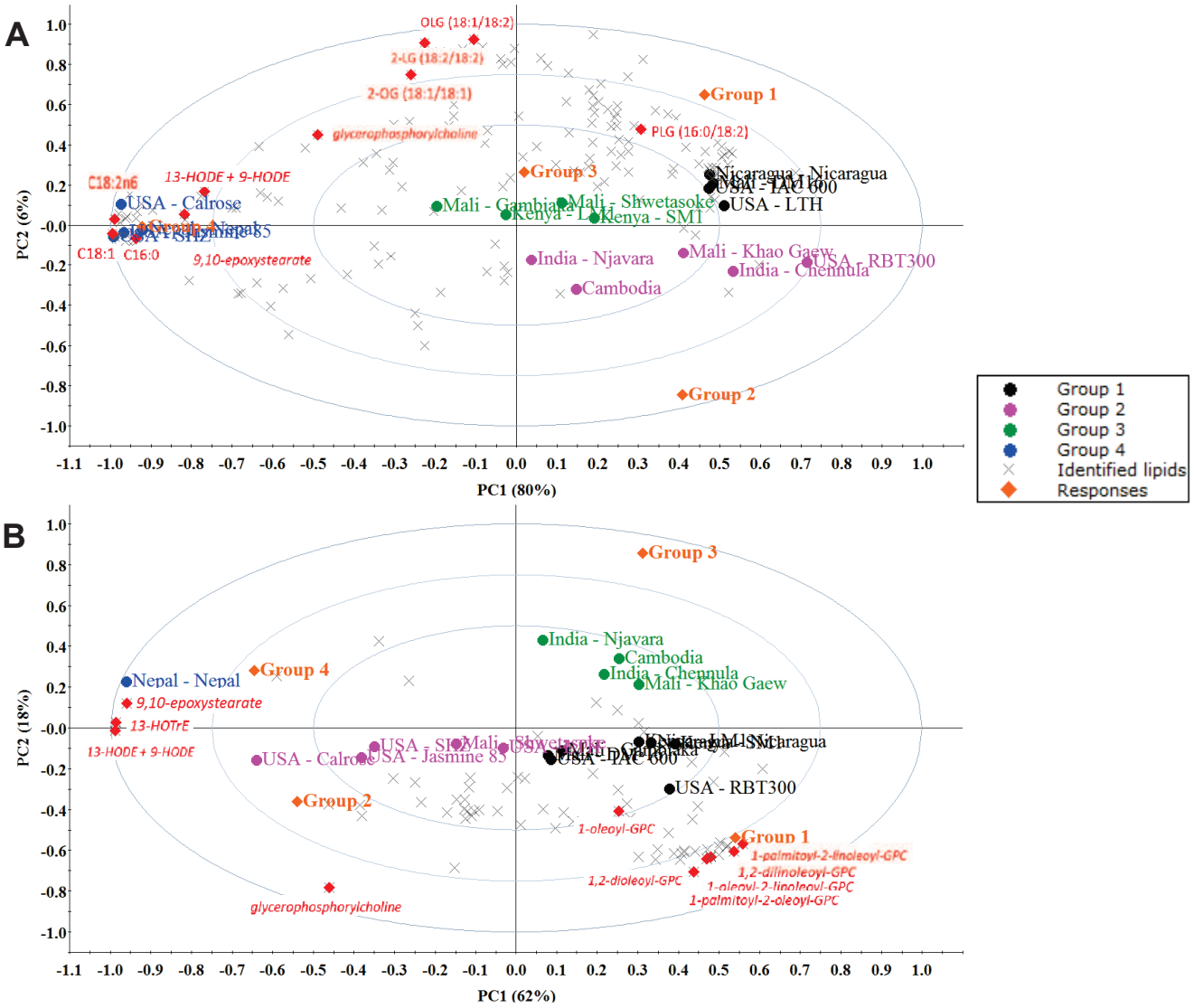


Figure 5 - Principal component analysis (PCA) from the total median scaled-relative abundance of ions for 163 identified lipids. A) Considering all lipids/subgroups identified; b) selected polar lipids and oxylipins groups. (Group 1: Nicaragua, Mali-DM16, USA-IAC600, USA-LHT; Group 2: USA-RBT300, Mali-Khao Gaew, India-Chennula, India-Njavara, Cambodia; Group 3: Mali-Shwetasoke, Kenya-SM1, kenya-LM1, Mali-Gambiaka; Group 4: USA-Calrose, Nepal, USA-Jasmine85, USA-SHZ)

Untargeted lipidomics supported that there was diversity between tested RB and highlighted the importance for presence of smaller lipid classes to the core lipidome. This work suggests that low abundance oxylipins and glycerolipid molecules should be considered for bioactivity importance with potential for synergies with higher abundance fatty acids and phospholipids. RB as a source of synergistic bioactive lipids (phospholipids and oxylipins) has been limited prior to these comprehensive profiling and targeted lipid analysis.

3.2. Plasma lipidome in children after rice bran consumption

To evaluate whether lipids from a diet enriched with RB could positively modulate blood lipid metabolism in humans, we analyzed plasma from a study completed with moderately

hypercholesterolemic children (Borresen et al., 2017; Li et al., 2018b). Plasma lipidomics revealed a total of 406 plasma lipids for all the children involved in the study. Nine groups of compounds were identified for strong association to lipid metabolism (Figure 6). Among these nine groups, the major classes by decreasing amount of total diversity were: a class of 26 % of total diversity gathering metabolites related to fatty acids/bile salts or vitamin metabolism, a class of 19 % of total diversity gathering fatty acids, a class of 18 % of total diversity gathering phospholipids, plasmalogens and their products of hydrolysis, a class of 12% of total diversity gathering sphingolipids and their derivatives, that is to say ceramides (Figure 6).

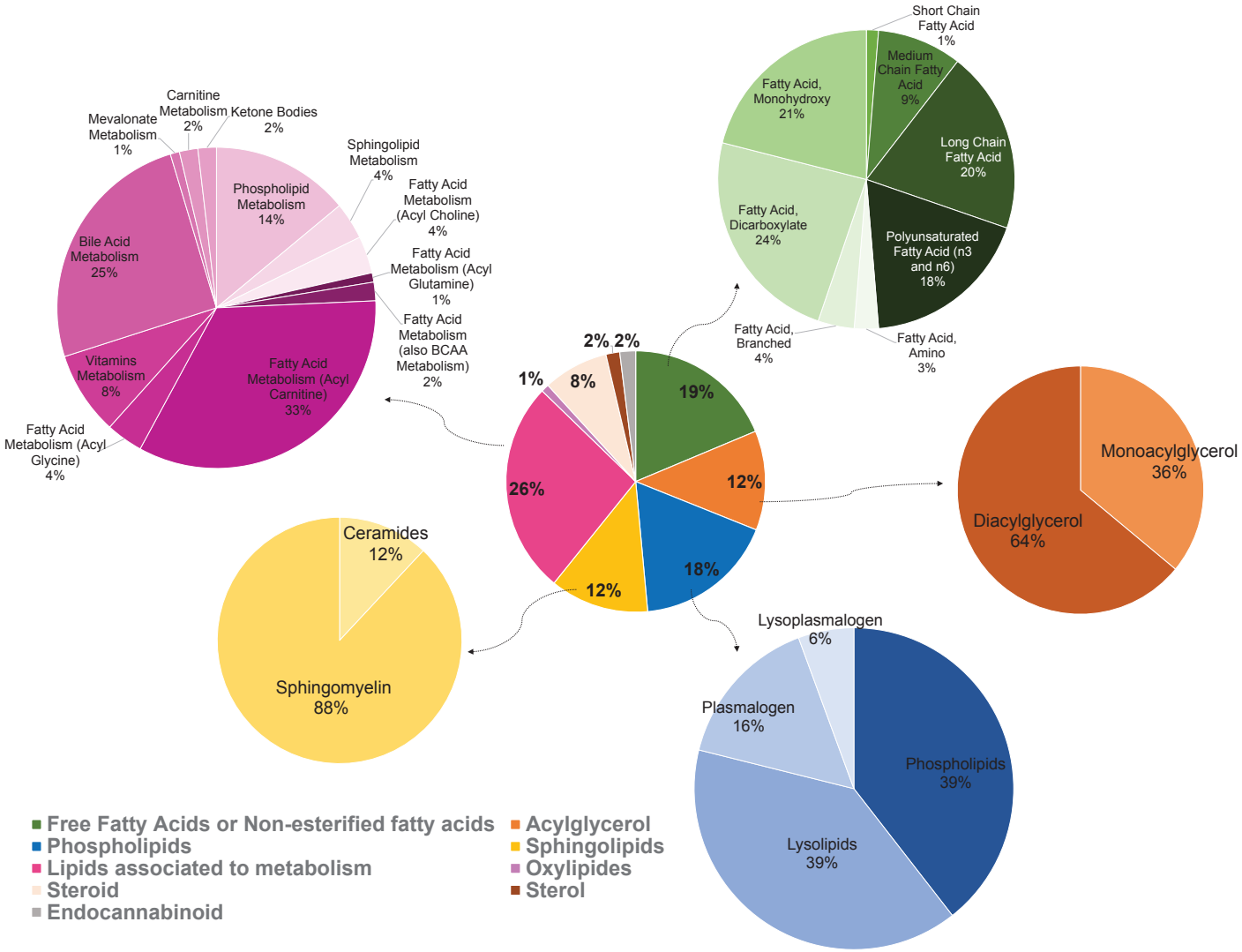


Figure 6 – Plasma lipidome and chemical subgroups from children before and after daily RB consumption for 28 days by UPLC-MS-MS.

Among the identified groups, the presence of long-chain fatty acids, as well as omega-3 and omega-6 fatty acids is noteworthy and reflect their important part in lipid metabolism. PUFA represents 18% of the fatty acids identified in this study (Figure 6).

Other important groups both in terms of diversity and functionally are phospholipids and sphingolipids that contribute to lipoproteins assembly and transport of lipid to target organs and will be considered throughout this discussion.

The multivariate analysis of plasma from children after the diet was supplemented with RB did not show major difference in terms of molecules present or strong associations between the molecules profile studied in overall lipidome. However, we next applied a focus on phospholipids and important lipids metabolites, including sphingolipids, fatty acids, oxylipins, sterols, and endocannabinoids for scrutiny in the blood (decreasing the number of identified metabolites to 285 lipids).

The PCA performed on the plasma metabolomes between RB baseline and RB after 4 weeks intervention was not able to project lipids as the maximum variance between the individuals in the study (n=18) (Figure 7A). To assist with maximizing the variance captured in the first dimension (x-axis), these data were submitted to an Orthogonal partial least squares discriminant analysis (OPLS-DA) model capturing the maximum difference between the 2 groups, shown in Figure 7B.

In Figure 7b it is possible to observe a great variability of the lipid profile among the children studied. In order to find compounds that are able to distinguish the two groups the covariance p[1] and correlation p[1] loadings from a two class OPLS-DA model (RB Baseline vs. RB 4 weeks) are shown in S-Plot format (Figure 7C). The points are Exact Mass/Retention Time pairs (EMRTs). The upper right quadrant of the S-plot shows those components which are elevated in RB Baseline, the control group, while the lower left quadrant shows EMRTs elevated in RB after 4 weeks, the treated group.

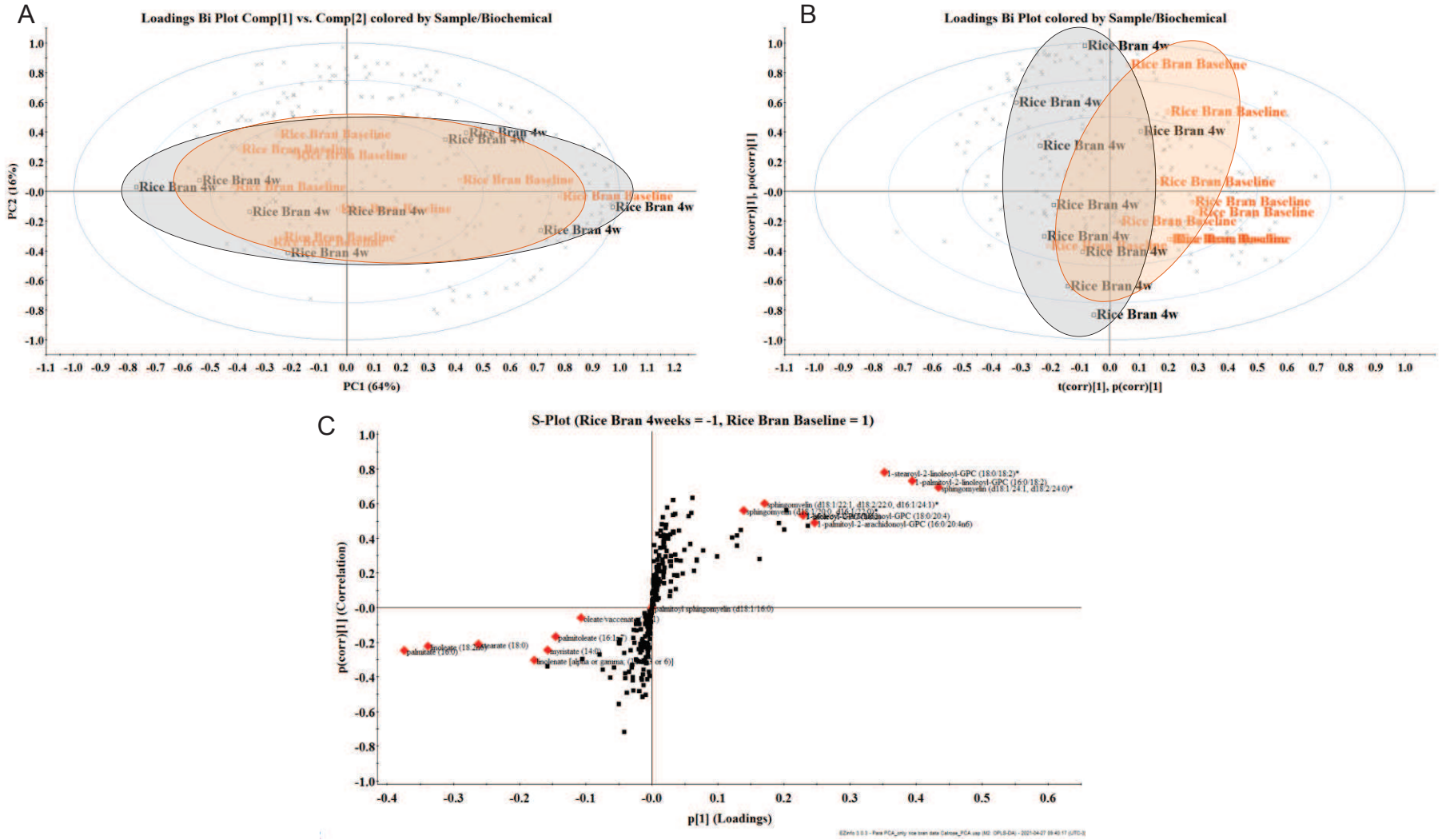


Figure 7 – Multivariate analysis from hypercholesteremic children comparing the lipid profile in plasma at the beginning of this study and after 4 weeks of RB supplementation. A) Principal Component Analysis (PCA); B) Orthogonal partial least squares discriminant analysis (OPLS-DA) model; C) S-plot from the OPLS-DA showing the most important lipids to discriminate the difference between the groups.

The further along the x-axis the greater the contribution to the variance between the groups (baseline vs. 4 weeks), while the further the y-axis the higher the reliability of the analytical result. The measured intensities and factor of change are based on the average of the measured values for each EMRT in the group. Some of the most important compounds responsible to discriminate the groups are plotted in red in figure 7C. Among them, we can highlight the presence of circulating phospholipids and sphingolipids in the plasma of the patients at the beginning of the study, against the presence of long-chain fatty acids (tails of 13 to 21 carbons), very long-chain (tails of 22 or more carbons) and also mono and polyunsaturated (oleic, linoleic and linolenic) fatty acids. Note that very long-chain FA (C22:0, C24:0) are also present in low quantity in RB (Figure 3.A). A paired comparison of the discriminating compounds for each child studied again remarks the variability of the lipid profile. Only 56% of the patients showed changes according to the discriminating lipids.

In order to clarify the results and make correlations between lipid profiles between patients, heat maps were performed and are displayed in Figure 8.

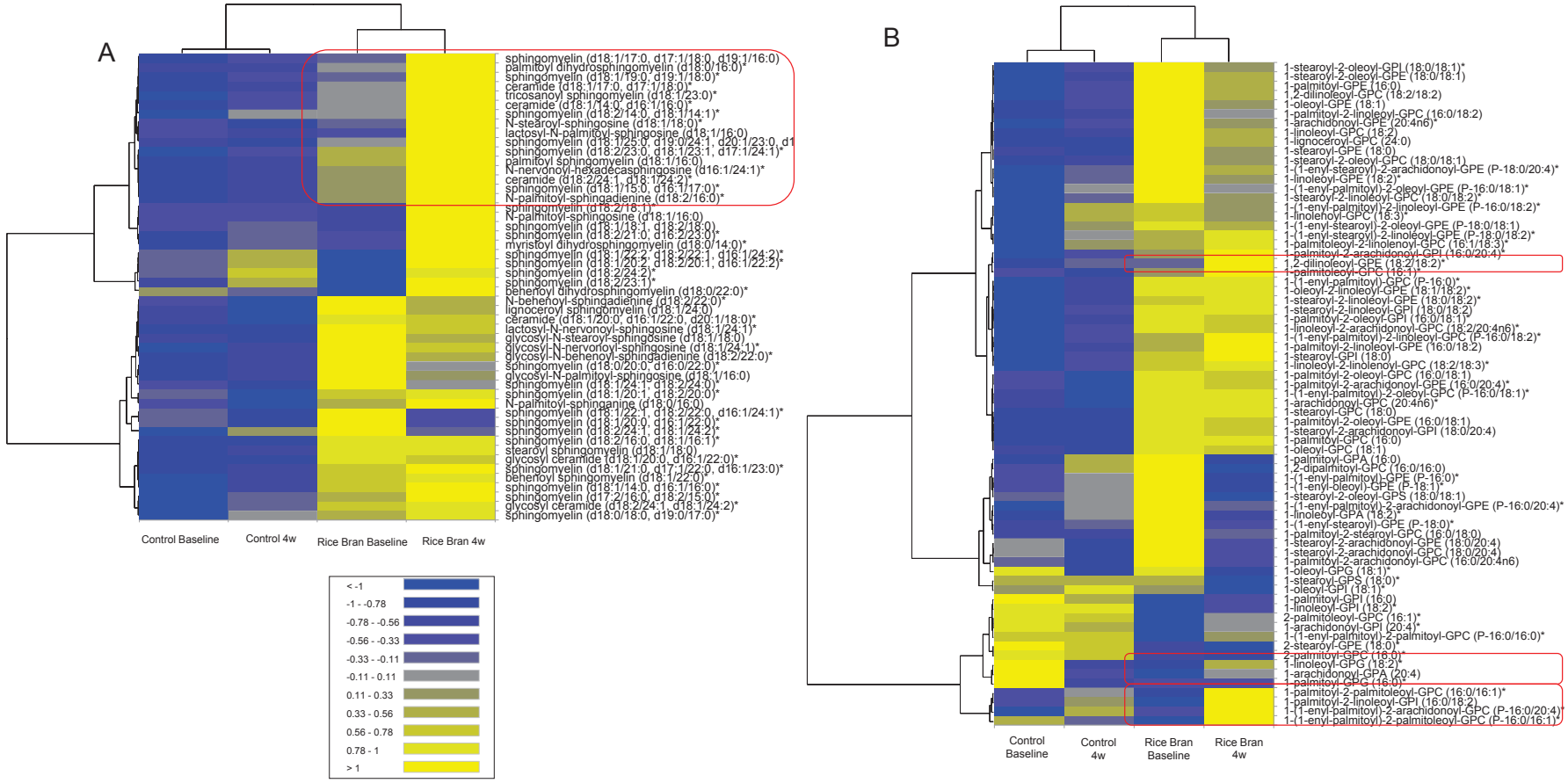


Figure 8 - Heatmaps of different groups of lipids to compare the presence/abundance in patients between control baseline vs control 4 weeks vs RB baseline vs RB 4 weeks. A) Group of sphingolipids; B) Group of phospholipids.

In Figure 8A, comparing the supplemented patients before and after 4 weeks and focusing first on sphingolipids, there is a perceptible increase after supplementation. Control patients had absent or low presence of these lipids and this may be associated with hypercholesterolemia.

In Figure 8B, focusing this time on phospholipids, it is possible to highlight some phospholipids that can be related to the supplementation. Among them, we find PUFA in position 2 and phosphate groups as PC, PE, and PI. Also noteworthy is the increase in circulating PUFA in plasma after supplementation. This fact may be related to the diversity of molecules in which they may be esterified and increasing their bioavailability (phospholipids and acylglycerols). Cuenoud et al. (2020) showed that monoacylglycerols can act as protectors against digestive actions, carrying omega 3 fatty acids and then modify their bioavailability.

To be more focused on the groups of oxylipins, endocannabinoids and fatty acids, another heatmap was performed and is displayed in Figure 9A. It is possible to highlight positive changes on these groups in children plasma after supplementation. Regarding oxylipins, we underline the decrease of two oxylipins: 9,10-DiHOME and 12-HETE. Some oxylipins can also have a proinflammatory action, signaling a lipid disorder and these oxilipins are characterized to their potential prooxidant effect (Putman et al., 2019).

Daily supplementation for 4 weeks also induce an increase in endocannabinoids (Figure 9A), one of the classes that showed the lowest number of identifications but which are key messengers of the gut-brain axis (DiPatrizio, 2021). Endocannabinoids are endogenous neurotransmitters, lipid-derived, produced from the consumption of the essential fatty acids and can act as a signal for a metabolic disorder (Hillard, 2018). Among the endocannabinoids whose presence increase post RB we can notice specifically: arachidonoyl ethanolamide (synthesized from arachidonic acid) and stearoyl ethanolamide.

To finish, still in figure 9A, we can highlight the presence of available PUFAs in the plasma of the children after the 4-week diet. It is important to signal the presence of some fatty acids that can be directly related to RB diet such as oleic, linoleic and linolenic acids, behenic acid, docosadieoic, docohexaecoic acids (DHA), as they showed an increase over the RB baseline.

A paired analysis was performed from the identified PUFAs, following the synthesis pathway according to Balić et al. (2020) and Sergeant et al. (2016). In figure 9B, 5 children

showed an increase in the PUFA detected that were present in the metabolism of omega-3 pathway such as linoleic, gamma-linolenic acid, dihomo-linolenic, arachidonic, docosatetraenoic and docosapentanoic acids and in the metabolism of omega-6 pathway such as alpha-linolenic, stearidonic, eicosapentaenoic acid, docosapentaenoic and docohexaecoic acids.

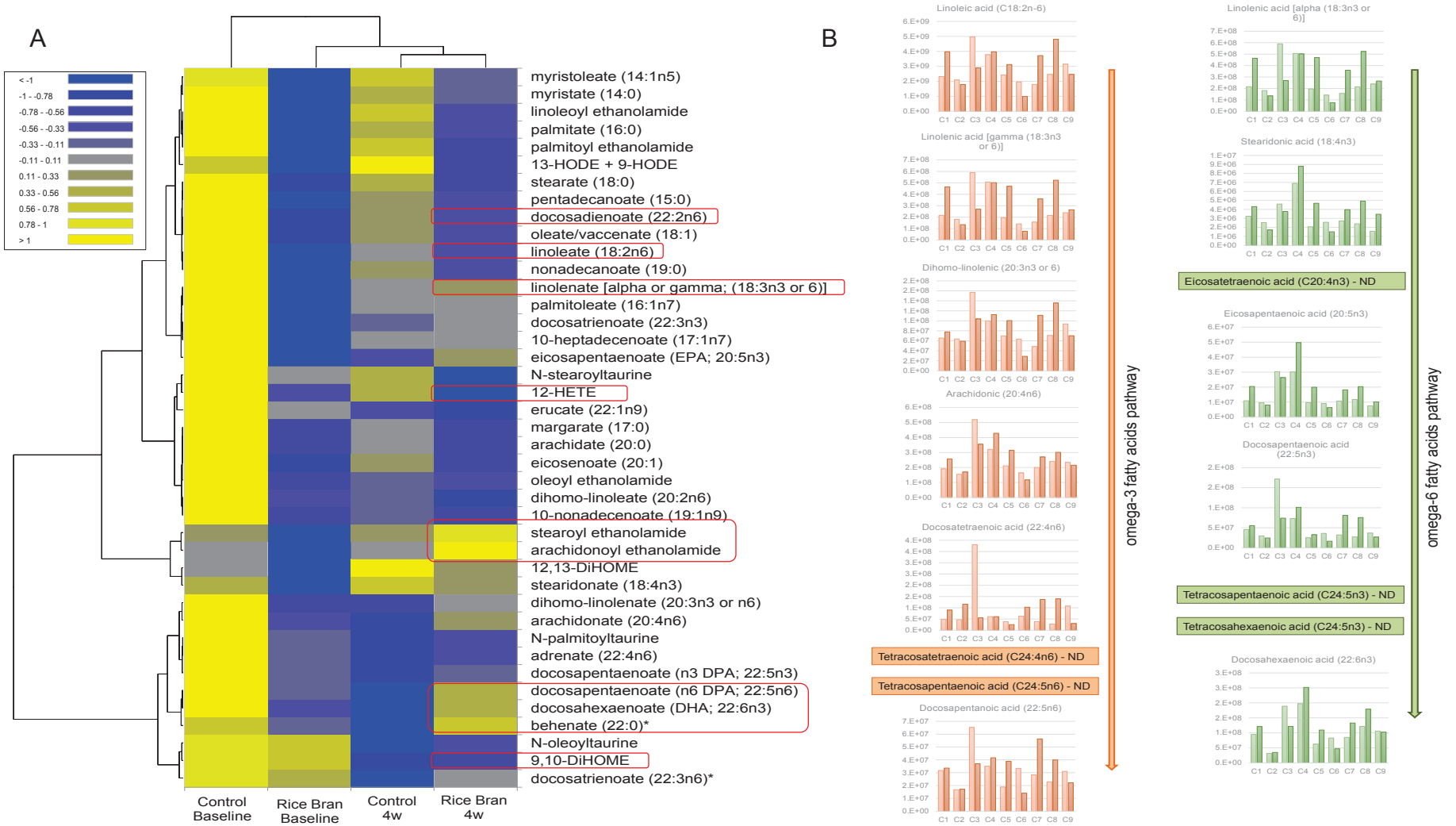


Figure 9 – A) Heatmap to compare the group of fatty acids, oxylipins and endocannabinoids the presence/abundance in children between control baseline vs control 4 weeks vs RB baseline vs RB 4 weeks. B) Total abundance of identified PUFAs from omega-3 and omega-6 pathway in children between RB baseline vs RB 4 weeks (ND: Not detected; C: children; light colors = baseline, dark colors = 4 weeks)

In this study, omics tools helped us not only to characterize the lipid profile of methanolic extracts of RB, but also to reanalyze a clinical study to find lipid markers that could be linked to the consumption of this coproduct when incorporated into the diet. However due to the complexity of lipid metabolism, an additional research targeting specifically lipid clinical makers and extractions that allow the whole lipidome to be analyzed is compulsory to check the putative modulations of circulating phospholipids, oxylipins, endocannabinoids and type of PUFA evidenced by omic tools.

4. Conclusion

Lipidomic had utility to elucidate the RB specific lipids alongside novel markers in human plasma that may be linked to RB consumption. The RB food analysis revealed a highly conserved core lipidome that did not significantly differ in ratios by variety or growing conditions but was useful to identify novel subclasses of lipids (oxylipins: PhytoPs and PhytoFs) that have lower levels of expression than classical triacyclglycerols or even phospholipids but may be functionally important. Oxylipins levels were not correlated with PUFA contents in RB. The novel RB polar lipids and oxylipins merit targeted attention in future in vivo research for exhibiting additive and synergistic impacts on health. The content and the profile of PhytoPs and PhytoFs recovered in RB suggest that these coproducts are an important natural vegetal source of oxylipins. A RB supplementation study in children indicated some changes in lipid profiles including polyunsaturated fatty acids and phospholipids. Further, the endocannabinoids that are important regulators of lipid metabolism were increased after the RB supplementation. Future studies to advance knowledge of the RB lipidome composition and function for impacts on health and for functional food industry applications should become a priority.

5. Author Contributions:

Millena Cristina Barros Santos: Conceptualization, Formal analysis, Investigation, Writing – original draft and Writing - review and editing. Nathalie Barouh: Conceptualization, Formal analysis, Investigation and Supervision. Valérie Lullien: Conceptualization and Supervision. Valérie Micard: Supervision. Pierre Villeneuve: Conceptualization and Supervision. Bingqing Zhou: Formal analysis and Investigation. Camille Oger: Formal analysis and Investigation. Claire Vigor: Conceptualization, Investigation and Supervision. Thierry Durand: Conceptualization, Investigation and Supervision. Mariana Simões Larraz Ferreira: Conceptualization, Writing - review and editing, Supervision, Project administration. Claire

Bourlieu-Lacanal: Conceptualization, Writing - review and editing, Supervision, Project administration. Elizabeth P Ryan: Conceptualization, Writing - review and editing, Supervision, Project administration, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

6. Acknowledgements

We appreciate Adam Jaskowiak, Laura Dvorak, Katie Hansen, and Meghan Willis from Medical Center of the Rockies for helping with participant recruitment and assistance with study implementation. We thank Dr Anna McClung from the USDA Agricultural Research Service Dale Bumpers National Rice Research Center and Gordon Gregory from Archer Daniels Midland Edible Bean Specialties for supplying the RB and cooked navy bean powder. We also thank Genevieve Forster, Nora Jean Nealon, and Allie Reava for assistance in study snack preparations, as well as all of our study participants and their families.

