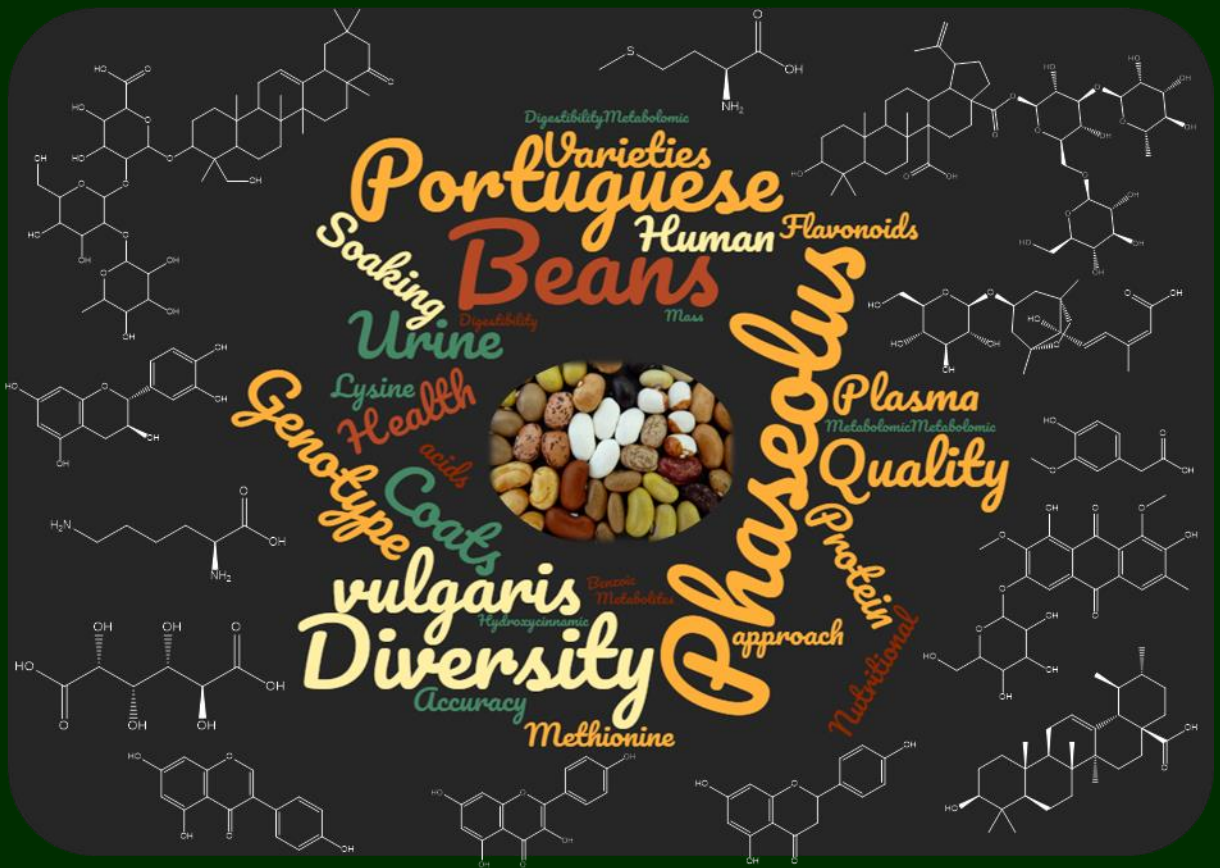
# *Phaseolus vulgaris L.* A contribution for the valorization of Portuguese varieties

*Elsa Velez Mecha*




*Dissertation presented to obtain the Ph.D degree in Molecular Biosciences*

*Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa*

*Oeiras, June, 2021*

UNIVERSIDADE NOVA DE LISBOA

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*"Do the best you can until you know better. Then, when you know better, do better"*

*Maya Angelou*

**DISSERTATION**: June 2021

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# Acknowledgments

On the top of my gratitude I must thank to my parents and my sister. They always showed me the value of education and gave me the emotional support I needed to pursue my PhD. Thank you for all the support, help and understanding, even when I was absent. Hope to compensate you after this long journey.

Then, I would like to express my sincere gratitude to all the ones that believed in this PhD and in this project as a knowledge generator for nutrition, agriculture and science fields.

Looking back to this period of my life, I will try to do my best in acknowledging all the supervisors, colleagues and friends.

During a stage period on the lab and by Professor Maria do Rosário Bronze, Doctor Carlota Vaz Patto and Professor Maria Eduardo Figueira suggestion I decided, fortunately, to develop my PhD project. To them, I must thank the PhD proposal and suggestions.

I must give my special thanks to Professor Maria do Rosário Bronze for the guidance, resources, understanding, motivation and patience. Thank you for showing me that it was possible to start and finish my PhD and for all the unconditional support during this long process and to Doctor Carlota Vaz Patto thank you for the enthusiasm, motivation, scientific support and discussions. They were key players in this PhD with comments and suggestions that enable the writing of the research articles and finally this thesis. Thanks to them I was able to explore methodologies and acquire knowledge in the field of food characterization, which exceeded my expectations and improved my academic formation. They

gave me several opportunities to present in national and international meetings the main achievements of my work. Looking back I really appreciated those times when the message was transmitted in person and the communication performed in its real assessment through verbal and non-verbal communication.

Of course I need also to thank FCT for accepting my PhD project on the right time allowing my growth at experimental and scientific levels through the embracement of this amazing PhD project.

Part of the beauty of my PhD project is related to its multidisciplinary approach. It gave me the opportunity to act as a “bridge student” in consequence of multiple interactions with different research groups.

To the Food and Health Division, iBET - Instituto de Biologia Experimental e Tecnológica, especially to the Natural Bioactives and Nutraceuticals Characterization Lab I need to thank all the colleagues that shared my PhD experience, making it a real team work.

To avoid missing someone I would like to thank everyone who was present in the lab during my PhD period, for their partnership and collaboration from the start until the end. My special acknowledgements go to Professor Luis Vilas Boas and to the late Professor Antero Ramos. To them I owe the time, the patience, the resilience, the knowledge and the friendship during my starting period on the lab. For sure I need to thank Ana Teresa Serra, Sheila Alves, Leonor Costa, Beatriz Anacleto, Andreia Bento da Silva, Inês Barbosa, Sofia Natallelo, Verónica Correia, Elsa Brito, Ana Bárbara Pereira, António Ferreira, Sandra Silva and Joana Alves, for

all the help in the lab work, support, strength, friendship, partnership, respect, enthusiastic conversations and contagious joy.

To all PlantX and BCV members, particularly to Mara Lisa Alves, Susana Leitão and Letice Gonçalves, thank you for your cheerful friendship and understanding. I enjoyed every lunch and “chicharada” we shared together, as well as the many meetings we had the opportunity to go. I will always remember the team work, unity and commitment on the many meetings we join together. I real appreciate the companionship, the help and the collaborative efforts.

To INIAV, especially to Doctor Maria Manuela Veloso I have to thank the common bean seeds for my PhD work, as well as the scientific knowledge in common bean accessions.

To the late Professor Judite Costa and Professor Maria de Fátima Cabral, from FFUL, I must thank for their noteworthy guidance, interest, inclusion, partnership and commitment.

To Ana Rodriguez-Mateos, Rodrigo Feliciano and Geoffrey Istas I must thank the availability in receiving me at Düsseldorf University and also for the time, guidance and help.

To iBET - Instituto de Biologia Experimental e Tecnológica I need to thank the possibility of using the facilities and equipment/ reagents that contributed for the work developed in this thesis.

To UniMS, ITQB NOVA/iBET, I would like to acknowledge all the team, especially, Ricardo Gomes, Ana Catarina Guerreiro, Catarina Correia and Maria Conceição Almeida, for all the help with equipment and software.

To Faculty of Engineering of Oporto, LEPABE, many thanks to Guillaume Erny for the crucial collaboration in data mining.

Finally, I would like to give my deepest acknowledgment to ITQB-NOVA, for accepting my PhD work, and providing the resources/conditions to accomplish this project.

# Summary

Common beans (*Phaseolus vulgaris* L.) are one of the most important foods to populations' survival worldwide, being fundamental for the development of sustainable farming systems, environment protection, human/ animal nutrition improvement and health promotion. Despite of the several advantages, common beans' production and consumption are far behind the recommendations that aim to counteract malnutrition and support food security in the world. Throughout this thesis several studies aiming to fulfil gaps of knowledge, from common beans' production to common beans' consumption and applicability by food industry were pursued, bringing through a multidisciplinary view new insights that ultimately will enhance common beans' production and consumption. At the production level, heat stress represents one of the major constraints. Expectable rising temperatures, from 2030 onwards, will impair plant growth, seed yield and quality. Understanding the impact of warming environments in common beans' nutritional composition and metabolomics is the way forward to unravel potential mechanisms of temperature stress tolerance in common bean seeds. With such purpose, 107 underexploited Portuguese common bean accessions were cropped under different environmental conditions (traditional *versus* heat stress environments). Regarding their nutritional composition (protein, carbohydrates, fat, dietary fiber, ash and moisture) higher environmental temperatures, clearly affected photosynthetic machinery and the mobilization of nitrogen, being responsible by decreased carbohydrates and increased protein contents in common bean seeds. Taking in consideration the natural variability in the

protein quality of common beans cropped under heat stress environment, future breeding programs for protein quality improvement should focus in the selection of accessions with higher individual amino acid contents rather than with higher protein content. By bridging the gap between genotype and phenotype, metabolomics can be applied to monitor plant responses to abiotic stress. The use of high-resolution mass spectrometry for untargeted metabolomics, combined with data mining extended the existent common beans metabolites identification. The recognition of specific metabolites associated to heat stress tolerant accessions unveiled molecular fingerprints that will allow common beans' production, even under environmental challenging conditions. Despite of the huge genotypic variability in metabolites content, the common beans cropped under the heat stress environment were characterized by higher levels of secondary metabolites, such as benzenoids (e.g. salycilic acid). To build the foundations that will enable a deeper understanding of common beans' phenolic compounds' impact in human health and to understand the influence of processing methods in common beans' phenolic composition, a study regarding the characterization of common beans' fractions after the soaking process was conducted. In this study developed with 31 under exploited accessions, it was clear the qualitative diversity of phenolic compounds found in the different common bean fractions, namely in the soaking water and soaked coats of coloured varieties, particularly rich in flavanols and flavonols, respectively. White varieties were mostly rich in phenolic acids (hydroxycinnamic acids) at the cotyledons fraction. Characterizing the common beans' phenolic compounds, considering the different fractions, paves the way for a better understanding of common

beans' phenolic compounds accessibility and how processing methods can be adjusted to take advantage of these compounds in a daily basis diet. Once accessible, common beans' phenolic compounds only exert their action in human body if bioavailable in the form of functional metabolites, which requires metabolic reactions throughout the gastrointestinal tract. In a human intervention study with seven volunteers, the metabolites obtained from phenolic compounds in common beans were studied in biological fluids (plasma and urine) after cooked common beans' consumption. Using UPLC-Q-TOF-MS methodology with commercial standards several different metabolites were identified and proposed as potential molecular markers of common beans' consumption in challenging matrices of plasma and urine. Such metabolites should be further studied through *in vitro* and *in vivo* disease models. Since a plant-based diet is associated to chronic diseases prevention, food industry and food marketing should be focused in dissemination strategies involving legumes, in general, and common beans, in particular, to increase consumption of affordable, tasty and healthier plant-based foods. In a human intervention study with sixteen volunteers, common bean flour was applied in partial substitution of wheat flour to produce alternative cookies. These alternative cookies, compared with the traditional ones, showed advantages in human capillary glycaemia and satiety perception. Finally this PhD thesis contributed, through the application of several different analytical approaches (spectrophotometry, near-infrared spectroscopy and high-resolution mass spectrometry techniques), for the characterization of common beans' nutritional diversity, for the improvement of existent human bioavailability studies with phenolic compounds derived from cooked

common beans and for a better understanding about the importance of adding common beans as an alternative ingredient in bakery products for human glycemic response and satiety perception. In an integrated legume-centred strategy, the characterization of diversity is the building block for a better quality seed production ensuring worldwide public health, in challenging times where climate changes, food security and malnutrition have huge economic and social burdens.

# Sumário

O feijão (*Phaseolus vulgaris* L.) é um dos alimentos mais importantes para a sobrevivência das populações em todo o mundo, sendo fundamental para o desenvolvimento de sistemas agrícolas sustentáveis, proteção do meio ambiente, melhoria da nutrição e promoção da saúde humana/ animal. Apesar das diversas vantagens, a produção e o consumo do feijão estão muito aquém das recomendações que visam combater a desnutrição e apoiar a segurança alimentar no mundo. Ao longo desta tese vários estudos foram desenvolvidos com o objetivo de preencher algumas lacunas de conhecimento, desde a produção ao consumo e aplicabilidade do feijão pela indústria alimentar, trazendo através de uma visão multidisciplinar novas abordagens que, em última análise, irão aumentar a produção local e o consumo do feijão. Ao nível da produção, o aumento das temperaturas representa uma das principais limitações. Aumentos de temperatura, de 2030 em diante, prejudicarão o crescimento das plantas, o rendimento e a qualidade das sementes. Para desvendar os mecanismos potenciais de tolerância a temperaturas elevadas no feijão será necessário compreender o impacto do aquecimento ambiental na composição nutricional e metabolómica do feijão. Com esse propósito, 107 acessos de feijoeiro português foram cultivados em diferentes condições ambientais (ambiente tradicional *versus* ambiente com *stress* térmico). Em relação à composição nutricional (proteínas, hidratos de carbono, gordura, fibra alimentar, cinzas e

humidade), as temperaturas ambientais mais elevadas afetaram claramente a fotossíntese e a mobilização de nitrogénio, sendo responsáveis pela diminuição do teor de hidratos de carbono e aumento do teor de proteína nas sementes de feijão. Tendo em consideração a variabilidade natural na qualidade da proteína do feijão cultivado em condições de temperaturas elevadas, os futuros programas de melhoramento para a qualidade da proteína devem-se focar na seleção de acessos com maiores teores de aminoácidos individuais ao invés de com maior teor de proteínas. Preenchendo a lacuna entre o genótipo e o fenótipo, a metabolómica pode ser aplicada para monitorizar as respostas das plantas ao *stress* abiótico. O uso de espetrometria de massa de alta resolução aplicada à metabolómica não direcionada, combinada com análise de dados, contribuiu para a identificação de metabolitos do feijão. O reconhecimento da síntese de metabolitos específicos, associados a acessos tolerantes a temperaturas elevadas, irá possibilitar a produção de feijão, mesmo em condições ambientais desafiadoras. Apesar da enorme variabilidade genotípica no teor de metabolitos, os feijões cultivados em condições de *stress* térmico (temperaturas elevadas) foram caracterizados por níveis mais elevados de metabolitos secundários, como os benzenóides (por exemplo, ácido salicílico). Com vista a compreender o impacto dos compostos fenólicos do feijão na saúde humana e entender a influência dos métodos de processamento na composição fenólica do feijão, foi desenvolvido um estudo sobre a caracterização das frações do feijão após o

processo de demolha. Neste estudo 31 acessos foram caracterizados, tendo sido evidente a diversidade qualitativa de compostos fenólicos encontrados nas diferentes frações do feijão, nomeadamente na água de demolha e nas cascas demolhadas das variedades coloridas, particularmente ricas, respetivamente, em flavanóis e flavonóis. As variedades brancas destacaram-se pela sua riqueza em ácidos fenólicos (ácidos hidroxicinâmicos), sobretudo na fração dos cotilédones. Caracterizar os compostos fenólicos do feijão considerando as diferentes frações possibilita uma melhor compreensão da acessibilidade dos compostos fenólicos do feijão e permite que os métodos de processamento possam ser ajustados de modo a retirar o máximo proveito destes compostos na dieta. Uma vez acessíveis, os compostos fenólicos do feijão apenas exercem a sua ação no corpo humano se biodisponíveis na forma de metabolitos funcionais, o que requer reações metabólicas ao longo do trato gastrintestinal. Num estudo de intervenção humana com sete voluntários, os metabolitos obtidos de compostos fenólicos no feijão foram estudados em fluidos biológicos (plasma e urina) após o consumo de feijão cozido. Usando a metodologia de UPLC-Q-TOF-MS com padrões comerciais, diferentes metabolitos foram identificados e propostos como potenciais marcadores do consumo de feijão. Esses metabolitos devem ser mais estudados recorrendo a modelos de doença *in vitro* e *in vivo*. Uma vez que a dieta à base de plantas está associada à prevenção de doenças crônicas, a indústria alimentar e o *marketing* alimentar devem focar-se em estratégias

de divulgação das leguminosas, em geral, e do feijão, em particular, para aumentar o consumo de alimentos à base de plantas, acessíveis, saborosos e mais saudáveis. Num estudo de intervenção humana com dezasseis voluntários, a farinha de feijão foi aplicada na substituição parcial da farinha de trigo para produzir bolachas alternativas. Essas bolachas, comparadas com as tradicionais, mostraram vantagens na glicemia capilar humana e na perceção da saciedade. Finalmente esta tese de doutoramento contribuiu, através da aplicação de diferentes abordagens analíticas (espectrofotometria, espectroscopia de infravermelho próximo e técnicas de espectrometria de massa de alta resolução), para a caracterização da diversidade nutricional de acessos de feijão, para avanços nos estudos de biodisponibilidade humana existentes com compostos fenólicos derivados do feijão cozido e para uma maior compreensão da importância do feijão como ingrediente alternativo em produtos de confeitaria na resposta glicémica humana e perceção da saciedade. Numa estratégia integrada centrada em leguminosas, a caracterização da diversidade é o alicerce para a produção de sementes de melhor qualidade, assegurando a nível mundial a saúde pública, em tempos desafiantes onde as alterações climáticas, a segurança alimentar e a má-nutrição têm enorme carga económica e social.

# Abbreviation list

AACC – American Association for Clinical Chemistry

AAPH – 2,2'-Azobis(2-amidinopropane)

AAS – Amino Acid Score

ACE – Angiotensin Converting Enzyme

AGC – Automatic Gain Control

AMPK – Adenosine Monophosphate-activated Protein Kinase

AUC – Area under the Curve

BAPA – Benzoyl-L-arginine-p-nitroanilide

BMI – Body Mass Index

CCK – Cholecystokinin

CE – Catechin Equivalent

COD – Center of Domestication

CV – Coefficient of Variation

CVD – Cardiovascular Diseases

DMSO – Dimethyl sulfoxide

DPPH – Dipeptidyl Peptidase

DW – Dry Weight

E – Environment

EAAs – Essential Amino Acids

EDTA – Ethylenediamine tetraacetic acid

ESI – Electro Spray Ionization

EU – European Union

F3H – Flavanone-3-Hydroxylase

FAO – Food and Agriculture Organization

FID – Flame Ionization Detector

FISh – Fragment Ion Search

FL – Fluorescein

FRAP – Ferric Reducing Antioxidant Power Assay

FU – 5-Fluorouracyl

FW – Fresh Weight

G – Genotype

G x E – Genotype x Environment interaction

GAE – Gallic Acid Equivalent

GC – Gas Chromatography

GI – Glycemic Index

GIP – Gastric Inhibitory Peptide

GL – Glycemic Load

GLP-1 – Glucagon-Like Peptide

GPS – Global Positioning System

HCl – Chloride Acid

HDL – High Density Lipoprotein

HORAC – Hydroxyl Radical Antioxidant Capacity

HPLC – High Performance Liquid Chromatography

I – Inca

IDA – Information Dependent Acquisition

IgE – Imunoglobulin E

IVPD – *In Vitro* Protein Digestibility

IVPDCAAS – *In Vitro* Protein Digestibility Corrected Amino Acid Score

LC-MS/MS – Liquid Chromatography with tandem Mass Spectrometry

LDL – Low Density Lipoprotein

LEA – Late embryogenesis abundant

LoQ – Limit of Quantification

MQL – Method Quantification Limit

MRM – Multiple Reaction Monitoring

mTORC1 – mammalian Target of Rapamycin Complex 1

N – Nitrogen

NCD – Non-Communicable Diseases

NEAAs – Non-Essential Amino Acids

NIR – Near InfraRed Spectroscopy

NPK – Nitrogen, phosphorus, and potassium

NSP – Non Starch Polysaccharide

ORAC – Oxygen Radical Absorbance Capacity

PBS – Phosphate Buffered Saline

PCA – Principal Component Analysis

PER – Protein Efficiency Ratio

PHA – Phytohemagglutinin

PLS-DA – Partial Least Square-Discriminant Analysis

PUFAs – Polyunsaturated Fatty Acids

PURE – Prospective Urban Rural Epidemiology

PYY – Peptide YY

RI – Refractive index

RMSEC – Root Mean Square Error of Calibration

RMSECV – Root Mean Square Error of Validation

ROS – Reactive Oxygen Species

RT – Retention Time

S – Sanilac

SD – Standard Deviation

SEM – Standard Error of Mean

SI – Satiety Index

SQ – Satiety Quotient

T – Tendergreen

TEAC – Trolox Equivalent Antioxidant Capacity

TFC – Total Flavonoids Content

Th2 – Type 2 helper

TIA – Trypsin inhibitor Activity

TPAC –Total Proanthocyanins Content

TPC – Total Phenolic Content

UGTs – UDP-Glycosyl Transferases

UPLC-Q-TOF-MS – Ultra Performance Liquid Chromatography-Quadrupole-Time Of Flight-Mass Spectrometry

USDA – United States Department of Agriculture

VAS – Visual Analogue Scale

VIP – Variable Importance in Projection

VLCFA – Very Low Chain Fatty Acids

# Chapter I: General Introduction

This chapter was submitted and published by InTechOpen as,

**Mecha, E**.; Figueira, M.E.; Vaz Patto, M.C.; Bronze, M.R. Two sides of the same coin: the impact of grain legumes on human health – Common bean (*Phaseolus vulgaris* L.) as a case study. In *Legume Seed Nutraceutical Research*; Jimenez-Lopez, J.; Clemente, C.; Eds.; InTechOpen: UK, England, 2018; DOI:10.5772/intechopen.78737.

In this Chapter, Elsa Mecha participated in the bibliographic research, drafted the manuscript and contributed to the final revision of the manuscript

# Two sides of the same coin: the impact of grain legumes on human health – Common bean (*Phaseolus vulgaris* L.) as a case study

## Abstract

Data from Food and Agriculture Organization indicate the worrying scenario of severe food insecurity in the world and the contrasting high prevalence of obesity (13% of the world adult population) in both developing and developed countries. Sustainable agriculture systems with increased inclusion of grain legume species and the boosting of public awareness about legume importance on diet should be a priority issue to eradicate malnutrition and promote public health. However, grain legume production and consumption are in constant state of decline, especially in the European Union. Assigned as the “poor man’s meat”, “promoters of flatulence”, or incorrectly classified as “starchy foods”, grain legumes have a negative image in modern societies. In fact, legumes represent an important source of protein, fiber, vitamins (e.g. folate) and minerals (e.g. magnesium). Moreover, legumes are rich in bioactive compounds (e.g. phenolic compounds, protease and α-amylase inhibitors) acting as a “double-edged sword” in human health. They may impair nutrients availability exerting at the same time beneficial biological activities in lipid profile, inflammation, glycaemia and weight. The present chapter is focused on the advantages of a legume-rich diet for health promotion at a global scale, reviewing legume nutritional and bioactive compounds, with particular emphasis on common bean.

**Keywords:** grain legumes; nutritional value; bioactive compounds; health benefits

## 1. Introduction

Grain legumes have been neglected, regardless of their potential to ensure nutrition and food security. Nutritionally rich in protein, fiber, carbohydrates, vitamins and minerals, grain legumes are key dietary components to eradicate hunger, as well as, malnutrition [1].

The ignorance regarding grain legume nutritional composition and food preparation techniques, allied with the negative image of legumes in modern societies, contributes to decrease legumes' consumption. Besides nutrients, legumes are also a rich source of bioactive compounds which can act as a "double-edged sword", since they can impair nutrients' bioavailability (as anti-nutritional factors), acting simultaneously, as health promoting compounds in the prevention of non-communicable diseases (e.g. cardiovascular diseases, inflammatory diseases and cancer) [2]. In order to balance negative and positive effects of these bioactive compounds, crops diversity should be preserved and characterized to give valid information to breeders and molecular biologists, who can manipulate the levels of these compounds through the selection of interesting varieties.

The present chapter aims to give a general overview of the current state of the art of grain legume production, consumption and impact on world food security. It also shows the nutritional value and the bioactive composition considering some *in vitro*, *in vivo* and epidemiological studies

conducted to analyse the potential health benefits associated with legumes consumption.

## 2. Legumes diversity

Legumes are dicotyledons plants, which belong to Leguminosae or Fabaceae family, with edible seeds developed in pods. By definition, it includes the fresh legumes, pulses and the seeds with high fat content (e.g. soybeans and peanuts). Pulses, also known as grain legumes, refer only to the dried seeds with virtually no fat, which excludes the fresh legumes, soybeans and peanuts. Common bean (*Phaseolus vulgaris* L.), pea (*Pisum sativum* L.), faba beans (*Vicia faba* L.), chickpea (*Cicer arietinum* L.), lentils (*Lens culinaris* L.) and grass pea (*Lathyrus sativus* L.) are examples of legumes well-adapted to several regions of the world, from semi-arid, subtropical to temperate areas.

The wild form of P. vulgaris is originally from Mesoamerica (which extends from northern Mexico to Colombia). Since its expansion, two independent domestication centers were formed in Mesoamerica and Andes (from southern Peru to northwestern Argentina) [3].

In Europe, particularly in Portugal [4], Spain, Italy and central-northern Europe, common bean germplasm derives mostly from the Andean domestication center (67%) and in the Eastern Europe there is a higher predominance of the Mesoamerican type [3].

Despite the large genetic diversity in grain legume seeds held in gene banks, the genetic resources are not intensively used in breeding programs. Preservation, characterization and evaluation of the genetic variability, in what concerns agronomic performance and quality traits, is a

useful approach to ensure *in situ* conservation and future breeding programs to cope with consumers' demands and environmental challenges [5].

## 3. Legumes production and consumption

Diversifying agriculture, instead of adopting an intensive specialized production system, is one of the goals to achieve a sustainable development. Grain legumes bring diversity, nutrient supply and disease control to cropping systems. In opposition to the American continent, Africa, Asia and Oceania, in the European Union, common bean production decreased drastically (−80.42%) between 1961 (817,000 tonnes) and 2013 (160,000 tonnes) [6]. During this period of time, there was a shift in land use toward an intensive cereals production [6], which contributed to the Europeans' dependence in imported grain legumes, compromising sustainability of the actual food farming system. Parallel to the decrease in common bean production data from FAOSTAT, relative to food balance, also indicate, in European Union (EU), a dramatic decrease on its consumption from 1.5 kg/capita/ year (1961) to 0.78 kg/capita/year (2013) [6].