This research was financed by the Foundation for Research Support of State of Rio de Janeiro (FAPERJ) (26/201.848/2019), and supported by UNIRIO, Coordination for Improvement of Personnel with Higher Education (CAPES) (financial code 001).

7. Data Availability:

Dataset is available publicly through the Frontiers in Nutrition paper: Li et al. (2018b).

Part IV

Application and valorization of pigmented rice bran coproduct extracts in heterogeneous media

For this last step, all the characterization previously performed was taken into account to select the ideal coproduct to proceed with the further analyses. Thus, performance and stability tests of the CALB enzyme were performed on different NaDES formulations prepared from model reactions between vinyl laurate and octanol. These tests were crucial to select the NaDES that represent the most suitable media to the enzymatic reaction for the synthesis of the new biomolecules. Next, the extraction and the biotransformation process were performed with red RB. And after this process, the biotransformed extracts were evaluated in heterogeneous medium (nanoemulsion) to compare the antioxidant efficacy between the non-biotransformed extracts.

Aim: To valorize the pigmented RB extracts from NaDES and selected the most suitable for the *in vitro* biotransformation between the bioactive compounds present in the RB (PC + lipids).

Based on the following publications:

- Chapter 8 – One-pot NaDES assisted extraction and biotransformation of RB. A new strategy to boost antioxidant activity of natural extracts. Short communication submitted to Process Biochemistry (Impact factor: 3.757)

Main results

- NaDES can be used as reaction medium that remain in the formulation
- Pro-oxidant effect of the pure NaDES tested was evidenced
- Chromatographic changes after biotransformation suggests a structural rearrangement between PC and lipids
- NaDES components can react with RB compounds

Chapter 8 – One-pot NaDES assisted extraction and biotransformation of rice bran. A new strategy to boost antioxidant activity of natural extracts

Millena Cristina Barros Santos[1], Nathalie Barouh[2,3], Bruno Baréa[2,3], Pierre Villeneuve[2,3], Claire Bourlieu-Lacanal[4], Mariana Simões Larraz Ferreira[1*] and Erwann Durand[2,3*]

[1]Laboratory of Bioactives (LabBio), Food and Nutrition Graduate Program (PPGAN), Federal University of State of Rio de Janeiro (UNIRIO), Rio de Janeiro, Brazil

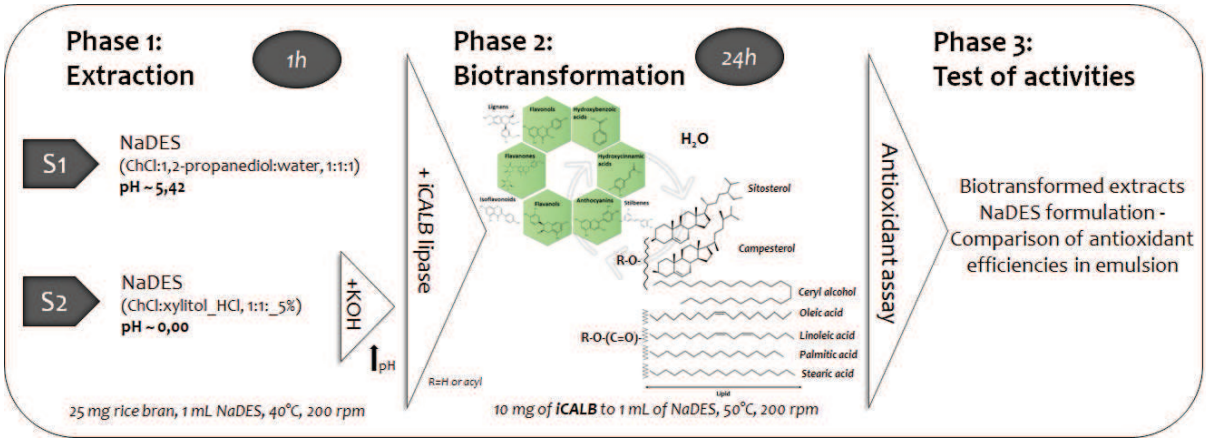
[2]CIRAD, UMR QualiSud, F-34398, France

[3]Qualisud, Univ Montpellier, CIRAD, Institut Agro, IRD, Université de la Réunion, Montpellier, France

[4]IATE, Univ Montpellier, INRAE, Institut Agro, Montpellier, France

---

Short communication submitted on Process Biochemistry, 2021.



Highlights

- Original one-pot extraction and enzymatic biotransformation of RB using NaDES.
- Confirmation of the NaDES extraction efficiency for phenolics but also for minor lipids.
- Chromatographic changes after biotransformation suggest structural rearrangements.
- NaDES showed a strong pro-oxidative effect in oil-in-water emulsion.
- NaDES can produce improved ready-to-use natural extracts.

Abstract

Natural deep eutectic solvents (NaDES) were used as sustainable one-pot extraction and enzymatically biotransformation of bioactive compounds from pigmented rice bran (RB). In this study, we evidenced the use of NaDES media to produce ready-to-use formulations, combining phenolics and lipids to promote antioxidant enrichment and to enhance the oxidative stability of lipids in an oil-in-water emulsion. Two NaDES were selected for the biotransformation step. Phenolics content varied according to the NaDES formulation. NaDES formulated with ChCl:xylitol:HCl 2M + KOH (1:1:1) stands out by showing a content of 1525 mg eq GA/100 g RB and decreased to 402 mg eq GA/100 g RB after biotransformation. Biotransformation process could be confirmed by lipids variation (e.g. lipolysis of triacylglycerols). The nature and amount of reaction products remain to be elucidated. These enriched extracts could be used as natural additives or ready-to-use extracts once fully characterized. Omics tools should be applied to elucidate the pool of extracted antioxidant molecules that may develop synergies and the potential structural rearrangement between the RB compounds that explain the protection against lipid oxidation.

Keywords: NaDES, iCALB lipase, rice coproducts, read-to-use extracts, antioxidant, lipid oxidation.

1. Introduction

Natural deep eutectic solvents (NaDES) are liquid mixtures resulting of metabolites association such as sugars, organic acids, amino acids, organic bases, or vitamins (Huber et al., 2021). Besides the natural origin of their constituents, NaDES present singular properties in the liquid state, along with their possible presence and role in living organisms (Radošević et al., 2016). The strong solubilization capacity of NaDES was already used to provide enriched extracts with high phytochemical concentration and molecular diversity (Dai et al., 2013; Duan et al., 2016). NaDES showed several advantages over conventional solvents, such as an enhanced biological activity or an excellent capacity to stabilize and protect (macro)molecules from degradation, mostly due to the supramolecular network of tightly interconnected molecules (da Silva et al., 2021). These liquid properties make NaDES very attractive for extraction processes of bioactive compounds. However, the difficulty to separate, and to recover dissolved molecules from NaDES, mostly due to their low vapor pressure and strong intermolecular connections, have limited up to new applications and industrial transfers. NaDES offer all characteristics to design atom economy, efficient, low cost and sustainable development for ready-to-use formulation, and depending on the nature of starting components, they may be fully compatible with food and feed applications (Panić et al., 2019). NaDES were already used to provide ready-to-use extracts with higher interest (da Silva et al., 2021; Panić et al., 2019). Besides the great capacity to extract bioactive compounds, these solvents can also be used as medium for enzymatic activity (Durand et al., 2012; Durand, Lecomte, & Villeneuve, 2013).

Naturally occurring antioxidants obtained from agroindustry processing may be efficiently used to prevent lipid oxidation in food (*e.g.* fruits (Vidal et al., 2022) and cereals(Rohfritsch et al., 2021)) and cosmetic products. Rice is one of the most important staple food and its processing lead to millions of tons of rice bran (RB) annually. RB is usually underutilized, destined to animal feed. The sustainable valorization of rice coproducts for improving human health has become a priority due to the presence of bioactive phytochemicals, such as phenolic compounds (PCs), vitamins, fibers, proteins, minerals such as iron, but also γ-oryzanol and the presence of unsaturated lipids (Pan et al., 2019). Natural extracts from RB can generate different industrial applications, especially for human consumption. Even more so, for the pigmented rice, which presents the highest total phenolic content and antioxidant capacity (Pang et al., 2018). Recently, NaDES were used to extract

PCs from pigmented RB and showed specificity, confirmed by omics tools, to extract distinct compounds in comparison to the ethanol/water mixture (Santos et al., 2021).

In addition, the structural modification of antioxidant molecules has been recognized as an efficient strategy to strengthen their capacity to retard oxidation of heterogeneous lipid-based systems. Increasing the lipophilic nature (so-called lipophilization) of antioxidants dispersed in water phase lead to a positive change in their capacity to stabilize lipid against oxidation in oil-in-water (O/W) emulsions (Lopez Giraldo, 2009; Lopez Giraldo et al., 2007). This has been observed with chlorogenate (Laguerre et al., 2009; López Giraldo et al., 2007), gallate (Phonsatta et al., 2017), ferulate (Qiu et al., 2017), or caffeate (Laguna et al., 2020) alkyl esters, in various colloidal systems. Actually, the physical location of antioxidants, and thus, their distribution and mobility when different phases co-exist (e.g., emulsions) is fundamental and matrices-dependent. This structural modification of PCs can be even more interesting, although more challenging, if the modification takes place enzymatically (e.g., with lipase EC 3.1.1.3) because of milder reaction conditions, higher selectivity, less by-products and purification steps, in line with this green chemistry era (Durand, Lecomte, Baréa, et al., 2013; Kahveci et al., 2015; Villeneuve, 2007). *Candida antarctica* lipase B has been pointed out as a great and versatile biocatalyst for lipophilization reaction of PCs in different media, including DES (Durand, Lecomte, Baréa, et al., 2013).

Although the increasing use of NaDES in food research (Jablonský et al., 2018; Mišan et al., 2020; Radošević et al., 2016; Ruesgas-Ramón et al., 2017), its scale-up is hardly explored and the possibilities for its application for human consumption still need to be developed. In this context, we went to investigate how the antioxidant activity of natural occurring molecules in RB may be improved by NaDES. The strategy was based on NaDES as a potential media to develop a sustainable one-pot extraction and biotransformation environment to (i) promote antioxidant enrichment of a natural extract, and (ii) enzymatically modify the structure of extracted compounds by lipase-catalyzed reactions. Therefore, the samples were tested and compared for their ability to prevent lipid oxidation in an O/W emulsion system.

2. Material and methods

2.1. Materials and chemicals

Choline chloride (≥ 99 %), lactic acid (~90%), 1.2-propanediol (≥ 99%), xylitol (≥ 99%), methanol (HPLC grade), water (HPLC grade), ethanol, isooctane, isopropanol, sodium

dodecyl sulfate (SDS), potassium hydroxide, iron sulfate, ammonium thiocyanate, 1,1,3,3-tetramethoxypropane, cumene hydroperoxide, trichloroacetic acid, thiobarbituric acid, mono and dibasic sodium phosphate, 1-octanol (anhydrous ≥99%), hydrochloric acid, immobilized lipase B from *C. antarctica* (Novozym® 435) (iCALB), the lipids standards: triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), free fatty acids (FFA), monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG), phosphatidylcholine (Martillanes et al.), and phosphatidylserine (Oliveira et al.), and gallic acid (GA) were obtained from Sigma-Aldrich (St. Louis, USA). Hexane (99%) was obtained from Labover (Montpellier, France) and vinyl laurate (≥99%) from Fluka (Saint Quentin, France).

2.2. Pigmented rice bran

The red RB from Madagascar were cultivated in Ampitatafika (-19.565717, 47.362911), obtained by abrasive dehulling (polishing) in a single pass rice mill and stabilized (100/110 °C - 5 min). The RB was sieved to eliminate particles larger than 1 mm size and then milled for 5 min with a ball mill (Fritsch Pulverizer Mill, Germany) to obtain a homogeneous fraction.

2.3. Preparation of Natural Deep Eutectic Solvents

Choline-chloride (ChCl) based NaDES in combination with lactic acid, 1.2-propanediol and xylitol were selected based on GRAS (Generally Recognized as Safe) components and according to the previous study from Ruesgas-Ramón *et al.* (2017). NaDES1 (ChCl:1,2-propanediol:water, 1:1:1), NaDES2 (ChCl:lactic acid, 1:10), NaDES3 (ChCl:xylitol:water, 1:1:1), NaDES3-HCl (ChCl:xylitol:HCl 2M, 1:1:1) and NaDES3-KCl (ChCl:xylitol:HCl 2M + KOH, 1:1:1). NaDES were prepared by the heating method at 50 °C, with orbital shaking at 400 rpm (IKA KS 4000 I control, Staufen, Germany) after mixing the components (according molar ratios) in a closed bottle for 45 min until a clear liquid is formed. NaDES3-KCl was obtained after adding KOH (9.59 mg per mL of NaDES) in NaDES3-HCl.

2.4. Assessment of the lipase activity and stability in NaDES

The lipase-catalyzed activity and stability evaluated after 24 h of iCALB pre-incubation were carried out in all NaDES, during 30 min at 50 °C according to Durand et al. (2012).

2.5. One-pot extraction and biotransformation of phenolic compounds and lipids

RB were extracted (25 mg/mL) with NaDES in a closed amber glass flask (60 min, 40 °C, 200 rpm orbital agitation) (Thermo Scientific Poly 15, Ville, China). Then, samples were cooled to room temperature (RT, ~20 °C) and centrifuged (5 min, 4000 rpm) (CR412 centrifuge; Jouan, Winchester, VA). The iCALB (10 mg) was directly added for the biotransformation reaction (biotransformed extracts) and left for 24h of reaction. Control samples without addition of the enzyme were also performed in this step. After centrifugation, aliquots (250 µL) of the supernatants of each extract were immediately extract by the Folch method (Folch et al., 1957), where aqueous and organic phases were separated, collected, and evaporated with a nitrogen flow. The aqueous phase was destinated for PCs characterization by HPLC and the organic phase for lipids identification to by thin layer chromatography (TLC). Beside this, aliquots of 500 µL of the supernatants, after centrifugation for biotransformed extracts, were destinated to the antioxidant activity in O/W emulsion.

2.6. Phenolic compound analysis with high performance liquid chromatography (HPLC)

The aqueous phase of the Folch extraction was evaporated with nitrogen flow, resuspended with 1 mL of MeOH/water (2:1, v/v), and filtered (0.45 µm, cellulose filter). Samples (40 µL) were injected in the HPLC (LC-20AD equipped with oven CTO-10ASvp and detector DAD SPO-M20, Shimadzu, Noisiel, France) (Ruesgas-Ramón et al., 2020) with the gradient method (mobile phase A: MeOH and B: water, both with 0.1% acetic acid): 0-5 min: isocratic at 10% of B; 5-20 min: linear gradient up to 100% B; 20-30 min isocratic at 100 % B; 30-35 min linear gradient up to 10% B; 35-42 min: equilibration at 10% B. The detection was performed at 280 nm and 330 nm. GA was used for the calibration curve quantification, and data were expressed in mg eq of GA.

2.7. Lipids analysis with thin layer chromatography (TLC)

Organic phases from Folch extraction method were resuspended with 500 µL of $ChCl_3$:MeOH (2:1, v/v) and spotted on silica gel HPTLC plates (Merck, Saint-Quentin Fallavier, France) using an automatic sampler (ATS4, Camag, Muttenz, Switzerland). Lipid standards (TAG, DAG, MAG, FFA, MGDG, DGDG, PC, and PS) were also spotted. The elution was performed in double migration using an automatic developing chamber ADC2 (Camag, Muttenz, Switzerland) as follows: 40 mm with $CHCl_3$/MeOH/water (19:4:0.5, v/v/v) and 80 mm with hexane/diethyl ether/formic acid (14:6:0.2, v/v/v). After elution, plates were

dried (RT), dipped into $CuSO_4/H_3PO_4$ reagent using a Chromatogram Immersion Device 3 (Camag, Muttenz, Switzerland), dried again (RT), and placed in an oven (180 °C, 10 min). Absorbances of revealed spots were measured at 500 nm (TLC Scanner 3, Camag, Muttenz, Switzerland), analyzed by VISIONCAT software, and qualitatively evaluated in comparison with lipid standards.

2.8. Antioxidant activity in oil-in-water (O/W) emulsion

An O/W nanoemulsion of rapeseed oil in PBS (10 mM, pH 7.0) with SDS 20 mM and 0.02 % sodium azide (Ferreira da Silveira et al., 2021). Aliquots (500 µL) of NaDES extracts and biotransformed NaDES extracts (after centrifugation) were added to 25 mL of nanoemulsions. RB (12.5 mg), considering that all rice samples had the final concentration of 500 ppm in the emulsion, and NaDES alone (500 µL), were used as control. The oxidation assays were carried out in triplicate into 25-mL screw-capped amber vials and incubated at 40 ºC for 20 days under gentle orbital stirring (110 rpm) (IKA KS 4000i control, Germany). Samples were collected at 1, 3, 6, 9, 13, 16, and 20 days to follow lipid oxidation.

2.9. Determination of lipid oxidation

The evaluation of lipid oxidation in the nanoemulsions was carried out by monitoring the formation of primary (peroxide value, PV) and secondary (2-thiobarbituric acid reactive substances, TBARS) oxidation products (Ferreira da Silveira et al., 2021).

Briefly, PV were determined on 100 mg of extract with isooctane/isopropanol solution (3:1 v/v). An aliquot of this extract was then added to MeOH/BuOH (3:7 v/v) to give a final volume of 260 µL in the microplate well (ThermoFischer, Courtaboeuf, France). Then, 2.5 µL of 30% ammonium thiocyanate solution and 2.5 µL of ferrous solution (0.144 M) were added, and the mixture was incubated (RT, 10 min). After incubation, the absorbance was measured at 510 nm (Infinite M1000 microplate reader, Tecan, Gröedig, Austria). A standard curve was prepared with cumene hydroperoxide. Analyses were performed in triplicate and results were expressed as meq O_2/kg oil.

For TBARS, briefly, 200 mg of nanoemulsions were mixed with 400 µL of TBARS solution (15% trichloroacetic acid, w/v; 1:2 (v:v) of 0.375% w/v of thiobarbituric acid and 0.25 M HCl) and then heated (90 ºC, 15 min). Then, samples were cooled in an ice bath and centrifuged. The absorbance of the collected supernatant was measured at 532 nm. The TBARS content was determined according to a calibration curve obtained using 1,1,3,3-tetramethoxypropane as external standard and expressed as mmol eq MDA/kg oil.

2.10. Statistical analysis

The XLSTAT software (Addinsoft, France) was used to perform the statistical analysis (Tukey's test, $p<0.05$, and one-way ANOVA). The graphical representation of the results was created using the GraphPad Prism (5.0) software.

3. Results and discussion

3.1. Evaluation of the NADES extraction capacity of phenolic compounds from RB.

Initially, NaDES1 (ChCl:1,2-propanediol:water, 1:1:1), NaDES2(ChCl:lactic acid, 1:10) and NaDES3 (ChCl:xylitol:water, 1:1:1), were only tested for their capacity to extract PC from RB. NaDES2 showed the highest capacity with 39.97 ± 2.30 and 303.88 ± 16.28 mg GA eq/100 g of RB at 280 and 330 nm, respectively (Fig. 1). As already highlighted in the literature (Loypimai et al., 2017; Santos et al., 2021), acidity of NaDES is an important attribute to promote the extraction of PCs. To confirm this hypothesis, NaDES3 was acidified (NaDES3_HCl) and tested for the RB extraction, a strategy that has already proven to be effective to release bound PCs. As expected, NaDES3_HCl significantly increased the phenolic content, by a factor of ten and two, at 280 and 330 nm, respectively. Indeed, NaDES3_HCl appeared as the best solvent of PCs extraction in this study, reaching 404.23 ± 41.17 and 715.05 ± 102.93 mg GA eq/100 g of RB at 280 and 330 nm, respectively.

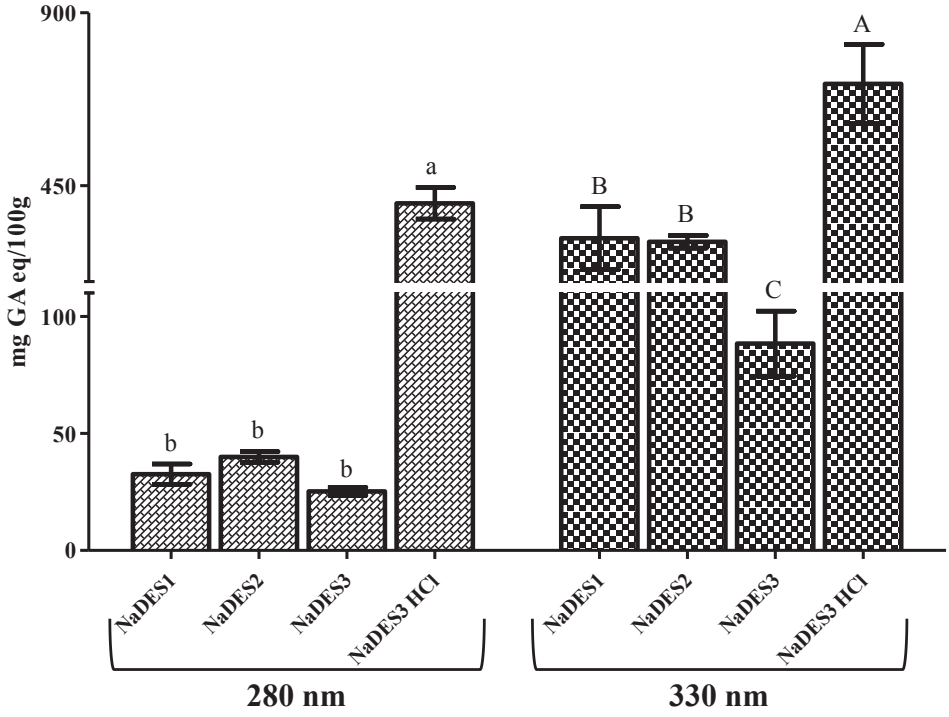


Figure 1 - Total content of phenolic in mg GA eq/100 g of RB. Lowercase letters are significantly different at $p < 0.05$ according to Tukey test between the different extractions for each NaDES at 280 nm and uppercase at 330 nm.

3.2. Evaluation of the lipase-catalyzed reaction in the selected NaDES

Some NaDES formulations may present limitations for enzymatic reactions, decreasing or inhibiting the lipase activity (Durand, Lecomte, Baréa, et al., 2013; Villeneuve, 2007). To determine whether NaDES could be adapted for iCALB lipase-catalyzed reactions, a model reaction based on the alcoholysis of vinyl laurate (VL) with octanol was employed. The use of vinyl ester as acyl donors is a fast and effective way to evaluate lipase initial activities (Durand, Lecomte, & Villeneuve, 2013), and has been successfully applied for estimating the iCALB activity and stability in DES (Durand et al., 2012). Conversion was calculated based on the VL decay, while the selectivity for octanol was assessed based on the octyl laurate (C8L) formation (Fig. 2). NaDES1 and NaDES3 allowed for a complete conversion of the VL substrate. Conversely, NaDES2 and NaDES3_HCl did not provide suitable environment for lipase activity, with a poor selectivity for transesterification, ~5 % and ~50 %, respectively. Low pH values in NaDES2 and NaDES3_HCl seems to inhibit the lipase activity and to promote the hydrolysis competitive reaction instead of the ester bond formation. Considering the high extraction efficiency of NaDES3_HCl, we have investigated its capacity to promote the lipase-catalyzed reaction after increasing pH obtaining the

NaDES3-KCl. This switchable-NaDES could be seen as a new and efficient strategy to move toward a one-pot NaDES-assisted extraction and biotransformation of natural extracts. Interestingly, NaDES3-KCl allowed for both a very high activity and high yield of conversion of VL into C8L (>90 %) (Fig. 2). Regarding the stability of iCALB, NaDES1, NaDES3 and NaDES3_KCl seemed to preserve, at least on the short term, the protein conformation since the lipase catalysis was hold over 24 hours, which confirms that media could be used for long-term lipase reactions.

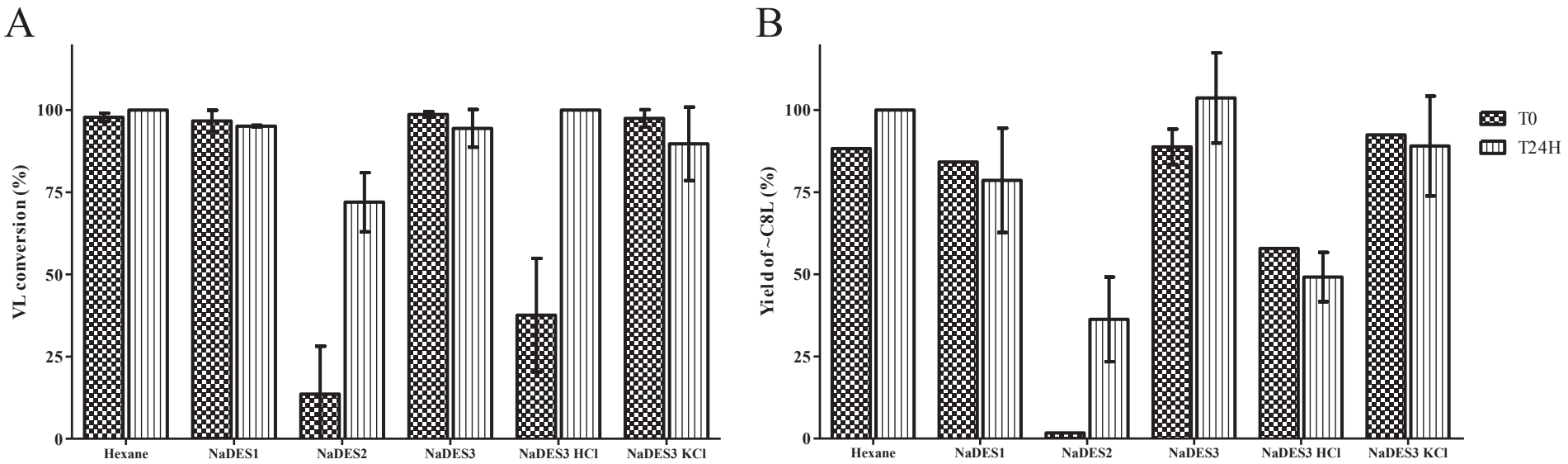


Figure 2 – A) Conversion of vinyl laurate (VL) and B) yield of octyl laurate (C8L) in each NaDES, and in hexane as a control.

3.3. One-pot NaDES assisted extraction and biotransformation of rice bran

Two distinct strategies were developed (S1 and S2) (Fig. 3): 1) with NaDES1, and 2) with the switchable NaDES3 (obtained from the NaDES3_HCl to the NaDES3_KCl, noted swNaDES3). NaDES1 was chosen because it offered a suitable environment for lipase-catalyzed reaction with moderate extraction capacity. While swNaDES3 showed the highest yield of PCs extraction (NaDES_HCl) and represented a potential, assessed by VL substrate, media for biotransformation activity with iCALB (NaDES_KCl). Thus, NaDES1 and swNaDES3 were evaluated according to their capacity to promote both, the extraction of bioactive molecules and subsequent biotransformation with the iCALB (Fig. 3).

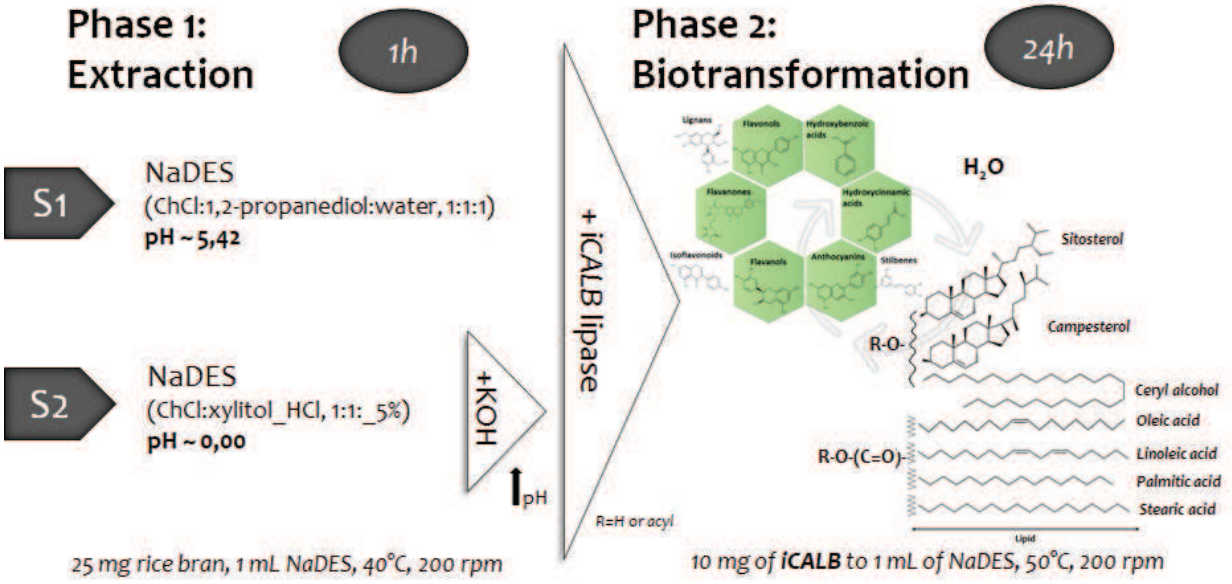


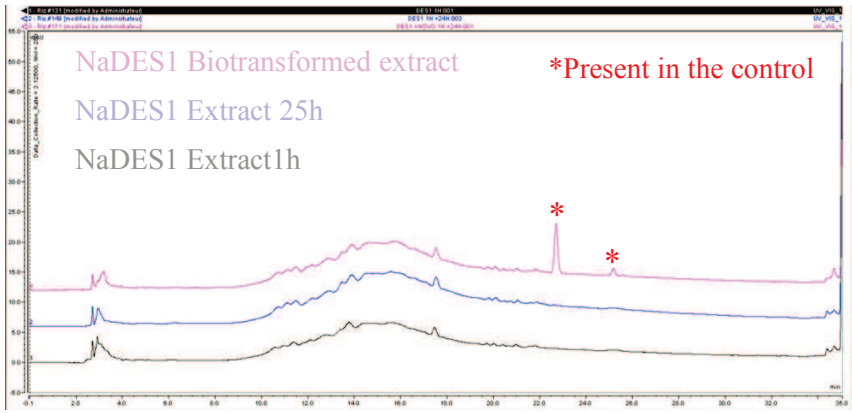
Figure 3 - Global strategy to develop a sustainable one-pot extraction and biotransformation environment. S1: strategy 1 and S2: strategy 2.

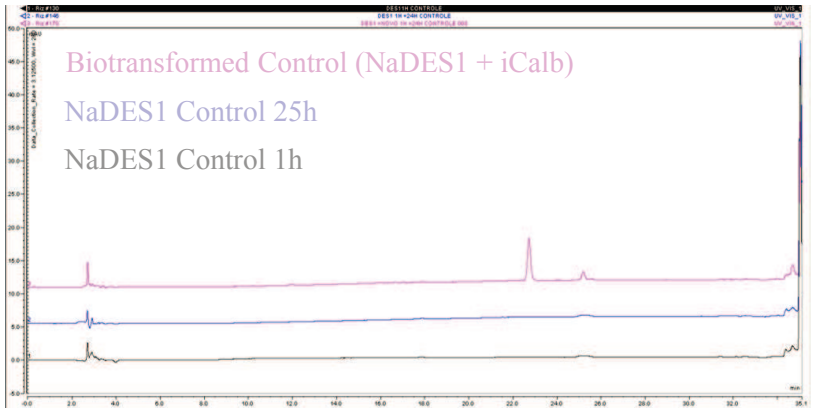
3.3.1. Evaluation of the PC and lipid profiles after extraction and biotransformation of rice bran

NADES1 and swNaDES3 extracts showed a slight variation in PC profile after potential biotransformation (Sup. Fig. 1). Especially, swNaDES3 presented a difference in chromatographic profile and in the total phenolic content (p<0.05) after the enzymatic reaction (Fig.4).

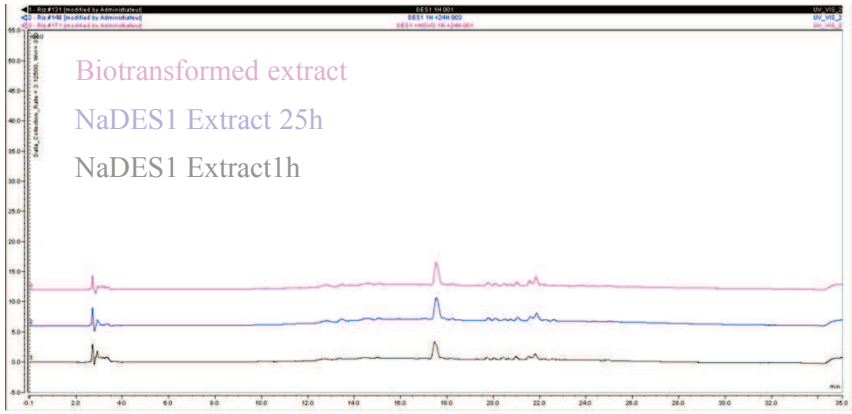
NaDES1
(ChCl:1,2-propanediol:water, 1:1:1)

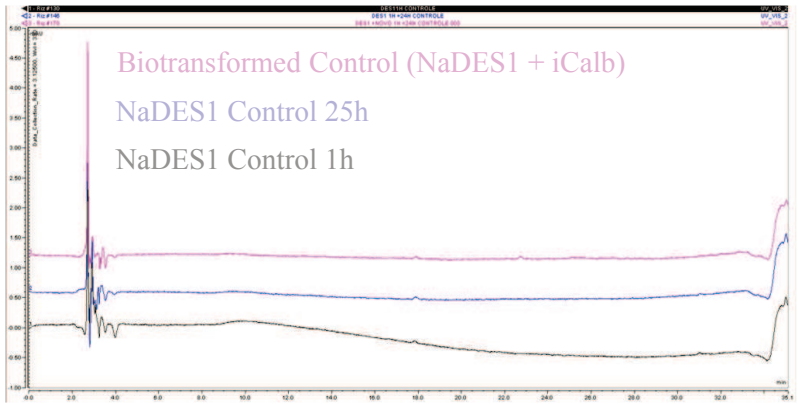
280 nm





330 nm

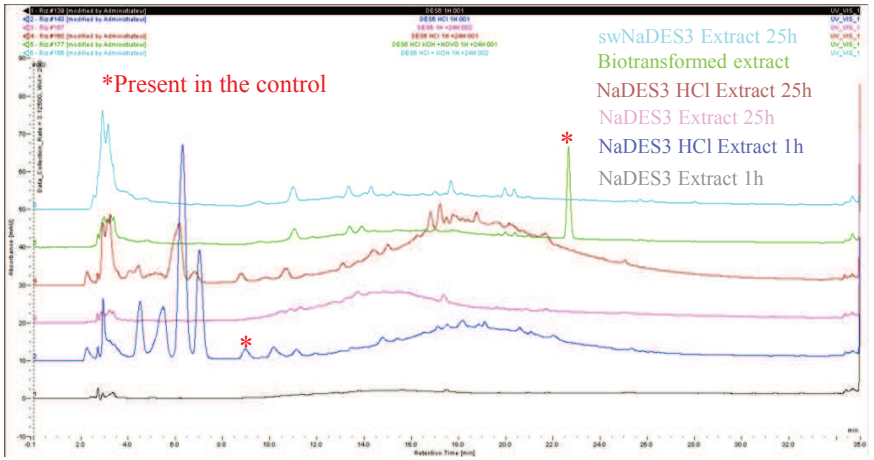


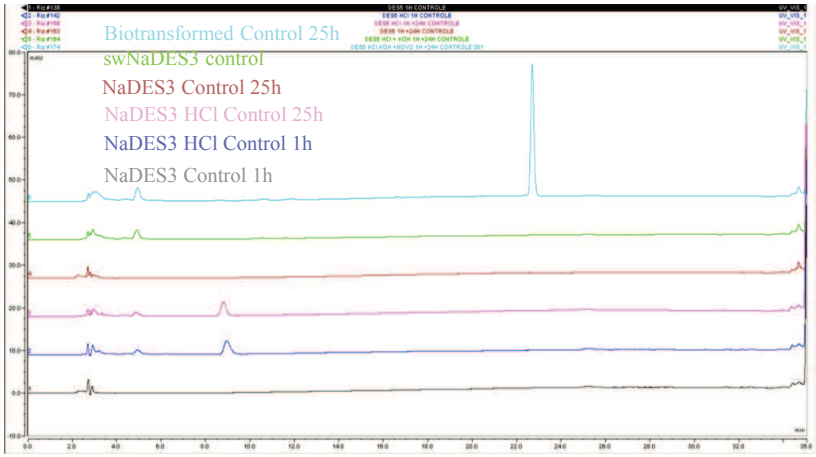


Supplementary Figure 1A - Chromatograms of NaDES1 extracts and controls at 280 nm and 330 nm.

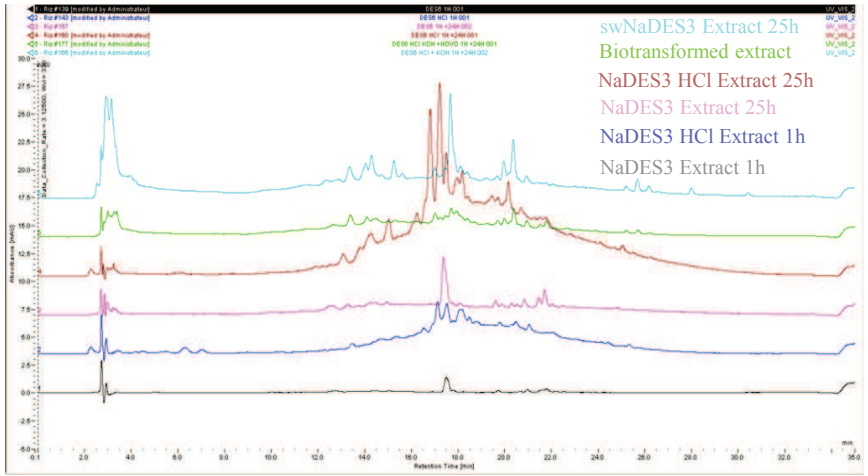
NaDES3
(ChCl:xylitol_HCl, 1:1:_5%)

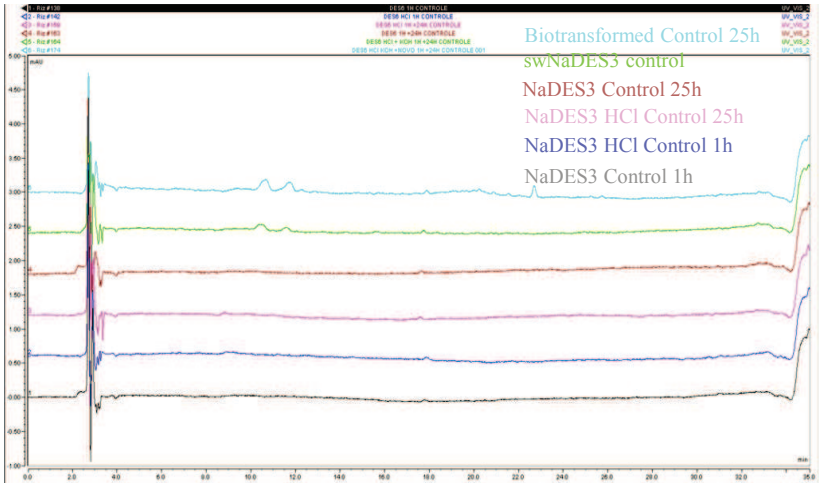
280 nm



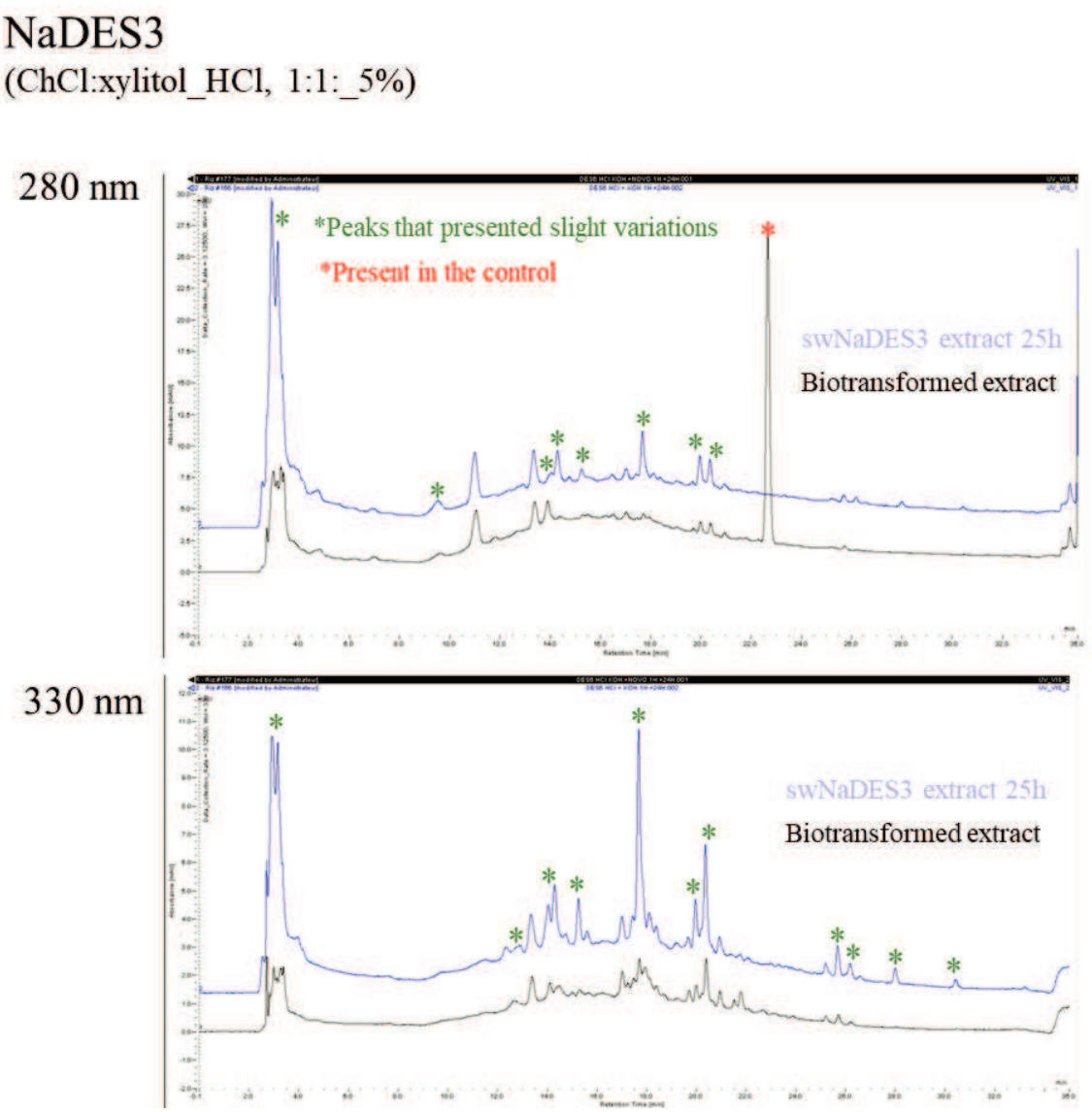


330 nm





Supplemetary Figure 1B - Chromatograms of NaDES3 extracts and controls at 280 nm and 330 nm.



Supplementary Figure 1C – Chromatogram comparison of swNaDES3 extract 25h and biotransformed extract at 280 nm and 330 nm.

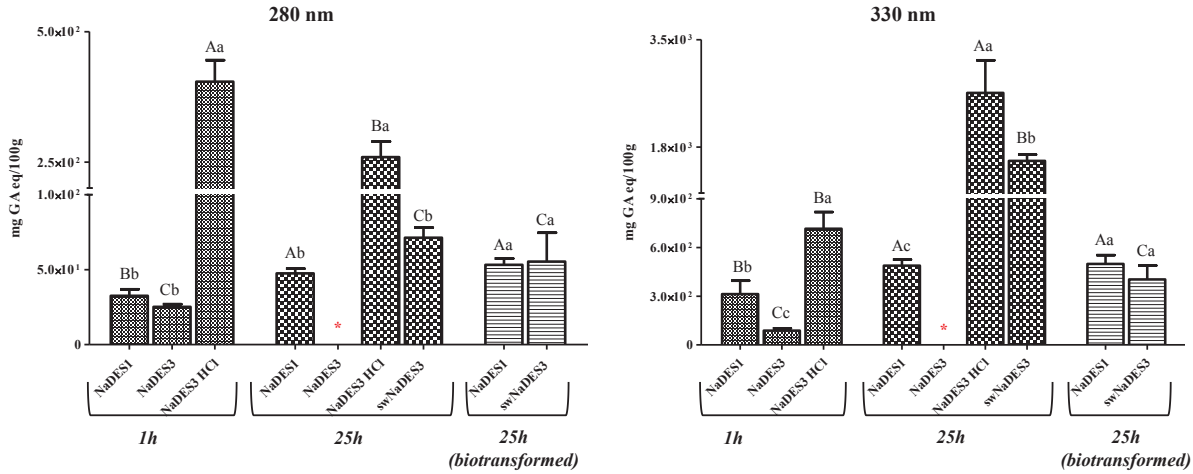
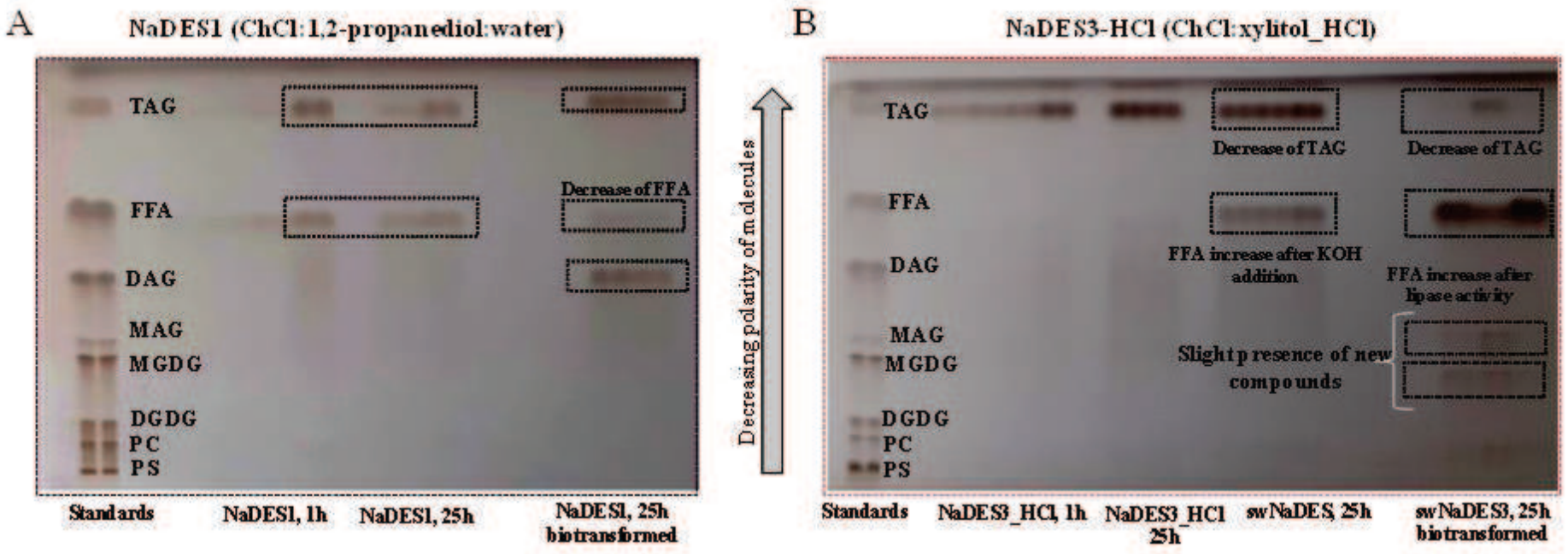


Figure 4 - Total content of phenolic compounds of RB extracts at 280 and 330 nm. Time of extraction are represented below: 1h of extraction, 25h: 1h of extraction and 24h of reaction without enzyme (control) and 25h biotransformed: 1h of extraction plus 24h of iCALB reaction. Uppercase letters compare the different time of extraction for the same NaDES extracts, while lowercase letters compare the extraction time for each NaDES (p<0,05 according to Tukey test). *Below the limit of detection.

Although hydrophilic NaDES have a great capacity to extract polar molecules, studies have revealed that they may also extract non-water-soluble molecules (Mišan et al., 2020). For instance, hydrophilic glycerol-based NaDES have been already shown to extract FFA from microalgae (Mehariya et al., 2021). In the current study, we performed a qualitative analysis of lipids, extracted from the RB with the NaDES1 and the swNaDES3 (Sup Fig. 2). Assessment was performed after 1h extraction, and after 25h (1h + 24h) with or without the addition of iCALB.

NaDES1 and swNaDES3 were able to extract TAG and FFA in different concentrations. A decrease in FFA and the appearance of new compounds (~ same retention factor- Rf as DAG) were observed (Fig. 5A). were observed after the biotransformation step (Sup. Fig. 2A). The mono- and di- acylation with the 1,2-propanediol (present in the NaDES1 formulation) with lipids could explain the formation of new compounds. Regarding the lipid profile with swNaDES3, an important decrease in TAG with a concomitant increase in FFA were observed (Sup. Fig. 2B). In addition, small spots corresponding to new compounds with higher polarity were observed after the lipase-catalyzed reaction. We can hypothesize that these new faintly spots are phenolic esters formed by esterification with the newly released fatty acids with the alcohol moiety of a flavonoid, lignin, stilbene or between a fatty alcohol with a phenolic acid. This biotransformation effect on the lipids profile, was confirmed by the control experiment (without iCALB), where the lipolysis was much less pronounced with a

slight increase in FFA content after KOH addition (Sup. Fig. 2B). In addition, in this control extract, no new compound with higher polarity was observed.



Supplementary Figure 2 - Thin layer chromatography showing the profile of lipid classes after NaDES extractions and the biotransformation. A) NaDES1 and B) NaDES3_HCl. TAG: triacylglycerol, DAG: diacylglycerol, MAG: monoacylglycerol, FFA: free fatty acids, MGDG: Monogalactosyldiacylglycerol, DGDG: digalactosyldiacylglycerol, PC: phosphatidylcholine and PS: phosphatidylserine.

In conclusion, slight variations in the lipid profile after biotransformation were observed, such as the increase in new compounds with same Rf of DAG and FFA and the appearance of more polar lipids. The hypothesis that these lipids with higher polarity are newly formed compounds from bonds with PC corroborates the HPLC results, where was observed the decrease of some peaks and the decrease of the phenolic content in the biotransformed extracts. Nevertheless, mass spectrometry analyses should be done to point out the enzymatic biotransformation and elucidate the structural rearrangement between PC and lipids.

3.4. Antioxidant activity of rice bran extracts in oil-in-water emulsion.

Extracts obtained with NaDES1 and swNaDES3, with or without *in-situ* biotransformation with iCALB, were tested and compared for their ability to prevent lipid oxidation in an O/W emulsion system, stored for 20 days at 40 °C, represented by PV and TBARS values (Fig. 5). Pure NaDES1 and swNaDES, along with the powder of RB were also tested.

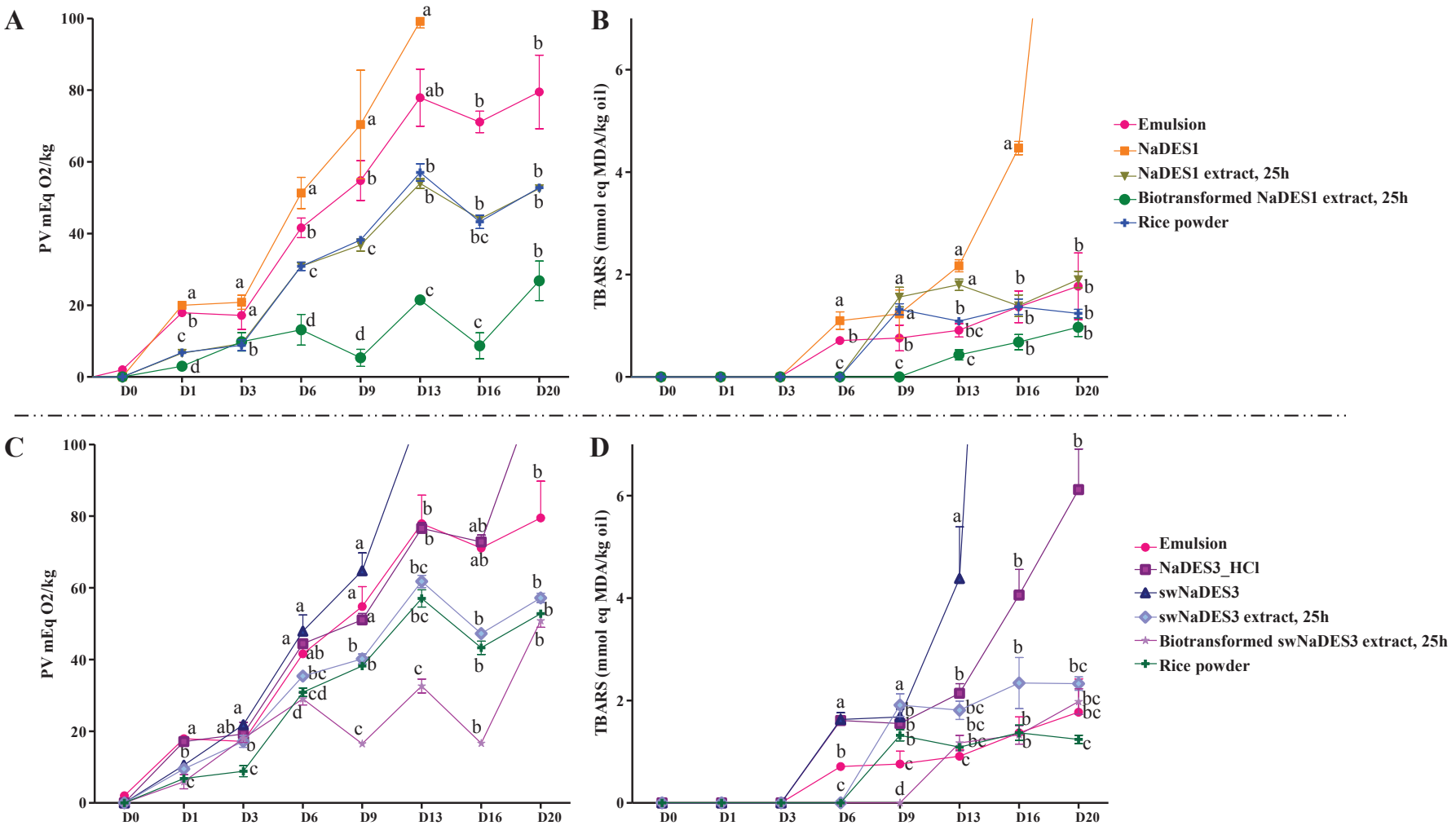


Figure 5 - Oxidative stability of nanoemulsions measured by peroxide value (PV) and 2-thiobarbituric acid reactive substances (TBARS). A) PV results from S1 strategy, with controls and NaDES1 extracts, B) TBARS results from S1 strategy, with controls and NaDES1 extracts, C) PV results from S2 strategy, with controls and swNaDES3 extracts. D) TBARS results from S2 strategy, with controls and swNaDES3 extracts. Different letters significantly mean difference ($p < 0.05$, Tukey test) between samples at the same incubation time.

Contrary to expectations, NaDES showed a strong pro-oxidative effect. Indeed, very high PV (>100 meq O_2/kg) and TBARS (> 6 mmol eq MDA/kg oil) values after 20 days were observed in comparison to the raw emulsion (PV = 79 meq O_2/kg, TBARS = 1.77 mmol eq MDA/kg oil). However, NaDES extracts of RB presented a good antioxidant activity, meaning that the compounds extracted from RB, or the leftover powder, provided antioxidant action and/or reduction of pro-oxidative effects of NaDES. With NaDES extracts, along with the powder of RB, the secondary products of oxidation appeared only after day 6, while they appeared after day 3 for others (Fig. 5B and 5D). Defatted RB showed a protective effect over fish oil and could be used as a natural additive (Rohfritsch et al., 2021). RB extracts have also shown the capacity to decrease PV in food product susceptible to oxidation (mayonnaise-type emulsion) and thus to extend food shelf life (Martillanes et al., 2020). Such extract can be a sustainable alternative as a food additive to guarantee food quality and safety. In addition, and interestingly, the biotransformation step in NaDES seems to promote a protective activity. Although at 20 days, the PV showed no difference between the extracts and controls, especially for the biotransformed extract NaDES1 that showed in day 3 a value of 9.8 meq O_2/kg (Fig. 5A) that stands out from the others (p<0.05). This tendency could be attributed to the molecular restructuration of PCs with lipids, a mechanism known to favor their antioxidant power compared to parent molecules (Figueroa-Espinoza & Villeneuve, 2005; Lopez Giraldo, 2009). In addition, the change in the pool of molecules after enzymatic action could also change the physical properties of extracts, improving the stability of lipid emulsion droplets.

4. Conclusion

This is the first study to enzymatically biotransform compounds present in RB based on a green approach to produce enriched antioxidant extracts to be applied in emulsion media. These results confirmed the efficiency of NaDES to extract PCs, but also in minor part of lipids. Acidic NaDES appear to promote higher extraction yields in PCs, but on the other hand, they do not offer lipase friendly environment. Thus, two strategies for the sustainable development of ready-to-use extracts from NaDES were implemented: one with NaDES allowing a moderate extraction and a good enzymatic activity in a model reaction (NaDES1), and other with a switchable NADES (swNaDES3), starting from an acidic medium to optimize the extraction step, before moving to a non-acid environment to recover an enzymatic activity. Although a strong pro-oxidant activity of NaDES could be observed in O/W emulsion, RB extracts in NaDES demonstrated an antioxidant capacity. In addition, the

biotransformation of extracts with iCALB seems to promote this antioxidant efficiency, but the nature of the potentially biotransformed products remains to be elucidated. There are still several challenges in developing sustainable analytical methodologies and application with NaDES, but this innovative preliminary work brings valuable results for the scientific community. To sum up, these biotransformed extracts should be characterized by omics tools to elucidate the structural rearrangement, along with to study the physical stability of emulsions in the presence of NaDES.