Several factors related to crop productivity, government policies and consumers' preferences can explain the reduced investment of European farmers in grain legumes production. The promotion of breeding programs to increase genetic diversity and the development of more attractive varieties adapted to the local growing conditions and to the consumers' demands (high quality varieties) must be pursued.

### 3.1. Food security

The Food and Agriculture Organization (FAO) of the United Nations declared 2016 as the International year of Pulses focusing on hunger and malnutrition eradication [7]. According to the second sustainable development goal of FAO, by 2030, countries should “end hunger”, adopt sustainable agriculture systems and provide food security to all population [8]. Several factors can affect food security worldwide: extreme weather events (e.g. droughts, floods and hurricanes), conflicts with violence affecting rural areas and economic recessions with increased unemployment [8]. Worrying data from FAO indicate that, in 2016, 815 million people suffer from chronic food deprivation and around 698 million people from severe food insecurity [8].

To avoid the financial pressure of malnutrition on health care systems and the economic burden of the co-morbidities related with malnutrition, governments should support sustainable agriculture practices with inclusion of legumes in cropping systems and subsidies to small farmers, especially in low- and middle-income countries dependent on agriculture [9]. Nutritional initiatives to eradicate malnutrition and protein deficiency should include public awareness about inclusion of vegetable protein in daily diet [8].

## 4. Nutritional value

Legumes are within the food items with a high nutrient value (330 ± 217 kcal/100 g) for a low cost value (0.26 ± 0.22 $/serving) [10].

Grain legumes are distinguished as a rich source of vegetable protein, soluble and insoluble fiber, resistant starch, micronutrients

(minerals and vitamins) and several bioactive compounds [11]. When complemented with the cereals' protein, grain legumes can be consumed as a sustainable alternative to animal protein. Despite of the American Cancer Society, the Centers for Disease Control and Prevention and the US Dietary guidelines who classify beans as vegetables, many consumers continue to associate grain legumes to starchy foods, like rice, pasta and tubers [12]. The major differences between legumes and starchy food (cereals) are related with macro- and micronutrients composition.

## 4.1. Macronutrients

The macronutrients should be provided by diet in large amounts to supply the energy and the molecular units that sustain the basal metabolism, physical activity, growth, pregnancy and lactation. The carbohydrates' contribution to total food energy is higher in cereals than in beans and there is an inverse situation for the protein contribution, with beans showing higher protein content than cereals [13].

### *4.1.1. Protein*

In legumes, proteins are stored in the parenchyma cells of cotyledons and are classified according to their solubility in different solvents as albumins, water extractable, globulins, extractable in salt solutions, prolamins, extractable in aqueous alcohol and glutelins extractable in weak acid/ alkaline solutions. In common bean, globulins are the most predominant fraction of storage proteins (54–79%), followed by albumins (12–30%), glutelins (20–30%) and prolamins (2–4%). The most abundant globulin in common bean is the phaseolin (40–50% of the total globulins) [14].

The structural units of the proteins known as amino acids can be classified as essential and non-essential. The essential ones must be necessarily provided by diet. If some of the eight essential amino acids is lacking, the missing one is named as a “limiting amino acid”. In legumes, the limiting amino acids are sulfur-containing amino acids (methionine and cysteine) and in cereals lysine is the limiting one. In order to increase the protein quality of legumes and cereals, both food items must be combined in a daily diet to provide all the essential amino acids and to prevent protein malnutrition [15].

The presence of anti-nutritional factors (trypsin inhibitors, phytic acid and tannins) in grain legumes, detailed below in this chapter, and the processing method used before consumption can influence protein digestibility and protein quality [16].

*4.1.1.1. Legume proteins as potential allergens*

As a rich protein source, legumes may cause allergenic reactions. More than 90% of the food allergies are caused by proteins of vegetable and animal origin [17]. Genetic factors and exposure to new allergenic food products, early in life, can explain the immune response of some individuals to one or more food proteins [18].

In developed countries, more than 6% of the children and around 4% of the adults have food allergies [19]. In developing countries and emerging economies (e.g. Brazil, China and India), the prevalence of food allergies is misreported and under-diagnosed [20].

The food allergy induced by legumes is an IgE immune reaction, characterized by activation of Th2-type lymphocytes [21]. In sensitized individuals, mild (cutaneous rash, diarrhea, vomiting, abdominal pain,

hypotension, arrhythmia, repetitive cough, tongue swelling, angioedema, rhinitis and asthma) to severe threatening-life symptoms can occur. The most severe reactions, rarely reported with pulses, include anaphylaxis and death [17].

Since legumes share common antigen determinants (epitopes) with other plants, the risk of an allergenic reaction, in sensitized individuals increases if cross-reactive foods were not eliminated from diet/ environment. For example, pea and common beans have cross-reactivity with pollens of *Olea europaea*, *Lolium perenne* and *Betula alba* [22]. In kidney bean, the major allergens were identified as defense proteins against biotic stress (lectin and α-amylase inhibitor), storage proteins (phaseolin) and stress tolerant proteins (late embryogenesis abundant, LEA, protein). These proteins also showed cross-reactivity with other legumes such as peanut and pigeon pea [18].

To prevent the development of food allergies, the pediatric nutrition authorities recommend exclusive breastfeeding until six months of age. Legumes and protein-rich foods (e.g. meat, egg, milk and yoghurt) should be only introduced at the age of 6–8 months [23]. At the agriculture level, promising strategies involving the breeding of crop varieties with reduced content of allergenic proteins are being put into action. Nevertheless, the development of such crops represents a challenge for farmers, who need to deal with compromised plant feasibility [24] and does not represent the appropriate strategy for consumers with severe allergies, since immune reactivity to legumes may occur, even with minimum quantity of allergens. In these patients, the clinical approach to manage allergies should focus on the patients' awareness of a list of food items that must be avoided, and on

a personalized nutritional intervention with indication of nutritive food alternatives.

### *4.1.2. Carbohydrates*

Legume carbohydrates include starch, fiber and oligosaccharides.

#### *4.1.2.1. Starch*

Starch represents the main carbohydrate reserve (22–45% of total carbohydrates) in legume seeds and is used by the plant as a source of glucose and energy [25]. Chemically, it is composed by two types of polymers: the amylose and the amylopectin. Amylopectin is a highly branched polymer characterized by a linear chain of glucose moieties linked by α-1,4-glycosidic bonds with several smaller glucose chains at α-1,6 positions. Amylose is a long unbranched linear chain of α-1,4-glucans. A comparative study of the starch structure of a legume (e.g. chickpea) and a cereal (e.g. wheat) revealed the higher content of amylose in chickpea's starch [26]. Starches with high amylose content have low glycemic index and therefore can be more adequate to type 2 *diabetes mellitus* populations [27].

#### *4.1.2.2. Dietary fiber*

Dietary fiber include the total non-starch polysaccharide (NSP), divided into soluble and insoluble NSP, resistant starch and fructooligosaccharides. Soluble fiber is defined as the fermentable fiber with prebiotic action. The insoluble fiber is poorly fermented and has a bulking function in colon [28]. Compared with cooked corn, cooked beans have higher content of dietary fiber (2.4/100 g in corn against 6.3–10.4/100 g in cooked beans) [13].

Besides total dietary fiber, legumes are also a rich source of resistant starch, which is defined as a portion of starch that passes through the duodenum and jejunum without being digested [28]. In colon, resistant starch is fermented, by the local microbiota, into several products, including short-chain fatty acids (acetate, propionate and butyrate), which are responsible to maintain gut integrity, improve intestinal microflora, reinforce immune system preventing intestinal colonization by pathogens, improve blood lipid profile by reducing plasma triglycerides and LDL cholesterol, control satiety by increasing the secretion of satiety hormones and contribute to prevent several diseases from allergies and autoimmune diseases to bowel cancer [29, 30]. Legumes show higher levels of resistant starch (e.g. 4.3% in kidney beans) than cereals (e.g. 1.4% in rice) and tubers (e.g. 1.8% in potato) in a dry weight basis [31].

*4.1.2.3. Fructooligosaccharides*

Grain legumes are particularly rich in oligosaccharides such as raffinose, stachyose and verbascose, which are likely to be fermented by colonic bacteria. As a consequence of bacterial fermentation, rectal gas is produced, which may be responsible for abdominal discomfort, bloating and flatulence. Since individual gas production is dependent on the individual microflora composition and consumption habits, beans are not necessarily responsible for increased flatulence [32].

Similarly to resistant starch, the colonic fermentation of oligosaccharides is also responsible for the production of short-chain fatty acids, acetate, propionate and butyrate, related to several health benefits [33]. To control the flatulence and reduce the content of oligosaccharides in

legumes, many populations, especially in Asia and Africa, consume fermented legumes as an interesting nutritive food alternative [34].

*4.1.3. Lipids*

Lipids represent 2–21% of the macronutrients present in legumes [35]. The content in the different fatty acids is quite variable among the different legume species. By increasing order of the monounsaturated fatty acid (oleic acid) content, common bean has the low amount (5.1–17.2%) followed by lentils (23.5–39.6%), faba beans (25.2–32.4%), peas (26.3–36%) and chickpeas (31.4–34.8%) [36]. However, common beans are particularly rich in polyunsaturated fatty acids (PUFAS), 48.4–68.7% of the lipid content, revealing an higher content of linolenic acid (9,12,15-(Z,Z,Z)-octadecatrienoic acid or C18:3, n-3) than linoleic acid (9,12-(Z,Z)-octadecadienoic acid or C18:2, n-6), ratio n6/n3 between 0.5 and 0.9, which is an indication of the common beans' protective effect against degenerative diseases, such as cardiovascular diseases and inflammatory diseases [36, 37].

4.2. Micronutrients

Contrarily to macronutrients, micronutrients are required, by human body, in small amounts performing crucial physiological roles (e.g. metabolism, hormone and enzyme synthesis, immune homeostasis and cell division). Legumes are particularly rich in B-complex vitamins, folate, vitamin E and minerals such as iron, calcium, phosphorus, magnesium, potassium, zinc, copper and selenium [38]. In low- and middle-income countries, highly dependent on legume proteins, the malnutrition by iron deficiency is one of the major worrying public health issues [8]. Although

the iron content of a vegetarian diet may be equal to the iron content of a mixed diet, in a nonvegetarian diet, with red meat, the heme iron, mostly present in the form of hemoglobin and myoglobin (10–12% of the total iron) [39] can be absorbed at a rate of 5–35% in the gut. However, in a vegetarian diet (rich in legumes, vegetables and cereals) where the main form of iron is the nonheme, the intestinal absorption decreases to 2–10% [40].

In countries where legumes are staple food products, consumption of biofortified legumes with iron and other micronutrients, such as zinc, with sources of vitamin C can be a solution for micronutrient malnutrition. The fortification of bean varieties with iron is currently a common practice in several countries, such as Rwanda, Uganda, Democratic Republic of Congo and Brazil, in order to control women and childhood iron deficiencies [41].

## 5. Bioactive compounds

In addition to the nutritional value of legumes in human health, legumes are also a rich source of several minor bioactive compounds (e.g. lectins, enzymatic inhibitors, saponins, phytates, oligosaccharides, sterols and phenolic compounds), whose presence has been linked to several nutraceutical properties [42].

### 5.1. Lectins

Lectins are proteins, globulins, accumulated in the cotyledons' vacuoles, with at least one non-catalytic domain which bind reversibly to carbohydrates or glycoproteins [43].

Many lectins present in raw or under-cooked beans are resistant to acidic and enzymatic proteolysis being absorbed into the blood stream of the animals. The affinity of some lectins (phytohemagglutinin) to the red blood cells results in red blood cells agglutination and hemolytic anaemia [38]. The levels of lectins are not influenced by the soaking process and cooking until getting soft beans (60 minutes) seems to be adequate to eliminate lectins' hemagglutinating activity [44].

*In vitro* studies with the phytohemagglutinin (PHA) of *Phaseolus vulgaris* in cancer cell lines, such as SK-MEL-28, HT-144 and C32 human melanoma, showed the potential of *Phaseolus vulgaris*' lectin in inhibiting cancer cells [45]. *In vivo* studies with mice pre-treated with 0.2 g of PHA/kg, before starting oral 5-fluorouracyl (FU) revealed higher survival of intestinal epithelium functional cells than mice not pre-treated with lectin [46].

5.2. Phaseolin and small bioactive peptides

Phaseolin is a trimeric glycoprotein, highly resistant to *in vitro* and *in vivo* digestion, as a consequence of the compact structure given by the high percentage of β-strands, high glycosylation pattern and hydrophobicity. Heat treatment promotes structural changes in the tertiary and quaternary structures of the protein, increasing susceptibility to enzymatic proteolysis and digestibility [47]. Depending on the molecular weight of phaseolin subunits, phaseolin can be classified as S (Sanilac), T (Tendergreen) and I (Inca) [48].

The small peptides obtained from phaseolin hydrolysis have potential antioxidant and iron chelating activities. After hydrolysis, the

phaseolin chelating activity increases highly, from 18%, before hydrolysis, to more than 81% after the hydrolytic treatment [49].

Besides the antioxidant activity, the common bean's bioactive peptides have also anti-hypertensive, through angiotensin-converting enzyme (ACE) inhibition, hypoglycemic, through α-amylase, α-glucosidase and dipeptidyl peptidase-IV (DPP-IV) inhibition and anti-carcinogenic properties, through cell apoptosis induction [50, 51].

### 5.3. Protease inhibitors

Serine protease inhibitors are traditionally divided into two families: the Kunitz trypsin inhibitors and the Bowman-Birk trypsin/chymotrypsin inhibitors. The Kunitz trypsin inhibitor is predominantly found in soybeans and the Bowman-Birk family is widely present in legume seeds. The Protease inhibitors of common bean (*Phaseolus vulgaris*) are included in the Bowman-Birk family [52]. Similar to the lectins, protease inhibitors protect plant from insects and predators and also protect the seed against fungi and microorganisms after harvesting, extending seeds' shelf life [53].

Protease inhibitors of raw or barely cooked legumes resist to the acidic pH of stomach and to the proteolytic enzymes (pepsin) and reach to the duodenum, interfering with digestion through irreversible inhibition of trypsin and chymotrypsin. Since, in duodenum, protease levels are reduced, protein digestibility is compromised and the absorption of amino acids decreases [54]. Despite the negative impact in serine proteases, the denaturated protease inhibitors have several health-promoting benefits in human health, mostly as anti-inflammatory and anticarcinogenic compounds in *in vitro* and *in vivo* models [55]. Until now the molecular

mechanism underlying Bowman-Birk inhibition in colorectal chemoprevention remains unknown [56].

### 5.4. α-Amylase inhibitors

The α-amylase inhibitors are mostly found in the embryonic axes and cotyledons of the seed as a defensive strategy against predators. These inhibitors prevent starch digestion by blocking the active site of the α-amylase enzyme [57]. The traditional cooking process at 100 °C during 10 minutes inactivates α-amylase inhibitors [57]. Several clinical studies with humans, conducted to characterize the effect of α-amylase inhibitor from raw white beans in weight loss and blood glucose levels, clearly showed the potential of a concentrated extract of white bean, with 3000 α-amylase inhibiting units per gram (before meals with carbohydrates) in reducing body weight, body mass index (BMI), fat mass, waist/hip circumferences, systolic/ diastolic blood pressure, triglycerides and post-prandial spikes in blood sugar, maintaining the lean body mass [58, 59].

### 5.5. Phytosterols

Phytosterols include plant sterols and stanols. Plant sterols are the most predominant sterols in plants, corresponding to unsaturated compounds with a double bond in the sterol ring. β-sitosterol, campesterol and stigmasterol are examples of sterols. Stanols represent only 10% of the total dietary phytosterols and are distinguished from sterols based on the absence of double bonds on the sterol ring (saturated molecules) [60].

Since humans cannot synthesize phytosterols, it must be achieved through the consumption of cereals, legumes, vegetables, fruits and nuts. In legumes, the sterols content is quite variable ranging from 134 mg/100

g, in kidney beans, to 242 mg/100 g, in peas [61]. Common bean show high levels of stigmasterol, 86.2 mg/100 g and 41.4 mg/100 g, in butter and kidney beans, respectively [61]. Dietary phytosterols intake normally ranges between 78 and 500 mg/day [62]. Some negative effects have been related to phytosterols consumption up to one year and include nausea, diarrhea or constipation. However, *in vivo* studies with rats associate phytosterols with several beneficial biological effects including anti-inflammatory and anticarcinogenic effects [60]. Phytosterols have been extensively studied as compounds with the ability to decrease cholesterol levels in the gut [42].

*5.5.1. Phytates*

Phytic acid is accumulated in plant seeds in the form of a salt associated with magnesium, calcium and copper, during the maturation stage. It represents 60–90% of the total phosphorus in the seed [63]. The phytate content in legumes is higher than in cereal-based food items. For instance, in cooked kidney beans it ranges from 8.3 to 13.4 mg/g dry weight (DW) while in wheat bread, the levels are considerably low (3.2–7.3 mg/g DW) [64].

Monogastric animals, including poultry and humans are unable to metabolize phytic acid as a consequence of the lack of the phytase degrading enzymes at gastrointestinal level [65].

The main anti-nutritional effects of phytates result from phytate capacity to chelate minerals such as calcium, zinc, copper and magnesium, reducing the minerals bioavailability on diet [66]. Phytates can also establish non-specific complexes with proteins which are less prone to

digestion by proteolytic enzymes [67]. Processing strategies, such as soaking [68], germination [69], fermentation [64] and the addition of phytases in animal feed [70] and as food additives [71] promote dephosphorylation of phytate improving the nutritional value of legumes. Due to phytate heat-stability, cooking process does not affect phytate content [72]. Despite the anti-nutritional effects, phytates have been related to antioxidant effects [73], anticarcinogenic activity [74], hypolipidemic [75] and hypoglycemic effects [76].

Regarding the impact of phytate in human health and the dose to ensure beneficial/ negative effects, more studies are required and should be a priority for new research lines.

### 5.6. Saponins

Chemically referred as triterpene and steroid glycosides, saponins are formed by one or more carbohydrate units attached to a triterpenoid or steroidal aglycone (sapogenin) [77]. Saponins are soluble in water and its content is reduced during soaking process [78]. The lowest saponin content was obtained when beans were only soaked for 6 h [78]. Saponins can be responsible for a bitter taste and astringency that compromises food intake.

Recognized as anti-nutritional compounds, saponins may reduce nutrients' bioavailability and decrease trypsin and chymotrypsin activity [79]. Despite of the anti-nutritional effects, saponins have been explored as hypocholesterolemic [80] and hypoglycemic compounds [81]. Saponins have also been studied for their anticarcinogenic activity, considering in cell based assays with hepatocellular carcinoma cells (HepG2), fibrosarcoma

cells (HT1080), cervical cancer cells (HeLa), promyelocytic leukemia cells (HL60) and breast cancer cells (MDA-MB-453) [82].

5.7. Phenolic compounds

Phenolic compounds, in common bean, include a huge diversity of secondary metabolites (phenolic acids such as hydroxybenzoic and hydroxycinnamic acids, flavonoids and stilbenes) synthesized from the amino acids phenylalanine or tyrosine, in the phenylpropanoid pathway. The C6-C1 skeleton of benzoic acids is generated by shortening of the hydroxycinnamic acids, **Figure 1**. Flavonoids are characterized by a C6-C3-C6 general structure, formed by two benzene rings (A and B) linked by a three carbon chain (a heterocyclic ring with an oxygen, the C ring), **Figure 1**. Stilbenes have a general structure C6-C2-C6, **Figure 1** [83].

Based on the chemical structure, flavonoids can be classified into six different classes, the flavones, flavanones, flavonols, flavanols, anthocyanins and isoflavones [84]. In **Table 1**, the major differences in the chemical structure of compounds included into the different flavonoids' classes are summarized [84].

In dry beans such as common bean, the majority of phenolic compounds are classified as phenolic acids and flavonoids (including proanthocyanidins). The anthocyanins, isoflavones, flavanols and flavonols are mostly located in the seed coat. The cotyledons are particularly rich in phenolic acids such as the hydroxycinnamic acids (e.g. ferulic and sinapic acids), mostly in esterified and glycosylated forms [85].

The content of phenolic compounds is quite variable depending on the legumes species, cultivar, seed's coat colour pattern, maturity, growing

location, environmental characteristics, storage conditions and processing techniques (e.g. boiling, germination and fermentation) [86]. The dark-coloured varieties have higher qualitative and quantitative diversity of phenolic compounds, especially anthocyanins and proanthocyanidins, than lighter varieties [85]. Flavonols such as quercetin and kaempferol glycoside derivatives have been described in black, pinto, dark red kidney, light red kidney and small red beans collected in the USA [87], in Mexican black, mottled gray, caffeto and pale beans [88] and in the Italian yellow and black seed coat beans [89]. Nonglycosylated isoflavones (daidzein and genistein) have been identified, by LC-ESI-QTOFMS, in Brazilian black varieties of common bean [90]. The phenolic acids derived from benzoic and hydroxycinnamic acids have been studied in Mexican varieties of common beans [88]. The ferulic, sinapic, vanillic and p-hydroxybenzoic acids were the most abundant phenolic acids in the Mexican varieties, regardless of the seed coats' colour [88].

Stilbene compounds, such as resveratrol glucoside, were identified and quantified, by mass spectrometry, in germinated black beans [91].

The anti-nutritional impact of phenolic compounds in human health is related to its inhibitory effect in the digestion enzymes (e.g. α-amylase and pancreatic lipase) [92]. In legumes, particularly rich in tannins, phenolic compounds may also interact with dietary proteins, promoting proteins' precipitation or reducing protease (e.g. pepsin, trypsin and chymotrypsin) accessibility to the hydrophobic sites on the proteins [93], which impairs protein digestibility.

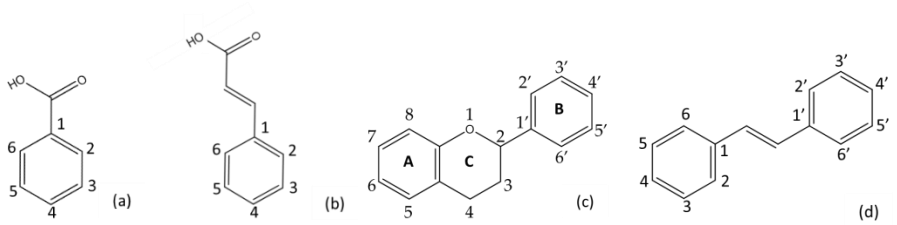


**Figure 1.** General structure of the most predominant phenolic compounds' families in common bean (a) p-hydroxybenzoic acids; (b) hydroxycinnamic acids; (c) flavonoids; (d) stilbenes.

**Table 1.** Chemical structure of compounds included into the different flavonoids' classes.

| Flavonoids Class | Chemical structure characteristics | Examples |
|---|---|---|
| Flavones | • Double bond C2-C3 (unsaturated C ring)<br>• Ketone at C4 of the C ring | Apigenin |
| Flavanones (Dihydroflavones) | • Saturated C ring<br>• Ketone at C4 of the C ring | Naringenin |
| Flavonols | • Double bond C2-C3 (unsaturated C ring)<br>• Ketone at C4 of the C ring<br>• OH- group at C3 of the C ring | Quercetin |
| Flavanols (Flavan-3-ols or Catechins) | • Saturated C ring<br>• OH- group at C3 of the C ring<br>• Ability to form polymers | Catechin<br>Procyanidin B1 |
| Anthocyanins | • Flavylium cations<br>• Majority in glycosidic form (sugars attached at C3) | Cyanidin<br>Cyanidin-3-glucoside |
| Isoflavones | • Double bond C2-C3 (unsaturated C ring)<br>• Ketone at C4 of the C ring<br>• B ring attached to C ring at C3 | Daidzein |

The negative impact of phenolic compounds in nutrients' digestibility can be glimpsed as a potential property of legumes to manage body weight and prevent obesity [93]. The health benefits of phenolic compounds are dependent on phenolic compounds' absorption and metabolism, which is influenced by several factors related to phenolic

compounds' structure, molecular size, solubility, concentration in food, degree of glycosylation, phenolic compounds interaction and phenolic compounds matrix binding interaction, cell wall structure, as well as, by individual factors such as enzyme activity, intestinal transit time, genetics, gender, age, microflora composition and gastrointestinal pathologies [94]. The cluster of mentioned factors indicates that the most concentrated compounds are not necessarily the most bioavailable, in fact regardless of the abundance, most of the hydroxycinnamic acids are in the esterified form which compromises hydroxycinnamic acids' intestinal absorption and bioavailability [95].

The health-promoting effects of common bean phenolic compounds include the antioxidant [96], anti-inflammatory [97], anti-hyperglycemic [98], anti-hyperlipidemic [99] and anticarcinogenic [100] activities. The molecular mechanisms responsible for such biological activities need further study. Moreover, long-term clinical studies are required to establish common beans bioactive compounds' benefits on human body.

## 6. Innovative food products

New "ready-to-eat" food products with inclusion of legumes as ingredients have been flooding the market. In the European Union, 3593 new products have been released between 2010 and 2014 [101].

In what regards common bean innovative food products, bean flour has been incorporated mostly in bakery products and snacks. In Mexico, whole wheat bread has been supplemented with 0.5% of freeze-dried black bean seed coat extract [102]. In Brazil, common bean flour has been added to rice flour and sugar in a proportion of 30:70:5%, respectively, to

produce extruded breakfast flakes [103]. In Canada, a new bean snack, similar to pretzels, composed by 34% of navy bean flour has been developed. In North America, common bean flour has also been incorporated in other snacks such as potato chips and tortilla chips [104]. In Italy, biscuits have been prepared with wheat, maize and common bean flour at different proportions (26.7, 32.1, 50.0, 53.6 and 64.3% of bean flour). The biscuits prepared with a bean flour percentage of 26.7 and 32.1% were accepted with a score similar to the traditional biscuit [105].

The improved quality of the new food products that include legumes as ingredients represent a new market challenge and a concerted action between research community and food industry with divulgation of the potential health benefits should be mandatory to increase legumes consumption.