Author contributions

Conceptualization, M.C.B.S., C.B.-L., M.S.L.F., E.D.; data curation, M.C.B.S., N.B., B.B., C.B.-L., E.D.; formal analysis, M.C.B.S., B.B., N.B. and E.D.; funding acquisition, M.S.L.F. and C.B.-L.; investigation, M.C.B.S., N.B., B.B. and E.D.; methodology, M.C.B.S., B.B., and E.D.; project administration, P.V., C.B.-L., M.S.L.F. and E.D.; resources, P.V., C.B.L., M.S.L.F. and E.D; supervision, P.V., C.B.-L., M.S.L.F. and E.D.; validation, M.C.B.S., N.B., B.B., P.V., C.B.-L., M.S.L.F. and E.D.; visualization, M.C.B.S., N.B., C.B.-L., M.S.L.F. and E.D.; writing—original draft, M.C.B.S.; writing—review and editing, M.C.B.S., C.B-L., M.S.L.F and E.D. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: The authors are grateful to Dr Youna Hemery (Institute of Research for Development (IRD); UMR 204 Food and Nutrition Research in the Global South (NUTRIPASS), IRD/University of Montpellier/SupAgro, Montpellier, France) for providing the red RB for analysis and Patrice Autfray (UPR AIDA; CIRAD) for the collection, processing, and transportation of samples.

Funding: This research was financed by the Foundation for Research Support of State of Rio de Janeiro (FAPERJ) (26/010.100.988/2018; 26/202.709/2018; 26/201.848/2019), National Council for Scientific and Technological Development (CNPq) (427.116/2018; 310.34/2019-4) and supported by UNIRIO, Coordination for Improvement of Personnel with Higher Education (CAPES) (financial code 001).

Part V

Discussion and conclusions of main results

Chapter 9 – General discussion, conclusions and perspectives

1. Background and reminder of the objectives

In the present work, we aimed to reach three main objectives: (i) the exhaustive characterization, focusing on PC and lipids, by untargeted metabolomics approaches of wheat and rice, considering different stages of grain maturation, genetic diversity and the resulting coproducts obtained from the industrial milling; (ii) the evaluation of different solvents (conventional and deep eutectic) as extraction media for wheat and rice PC from coproducts and (iii) the development of enzymatically biotransformed PC rich extracts combined with lipids from pigmented RB, with potentially enhanced antioxidant activity. These objectives were reached, and our main conclusions are going to be summarized in this conclusive section of the manuscript.

The two chosen cereals, wheat and rice, have similar structures and some common characteristics (Serna-Saldivar, 2010b). Both of them are important contributors to human diet in terms of calories, especially for polysaccharides intake. In addition, the removed parts in the grain milling, i.e., the bran and the germ, can be used to improve technological and sensorial properties of grain derived products. In this thesis, we have characterized mature or immature (and at several immaturity stages) wheat grains, wheat bran and aleurone layer, and eventually ground mature grains under the form of refined flour (Table 1). On rice however, we have decided to focus only on bran which comprises aleurone, pericarp, subaleurone layers and germ, since it is the most important rice coproduct in terms of volume and nutritional properties. Indeed, RB contains lipids including essential fatty acids, phospholipids, sterols, vitamins but also PC, minerals, and fibers.

Table 1 - Overview of the studied cereal samples.

Samples	Cereal			
	Wheat	Number of varieties	Rice	Number of varieties
Stages	*Immature grains*			
Milky	x	7	-	
Softy	x	7	-	
Physiological maturity	x	7	-	
Mature	x	7	-	
	Final product (refined flour)			
Endosperm	x	14	-	
	Coproducts			
Aleurone	x	1	-	
Bran	x	1	x	34

The untargeted metabolomics was the main analytical tool applied in this thesis for the characterization of PC primarily. It was also used to unveil minor functional lipids present in in a collection of RB gathering diverse varieties from several continents. Complementary analyses, from centesimal composition to *in vitro* tests were also conducted on these samples. Although currently very criticized, the *in vitro* antioxidant assays performed in this thesis (example: Folin-Ciocalteu, DPPH), allowed us to get an indication and estimate of reducing compounds translated as phenolic content or free radical scavenging activity (Granato et al., 2018), that showed positive correlations with our results based on metabolomics.

Globally, all along this work, the PC were extracted with conventional organic solvents (presented as CS in Table 2). Finally, in the last part of the work, we have also compared CS with new green solvents to extract PC and minor bioactives from RB and wheat coproducts; we have also explored how these new green solvents may be used at the same time as means of extraction and functionalization of a pigmented RB sample.

Table 2 - Overview of solvents used for extraction of phenolic compounds in the present study.

	Wheat		Rice	
Samples	**CS***	**NaDES**	**CS**	**NaDES**
Immature grains	x	-	-	-
Flour	x	-	-	-
Coproducts	x	x	x	x

*CS: conventional organic solvents

2. Main results

Table 3 - Synthetic table to present the objectives that have been reached.

Objective	Main results	Valorization
1. Exhaustive characterization, focusing on PC and lipids, by untargeted metabolomics approaches of rice and wheat, considering different stages of grain maturation, genetic diversity and the resulting coproducts obtained from the industrial milling	Different profile immature *vs*. mature grains Decreasing number of PC towards maturation in wheat 43 PC in refined wheat flour 44 PC in wheat bran and aleurone 89 PC in red and black rice Wide presence of PC isomers 163 lipids in RB Presence of essential fatty acids, polar lipids and oxylipins	4 papers, 6 oral communications, 7 posters
2. Evaluation of different solvents (conventional and deep eutectic) as extraction media for rice and wheat PC from coproducts	CS extracts with higher number of free PC NaDES extraction result in specific PC profile NaDES cannot be separated from molecules nor easily analyzed	2 papers, 3 communications
3. Development of enzymatically biotransformed PC rich extracts combining with lipids from pigmented RB, with potentially enhanced antioxidant activity	NaDES can be used as reaction medium and remain in the formulation, unlike CS Pro-oxidant effect of the NaDES tested was evidenced Chromatographic changes after biotransformation suggests a structural rearrangement between PC and lipids NaDES components can react with RB compounds	1 paper, 1 communication

2.1. Overview of the phenolic profile between all analyses - immature grains (each stage), wheat flour, aleurone, bran and pigmented rice brans (red and black)

To find a natural grouping structure in the data, an exploratory technique was performed considering all the identified and relatively quantified free PC obtained from metabolomics analysis. This allowed us to evaluate the dimensionality and suggest hypotheses about the structure of relationships, seeking to group individuals based on distances (dissimilarities). According to the dendrogram illustrated in Figure 1, it is possible to highlight two main clusters: wheat *vs*. pigmented rices. The wheat samples formed four new clusters: wheat bran *vs.* immature grains (milky – 10 DPA and softy – 15 DPA) *vs.* refined wheat flour *vs.* mature grains (physiological maturity - 20 DPA and mature – 45 DPA) and aleurone. These results show the great and important difference in phenolic profile, not only evidencing the stages of maturity, but between the fractions of the grain. It is possible to highlight the proximity of the wheat bran and immature grain clusters, and thus be able to bring options of valorization of these matrices for food applications.

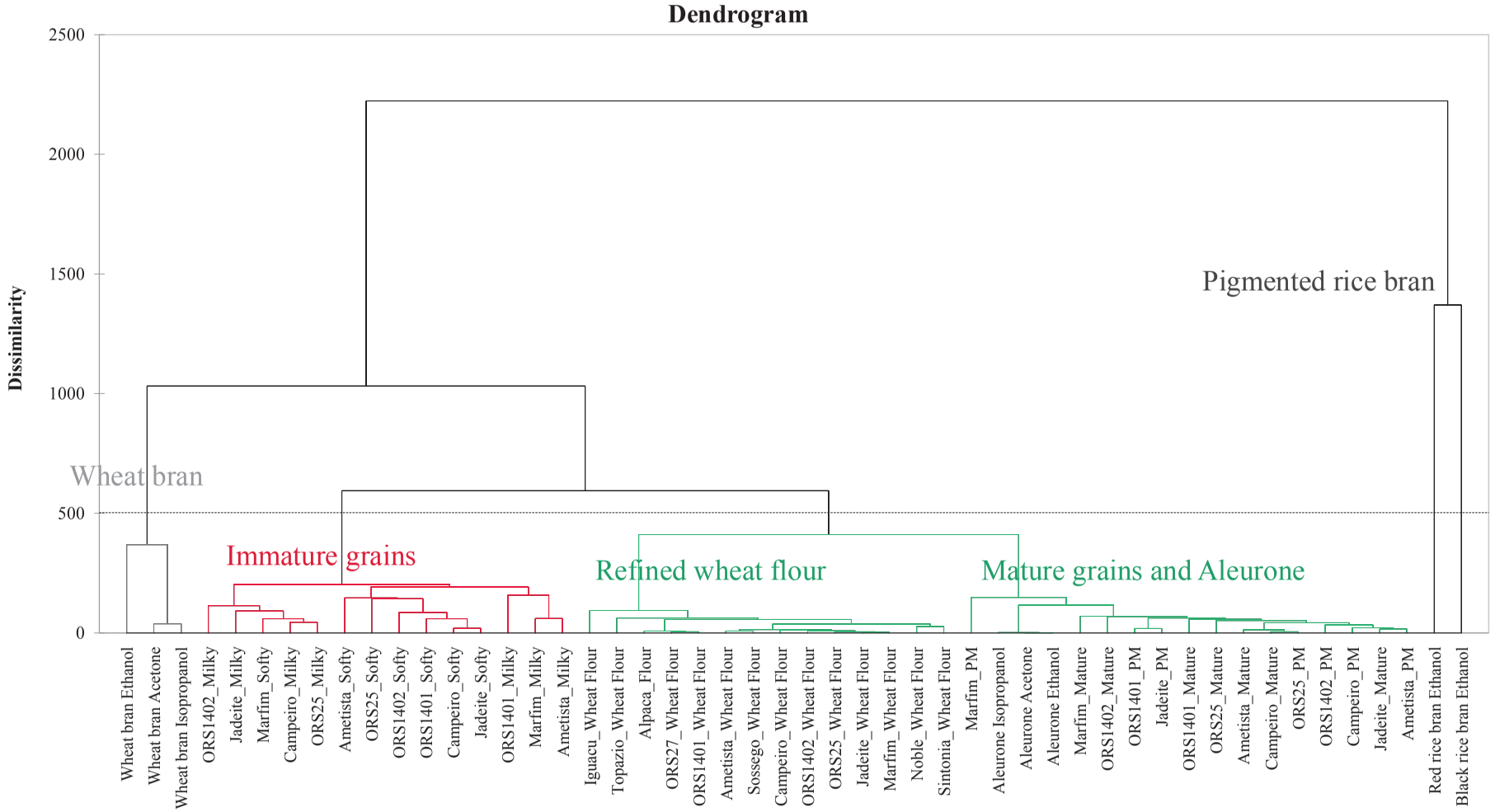


Figure 1 - Dendrogram of cluster analysis based on free phenolics data of the global data.

The starting part of this study was the characterization of wheat throughout grain development, evaluating four stages of maturity. Recent studies have shown from non-omics analyses a higher presence of nutrients and bioactive compounds in immature grains, such as dietary fiber, fructo-oligosaccharides and PC (Özkaya et al., 2018), as well as higher antioxidant and antiproliferative activities (Kim & Kim, 2016). Variations in the accumulation of minerals and nutrients are explained by physiological variations that take place during seed development and maturation. Such appealing functional/nutritional properties of immature grains could be perceived as a formidable lever for food product innovation. However, immature grains present some drawbacks such as low yield and sensory limitations, such as green color and after taste (Kim & Kim, 2017). The interesting nutritional properties of immature rice-based products has also recently underlined by (Miraji et al., 2020). These studies strengthened our interest to characterize and elucidate, what had not been evaluated until then, and to contribute to fill the gap in the knowledge of wheat grain development from different genotypes, by applying modern omics tools.

Hence, in chapter 2, we were able to identify a great diversity of PC structures in the studied grains. The identified PC showed a remarkable number of isomers, since the possibilities of linkages are substantial, made from the hydroxyls present. It was also possible to follow the biosynthesis of molecules (e.g., glycosylated phenolic acids or flavonoids; chlorogenic acids – phenolic acid + quinic acid; ferulic dimers and polymers of PC), as well as the decrease (free PC such as ferulic, *p*-coumaric acids) or increase (bound PC such as caffeic and cinnamic acid) of several compounds. The presence of PC in the immature grains was higher than in the mature grains, which is destined to produce refined flour, as final product. This fact may be associated to the phenolic profile itself that changes throughout maturation, especially during the physiological maturation stage. The same change pattern was found for the components belonging to the primary metabolism of the plant (Zhen et al., 2016).

In chapter 3, we used the same untargeted metabolomics approach to evaluate refined flours and pointed out, as expected, a significant reduction of compounds (-74%). Indeed, the industrial processing of milling includes a stage where the grain is literally stripped off from its external layers, leading to the reduction of these compounds, mostly present in the outermost layers of the grain. What was in fact unexpected, was to find an expressive profile of PC in the refined flour. The contribution of the aleurone layer at this point cannot be neglected.

If we balance the different levers that modify PC profile, we can conclude that: considering first natural levers i) wheat grain stage of maturation was the predominant factors that modulated PC profile in our study, ii) phenotype and growth conditions were the second most important factors, iii) eventually genotype was the least important factor probably due to the very diversified PC profiles affected by external factors. Now, considering intentional levers, such as industrial processing, we can say that it was a very powerful lever at least in terms of reduction in the number of PC than the natural lever.

Taken these results together we can suggest and strengthen the use of immature grains, considering the richness of PC in these stages, either as a complement in the production of flours, or in the production of concentrated extracts for pharmaceutical or technological application. In addition, this approach may represent a way to take advantage of and to prevent pre-harvest loss in case of disasters in the wheat crop. Of course, the color and sensory properties of the fortified product must be managed but this lever of innovation seem very appealing.

2.2. Are eutectic solvents a good alternative for extraction and subsequent analyses by metabolomics tools? - Comparative phenolic profile between conventional and natural deep eutectic solvents

Bound PC are generally the fraction predominant in plant derived samples. Indeed, PC can present intermolecular bonding forming polymers also known as polyphenols, but also with other macromolecules or even with the plant cell wall. Such chemical linking apparently reduces the presence of PC when direct extraction, *i.e.,* without prior hydrolysis, is undertaken. However, from an analytical point of view, the diversity of PC structures and partial binding within biological matrices make the extraction and characterization of these compounds a challenge. It is necessary to have extraction techniques that allow the recovery of these compounds and robust analysis techniques that produce reproducible and high-resolution data. It is important to underline that every analytical method has a limitation and to reach the most complete recovery of PC from a biological matrix often requires many precautions such as: sequential extractions, combinations of different solvents, application of physical stresses such as ultrasound, temperature or pression, selection and concentration on SPE columns, and also work with high-resolution hyphenated techniques such as liquid chromatography coupled to mass spectrometer.

CS are still widely used because they are particularly effective and easily to apply for for extraction. However, the use of pure solvents does not guarantee a good extraction, mainly due to the varied polarity of these compounds, and thus it is generally more effective to use a proportion of organic solvents or alcohol and mix it with water. Chapter 4 gives a good illustration of the diversity of profiles in PC and minor bioactive compounds when using different CS on wheat aleurone and wheat brans extracts.

In the point of view of sustainable development, it is extremely important to develop techniques that allow the extraction of compounds of interest without harming the environment and health of the analyst. Natural deep eutectic solvents (NaDES) have been highlighted in the literature for having, besides others, such properties.

In this thesis, it was possible to make a comparative analysis between CS and NaDES. For the discussion of the efficacy and specificity of these solvents, the profiles of identified PC from all the metabolomic characterization of wheat and rice coproduct extracts presented in this manuscript were considered. For CS, ethanol/water, acetone/water, or isopropanol/water extracts were considered, while NaDES formulations were based on choline chloride with 1,2-propanediol (NaDES1) or lactic acid (NaDES2).

Overall in this enlarged set of data, 127 PC were tentatively identified. From the Venn diagram shown in Figure 2, we can draw conclusions about the efficacy of the type of solvent to extract diversified molecules. With CS it was possible to extract a higher number of PC (110) than with NaDES, which were able to extract 66 PC. A total of 49 PC were extracted regardless of the type of solvent used, so they could be present in any extract, from any matrix (wheat or rice coproduct). CS were more efficient in extracting a greater structural diversity of molecules than NaDES and showed a total of 61 PC uniquely extracted in these solvents (Fig. 2). In fact, different extraction efficacies of CS were also observed when analyzing the coproducts separately (Chapter 3: article 3 and 4).

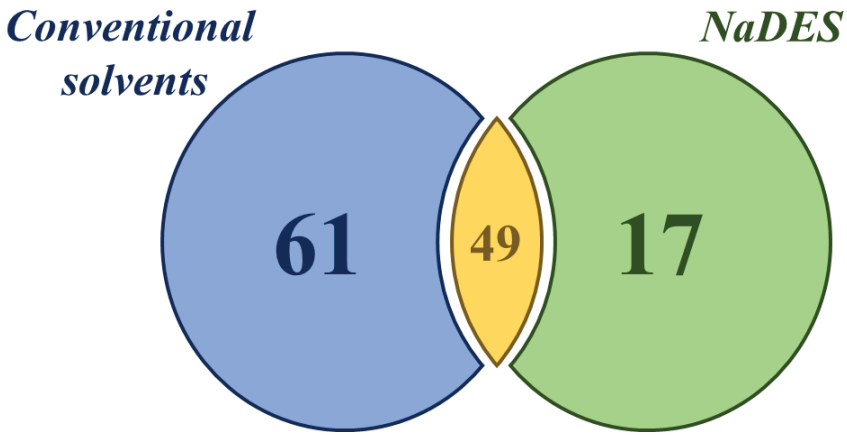


Figure 2 - Comparison of the number of identified compounds between conventional (CS: ethanol/water, acetone or isopropanol) and natural deep eutectic solvents (NADES: based on choline chloride with 1,2-propanediol (NaDES1) or lactic acid (NaDES2)) on wheat and rice coproduct extracts presented in this manuscript.

Although the NaDES used in this study were not so efficient as CS, their selectivity, i.e., ability to extract unique molecules must be pointed out. Especially when these unique molecules deserve attention due to specific functional moieties. Indeed, the 17 unique PC extracted by NaDES in this thesis, stand out for having methyl groups, or having furans in the chain, or glycosides.

To get an overview of our wheat and rice coproducts extracts, the data were submitted to an unsupervised multivariate analysis by PCA (Fig. 3). The biplot considered the relative ion abundance of each putative identified PC as a variable and the score of each sample was calculated. As displayed in Figure 3, across different extracts, the PC1 accounted for 34.73% and PC2 for 19.08% of the total variation in the dataset. As expected, it is possible to observe and confirm an important difference between wheat and rice coproducts (further discussed in section 2.4) and, also, that the characterization of the phenolic profile is very dependent on the choice of solvent. However, PCA was not able to group the extracts according to solvents.

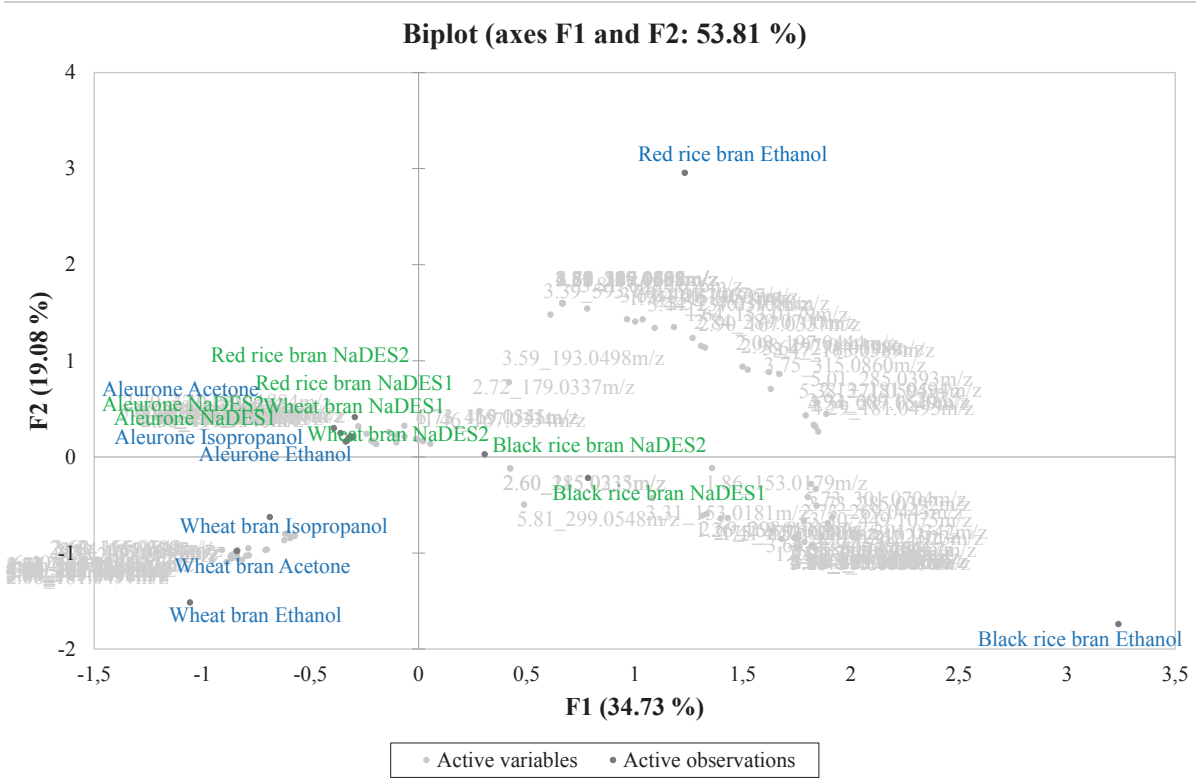


Figure 3 - Principal Component Analysis of wheat and rice coproduct extracted with conventional (CS in blue color) and natural deep eutectic solvents (NaDES in green color).

To further investigate the differences or similarities between the two types of solvents (CS or NaDES), the data (considering now the relative ion abundance) was submitted to the supervised multivariate analysis by using an OPLS-DA model (Figure 4). After data pretreatment by Pareto scaling, 66 putatively identified PC were excluded. The scores are weighted averages of the original ones, hence providing a good summary. In addition, these scores display the separation of the groups. In Figure 4, the upper right quadrant of the S-plot shows the elevation of PC, in NaDES, while the lower-left quadrant presents a comparison of the elevation of PC in CS extracts. Each S-plot evaluated the discriminant PC by variable importance in projection (VIP >1.5), these discriminant PC are shown in red.

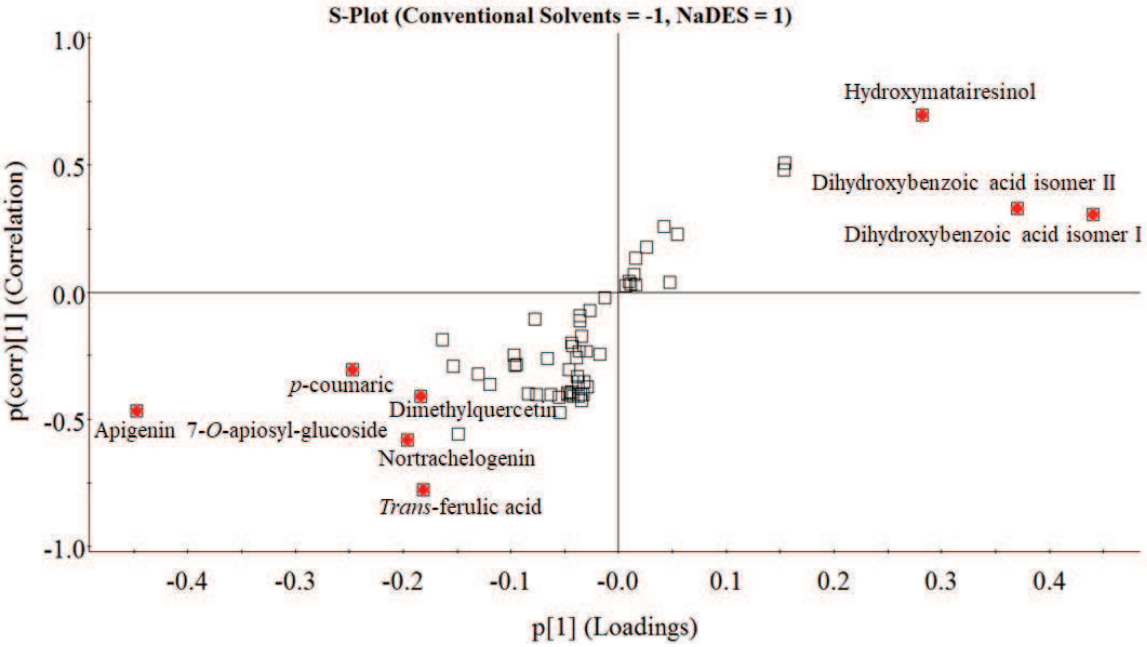


Figure 4 -The covariance p[1] and correlation p[1] loadings from a two-class OPLS-DA are displayed in an S-plot format (CS *vs*. NaDES).

2.3. What are the methodological limits in the use of NADES as extraction solvents?

It is noteworthy that although promising, eutectic solvents are still a challenge from the analytical point of view due to their viscosity and low vapor pressure, making them a difficult media to recover the extracted compounds. In this thesis, to enable the analysis of these extracts, resuspension steps with CS (MeOH/water, 2:1, v/v) were necessary prior to injection into the chromatographic system coupled to the mass spectrometer. Even so, our chromatograms showed interfering peaks that caused the suppression of ions of interest, or noises, such as peaks of lactic acid and its polymers formed from the NaDES component during ionization (Fig. 4).

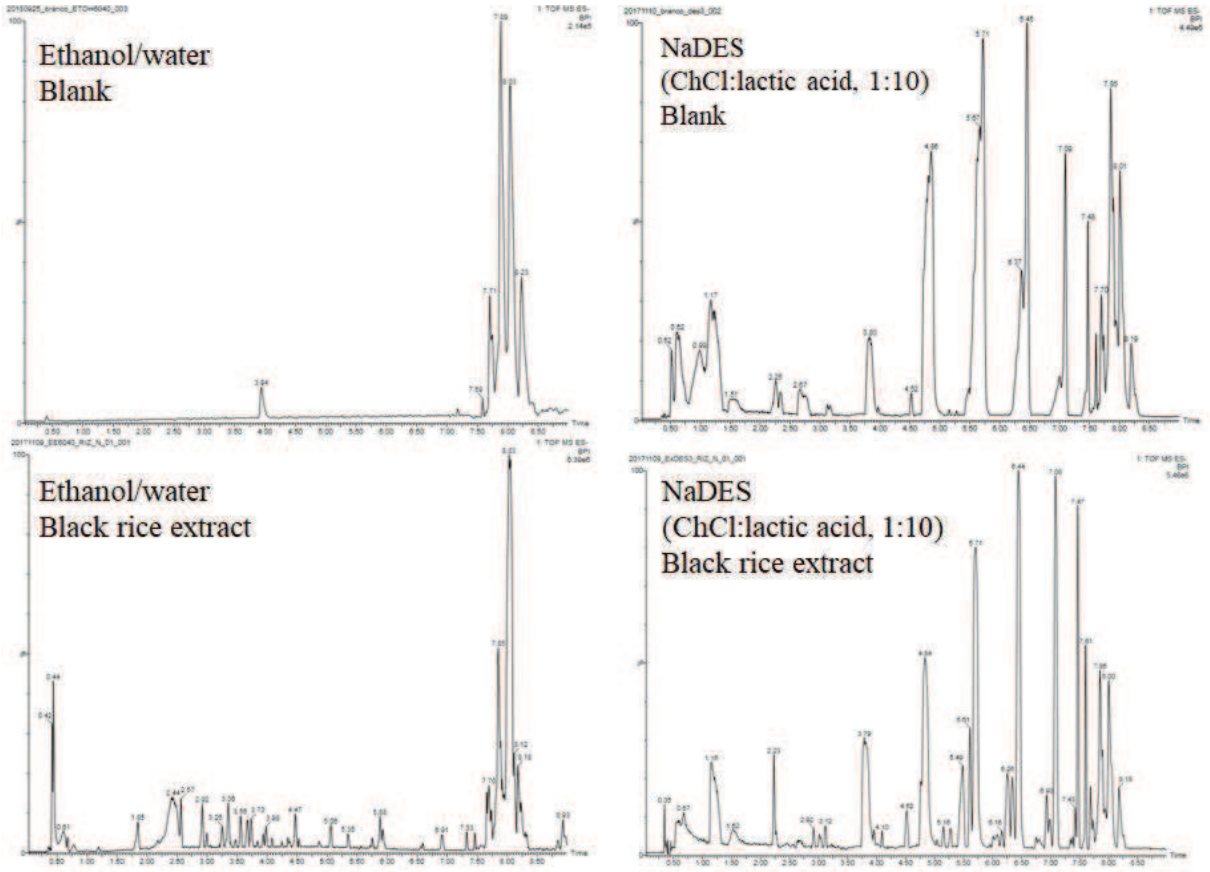


Figure 5 - Comparison of chromatograms of black RB extracted by conventional solvent (ethanol/water) and NaDES2 (ChCl:lactic acid, 1:10 v/v).

This fact can be explained by the exposure to heat from the ESI source (150 °C at ion block) and the formation of polymers of lactic acids so intense that they suppress the signal intensity of our compounds of interest (Södergård & Stolt, 2002). In Figure 6, it is possible to see in the spectrum of each peak the lactic acid (*m/z* = 89.027, [M-H]-) and probable their isomers. Many studies that analyze food extracts obtained using NaDES by LC-MS did not show their chromatograms leaving open the question whether it is really a problem to analyze extract using MS tools. Most of the time, simple HPLC chromatograms of NaDES extracts are displayed in publications and the mass chromatogram is not reported (Huang et al., 2017a; Khezeli et al., 2016).

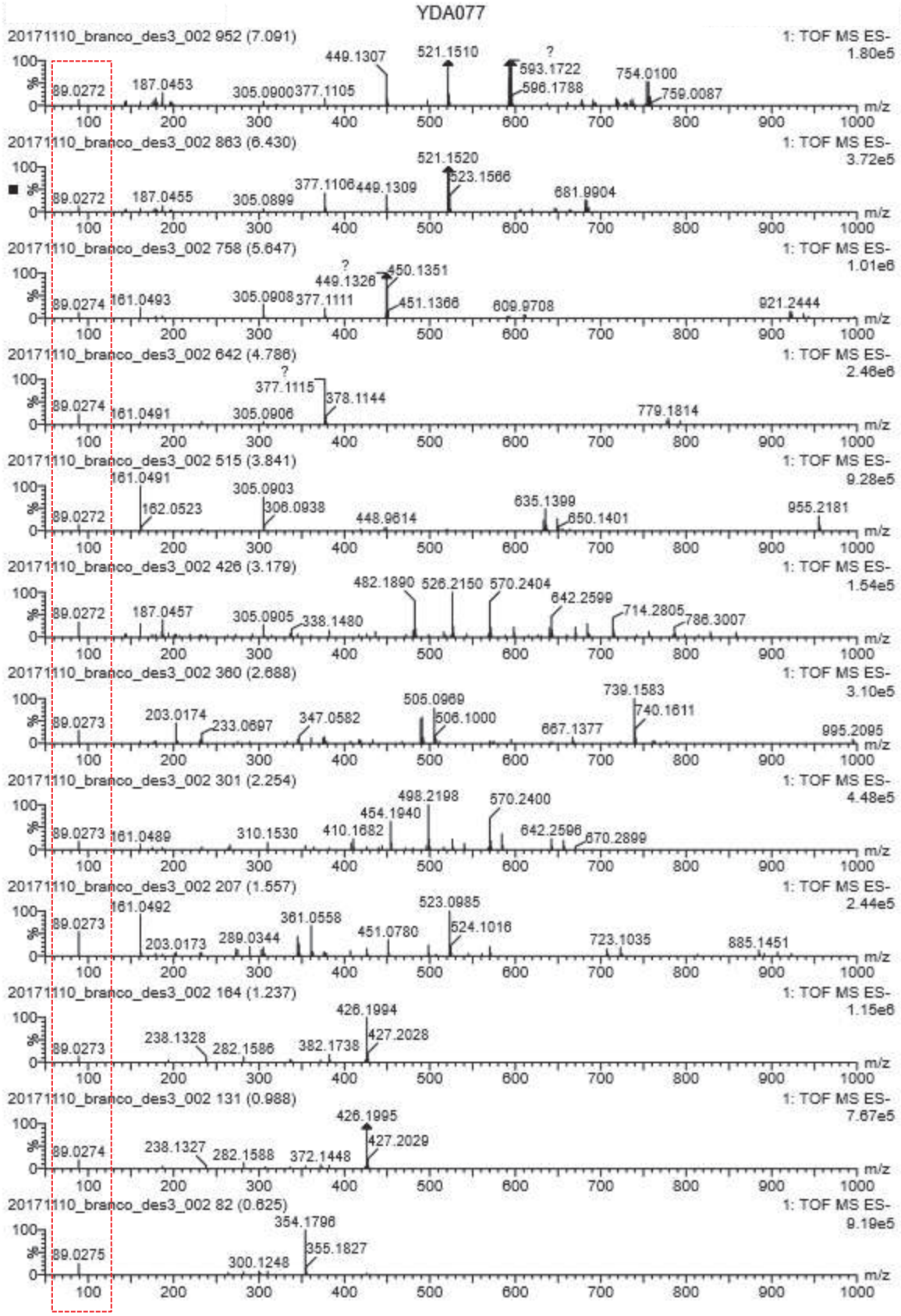


Figure 6 – MS Spectra of different peaks in the NaDES2 (ChCl/lactic acid, 1:10 v/v) blank extracts. In red is highlighted the ion of the lactic acid.

However, as part of the workflow analyses, extraction blanks were performed in all analyses to avoid false positive identifications and to eliminate these undesirable compounds from our study on data processing and putatively identify the PC in our samples.

Based on the literature, we know that NaDES are considered a great extraction media since their components can form hydrogen bonds with the molecules to be extracted. Because of this H-bond network, the solubility of the molecule is increased (Dai et al., 2013; Morrison et al., 2009). However, it is so strong that even after dilution (Dai et al., 2015) these bonds do not break. During this thesis, we tested different means to dilute or eliminated NaDES from extracts using methods such as: playing on the ratios of dilution (1:5, 1:10, 1:50, 1:100, 1:200 v/v), precipitation, clean up and SPE columns, but we did not reach satisfactory results. Thus, we can say that extraction by NaDES can difficult the subsequent analyses and we need to optimize the NaDES components, extraction, compounds recovery and check their behavior on MS analysis. In addition, to overcome the recovery step of the compounds the NADES can be used as a formulation medium in the final product or going a step further using it also as a reaction medium.

2.4. Characterization of wheat and rice coproducts extracts: a crucial preliminary stage for the development of biotransformed ready-to-use extracts

As mentioned before, the consumption of wheat and rice, especially in the refined and polished form, lead to a huge production of coproducts. The bran is the most significant fraction among the coproducts. We must remind that the production of both, wheat bran and RB biomasses, represents around 150 and 50 million/t/year, respectively. Although in recent years there has been an increase in the consumption of whole grain cereals, precisely because of their rich composition of bioactive nutrients beneficial for human health, the efficient and sustainable valorization of cereal coproducts generated by food industries remains a priority.

In this section, to deepen the analysis and comparison between all the wheat and rice coproduct extracts obtained in this thesis, a heatmap was performed with all compounds identified from the UPLC-MS analyses (Figure 7). For this figure, the relative abundances of ions are considered and rescaled varying between -1 and 1, with a color code variation between blue and yellow. This heatmap formed two large clusters separating the ethanolic extracts of pigmented RB (red and black) from the other extracts (NaDES extracts of pigmented RB and all wheat bran and aleurone extracts). Evaluating this second cluster, the

wheat bran extracts obtained using CS are well separated from the others. It is further evidenced that overall, the profile and abundance of identified PC in the extracts are different not only among solvents, but also among the different coproducts used. Regarding the classes, we could not associate a specific class with a specific coproduct.

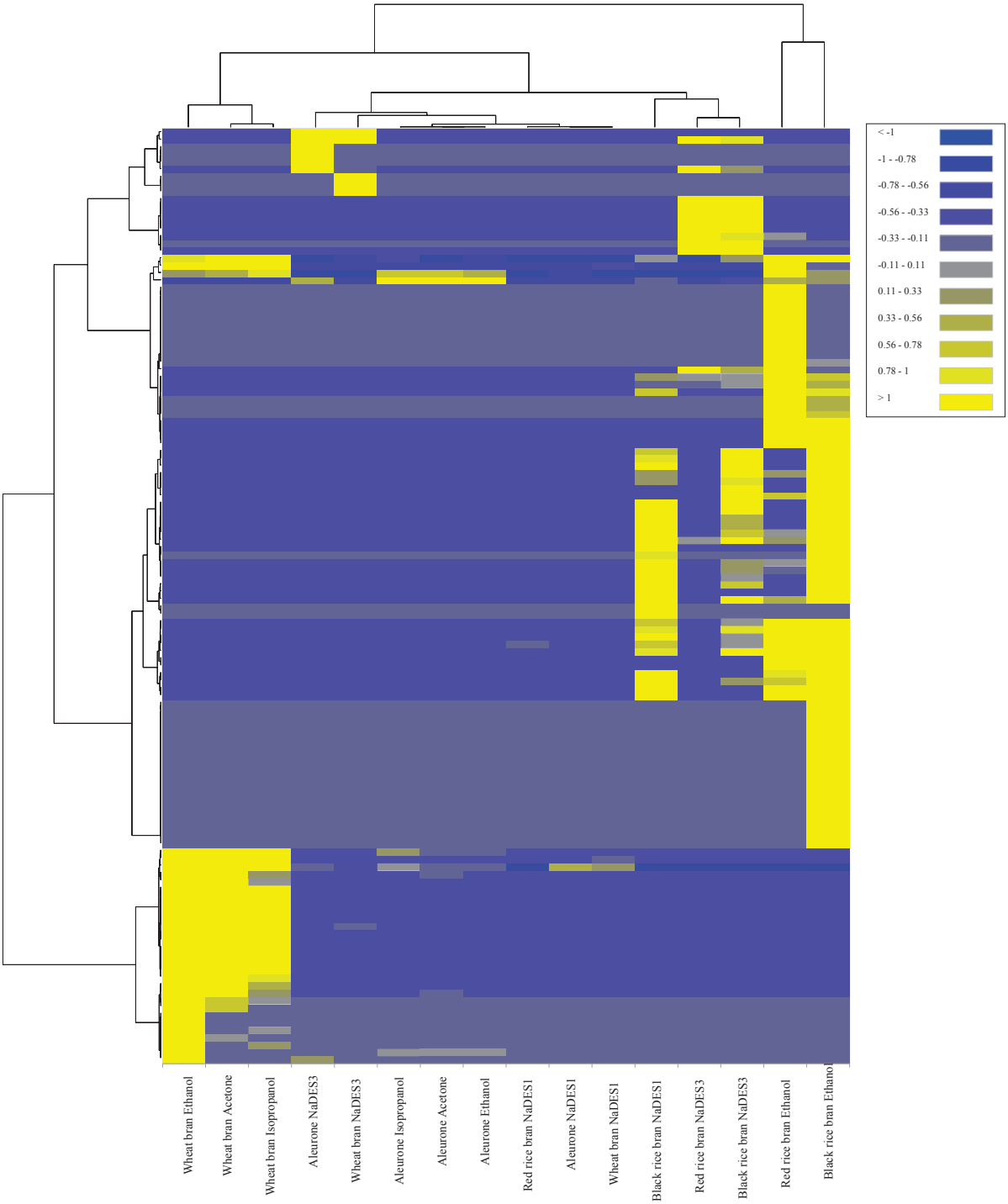


Figure 7 - Heatmap comparing the abundance of PC in all the wheat and rice coproducts extracted using conventional solvents (Bucsella et al.) and NaDES. The relative abundances of ions are considered and rescaled varying between -1 and 1, with a color code variation between blue and yellow. The color code is as follows: the

absence of the compound stands in stronger blue (-1) and its presence in higher abundance in stronger yellow tone (1).

Although the metabolomics results of this thesis showed CS as the most effective in extracting an abundant and diversified profile of PC, one of the objectives was to produce extracts with enhanced bioactivity using NaDES as an extraction and bioacatalysis medium. To select the NADES extract for such one-pot biotransformation, a comparison of the various wheat and rice coproducts obtained using NADES was undertaken. Thus, a new heatmap was performed (Figure 8), considering on this time only the NaDES extracts of all coproducts. The clusters formed highlight the black RB extracted by NaDES1 (ChCl:1,2-propanediol) but also underline a difference in compound profile between NaDES1 and NaDES2 (ChCl:lactic acid).

As discussed in the chapters 4, 5 and 8 and already described in the literature, acidic NaDES showed the best extraction efficiency for PC. This efficacy is associated with hydrolysis, thus allowing the release of bound compounds. Our results clearly show the difference in profile between the co-products, but the extracts regroup in a way that makes it possible to visualize four classes: 1) black RB, 2) red RB, 3) aleurone, and 4) wheat bran.

Considering the number of identifications, RB showed a higher diversity of PC than wheat bran, being thus selected for NaDES one-pot biotransformation. Considering now other chemical characterization conducted on the CS extracts from RB collection (chapter 6), and the outstanding quality of lipid profile (low quantity of FFA) of Madagascar red RB. This latter was selected among other colored RB.

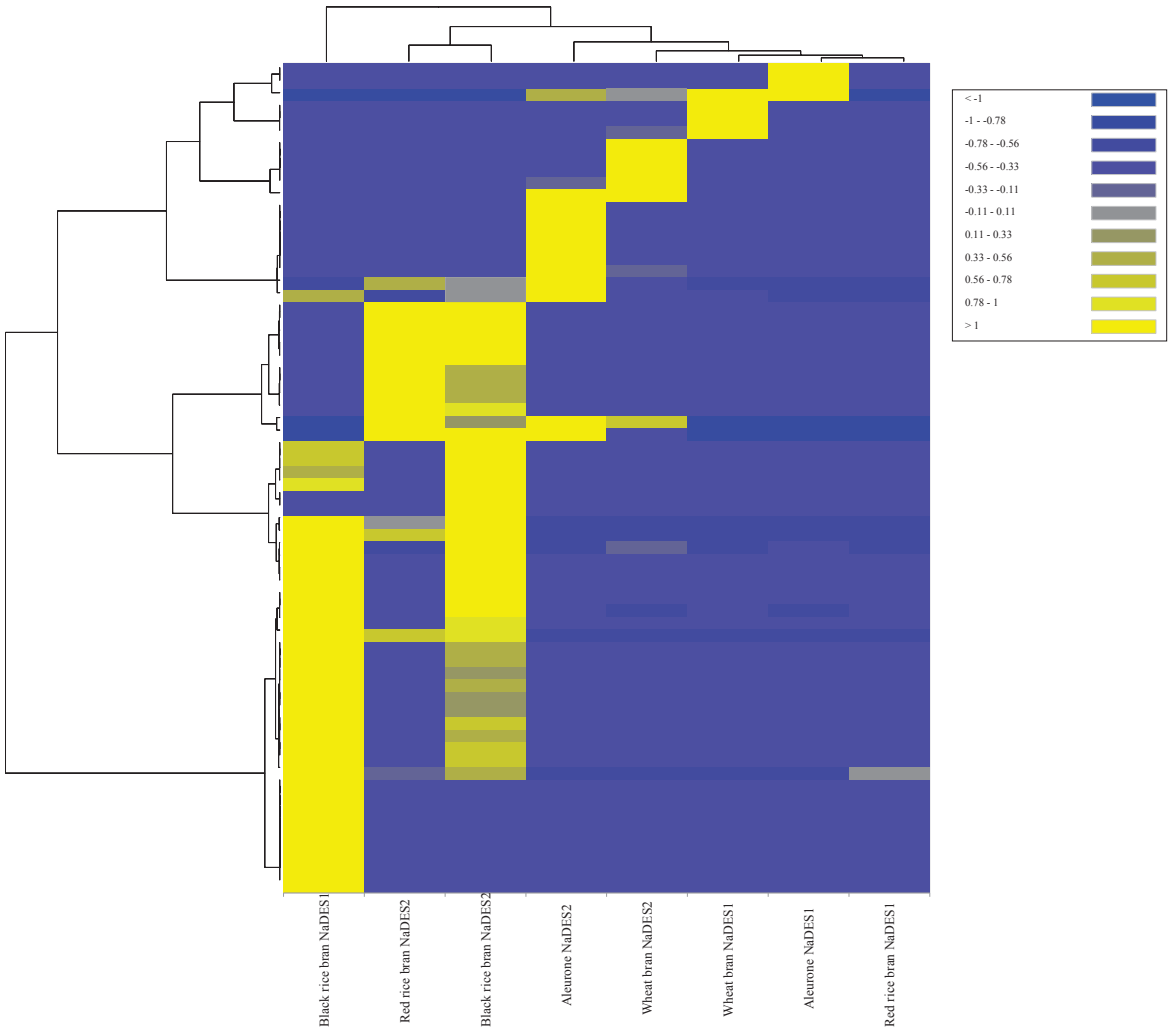


Figure 8 - Heatmap comparing PC coproducts extracted only considering NaDES such as the most efficients showed in this study.

2.5. Originality, pros, and cons of our one-pot rice bran biocatalysed transformation in natural deep eutectic solvents

One of the advantages of some DES is that, besides their high extraction power, they can be compatible with enzymatic reactions. What this means on a catalytic point of view, that the solvent will solubilize the substrate and preserve the active conformation of the enzyme (i.e. the level of hydration of the molecule is a key factor). Some CS are also compatible with biocatalysis but a balance must be found between to solubilize substrate and do not denaturate enzyme or at least not too fast. For this model biotranformation, we selected a very robust and non-specific lipase from *Candida Antarctica* lipase B, used under the form of Novozym® 435 *i.e.,* immobilized on a hydrophobic carrier (acrylic resin) that eases enzyme removal from the reaction media.

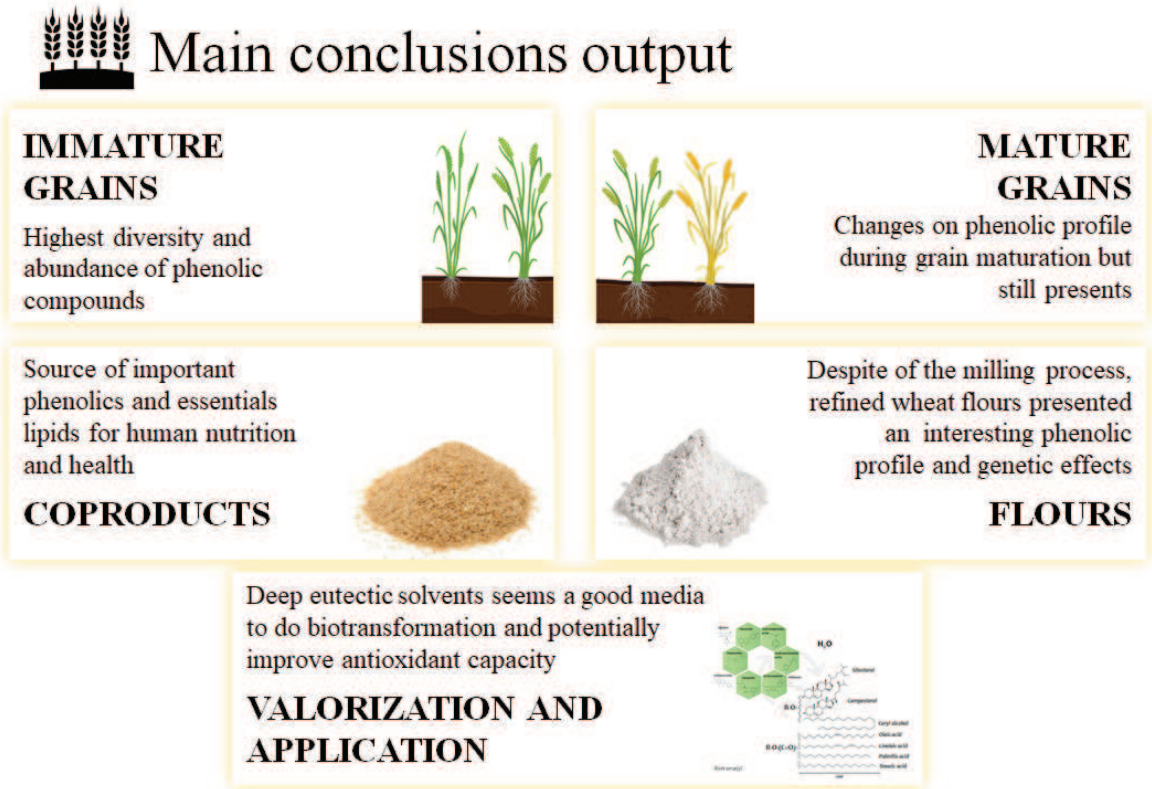
We targeted several potential reactions, and the originality of our approach is to have realized extraction and putative biotransformation in one-pot avoiding recovery steps. However, our imprecision in identifying the key reactions and potentials products formed during this putative biotransformation is also an important limit in our strategy.

2.6. Going beyond phenolic compounds metabolomics analyses on rice coproducts

As mentioned before, RB stands out for their peculiar richness in PC and important amount of residual lipids (15-20%, cf chapter 6). In this thesis, we realized that these RB lipids had been characterized in terms of major classes but not by minor classes of high biological interest (polar lipids such as phospho- and sphingolipids, oxylipins). In addition, we had the opportunity of having metabolomics data about an intervention study on infant supplementation with one type of RB that could be explored with a focus on lipid variation. We could identify such polar lipids and oxylipins (especially the non-enzymatic ones: phytoprostanes and phytofurans) in RB. In children plasma, we could highlight modulations in the plasma lipid profile associated with RB consumption, such as the presence of PUFA, increase of fatty acids related to the metabolism of omega-3 and omega-6 pathway and increase of some endocannabinoids (notably from arachidonic acid) in children after the intervention. Of course, this data must be considered with precaution especially for the intervention trial, and the limitation of having used methanol extracts. However, are of interest to advance knowledge of the RB lipidome composition and function for positive impacts on lipid modulation and health as well as opens perspectives of new intervention trial.

3. Conclusion

To conclude, the extensive characterization performed in this thesis allows us to ensure the bioactive potential of the cereals most consumed by humans, as well as of their main coproducts. Thus, they may be sources of important (and essentials) phytochemicals for human nutrition and health, especially in developing countries whose food base is cereals. Moreover, the possibility of application based on green strategies and with reduced steps, can be promising for the development of supplementations by the food industry and non-food applications by cosmetic and pharmaceutical industries.



Perspectives

From our results, new questions appear necessary to be raised. Thus, some perspectives for further research are proposed bellow.

Ion mobility

Recent studies have addressed limitations in the identification of PC from food matrices. Although some techniques have been widely applied for this purpose, such as tandem mass spectrometry. The presence of a great structural diversity either by bonds with other molecules (e.g., glycosides) or by isomers makes the identification difficult since parameters used as mass/charge ratio, fragments, among others, end up not being able to ensure the identification. Ion mobility is an omics tool that allows the differentiation of positional isomer compounds from their three-dimensional structure in the gas phase (Souza et al, 2017). The application of this new dimension is commonly highlighted in proteomics papers (i.e. proteomic-based studies of metabolic proteins of wheat, (Victorio et al, 2018) and more recently proposed for metabolomics studies showing the effectiveness to separate low molecular weight compounds (Feuerstein et al., 2021; Paglia et al., 2021).

In this sense, one of the main perspectives that would bring significant advancement is the use of ion mobility to separate isomers of PC and to contribute to online annotation databases with specific data (drift-time, specific fragments, specific glycosidic bond sites, and even determine glycosylated phenolics most likely to be formed) for signature and identification of these compounds.

Molecular simulation

Fragmentation is also an important technique in mass spectrometry since it allows us to identify a compound with greater reliability. The identification is allowed thanks to the comparison with spectral banks using experimental versus theoretical data (such as fragments), due to the fact that there are no analytical standards for the multitude of existing molecules, besides the high cost that these analyses would represent. However, the databases still lack information about some compounds, since each molecule will present specific fragments linked to its conformational molecular structure.

In this context, another perspective that can be allied to mass spectrometry is Molecular Modeling. Molecular simulations can be key to predicting, from computational approaches, possible structures, isomers and interactions, molecular conformation, more stable protonation and deprotonation sites, as well as molecular dynamics simulation of

collisions and generation of fragment spectra (Koopman & Grimme, 2021), which can improve databases and consequently bring more reliable comparison/identifications, especially to untargeted metabolomics.

NaDES formulations

We have obtained promising results with application of NaDES as extractor of bioactive compounds. However, it is essential to perform further omics analysis to visualize the structural rearrangements formed based on the hypotheses we were able to form. However, this thesis also draws attention to the limitations found by metabolomic analyses of NaDES extracts, being necessary to develop and make compatible chromatographic methods hyphenated to mass spectrometry for the analysis of NaDES-based extracts.

As a perspective regarding the green solvents and their applications, it is important that new formulations of NaDES and optimizations be performed to allow: 1) higher yields of the compounds of interest, in our case the PC, 2) a viable recovery of the compounds to perform analyses and further applications, and 3) to be used as extraction and reaction medium without the need to adjust pH (e.g. perform physical hydrolysis, or enzymatic hydrolysis) and that can be consequently reanalyzed in order to verify the structures formed.

Cell and clinical trials

It is well known that *in vitro* analyses can have limitations, and that the action of a molecule *in vivo* can be influenced by several factors: i) the molecule of interest may not be in physiological concentration required to obtain bioactivity, ii) interactions with another molecule causing a negative effect, iii) the generated metabolite has no bioactivity or has a negative effect, or even greatest bioactivity, or iv) be excreted directly without being metabolized.

Recent studies have evaluated the absorption in different stages of the digestive tract and the potential of PC to modulate the intestinal microbiota. However, to overcome the obstacles and limitations imposed by *in vitro* analyses and to confirm the bioactive potential of our extracts and to increase even more after the biotransformation step, specific studies need to be performed such as cell testing or clinical studies.

References

A. Di Loreto, S. B., L. Montero, V. Bregola, I. Marotti, R.E. Sferrazza, G. Dinelli, M. Herrero, A. Cifuentes. (2018). Determination of phenolic compounds in ancient and modern durum wheat genotypes. *ELECTROPHORESIS, 0*(0). doi:https://doi.org/10.1002/elps.201700439

AACCI. (2000). Approved methods. In 10th ed.). Minneapolis, MN: American Association of Cereal Chemists International.

Abdel-Aal, E.-S. M., & Rabalski, I. (2013). Effect of baking on free and bound phenolic acids in wholegrain bakery products. *Journal of Cereal Science, 57*(3), 312-318. doi:https://doi.org/10.1016/j.jcs.2012.12.001

ABITRIGO. (2018). *Associação Brasileira das Indústrias de Trigo*. Retrieved from http://www.abitrigo.com.br/estatisticas-global.php

Abozed, S. S., El-kalyoubi, M., Abdelrashid, A., & Salama, M. F. (2014). Total phenolic contents and antioxidant activities of various solvent extracts from whole wheat and bran. *Annals of Agricultural Sciences, 59*(1), 63-67. doi:https://doi.org/10.1016/j.aoas.2014.06.009

Acosta-Estrada, B. A., Gutiérrez-Uribe, J. A., & Serna-Saldívar, S. O. (2014). Bound phenolics in foods, a review. *Food Chem, 152*, 46-55. doi:https://doi.org/10.1016/j.foodchem.2013.11.093

Adom, K. K., & Liu, R. H. (2002). Antioxidant Activity of Grains. *Journal of Agricultural and Food Chemistry, 50*(21), 6182-6187. doi:10.1021/jf0205099

Akkarach Bumrungpert, Rewadee Chongsuwat, Phosat, C., & Butacnum, A. (2019). Rice Bran Oil Containing Gamma-Oryzanol Improves Lipid Profiles and Antioxidant Status in Hyperlipidemic Subjects: A Randomized Double-Blind Controlled Trial. *The Journal of Alternative and Complementary Medicine, 25*(3), 353-358. doi:10.1089/acm.2018.0212

Alvarez-Jubete, L., Wijngaard, H., Arendt, E. K., & Gallagher, E. (2010). Polyphenol composition and in vitro antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. *Food Chem, 119*(2), 770-778. doi:https://doi.org/10.1016/j.foodchem.2009.07.032

Álvarez-Sánchez, B., Priego-Capote, F., & Castro, M. D. L. d. (2010). Metabolomics analysis II. Preparation of biological samples prior to detection. *TrAC Trends in Analytical Chemistry, 29*(2), 120-127. doi:https://doi.org/10.1016/j.trac.2009.12.004

Andreasen, M. F., Kroon, P. A., Williamson, G., & Garcia-Conesa, M.-T. (2001). Intestinal release and uptake of phenolic antioxidant diferulic acids. *Free Radical Biology and Medicine, 31*(3), 304-314. doi:https://doi.org/10.1016/S0891-5849(01)00585-8

Angelino, D., Cossu, M., Marti, A., Zanoletti, M., Chiavaroli, L., Brighenti, F., . . . Martini, D. (2017). Bioaccessibility and bioavailability of phenolic compounds in bread: a review. *Food & function, 8*(7), 2368-2393. doi:10.1039/C7FO00574A

Anson, N. M., van den Berg, R., Havenaar, R., Bast, A., & Haenen, G. R. (2009). Bioavailability of ferulic acid is determined by its bioaccessibility. *Journal of Cereal Science, 49*(2), 296-300.

Antoine, C., Peyron, S., Lullien-Pellerin, V., Abecassis, J., & Rouau, X. (2004). Wheat bran tissue fractionation using biochemical markers. *Journal of Cereal Science, 39*(3), 387-393. doi:https://doi.org/10.1016/j.jcs.2004.02.001

Antonelli, M., Benedetti, B., Cavaliere, C., Cerrato, A., Montone, C. M., Piovesana, S., . . . Capriotti, A. L. (2020). Phospholipidome of extra virgin olive oil: Development of a solid phase extraction protocol followed by liquid chromatography–high resolution mass spectrometry for its software-assisted identification. *Food Chem, 310*, 125860. doi:https://doi.org/10.1016/j.foodchem.2019.125860

AOAC. (1984). *Official methods of analysis*. Association off Official Analytical Chemists.

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem, 99*(1), 191-203. doi:http://dx.doi.org/10.1016/j.foodchem.2005.07.042

Balić, A., Vlašić, D., Žužul, K., Marinović, B., & Bukvić Mokos, Z. (2020). Omega-3 Versus Omega-6 Polyunsaturated Fatty Acids in the Prevention and Treatment of Inflammatory Skin Diseases. *International Journal of Molecular Sciences, 21*(3), 741. Retrieved from https://www.mdpi.com/1422-0067/21/3/741

Balmer, D., Flors, V., Glauser, G., & Mauch-Mani, B. (2013). Metabolomics of cereals under biotic stress: current knowledge and techniques. *Frontiers in Plant Science, 4*(82). doi:10.3389/fpls.2013.00082

Barron, C., Samson, M.-F., Lullien-Pellerin, V., & Rouau, X. (2011). Wheat grain tissue proportions in milling fractions using biochemical marker measurements: application to different wheat cultivars. *Journal of Cereal Science, 53*(3), 306-311.