## 7. Conclusions

The Fabaceae family includes a huge number of species that can bring diversity, nutrient supply and disease control to cropping systems. In Europe, beans production and consumption decreased drastically in the last decades. Nutritionally different from starchy foods, legumes have higher protein, amylose, fiber, folate and minerals contents. Therefore, the inclusion of legumes in a daily diversified diet is one of the best nutritional strategies to prevent malnutrition. Legumes are also a rich source of bioactive compounds (e.g. enzymatic inhibitors and phenolic compounds). The content of such compounds in plants is quite variable depending on the plant genotype and on the environmental and processing conditions. In fact, most of the anti-nutritional effects can be inactivated toward

preparation and processing techniques (e.g. soaking, peeling, boiling, fermentation and germination). Recent research on the impact of bioactive compounds on health showed their potential to exert biological actions as antioxidant, anti-inflammatory, anti-hyperlipidemic, anti-hyperglycemic and anticarcinogenic compounds.

Future research lines should focus on the characterization of legume genetic diversity, development of reliable and quick screening assays of quality-related traits to improve varieties in legume breeding programs, update of legume consumption in each country and bioavailability studies (including assays regarding the effective doses of bioactive compounds responsible for significant biological actions in clinical studies).

## Thesis Objectives

In order to overcome gaps of knowledge related to the nutritional and anti-nutritional composition of common beans, the work developed in the present thesis aimed to:

**(1)** Study the nutritional and the metabolomics diversity of underexploited common bean accessions, such as the ones in the Portuguese germplasm, cropped under contrasting environments;

**(2)** Evaluate the impact of processing (soaking and peeling) in common beans' phenolic composition;

**(3)** Characterize the metabolites present in human plasma and urine after cooked common beans consumption;

**(4)** Study the impact of common bean flour as alternative ingredient in bakery products (cookies) nutritional quality, human glycaemia and satiety perception.

## Thesis Outline

To cope with the thesis objectives, the following chapters of the thesis were written in the form of research articles, respecting the structure required by the corresponding journal for submission.

In the second chapter the nutritional and protein quality of underexploited Portuguese common bean varieties cropped under contrasting environments (traditional *versus* stressful) was described. For the first time the diversity of a representative collection of Portuguese varieties regarding their nutritional value was revealed. In this chapter and taking in account the future challenge of growing common bean varieties in heat stress conditions, the protein quality of the Portuguese common bean accessions was further analyzed for the accessions cropped under the warmest environmental conditions.

In the third chapter an untargeted metabolomics approach to the different Portuguese common bean accessions cropped under two contrasting environments (traditional *versus* stressful) was pursued. In this chapter, the different metabolites responsible for samples' discrimination were identified and their relative quantification performed to distinguish the different Portuguese common bean accessions.

In the fourth chapter a characterization of the phenolic composition in different common bean fractions (soaking water, soaked coats, soaked cotyledons and whole flour) was made.

In the fifth chapter, a human bioavailability study conducted to characterize, by targeted metabolomics, some metabolites in human plasma and urine, derived from the metabolism of phenolic compounds after common beans consumption, was reported.

In the sixth chapter, the capillary glycaemia and satiety perception of healthy volunteers after consumption of common bean enriched cookies was evaluated to show the potential of common bean flour as an alternative ingredient in healthier ready-to-eat food products.

Finally the seventh chapter presents a general discussion, which aims to articulate the previous chapters of this thesis and propose future perspectives considering the work performed so far.

Overall through the use of multiple chemical characterization approaches this thesis contributed for the valorization of underexploited common bean accessions, generating meaningful knowledge to researchers, breeders, farmers, consumers and food industry, highlighting the importance of common beans consumption, in a daily basis diet.

## Acknowledgements

The authors acknowledge the financial support provided by the FP7-EU project Strategies for Organic and Low-input Integrated Breeding and Management (SOLIBAM), FCT, Portugal for the funded project – “Exploiting Bean Genetics for food Quality and Attractiveness Innovation” (BEGEQA), PTDC/AGR-TEC/3555/2012BEGEQA and Elsa Mecha PhD fellowship (SFRH/BD/89287/2012), Maria Carlota Vaz Patto, FCT Investigator Program Development Grant (IF/01337/2014), and through R&D unit, UID/Multi/04551/2013 (GreenIT).

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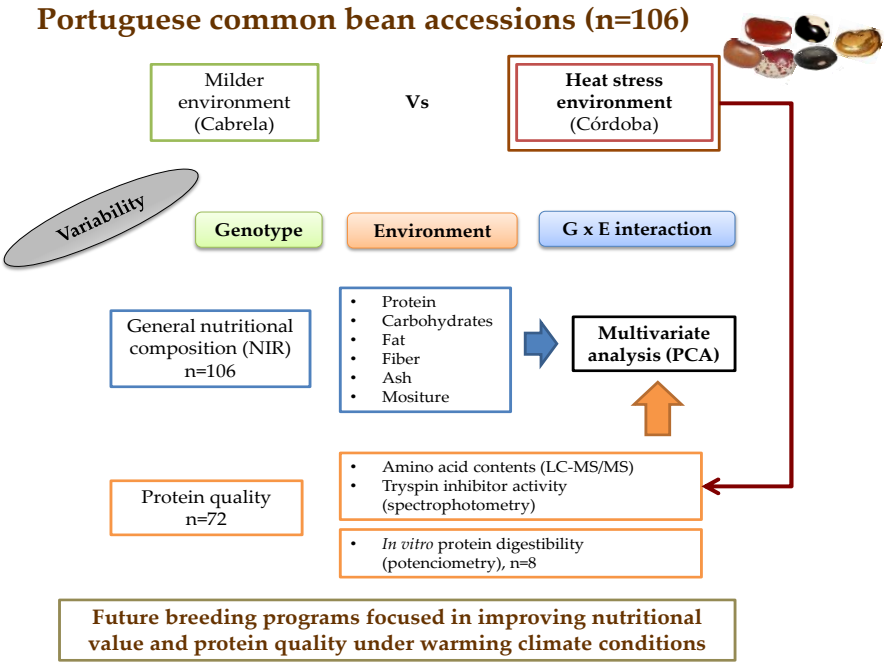
# Chapter II




This Chapter was submitted and accepted by Agronomy *as*,

**Mecha, E.**, Natalello, S., Carbas, B., Bento da Silva, A.; Leitão, S.T., Brites, C., Veloso, M. M., Rubiales, D., Costa, J., Cabral, M.d.F., Figueira, M.E., Vaz Patto, M.C. & Bronze, M.R. Disclosing the nutritional quality diversity of Portuguese common beans—The missing link for their effective use in protein quality breeding programs. *Agronomy* **2021**, 11(2), 221; DOI:10.3390/agronomy11020221.

In this Chapter, Elsa Mecha participated in the experimental work, data analysis, manuscript drafting and final manuscript writing.

## Disclosing the nutritional quality diversity of Portuguese common beans — The missing link for their effective use in protein quality breeding programs

### Abstract

The common bean (*Phaseolus vulgaris* L.) represents a sustainable and affordable source of protein, namely to populations with vegetarian dietary habits. Despite the national germplasm genetic diversity, little is known about the Portuguese accessions' nutritional and protein quality, leading to their underuse in breeding programs. To fill this gap, a representative collection (106 accessions) was cropped under two contrasting environments (traditional *versus* heat stress) and evaluated in terms of nutritional quality by near-infrared spectroscopy. Protein quality was assessed, under the stressful environment, considering the individual amino acid contents and the activity of trypsin inhibitors, through mass spectrometry (LC-MS/MS) and spectrophotometry, respectively. On top of strong genotypic control, the nutritional composition (protein, fat, fiber, moisture and ash) was also highly influenced by the environment and by genotype × environment interaction, with a clear nutritional quality ranking change for the accessions in heat stress conditions. Classified into three clusters, the accessions from the cluster with the highest individual amino acid and protein contents also showed higher trypsin inhibitor activity (TIA). Since different levels of TIA had no translation into contrasting protein digestibility, breeders focusing on common beans' protein quality improvement, especially under challenging warming climate conditions, may take advantage of this group of accessions.

**Keywords:** common bean; variability; food sustainability; nutritional value; protein quality; amino acid; trypsin inhibitors; protein digestibility

## 1. Introduction

According to the World Health data platform, worldwide, in 2019, 21.3% (144 million), 6.9% (47 million) and 5.6% (38 million) of all children under 5 years old were suffering, respectively, from stunting (low height for age), wasting (low weight for height) or overweight (excess weight for height) [1]. Factors such as limited natural resources and vital cropland, poor investment in affordable sustainable food systems and the lack of access to local food diversity are among the major causes of malnutrition [2, 3]. To avoid malnutrition and its negative impacts at individual and social levels, populations should be aware of the importance of a healthy diet [4], and have access to affordable dietary sources of protein such as legumes. Despite legumes' undeniable ecological and nutritional value in food and feed systems [5, 6], through enhancement of soil's nutritional state and diversification of farming systems [5, 6], in Europe, between 1961 and 2013, governmental policies encouraged farmers to produce cereals intensively, thereby reducing the legume cropland. This led to increased external dependence on vegetable protein for human and animal consumption [7–9]. Furthermore, at a global scale, climate change is one of the most important challenges that affect food production, including legumes. Prediction models anticipate an increase of temperature in the order of 2–4 °C over the next century, which will affect crops, especially at the reproduction stage (flowering and seedling) [10, 11]. The aggravation

of existing agronomic problems due to climate change will be particularly important for the Mediterranean basin [12].

Under stressful environmental conditions (e.g. water deficit, nitrogen-deficient soils) the interactions established between ureidic legume species such as *Phaseolus vulgaris* L. and soil bacteria will mediate crop productivity [13]. As a rhizobial, nodulated, $N_2$-fixing legume, the symbiotic relationships of Actinobacteria, Bacteroidetes and Firmicutes facilitate nutrient uptake and seeding under challenging environments. The fixed atmospheric nitrogen is reduced by nodule bacteria into ammonia which subsequently participates in purine synthesis (e.g. uric acid) in the form of glutamine. The hydrolysis of uric acid gives rise to ureides (allantoin and allantoic acid) that represent 86% of the nitrogen in plant xylem [14]. Although the interactions of soil microbiome and plant genotypes will require further investigation, the value of these ureides as the main forms of nitrogen transport and storage in the nodulated nitrate fed-plants is well recognized. Nevertheless, after nitrogen fertilization (nitrate fed-plants), the amides asparagine and glutamine are the major compounds responsible by the transport and storage of nitrogen [14]. Studies dedicated to the identification of genotypes capable of producing high quality seeds at supra-optimal temperatures, making use of genotype (G) × environment (E) interactions by breeding for specific adaptations, are still scarce and should be a priority for grain legume breeders to ensure viable adapted crops with nutritional quality for future generations [10, 11]. Grain legumes represent a rich source of protein [5, 6]. In fact for some communities, particularly those with vegetarian dietary habits, grain legumes are the main source of dietary protein.

The improvement of protein yield has been one of the major breeding goals for legumes, relegating protein quality to a secondary position [15]. Protein quality, defined by Food and Agriculture Organization (FAO) as the capacity of a food protein source and diet to meet the protein and essential amino-nitrogen requirements [16], can be evaluated in terms of amino acid composition and protein digestibility. Amino acids have been traditionally classified as essential and "non-essential" (**Table S1**) [17]. Contrarily to "non-essential" amino acids (NEAAs), the essential amino acids (EAAs) must be provided by the diet, since their carbon skeleton cannot be synthesized in living organisms or the synthesis rate is not adequate to sustain normal growth and health. Nevertheless, the NEAAs have recently been considered indispensable for living organisms' survival, rendering the term "non-essential" as inadequate [18].

To evaluate protein digestibility, several *in vitro* and *in vivo* methods have been described. Due to the high correlation with *in vivo* protein digestibility, *in vitro* methods have gained researchers attention due to their simpler and less expensive application [19]. In a plant genotype-dependent way, the protein quality of legumes can be impaired by the reduced content of sulfur amino acids (methionine and cysteine) and by the presence of anti-nutritional factors (such as enzymatic inhibitors, saponins and tannins) that interfere with protein digestibility. Other factors that influence legumes' protein quality include environmental conditions during the growing season and food preparation methods (e.g., traditional cooking or microwaving) [20]. Strategies such as blending legumes and cereals in the diet to balance amino acid intake and/or selecting through breeding programs, from locally adapted legume collections, the most promising genotypes in

terms of amino acid contents, represent effective approaches for protein quality improvement in foods [21].

The breeding strategy should be promoted to ensure the future access of human populations to viable legume varieties characterized by higher protein quality. In Portugal, despite the high genetic diversity among common bean accessions [22], there is a lack of information regarding their nutritional and protein quality, hampering their exploitation in national or worldwide breeding programs and their contribution to a sustainable high quality diet. The present research was conducted to overcome this gap in knowledge. The initial focus was on the overall nutritional composition of a representative collection of Portuguese common bean accessions cropped under contrasting environments (traditional *versus* heat stress environment) and afterwards on the protein quality (amino acid composition, trypsin inhibitor activity and *in vitro* protein digestibility) of the accessions cropped under the most stressful environment. Regarding the nutritional parameters, this research was designed to characterize the existent variability among Portuguese common bean accessions, identifying for the first time the most promising common beans accessions, sources of high protein quality in a stressful environment, mimicking future climatic changes. Moreover, this research aimed to enrich the existent worldwide legume composition databases, which are currently missing a detailed characterization of the nutritional parameters and amino acid contents in the national common bean accessions [19]. By doing so, we will be also promoting consumption, preservation and the introduction of Portuguese common bean accessions into future national or worldwide

breeding programs more focused on the improvement of the common bean's protein quality.

## 2. Materials and Methods

### 2.1. Chemicals

Milli-Q® water (18.2 MΩ·cm) was obtained from a Millipore–Direct Q3 UV System (Molsheim, France). Chloride acid (HCl), formic acid (98% p.a) and acetonitrile for HPLC Plus gradient grade were purchased from Carlo Erba Reagents SAS (Val de Reuil, France). Phenol BioXtra ≥ 99.5% (GC), nonafluoropentanoic acid, bovine trypsin from bovine pancreas ≥ 10,000 BAEE units/ mg protein, calcium chloride dehydrated ($CaCl_2.2H_2O$), benzoyl-L-arginine-p-nitroanilide (L-BAPA), dimethyl sulfoxide (DMSO), tris(hydroxymethyl)aminomethane, glacial acetic acid (≥99%), sodium hydroxide (NaOH), porcine trypsin type IX-S, bovine α-chymotrypsin, type II, *Streptomyces griseus* protease, type XIV and casein from bovine milk were obtained from Sigma-Aldrich (St. Louis, MI, USA). Amino acids standard H was purchased from Thermo Fisher Scientific (Rockford, MA, USA).

### 2.2. Plant Material

A collection of 106 common bean (*Phaseolus vulgaris* L.) accessions was provided by the Research Unit of Biotechnology and Genetic Resources germplasm bank, located at INIAV, Oeiras, Portugal. These accessions were selected to represent varieties collected from all traditional common bean growing regions in Portugal. In order to study the impacts of genotype and environment on the general nutritional

composition, meaning macronutrients (protein, carbohydrate and fat), fiber, ash and moisture contents, the different accessions were cropped in field trials using a randomized complete block design with two replicates in two different environments. Cabrela represented a standard common bean production area in Portugal (Global Positioning System, GPS, coordinates: latitude–38º52'6.816" N and longitude–9º21'5.804" W) and Córdoba, a heat stress prone production area in Spain (GPS coordinates: latitude–37º53'29.58" N and longitude–4º46'21.90" W). The two environments were characterized by different average temperature ranges (18–21 °C in Cabrela and 15–32 °C in Córdoba), different average ranges of relative humidity (66–80% in Cabrela and 31–63% in Córdoba) during the growing season [23] (**Figure S2**) and different soil types. In Cabrela the soil was classified as eutric cambisol and in Córdoba as fluvisol [24]. In Cabrela, the growing season extended from May to September 2014 and in Córdoba from March to July 2015. The two field trials were established under artificial irrigation and a NPK fertilizer was applied at sowing in a rate of 250 kg/ha. Mature dried seeds (from a total of 106 accessions) were collected—66 accessions from the two field trials, 12 and 28 exclusively collected from Cabrela and Córdoba, respectively. The mature seeds collected from Cabrela were artificially dried in a seed drying room under continuous air flow. Each accession's final yield per plot was measured at harvest and expressed as kg/ha. Protein quality was evaluated only for the accessions cropped under the most stressful environment (Córdoba). Data relative to seed colour and pattern, seed size, gene pool and geographical origin were detailed previously [22], and summarized in **Table S2**.

## 2.3. Sample Preparation

The mature dried seeds were milled (Falling nº 3100–Perten, Sweden) to a particle size of 0.8 mm and stored at −20 °C, until further analysis.

## 2.4. General Nutritional Composition

### *2.4.1. Total Protein, Fat, Fiber, Moisture and Ash Content*

Total protein, fat, fiber, moisture and ash (%) were determined by a near-infrared (NIR) analyzer (MPA; Bruker, Billerica, MA, USA), using the flour calibrations for grain legumes provided by Bruker, as described by Serrano et al. [25].

### *2.4.2. Calculated Total Carbohydrate Content*

As determined in FoodData Central, by USDA [26], the total carbohydrates were determined following the equation (Equation **(1)**)

$$\text{Total Carbohydrates (calculated)} = 100\text{-(Total Protein} + \text{Total Fat} + \text{Moisture} + \text{Ash)} \quad \textbf{(1)}$$

## 2.5. Protein Quality

### *2.5.1. Amino Acids' Extraction*

The extraction of amino acids was performed according to Jajic et al. [27]. Briefly, 0.5 g of common bean seed whole flour was hydrolyzed, in a solution of HCl 6 M with 0.1% of phenol (7 mL), during 6 h at 150 °C. After dryness in a Speedvac concentrator (Labconco®, Kansas City, MO, USA), HCl 0.1 M (5 mL) was added to each tube and vortexed. The mixture was then centrifuged at 5000× g during 15 min. The supernatant

was collected and filtered using cellulose acetate filters 0.20 µm. The final extract was preserved at −20 °C, until analysis. The extraction was performed in duplicate.

*2.5.2. Amino Acid Content*

The LC-MS/MS system used was a Waters Alliance 2695 HPLC system coupled to a triple quadrupole mass spectrometer, Micromass® Quattro micro API (Micromass, Waters, Milford, MA, USA), equipped with an electro spray ionization source (ESI). The chromatographic separation was performed in a Mediterranean Sea 18, 5 µm 20 × 0.21 cm, 1.8 µm, (Teknokroma®, Barcelona, Spain) column at 45 °C. The eluents, an aqueous solution of 0.1% formic acid with 0.15% of nonafluoropentanoic acid (eluent A) and an acetonitrile solution of 0.1% formic acid with 0.15% of nonafluoropentanoic acid (eluent B), were applied in a gradient mode during 45 min at a flow rate of 0.3 mL/min. The gradient elution started with 2% of eluent B and kept at such concentration for three minutes. Then the percent of eluent B increased to 25% in 22 min, remaining at this concentration during two minutes. The initial conditions, 2% of eluent B, were re-established in 18 min. The amino acids' extracts were diluted 1:1000 in eluent A before analysis and kept in the auto sampler at 10 °C, until injection. The sample injection volume was 20.0 µL. The ionization source temperature was set at 130 °C with a cone voltage of 20.0 V and capillary voltage of 2.70 kV. Nitrogen ($N_2$) was used as drying and nebulizing gas and Argon (Ar) as the collision gas. Overall, 16 amino acids (Ser; Asp; Gly; Glu; Thr; Ala; Pro; Val; Met; His; Tyr; Lys; Ile; Leu; Arg and Phe) were analyzed by multiple reaction monitoring (MRM) mode, using an

ESI source operating in ion positive mode, **Figure S1**. Distinct MRM transitions allowed the quantification of the different amino acids. The most abundant product ion was selected for quantification and the second most abundant as the qualifier ion, **Table S3**. Amino acids were identified by comparison with the amino acids' standards retention time and corresponding *m/z* values. For quantification purposes, calibration curves were prepared with solutions of amino acids' standards in eluent A at different concentrations (between 3.8 and 30 µM). The lowest concentration used for quantification was above the limit of quantification (LoQ) defined as a signal-to-noise ratio of ten [28]. MassLynx software, version 4.1, (Waters, Milford, MA, USA) was used to acquire and process data. The final amino acid content was expressed as g/16 g of nitrogen (N).

*2.5.3. Trypsin Inhibitor Activity*

The trypsin inhibitor activity of common bean accessions was determined according to the ISO 14902:2001(E) [29]. Briefly the activity of the trypsin inhibitors was measured against a bovine trypsin stock solution preserved at 10 °C during a maximum period of 5 days (27 mg in 100 mL of $CaCl_2 \cdot 2H_2O$–735 mg: 1 L HCl 0.001 M, pH 3.0 ± 0.1) which was diluted (1:20) in $CaCl_2.2H_2O$. After preparing 100 mL of L-BAPA solution, in the assay day, with 60 mg of L-BAPA in 1 mL of DMSO and 99 mL of Tris buffer/$CaCl_2.2H_2O$ (prepared by adding 6.05 g, Tris-buffer and 735 mg $CaCl_2.2H_2O$ to 900 mL Milli-Q® water, adjusted to pH 8.2 ± 0.1 with HCl 6M and diluted to 1 L with Milli-Q® water), the trypsin activity was evaluated after adding, L-BAPA, Milli-Q® water, trypsin solution and acetic acid in a proportion of 1:0.6:0.2:0.2, and compared to a blank prepared in the same

conditions, which only difference was the addition of acetic acid before the trypsin solution. Before adding the trypsin solution, the mixture was incubated at 37 ± 0.25 °C for 10 min in a water bath with a controlled temperature. After centrifugation at 1500× g, during 10 min, the absorbance of the mixtures was evaluated against Milli-Q® water, at 410 nm, in a Genesys 10UV Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The enzymatic activity of the bovine trypsin was considered acceptable if, Trypsin absorbance − Blank absorbance = 0.380 ± 0.050

To extract the inhibitors of trypsin, 0.5 g of common bean flour was added to 25 mL of NaOH 0.01M and the pH adjusted to 9.5 ± 0.1 with HCl 1M or HCl 0.1M. The mixture was kept at 0–4 °C for 15–24 h in Milli-Q® water. On the analysis day the refrigerated water was added to the mixture to complete a final volume of 50 mL. After 15 min, a dilution range between 4 and 10 mg/g was tested, for each sample, with Milli-Q® water at the room temperature, in order to estimate a value of inhibitor trypsin activity between 40% and 60%. For each sample it was prepared a sample tube and a blank according to the following scheme: 1 mL of L-BAPA: 0.2 mL of diluted sample: 0.4 mL of Milli-Q® water: 0.2 mL of trypsin: 0.2 mL of acetic acid. In the blank the acetic acid was added before the trypsin solution, as mentioned previously. Once again the sample and the blank sample were kept at 37 °C, during 10 min, before adding the trypsin solution, remaining at such temperature during an additional period of 10 min. After centrifugation at 1500× g, during 10 min, the absorbance was measured at 410 nm and the inhibition percentage calculated according to the formula (Equation **(2)**):

$$i\ (\%) = \frac{(\text{Trypsin absorbance} - \text{Trypsin blank absorbance}) - (\text{Sample absorbance} - \text{Sample blank absorbance})}{\text{Trypsin absorbance} - \text{Trypsin blank absorbance}} x\ 100 \quad \textbf{(2)}$$

Based on the inhibition percentage it was possible to determine the trypsin inhibitor activity (TIA), Equation **(3)**:

$$TIA\left(\frac{mg}{g}\right) = \frac{i}{100} x \frac{trypsin\ mass\ (mg)\ x\ \left(\frac{100}{volume\ of\ diluted\ sample}\right)\ x\ 0.00028}{\text{Sample}'s\ mass\ (g)} \quad \textbf{(3)}$$

where, *volume of diluted sample* was the tested volume of sample (mL) responsible by 40–60% inhibition. Analyses were performed in duplicate for each sample. Values below 0.5 mg/g were below the detection limit of the method [29].