Barron, C., Surget, A., & Rouau, X. (2007). Relative amounts of tissues in mature wheat (Triticum aestivum L.) grain and their carbohydrate and phenolic acid composition. *Journal of Cereal Science, 45*(1), 88-96. doi:http://dx.doi.org/10.1016/j.jcs.2006.07.004

Bijttebier, S., Van der Auwera, A., Foubert, K., Voorspoels, S., Pieters, L., & Apers, S. (2016). Bridging the gap between comprehensive extraction protocols in plant metabolomics studies and method validation. *Analytica Chimica Acta, 935*, 136-150. doi:https://doi.org/10.1016/j.aca.2016.06.047

Bixi, T., Cai-Yun, P., Kwame Oteng, D., Ting, T., Yanjun, H., Qiongli, S., & Xiaoping, Y. (2016). Paeonol, a Powerful Natural Product with Broad Biological Spectra by Inhibiting Inflammatory Pathway. *Current Traditional Medicine, 2*(2), 72-79. doi:http://dx.doi.org/10.2174/2215083802666161025162252

Borrás-Linares, I., Herranz-López, M., Barrajón-Catalán, E., Arráez-Román, D., González-Álvarez, I., Bermejo, M., . . . Segura-Carretero, A. (2015). Permeability Study of Polyphenols Derived from a Phenolic-Enriched Hibiscus sabdariffa Extract by UHPLC-ESI-UHR-Qq-TOF-MS. *International Journal of Molecular Sciences, 16*(8), 18396-18411. doi:10.3390/ijms160818396

Borresen, E. C., Jenkins-Puccetti, N., Schmitz, K., Brown, D. G., Pollack, A., Fairbanks, A., . . . Ryan, E. P. (2017). A Pilot Randomized Controlled Clinical Trial to Assess Tolerance and Efficacy of Navy Bean and RB Supplementation for Lowering Cholesterol in Children. *Glob Pediatr Health, 4*, 2333794x17694231. doi:10.1177/2333794x17694231

Borresen, E. C., & Ryan, E. P. (2014). Chapter 22 - Rice Bran: A Food Ingredient with Global Public Health Opportunities. In R. R. Watson, V. R. Preedy, & S. Zibadi (Eds.), *Wheat and Rice in Disease Prevention and Health* (pp. 301-310). San Diego: Academic Press.

Bourlieu, C., Barouh, N., Kergomard, J., Ménard, O., Dupont, D., Villeneuve, P., . . . Michalski, M.-C. (2021). Polar Lipids *Handbook of Dairy Foods Analysis*. Boca Raton Etats-Unis: CRC Press.

Bourlieu, C., Paboeuf, G., Chever, S., Pezennec, S., Cavalier, J.-F., Guyomarc'h, F., . . . Vié, V. (2016). Adsorption of gastric lipase onto multicomponent model lipid monolayers with phase separation. *Colloids and Surfaces B: Biointerfaces, 143*, 97-106. doi:https://doi.org/10.1016/j.colsurfb.2016.03.032

Box, J. D. (1983). Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. *Water Research, 17*(5), 511-525. doi:https://doi.org/10.1016/0043-1354(83)90111-2

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology, 28*(1), 25-30. doi:https://doi.org/10.1016/S0023-6438(95)80008-5

BRASIL. (2005). *Instrução Normativa 8/2005: Regulamento Técnico de Identidade e Qualidade da Farinha de Trigo*.

BRASIL. (2010). Instrução Normativa nº 38 de 30 de novembro de 2010. *Ministério da Agricultura, Pecuária e Abastecimento*.

Brouns, F., Hemery, Y., Price, R., & Anson, N. M. (2012). Wheat Aleurone: Separation, Composition, Health Aspects, and Potential Food Use. *Critical Reviews in Food Science and Nutrition, 52*(6), 553-568. doi:10.1080/10408398.2011.589540

Brown, D. G., Borresen, E. C., Brown, R. J., & Ryan, E. P. (2017). Heat-stabilised rice bran consumption by colorectal cancer survivors modulates stool metabolite profiles and metabolic networks: a randomised controlled trial. *British Journal of Nutrition, 117*(9), 1244-1256. doi:10.1017/S0007114517001106

Brunschwiler, C., Heine, D., Kappeler, S., Conde-Petit, B., & Nyström, L. (2013). Direct measurement of RB lipase activity for inactivation kinetics and storage stability prediction. *Journal of Cereal Science, 58*(2), 272-277. doi:https://doi.org/10.1016/j.jcs.2013.06.007

Bucsella, B., Molnár, D., Harasztos, A. H., & Tömösközi, S. (2016). Comparison of the rheological and end-product properties of an industrial aleurone-rich wheat flour, whole grain wheat and rye flour. *Journal of Cereal Science, 69*, 40-48. doi:https://doi.org/10.1016/j.jcs.2016.02.007

Carson, G., & Edwards, N. (2009). Criteria of wheat and flour quality. *Wheat Chemistry and Technology*, 97-118. doi:10.1094/9781891127557.004

Castanho, A., Lageiro, M., Calhelha, R. C., Ferreira, I. C. F. R., Sokovic, M., Cunha, L. M., & Brites, C. (2019). Exploiting the bioactive properties of γ-oryzanol from bran of different exotic rice varieties. *Food & function, 10*(5), 2382-2389. doi:10.1039/C8FO02596G

Chakuton, K., Puangpronp, D., & Muntana, N. (2012). Phytochemical Content and Antioxidant Activity of Colored and Non-colored Thai Rice Cultivars. *Asian Journal of Plant Sciences, 11*, 285-293. doi:10.3923/ajps.2012.285.293

Chehab, E. W., Perea, J. V., Gopalan, B., Theg, S., & Dehesh, K. (2007). Oxylipin Pathway in Rice and Arabidopsis. *Journal of Integrative Plant Biology, 49*(1), 43-51. doi:https://doi.org/10.1111/j.1744-7909.2006.00405.x

Chemat, F., Abert Vian, M., Ravi, H. K., Khadhraoui, B., Hilali, S., Perino, S., & Fabiano Tixier, A.-S. (2019). Review of Alternative Solvents for Green Extraction of Food and Natural Products: Panorama, Principles, Applications and Prospects. *Molecules, 24*(16), 3007. Retrieved from https://www.mdpi.com/1420-3049/24/16/3007

Chen, L.-J., & Hrazdina, G. (1981). Structural aspects of anthocyanin-flavonoid complex formation and its role in plant color. *Phytochemistry, 20*(2), 297-303. doi:https://doi.org/10.1016/0031-9422(81)85111-4

Chen, X. Q., Nagao, N., Itani, T., & Irifune, K. (2012). Anti-oxidative analysis, and identification and quantification of anthocyanin pigments in different coloured rice. *Food Chem, 135*(4), 2783-2788. doi:https://doi.org/10.1016/j.foodchem.2012.06.098

Cifuentes, A. (2009). Food analysis and Foodomics. *Journal of Chromatography A, 1216*(43), 7109. doi:https://doi.org/10.1016/j.chroma.2009.09.018

Cozzano Ferreira, S., Fernández, A. M., del Castillo Bilbao, M. D., & Medrano Fernández, A. (2019). New Functional Ingredients From Agroindustrial By-Products for the Development of Healthy Foods. In P. Ferranti, E. M. Berry, & J. R. Anderson (Eds.), *Encyclopedia of Food Security and Sustainability* (pp. 351-359). Oxford: Elsevier.

Cuenoud, B., Rochat, I., Gosoniu, M. L., Dupuis, L., Berk, E., Jaudszus, A., . . . Cruz-Hernandez, C. (2020). Monoacylglycerol Form of Omega-3s Improves Its Bioavailability in Humans Compared to Other Forms. *Nutrients, 12*(4). doi:10.3390/nu12041014

Czaja, T., Sobota, A., & Szostak, R. (2020). Quantification of Ash and Moisture in Wheat Flour by Raman Spectroscopy. *Foods (Basel, Switzerland), 9*(3), 280. doi:10.3390/foods9030280

da Silva, D. T., Smaniotto, F. A., Costa, I. F., Baranzelli, J., Muller, A., Somacal, S., . . . Emanuelli, T. (2021). Natural deep eutectic solvent (NADES): A strategy to improve the bioavailability of blueberry phenolic compounds in a ready-to-use extract. *Food Chem, 364*, 130370. doi:https://doi.org/10.1016/j.foodchem.2021.130370

Dai, Y., Rozema, E., Verpoorte, R., & Choi, Y. H. (2016). Application of natural deep eutectic solvents to the extraction of anthocyanins from Catharanthus roseus with high extractability and stability replacing conventional organic solvents. *Journal of Chromatography A, 1434*, 50-56. doi:https://doi.org/10.1016/j.chroma.2016.01.037

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., & Choi, Y. H. (2013). Natural deep eutectic solvents as new potential media for green technology. *Analytica Chimica Acta, 766*, 61-68. doi:https://doi.org/10.1016/j.aca.2012.12.019

Dai, Y., Verpoorte, R., & Choi, Y. H. (2014). Natural deep eutectic solvents providing enhanced stability of natural colorants from safflower (Carthamus tinctorius). *Food Chem, 159*, 116-121. doi:https://doi.org/10.1016/j.foodchem.2014.02.155

Dai, Y., Witkamp, G.-J., Verpoorte, R., & Choi, Y. H. (2015). Tailoring properties of natural deep eutectic solvents with water to facilitate their applications. *Food Chemistry, 187*, 14-19. doi:https://doi.org/10.1016/j.foodchem.2015.03.123

Deng, G.-F., Xu, X.-R., Zhang, Y., Li, D., Gan, R.-Y., & Li, H.-B. (2013). Phenolic Compounds and Bioactivities of Pigmented Rice. *Critical Reviews in Food Science and Nutrition, 53*(3), 296-306. doi:10.1080/10408398.2010.529624

Dhiraj, B., & Prabhasankar, P. (2013). Influence of Wheat-Milled Products and Their Additive Blends on Pasta Dough Rheological, Microstructure, and Product Quality Characteristics. *International Journal of Food Science, 2013*. doi:10.1155/2013/538070

Di Loreto, A., Di Silvestro, R., Dinelli, G., Bregola, V., Stenico, V., Sferrazza, R. E., . . . Bosi, S. (2017). Nutritional and nutraceutical aspects of KAMUT® khorasan wheat grown during the last two decades. *The Journal of Agricultural Science, 155*(6), 954-965. doi:10.1017/S002185961700003X

Di Silvestro, R., Di Loreto, A., Bosi, S., Bregola, V., Marotti, I., Benedettelli, S., . . . Dinelli, G. (2017). Environment and genotype effects on antioxidant properties of organically grown wheat varieties: a 3-year study. *Journal of the Science of Food and Agriculture, 97*(2), 641-649. doi:https://doi.org/10.1002/jsfa.7782

Dinelli, G., Segura-Carretero, A., Di Silvestro, R., Marotti, I., Arráez-Román, D., Benedettelli, S., . . . Fernadez-Gutierrez, A. (2011). Profiles of phenolic compounds in modern and old common wheat varieties determined by liquid chromatography coupled with time-of-flight mass spectrometry. *Journal of Chromatography A, 1218*(42), 7670-7681. doi:https://doi.org/10.1016/j.chroma.2011.05.065

Dinelli, G., Segura Carretero, A., Di Silvestro, R., Marotti, I., Fu, S., Benedettelli, S., . . . Fernández Gutiérrez, A. (2009). Determination of phenolic compounds in modern and old varieties of durum wheat using liquid chromatography coupled with time-of-flight mass spectrometry. *Journal of Chromatography A, 1216*(43), 7229-7240. doi:https://doi.org/10.1016/j.chroma.2009.08.041

DiPatrizio, N. V. (2021). Endocannabinoids and the Gut-Brain Control of Food Intake and Obesity. *Nutrients, 13*(4), 1214. Retrieved from https://www.mdpi.com/2072-6643/13/4/1214

Duan, L., Dou, L.-L., Guo, L., Li, P., & Liu, E. H. (2016). Comprehensive Evaluation of Deep Eutectic Solvents in Extraction of Bioactive Natural Products. *ACS Sustainable Chemistry & Engineering, 4*(4), 2405-2411. doi:10.1021/acssuschemeng.6b00091

Durand, E., Lecomte, J., Baréa, B., Dubreucq, E., Lortie, R., & Villeneuve, P. (2013). Evaluation of deep eutectic solvent–water binary mixtures for lipase-catalyzed lipophilization of phenolic acids. *Green Chemistry, 15*(8), 2275-2282.

Durand, E., Lecomte, J., Baréa, B., Piombo, G., Dubreucq, E., & Villeneuve, P. (2012). Evaluation of deep eutectic solvents as new media for Candida antarctica B lipase catalyzed reactions. *Process Biochemistry, 47*(12), 2081-2089. doi:https://doi.org/10.1016/j.procbio.2012.07.027

Durand, E., Lecomte, J., & Villeneuve, P. (2013). Deep eutectic solvents: Synthesis, application, and focus on lipase-catalyzed reactions. *European Journal of Lipid Science and Technology, 115*(4), 379-385. doi:doi:10.1002/ejlt.201200416

Durand, E., Lecomte, J., & Villeneuve, P. (2017). The Biological and Antimicrobial activities of Phenolipids. *Lipid Technology, 29*(7-8), 67-70. doi:doi:10.1002/lite.201700019

Durazzo, A., Zaccaria, M., Polito, A., Maiani, G., & Carcea, M. (2013). Lignan Content in Cereals, Buckwheat and Derived Foods. *Foods, 2*(1), 53-63. doi:10.3390/foods2010053

Fanali, C., Posta, S. D., Dugo, L., Russo, M., Gentili, A., Mondello, L., & De Gara, L. (2020). Application of deep eutectic solvents for the extraction of phenolic compounds from extra-virgin olive oil. *Electrophoresis, 41*(20), 1752-1759.

FAOSTAT. (2018). OECD-FAO Agricultural Outlook 2018-2027 (pp. 109 - 125).

FAOSTAT. (2020). OECD-FAO Agricultural Outlook 2020.

Fardet, A. (2010). New hypotheses for the health-protective mechanisms of whole-grain cereals: what is beyond fibre? *Nutrition Research Reviews, 23*(1), 65-134. doi:10.1017/S0954422410000041

Fereidoon, S., Renan, D., & Chiaki, I. (2021). Phenolic compounds in cereal grains and effects of processing on their composition and bioactivities: A review. *Journal of Food Bioactives, 15*(0). doi:10.31665/JFB.2021.15281

Fernández-Ochoa, Á., Leyva-Jiménez, F. J., De la Luz Cádiz-Gurrea, M., Pimentel-Moral, S., & Segura-Carretero, A. (2021). The Role of High-Resolution Analytical Techniques in the Development of Functional Foods. *International Journal of Molecular Sciences, 22*(6), 3220. Retrieved from https://www.mdpi.com/1422-0067/22/6/3220

Ferreira da Silveira, T. F., Laguerre, M., Bourlieu-Lacanal, C., Lecomte, J., Durand, E., Figueroa-Espinoza, M. C., . . . Villeneuve, P. (2021). Impact of surfactant concentration and antioxidant mode of incorporation on the oxidative stability of oil-in-water nanoemulsions. *LWT, 141*, 110892. doi:https://doi.org/10.1016/j.lwt.2021.110892

Ferreira, M. S. L., Samson, M.-F., Bonicel, J., & Morel, M.-H. (2012). Relationship between endosperm cells redox homeostasis and glutenin polymers assembly in developing durum wheat grain. *Plant Physiology and Biochemistry, 61*, 36-45. doi:http://dx.doi.org/10.1016/j.plaphy.2012.08.015

Feuerstein, M. L., Kurulugama, R. T., Hann, S., & Causon, T. (2021). Novel acquisition strategies for metabolomics using drift tube ion mobility-quadrupole resolved all ions time-of-flight mass spectrometry (IM-QRAI-TOFMS). *Analytica Chimica Acta, 1163*, 338508. doi:https://doi.org/10.1016/j.aca.2021.338508

Figueroa-Espinoza, M.-C., & Villeneuve, P. (2005). Phenolic Acids Enzymatic Lipophilization. *Journal of Agricultural and Food Chemistry, 53*(8), 2779-2787. doi:10.1021/jf0484273

Folch, J., Lees, M., & Stanley, G. H. S. (1957). A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES. *Journal of Biological Chemistry, 226*(1), 497-509. doi:https://doi.org/10.1016/S0021-9258(18)64849-5

Friedman, M. (2013). RBs, RB Oils, and Rice Hulls: Composition, Food and Industrial Uses, and Bioactivities in Humans, Animals, and Cells. *Journal of Agricultural and Food Chemistry, 61*(45), 10626-10641. doi:10.1021/jf403635v

Gabbs, M., Leng, S., Devassy, J. G., Monirujjaman, M., & Aukema, H. M. (2015). Advances in Our Understanding of Oxylipins Derived from Dietary PUFAs. *Advances in Nutrition, 6*(5), 513-540. doi:10.3945/an.114.007732

Gakh, A. A., Anisimova, N. Y., Kiselevsky, M. V., Sadovnikov, S. V., Stankov, I. N., Yudin, M. V., . . . Sosnov, A. V. (2010). Dihydro-resveratrol—A potent dietary polyphenol. *Bioorganic &*

Medicinal Chemistry Letters, 20(20), 6149-6151. doi:https://doi.org/10.1016/j.bmcl.2010.08.002

Galano, J.-M., Lee, Y. Y., Oger, C., Vigor, C., Vercauteren, J., Durand, T., . . . Lee, J. C.-Y. (2017). Isoprostanes, neuroprostanes and phytoprostanes: An overview of 25years of research in chemistry and biology. *Progress in Lipid Research, 68*, 83-108. doi:https://doi.org/10.1016/j.plipres.2017.09.004

Galano, J.-M., Roy, J., Durand, T., Lee, J. C.-Y., Le Guennec, J.-Y., Oger, C., & Demion, M. (2018). Biological activities of non-enzymatic oxygenated metabolites of polyunsaturated fatty acids (NEO-PUFAs) derived from EPA and DHA: New anti-arrhythmic compounds? *Molecular Aspects of Medicine, 64*, 161-168. doi:https://doi.org/10.1016/j.mam.2018.03.003

Garcia-Conesa, M., Wilson, P., Plumb, G., Ralph, J., & Williamson, G. (1999). Antioxidant properties of 4,4′-dihydroxy-3,3′-dimethoxy-β,β′-bicinnamic acid (8-8-diferulic acid, non-cyclic form). *Journal of the Science of Food and Agriculture, 79*(3), 379-384. doi:doi:10.1002/(SICI)1097-0010(19990301)79:3<379::AID-JSFA259>3.0.CO;2-V

Garcia-Conesa, M. T., Plumb, G. W., Waldron, K. W., Ralph, J., & Williamson, G. (1997). Ferulic acid dehydrodimers from wheat bran: isolation, purification and antioxidant properties of 8-0-4-diferulic acid. *Redox Report, 3*(5-6), 319-323. doi:10.1080/13510002.1997.11747129

Gassi, J. Y., Blot, M., Beaucher, E., Robert, B., Leconte, N., Camier, B., . . . Gaucheron, F. (2016). Preparation and characterisation of a milk polar lipids enriched ingredient from fresh industrial liquid butter serum: Combination of physico-chemical modifications and technological treatments. *International Dairy Journal, 52*, 26-34. doi:https://doi.org/10.1016/j.idairyj.2015.08.012

Ghasemzadeh, A., Jaafar, H. Z. E., Juraimi, A. S., & Tayebi-Meigooni, A. (2015). Comparative Evaluation of Different Extraction Techniques and Solvents for the Assay of Phytochemicals and Antioxidant Activity of Hashemi Rice Bran. *Molecules, 20*(6), 10822-10838. Retrieved from https://www.mdpi.com/1420-3049/20/6/10822

Goodyear, A., Kumar, A., Ehrhart, E. J., Swanson, K. S., Grusak, M. A., Leach, J. E., . . . Ryan, E. P. (2015). Dietary Rice Bran supplementation prevents Salmonella colonization differentially across varieties and by priming intestinal immunity. *Journal of Functional Foods, 18*, 653-664. doi:https://doi.org/10.1016/j.jff.2015.08.027

Gopala Krishna, A. G., Raja Rajan, R. G., & Bhatnagar, A. S. (2012). Rice Bran : Chemistry, Production and Applications - A Revievv. *Beverage & Food World*, 31-36.

Górnaś, P., Dwiecki, K., Siger, A., Tomaszewska-Gras, J., Michalak, M., & Polewski, K. (2016). Contribution of phenolic acids isolated from green and roasted boiled-type coffee brews to total coffee antioxidant capacity. *European Food Research and Technology, 242*(5), 641-653. doi:10.1007/s00217-015-2572-1

Gorrochategui, E., Jaumot, J., Lacorte, S., & Tauler, R. (2016). Data analysis strategies for targeted and untargeted LC-MS metabolomic studies: Overview and workflow. *TrAC Trends in Analytical Chemistry, 82*, 425-442. doi:https://doi.org/10.1016/j.trac.2016.07.004

Goufo, P., & Trindade, H. (2014). Rice antioxidants: phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ-oryzanol, and phytic acid. *Food Sci Nutr, 2*(2), 75-104. doi:10.1002/fsn3.86

Goufo, P., & Trindade, H. (2017). Factors influencing antioxidant compounds in rice. *Critical Reviews in Food Science and Nutrition, 57*(5), 893-922. doi:10.1080/10408398.2014.922046

Granato, D., Shahidi, F., Wrolstad, R., Kilmartin, P., Melton, L. D., Hidalgo, F. J., . . . Finglas, P. (2018). Antioxidant activity, total phenolics and flavonoids contents: Should we ban in vitro screening methods? *Food Chem, 264*, 471-475. doi:https://doi.org/10.1016/j.foodchem.2018.04.012

Graziani, V., Scognamiglio, M., Belli, V., Esposito, A., D'Abrosca, B., Chambery, A., . . . Fiorentino, A. (2018). Metabolomic approach for a rapid identification of natural products with cytotoxic activity against human colorectal cancer cells. *Scientific Reports, 8*(1), 5309. doi:10.1038/s41598-018-23704-9

Guyot, B., Bosquette, B., Pina, M., & Graille, J. (1997). Esterification of phenolic acids from green coffee with an immobilized lipase from Candida antarctica in solvent-free medium. *Biotechnology Letters, 19*(6), 529-532. doi:10.1023/a:1018381102466

Hayyan, M., Hashim, M. A., Al-Saadi, M. A., Hayyan, A., AlNashef, I. M., & Mirghani, M. E. S. (2013). Assessment of cytotoxicity and toxicity for phosphonium-based deep eutectic solvents. *Chemosphere, 93*(2), 455-459. doi:https://doi.org/10.1016/j.chemosphere.2013.05.013

Hayyan, M., Hashim, M. A., Hayyan, A., Al-Saadi, M. A., AlNashef, I. M., Mirghani, M. E. S., & Saheed, O. K. (2013). Are deep eutectic solvents benign or toxic? *Chemosphere, 90*(7), 2193-2195. doi:https://doi.org/10.1016/j.chemosphere.2012.11.004

Heim, K. E., Tagliaferro, A. R., & Bobilya, D. J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *J Nutr Biochem, 13*(10), 572-584. Retrieved from http://dx.doi.org/

Hemery, Y., Chaurand, M., Holopainen, U., Lampi, A.-M., Lehtinen, P., Piironen, V., . . . Rouau, X. (2011). Potential of dry fractionation of wheat bran for the development of food ingredients, part I: Influence of ultra-fine grinding. *Journal of Cereal Science, 53*(1), 1-8. doi:https://doi.org/10.1016/j.jcs.2010.09.005

Hemery, Y., Lullien-Pellerin, V., Rouau, X., Abecassis, J., Samson, M.-F., Åman, P., . . . Barron, C. (2009). Biochemical markers: efficient tools for the assessment of wheat grain tissue proportions in milling fractions. *Journal of Cereal Science, 49*(1), 55-64.

Hemery, Y., Rouau, X., Lullien-Pellerin, V., Barron, C., & Abecassis, J. (2007). Dry processes to develop wheat fractions and products with enhanced nutritional quality. *Journal of Cereal Science, 46*(3), 327-347. Retrieved from http://www.sciencedirect.com/science/article/B6WHK-4PRYFYP-1/2/10e6861717412a96b781d05c49d6145a

Hemery, Y. M., Anson, N. M., Havenaar, R., Haenen, G. R. M. M., Noort, M. W. J., & Rouau, X. (2010). Dry-fractionation of wheat bran increases the bioaccessibility of phenolic acids in breads made from processed bran fractions. *Food Research International, 43*(5), 1429-1438. doi:https://doi.org/10.1016/j.foodres.2010.04.013

Henderson, A. J., Ollila, C. A., Kumar, A., Borresen, E. C., Raina, K., Agarwal, R., & Ryan, E. P. (2012). Chemopreventive Properties of Dietary Rice Bran: Current Status and Future Prospects. *Advances in Nutrition, 3*(5), 643-653. doi:10.3945/an.112.002303

Herrero, M., Simó, C., García-Cañas, V., Ibáñez, E., & Cifuentes, A. (2012). Foodomics: MS-based strategies in modern food science and nutrition. *Mass Spectrometry Reviews, 31*(1), 49-69. doi:doi:10.1002/mas.20335

Hillard, C. J. (2018). Circulating Endocannabinoids: From Whence Do They Come and Where are They Going? *Neuropsychopharmacology, 43*(1), 155-172. doi:10.1038/npp.2017.130

Holčapek, M., Liebisch, G., & Ekroos, K. (2018). Lipidomic Analysis. *Analytical Chemistry, 90*(7), 4249-4257. doi:10.1021/acs.analchem.7b05395

Huang, D., Ou, B., & Prior, R. L. (2005). The Chemistry behind Antioxidant Capacity Assays. *Journal of Agricultural and Food Chemistry, 53*(6), 1841-1856. doi:10.1021/jf030723c

Huang, Y., Feng, F., Jiang, J., Qiao, Y., Wu, T., Voglmeir, J., & Chen, Z.-G. (2017a). Green and efficient extraction of rutin from tartary buckwheat hull by using natural deep eutectic solvents. *Food Chemistry, 221*, 1400-1405. doi:https://doi.org/10.1016/j.foodchem.2016.11.013

Huang, Y., Feng, F., Jiang, J., Qiao, Y., Wu, T., Voglmeir, J., & Chen, Z. G. (2017b). Green and efficient extraction of rutin from tartary buckwheat hull by using natural deep eutectic solvents. *Food Chem, 221*, 1400-1405. doi:10.1016/j.foodchem.2016.11.013

Huber, V., Häckl, K., Touraud, D., & Kunz, W. (2021). Chapter Two - Natural deep eutectic solvents: From simple systems to complex colloidal mixtures. In R. Verpoorte, G.-J. Witkamp, & Y. H. Choi (Eds.), *Advances in Botanical Research* (Vol. 97, pp. 17-40): Academic Press.

Irakli, M., Kleisiaris, F., Mygdalia, A., & Katsantonis, D. (2018). Stabilization of rice bran and its effect on bioactive compounds content, antioxidant activity and storage stability during infrared radiation heating. *Journal of Cereal Science, 80*, 135-142. doi:https://doi.org/10.1016/j.jcs.2018.02.005

Irakli, M. N., Samanidou, V. F., Biliaderis, C. G., & Papadoyannis, I. N. (2012a). Development and validation of an HPLC-method for determination of free and bound phenolic acids in cereals after solid-phase extraction. *Food Chem, 134*(3), 1624-1632. doi:https://doi.org/10.1016/j.foodchem.2012.03.046

Irakli, M. N., Samanidou, V. F., Biliaderis, C. G., & Papadoyannis, I. N. (2012b). Simultaneous determination of phenolic acids and flavonoids in rice using solid-phase extraction and RP-HPLC with photodiode array detection. *Journal of Separation Science, 35*(13), 1603-1611. doi:10.1002/jssc.201200140

Jablonský, M., Škulcová, A., Malvis, A., & Šima, J. (2018). Extraction of value-added components from food industry based and agro-forest biowastes by deep eutectic solvents. *Journal of Biotechnology, 282*, 46-66. doi:https://doi.org/10.1016/j.jbiotec.2018.06.349

Janvier, M., Hollande, L., Jaufurally, A. S., Pernes, M., Ménard, R., Grimaldi, M., . . . Allais, F. (2017). Syringaresinol: A Renewable and Safer Alternative to Bisphenol A for Epoxy-Amine Resins. *ChemSusChem, 10*(4), 738-746. doi:10.1002/cssc.201601595

Johnson, J. B., Mani, J. S., Broszczak, D., Prasad, S. S., Ekanayake, C. P., Strappe, P., . . . Naiker, M. (2021). Hitting the sweet spot: A systematic review of the bioactivity and health benefits of phenolic glycosides from medicinally used plants. *Phytotherapy Research, 35*(7), 3484-3508. doi:https://doi.org/10.1002/ptr.7042

Junmarkho, K., & Hansakul, P. (2019). Thai pigmented Rice Bran extracts inhibit production of superoxide, nitric oxide radicals and inducible nitric oxide synthase in cellular models. *Asian Pacific Journal of Tropical Biomedicine, 9*(7), 291-298. doi:10.4103/2221-1691.261809

Kahveci, D., Laguerre, M., & Villeneuve, P. (2015). 7 - Phenolipids as New Antioxidants: Production, Activity, and Potential Applications. In M. U. Ahmad & X. Xu (Eds.), *Polar Lipids* (pp. 185-214): Elsevier.

Karboune, S., Yebaoh, F. K., & Kermasha, S. (2005). Enzymatic esterification of dihydrocaffeic acid with linoleyl alcohol in organic solvent media AU - Sabally, Kebba. *Biocatalysis and Biotransformation, 23*(1), 37-44. doi:10.1080/10242420500043762

Katyal, M., Virdi, A. S., Kaur, A., Singh, N., Kaur, S., Ahlawat, A. K., & Singh, A. M. (2016). Diversity in quality traits amongst Indian wheat varieties I: flour and protein characteristics. *Food Chem, 194*, 337-344. doi:10.1016/j.foodchem.2015.07.125

10.1016/j.foodchem.2015.07.125. Epub 2015 Aug 6.

Kersting, H.-J., Lindhauer, M. G., & Bergthaller, W. (1994). Application of wheat gluten in non-food industry — wheat gluten as a natural cobinder in paper coating. *Industrial Crops and Products, 3*(1), 121-128. doi:https://doi.org/10.1016/0926-6690(94)90085-X

Khakimov, B., Bak, S., & Engelsen, S. B. (2014). High-throughput cereal metabolomics: Current analytical technologies, challenges and perspectives. *Journal of Cereal Science, 59*(3), 393-418. doi:https://doi.org/10.1016/j.jcs.2013.10.002

Khammanit, R., Lomarat, P., Anantachoke, N., Sato, V. H., Ungsurungsie, M., & Mangmool, S. (2017). Inhibition of Oxidative Stress through the Induction of Antioxidant Enzymes of Pigmented RB in HEK-293 Cells. *Natural Product Communications, 12*(7), 1934578X1701200727. doi:10.1177/1934578x1701200727

Khezeli, T., Daneshfar, A., & Sahraei, R. (2016). A green ultrasonic-assisted liquid–liquid microextraction based on deep eutectic solvent for the HPLC-UV determination of ferulic, caffeic and cinnamic acid from olive, almond, sesame and cinnamon oil. *Talanta, 150*, 577-585. doi:https://doi.org/10.1016/j.talanta.2015.12.077

Khir, R., & Pan, Z. (2019). Chapter 2 - Rice. In Z. Pan, R. Zhang, & S. Zicari (Eds.), *Integrated Processing Technologies for Food and Agricultural By-Products* (pp. 21-58): Academic Press.

Kim, M. J., & Kim, S. S. (2016). Antioxidant and antiproliferative activities in immature and mature wheat kernels. *Food Chem, 196*, 638-645. doi:https://doi.org/10.1016/j.foodchem.2015.09.095

Kim, M. J., & Kim, S. S. (2017). Utilisation of immature wheat flour as an alternative flour with antioxidant activity and consumer perception on its baked product. *Food Chem, 232*, 237-244. doi:https://doi.org/10.1016/j.foodchem.2017.04.007

Koopman, J., & Grimme, S. (2021). From QCEIMS to QCxMS: A Tool to Routinely Calculate CID Mass Spectra Using Molecular Dynamics. *J Am Soc Mass Spectrom, 32*(7), 1735-1751. doi:10.1021/jasms.1c00098

Kullenberg, D., Taylor, L. A., Schneider, M., & Massing, U. (2012). Health effects of dietary phospholipids. *Lipids in Health and Disease, 11*(1), 1-16. Retrieved from http://dx.doi.org/10.1186/1476-511X-11-3

Kumar, A., Henderson, A., Forster, G. M., Goodyear, A. W., Weir, T. L., Leach, J. E., . . . Ryan, E. P. (2012). Dietary Rice Bran promotes resistance to Salmonella enterica serovar Typhimurium colonization in mice. *BMC Microbiology, 12*(1), 71. doi:10.1186/1471-2180-12-71

Kusano, M., Yang, Z., Okazaki, Y., Nakabayashi, R., Fukushima, A., & Saito, K. (2015). Using Metabolomic Approaches to Explore Chemical Diversity in Rice. *Molecular Plant, 8*(1), 58-67. doi:https://doi.org/10.1016/j.molp.2014.11.010

Lacalle-Bergeron, L., Izquierdo-Sandoval, D., Sancho, J. V., López, F. J., Hernández, F., & Portolés, T. (2021). Chromatography hyphenated to high resolution mass spectrometry in untargeted metabolomics for investigation of food (bio)markers. *TrAC Trends in Analytical Chemistry, 135*, 116161. doi:https://doi.org/10.1016/j.trac.2020.116161

Laddomada, B., Durante, M., Mangini, G., D'Amico, L., Lenucci, M. S., Simeone, R., . . . Blanco, A. (2017). Genetic variation for phenolic acids concentration and composition in a tetraploid wheat (Triticum turgidum L.) collection. *Genetic Resources and Crop Evolution, 64*(3), 587-597. doi:10.1007/s10722-016-0386-z

Laguerre, M., López-Giraldo, L. J., Lecomte, J., Baréa, B., Cambon, E., Tchobo, P. F., . . . Villeneuve, P. (2008). Conjugated autoxidizable triene (CAT) assay: A novel spectrophotometric method for determination of antioxidant capacity using triacylglycerol as ultraviolet probe. *Analytical Biochemistry, 380*(2), 282-290. doi:https://doi.org/10.1016/j.ab.2008.06.006

Laguerre, M., López Giraldo, L. J., Lecomte, J., Figueroa-Espinoza, M.-C., Baréa, B., Weiss, J., . . . Villeneuve, P. (2009). Chain Length Affects Antioxidant Properties of Chlorogenate Esters in Emulsion: The Cutoff Theory Behind the Polar Paradox. *Journal of Agricultural and Food Chemistry, 57*(23), 11335-11342. doi:10.1021/jf9026266

Laguna, O., Durand, E., Baréa, B., Dauguet, S., Fine, F., Villeneuve, P., & Lecomte, J. (2020). Synthesis and Evaluation of Antioxidant Activities of Novel Hydroxyalkyl Esters and Bis-Aryl Esters Based on Sinapic and Caffeic Acids. *Journal of Agricultural and Food Chemistry, 68*(35), 9308-9318. doi:10.1021/acs.jafc.0c03711

Large, E. C. (1954). Growth stages in cereals-illustration of the Feekes scale. *Plant Pathology, 3*, 128-129.

Leung, K. S., Oger, C., Guy, A., Bultel-Poncé, V., Vigor, C., Durand, T., . . . Lee, J. C.-Y. (2021). Alpha-linolenic acid, phytoprostanes and phytofurans in plant, algae and food.

Li, C.-z., Jin, H.-h., Sun, H.-x., Zhang, Z.-z., Zheng, J.-x., Li, S.-h., & Han, S.-h. (2016). Eriodictyol attenuates cisplatin-induced kidney injury by inhibiting oxidative stress and inflammation. *European Journal of Pharmacology, 772*, 124-130. doi:https://doi.org/10.1016/j.ejphar.2015.12.042

Li, K. J., Borresen, E. C., Jenkins-Puccetti, N., Luckasen, G., & Ryan, E. P. (2018a). Navy Bean and RB Intake Alters the Plasma Metabolome of Children at Risk for Cardiovascular Disease. *Frontiers in nutrition, 4*, 71-71. doi:10.3389/fnut.2017.00071

Li, K. J., Borresen, E. C., Jenkins-Puccetti, N., Luckasen, G., & Ryan, E. P. (2018b). Navy Bean and RB Intake Alters the Plasma Metabolome of Children at Risk for Cardiovascular Disease. *Frontiers in Nutrition, 4*(71). doi:10.3389/fnut.2017.00071

Li, L., Shewry, P. R., & Ward, J. L. (2008). Phenolic Acids in Wheat Varieties in the HEALTHGRAIN Diversity Screen. *Journal of Agricultural and Food Chemistry, 56*(21), 9732-9739. doi:10.1021/jf801069s

Liu, L., Waters, D. L. E., Rose, T. J., Bao, J., & King, G. J. (2013). Phospholipids in rice: Significance in grain quality and health benefits: A review. *Food Chem, 139*(1), 1133-1145. doi:https://doi.org/10.1016/j.foodchem.2012.12.046

Liu, Y. Q., Strappe, P., Zhou, Z. K., & Blanchard, C. (2019). Impact on the nutritional attributes of RB following various stabilization procedures. *Critical Reviews in Food Science and Nutrition, 59*(15), 2458-2466. doi:10.1080/10408398.2018.1455638

Liyana-Pathirana, C., Dexter, J., & Shahidi, F. (2006). Antioxidant properties of wheat as affected by pearling. *Journal of Agricultural and Food Chemistry, 54*(17), 6177-6184. doi:10.1021/jf060664d

Liyana-Pathirana, C. M., & Shahidi, F. (2006). Importance of insoluble-bound phenolics to antioxidant properties of wheat. *J Agric Food Chem, 54*(4), 1256-1264. doi:10.1021/jf052556h

Lopez Giraldo, L. J. (2009). Lipophilisation enzymatique de composés phénoliques et évaluation de leurs propriétés antioxydantes. *OCL, 16*(4-5-6), 273-275. Retrieved from https://doi.org/10.1051/ocl.2009.0274

López Giraldo, L. J., Laguerre, M., Lecomte, J., Figueroa-Espinoza, M.-C., Barouh, N., Baréa, B., & Villeneuve, P. (2007). Lipase-catalyzed synthesis of chlorogenate fatty esters in solvent-free medium. *Enzyme and Microbial Technology, 41*(6), 721-726. doi:https://doi.org/10.1016/j.enzmictec.2007.06.004

Lopez Giraldo, L. J., Laguerre, M., Lecomte, J., Figueroa-Espinoza, M.-C., Pina, M., & Villeneuve, P. (2007). Lipophilisation de composés phénoliques par voie enzymatique et propriétés antioxydantes des molécules lipophilisées. [archive]. *OCL. Oléagineux Corps gras Lipides, 14*(1), 51-59. doi:10.1684/ocl.2007.0100

Loypimai, P., Moongngarm, A., & Chottanom, P. (2017). Extraction solvents affecting phytochemicals in food colorant prepared from purple glutinous RB. *Applied Biological Chemistry, 60*(2), 181-189. doi:10.1007/s13765-017-0266-z

Lu, Y., Luthria, D., Fuerst, E. P., Kiszonas, A. M., Yu, L., & Morris, C. F. (2014). Effect of Processing on Phenolic Composition of Dough and Bread Fractions Made from Refined and Whole Wheat Flour of Three Wheat Varieties. *Journal of Agricultural and Food Chemistry, 62*(43), 10431-10436. doi:10.1021/jf501941r

Lu, Y., Memon, A., Fuerst, P., Kizonas, A., Morris, C., & Luthria, D. (2017). Changes in the phenolic acids composition during pancake preparation: Whole and refined grain flour and processed food classification by UV and NIR spectral fingerprinting method—Proof of concept. *Journal of Food Composition and Analysis, 60*, 10-16. doi:https://doi.org/10.1016/j.jfca.2017.03.001

Luo, C., Zou, L., Sun, H., Peng, J., Gao, C., Bao, L., . . . Sun, S. (2020). A Review of the Anti-Inflammatory Effects of Rosmarinic Acid on Inflammatory Diseases. *Frontiers in Pharmacology, 11*(153). doi:10.3389/fphar.2020.00153

Luthria, D. L., Lu, Y., & John, K. M. M. (2015). Bioactive phytochemicals in wheat: Extraction, analysis, processing, and functional properties. *Journal of Functional Foods, 18, Part B*, 910-925. doi:https://doi.org/10.1016/j.jff.2015.01.001

Lv, P., Yu, J., Xu, X., Lu, T., & Xu, F. (2019). Eriodictyol inhibits high glucose-induced oxidative stress and inflammation in retinal ganglial cells. *Journal of Cellular Biochemistry, 120*(4), 5644-5651. doi:10.1002/jcb.27848

MAPA. (2005). Instrução Normativa Nº 8, de 2 de junho de 2005, do Ministério da Agricultura - Regulamento Técnico de Identidade e Qualidade da farinha de trigo. *Ministério da Agricultura, Pecuária e Abastecimento.*

Martillanes, S., Rocha-Pimienta, J., Gil, M. V., Ayuso-Yuste, M. C., & Delgado-Adámez, J. (2020). Antioxidant and antimicrobial evaluation of RB (Oryza sativa L.) extracts in a mayonnaise-type emulsion. *Food Chem, 308*, 125633. doi:https://doi.org/10.1016/j.foodchem.2019.125633

Mateo Anson, N., van den Berg, R., Havenaar, R., Bast, A., & Haenen, G. R. M. M. (2009). Bioavailability of ferulic acid is determined by its bioaccessibility. *Journal of Cereal Science, 49*(2), 296-300. doi:https://doi.org/10.1016/j.jcs.2008.12.001

Mbanjo, E. G. N., Kretzschmar, T., Jones, H., Ereful, N., Blanchard, C., Boyd, L. A., & Sreenivasulu, N. (2020). The Genetic Basis and Nutritional Benefits of Pigmented Rice Grain. *Frontiers in Genetics, 11*(229). doi:10.3389/fgene.2020.00229

Mbous, Y. P., Hayyan, M., Wong, W. F., Looi, C. Y., & Hashim, M. A. (2017). Unraveling the cytotoxicity and metabolic pathways of binary natural deep eutectic solvent systems. *Scientific Reports, 7*, 41257. doi:10.1038/srep41257

https://www.nature.com/articles/srep41257#supplementary-information

McCallum, J. A., & Walker, J. R. L. (1990). Phenolic biosynthesis during grain development in wheat: changes in phenylalanine Ammonia-lyase activity and soluble phenolic content. *Journal of Cereal Science, 11*(1), 35-49. doi:http://dx.doi.org/10.1016/S0733-5210(09)80179-3

McCallum, J. A., & Walker, J. R. L. (1991). Phenolic biosynthesis during grain development in wheat (Triticum aestivum L.) III. Changes in hydroxycinnamic acids during grain development. *Journal of Cereal Science, 13*(2), 161-172. doi:http://dx.doi.org/10.1016/S0733-5210(09)80033-7

Mehariya, S., Fratini, F., Lavecchia, R., & Zuorro, A. (2021). Green extraction of value-added compounds form microalgae: A short review on natural deep eutectic solvents (NaDES) and related pre-treatments. *Journal of Environmental Chemical Engineering, 9*(5), 105989. doi:https://doi.org/10.1016/j.jece.2021.105989

Melini, V., Panfili, G., Fratianni, A., & Acquistucci, R. (2019). Bioactive compounds in rice on Italian market: pigmented varieties as a source of carotenoids, total phenolic compounds and anthocyanins, before and after cooking. *Food Chem, 277*, 119-127. doi:https://doi.org/10.1016/j.foodchem.2018.10.053

Min, B., McClung, A. M., & Chen, M.-H. (2011). Phytochemicals and Antioxidant Capacities in RBs of Different Color. *Journal of Food Science, 76*(1), C117-C126. doi:doi:10.1111/j.1750-3841.2010.01929.x

Miraji, K. F., Linnemann, A. R., Fogliano, V., Laswai, H. S., & Capuano, E. (2020). Nutritional quality and in vitro digestion of immature rice-based processed products. *Food & function, 11*(9), 7611-7625. doi:10.1039/D0FO01668C

Mišan, A., Nađpal, J., Stupar, A., Pojić, M., Mandić, A., Verpoorte, R., & Choi, Y. H. (2020). The perspectives of natural deep eutectic solvents in agri-food sector. *Critical Reviews in Food Science and Nutrition, 60*(15), 2564-2592. doi:10.1080/10408398.2019.1650717

Moongngarm, A., Daomukda, N., & Khumpika, S. (2012). Chemical Compositions, Phytochemicals, and Antioxidant Capacity of RB, Rice Bran Layer, and Rice Germ. *APCBEE Procedia, 2*, 73-79. doi:https://doi.org/10.1016/j.apcbee.2012.06.014

Moore, J., Hao, Z. G., Zhou, K. Q., Luther, M., Costa, J., & Yu, L. L. (2005). Carotenoid, tocopherol, phenolic acid, and antioxidant properties of Maryland-grown soft wheat. *Journal of Agricultural and Food Chemistry, 53*(17), 6649-6657. doi:10.1021/jf050481b

Morrison, H. G., Sun, C. C., & Neervannan, S. (2009). Characterization of thermal behavior of deep eutectic solvents and their potential as drug solubilization vehicles. *International Journal of Pharmaceutics, 378*(1), 136-139. doi:https://doi.org/10.1016/j.ijpharm.2009.05.039

Nadeem, m. k., Anjum, F., Amir, R. A. I., Khan, M. R., Hussain, S., & Javed, M. S. (2010). *An overview of anti-nutritional factors in cereal grains with special reference to wheat-A review* (Vol. 20).

Nardini, M., Cirillo, E., Natella, F., Mencarelli, D., Comisso, A., & Scaccini, C. (2002). Detection of bound phenolic acids: prevention by ascorbic acid and ethylenediaminetetraacetic acid of degradation of phenolic acids during alkaline hydrolysis. *Food Chem, 79*(1), 119-124. doi:https://doi.org/10.1016/S0308-8146(02)00213-3

Nascimento, T. P., Santos, M. C. B., Lima, L. R. d. S., Nascimento, F. R., Cameron, L. C., & Ferreira, M. S. L. (2018). Dataset on phenolic profile of seven wheat genotypes along maturation. *Data in Brief, 21*, 284-288. doi:https://doi.org/10.1016/j.dib.2018.09.111

Nascimento, T. P., Santos, M. C. B., Lima, L. R. S., Nascimento, F. R., Cameron, L. C., & Ferreira, M. S. L. (submitted). Dataset on phenolic profile of seven wheat genotypes along maturation. *Data in brief*.

Nascimento, T. P. d., Santos, M. C. B., Luciana Ribeiro da Silva, L., Nascimento, F. R., Cameron, L. C., & Ferreira, M. S. L. (2018). Dataset on phenolic profile of seven wheat genotypes along maturation. *Data in Brief*. doi:https://doi.org/10.1016/j.dib.2018.09.111

Ncube, E., Mhlongo, M., Piater, L., Steenkamp, P., Dubery, I., & Madala, N. (2014). Analyses of chlorogenic acids and related cinnamic acid derivatives from Nicotiana tabacum tissues with the aid of UPLC-QTOF-MS/MS based on the in-source collision-induced dissociation method. *Chemistry Central journal, 8*, 66. doi:10.1186/s13065-014-0066-z

Nealon, N. J., Worcester, C. R., & Ryan, E. P. (2017). Lactobacillus paracasei metabolism of RB reveals metabolome associated with Salmonella Typhimurium growth reduction. *Journal of Applied Microbiology, 122*(6), 1639-1656. doi:10.1111/jam.13459

Nicoletti, I., Martini, D., De Rossi, A., Taddei, F., D'Egidio, M. G., & Corradini, D. (2013). Identification and Quantification of Soluble Free, Soluble Conjugated, and Insoluble Bound Phenolic Acids in Durum Wheat (Triticum turgidum L. var. durum) and Derived Products by

RP-HPLC on a Semimicro Separation Scale. *Journal of Agricultural and Food Chemistry, 61*(48), 11800-11807. doi:10.1021/jf403568c

Nignpense, B. E., Francis, N., Blanchard, C., & Santhakumar, A. B. (2021). Bioaccessibility and Bioactivity of Cereal Polyphenols: A Review. *Foods (Basel, Switzerland), 10*(7), 1595. doi:10.3390/foods10071595

Oliveira, J. P. S., Hakimi, O., Murgu, M., Koblitz, M. G. B., Ferreira, M. S. L., Cameron, L. C., & Macedo, A. F. (2018). Tissue culture and metabolome investigation of a wild endangered medicinal plant using high definition mass spectrometry. *Plant Cell, Tissue and Organ Culture (PCTOC)*. doi:10.1007/s11240-018-1408-7

Oliveira, M. d. S., Feddern, V., Kupski, L., Cipolatti, E. P., Badiale-Furlong, E., & de Souza-Soares, L. A. (2011). Changes in lipid, fatty acids and phospholipids composition of whole Rice Bran after solid-state fungal fermentation. *Bioresource Technology, 102*(17), 8335-8338. doi:https://doi.org/10.1016/j.biortech.2011.06.025

Onipe, O. (2015). *Composition and functionality of wheat bran and its application in some cereal food products* (Vol. 50).

Ozdal, T., Capanoglu, E., & Altay, F. (2013). A review on protein–phenolic interactions and associated changes. *Food Research International, 51*(2), 954-970. doi:https://doi.org/10.1016/j.foodres.2013.02.009

Özkaya, B., Turksoy, S., Özkaya, H., Baumgartner, B., Özkeser, İ., & Köksel, H. (2018). Changes in the functional constituents and phytic acid contents of firiks produced from wheats at different maturation stages. *Food Chem, 246*, 150-155. doi:https://doi.org/10.1016/j.foodchem.2017.11.022

Paglia, G., Smith, A. J., & Astarita, G. (2021). Ion mobility mass spectrometry in the omics era: Challenges and opportunities for metabolomics and lipidomics. *Mass Spectrometry Reviews, n/a*(n/a). doi:https://doi.org/10.1002/mas.21686

Pan, Z., Zhang, R., & Zicari, S. (2019). *Integrated Processing Technologies for Food and Agricultural By-Products*: Academic Press.

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative medicine and cellular longevity, 2*(5), 270-278. doi:10.4161/oxim.2.5.9498

Pang, Y., Ahmed, S., Xu, Y., Beta, T., Zhu, Z., Shao, Y., & Bao, J. (2018). Bound phenolic compounds and antioxidant properties of whole grain and bran of white, red and black rice. *Food Chem, 240*, 212-221. doi:https://doi.org/10.1016/j.foodchem.2017.07.095

Panić, M., Radić Stojković, M., Kraljić, K., Škevin, D., Radojčić Redovniković, I., Gaurina Srček, V., & Radošević, K. (2019). Ready-to-use green polyphenolic extracts from food by-products. *Food Chem, 283*, 628-636. doi:https://doi.org/10.1016/j.foodchem.2019.01.061

Park, B.-Y., Oh, S.-R., Ahn, K.-S., Kwon, O.-K., & Lee, H.-K. (2008). (–)-Syringaresinol inhibits proliferation of human promyelocytic HL-60 leukemia cells via G1 arrest and apoptosis. *International Immunopharmacology, 8*(7), 967-973. doi:https://doi.org/10.1016/j.intimp.2008.02.012

Park, H.-Y., Lee, K.-W., & Choi, H.-D. (2017). Rice bran constituents: immunomodulatory and therapeutic activities. *Food & function, 8*(3), 935-943. doi:10.1039/C6FO01763K

Peanparkdee, M., Patrawart, J., & Iwamoto, S. (2019). Effect of extraction conditions on phenolic content, anthocyanin content and antioxidant activity of bran extracts from Thai rice cultivars. *Journal of Cereal Science, 86*, 86-91. doi:https://doi.org/10.1016/j.jcs.2019.01.011

Pekkinen, J., Rosa, N. N., Savolainen, O.-I., Keski-Rahkonen, P., Mykkänen, H., Poutanen, K., . . . Hanhineva, K. (2014). Disintegration of wheat aleurone structure has an impact on the bioavailability of phenolic compounds and other phytochemicals as evidenced by altered urinary metabolite profile of diet-induced obese mice. *Nutrition & Metabolism, 11*(1), 1. doi:10.1186/1743-7075-11-1

Pereira-Caro, G., Cros, G., Yokota, T., & Crozier, A. (2013). Phytochemical Profiles of Black, Red, Brown, and White Rice from the Camargue Region of France. *Journal of Agricultural and Food Chemistry, 61*(33), 7976-7986. doi:10.1021/jf401937b

Petroni, K., Landoni, M., Tomay, F., Calvenzani, V., Simonelli, C., & Cormegna, M. (2017). Proximate Composition, Polyphenol Content and Anti-inflammatory Properties of White and Pigmented Italian Rice Varieties. *Universal Journal of Agricultural Research, 5*, 312-321. doi:10.13189/ujar.2017.050509

Phelix, C. F., & Feltus, F. A. (2015). Plant stress biomarkers from biosimulations: the Transcriptome-To- Metabolome (TM) (TTM (TM)) technology - effects of drought stress on rice. *Plant Biology, 17*(1), 63-73. doi:10.1111/plb.12221

Phonsatta, N., Deetae, P., Luangpituksa, P., Grajeda-Iglesias, C., Figueroa-Espinoza, M. C., Le Comte, J., . . . Panya, A. (2017). Comparison of Antioxidant Evaluation Assays for Investigating Antioxidative Activity of Gallic Acid and Its Alkyl Esters in Different Food Matrices. *Journal of Agricultural and Food Chemistry, 65*(34), 7509-7518. doi:10.1021/acs.jafc.7b02503

Pinciroli, M., Domínguez-Perles, R., Abellan, A., Bultel-Poncé, V., Durand, T., Galano, J., . . . Gil-Izquierdo, Á. (2018). Statement of foliar fertilization impact on yield, composition, and oxidative biomarkers in rice. *Journal of Agricultural and Food Chemistry, 67*(2), 597-605.

Pinciroli, M., Domínguez-Perles, R., Abellán, A., Guy, A., Durand, T., Oger, C., . . . Gil-Izquierdo, A. (2017). Comparative Study of the Phytoprostane and Phytofuran Content of indica and japonica Rice (Oryza sativa L.) Flours. *Journal of Agricultural and Food Chemistry, 65*(40), 8938-8947. doi:10.1021/acs.jafc.7b03482

Pinciroli, M., Domínguez-Perles, R., Garbi, M., Abellán, A., Oger, C., Durand, T., . . . Gil-Izquierdo, A. (2018). Impact of Salicylic Acid Content and Growing Environment on Phytoprostane and Phytofuran (Stress Biomarkers) in Oryza sativa L. *Journal of Agricultural and Food Chemistry, 66*(47), 12561-12570.

Putman, A. K., Brown, J. L., Gandy, J. C., Abuelo, A., & Sordillo, L. M. (2019). Oxylipid profiles of dairy cattle vary throughout the transition into early mammary gland involution. *Journal of Dairy Science, 102*(3), 2481-2491. doi:https://doi.org/10.3168/jds.2018-15158

Qiu, X., Jacobsen, C., Villeneuve, P., Durand, E., & Sørensen, A.-D. M. (2017). Effects of Different Lipophilized Ferulate Esters in Fish Oil-Enriched Milk: Partitioning, Interaction, Protein, and

Lipid Oxidation. *Journal of Agricultural and Food Chemistry, 65*(43), 9496-9505. doi:10.1021/acs.jafc.7b02994

Radošević, K., Ćurko, N., Gaurina Srček, V., Cvjetko Bubalo, M., Tomašević, M., Kovačević Ganić, K., & Radojčić Redovniković, I. (2016). Natural deep eutectic solvents as beneficial extractants for enhancement of plant extracts bioactivity. *LWT, 73*, 45-51. doi:https://doi.org/10.1016/j.lwt.2016.05.037

Radošević, K., Cvjetko Bubalo, M., Gaurina Srček, V., Grgas, D., Landeka Dragičević, T., & Radojčić Redovniković, I. (2015). Evaluation of toxicity and biodegradability of choline chloride based deep eutectic solvents. *Ecotoxicology and Environmental Safety, 112*, 46-53. doi:https://doi.org/10.1016/j.ecoenv.2014.09.034

Rajan, V. K., Muraleedharan, K., & Hussan, K. P. S. (2018). Chapter 5 - Structural Evaluation and Toxicological Study of a Bitter Masking Bioactive Flavanone, 'Eriodictyol'. In R. R. Watson, V. R. Preedy, & S. Zibadi (Eds.), *Polyphenols: Prevention and Treatment of Human Disease (Second Edition)* (pp. 45-60): Academic Press.

Rascio, A., Beleggia, R., Platani, C., Nigro, F., Codianni, P., De Santis, G., . . . Fragasso, M. (2016). Metabolomic diversity for biochemical traits of Triticum sub-species. *Journal of Cereal Science, 71*, 224-229. doi:https://doi.org/10.1016/j.jcs.2016.08.009

Robert, C., Couëdelo, L., Vaysse, C., & Michalski, M.-C. (2020). Vegetable lecithins: A review of their compositional diversity, impact on lipid metabolism and potential in cardiometabolic disease prevention. *Biochimie, 169*, 121-132. doi:https://doi.org/10.1016/j.biochi.2019.11.017

Rodrigues, F., Nunes, M. A., Alves, R. C., & Oliveira, M. B. P. P. (2017). Chapter 7 - Applications of recovered bioactive compounds in cosmetics and other products. In C. M. Galanakis (Ed.), *Handbook of Coffee Processing By-Products* (pp. 195-220): Academic Press.

Rohfritsch, Z., Canelli, G., Pollien, P., & Bel-Rhlid, R. (2021). Wheat and Rice Bran as Natural Additives for the Protection of Fish Oil from Oxidation. *ACS Food Science & Technology, 1*(7), 1160-1168. doi:10.1021/acsfoodscitech.1c00054

Rosa-Sibakov, N., Poutanen, K., & Micard, V. (2015). How does wheat grain, bran and aleurone structure impact their nutritional and technological properties? *Trends in Food Science & Technology, 41*(2), 118-134. doi:https://doi.org/10.1016/j.tifs.2014.10.003

Rosa, N. N., Dufour, C., Lullien-Pellerin, V., & Micard, V. (2013). Exposure or release of ferulic acid from wheat aleurone: Impact on its antioxidant capacity. *Food Chem, 141*(3), 2355-2362. doi:https://doi.org/10.1016/j.foodchem.2013.04.132

Ruesgas-Ramón, M., Figueroa-Espinoza, M. C., & Durand, E. (2017). Application of Deep Eutectic Solvents (DES) for Phenolic Compounds Extraction: Overview, Challenges, and Opportunities. *Journal of Agricultural and Food Chemistry, 65*(18), 3591-3601. doi:10.1021/acs.jafc.7b01054

Ruesgas-Ramón, M., Suárez-Quiroz, M. L., González-Ríos, O., Baréa, B., Cazals, G., Figueroa-Espinoza, M. C., & Durand, E. (2020). Biomolecules extraction from coffee and cocoa by- and co-products using deep eutectic solvents. *Journal of the Science of Food and Agriculture, 100*(1), 81-91. doi:https://doi.org/10.1002/jsfa.9996

Sabir, A., Rafi, M., & Darusman, L. K. (2017). Discrimination of red and white rice bran from Indonesia using HPLC fingerprint analysis combined with chemometrics. *Food Chem, 221*, 1717-1722. doi:https://doi.org/10.1016/j.foodchem.2016.10.114

Sahaka, M., Amara, S., Wattanakul, J., Gedi, M. A., Aldai, N., Parsiegla, G., . . . Carrière, F. (2020). The digestion of galactolipids and its ubiquitous function in Nature for the uptake of the essential α-linolenic acid. *Food & function, 11*(8), 6710-6744. doi:10.1039/D0FO01040E

Saltveit, M. E. (2017). Synthesis and metabolism of phenolic compounds. *Fruit Veget. Phytochem. Chem. Human Health, 2*, 115.