*2.5.4. In Vitro Protein Digestibility*

After hierarchical cluster analysis (Ward's method) of TIA values, eight selected accessions characterized by belonging to two distinct clusters of TIA (four accessions from each TIA cluster) were evaluated in terms of *in vitro* protein digestibility (IVPD) according to the pH-drop procedure proposed by Tinus et al. [30] and by comparison with the *in vitro* digestibility of bovine casein. Briefly, common bean raw whole flour, equivalent to 62.5 mg of protein, was weighted. Milli-Q® water (10 mL) was added to the flour and mixed with a magnetic stirrer bar. The mixture was incubated at 37 °C, during 1 h and the initial pH adjusted to 8.0 with 0.1 M NaOH and/or HCl. A multi-enzyme solution (10 mL) of porcine trypsin (16 mg), bovine chymotrypsin (31 mg) and *Streptomyces griseus* protease (13 mg) was prepared, on the analysis day, and kept at 37 °C. The pH of the multi enzyme solution was also adjusted to 8.0. The pH drop was automatically recorded as a digestogram, every five seconds during 15

min, after adding 1 mL of the multi-enzyme solution to the 10 mL sample dispersion, in a pH meter Metrohm 703 Ti Stand with stirrer and pump (Metrohm, Herisau, Switzerland). For the cooking process, after mixing the common bean whole flour with milli-Q® water, and before pH adjustment, samples were cooked in boiling water (100 °C) [31], during 2 h [32] in an oil bath with controlled temperature. After 2 h, the samples were left at room temperature in order to proceed with the pH adjustment at 37 °C and multi-enzyme digestion, as described above for the raw whole flour. The analyses were performed in single trials for each sample, considering raw and corresponding cooked common bean accessions, with two field replicates for each sample. The *in vitro* protein digestibility (IVPD%) was calculated following the equation (Equation **(4)**):

$$IVPD\ (\%) = 65.66 + 18.10 \times (\Delta pH\ 10\ min) \quad \textbf{(4)}$$

*2.5.5. Calculated Protein Quality*

The calculated protein quality was evaluated in terms of amino acid score by comparison of the EAAs content with the scoring pattern recommended for children from 2 to 5 years old [33]. The limiting amino acid was defined as the amino acid with the lowest score [16]. The theoretical protein efficiency ratios (PER) values were determined according to the equations described by Pastor-Cavada et al. [34].

$$PER\ 1 = -0.684 + 0.456 \times Leu\ - 0.047 \times Pro \quad \textbf{(5)}$$

$$PER\ 2 = -0.468 + 0.454 \times Leu - 0.105 \times Tyr \quad \textbf{(6)}$$

$$PER\ 3 = -1.816 + 0.435 \times Met + 0.78 \times Leu + 0.211 \times Hys - 0.944 \times Tyr$$ **(7)**

The *in vitro* protein digestibility corrected amino acid score (IVPDCAAS) was determined according to Equation **(8)**.

$$IVPDCAAS\ score = Lowest\ amino\ acid\ \times IVPD$$ **(8)**

2.6. Statistical Analyses

Assumptions of normality (Kolmogorov–Smirnov test and normal Q-Q plots) and variance homogeneity (Levene's test) were tested at a significance level of 1%. The accessions final yield was transformed by square root transformation to improve the normality of residuals. The yield frequency distribution on the two environments was compared and represented by histogram plots. The main effects of G, E and G × E interactions were tested by nested ANOVA for each nutritional parameter (protein, total carbohydrates, fat, fiber, ash and moisture), at a significance level of 5%. Significant differences were defined by *post-hoc* Tukey HSD test. $Eta^2$ (%), defined as a measure of the effect size (proportion of variance between explained and predictor variables, after controlling for other predictor variables) [35] was used to analyze the contribution of the different factors (G, E and G × E interaction) to nutritional parameters' variability. The adjusted $R^2$ associated with the ANOVA models was applied to evaluate the quality of the models used to explain the nutritional parameters' variability. For each environment, the mean values of the different nutritional parameters were compared by one-way ANOVA considering the geographical origin, the gene pool and the morphological

characteristics, namely seed coat colour and seed size of each accession, as fixed factors, at a significance level of 5%. A t-test for independent samples was performed to compare accessions' mean yields using the environment as a grouping variable, at a significance level of 5%. Multivariate principal component analysis (PCA) was performed with the accessions collected from both environments, considering the general nutritional parameters (protein, total carbohydrates, fat, fiber, ash and moisture) in order to establish differences in the nutritional composition between accessions and the effect of the two contrasting environments. The accessions cropped under the heat stress environment (Córdoba) were selected for further analysis of protein quality. The PCA analysis with accessions collected from Córdoba, analyzed in terms of amino acid content, TIA and protein content, was carried out after expressing all the parameters as g/100 g, and gathering suggested genotypes' spatial distributions, based on protein content and quality. The number of retained components was based on Kaiser's criterion (eigenvalues higher than one), and was further used in articulation with cluster analysis. The number of clusters was defined after saving the range of solutions, K = 2 to K = 6, obtained by Ward's hierarchical clustering analysis method, based on an explained variance ($R^2$) higher than 50%. K-means algorithm was applied for cluster analysis' optimization. Differences between clusters were established by ANOVA, at a significance level of 5%, and *post-hoc* analysis established by Scheffé's test.

## 3. Results and Discussion

The nutritional and protein quality of a common bean variety influences its economic value [36], and so these criteria are gaining supporters among legume breeders for common bean selection [21], especially in developing countries where the priority should be the development of adapted nutritionally-rich legumes to supply populations' dietary needs. In Portugal, despite the high genetic and morphological diversity detected among traditional common bean accessions [22], indicating a promising breeding potential, the nutritional and protein quality of Portuguese accessions have been underexplored. This has hampered their effective use in breeding programs, either for wide or for specific adaptation, reducing their contribution for agriculture and food sustainability, worldwide. This study aimed to overcome this gap of knowledge by characterizing the nutritional composition diversity in Portuguese common bean accessions, and the environmental effect on this diversity by cropping these accessions in two contrasting environments. The protein quality diversity of Portuguese common beans was also investigated under the most stressful environment, mimicking the expected increase in temperature due to climate change, and all the knowledge will be fundamental for efficient and effective use of this germplasm in common bean breeding programs.

### 3.1. Diversity in the Nutritional Composition of Portuguese Common Beans

As shown in supplementary material, **Table S4**, by the number of homogeneous subsets of common bean accessions for each evaluated nutritional parameter, the Portuguese accessions were characterized by

high variability in the macronutrients (protein, carbohydrates and fat), and in the fiber, ash and moisture contents.

Multivariate PCA obtained with the nutritional data collected from Cabrela (milder traditional environment) and Córdoba (heat stress environment) field trials showed that 75.4% of the total variability was explained by the two first principal components, **Figure 1**.

With the exception of moisture, whose variance was roughly explained by the first two principal components (communality lower than 0.4), all the remaining parameters contributed significantly to common beans' dispersion along the two first principal components. Protein, ash and carbohydrate contents were the major nutritional parameters responsible for common bean accessions' spatial distribution along the first principal component. By comparison with the traditional environment for growing common beans, the stressful environment of Córdoba, characterized by higher fluctuations in ambient temperature and relative humidity (**Figure S2**) imposed changes in common beans' nutritional composition. On average the common beans cropped under the heat stress environmental conditions of Córdoba were characterized by higher protein, ash, fiber and moisture contents, but also by lower carbohydrate content than the accessions collected from the milder, traditional environment of Cabrela, $p < 0.05$, **Table 1**.

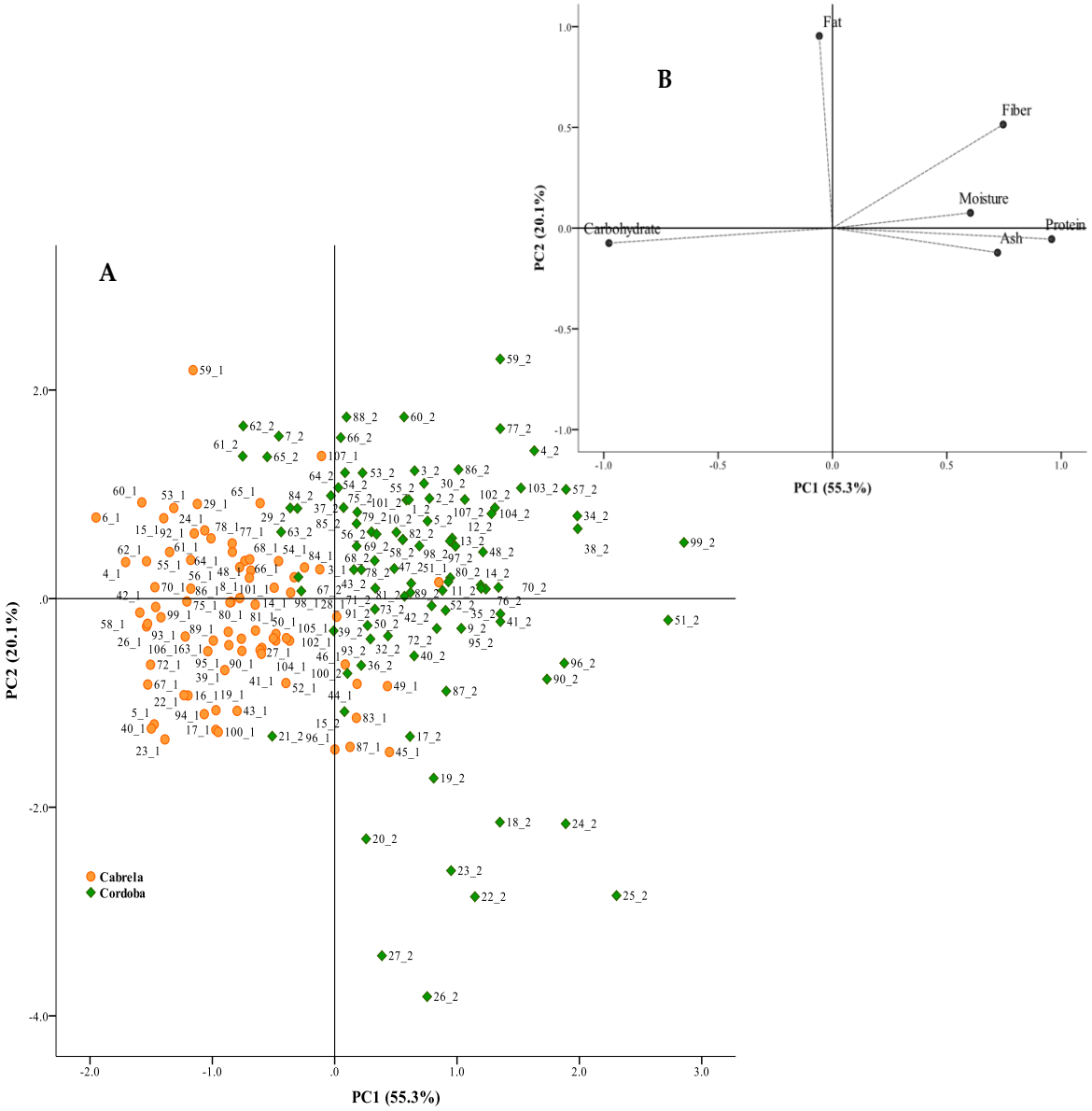



**Figure 1. (A)** Score plot of Portuguese common bean accessions (n = 66) cropped in Cabrela and Córdoba, in a bi-dimensional space (PC1 and PC2), which accounted to 75.4 % of the total variance. Different common bean accessions were identified according to **Table S2**, followed by numbers 1 or 2 to indicate the corresponding environment. 1−Cabrela; 2−Córdoba. **(B)** Projection of the variables responsible for accessions' dispersion.

**Table 1.** Averages ± standard deviations of protein, carbohydrates, fat, fiber, ash and moisture contents (g/100 g) obtained for common bean accessions cropped in the two contrasting environments (Cabrela and Córdoba). In each row different letters indicate significant differences ($p < 0.05$) and equal letters the absence of differences ($p > 0.05$).

| Parameters | Cabrela, 2014 (*n* = 79) | Córdoba, 2015 (*n* = 93) |
|---|---|---|
| Protein | 21.26 ± 1.42 [a] | 24.10 ± 1.66 [b] |
| Carbohydrates | 60.58 ± 1.50 [b] | 56.68 ± 1.75 [a] |
| Fat | 1.44 ± 0.23 [a] | 1.49 ±0.47 [a] |
| Fiber | 5.75 ± 0.44 [a] | 6.77 ± 0.72 [b] |
| Ash | 3.15 ± 0.08 [a] | 3.25 ± 0.13 [b] |
| Moisture | 13.56 ± 0.45 [a] | 14.49 ± 0.51 [b] |

Nevertheless, a significant G × E cross-over interaction led to an accession ranking change between the two environments for the evaluated nutritional traits, **Figure 2**. Since no significant differences ($p > 0.05$) were detected for average yield productions between the two environments—although there was higher variability at Córdoba, as depicted in **Figure S3**—variations in seed nutritional contents between Cabrela and Córdoba could not be due to the commonly known concentration or dilution seed yield effects. Nevertheless, in both environments, moderate negative correlations between yield and protein content were detected (Pearson's R coefficient of −0.402 in Cabrela and −0.407 in Córdoba, $p < 0.05$), which is in accordance with the inverse relationships found between seed yield and protein content in other legumes [37, 38]. Artificial irrigation probably contributed to reduced average yield differences between both environments. Therefore, the higher contents of carbohydrates and protein, respectively, in Cabrela and Córdoba field trials, suggested that heat environmental differences could be the main factor responsible for the ratio of carbon/ nitrogen in legumes seeds. A strong negative correlation between carbohydrate and protein contents was detected in both

environments (Pearson's R coefficient of −0.970 in Cabrela and −0.954 in Córdoba) in agreement with what was previously reported for Spanish varieties [39]. As shown in **Figure 3**, the environment was one of the most important sources of variation in carbohydrates ($Eta^2$ = 50%). Supporting these findings, previous literature has reported the effect of rising temperature (30–35 °C) in the repression of photosynthetic enzymatic machinery and consequent carbohydrates production [39, 40]. Additionally, under high ambient temperature and reduced air humidity, common bean seeds tend to accumulate soluble amino acids/proteins and/or assimilate/remobilize nitrogen from the vegetative parts of the plant in order to preserve the nutritional supply of nitrogen for the normal development of the embryo kept inside the seed [11, 40]. Beyond the genotype and environmental impacts on moisture variability, the higher content determined in common bean seeds collected from Córdoba could be also attributed to the natural drying process of the mature seeds without additional artificial dryness. The fat content was not significantly different among common bean accessions cropped in the two contrasting environments. Nevertheless, this parameter allowed accessions' dispersion along the second principal component, in response to the presence of genotypic variability among the Portuguese common bean accessions.

From all sources of variation (G, E and G × E interaction effects), G was the only one relevant for the variability of all the studied parameters, particularly of the fat content ($Eta^2$ = 75.8%), **Figure 3**, **Table S5**.

Compared with the nutritional value of white common beans described by USDA [26], the Portuguese white accessions stood out by their higher fat content (1.79 ± 0.20 g/100 g in Cabrela and 1.74 ± 0.33

g/100 g in Córdoba, against 0.85 g/100 g in USDA database). On average, the Portuguese white accessions showed higher fat content than the coloured ones (1.79 ± 0.20 g/100 g against 1.40 ± 0.20 g/100 g in Cabrela and 1.74 ± 0.34 g/100 g against 1.43 ± 0.29 g/100 g in Córdoba) and the small seed accessions characterized by higher fat content than the large-seeded ones (1.57 ± 0.15 g/100 g against 1.40 ± 0.20 g/ 100 g in Cabrela and 1.64 ± 0.20 g/100 g against 1.41 ± 0.32 g/100 g in Córdoba). With exception of fat, in both environments, there were no significant differences for the remaining nutritional parameters (protein, carbohydrates, fiber, ash and moisture) among morphologically distinct accessions (different seed coat colours and seed sizes). The different gene pools or geographical origins did not differ significantly in the studied nutritional parameters' contents measured in the two contrasting environments. The traditional seed exchange between Portuguese farmers over centuries might have contributed to a dilution of particular differences within each gene pool or each geographical origin. Indeed, the Portuguese common bean germplasm is characterized by a considerable percentage of accessions with a mixed origin between the main common bean gene pools [22]. The higher dispersion of common bean accessions collected from Córdoba field trial supported the existence of high variability among accessions obtained from the most stressful environment as a consequence of the environmental impact on the common bean's quality. Grain legume breeders can take advantage of such variability and the existent G × E interaction to select accessions with interesting nutritional composition for heat stress environments. Indeed, future warming in the Mediterranean region, due to climate change, is expected to exceed worldwide rates by

20%, with summer warming surpassing the global rate's mean by 40% [41]. Since temperature rises are causing changes in the quality ranking of common bean accessions, specifically for genotype adaptation, selection conducted in a stressful environment, to achieve significant yield gains under such conditions, represents the way forward for common bean quality breeding based on the systematic exploitation of G × E effects [42].

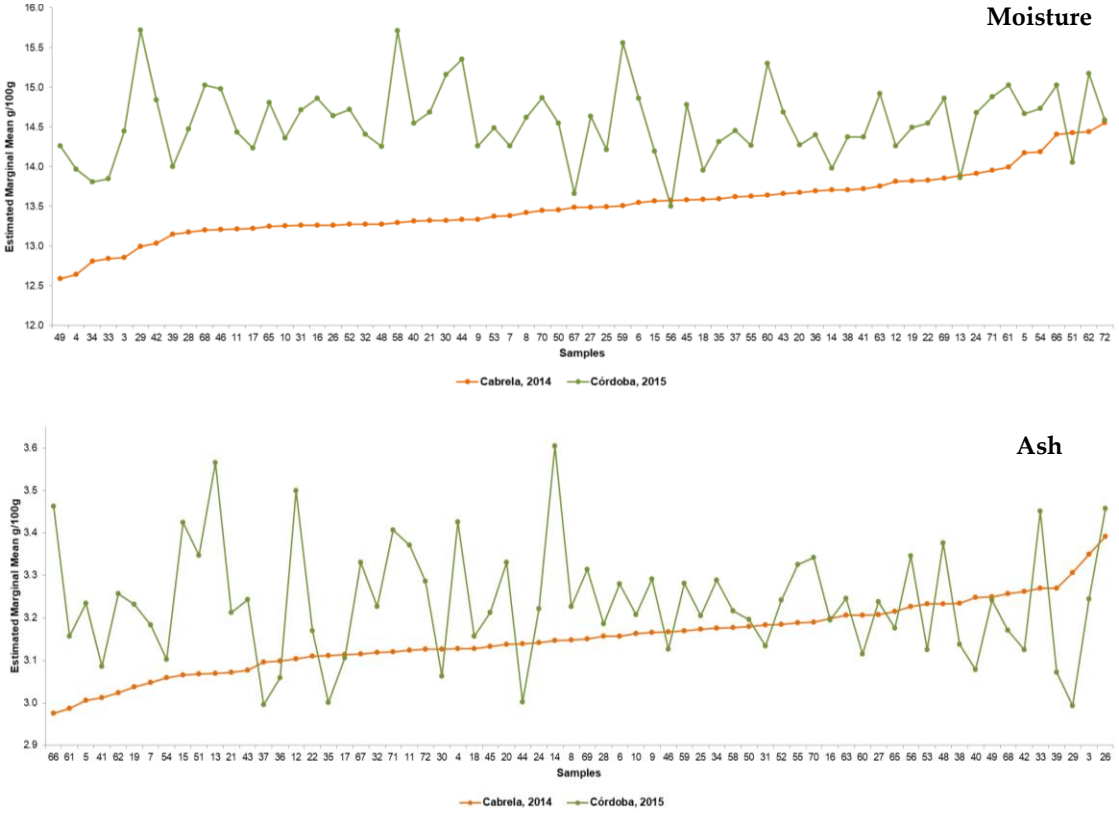
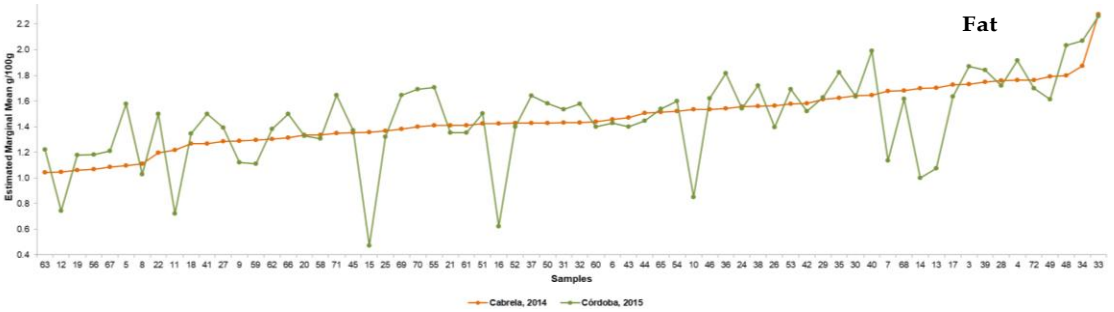
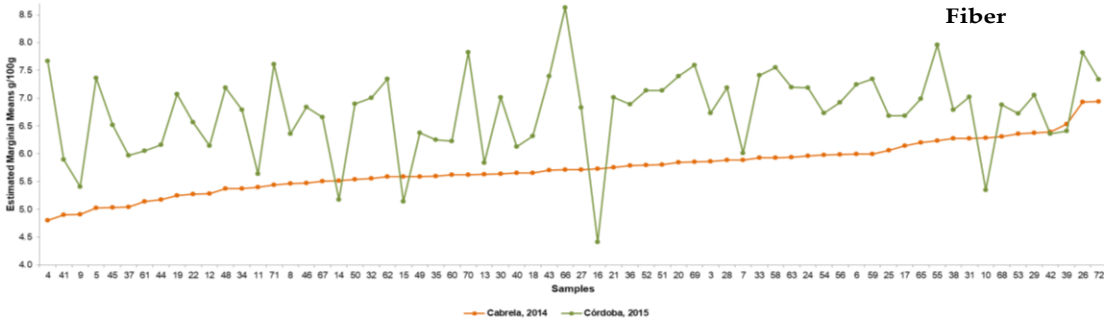
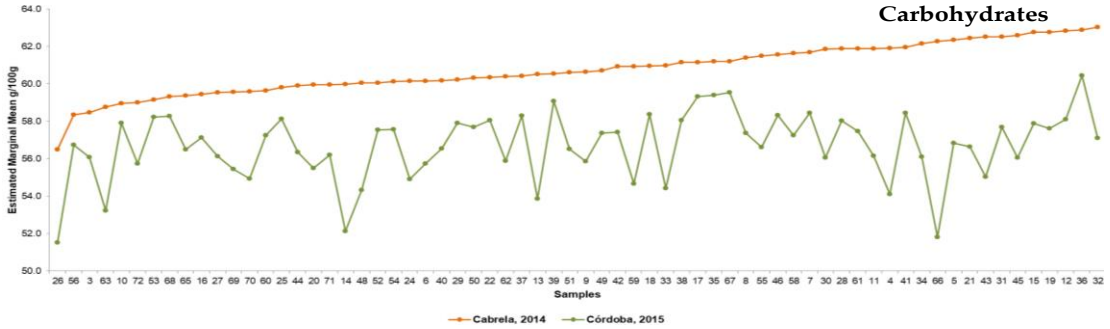



**Figure 2.** Graphic representations of the genotype × environment interaction effects in the nutritional parameters analyzed. Common bean samples collected from Cabrela field trial were ranked according to increasing values of moisture, ash, fat, fiber, carbohydrates and protein contents.



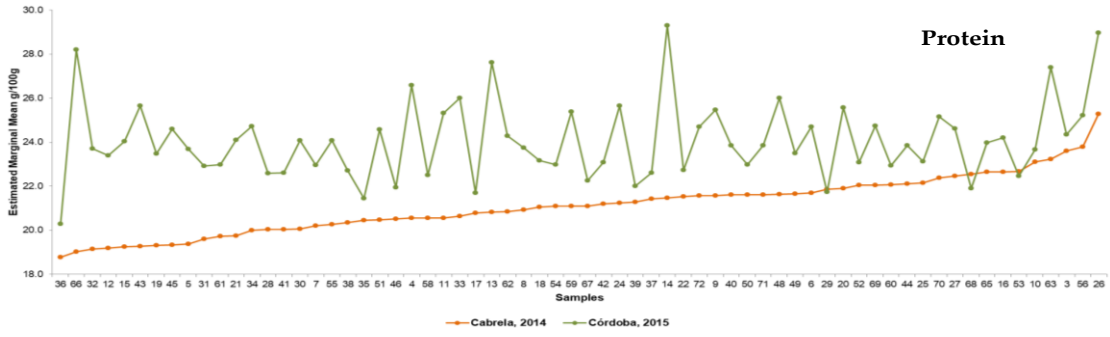




**Figure 2.** *Cont.*

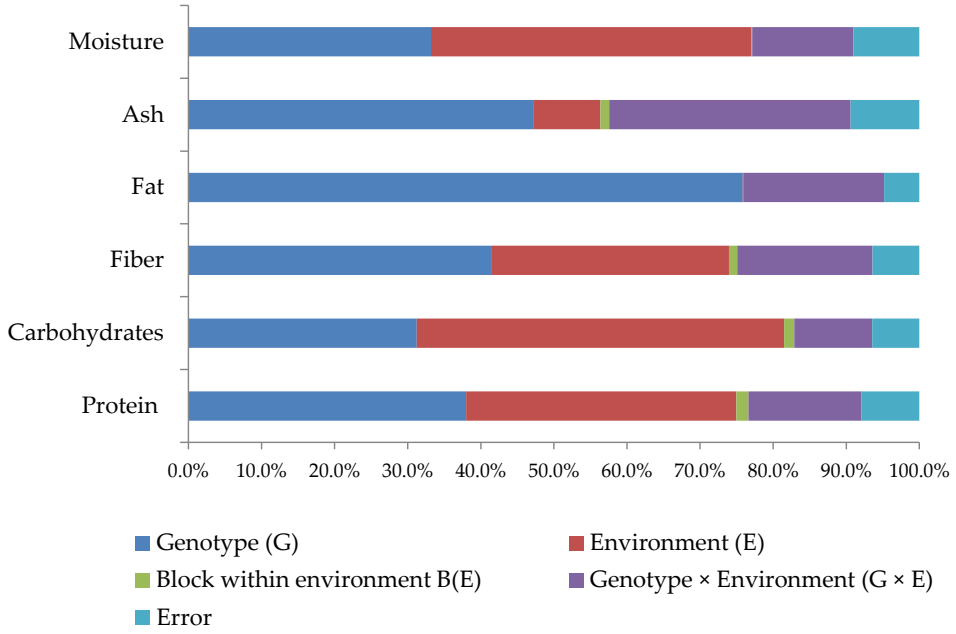



**Figure 3.** Genotype, environment and genotype × environment interaction's contribution ($Eta^2$ %) to general nutritional parameters' variability in common bean accessions.

## 3.2. Diversity in the Protein Quality of Portuguese Common Beans

In order to mimic the expected warming in the Mediterranean region and the need to characterize the potential of the varieties under these more marginal environments to better exploit them in future specific adaptation breeding, the common bean accessions cropped under the heat stress environment (Córdoba) were chosen to be further analyzed in terms of protein quality. The protein quality was accessed considering the amino acid content, the TIA and the protein digestibility. Multivariate PCA, taking into account the amino acid contents, the trypsin inhibitor activity and the protein content, in common bean accessions collected from Córdoba, explained 79.1% of the total variance in a bi-dimensional space defined by

the first two principal components, **Figure 4**. From all the parameters, protein content was the only one in this statistical model with low contribution for accessions' variability (communality lower than 0.4). The spatial distribution along the first principal component was mostly related to amino acid contents, highlighting the presence of considerable diversity among the Portuguese accessions. The spatial distribution of common bean accessions suggested the existence of three clusters accumulating 53.9% of the total variability. Cluster 1 included the accessions characterized by the highest content of the different evaluated amino acids, including the limiting amino acid, methionine, and the highest contents of protein and TIA. Cluster 2 grouped the samples with intermediate content of protein, sharing with cluster 1 higher TIA values and with cluster 3 lower contents for the majority of the amino acids. Cluster 3 was globally characterized by the lowest contents of protein, TIA and amino acids, with exception of Gly and Ser, which brought cluster 3 closer to cluster 1, **Table 2**. This cluster analysis supports the relevance of studying the individual amino acid contents to characterize the protein quality and not only the overall protein content. As shown in **Figure 4**, the accessions with higher protein content and higher individual amino acid contents were also the ones with higher activity of trypsin inhibitors. Although antinutritional factors such as trypsin inhibitors may interfere in the breakdown of peptide bonds, influencing protein availability, trypsin inhibitors classified as proteins (serine proteases) can be inactivated during the cooking process. A breeding program focused in the improvement of common beans' protein quality could take advantage of accessions grouped in cluster 1 since they have higher individual amino acid contents.

Considering the total quantified amino acids, the EAAs represented, on average, 40% of the total value, which was in accordance with the % of EAAs found in other legume species [34]. Leu, Lys and Phe were the most abundant ones. As described for other legumes [43–46], in the Portuguese common bean accessions, Met was the limiting amino acid of the measured ones, representing the less abundant EAAs among the different accessions, **Table S6**. By comparison with other authors [44–46], the content of Met in Portuguese accessions was similar to the reported values for other legumes spp. such as the ones from *Lathyrus* (0.58 ± 0.26 g/100 g protein), *Pisum* (0.53 ± 0.05 g/100 g protein) and *Vicia* (0.56 ± 0.23 g/100 g protein) genus [34].

Although Met has been considered a limiting factor for the beans protein quality, recent studies reviewed by Kitada et al. [47] regarding longevity and animal lifespan, indicate the advantage of restricting methionine on lifespan extension through several mechanisms that involve intracellular suppression of mTORC1 and removal of reactive oxygen species (ROS) [47], and in the prevention of cardiovascular diseases. In fact, a prospective cohort study, conducted by Virtanen et al. [48], during a follow-up period of 14 years, with 1981 Finnish men aged 42–60 years, concluded that low levels of Met (<1.7 g of Met/day) were associated with a reduced relative risk of developing acute coronary disease [48].

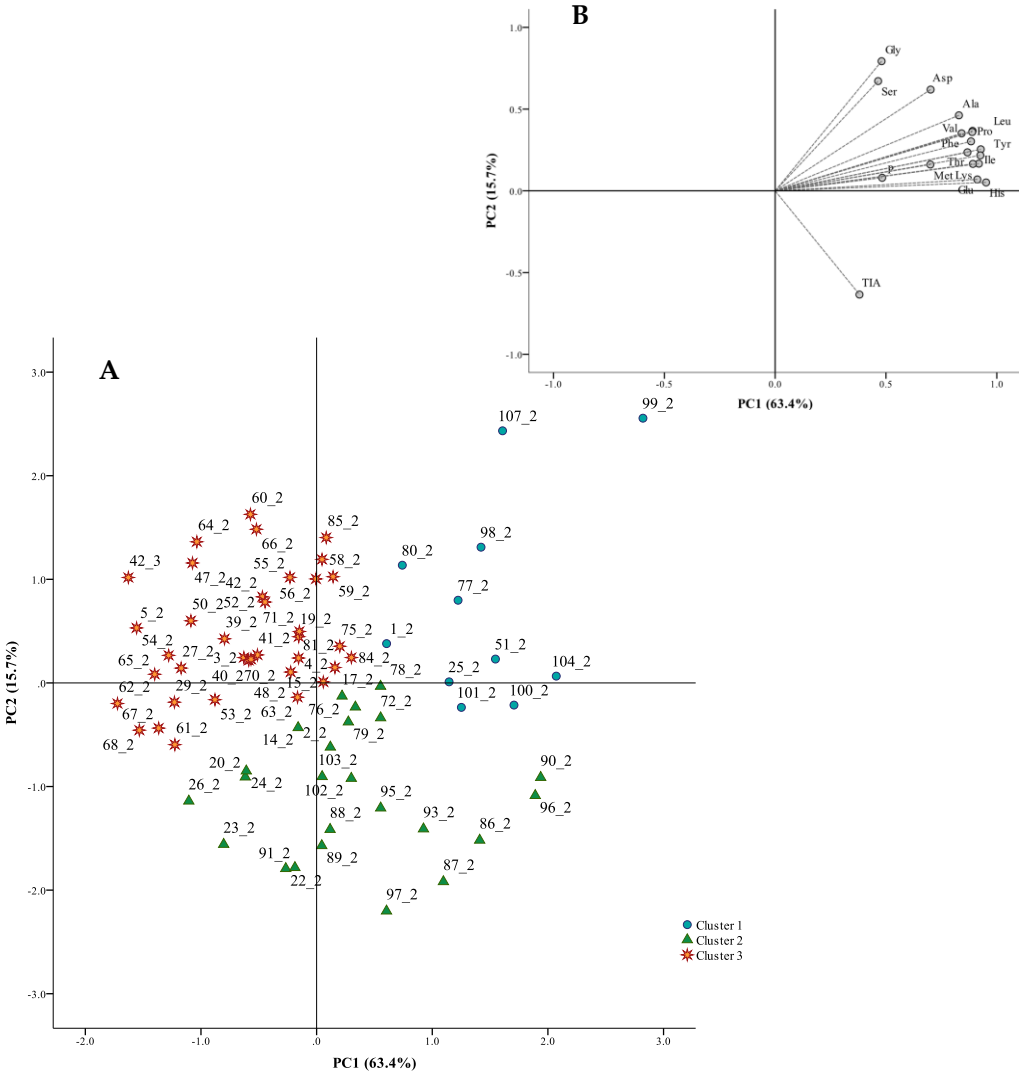



**Figure 4.** (A) Projection of Portuguese common bean accessions (n = 72) collected from Córdoba field trial, considering the variables directly related to protein analysis (B) P−Protein; Gly−glycine; Ala−alanine; Ser−serine; Pro−proline; Val−valine; Thr−threonine; Ile−isoleucine; Leu−leucine; Asp−aspartic acid; Glu−glutamic acid; Met−methionine; His−histidine; Phe−phenylalanine; Tyr−tyrosine; Lys−lysine; Arg−arginine; TIA−trypsin inhibitor activity, in a bi-dimensional space (PC1 and PC2), responsible by 79.1% of the total variance. The clusters were highlighted by different colours and symbols.

**Table 2.** Protein quality (protein content, amino acid content, protein efficiency ratio, PER and amino acid score, AAS), and trypsin inhibitor activity, TIA, considering the clusters established for common bean accessions cropped in Córdoba (*n* = 72). Results were shown as the average ± standard deviation. In each row, different letters indicate significant differences between clusters ($p < 0.05$) and equal letters the absence of differences ($p > 0.05$).

| Parameters | Cluster 1 | Cluster 2 | Cluster 3 |
|---|---|---|---|
| Protein content, g/100g | 25.33 ± 2.53 $^{b}$ | 24.35 ± 1.32 $^{a,b}$ | 23.48 ± 1.40 $^{a}$ |
| Amino acids composition, g/100 g (g/16g N) | | | |
| Gly | 1.27 ± 0.11 $^{c}$ (5.06 ± 0.60) | 1.00 ± 0.09 $^{a}$ (4.12 ± 0.39) | 1.09 ± 0.09 $^{b}$ (4.67 ± 0.38) |
| Ala | 1.32 ± 0.12 $^{b}$ (5.24 ± 0.63) | 1.07 ± 0.08 $^{a}$ (4.41 ± 0.41) | 1.05 ± 0.09 $^{a}$ (4.46 ± 0.40) |
| Ser | 1.52 ± 0.15 $^{c}$ (6.05 ± 0.80) | 1.19 ± 0.16 $^{a}$ (4.89 ± 0.71) | 1.31 ± 0.13 $^{b}$ (5.59 ± 0.62) |
| Pro | 1.12 ± 0.10 $^{b}$ (4.45 ± 0.51) | 0.94 ± 0.09 $^{a}$ (3.86 ± 0.41) | 0.90 ± 0.07 $^{a}$ (3.86 ± 0.31) |
| Val | 1.44 ± 0.13 $^{b}$ (5.71 ± 0.55) | 1.22 ± 0.12 $^{a}$ (5.01 ± 0.54) | 1.17 ± 0.11 $^{a}$ (4.99 ± 0.42) |
| Thr | 1.08 ± 0.14 $^{b}$ (4.30 ± 0.62) | 0.87 ± 0.12 $^{a}$ (3.52 ± 0.47) | 0.81 ± 0.10 $^{a}$ (3.40 ± 0.35) |
| Ile | 1.37 ± 0.12 $^{b}$ (5.45 ± 0.69) | 1.13 ± 0.13 $^{a}$ (4.64 ± 0.50) | 1.06 ± 0.10 $^{a}$ (4.53 ± 0.37) |
| Leu | 2.40 ± 0.22 $^{b}$ (9.56 ± 1.16) | 1.96 ± 0.22 $^{a}$ (8.06 ± 0.84) | 1.89 ± 0.19 $^{a}$ (8.06 ± 0.69) |
| Asp | 2.98 ± 0.21 $^{b}$ (11.85 ± 1.25) | 2.29 ± 0.23 $^{a}$ (9.42 ± 0.93) | 2.39 ± 0.23 $^{a}$ (10.17 ± 0.91) |
| Glu | 5.87 ± 0.50 $^{c}$ (23.38 ± 3.06) | 5.01 ± 0.50 $^{b}$ (20.61 ± 1.92) | 4.56 ± 0.39 $^{a}$ (19.46 ± 1.77) |
| Met | 0.15 ± 0.03 $^{b}$ (0.61 ± 0.10) | 0.13 ± 0.03 $^{a}$ (0.53 ± 0.10) | 0.12 ± 0.02 $^{a}$ (0.50 ± 0.07) |
| His | 0.83 ± 0.07 $^{c}$ (3.30 ± 0.49) | 0.71 ± 0.07 $^{b}$ (2.92 ± 0.31) | 0.64 ± 0.06 $^{a}$ (2.74 ± 0.25) |
| Phe | 1.67 ± 0.11 $^{b}$ (6.63 ± 0.61) | 1.42 ± 0.15 $^{a}$ (5.82 ± 0.54) | 1.36 ± 0.12 $^{a}$ (5.81 ± 0.47) |
| Tyr | 1.00 ± 0.09 $^{b}$ (4.00 ± 0.53) | 0.84 ± 0.09 $^{a}$ (3.46 ± 0.40) | 0.78 ± 0.08 $^{a}$ (3.34 ± 0.33) |
| Lys | 1.76 ± 0.10 $^{b}$ (7.01 ± 0.60) | 1.57 ± 0.17 $^{a}$ (6.45 ± 0.75) | 1.45 ± 0.13 $^{a}$ (6.19 ± 0.59) |
| Arg | 1.36 ± 0.16 $^{b}$ (5.39 ± 0.54) | 1.09 ± 0.14 $^{a}$ (4.47 ± 0.52) | 0.99 ± 0.13 $^{a}$ (4.22 ± 0.43) |
| PER | | | |
| PER1 | 3.46 ± 0.50 $^{b}$ | 2.81 ± 0.37 $^{a}$ | 2.81 ± 0.30 $^{a}$ |
| PER2 | 3.45 ± 0.47 $^{b}$ | 2.83 ± 0.35 $^{a}$ | 2.84 ± 0.29 $^{a}$ |
| PER3 | 2.82 ± 0.54 $^{b}$ | 2.05 ± 0.45 $^{a}$ | 2.12 ± 0.38 $^{a}$ |
| AAS, % | | | |
| AAS_Val | 163.07 ± 15.74 $^{b}$ | 143.13 ± 15.30 $^{a}$ | 142.50 ± 12.01 $^{a}$ |
| AAS_Thr | 126.38 ± 18.30 $^{b}$ | 105.11 ± 14.03 $^{a}$ | 101.44 ± 11.56 $^{a}$ |
| AAS_Ile | 194.60 ± 24.69 $^{b}$ | 165.70 ± 17.97 $^{a}$ | 161.84 ± 13.11 $^{a}$ |
| AAS_Leu | 144.78 ± 17.52 $^{b}$ | 122.12 ± 12.76 $^{a}$ | 122.14 ± 10.50 $^{a}$ |
| AAS_Met | 24.39 ± 4.07 $^{b}$ | 21.37 ± 4.01 $^{a}$ | 19.77 ± 2.96 $^{a}$ |
| AAS_His | 173.78 ± 25.85 $^{c}$ | 153.49 ± 16.11 $^{b}$ | 144.07 ± 13.29 $^{a}$ |
| AAS_Phe+Tyr | 168.76 ± 17.92 $^{b}$ | 147.25 ± 14.39 $^{a}$ | 145.20 ± 12.04 $^{a}$ |
| AAS_Lys | 120.92 ± 10.29 $^{b}$ | 111.15 ± 12.85 $^{a}$ | 106.79 ± 10.21 $^{a}$ |
| Protease inhibitors | | | |
| TIA, mg/g | 9.68 ± 2.75 $^{b}$ | 10.95 ± 2.06 $^{b}$ | 7.47 ± 2.00 $^{a}$ |

The values of protein efficiency ratio (PER3), calculated considering the amounts of Met, Leu, His and Tyr, were, on average, lower than the values determined for PER1 and PER2, **Table S7**. The same trend has been described by Pastor-Cavada et al. [34] for *Pisum* species and stemmed from the limited amount of Met in the different common bean accessions. In fact, the average PER3 value of 2.20 ± 0.50, below the standardized PER value for casein, 2.5, confirms, as expected, by comparison with casein, the lower quality of common bean protein [49].

Moreover, taking in account the recommendations of the different EAAs for pre-school children (2–5 years old) [33] Met was, in the present study, the only amino acid below the suggested pattern, representing on average 21.01 ± 3.82% of the recommended value, **Table S8**. This amino acid score was lower than the one determined by Khattab et al. [46] for Canadian and Egyptian beans, 39.07% and 57.04%, respectively. The difference could be attributed to the study of distinct common bean accessions but also to differences in the used hydrolysis process, which was conducted by Khattab et al. [46] for sulfur-containing amino acids only after oxidation with performic acid [46]. Such procedure is described as preserving Met in the form of methionine sulphone, reducing consequently Met losses during the acidic hydrolysis process. However, most of the amino acids are partly decomposed with the performic acid oxidation [50], and therefore in the present study to preserve the maximum amount of the different amino acids, the acidic hydrolysis was performed without previous performic acid oxidation. Regarding the NEAAs, the most abundant amino acids were Glu and Asp, and the less abundant ones were Pro and Tyr, **Table S9**. The use of nitrogen fertilization may have contributed to the

common bean seeds' nitrogen storage in the form of amino acids Glu and Asp [14]. Besides the amino acid content/proportion, the protein quality is influenced by protein digestibility, which depends on the anti-nutritional composition (e.g., protease inhibitors content) [51]. Legumes have been described as food products rich in dietary trypsin inhibitors [52]. Such food components correspond to serine protease inhibitors that adversely affect protein digestibility in mammals, birds and insects. These compounds present in raw seeds promote pancreas enlargement increasing the secretion of enzymes trypsin and chymotrypsin. Once used for enzymatic synthesis, the sulfur-containing amino acids are no longer available for protein body synthesis, which slows down animal body growth [53]. Even so, recent evidence suggests the beneficial chemo preventive, anti-metastatic and anti-inflammatory properties of the protease inhibitors for human health and also for crop protection against insects [54]. Despite the detected variability in the TIA content among the Portuguese common bean collection (coefficient of variation of 29.6%), **Table S10**, with two contrasting TIA clusters (71.8% of the total variance), **Figure S4**, no significant TIA differences were found among different gene pools, coat colours, seed sizes, or different geographical origins, $p > 0.05$.

Moreover, there was no significant difference between *in vitro* protein digestibility (IVPD) or *in vitro* protein digestibility corrected amino acid score (IVPDCAAS) on the selected accessions, characterized by different TIA contents, **Table 3**. The values reported, herein, for IVPD, **Table 3**, were higher than the ones described in improved Brazilian varieties (50 ± 4–67 ± 0%) [55]. Nevertheless, similarly to Rezende et al. [55] in the present study, there was no correlation between IVPD and TIA

content determined in raw seeds (Pearson's R correlation of 0.215). This suggested that other factors (e.g. phytic acid), beyond trypsin inhibitors, may be present in concentrations that compensate TIA inter-variability, allowing constant IVPD and IVPDCAAS values among the different accessions. Trypsin inhibitors can be inactivated by thermal treatments [52]. In fact, cooking during 2 h, under controlled temperature (100 °C) allowed the common beans' digestogram to come closer to the casein's digestogram, **Figure 5**, and to increase significantly the IVPD values of the selected accessions, **Table 3**.

Such results could be attributed to the decrease of anti-nutritional factors such as trypsin inhibitors, tannins and saponins, during the cooking process [46, 53]. The IVPD values determined in cooked samples are in alignment with the reviewed values for the fecal protein digestibility, reported for pinto beans (72–79%) [53], and represent a clear indication for adopting IVPD as a reliable, less expensive and animal friendly methodology to access food products' protein digestibility.

**Table 3.** *In vitro* protein digestibility (IVPD) and *in vitro* protein digestibility corrected by the limiting amino acid score (IVPDCAAS) in raw common bean accessions (n = 8). The *in vitro* protein digestibility (IVPD) of cooked common bean accessions (n = 4) was also presented.

| # | Accessions | IVPD (Raw) % | IVPDCAAS (Raw) % | IVPD (Cooked) % |
|---|---|---|---|---|
| 19 | 698 | 69.66 ± 0.64 | 13.0 | - |
| 20 | 706 | 69.47 ± 0.81 | 11.8 | 75.55 ± 1.63 |
| 25 | 1644 | 69.22 ± 0.01 | 10.3 | 73.52 ± 1.75 |
| 29 | 1663 | 69.86 ± 0.18 | 13.0 | 76.78 ± 0.21 |
| 43 | 1984 | 69.06 ± 0.38 | 12.1 | - |
| 48 | 2179 | 68.78 ± 0.30 | 11.4 | 75.07 ± 0.41 |
| 64 | 4119 | 69.61 ± 0.52 | 11.0 | - |
| 65 | 4120 | 69.70 ± 0.13 | 10.3 | - |
| | Casein | 88.96 ± 1.90 | | |

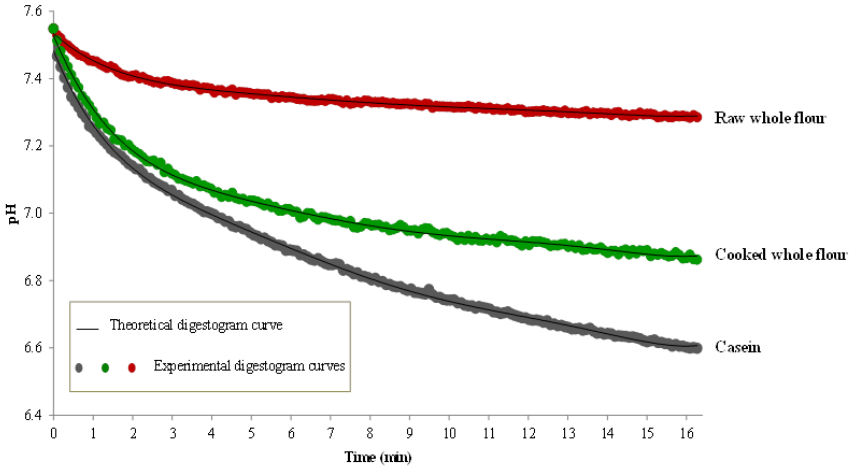



**Figure 5.** Example of a common bean (accession 29) digestogram obtained by *in vitro* multi-enzyme digestion of raw whole flour and corresponding cooked whole flour. Casein's digestogram was also included for data comparison.

## 4. Conclusions

Legumes represent affordable and sustainable nutritional sources of protein, especially in low-income countries, where the need for resilient and productive varieties represents a challenge for local farmers. Despite the recognized genetic diversity of the Portuguese common bean accessions, the lack of studies regarding their nutritional and protein value contributed to the downgrading and non-inclusion of this material in breeding programs. In the study reported herein, the data obtained from 106 representative Portuguese common bean accessions cropped in two contrasting environments showed that, under a heat stress prone environment the accessions have higher protein and lower carbohydrate contents, maintaining high diversity at the nutritional composition level, when compared with a more traditional production environment. The genotype × environment cross-over interaction effect, evident by accession

ranking changes in the nutritional quality between environments can be exploited for future selection of accessions with specific environmental adaptations within warming climate conditions. Taking advantage of such variability, and considering the protein content, the amino acid composition and the activity of trypsin inhibitors, three clusters of common bean accessions were defined. Cluster 1 assembled the most promising accessions for a future breeding program driven by protein quality improvement, since it gathered the accessions with higher individual amino acid contents. The most abundant EAAs in the Portuguese common bean accessions were Leu, Lys and Phe. Met, as expected, was the less abundant one, which contributed to decreasing the protein efficiency ratio and the amino acid score by comparison with the recommended requirements for pre-school children (2–5 years old). Despite this limitation, recent studies showed the importance of restricting Met in the diet as a way of extending longevity and preventing cardiovascular events, especially in developing countries where there is high prevalence of old-aged people and food availability is not a matter of concern. The characteristics of cluster 3, with common bean accessions with lower protein contents and TIA values but with Gly and Ser contents closer to cluster 1, reinforce the importance of studying not only the overall protein content but also the individual amino acid contents to characterize protein quality.

In this study, although previous reports attributed to trypsin inhibitors main responsibility for impairing common beans' protein digestibility, there were no significant differences in the *in vitro* protein digestibility of accessions characterized by contrasting TIA contents. This

suggested that different anti-nutritional factors (e.g. trypsin inhibitors, saponins, tannins, phytic acid) may occur in a balanced ratio contributing to stable IVPD values among the seeds of the different Portuguese common bean accessions. Regardless of trypsin inhibitors activity, after the cooking process, the common bean's IVPD increased to values closer to those of casein, showing the importance of the cooking process to common beans' protein digestibility.

Taken together, the information disclosed herein will be useful to enrich food composition databases and for the development of future breeding programs guided by the goal of protein content and quality improvement, through the selection of accessions with higher contents of individual amino acids.

## Supplementary Materials

The following are available online at https://www.mdpi.com/2073-4395/11/2/221/s1, Figure S1: Multiple reaction monitoring chromatograms of the 16 amino acids present in a standard mixture, 30 µM (conditions of analysis according to experimental section), Figure S2: Average ambient temperatures and air humidity in Cabrela and in Córdoba, during the growing season [23], Figure S3. Frequency distribution, for the average yield (kg/ha) of cropped accessions in Cabrela and Córboba, Figure S4: Dendogram obtained by hierarchical clustering (Ward's method) for common bean accessions, based on trypsin inhibitor activity (TIA) measurements. The underlined accessions were selected for protein digestibility assays, Table S1: Amino acid (AA) classifications and molecular structures [17], Table S2: Morphology, gene pools

(Mesoamerican; Andean; mixed) and geographical origins of Portuguese common bean seeds' accessions [22], Table S3: Retention time, precursor ion, MRM transitions and calibration curves of the amino acid standards used for the amino acid quantification in common bean accessions, Table S4: Protein (P), total carbohydrates (CH), fat, fiber, ash and moisture in g/100 g, evaluated in the Portuguese common bean accessions cropped in contrasting environments, 1−Cabrela and 2−Córdoba. For environment, different letters per parameter (row) indicate significant differences ($p < 0.05$) between values, Table S5: Nested ANOVA of genotype (G), environment (E), block within environment (B(E)) and genotype × environment (G × E) interaction effects in common bean variability, Table S6: Contents of essential amino acids, average ± SD (standard deviation, g/16gN (100g of protein), in the different Portuguese common bean accessions cropped in Córdoba (n = 72). Different letters per column indicate significant differences between accessions ($p < 0.05$), Table S7: Protein efficiency ratio (PER) calculated for the different Portuguese common bean accessions cropped in Córdoba (n = 72), Table S8: Amino acid scores of individual amino acids for the different common bean accessions cropped in Córdoba (n = 72) by comparison with the scoring pattern recommended for children from 2 to 5 years old [33], Table S9: Contents of non-essential amino acids, average ± SD (standard deviation), g/16gN (100g of protein), in the different Portuguese common bean accessions cropped in Córdoba (n = 72). Different letters per column indicate significant differences between accessions ($p < 0.05$), Table S10: Trypsin inhibitor activity (TIA), mg/g, measured in the different Portuguese

common bean accessions cropped in Córdoba (n = 72). Different letters indicate significant differences between accessions ($p < 0.05$).

## Author Contributions

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

## Funding

This research was funded by FCT, Portugal, through BEGEQA project (PTDC/AGRTEC/3555/2012), E.M. PhD fellowship (SFRH/BD/89287/2012 and R&D unit, UIDB/04551/2020 (GREEN-IT–Bioresources for sustainability), PORTUGAL 2020, grant number LISBOA-01-0145-FEDER-402-022125 and PDR2020 Operação 7.8.4, Recursos genéticos (PDR2020-784-042734).

## Acknowledgments

Thanks to the Research Unit of Biotechnology and Genetic Resources germplasm bank, INIAV, Oeiras, Portugal, for providing the common bean

samples. The authors also acknowledge to the Portuguese Mass Spectrometry Network.

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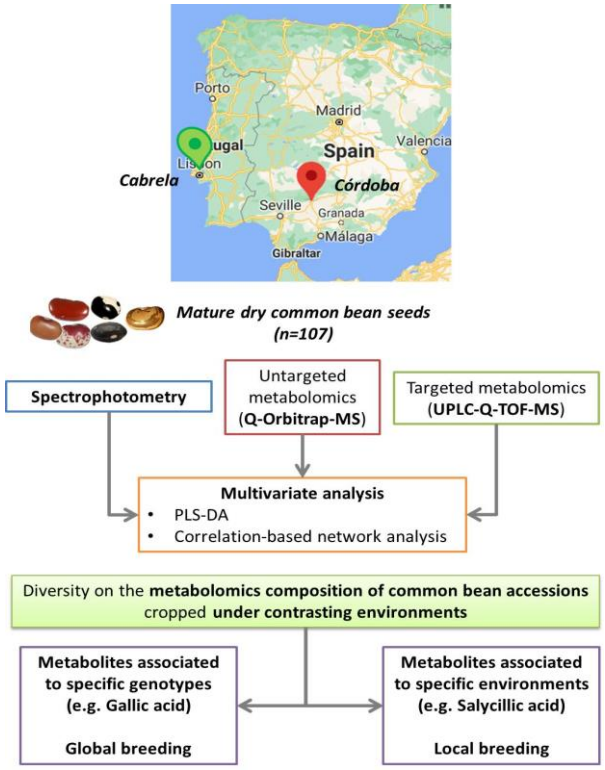
# Chapter III




This chapter was submitted to Food Chemistry as

**Mecha, E**., Erny, G.L., Guerreiro, A.C.L., Feliciano, R. P., Barbosa, I., Bento da Silva, A., Leitão, S.T., Veloso, M.M., Rubiales, D., Rodriguez-Mateos, A., Figueira, M.E., Vaz Patto, M.C., Bronze, M.R. Metabolomics profile responses to changing environments in a common bean (*Phaseolus vulgaris* L.) germplasm collection

In this Chapter, Elsa Mecha participated in the experimental work, data analysis, manuscript drafting and final manuscript writing.