Santos, M. C. B., Barouh, N., Durand, E., Baréa, B., Robert, M., Micard, V., . . . Bourlieu-Lacanal, C. (2021). Metabolomics of Pigmented Rice Coproducts Applying Conventional or Deep Eutectic Extraction Solvents Reveal a Potential Antioxidant Source for Human Nutrition. *Metabolites, 11*(2), 110. Retrieved from https://www.mdpi.com/2218-1989/11/2/110

Santos, M. C. B., Lima, L. R. d. S., Nascimento, F. R., do Nascimento, T. P., Cameron, L. C., & Ferreira, M. S. L. (2019). Metabolomic approach for characterization of phenolic compounds in different wheat genotypes during grain development. *Food Research International, 124*, 118-128. doi:https://doi.org/10.1016/j.foodres.2018.08.034

Sarfaraz, A., Azizi, M. H., Ahmadi Gavlighi, H., & Barzegar, M. (2017). Physicochemical and functional characterization of wheat milling co-products: Fine grinding to achieve high fiber antioxidant-rich fractions. *Journal of Cereal Science, 77*, 228-234. doi:https://doi.org/10.1016/j.jcs.2017.08.021

Saulnier, L., Sado, P.-E., Branlard, G., Charmet, G., & Guillon, F. (2007). Wheat arabinoxylans: Exploiting variation in amount and composition to develop enhanced varieties. *Journal of Cereal Science, 46*(3), 261-281. doi:http://dx.doi.org/10.1016/j.jcs.2007.06.014

Schymanski, E. L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H. P., & Hollender, J. (2014). Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. *Environmental Science & Technology, 48*(4), 2097-2098. doi:10.1021/es5002105

Sergeant, S., Rahbar, E., & Chilton, F. H. (2016). Gamma-linolenic acid, Dihommo-gamma linolenic, Eicosanoids and Inflammatory Processes. *European Journal of Pharmacology, 785*, 77-86. doi:https://doi.org/10.1016/j.ejphar.2016.04.020

Serna-Saldivar, S. O. (2010a). Chapter 1: Cereal Grains: The Staff of Life *Cereal Grains: Properties, Processing, and Nutritional Attributes* (pp. 1-40).

Serna-Saldivar, S. O. (2010b). Chapter 4: Grain Development, Morphology, and Structure *Cereal Grains: Properties, Processing, and Nutritional Attributes* (pp. 109-126).

Serna-Saldivar, S. O. (2010b). Chapter 17: Role of Cereals in Human Nutrition and Health *Cereal Grains: Properties, Processing, and Nutritional Attibutes* (pp. 565-665).

Shaheen, S. M., Shakil Ahmed, F. R., Hossen, M. N., Ahmed, M., Amran, M. S., & Ul-Islam, M. A. (2006). Liposome as a carrier for advanced drug delivery. *Pak J Biol Sci, 9*. doi:10.3923/pjbs.2006.1181.1191

Shahidi, F., & Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects – A review. *Journal of Functional Foods, 18*, 820-897. doi:https://doi.org/10.1016/j.jff.2015.06.018

Shakri, A. N. A., Kasim, K. F., & Rukunudin, I. B. (2021). Chemical Compositions and Physical Properties of Selected Malaysian Rice: A Review. *IOP Conference Series: Earth and Environmental Science, 765*(1), 012024. doi:10.1088/1755-1315/765/1/012024

Shamloo, M., Babawale, E. A., Furtado, A., Henry, R. J., Eck, P. K., & Jones, P. J. H. (2017). Effects of genotype and temperature on accumulation of plant secondary metabolites in Canadian and Australian wheat grown under controlled environments. *Scientific Reports, 7*(1), 9133. doi:10.1038/s41598-017-09681-5

Shao, Y., Xu, F., Sun, X., Bao, J., & Beta, T. (2014a). Identification and quantification of phenolic acids and anthocyanins as antioxidants in bran, embryo and endosperm of white, red and black rice kernels (Oryza sativa L.). *Journal of Cereal Science, 59*(2), 211-218. doi:https://doi.org/10.1016/j.jcs.2014.01.004

Shao, Y., Xu, F., Sun, X., Bao, J., & Beta, T. (2014b). Phenolic acids, anthocyanins, and antioxidant capacity in rice (Oryza sativa L.) grains at four stages of development after flowering. *Food Chem, 143*, 90-96. doi:https://doi.org/10.1016/j.foodchem.2013.07.042

Sharma, M., Rahim, M. S., Kumar, P., Mishra, A., Sharma, H., & Roy, J. (2020). Large-scale identification and characterization of phenolic compounds and their marker–trait association in wheat. *Euphytica, 216*(8), 127. doi:10.1007/s10681-020-02659-x

Sharma, M., Sandhir, R., Singh, A., Kumar, P., Mishra, A., Jachak, S., . . . Roy, J. (2016). Comparative Analysis of Phenolic Compound Characterization and Their Biosynthesis Genes between Two Diverse Bread Wheat (Triticum aestivum) Varieties Differing for Chapatti (Unleavened Flat Bread) Quality. *Frontiers in plant science, 7*, 1870-1870. doi:10.3389/fpls.2016.01870

Shearer, G. C., & Walker, R. E. (2018). An overview of the biologic effects of omega-6 oxylipins in humans. *Prostaglandins Leukot Essent Fatty Acids, 137*, 26-38. doi:10.1016/j.plefa.2018.06.005

Sheflin, A. M., Borresen, E. C., Kirkwood, J. S., Boot, C. M., Whitney, A. K., Lu, S., . . . Weir, T. L. (2017). Dietary supplementation with rice bran or navy bean alters gut bacterial metabolism in colorectal cancer survivors. *Molecular Nutrition & Food Research, 61*(1), 1500905. doi:10.1002/mnfr.201500905

Sheflin, A. M., Borresen, E. C., Wdowik, M. J., Rao, S., Brown, R. J., Heuberger, A. L., . . . Ryan, E. P. (2015). Pilot Dietary Intervention with Heat-Stabilized Rice Bran Modulates Stool Microbiota and Metabolites in Healthy Adults. *Nutrients, 7*(2), 1282-1300. Retrieved from https://www.mdpi.com/2072-6643/7/2/1282

Shewry, P. R., & Hey, S. J. (2015). The contribution of wheat to human diet and health. *Food and energy security, 4*(3), 178-202. doi:10.1002/fes3.64

Silva, G. G. D., Couturier, M., Berrin, J.-G., Buléon, A., & Rouau, X. (2012). Effects of grinding processes on enzymatic degradation of wheat straw. *Bioresource Technology, 103*(1), 192-200. doi:https://doi.org/10.1016/j.biortech.2011.09.073

Silvestro, R. D., Loreto, A. D., Bosi, S., Bregola, V., Marotti, I., Benedettelli, S., . . . Dinelli, G. (2016). Environment and genotype effects on antioxidant properties of organically grown wheat varieties: a 3-year study. *Journal of the Science of Food and Agriculture, 97*(2), 641-649. doi:10.1002/jsfa.7782

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology, 299*, 152-178. doi:http://dx.doi.org/10.1016/S0076-6879(99)99017-1

Södergård, A., & Stolt, M. (2002). Properties of lactic acid based polymers and their correlation with composition. *Progress in Polymer Science, 27*(6), 1123-1163. doi:https://doi.org/10.1016/S0079-6700(02)00012-6

Sosulski, F., Krygier, K., & Hogge, L. (1982). Free, esterified, and insoluble-bound phenolic acids. 3. Composition of phenolic acids in cereal and potato flours. *Journal of Agricultural and Food Chemistry, 30*(2), 337-340. doi:10.1021/jf00110a030

Souza, G. H. M. F., Guest, P. C., & Martins-de-Souza, D. (2017). LC-MSE, Multiplex MS/MS, Ion Mobility, and Label-Free Quantitation in Clinical Proteomics. In P. C. Guest (Ed.), *Multiplex Biomarker Techniques: Methods and Applications* (pp. 57-73). New York, NY: Springer New York.

Steinfath, M., Strehmel, N., Peters, R., Schauer, N., Groth, D., Hummel, J., . . . van Dongen, J. T. (2010). Discovering plant metabolic biomarkers for phenotype prediction using an untargeted approach. *Plant Biotechnology Journal, 8*(8), 900-911. doi:10.1111/j.1467-7652.2010.00516.x

Subbanagounder, G., Watson, A. D., & Berliner, J. A. (2000). Bioactive products of phospholipid oxidation: isolation, identification, measurement and activities. *Free Radical Biology and Medicine, 28*(12), 1751-1761. doi:https://doi.org/10.1016/S0891-5849(00)00233-1

Sugano, M., & Tsuji, E. (1997). Rice Bran Oil and Cholesterol Metabolism. *The Journal of Nutrition, 127*(3), 521S-524S. doi:10.1093/jn/127.3.521S

Sumczynski, D., Kotásková, E., Družbíková, H., & Mlček, J. (2016). Determination of contents and antioxidant activity of free and bound phenolics compounds and in vitro digestibility of commercial black and red rice (Oryza sativa L.) varieties. *Food Chem, 211*, 339-346. doi:https://doi.org/10.1016/j.foodchem.2016.05.081

Sumner, L. W., Amberg, A., Barrett, D., Beale, M. H., Beger, R., Daykin, C. A., . . . Viant, M. R. (2007). Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics : Official journal of the Metabolomic Society, 3*(3), 211-221. doi:10.1007/s11306-007-0082-2

Sunthonkun, P., Palajai, R., Somboon, P., Suan, C. L., Ungsurangsri, M., & Soontorngun, N. (2019). Life-span extension by pigmented RB in the model yeast Saccharomyces cerevisiae. *Scientific Reports, 9*(1), 18061. doi:10.1038/s41598-019-54448-9

Surget, A., & Barron, C. (2005). Histologie du grain de blé. *Industrie des céréales*(145), 3-7.

Ti, H., Li, Q., Zhang, R., Zhang, M., Deng, Y., Wei, Z., . . . Zhang, Y. (2014). Free and bound phenolic profiles and antioxidant activity of milled fractions of different indica rice varieties cultivated in southern China. *Food Chem, 159*, 166-174. doi:https://doi.org/10.1016/j.foodchem.2014.03.029

Tong, C., & Bao, J. (2019). 5 - Rice lipids and Rice Bran oil. In J. Bao (Ed.), *Rice (Fourth Edition)* (pp. 131-168): AACC International Press.

USDA. (2020). Dietary Guidelines for Americans, 2020-2025. *Department of Health and Human Services, U.S. Department of Agriculture, 9*.

Vadivel, V., Moncalvo, A., Dordoni, R., & Spigno, G. (2017). Effects of an acid/alkaline treatment on the release of antioxidants and cellulose from different agro-food wastes. *Waste Management*. doi:https://doi.org/10.1016/j.wasman.2017.03.010

van den Berg, R. A., Hoefsloot, H. C. J., Westerhuis, J. A., Smilde, A. K., & van der Werf, M. J. (2006). Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genomics, 7*(1), 142. doi:10.1186/1471-2164-7-142

Vázquez, L., Corzo-Martínez, M., Arranz-Martínez, P., Barroso, E., Reglero, G., & Torres, C. (2019). Bioactive Lipids (pp. 467-527).

Verma, B., Hucl, P., & Chibbar, R. N. (2009). Phenolic acid composition and antioxidant capacity of acid and alkali hydrolysed wheat bran fractions. *Food Chem, 116*(4), 947-954. doi:http://dx.doi.org/10.1016/j.foodchem.2009.03.060

Vermerris, W., & Nicholson, R. (2006). Phenolic Compounds and their Effects on Human Health *Phenolic Compound Biochemistry* (pp. 235-255). Dordrecht: Springer Netherlands.

Victorio, V. C. M., Alves, T. O., Souza, G. H. M. F., Gutkoski, L. C., Cameron, L. C., & Ferreira, M. S. L. (2021). NanoUPLC-MSE reveals differential abundance of gluten proteins in wheat flours of different technological qualities. *Journal of Proteomics, 239*, 104181. doi:https://doi.org/10.1016/j.jprot.2021.104181

Vidal, O. L., Santos, M. C. B., Batista, A. P., Andrigo, F. F., Baréa, B., Lecomte, J., . . . Ferreira, M. S. L. (2022). Active packaging films containing antioxidant extracts from green coffee oil by-products to prevent lipid oxidation. *Journal of Food Engineering, 312*, 110744. doi:https://doi.org/10.1016/j.jfoodeng.2021.110744

Villeneuve, P. (2007). Lipases in lipophilization reactions. *Biotechnology Advances, 25*(6), 515-536. doi:https://doi.org/10.1016/j.biotechadv.2007.06.001

Wang, L., & Chen, C. (2013). Emerging applications of metabolomics in studying chemopreventive phytochemicals. *The AAPS journal, 15*(4), 941-950. doi:10.1208/s12248-013-9503-5

Wang, L., Yao, Y., He, Z., Wang, D., Liu, A., & Zhang, Y. (2013). Determination of phenolic acid concentrations in wheat flours produced at different extraction rates. *Journal of Cereal Science, 57*(1), 67-72. doi:https://doi.org/10.1016/j.jcs.2012.09.013

Wang, Y.-X., Li, Y., Sun, A.-M., Wang, F.-J., & Yu, G.-P. (2014). Hypolipidemic and Antioxidative Effects of Aqueous Enzymatic Extract from Rice Bran in Rats Fed a High-Fat and -Cholesterol Diet. *Nutrients, 6*(9), 3696-3710. Retrieved from https://www.mdpi.com/2072-6643/6/9/3696

Wang, Y., Berhow, M. A., Black, M., & Jeffery, E. H. (2020). A comparison of the absorption and metabolism of the major quercetin in brassica, quercetin-3-O-sophoroside, to that of quercetin aglycone, in rats. *Food Chem, 311*, 125880. doi:https://doi.org/10.1016/j.foodchem.2019.125880

Wen, Q., Chen, J.-X., Tang, Y.-L., Wang, J., & Yang, Z. (2015). Assessing the toxicity and biodegradability of deep eutectic solvents. *Chemosphere, 132*, 63-69. doi:https://doi.org/10.1016/j.chemosphere.2015.02.061

Yang, X., Twitchell, E., Li, G., Wen, K., Weiss, M., Kocher, J., . . . Yuan, L. (2015). High protective efficacy of RB against human rotavirus diarrhea via enhancing probiotic growth, gut barrier function and innate immunity. *Scientific Reports, 5*(1), 15004. doi:10.1038/srep15004

Yang, Z., Guo, Z., & Xu, X. (2012). Enzymatic lipophilisation of phenolic acids through esterification with fatty alcohols in organic solvents. *Food Chem, 132*(3), 1311-1315. doi:https://doi.org/10.1016/j.foodchem.2011.11.110

Yousaf, S., Iqbal, H., Arif, S., Khurshid, S., Ul, Q., & Akbar, A. (2019). Alveograph rheological parameters in relation to physicochemical attributes as an indicator of wheat flour quality. *International Journal of Biology and Biotechnology*, 425-431.

Yu, L., Haley, S., Perret, J., & Harris, M. (2004). Comparison of wheat flours grown at different locations for their antioxidant properties. *Food Chem, 86*(1), 11-16. doi:https://doi.org/10.1016/j.foodchem.2003.08.037

Zadoks, J. C., Chang, T. T., & Konzak, C. F. (1974). A decimal code for the growth stages of cereals. *Weed Research, 14*(6), 415-421. doi:10.1111/j.1365-3180.1974.tb01084.x

Zarei, I., Brown, D. G., Nealon, N. J., & Ryan, E. P. (2017). Rice Bran Metabolome Contains Amino Acids, Vitamins & Cofactors, and Phytochemicals with Medicinal and Nutritional Properties. *Rice (New York, N.Y.), 10*(1), 24-24. doi:10.1186/s12284-017-0157-2

Zarei, I., Luna, E., Leach, J. E., McClung, A., Vilchez, S., Koita, O., & Ryan, E. P. (2018). Comparative RB Metabolomics across Diverse Cultivars and Functional Rice Gene–Bran Metabolite Relationships. *Metabolites, 8*(4), 63. doi:10.3390/metabo8040063

Zarei, I., Oppel, R. C., Borresen, E. C., Brown, R. J., & Ryan, E. P. (2019). Modulation of plasma and urine metabolome in colorectal cancer survivors consuming RB. *Integrative food, nutrition and metabolism, 6*(3), 10.15761/IFNM.1000252. doi:10.15761/IFNM.1000252

Zaupa, M., Calani, L., Del Rio, D., Brighenti, F., & Pellegrini, N. (2015). Characterization of total antioxidant capacity and (poly)phenolic compounds of differently pigmented rice varieties and their changes during domestic cooking. *Food Chem, 187*, 338-347. doi:https://doi.org/10.1016/j.foodchem.2015.04.055

Zha, J., & Koffas, M. A. G. (2017). Production of anthocyanins in metabolically engineered microorganisms: Current status and perspectives. *Synthetic and Systems Biotechnology, 2*(4), 259-266. doi:https://doi.org/10.1016/j.synbio.2017.10.005

Zhang, H., Shao, Y., Bao, J., & Beta, T. (2015). Phenolic compounds and antioxidant properties of breeding lines between the white and black rice. *Food Chem, 172*, 630-639. doi:https://doi.org/10.1016/j.foodchem.2014.09.118

Zhang, M. W., Zhang, R. F., Zhang, F. X., & Liu, R. H. (2010). Phenolic Profiles and Antioxidant Activity of Black Rice Bran of Different Commercially Available Varieties. *Journal of Agricultural and Food Chemistry, 58*(13), 7580-7587. doi:10.1021/jf1007665

Zhang, Y., Wang, L., Yao, Y., Yan, J., & He, Z. (2012). Phenolic acid profiles of Chinese wheat cultivars. *Journal of Cereal Science, 56*(3), 629-635. doi:https://doi.org/10.1016/j.jcs.2012.07.006

Zhen, S., Dong, K., Deng, X., Zhou, J., Xu, X., Han, C., . . . Yan, Y. (2016). Dynamic metabolome profiling reveals significant metabolic changes during grain development of bread wheat (Triticum aestivum L.). *Journal of the Science of Food and Agriculture, 96*(11), 3731-3740. doi:10.1002/jsfa.7561

Publications of collaborations conducted during the thesis

1. https://doi.org/10.1016/j.foodchem.2021.130644 Gabriela de Freitas Laiber Pascoal; Marta Angela de Almeida Sousa Cruz; Joel Pimentel de Abreu; **Millena Cristina Barros Santos**; Gustavo Bernardes Fanaro; Mário Roberto Maróstica Júnior; Otniel Freitas Silva; Ricardo Felipe Alves Moreira; Luiz Claudio Cameron; Mariana Simões Larraz Ferreira; Anderson Junger Teodoro. Evaluation of the antioxidant capacity, volatile composition and phenolic content of hybrid Vitis vinifera L. varieties sweet sapphire and sweet surprise. **Food Chemistry, 2022.**
2. https://doi.org/10.1016/j.jfoodeng.2021.110744 Oscar Lombo Vidal; **Millena Cristina Barros Santos**; Ana Paula Batista; Fernanda Franceschi Andrigo; Bruno Baréa; Jérôme Lecomte; Maria Cruz Figueroa-Espinoza; Nathalie Gontard; Pierre Villeneuve; Valérie Guillard; Claudia Moraes Rezende; Claire Bourlieu-Lacanal and Mariana Simões Larraz Ferreira. Active packaging films containing antioxidant extracts from green coffee oil by-products to prevent lipid oxidation. **Journal of Food Engineering, 2022.**
3. https://doi:10.1016/j.foodchem.2020.127882 Brito, T.B.N.; R.S. Lima, L.; **Santos, M.C.B.**; A. Moreira, R.F.; Cameron, L.C.; C. Fai, A.E.; S.L. Ferreira, M. Antimicrobial, antioxidant, volatile and phenolic profiles of cabbage-stalk and pineapple-crown flour revealed by GC-MS and UPLC-MS^{E}. **Food Chemistry, 2021.**
4. https://doi:10.1080/14786419.2020.1868459 Dos Santos, Mônica Cristine Pereira; Cavalcanti, Elisa D'avila Costa; **Santos, Millena C.B.**; Seljan, Mariana Pulmar; Cameron, Luiz Claudio; Ferreira, Mariana Simões Larraz; Gonçalves, Édira Castello Branco De Andrade. Profile of phenolic compounds in jabuticaba (*Myrciaria sp*.) a potential functional ingredient. **Natural Product Research, 2021.**
5. https://doi.org/10.3390/molecules25020342 Lima, L. G. B.; Montenegro, J. ; Abreu, J.P.; **Santos, M.C.B**.; Nascimento, T. P.; Santos, M. S.; Ferreira, A. G.; Cameron, L.C.; Ferreira, M.S.L.; Teodoro, A. J.. Metabolite Profiling by UPLC-MS^{E}, NMR, and Antioxidant Properties of Amazonian Fruits: Mamey Apple (*Mammea americana*), Camapu (*Physalis angulata*), and Uxi (*Endopleura uchi*). **Molecules, 2020.**
6. https://doi.org/10.1002/jsfa.10209 Nascimento, T. P.; **Santos, M. C. B.**; Abreu, J. P.; Almeida, I. L. G. T.; Feijo, M. B. S ; Teodoro, A. J.; Ferreira, M.S.L.; Cameron, L.C.; Koblitz, M. G. B. Effects of cooking on the phytochemical profile of breadfruit as revealed by high-resolution UPLC-MS^{E}. **Journal of the Science of Food and Agriculture, 2020**.

7. https://doi:10.1016/j.foodres.2020.109292 De Sá Mendes, Nathânia; Coimbra, Pedro P.S.; **Santos, Millena C.B.**; Cameron, Luiz C.; Ferreira, Mariana S.L.; Buera, María Del P.; Gonçalves, Édira C.B.A. *Capsicum pubescens* as a functional ingredient: Microencapsulation and phenolic profilling by UPLC-MS^{E}. **Food Research International, 2020**.
8. DOI: 10.37423/200601222 V.C.M. Victorio; **M.C.B. Santos**; C.T.S. D'Almeida; A.F. Macedo; G.H.M.F. Souza; L.C. Cameron; L.C. Gutkoski; M.S.L. Ferreira. Comparação de dois métodos de aquisição de espectrometria de massas aplicados para melhor caracterização das proteínas do trigo: HDMS^{E} X UDMS^{E}. **Chapter - Nutrição em Foco: uma abordagem holística, 2020**.
9. https://doi.org/10.1016/j.lwt.2019.05.107 Mendes, N.S.; Santos, M.C.P.; **Santos, M.C.B.**; Cameron, L.C.; Ferreira, M.S.L.; Goncalves, E.C B.A.. Characterization of Pepper (*Capsicum baccatum*) - A Potential Functional Ingredient. **LWT-Food Science and Technology, 2019**.
10. Dalmeida, C.T.S.; **Santos, M.C.B.**; Rodrigues, S.K.P.M.; Lima, Luciana.; Ferreira, M.S.L. Monitoramento da oxidação dos tióis proteicos em grãos de trigo imaturos e de diferentes aptidões tecnológicas. **SEMEAR: Revista de Alimentação, Nutrição e Saúde, v.1, p.31 - 42, 2019**.
11. https://doi.org/10.1016/j.foodchem.2017.07.049 Victorio, V.C.M.; Souza, G.H.M.F.; **Santos, M.C.B.**; Vega, A.R.; Cameron, L.C.; Ferreira, M.S.L.. Differential expression of albumins and globulins of wheat flours of different technological qualities revealed by nanoUPLC-UDMS^{E}. **Food Chemistry, 2018**.
12. https://doi.org/10.1016/j.dib.2018.09.111 Nascimento, T. P.; **Santos, M. C. B**.; Lima, L. R. S.; Nascimento, F. R.; Cameron, L.C.; Ferreira, M. S. L.. Dataset on phenolic profile of seven wheat genotypes along maturation. **Data In Brief, 2018**.
13. https://doi.org/10.1016/j.foodres.2018.09.050 Silva, C. P.; Soares-Freitas, R. A. M.; Sampaio, G. R.; **Santos, M. C. B.**; Nascimento, T. P.; Cameron, L. C.; Ferreira, M. S. L.; Areas, J. A. G. Identification and action of phenolic compounds of Jatobá-do-cerrado (*Hymenaea stignocarpa Mart*.) on α-amylase and α-glucosidase activities and flour effect on glycemic response and nutritional quality of breads. **Food Research International, 2018.**
14. *Submitted*. Nascimento, Talita; Ladeira, Karine; Bezerra, Fernanda; **Santos, Millena**; de Souza, Thaiza; L.C., Cameron; Ferreira, Mariana; Koblitz, Maria. Ecofriendly

enrichment of minority compounds from the aqueous extract of sunflower meal with activated charcoal. **Journal of the Science of Food and Agriculture, 2021**.

15. *On preparation*. Luciana Ribeiro da Silva Lima, **Millena Cristina Barros Santos**, Carolina Thomaz dos Santos D'Almeida, Luiz Claudio Cameron, Mariana Simões Larraz Ferreira. Title: Foodomics in whole wheat grain reveals phenolic profile of different genotypes and technological qualities.
16. *On preparation*. Gabriela Soster Santetti, Barbara Biduski, Luciana Ribeiro da Silva Lima, Carolina Thomaz dos Santos D'Almeida, **Millena Cristina Barros Santos**, Luiz Claudio Cameron, Mariana Simões Larraz Ferreira, Luiz Carlos Gutkoski, Renata Dias de Mello Castanho Amboni. Title: Untargeted metabolomics reveals improved phenolic profile in whole wheat breads with yerba mate and effects of the bread-making process.
17. *On preparation*. Tatiana Cauduro, Barbara Biduski, Carolina Thomaz dos Santos D'Almeida Alessandra dos Santos, **Millena Cristina Barros Santos**, Luiz Claudio Cameron, Mariana Simões Larraz Ferreira, Luiz Carlos Gutkoski. Title: Whole wheat flour replaced by sprouted wheat improves rheological and breadmaking properties.

Scientific oral communications

1. **SANTOS, M. C. B.** Are there antioxidant compounds in wheat flour? 2017. (Conference or lecture).
2. **SANTOS, M. C. B.**; Lima, L. R. S.; Nascimento, F. R.; Alves, T. O.; Cameron, L. C.; Ferreira, M. S. L. **Metabolomic approach for characterization of phenolic compounds in different Brazilian wheat varieties during grain development**, 2017. (International Symposium).
3. **SANTOS, M. C. B.**; Barron, C.; Lullien-Pellerin, V.; Durand, E. ; Villeneuve, P. ; Cameron, L.C. ; Bourlieu-Lacanal, C.; Ferreira, M. S. L. . **Metabolomics Of Pigmented Rice Coproducts Applying Conventional and Deep Eutectic Solvents**. 2019. (IV SIAN - International Symposium on Food and Nutrition, 2019, Rio de Janeiro. Sustainability and Innovation in Food Science - Impact on the Bioeconomy).
4. **SANTOS, M. C. B.** ; Durand, E. ; Barouh, N.; Lullien-Pellerin, V.; Barron, C.; Micard, V.; Villeneuve, P.; Bourlieu-Lacanal, C.; Ferreira, M.S.L**. Fonctionnalisation enzymatique et caractérisation métabolomique des composés phénoliques de blé et de riz obtenus par extraction classique ou solvant eutectique**. 2020 (Congrès Phloème - Biennales de l'innovation céréalière, Paris)

5. **SANTOS, M**. C. B.; Barouh, N.; Durand, E.; Micard, V.; Lullien-Pellerin, V.; Villeneuve, P.; Ryan, Elizabeth P.; Ferreira, M. S. L.; Bourlieu-Lacanal, C.. **Lipidomics of RB**. 2020. (Journées Chevreul de la SFEL- Les lipides au cœur d'enjeux de transition).
6. **SANTOS, M. C. B.;** Barouh, N.; Durand, E.; Barea, B.; Robert, M.; Micard, V.; Lullien-Pellerin, V.; Cameron, L. C.; Villeneuve, P.; Ryan, E.P.; Ferreira, M. S. L.; Bourlieu-Lacanal, C. . **Physicochemical characterization of rice bran from different locations by classical and omics analysis**. 2020. (Valorization of Rice Bran Mini-symposium - 4th Global Food Security Conference 2020).
7. **Millena Cristina Barros Santos**; Nathalie Barouh; Bruno Baréa, Mélina Robert, Pierre Villeneuve, Luiz Cláudio Cameron; Valérie-Lullien, Claire Bourlieu-Lacanal, Mariana Simões Larraz Ferreira and Erwann Durand - **Could NaDES assisted one-pot extraction and biotransformation be a relevant strategy to boost activity of natural extracts?** 2021. (2nd International Meeting on Deep Eutectic Systems).

Awards

2020 – Promising Thesis in Congress Phloéme - « Les biennales de l'innovation céréalière » ARVALIS – Institut du végétal. Paris, France.

2019 - VI SIAN - 3rd Place best work presented as a poster. PPGAN - UNIRIO.

2018 - Best paper of the 17th Scientific Initiation Day (JIC) - Food Sciences area, UNIRIO.

2017 - III SIAN - 3rd place best work presented in poster. PPGAN, UNIRIO.

2017 - Best work of the 16th Conference of Scientific Initiation, Food Science area, UNIRIO.