# Metabolomics profile responses to changing environments in a common bean (*Phaseolus vulgaris* L.) germplasm collection

## Abstract

Metabolomics is one of the most powerful -*omics* to assist plant breeding. Despite the recognized genetic diversity in Portuguese common bean germplasm, details on its metabolomics profiles are still missing. Aiming to promote their use and to understand the environment's effect in bean metabolomics profiles, 107 Portuguese common bean accessions, cropped under contrasting environments, were analysed using spectrophotometric, untargeted and targeted mass spectrometry approaches. Although genotype was the most relevant factor on bean metabolomics profile, a clear genotype × environment interaction was also detected. Multivariate analysis highlighted, on the heat-stress environment, the existence of higher levels of salicylic acid, and lower levels of triterpene saponins. Three clusters were defined within each environment. White accessions presented the lowest content and the coloured ones the highest levels of prenol lipids and flavonoids. Sources of interesting metabolomics profiles are now identified for bean breeding, focusing either on local or on broader adaptation.

**Keywords:** *Phaseolus vulgaris*; metabolomics; diversity; mass spectrometry; multivariate analysis; correlation-based network analysis

## 1. Introduction

In the plant kingdom, there is a vast diversity of metabolites, up to 1 000 000 compounds, characterized by distinct chemical structures and present in a large range of concentrations [1].

These plant metabolites can be classified as primary and secondary metabolites. Although this classification has been considered ambiguous (since the primary metabolites can also participate in plant metabolism as secondary metabolites) traditionally the term primary corresponds to molecules involved in living organisms' growth and survival. The term secondary concerns metabolites formed from the primary ones exerting functions related to environmental conditions' adaptability, such as defense against biotic and abiotic stresses, signaling and metal transport.

These secondary metabolites, including phenolic compounds (described as the most representative secondary metabolites found in plants [2]), may also exert antioxidant, anti-inflammatory and anticarcinogenic activities in animal and human health, possessing undeniable economical value for pharmaceutical, nutraceutical and agro-industries [3].

Plant metabolomics, as a systematic, untargeted profiling of plant metabolites involved in core essential functions and in plant interactions with their environment have been also used to access the natural variance in metabolite content between individual plants, representing a powerful tool to assist the improvement of crops' compositional quality [4, 5].

Metabolomics studies have been mostly applied to model plant species and major crops such as tomato, rice, maize, wheat [6, 7]. Only a

few published studies reported the use of –*Omics* in the study of legumes' metabolomics profile, such as common bean [8–10].

Common bean (*Phaseolus vulgaris* L.) represent one of the major grain legumes consumed worldwide for its edible seeds and pods [11], being an important source of dietary protein and metabolites with potential health promoting effects, e.g. phenolic compounds and terpenoids [5]. Portugal as part of the Iberian Peninsula is considered a secondary center of common bean genetic diversity [12], with many bean landraces still in cultivation [13].

The first study on common bean metabolomics, using a non-targeted metabolite profiling approach conducted by gas chromatography – mass spectrometry, characterized metabolite profile changes in common bean roots, under phosphorus deficient soil conditions [8]. A second study conducted only with six cultivars and not focused on metabolites identification, associated small molecules to distinct common bean centers of domestication (COD) [9]. The most recent study dedicated to the metabolite profiling of different common bean organs (seedlings, roots, leaves, flowers, pods) established, through an integrative network analysis, the tissue and accession specific metabolic diversity [10].

Although the genetic diversity of Portuguese common bean germplasm has been extensively recognized [13], so far no study has focused on this germplasm metabolite diversity and/or on the impact of the environment in common bean metabolomics profile diversity.

The present study aimed to overcome the lack of knowledge regarding the natural variance in metabolites content of Portuguese common beans, in particular, and on common beans' metabolome

variability under challenging environmental conditions, in general. To fulfill these goals common bean dry seeds from a Portuguese germplasm collection (n=107 accessions), cropped under two contrasting environments (traditional *versus* heat stress), were studied.

Disclosing the common bean seeds metabolomics profile, under contrasting environments, will provide useful information to breeders focused on improving common bean crop yields and quality, as well as to farmers facing climate change. This information will be useful to understand the impact of the environment on the beans' metabolome and therefore to predict specific metabolite levels under different environmental conditions. This can have implications on some future cropping adopted measures (e.g. sun exposure, irrigation conditions) in order to obtain an adequate level of specific metabolites. Characterizing Portuguese common beans' metabolome will create the opportunity to introduce the Portuguese common beans into breeding programs with the aim of giving response to a multitude of challenges, such as future warming climate conditions, crop productivity, resilience to biotic and abiotic stresses and the demand of processors and consumers for accessions with attractive nutritional, nutraceutical and sensorial characteristics.

## 2. Materials and Methods

### 2.1. Chemicals

Folin-Ciocalteu's phenol reagent, sodium carbonate (99%), *(+)*-catechin (98%), sodium nitrite (97%), aluminium chloride (99.9%), and vanillin (99%) were purchased from Sigma-Aldrich (St. Louis, USA). Sulphuric acid (95–97%) was purchased from Fluka (Seelze, Germany).

Sodium hydroxide (98%) was purchased from Merck (Darmstadt, Germany). Methanol (99.9%) was purchased from Carlo Erba Reagents (Rodano, Italy). Acetonitrile for LC-MS Ultra Chromasolv was purchased from Honeywell Riedel-de Haën™ (Seelze, Germany). Milli-Q® water (18.2 MΩ.cm) was obtained in a Millipore – Direct Q3 UV System equipment (Molsheim, France). Formic acid (98%) was obtained from Carl Roth (Karlsruhe, Germany). Eluents A and B used for Q-Orbitrap were from Optima™ LC/MS Grade, Fisher Scientific (NH, USA). Gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, sinapic acid, catechin, epicatechin, caffeic acid, *p*-coumaric acid, *t*-ferulic acid, kaempferol, quercetin were obtained from Sigma-Aldrich Co. (Steinheim, Germany).

## 2.2. Plant material

A total of 107 different common beans (*Phaseolus vulgaris* L.) accessions was provided by the Research Unit of Biotechnology and Genetic Resources germplasm bank at INIAV (PRT05) Portugal, covering different morphological characteristics (seeds colour and size) as well as different gene pool of origin, as described previously [13], **Table S1**. The different accessions were cropped in two contrasting environments, a traditional common bean cultivation environment at Cabrela, in Portugal (latitude – 38°52'6.816''N and longitude – 9°21'15.804''W) and a stressful environment at Córdoba, in Spain (latitude – 37°53'29.58''N and longitude – 4°46'21.90''W), following a randomized complete block design with two blocks (two biological replicates per accession, each containing 20 plants). The two environments were characterized by different average temperatures (18–21 °C, in Cabrela, and 15–32 °C, in Córdoba), different

average relative humidity (66–80%, in Cabrela, and 31–63%, in Córdoba) and different types of soil (eutric cambisol in Cabrela and fluvisol in Córdoba) [14].

2.3. Samples' preparation and extraction

The mature dried seeds of the viable plants were collected and milled (Falling nº 3100 – Perten, Sweeden) to a particle size of 0.8 mm and stored at -20 °C, until further analysis. Extracts of the milled common bean seeds were prepared in duplicate (technical replicates), as previously described [15]. Briefly, one gram of dry whole seed flour was extracted with 20 mL of methanol: water (60:40, v/v) solution, followed by sonication for 60 minutes. After centrifugation at 420× g during 15 minutes, the supernatant was collected and the final volume adjusted to 20 mL. The prepared extracts were filtered through a 0.22 µm 13 mm CA syringe filter (GE WhatmanTM, Malborough, MA, USA) and kept at -20 ºC, until analysis.

2.4. Total phenolic content and total flavonoids content

Total phenolic content (TPC) and total flavonoids content (TFC) were measured by spectrophotometric methods as previously described [15]. For TPC, after testing for the appropriate dilution, 3.5 mL of diluted extract were mixed to 0.100 mL of *Folin-Ciocalteu's* reagent. Sodium carbonate solution (35% w/v), 0.400 mL, was added to the mixture 3 min after. The absorbance was measured, after keeping the mixture during one hour in the dark, at 725 nm, in a Spectrophotometer DU-70 (Beckman®, Brea, CA, USA). A blank of water was also prepared in the same

conditions and the gallic acid used, as the external standard, in a concentration range of 1 – 6 mg/L. The final results were expressed as mg of Gallic acid equivalents (GAE) per g of flour dry weight.

For TFC, after testing the appropriate dilution, 1 mL of diluted extract was added to 4 mL of Milli-Q® water and 0.300 mL of sodium nitrite (5%, w/v). After 5 min, 0.300 mL of aluminum chloride (10%, w/v) was added and to complete the reaction, after 6 minutes, 2 mL of 1 M sodium hydroxide solution were added. Milli-Q® water was added to complete a final volume of 10 mL. Absorbance was measured in a Spectrophotometer DU-70 (Beckman®, Brea, CA, USA), at 510 nm, against water. *(+)*-Catechin was applied as the external standard in a concentration range of 20 to 100 mg/L and the final results expressed as mg of *(+)*-catechin equivalents (CE) per g of flour dry weight (DW). The moisture content (%) of the raw flour used in the present study was retrieved from [21] and determined by Near Infrared (NIR) analyser (MPA; Bruker, Billerica, MA, USA).

## 2.5. Untargeted metabolomics analysis by Q Exactive™ Focus Hybrid Orbitrap

The analysis of metabolites by untargeted metabolomics, in common bean extracts, was achieved by Orbitrap high-resolution mass spectrometry using a Q Exactive™ Focus Hybrid Q-Orbitrap (Thermo Scientific, MA, USA). For metabolites separation a XBridge BEH C18 (130 Å, 3.5 µm, 2.1 x 150 mm) column (Waters, MA, USA) was used. The elution was ensured with a binary system consisting of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B), at a constant flow rate of 400 µL/min, during 20 minutes. The following gradient

elution program was applied: gradual increase of eluent B percent (from 1% of B at 1 minute to 99% of B at 13 minutes), followed by a steady percent of 99% of B during 2 minutes. At 15 minutes, the percent of B eluent returned to the initial conditions (1% of B) in one minute (from 99% of B at 15 minutes to 1% of B at 16 minutes). These conditions were maintained during 4 minutes before the next analysis starting. The UHPLC system (Dionex UltiMate 3000, Thermo Scientific, MA, USA) was coupled with a Q-Orbitrap mass spectrometer equipped with an electrospray ionization source working in negative mode. The data were acquired in full-MS scan mode (scan range from 75 – 1125 *m/z*) with a resolution of 70 000 (at 200 *m/z*), $1x10^6$ automatic gain control (AGC) and internal calibration with lock mass (112.98550 *m/z*). The identification of compounds was fulfilled by Data-dependent method (ddMS2). The three most intense ions were subjected to higher energy collisional dissociation, HCD, 17 500 resolution, 20, 40, 60 normalized collision energy (NCE) and $1x10^5$ AGC. The maximum injection time was set at 100 ms and 6 s of dynamic exclusion. A quality control (QC) sample was prepared as a pool of distinct common bean extracts obtained from different common bean accessions (n=32). The selected accessions were characterized by distinct colours, seed size and gene pool of origin, representing 30% of the total number of analysed accessions. In the sequence analysis, the quality control (QC) was analysed at every 63 injections, maximum once per day.

### *2.5.1. Data processing, identification and relative quantification of compounds*

The collected data were analysed using the *Finnee2016* toolbox for untargeted metabolomics analysis [16]. The data obtained from the QC sample analyses were processed in order to correct for baseline drift and remove background noise. The data were mined for chromatographic peaks. After, peaks obtained from the quality control (QC) sample analyses were aligned in order to find the peaks that were common to at least 75% of the samples. De-isotoping and de-clustering based on peak shapes (Pearson correlation coefficient) were performed to remove duplicate peaks. Samples were analysed by generating extracted ion chromatograms (EIC) based on the markers obtained using the QC samples' ions (defined by *m/z* values and retention time). An algorithm to detect and quantify the peaks in each EIC was developed. For that, peak limits were obtained using the first derivative of signal and the chromatographic parameters (peak area, migration time and peak variance) were calculated using the chromatographic statistical moment [17]. The quantified compounds were characterized by a ratio signal-to-noise higher than 10. A peak area greater than 100 counts x min was considered for quantification purposes. The final excel file included a total of 1122 compounds (defined by *m/z* values) aligned accordingly to the retention time for further statistical analysis. For each feature (compound) the coefficient of variation between the obtained areas of the QC samples was lower than 20%.

The final excel file was exported to MetaboAnalyst (version 4.0) freely available at https://www.metaboanalyst.ca/, for statistical analysis and metabolites selection [18]. The data were log transformed and pareto-scaled. Multivariate analysis by partial least square-discriminant analysis

(PLS-DA) allowed to select the most relevant compounds responsible by genotype, gene pool of origin and environment differences, based on values of variable importance in projection (VIPs) higher than 0.8, as reviewed elsewhere [19]. A *Venn* diagram was performed by *Venny* 2.1 (freely available at https://bioinfogp.cnb.csic.es/tools/venny/ [20]) applied as a tool to quickly distinguish the compounds exclusively responsible by genotype, gene pool of origin or environment distinction and the ones shared by the different groups.

After confirming the mass of the most abundant isotopes using XCalibur software (Thermo Fisher Scientific, MA, USA), the compounds were identified using the Compound Discoverer software, version 2.1, (Thermo Scientific™, MA, USA). Considering the complexity of *Phaseolus* spp. metabolism, it is worthy to notice that a clear identification by untargeted metabolomics can be difficult to attain, since the availability of authentic standards is often limited and the online databases are frequently incomplete or inconsistent. Therefore, to increase accuracy, for the putative identification (annotation) of metabolites, multiple databases were used [21]. The proposed annotations were considered acceptable if there was at least match with one of the online databases (m/zCloud and/or Chemspider); a mass accuracy, $\frac{Predicted\ m/z - Observed\ m/z}{Predicted\ m/z} x\ 1000\,000,$ ≤ 1 ppm; at least one fragment with relation signal-to-noise higher than three, different from the parent ion, in common with the described fragmentation pattern (m/zCloud match score indicating similarity between experimental and described fragmentation spectra and Fragment Ion Search (FISh) scoring algorithm explaining fragment ions based on literature defined chemical reactions) and/or the compound was previously identified in Plantae

kingdom and preferentially related to Fabaceae family. Whenever possible, freely available databases and published articles were used for data comparison (**Table 1**). Since m/zCloud is a curated database of high-resolution tandem mass spectra, primacy was given to the identification made by this database. All the retained fragment ions were characterized by intensity values higher than 10 000 counts. Classification of compounds into SuperClass level, Class level and categories within the Class was automated using the web-based application ClassyFire as described elsewhere [22], freely available at http://classyfire.wishartlab.com/ [22].

The relative quantification was conducted by comparison of the percent area of individual identified compounds, compound classes and superclasses, considering the different analysed common bean accessions.

### 2.6. Targeted metabolomics by UPLC-Q-TOF-MS

For quantification of individual phenolic compounds, the common bean extracts were analysed by targeted metabolomics using UPLC-Q-TOF-MS, in an Agilent 6550 iFunnel Accurate-Mass Q-TOF MS (Agilent, Waldbronn, Germany) equipment, with commercially available standards, following the procedure described elsewhere [23]. Metabolites' separation was carried on a 1290 Infinity UPLC system (Agilent, Waldbronn, Germany) using a Zorbax Eclipse Plus RRHD column 2.1 x 50 mm, 1.8 mm with a compatible Eclipse Plus guard column 2.1 x 5 mm, 1.8 mm (Agilent, Waldbronn, Germany). The mobile phases used for samples' analysis included 0.1% formic acid (eluent A) and acetonitrile with 0.1% formic acid (eluent B). With a flow rate of 0.4 mL/min, the analysis run was

performed during 10 minutes according to the following scheme: after a gradual increase of eluent B from 1 to 10% on the first 5 minutes, to 25% at 8 minutes and to 99% at 9.1 minutes, eluent B concentration remained constant during 0.9 minutes, returning to 1% during 2 minutes to equilibrate the column. The samples' ionization occurred through an electrospray interface with Jet Stream technology in the negative mode. MassHunter Workstation Quantitative Analysis software, version B.06.00 (Agilent, Waldbronn, Germany) was used for quantification. Calibration curves of standards (gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, catechin, epicatechin, *p*-coumaric acid, *t*-ferulic acid, sinapic acid, quercetin and kaempferol) were prepared and analysed under the same analytical conditions. All the standards were prepared in Milli-Q® water, with exception of the flavonoids, quercetin and kaempferol, prepared in aqueous methanol (50%, v/v). The calibration curve parameters (range of concentration, slope, intercept, and coefficient of determination) were previously described [24]. The quantified compounds were identified by comparison with the retention time and *m/z* values of the standards. Contents were expressed as µg per gram of raw flour in DW. The quantified concentrations were higher than the MQL previously described [25]. As described above classification of the targeted compounds was also conducted by application of a web-based application, ClassyFire, freely available at http://classyfire.wishartlab.com/ [22].

## 2.7. Statistical analysis

Using IBM® SPSS® Statistics, version 22 (IBM®, NY, USA), normality assumption was tested for each analysed parameter

(Kolmogorov-Smirnov test at a significance level of 1%, variable's distribution in histogram and normal Q-Q plots) and when necessary different transformations were tested (logarithmic, inverse, square root, cubic root, fourth root and fractional ratio transformations) in order to achieve residuals' normality. Levene's test was used to test homoscedasticity at a significance level of 1%. The impact of factors, such as genotype and environment, as well as the genotype x environment interaction effect were tested by nested ANOVA at a significance level of 5%. Significant differences were defined by *post-hoc* Scheffe's test or Games-Howell test, depending, respectively, on the presence or absence of equal variances. $Eta^2$ (%) was used to analyse the contribution of the different factors for parameters' variability. The adjusted $R^2$ indicated the quality of the models. One-way ANOVA was applied to test significant differences in the studied parameters considering the environmental conditions as a fixed factor and, for each environment, to test the existence of significant differences among the morphological aspects of common bean seeds, such as seed coat colour and seed size, as well as the gene pool of origin, at a significant level of 1%.

Multivariate analysis by principal component analysis (PCA) was performed to explore accessions' spatial grouping. The number of retained components was based on the Kaiser's criterion, eigenvalues higher than one, and the retained components applied in articulation with cluster analysis (K-means cluster analysis) to predict clusters' membership. For multivariate analysis purposes, only the analysed parameters with communalities above 0.4 in the retained components were considered. The number of clusters was defined by a percentage of explained variance

higher than 50%. To sharpen groups' separation and establish correlations between the studied parameters and the defined clusters Partial Least Square – Discriminant Analysis (PLS-DA) was applied using Unscrambler® X 10.4.1, Camo Analytics Software (Oslo, Norway). After full cross-validation, quality parameters such as $R^2(X)$, correlation coefficient of multiple determination for X, $R^2(Y)$, correlation coefficient of multiple determination for Y, RMSEC, root mean square error of calibration, RMSECV, root mean square error of validation and $Q^2$, cross-validated correlation coefficient were evaluated [26]. Cytoscape software (Seattle, WA) version 3.7.1, free downloadable from cytoscape.org [27] was applied for network data integration. For the correlation-based network, the data collected by untargeted and targeted metabolomics were processed using the Correlation Calculator for Metabolomics data, freely available in the Metscape website [28]. Partial correlations were calculated using DSPC (Debiased Sparse Partial Correlation) in order to measure the association between two metabolites without the confounding effect of all other metabolites related to them [28]. The saved CSV (common separated values) file containing the partial correlations and corresponding significance levels was imported into Cytoscape software. A circular layout was selected to represent the partial correlations (edges) established between the analysed parameters (nodes).

## 3. Results and Discussion

Only few metabolomics studies have been dedicated to the qualitative and quantitative diversity in common bean dry seeds [29] or to the environmental effects, e.g. site of growth [30], in their metabolomics

profiles. In order to enlarge the existent knowledge to increase the efficiency of common bean breeding and production, the present study was conducted with 107 different underexplored Portuguese common bean accessions cropped in two contrasting environments and the metabolite profiles, from the harvested common bean dry seeds, were further analysed by spectrophotometric and LC-Mass spectrometry methodologies.

3.1. Metabolic diversity of common bean dry seeds

The annotation of metabolites using Q-Orbitrap-MS was carried out using available online databases by comparison with mass accuracy, MS spectra and MS/MS fragmentation spectra. By using Q-Orbitrap-MS, 70 compounds, **Table 1**, from a dataset of 827 selected compounds were annotated, **Figure S1**. For the compounds' selection, PLS-DA analysis of an initial dataset of 1122 compounds was performed considering the environment, the accession and the gene pool of origin as fixed factors. Only the compounds with VIP scores higher than 0.8 [19] were selected.

As shown in *Venn's* diagram, only 35.6% of the selected compounds were responsible for the common bean accessions' discrimination considering genotype, gene pool of origin or environmental conditions, **Figure S1.** As shown in **Table 1**, 42 compounds were described, for the first time, in Fabaceae species, namely in common bean.

Although multiple databases were used for compounds identification [21], compounds annotation was impaired by the quality of the MS spectra and MS/MS fragmentation spectra published online. In fact, the previous poor investment in the legume metabolomics research field (only

9768 articles of plant metabolomics from a universe of 1223442 articles dedicated to plants at the date of manuscript writing, 27 February 2021) [31] has overall hampered compounds annotation. The annotated compounds, **Figure 1A, Figure S2**, were classified, accordingly to the web-based application, ClassyFire, into seven different superclasses: organoheterocyclic compounds; phenylpropanoids and polyketides; organic oxygen compounds; benzenoids; lipids and lipid-like molecules; nucleosides, nucleotides and analogues and into the superclass of organic acids and derivatives. Most of the newly described compounds belonged to phenylpropanoids and polyketides (Cp4, Cp12, Cp14, Cp16, Cp17, Cp20, Cp24, Cp25, Cp27, Cp34, Cp35, Cp36, Cp40, Cp41, Cp48, Cp49, Cp51, Cp56 and Cp66) as well as to lipids and lipid-like molecules superclasses (Cp23, Cp31, Cp38, Cp55, Cp57, Cp58, Cp59, Cp60, Cp61, Cp63, Cp64, Cp65, Cp67, Cp68, Cp69, Cp70). Other new compounds were classified into organic oxygen compounds (Cp13, Cp19), benzenoids (Cp28, Cp37 and Cp45), nucleosides, nucleotides and analogues (Cp26) and organic acids and derivatives superclasses (Cp53). The phenylpropanoid and polyketides superclass was the one with higher diversity of compounds (34 of 70 compounds). This vast superclass of compounds comprises the largest pool of secondary metabolites representing 20% of the total carbon in biosphere [32]. Characterized by an aromatic ring linked to a three-carbon propene chain these compounds derived from deamination of phenylalanine [33], **Figure S3**. With strong effects on plant growth and development, these compounds are also involved in the plant response to biotic and abiotic stresses, contributing to plant environmental adaptability and survival [34]. As shown in **Figure 1B**, the phenylpropanoid and

polyketides superclass shares with the benzenoids superclass several metabolic pathways including the alkaloids and terpenoids biosynthesis. Additionally, phenylpropanoid and polyketides participate through the AMPK signalling pathway, **Figure 1B**, on downregulation of processes such as gluconeogenesis, lipid and protein synthesis, promoting fatty acid oxidation and autophagy, which may have interest for the treatment of type II diabetes, obesity and cancer [3, 35]. Within this superclass, the flavonoids class was the most abundant with a total of 21 identified compounds. Flavonoids are known to play several key roles in plants, contributing for the establishment of symbiotic relationships between plants and microorganisms as well as in plant survival through the action of compounds that may induce insects and herbivores repelling and/or pollinators' attractiveness, e.g. anthocyanins [36].

The second most abundant superclass was the one named as lipids and lipid-like molecules, which included a total of 21 compounds. Into this superclass the prenol lipids class was one of the most diversified classes with a total of 15 annotated compounds. Eleven of the 15 compounds were classified as triterpene saponins and triterpenoid compounds. Triterpenes are ubiquitous compounds in the plant kingdom, comprising six isoprene units in their structure. They can act as signalling molecules or as in the case of glycosylated triterpenes (saponins) as protecting compounds against pathogens [37]. Triterpenes can be biosynthesized through the cytosolic mevalonate (MVA) pathway or alternatively by the plastidial non-mevalonate pathway (2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate), MEP/DOXP pathway, **Figure S3**. Several factors such as light and heat stress affect metabolic routes involved in the

delivery and/or competition for carbon precursors [38], including the lipid and phenylpropanoid biosynthesis, **Figure S3**. Although a possible connection between the metabolic routes of terpenoids and phenylpropanoids exist, further studies are required to understand the regulation of both pathways [39].

Represented by nine compounds, the benzenoids superclass includes compounds described in Kegg and MetaCyc databases as metabolites involved in the shikimate pathway, which participate in the synthesis of compounds with several essential roles in plant physiology (e.g. hormones, folate, amino acids and secondary metabolites biosynthesis). Involved in siderophore group biosynthesis, salicylic acid attenuates plant iron deficiency, especially in calcareous soil, where the availability of Fe (II) is impaired [40]. Salicylic acid as a metabolite produced by the family Fabaceae has also an ecological role in the phytoremediation of contaminated soils participating in the degradation pathways of several soil contaminants/ pollutants such as polycyclic aromatic hydrocarbons, dioxins, toluene, naphthalene and bisphenol, **Figure 1B** [41, 42]. The high qualitative diversity of metabolites described above was identified in the common bean accessions regardless of the cropping environmental conditions.

**Table 1.** Tentative identification of metabolites in common bean accessions using *Compound Discoverer* software, m/zCloud and/or Chemspider (CS) identifications (ID) are presented. For more details about the references please consult the list provided in Supplementary material

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (matched fragments) | Database (m/z Cloud ID; CS ID) | Reference(Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Organoheterocyclic | Pteridines and derivatives (Flavins) | Cp1 | Riboflavin cyclic 4',5'-phosphate | 5.585 | 437.08676 | 438.09406 | 438.09399 | 0.16 | $C_{17}H_{19}N_4O_8P$ | - | 2.30 | 68.99525; 72.99273; 82.0294; 84.04554; 86.02475; 87.0081; 98.02467; 100.03986; 110.02464; 111.00861; 112.08742; 128.03522; 134.02806; 134.06094; 136.03976; 150.017; 153.06635; 167.04373; 171.07697; 200.1769; 221.09337; 222.04153; 235.106; 263.10025; 265.08267; 345.07666; 351.20087; 357.07608; 376.19833; 377.17981; 395.19055; 401.06747; 419.07706 ($C_{17}H_{18}N_4O_7P$); 437.21289 | CS (9201698) | [53] FDB031146 (Common bean) |
| Phenylpropanoids and polyketides | Flavonoids (Flavonoid-3-O-glycosides) | Cp2 | Quercetin-3β-D-glucoside or isomer | 6.037 | 463.08792 | 464.09548 | 464.09517 | 0.65 | $C_{21}H_{20}O_{12}$ | 74.2 | 10.43 | 57.03429; 119.01334; 123.00887; 124.01643; 125.02424 (C6H5O3); 135.00861; 137.02417; 143.04955; 144.05801; 147.00868; 148.01642; 149.02386; 159.04468; 161.02376; 163.00333; 164.01111; 171.04472; 173.02419; 175.03983; 177.01894; 183.04489; 186.0321; 187.03987; 188.04755; 189.01942; 190.99831; 191.03505; 198.03284; 199.04027; 201.01961; 203.03467; 204.04247; 205.05058; 211.04031; 215.03564; 216.04272; 226.0275; 227.03473; 228.04301; 229.01361; 233.04486; 239.03481; 243.02953; 254.02164; 255.02963; 256.03699; 271.0246; 272.03259; 273.04004 ($C_{14}H_9O_6$); 283.02457; 299.01965; 300.02734 ($C_{15}H_8O_7$); 301.03506 ($C_{15}H_9O_7$); 315.04919; 337.05704; 463.08783 ($C_{21}H_{19}O_{12}$) | m/z Cloud (1472) CS (4444361) | [54] (Common bean) |
| | | Cp4 | Isorhamnetin-3-glucoside | 6.360 | 477.10336 | 478.11112 | 478.11063 | 1.01 | $C_{22}H_{22}O_{12}$ | - | 12.24 | 57.0339; 124.01664; 125.02419 ($C_6H_5O_3$); 143.04958; 147.00874; 148.0162 ($C_8H_4O_3$); 149.0246; 155.04964; 159.04492; 163.00313; 171.0448; 177.01822; 183.0451; 185.02393; 187.03972; 197.02353; 198.03166; 199.03996; 201.01913; 203.03558; 211.03947; 213.01938; 214.02678; 215.03488; 225.01859; 226.02693; 227.03497; 229.01434; 242.02182; 243.0294; 253.01367; 254.02124; 255.02972; 256.03668; 257.00906; 270.01624; 271.02451; 272.03223; 281.00912; 282.01688; 283.02417; 298.01218; 299.0195; 300.02737 ($C_{15}H_8O_7$); 313.035; 314.04318 ($C_{16}H_{10}O_7$); 315.05106 ($C_{16}H_{11}O_7$); 329.06693; 477.10385 ($C_{22}H_{21}O_{12}$) | CS (4477169) | [55] (Strawberry) |
| | | Cp15 | Rutin | 7.224 | 609.14600 | 610.15338 | 610.15323 | 0.26 | $C_{27}H_{30}O_{16}$ | 90.7 | 22.86 | 63.02348; 65.00316; 71.0135; 83.01385 ($C_4H_3O_2$); 93.03432; 107.01379; 108.02193 ($C_6H_4O_2$); 109.02912; 119.05016; 121.02897; 125.02473 ($C_6H_5O_3$); 148.01617; 151.00362 ($C_7H_5O_4$); 163.00389; 165.01881; 177.01878; 178.99857; 185.06102; 187.04041; 199.03963; 211.04004; 226.02658; 227.03464; 243.02951; 245.04579 ($C_{13}H_{19}O_5$); 254.02158; 255.02982; 271.02478; 271.06067; 272.03287; 283.02481; 299.01941; 300.0275 ($C_{15}H_8O_7$); 301.03555 ($C_{15}H_9O_7$); 609.14624 ($C_{27}H_{29}O_{16}$) | m/z Cloud (28) CS (4444362) | [54] (Common bean) |
| | | Cp18 | Quercetin-3β-D-glucoside or isomer | 7.388 | 463.08798 | 464.09549 | 464.09526 | 0.49 | $C_{21}H_{20}O_{12}$ | 76.1 | 14.71 | 63.02364; 65.00301; 83.01347; 107.01371; 108.02141 ($C_6H_4O_2$); 109.02923; 121.02964; 135.00818; 148.01666; 151.0036 ($C_7H_5O_4$); 163.00349; 164.01115; 178.99829; 183.04454; 187.03999; 199.03976; 201.05544; 203.03491; 211.03943; 215.03441; 226.02731; 227.03473; 243.02954; 245.04408; 254.0224; 255.02965; 271.02454; 272.03134; 273.04132; 283.02463; 299.0209; 300.02744 ($C_{15}H_8O_7$); 301.03519 ($C_{15}H_9O_7$); 463.08813 ($C_{21}H_{19}O_{12}$) | m/z Cloud (1472) CS (4444361) | [54] (Common bean) |
| | | Cp21 | Astragalin (Kaempferol-3-O-glucoside) | 7.669 | 447.09302 | 448.10056 | 448.10031 | 0.55 | $C_{21}H_{20}O_{11}$ | 82.7 | 17.11 | 63.02389; 65.00321; 83.0136; 91.01852; 93.03452; 107.01379; 108.02133; 109.02919; 117.03447 ($C_8H_5O$); 125.02403; 132.02153; 135.00864; 137.02403; 143.05026; 145.02919; 151.00366; 153.019; 154.04243; 155.04982; 157.0659; 159.04495; 163.00334; 164.01151; 165.01952; 167.0497; 169.06581; 171.04466; 174.03226; 178.99818; 182.03697; 183.04501; 185.02484; 185.06064; 187.0399; 189.05603; 190.99911; 195.04393; 197.06096; 199.04019; 200.04802; 201.0553; 210.03252; 211.03999; 212.04787; 213.01994; 213.05571; 214.02745; 215.035; 226.02658; 227.03491; 228.04253; 229.05031 ($C_{13}H_9O_4$); 239.03462; 240.04268; 241.05019; 243.02991; 255.02974 ($C_{14}H_7O_5$); 256.03751; 257.04535 ($C_{14}H_9O_5$); 267.02914; 269.04529 ($C_{15}H_9O_5$); 283.02496; 284.0325 ($C_{15}H_{18}O_6$); 285.04034 ($C_{15}H_9O_6$); 299.05554; 327.05203; 447.09299 ($C_{21}H_{19}O_{11}$) | m/z Cloud (8165) CS (4445311) | [56] (Common bean) |

**Table 1.** *Cont*

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (matched fragments) | Database (m/z Cloud ID; CS ID) | Reference (Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phenylpropanoids and polyketides | Flavonoids (Flavonoid-3-O-glycoside) | Cp24 | 2"-acetylastragalin | 7.885 | 489.10345 | 490.11112 | 490.11071 | 0.82 | $C_{23}H_{22}O_{12}$ | - | 18.75 | 65.00303; 93.03423; 107.01376; 132.02142; 135.00865; 143.05003; 151.00375; 159.04501; 163.00388; 164.01094; 167.04965; 169.06551; 171.04497; 183.04509; 185.02477; 185.061; 187.04008; 189.05655; 195.04428; 199.03964; 211.04004; 213.05563; 227.03484; 229.05037; 239.03474; 255.02963 ($C_{14}H_7O_5$); 256.03796; 257.04526 ($C_{14}H_9O_5$); 283.02548; 284.03232 ($C_{15}H_8O_6$); 285.04016 ($C_{15}H_9O_6$); 489.10324 ($C_{23}H_{21}O_{12}$) | CS (26345009) | [57] (*Delphinium staphisagria*) |
| | | Cp36 | kaempferol 3-O-rutinoside | 8.218 | 593.15100 | 594.15845 | 594.15826 | 0.32 | $C_{27}H_{30}O_{15}$ | - | 28.57 | 57.03396; 59.01331; 65.0032; 83.01394 ($C_4H_3O_2$); 85.02898; 107.01376; 125.02415 ($C_6H_5O_3$); 135.04515; 151.00369; 161.02408; 287.05612; 449.10944; 491.11969 ($C_{23}H_{23}O_{12}$); 593.15259 ($C_{27}H_{29}O_{15}$) | CS (4588328) | [58] (*Costus spectabilis*) |
| | Flavonoids (Flavonoid-7-O-glycosides) | Cp11 | Eriodictyol-7-glucoside | 6.924 | 449.10864 | 450.11621 | 450.11592 | 0.65 | $C_{21}H_{22}O_{11}$ | - | 6.76 | 57.03432; 63.02388; 65.00315; 81.03445; 83.0138 ($C_4H_5O_2$); 93.03448; 95.0137; 97.0293; 105.03442; 107.01374; 107.05006; 108.02158 ($C_6H_4O_2$); 109.02948; 111.00852 ($C_5H_3O_3$); 117.03432; 119.05013; 121.02941; 123.04472; 124.01651; 125.02422 ($C_6H_5O_3$); 131.05013; 133.02969; 135.04507; 137.02425; 138.03223; 139.04008; 149.02426; 151.00352; 152.01115; 153.01912; 155.05061; 157.0659; 158.03677; 159.04491; 163.00356; 164.01105; 165.01915; 167.03529; 169.06598; 171.04485; 172.05258; 173.06067; 175.03958; 176.01114; 177.05537; 178.9984; 179.03442; 180.05777; 181.06584; 183.04486; 192.00645; 193.01407; 196.05367; 197.06018; 199.03995; 199.076; 200.04819; 201.05563; 213.056; 215.07103; 219.06615; 224.04634; 225.05563; 241.05089; 243.06552; 255.06644; 259.06073; 269.0452; 283.06039; 287.05627; 287.09149; 311.05554; 355.06595; 449.10843 ($C_{21}H_{21}O_{11}$) | CS (10186421) | [59] (Soybean and Mung bean) |
| | | Cp25 | Luteolin 7-O-(6-O-malonyl-beta-D-glucoside) | 7.885 | 533.09320 | 534.10095 | 534.10053 | 0.79 | $C_{24}H_{22}O_{14}$ | - | 7.41 | 93.03452; 107.01387; 132.02161; 135.00853; 143.05046; 151.00342; 159.04503; 163.00371; 183.04488; 185.06064; 187.03976; 189.05539; 197.0605; 199.04019; 211.04022; 213.05542; 227.0349; 229.05051; 239.03514; 241.05061; 255.02975; 256.03775; 257.04572; 267.02927; 284.0325 ($C_{15}H_8O_6$); 285.04031; 489.10379 ($C_{23}H_{21}O_{12}$) | CS (4444988) | [53] FDB000139 (Celery leaves) |
| | | Cp29 | Naringin | 8.000 | 579.17181 | 580.17921 | 580.17887 | 0.58 | $C_{27}H_{32}O_{14}$ | 38.7 | 12.50 | 57.03391; 85.02946 ($C_4H_5O_2$); 119.05054; 163.03984; 191.01912; 225.05534; 241.0862; 433.13412 | m/z Cloud (17) CS (390868) | [60] (common bean) |
| | | Cp48 | Diosmin (Diosmetin-7-O-rutinoside) | 9.194 | 607.16650 | 608.17413 | 608.17385 | 0.46 | $C_{28}H_{32}O_{15}$ | - | 8.33 | 57.03403; 65.00317; 89.00304; 97.02938 ($C_5H_5O_2$); 109.02921; 121.0298; 135.04507; 149.9957; 165.01912; 301.07156; 463.12524; 505.13818 | CS (4444932) | [53] FDB000693 (rosemaries, lemon, orange, vegetables) |

**Table 1.** *Cont*

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (matched fragments) | Database (m/z Cloud ID; CS ID) | Reference (Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phenylpropanoids and polyketides | Flavonoids (Flavonoid-O-glycosides) | Cp14 | Plantagoside | 7.074 | 465.10342 | 466.11112 | 466.11066 | 0.97 | $C_{21}H_{22}O_{12}$ | - | 9.09 | 57.03432; 63.02377; 65.00312 ($C_4HO$); 83.01381 ($C_4H_3O_2$); 93.03439; 95.01369; 107.01373; 109.0295; 111.00842; 121.02964; 123.00851; 123.04493; 124.01641; 125.02424 ($C_6H_5O_3$); 137.0242; 139.04001; 147.04515; 149.02431; 150.03209; 151.00352; 151.03984; 152.01135; 153.01924; 161.02457; 161.06064; 165.01904; 169.0146; 171.04501; 173.02383; 173.06082; 174.0322; 175.03983; 177.01903; 178.9984; 181.01329; 188.04738; 189.05534; 191.03505; 193.05054; 199.03989; 201.01947; 213.05559; 215.03482; 216.04341; 217.05051; 219.02946; 231.06541; 241.05032; 243.02931; 257.04471; 259.06085; 275.05643; 285.04013; 303.05066 ($C_{15}H_{11}O_7$); 465.10275 ($C_{21}H_{21}O_{12}$) | CS (151954) | [61] (Plantago major seeds) |
| | | Cp16 | Phloridzin or isomer | 7.245 | 435.12930 | 436.13693 | 436.13655 | 0.88 | $C_{21}H_{24}O_{10}$ | - | 19.05 | 57.03417; 69.03426; 83.01381 ($C_4H_3O_2$); 93.03437 ($C_6H_5O$); 107.05038; 109.0294 ($C_6H_5O_2$); 121.02936; 123.04499; 125.02429 ($C_6H_5O_3$); 134.03729; 135.0451; 137.02429; 147.04532; 151.03989; 161.02425; 175.0401; 179.03532; 191.03546; 273.07782; 299.07736; 435.13086 | CS (16498836) | [62] (Apple) [63] (Strawberry) |
| | | Cp20 | Phloridzin or isomer | 7.662 | 435.12930 | 436.13693 | 436.13659 | 0.78 | $C_{21}H_{24}O_{10}$ | - | 20.00 | 81.0345; 83.01353; 93.03456 ($C_6H_5O$); 95.05013; 97.02901; 99.04459; 119.05019; 123.04508; 125.02416 ($C_6H_5O_3$); 137.02454; 137.06082; 139.03992; 165.05559; 167.03481; 179.03523; 189.05548; 209.04544; 273.0762 ($C_{15}H_{13}O_5$); 315.08713 ($C_{17}H_{15}O_6$); 345.09686 | CS (16498836) | [62] (Apple) [63] (Strawberry) |
| | Flavonoids (Biflavonoids and polyflavonoids) | Cp7 | Procyanidin C1 or isomer | 6.420 | 865.19849 | 866.20581 | 866.20571 | 0.11 | $C_{45}H_{38}O_{18}$ | - | 5.66 | 83.01376; 93.03417; 95.04984; 97.02924; 107.05019; 108.02151 ($C_6H_4O_2$); 109.0293 ($C_6H_5O_2$); 121.02942; 123.04499; 125.02419 ($C_6H_5O_3$); 131.05003; 133.02939; 135.04512; 137.0242; 139.0401; 145.02977; 147.04529; 149.02426; 150.032 ($C_8H_6O_3$); 151.03989; 159.04503; 161.02417; 161.06015; 162.03165; 163.00357; 163.03984; 164.01118; 165.01909; 167.03531; 173.02504; 173.0607; 174.03212; 175.03981; 176.01134; 177.01907; 177.05571; 179.03462; 185.0609; 187.04024; 188.04822; 189.01938; 189.05592; 190.02737; 191.03552; 193.05087; 199.03976; 201.02002; 201.05553; 203.03485; 203.07097; 205.01476; 205.0498; 211.04074; 213.05591; 214.02777; 215.03568; 217.05016; 219.02963; 221.08192; 225.05585; 227.03564; 227.07188; 229.04942; 231.0294; 241.05106; 243.02937; 243.06783; 245.04514; 245.08142; 253.0511; 255.02989; 255.06816; 256.03751; 257.04645; 261.03961; 269.04471; 271.06204; 273.04019; 281.04514; 283.02469; 285.04071; 287.05545 ($C_{15}H_{11}O_6$); 289.07144; 299.0575; 315.08936; 339.0853; 341.0679; 391.04614; 405.06113; 407.07608; 413.0845; 423.07132; 425.08868; 449.08548; 451.1011; 525.08673; 543.09229; 559.13086; 561.10809; 575.11749; 577.13452; 587.12335; 695.13922 ($C_{37}H_{27}O_{14}$); 713.1593; 739.17334; 865.20331 | CS (148540) | [64] (Broad bean) |

**Table 1.** *Cont*

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (common fragments) | Database (m/z Cloud ID; CS ID) | Reference (Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phenylpropanoids and polyketides | Flavonoids (Flavanonols) | Cp22 | Taxifolin | 7.678 | 303.05084 | 304.05829 | 304.05809 | 0.65 | $C_{15}H_{12}O_7$ | - | 17.39 | 57.03432; 57.03698; 63.0237; 65.00314;81.03426; 83.01382; 93.03439 ($C_6H_5O$); 95.04997; 97.02924; 105.03426; 107.01367; 108.02151 ($C_6H_4O_2$); 109.02943 ($C_5H_4O_2$); 111.0086; 121.0295; 122.03725; 123.04501; 124.01653 ($C_6H_4O_3$); 125.02425 ($C_6H_5O_3$); 133.02928; 137.02403; 139.03999; 143.05017; 145.02937; 146.03773; 147.04495; 149.02443; 150.03212 ($C_8H_6O_3$); 151.00348 ($C_7H_3O_4$); 151.03989 ($C_8H_7O_3$); 152.01157 ($C_7H_4O_4$); 153.01927; 161.02477; 161.06131; 164.04774; 165.05553; 167.03525; 171.04514; 173.02477; 173.06114; 174.03221; 175.03992; 177.01921; 178.99815; 183.03001; 188.04781; 189.05548; 193.05051; 199.03996; 201.01956; 213.05603; 215.03563; 216.04346; 217.05046; 231.06696; 241.05031; 243.02992; 259.06058; 275.05646; 285.04025; 303.0509 ($C_{15}H_{11}O_7$) | CS (388626) | [65] (Common bean) |
| | Flavonoids (Flavone) | Cp43 | Luteolin | 8.602 | 285.04019 | 286.04774 | 286.04751 | 0.80 | $C_{15}H_{10}O_6$ | 69.6 | 11.76 | 59.01362; 63.02361; 65.0031; 83.01376; 109.02883; 133.02939 ($C_8H_5O_2$); 143.04993; 149.02365; 151.00299; 171.0448; 175.03999; 199.03998; 201.01891; 213.05498; 217.05048; 241.05124; 285.04016 ($C_{15}H_9O_6$) | m/z Cloud (1316) CS (4444102) | [66] (Lentils, common beans and chickpeas |
| | Flavonoids (Flavonols) | Cp46 | Quercetin | 8.761 | 301.03510 | 302.04265 | 302.04246 | 0.65 | $C_{15}H_{10}O_7$ | 94.2 | 15.38 | 63.02383; 65.00312; 65.00629; 83.01382; 93.03444; 107.01377; 109.02908; 121.02931; 124.01637; 139.03981; 149.02422 ($C_8H_5O_3$); 151.00354 ($C_7H_3O_4$); 159.04453; 161.02376; 164.01181; 169.01451; 178.99828; 187.03966; 193.0139; 201.05533; 227.03516; 229.04999; 245.04446; 255.02936; 273.03983 ($C_{14}H_9O_6$); 301.0351 ($C_{15}H_9O_7$) | m/z Cloud (27) CS (12269344) | [60] (Common bean) |
| | | Cp50 | Kaempferol | 9.404 | 285.04020 | 286.04773 | 286.04751 | 0.77 | $C_{15}H_{10}O_6$ | - | 7.77 | 63.02385; 65.0032; 67.01837; 79.01838; 83.01379; 89.03929; 91.01893; 93.03448; 95.01376; 107.01377; 108.02161; 109.02937; 117.03448 ($C_8H_5O$); 119.01375; 119.05044; 121.02923; 123.00876; 123.045; 129.03418; 130.04214; 131.05013; 132.02103; 133.02942 ($C_8H_5O_2$); 135.00873; 136.01604; 137.02411; 141.0708; 143.05017; 145.02954; 145.06511; 147.04497; 151.00359; 154.04231; 155.05006; 156.05782; 157.02946; 157.06583; 158.03758; 159.04498; 161.02426; 161.06017; 163.00366; 164.01125; 165.01886; 167.05006; 168.05783; 169.015; 169.02892; 169.0657; 171.04506; 173.02435; 173.06007; 174.03168; 175.03941; 183.04457; 184.05263; 185.06067; 187.03993; 189.05579; 190.9982; 191.03448; 192.006; 195.04509; 196.05235; 197.06126; 198.03209; 199.04024; 201.05562; 210.03152; 211.04008; 212.04738; 213.05579; 214.02721; 215.03493; 219.02966; 227.03459; 229.05055 ($C_{13}H_9O_4$); 239.03482; 240.04224; 241.05154; 243.02931; 255.0298 ($C_{14}H_7O_5$); 256.03781; 257.04572 ($C_{14}H_9O_5$); 267.02982; 268.03659; 284.03308 | CS (4444395) | [60] (Common bean) |
| | Flavonoids (4'-O-methylated flavonoids) | Cp51 | Diosmetin | 9.429 | 299.05591 | 300.06338 | 300.06318 | 0.69 | $C_{16}H_{12}O_6$ | - | 14.29 | 135.00819; 148.01659; 150.0318; 151.00337 ($C_7H_3O_4$); 176.01111; 183.0448; 195.04459; 199.03978; 200.04805; 211.04001; 212.0473; 227.03481; 228.04205; 239.03557; 240.04234; 255.02943; 256.03668; 267.02969; 283.0242; 284.03226 ($C_{15}H_8O_6$); 299.05579 ($C_{16}H_{11}O_6$) | CS (4444931) | [53] (citrus, common sage, common thyme) |

**Table 1.** *Cont*

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (matched fragments) | Database (m/z Cloud ID; CS ID) | Reference (Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phenylpropanoids and polyketides | Flavonoids (4'-O-methylated flavonoids) | Cp52 | Hesperetin (5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)chroman-4-one) | 9.567 | 301.07159 | 302.07904 | 302.07879 | 0.84 | $C_{16}H_{14}O_6$ | 76.9 | 6.25 | 63.02355; 65.00274; 93.0345; 107.01354; 108.02097; 109.02955; 121.02911; 134.03687; 136.01627; 137.02451; 151.00398; 165.01921; 177.019; 196.00069; 285.04175; 301.07184 ($C_{16}H_{13}O_6$) | m/z Cloud (6639) CS (65234) | [53] FDB002677 (citrus and Fabaceae) |
| | Isoflavonoids (Isoflavonoid-O-glycoside) | Cp17 | Daidzin 4'-O-glucuronide | 7.371 | 591.13488 | 592.14282 | 592.14247 | 0.59 | $C_{27}H_{28}O_{15}$ | - | 25.00 | 57.03398; 71.01355; 85.02953 ($C_4H_5O_2$); 111.00818; 129.01921 ($C_5H_5O_4$); 134.03728; 149.02454; 193.05032 | CS (30777617) | [67] (Soy) [68] HMDB0041719 (Food and plants) |
| | Isoflavonoids (Isoflavones) | Cp32 | Genistein | 8.098 | 269.04523 | 270.05282 | 270.05257 | 0.94 | $C_{15}H_{10}O_5$ | 87.3 | 13.33 | 91.01887; 133.0294; 135.00899; 153.01956; 157.06618; 169.06619; 180.05835; 181.06633; 183.04524; 197.06108; 199.03879; 207.04538; 225.05507; 241.05103 ($C_{14}H_9O_4$); 269.04535 ($C_{15}H_9O_5$) | m/z Cloud (24) CS (4444448) | [60] (Common bean) |
| | | Cp39 | Daidzein | 8.524 | 253.05034 | 254.05791 | 254.05766 | 0.96 | $C_{15}H_{10}O_4$ | 95.7 | 33.33 | 91.01878 ($C_6H_3O$); 132.0215; 133.02974; 135.00883 ($C_7H_3O_3$); 135.04501; 195.04478 ($C_{13}H_7O_2$); 196.05251; 197.06056; 208.05275; 209.06039; 223.04062 ($C_{14}H_7O_3$); 224.04826; 225.05556; 252.04214; 253.0504 ($C_{15}H_9O_4$) | m/z Cloud (680) CS (4445025) | [60] (Common bean) |
| | | Cp44 | Glycitein | 8.633 | 283.06097 | 284.06847 | 284.06824 | 0.81 | $C_{16}H_{12}O_5$ | 80.4 | 4.55 | 91.01885; 108.02149; 132.02179; 135.00879; 148.0166; 153.01938; 156.05812; 160.01695; 183.04543; 184.05299; 195.04518; 196.05325; 211.04012; 212.04794; 223.04013; 224.04764; 239.03484; 240.04282; 251.03471; 267.02979; 268.03754; 283.0611 ($C_{16}H_{11}O_5$) | m/z Cloud (428) | [69] (common beans) |
| | Aurone flavonoids (Auronols) | Cp34 | Maesopsin (2,4,6-Trihydroxy-2-(4-hydroxybenzyl)-1-benzofuran-3(2H)-one) | 8.206 | 287.05591 | 288.06339 | 288.06319 | 0.69 | $C_{15}H_{12}O_6$ | 94.1 | 2.78 | 57.03433; 63.02388; 65.0032; 81.03452; 83.01379; 93.034;107.0138; 107.04974; 109.02916; 121.02886; 123.04467; 124.01658; 125.0243; 131.0499; 133.0302; 134.03682; 135.04494; 151.00362; 152.01143; 153.01945; 156.05838; 157.06549; 159.04526; 172.05269; 173.06056; 177.05565; 178.99837; 199.07549; 201.05568; 213.05539; 215.07123; 241.05026; 243.06662; 259.06094; 269.04568; 287.056 ($C_{15}H_{11}O_6$) | m/z Cloud (7874) CS (141288) | [70] (*Artocarpus tonkinensis*) |

**Table 1.** *Cont*

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (matched fragments) | Database (m/z Cloud ID; CS ID) | Reference (Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phenylpropanoids and polyketides | Cinnamic acids and derivatives (Coumaric acid derivatives) | Cp12 | Calceolarioside B | 6.986 | 477.13998 | 478.14752 | 478.14709 | 0.91 | $C_{23}H_{26}O_{11}$ | - | 21.05 | 59.01365; 71.01389 ($C_3H_3O_2$); 83.01339; 85.02962 ($C_4H_5O_2$); 89.02396; 93.03415; 97.02933; 101.02424 ($C_4H_5O_2$); 113.02401; 120.98817; 123.04475; 135.04486 ($C_8H_7O_2$); 137.02422; 139.03951; 187.07664; 189.05528; 273.07678; 299.1138; 431.15784 | CS (4437970) | [71] (*Stauntonia brachyanthera*) |
| | | Cp27 | sinapoyl D-glucoside | 7.954 | 385.11365 | 386.12131 | 386.12096 | 0.90 | $C_{17}H_{22}O_{10}$ | - | 16.00 | 57.03432; 59.01359; 65.00309; 68.99546; 71.0136; 83.01378 ($C_4H_3O_2$); 85.0294 ($C_4H_5O_2$); 95.01377; 97.02946 ($C_5H_5O_2$); 121.02905; 123.00842 ($C_6H_3O_3$); 124.01656; 137.02432; 138.03214; 139.04015; 148.01682; 149.99597; 151.00362; 165.01909; 166.02693; 170.02153; 181.05046; 191.01953; 198.01698; 209.02983 | CS (13077484) | [72] (fruits; vegetables; rye; herbs and spices) |
| | Cinnamic acids and derivatives (Hydroxycinnamic acid glycoside) | Cp41 | 1-O-feruloyl-beta-D-glucose | 8.584 | 355.10321 | 356.11072 | 356.11047 | 0.68 | $C_{16}H_{20}O_9$ | - | 11.76 | 57.0341; 95.05016; 107.05035 ($C_7H_7O$); 108.02156 ($C_6H_4O_2$); 109.02872; 115.07603; 119.08636; 121.02932; 122.03713; 123.04459; 125.02396; 133.02934; 161.0242; 163.07628; 175.07626; 193.08672; 355.11871 | CS (26336946) | [53] FDB000238 (green and root vegetables) |
| | Coumarins and derivatives (7-Hydroxycoumarin) | Cp40 | 5,7-Dihydroxy-4-methylcoumarin or isomer | 8.549 | 191.03491 | 192.04226 | 192.04219 | 0.37 | $C_{10}H_8O_4$ | 79.5 | 4.17 | 63.0238; 65.00311; 79.05479; 81.03445; 83.01376; 85.0293; 87.00863; 87.92526; 91.05469; 102.9487; 103.05479; 103.91978; 105.03442; 107.01376; 111.00863; 119.05009; 121.02911; 123.04512; 132.02153; 147.04504; 149.02414; 176.01122; 191.03485 ($C_{10}H_7O_4$); 191.10751 | m/z Cloud (246) | [73] (Plants) |
| | Coumarins and derivatives (6,7-dihydroxycoumarin) | Cp56 | 6,7-Dihydroxy-4-methylcoumarin or isomer | 9.834 | 191.03480 | 192.04226 | 192.04216 | 0.52 | $C_{10}H_8O_4$ | 68.4 | 5.00 | 87.00814; 87.92508; 102.948; 111.0087; 121.02938; 147.04543; 191.03453 ($C_{10}H_7O_4$) | m/z Cloud (266) CS (4477791) | [53] FDB003829 (dill and plants) |

**Table 1.** *Cont*

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (matched fragments) | Database (m/z Cloud ID; CS ID) | Reference (Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phenylpropanoids and polyketides | Linear 1,3-diarylpropanoids (2'-hydroxy-dihydrocalchone) | Cp49 | Phloretin | 9.331 | 273.07660 | 274.08412 | 274.08389 | 0.84 | $C_{15}H_{14}O_5$ | 76.8 | 10.00 | 81.03459; 83.01334; 93.03407; 99.00835; 119.05022; 123.04514; 125.02432; 137.02371; 167.03496; 273.07703 ($C_{15}H_{13}O_5$) | m/z Cloud (542) CS (4624) | [53] FDB015553 (apples, Asian pears, oregano, huckleberries) |
| | Stilbenes (Stilbene glycoside) | Cp35 | Astringin or isomer | 8.210 | 405.11874 | 406.12637 | 406.12601 | 0.89 | $C_{20}H_{22}O_9$ | - | 30.00 | 69.03451; 95.01373; 97.02937; 107.01369; 108.02151 ($C_6H_4O_2$); 123.00861; 125.02425 ($C_6H_5O_3$); 151.00359; 242.05827; 243.06648 ($C_{14}H_{11}O_4$) | CS (4445028) | [74] (spruce *Picea* species) |
| | Macrolides and analogues (Milbemycins) | Cp66 | Avermectin A2a monossachari de | 11.872 | 759.43225 | 760.43976 | 760.43964 | 0.15 | $C_{42}H_{64}O_{12}$ | - | 13.33 | 57.03428; 59.01324; 69.03414; 71.01377 ($C_3H_3O_2$); 72.99249; 75.00868; 85.02948; 87.00806; 95.01328; 97.02869; 99.00817; 113.02435; 116.01108; 125.02401; 759.4325 ($C_{42}H_{63}O_{12}$) | CS (10128280) | [75] (Metabolite from soil microflora community) |
| Organic oxygen compounds | Organooxygen compounds (Glucuronic acid derivatives) | Cp3 | 2-(E)-O-feruloyl-D-galactaric acid or isomer | 6.334 | 385.07742 | 386.08490 | 386.08466 | 0.62 | $C_{16}H_{18}O_{11}$ | - | 55.56 | 55.01833; 57.03434; 59.01368 ($C_2H_3O_2$); 71.01378 ($C_3H_3O_2$); 72.99296 ($C_2HO_3$); 75.00828; 83.01343; 84.02168; 85.02946 ($C_4H_5O_2$); 89.02387; 111.00819; 129.01888; 133.0139 ($C_4H_5O_3$); 134.03738 ($C_8H_6O_2$); 147.02997 ($C_5H_7O_7$); 191.01982 ($C_6H_7O_7$); 193.05028 ($C_{10}H_9O_4$); 209.03011 ($C_6H_9O_8$) | CS (23107088) | [54] (Common bean) |
| | | Cp5 | 2-O-sinapoyl-D-glucaric acid | 6.374 | 415.08793 | 416.09549 | 416.09514 | 0.84 | $C_{17}H_{20}O_{12}$ | - | 47.37 | 55.01868; 57.03432; 59.01357; 71.01376 ($C_3H_3O_2$); 72.99287; 75.00844; 83.01366 ($C_4H_3O_2$); 84.02158; 85.02939 ($C_4H_5O_2$); 87.00838; 111.00867 ($C_5H_3O_3$); 129.01941 ($C_5H_5O_4$); 147.02991 ($C_5H_7O_5$); 149.02431; 164.04781 ($C_9H_8O_3$); 191.01959 ($C_6H_7O_7$); 209.03015 ($C_6H_9O_8$) | CS (21865649) | [54] (Common bean) |

**Table 1.** *Cont*

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (matched fragments) | Database (m/z Cloud ID; CS ID) | Reference (Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Organic oxygen compounds | Organooxygen compounds Glucuronic acid derivatives | Cp8 | 2-(E)-O-feruloyl-D-galactaric acid or isomer | 6.487 | 385.07733 | 386.08490 | 386.08468 | 0.58 | $C_{16}H_{18}O_{11}$ | - | 52.78 | 55.01826; 57.03432; 59.01363; 71.01382 ($C_3H_3O_2$); 72.993 ($C_2HO_3$); 75.00826; 83.01336; 84.02153; 85.02945 ($C_4H_5O_2$); 87.00814; 89.02438 ($C_3H_5O_3$); 111.00876 ($C_5H_3O_3$); 129.01898 ($C_5H_5O_4$); 133.01422 ($C_4H_5O_5$); 134.03722 ($C_8H_6O_2$); 147.02925; 191.01968 ($C_6H_7O_7$); 209.03021 ($C_6H_9O_8$) | CS (23107088) | [54] (Common bean) |
| | | Cp10 | D-Saccharic acid | 6.497 | 209.03012 | 210.03757 | 210.03740 | 0.80 | $C_6H_{10}O_8$ | 71.6 | 64.29 | 57.03432; 59.01361; 71.01379 ($C_3H_3O_2$); 72.99294 ($C_2HO_3$); 75.00864 ($C_2H_3O_3$); 83.01333; 85.02943 ($C_4H_5O_2$); 87.00817; 89.02429 ($C_3H_5O_3$); 111.00848 ($C_5H_3O_3$); 129.01921 ($C_5H_5O_4$); 133.01439 ($C_4H_5O_5$); 191.01888; 209.0302 ($C_6H_9O_8$) | m/z Cloud (1498) CS (30577) | [76] (Mung bean) |
| | Organooxygen compounds (Phenolic glycosides) | Cp13 | Paeonoside | 7.015 | 327.10840 | 328.11581 | 328.11566 | 0.46 | $C_{15}H_{20}O_8$ | - | 53.85 | 58.00575; 59.01365; 69.03429; 71.0138 ($C_3H_3O_2$); 72.99275; 73.02943 ($C_3H_5O_2$); 83.0139 ($C_4H_3O_2$); 85.02922; 89.02417 ($C_3H_5O_3$); 101.02434 ($C_4H_5O_3$); 113.02431 ($C_5H_5O_3$); 123.04494; 161.04581 ($C_6H_9O_5$) | CS (391227) | [77] (*Paeonia suffruticosa*) |
| | Organooxygen compounds (O-glycosyl compound) | Cp19 | Benzyl D-glucopyranoside | 7.415 | 269.10275 | 270.11035 | 270.11007 | 1.04 | $C_{13}H_{18}O_6$ | - | 26.67 | 57.03427; 71.01377 ($C_3H_3O_2$); 72.99303 ($C_2HO_3$); 107.05019 ($C_7H_7O$); 109.06584; 123.0814; 135.082; 137.09706; 147.08124; 153.12813; 163.11261; 179.10741; 207.10254; 225.11301; 269.10275 ($C_{13}H_{17}O_6$) | CS (9251641) | [78] (*Idesia polycarpa*) |
| Benzenoids | Benzene and substituted derivatives M-methoxybenzoic acids derivatives | Cp6 | Vanillic acid | 6.379 | 167.03491 | 168.04226 | 168.04216 | 0.57 | $C_8H_8O_4$ | 87.3 | 50.00 | 108.02164 ($C_6H_4O_2$); 123.0444; 152.01141 ($C_7H_4O_4$); 167.03424 | m/z Cloud (1471) CS (8155) | [79] (Common bean) |

**Table 1.** *Cont*

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (common fragments) | Database (m/z Cloud ID; CS ID) | Reference (Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Benzenoids | Benzene and substituted derivatives Gallic acid and derivatives | Cp9 | Syringic acid acetate | 6.489 | 239.05586 | 240.06338 | 240.06315 | 0.96 | $C_{11}H_{12}O_6$ | - | 30.77 | 59.01332; 87.00863; 106.04239; 107.05015; 108.02142 ($C_6H_4O_2$); 121.0289; 133.06569; 135.04497 ($C_8H_7O_2$); 149.06073; 177.05563; 179.03482; 195.0659 ($C_{10}H_{11}O_4$); 239.05597 ($C_{11}H_{11}O_6$) | CS (196206) | [56] (Common bean) |
| | Benzene and substituted derivatives (Salycilic acid) | Cp42 | Salicylic acid | 8.589 | 137.02420 | 138.03169 | 138.03158 | 0.84 | $C_7H_6O_3$ | 98.5 | 85.71 | 65.0396 ($C_5H_5$); 93.03452 ($C_6H_5O$); 136.86255; 137.02434 ($C_7H_5O_3$) | m/z Cloud (643) CS (331) | [80] (*Vicia faba*) |
| | Benzene and substituted derivatives (Phenyl pyruvic acid) | Cp45 | 4-Hydroxyphenylpyruvic acid | 8.639 | 179.03484 | 180.04226 | 180.04212 | 0.75 | $C_9H_8O_4$ | 72.7 | 7.14 | 79.9572; 90.99797; 91.01877; 93.03453; 95.01376; 108.02159; 121.02937; 122.95914; 134.9879; 136.01651; 137.02435; 150.95369; 151.03978; 179.03482 ($C_9H_7O_4$) | m/z Cloud (1301) CS (552441) | [81] (*Arabidopsis thaliana*) |
| | Phenols (Methoxyphenols) | Cp28 | Homovanillic acid | 7.956 | 181.05054 | 182.05791 | 182.05781 | 0.55 | $C_9H_{10}O_4$ | 59.3 | 13.04 | 65.00311; 68.99567; 83.01378; 86.98614; 89.00304; 92.99361; 95.01379; 97.02924; 112.98502; 121.02898; 123.00864; 124.01653; 136.9834; 137.02434 ($C_7H_5O_3$); 138.03212; 139.04005; 148.01669; 151.00372; 165.01888; 166.02692 ($C_8H_6O_4$); 180.97296; 181.05045 ($C_9H_9O_4$) | m/z Cloud (1296) CS (1675) | [68, 82] (HMDB) HMDB00001 18 (olives; beer; avocado; milk) |
| | Anthracenes (Hydroxyanthraquinone) | Cp37 | Aurantio-obtusin beta-D-glucoside | 8.243 | 491.11923 | 492.12677 | 492.12644 | 0.68 | $C_{23}H_{24}O_{12}$ | - | 26.32 | 59.01371 ($C_2H_3O_2$); 63.02394; 65.00314; 83.01385 ($C_4H_3O_2$); 107.01382; 108.02139; 109.02951; 125.02416 ($C_6H_5O_3$); 134.03719; 135.0451; 151.00356; 161.02412; 165.0192; 169.01419; 196.00067; 287.05588; 431.09366; 449.10675; 491.1189 ($C_{23}H_{23}O_{12}$) | CS (391073) | [83] (*Cassia tora*) |

**Table 1.** *Cont*

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (matched fragments) | Database (m/z Cloud ID; CS ID) | Reference (Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lipids and lipid-like molecules | Fatty acyls (Medium chain fatty acids) | Cp30 | Suberic acid | 7.452 | 173.08174 | 174.08921 | 174.08909 | 0.70 | $C_8H_{14}O_4$ | 93.3 | 66.67 | 57.03433; 83.05014; 109.0658 ($C_7H_9O$); 111.0814 ($C_7H_{11}O$); 129.0919; 173.0817 ($C_8H_{13}O_4$) | m/zCloud (1393) CS (10025) | [53] FDB003340 (Food and Fabaceae plants) |
| | | Cp31 | 2-hydroxycaproic acid | 8.013 | 131.07123 | 132.07864 | 132.07855 | 0.68 | $C_6H_{12}O_3$ | 87.0 | 50.00 | 68.99545; 85.0658 ($C_5H_9O$); 87.04478; 131.07123 ($C_6H_{11}O_3$) | m/z Cloud (153) CS (90191) | [53] FDB022697 (Food) |
| | | Cp33 | Azelaic acid | 8.118 | 187.09749 | 188.10486 | 188.10473 | 0.68 | $C_9H_{16}O_4$ | 96.3 | 60.00 | 57.03428; 69.03455; 83.05016; 95.05 ($C_6H_7O$); 97.06575; 123.08144 ($C_8H_{11}O$); 125.097 ($C_6H_{13}O$); 143.10765 ($C_9H_{15}O_2$); 169.08716 ($C_9H_{13}O_3$); 187.09734 ($C_9H_{15}O_4$) | m/z Cloud (331) CS (2179) | [53] FDB012192 (Food and Fabaceae plants) |
| | Fatty acyls (Fatty acid esters) | Cp55 | Glaurin | 9.810 | 287.22256 | 288.23007 | 288.22987 | 0.69 | $C_{16}H_{32}O_4$ | - | 40.00 | 99.08126; 141.1281; 269.21219; 285.20731 ($C_{16}H_{29}O_4$); 287.22266 ($C_{16}H_{31}O_4$) | CS (60661) | [84] (Wheat) |
| | Fatty acyls (Lineoilc acids and derivatives) | Cp65 | (9Z,12Z)-6,8-Dihydroxy-9,12-octadecadienoic acid | 11.152 | 311.22266 | 312.23006 | 312.22994 | 0.40 | $C_{18}H_{32}O_4$ | 65.7 | 38.10 | 57.03436; 58.00547; 79.95724; 85.02902; 87.04507 ($C_4H_7O_2$); 118.96644; 119.0498; 146.96109; 174.95631; 183.01202; 184.0204; 197.02704; 216.00964; 223.17024 ($C_{14}H_{23}O_2$); 235.17039 ($C_{15}H_{23}O_2$); 275.2002; 293.21243 ($C_{18}H_{29}O_3$); 311.16873; 311.22299 ($C_{18}H_{31}O_4$) | m/z Cloud (7971) CS (22842404) | - |

**Table 1.** *Cont*

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (matched fragments) | Database (m/z Cloud ID; CS ID) | Reference (Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lipids and lipid-like molecules | Fatty acyls (Long chain fatty acids) | Cp69 | 16-Hydroxyhexadecanoic acid | 15.352 | 271.22766 | 272.23514 | 272.23489 | 0.93 | $C_{16}H_{32}O_3$ | 91.2 | 55.56 | 116.92824; 223.20638; 225.22226 ($C_{15}H_{29}O$); 253.21724 ($C_{16}H_{29}O_2$); 271.22763 ($C_{16}H_{31}O_3$) | m/z Cloud (2551) CS (10034) | [68] HMDB0006294 [72] ChEBI 55328 (Plants) |
| | Prenol lipids Iridoid-O-glycoside | Cp23 | Negundoside | 7.803 | 495.15045 | 496.15808 | 496.15772 | 0.73 | $C_{23}H_{28}O_{12}$ | 48.7 | 28.57 | 83.01373 ($C_4H_3O_2$); 107.04993; 109.02956 ($C_6H_5O_2$); 123.04514; 125.02384; 134.03734; 137.02429 ($C_7H_5O_3$); 149.06071; 150.03152; 151.03998 ($C_8H_7O_3$); 151.05229; 161.02431; 287.09229; 449.14578 | m/z Cloud (7691) CS (8111189) | [85] (*Vitex negundo*) |
| | Prenol lipids (Terpene glycoside) | Cp38 | 2-[(2R,4aS,8aR)-7-(beta-D-Glucopyranosyloxy)-4a,8-dimethyl-6-oxo-1,2,3,4,4a,5,6,8a-octahydro-2-naphthalenyl]-2-propanyl beta-D-glucopyranoside | 8.521 | 575.27039 | 576.27820 | 576.27785 | 0.61 | $C_{27}H_{44}O_{13}$ | - | 50.00 | 57.03402; 59.01345; 71.01383 ($C_3H_3O_2$); 75.00824; 85.02936 ($C_4H_5O_2$); 113.0242 ($C_5H_5O_3$); 575.27057 ($C_{27}H_{43}O_{13}$) | CS (9105145) | [86] (African medicinal plants e.g. *Atractylis gumifera*) |
| | Prenol lipids (Diterpene glycoside) | Cp64 | Ciwujianoside C1 | 11.039 | 1041.52686 | 1042.53491 | 1042.53415 | 0.73 | $C_{52}H_{82}O_{21}$ | - | 70.83 | 72.99314 ($C_2HO_3$); 73.02936 ($C_3H_5O_2$); 75.00866 ($C_2H_3O_3$); 83.01373 ($C_4H_3O_2$); 85.0294 ($C_4H_5O_2$); 86.00049; 87.00864 ($C_3H_3O_3$); 87.04494; 89.02429 ($C_3H_5O_3$); 95.01384; 99.00865 ($C_4H_3O_3$); 101.02429 ($C_4H_5O_3$); 103.04016 ($C_4H_7O_3$); 112.01603; 113.02428 ($C_5H_5O_3$); 116.92839; 119.0349 ($C_4H_7O_4$); 131.03468 ($C_5H_7O_4$); 143.03479 ($C_6H_7O_4$); 145.04999; 163.06059; 205.07182 ($C_8H_{13}O_6$); 1023.51471 ($C_{52}H_{79}O_{20}$); 1041.52673 ($C_{52}H_{81}O_{21}$) | CS (143787) | [87] (Ginseng siberian) |
| | Prenol lipids (Triterpene saponin) | Cp47 | Cauloside D | 8.766 | 1073.55322 | 1074.56104 | 1074.56053 | 0.47 | $C_{53}H_{86}O_{22}$ | - | 46.15 | 157.01363; 161.04498; 163.06149 ($C_6H_{11}O_5$); 205.07161 ($C_8H_{13}O_6$); 247.06345; 569.3869; 589.41077; 747.43176 ($C_{41}H_{63}O_{12}$); 865.50403; 927.49725 ($C_{47}H_{75}O_{18}$); 1011.54614; 1055.54333 ($C_{53}H_{83}O_{21}$); 1073.55286 ($C_{53}H_{85}O_{22}$); 1074.552 | CS (10259282) | [88] (*Caulophyllum robustum* roots) |

**Table 1.** *Cont*

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (matched fragments) | Database (m/z Cloud ID; CS ID) | References (Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lipids and lipid-like molecules | Prenol lipids (Triterpene saponins) | Cp54 | Azukisaponin III | 9.744 | 809.43274 | 810.44019 | 810.44002 | 0.20 | $C_{42}H_{66}O_{15}$ | - | 50.00 | 59.0131; 71.01379 ($C_3H_3O_2$); 72.99263; 73.02892; 75.00867 ($C_2H_3O_3$); 83.01341; 85.02941 ($C_4H_5O_2$); 87.0088 ($C_3H_3O_3$); 95.01314; 99.0087 ($C_4H_3O_3$); 101.02361; 113.02429 ($C_5H_5O_3$); 471.34476; 633.40204 ($C_{36}H_{57}O_9$); 747.43256; 809.43219 ($C_{42}H_{65}O_{15}$) | CS (390484) | [71] (adzuki beans) [53] FDB018908 (Fabaceae) |
| | | Cp57 | Pitheduloside K | 9.962 | 1059.53748 | 1060.54541 | 1060.54486 | 0.51 | $C_{52}H_{84}O_{22}$ | - | 40.98 | 75.00865 ($C_2H_3O_3$); 81.03455; 83.0138 ($C_4H_3O_2$); 84.02168 ($C_4H_4O_2$); 85.02942 ($C_4H_5O_2$); 86.00093 ($C_3H_2O_3$); 87.00864 ($C_3H_3O_3$); 89.02433 ($C_3H_5O_3$); 95.01376; 97.02938; 99.00867 ($C_4H_3O_3$); 101.02429 ($C_4H_5O_3$); 111.00876 ($C_5H_3O_3$); 112.01645; 113.02433 ($C_5H_5O_3$); 115.00374 ($C_4H_3O_4$); 119.03483 ($C_4H_7O_4$); 125.02413 ($C_6H_5O_3$); 129.01918 ($C_5H_5O_4$); 131.03485 ($C_5H_7O_4$); 139.00357; 143.03484 ($C_6H_7O_4$); 149.04544; 157.01401 ($C_6H_5O_5$); 159.02965 ($C_6H_7O_5$); 161.04539 ($C_6H_9O_5$); 173.09769; 179.05579; 221.06644 ($C_8H_{13}O_7$); 249.06212; 263.07736 ($C_{10}H_{15}O_8$); 295.24222; 379.30145; 391.30185; 407.2941; 407.34058; 409.34879; 423.33176; 437.34924; 439.31479; 453.33926; 455.35892; 457.36761; 463.36087; 501.358; 511.38144; 541.39001; 553.35455; 557.38348; 559.40076; 597.34583; 673.43506; 699.41107; 717.42114 ($C_{40}H_{61}O_{11}$); 835.48798; 897.49213; 983.52588; 997.53613; 1041.52844 ($C_{52}H_{81}O_{21}$); 1059.53796 ($C_{52}H_{83}O_{22}$); 1060.53296; | CS (10197280) | [53] FDB008683 [68] HMDB0031990 (food and plants) |
| | | Cp59 | (3β,5ξ,9ξ)-3-{[2-O-(β-D-Glucopyranosyl)-β-D-glucopyranosyl]oxy}-23-hydroxyolean-12-en-28-oic acid | 10.421 | 795.45355 | 796.46091 | 796.46079 | 0.15 | $C_{42}H_{68}O_{14}$ | 66.6 | 57.89 | 57.03428; 58.00574; 59.0136; 68.99815 ($C_3HO_2$); 69.03454; 71.01374 ($C_3H_3O_2$); 72.993 ($C_2HO_3$); 73.0294 ($C_3H_5O_2$); 75.00864 ($C_2H_3O_3$); 83.01375 ($C_4H_3O_2$); 84.02148 ($C_4H_4O_2$); 85.0294 ($C_4H_5O_2$); 86.00089 ($C_3H_2O_3$); 87.00864 ($C_3H_3O_3$); 89.0243 ($C_3H_5O_3$); 95.01373; 97.0295; 99.00864 ($C_4H_3O_3$); 101.02426 ($C_4H_5O_3$); 111.00882; 112.01642; 113.0243 ($C_5H_5O_3$); 115.00326; 119.03476 ($C_4H_7O_4$); 129.0195 ($C_5H_5O_4$); 131.03496 ($C_5H_7O_4$); 139.00343; 143.03493 ($C_6H_7O_4$); 157.01405 ($C_6H_5O_5$); 161.04541 ($C_6H_9O_5$); 179.05646; 391.30066; 407.33292; 409.35068; 437.34088; 457.36981; 615.38953 ($C_{36}H_{55}O_8$); 795.4527 ($C_{42}H_{67}O_{14}$) | m/z Cloud (8152) CS (29814912) | - |
| | | Cp60 | (3β,5ξ,9ξ,18ξ)-22-Hydroxyolean-12-en-3-yl 6-deoxy-α-L-mannopyranosyl-(1->2)hexopyranosyl-(1->2)-β-D-glucopyranosiduronic acid | 10.430 | 925.51642 | 926.52390 | 926.52368 | 0.24 | $C_{48}H_{78}O_{17}$ | 94.8 | 65.12 | 67.0188 ($C_4H_3O$); 68.99813 ($C_2HO_2$); 69.03454; 71.01375 ($C_3H_3O_2$); 72.99297 ($C_2HO_3$); 73.02941 ($C_3H_5O_2$); 75.00863 ($C_2H_3O_3$); 83.01382 ($C_4H_3O_2$); 85.0294 ($C_4H_5O_2$); 86.00087 ($C_3H_2O_3$); 87.00861 ($C_3H_3O_3$); 87.04495; 89.02431 ($C_3H_5O_3$); 95.01371; 97.02949 ($C_5H_5O_2$); 99.00867 ($C_4H_3O_3$); 99.04505 ($C_5H_7O_2$); 101.02426 ($C_4H_5O_3$); 103.03989 ($C_4H_7O_3$); 111.00871; 112.01671; 113.02428 ($C_5H_5O_3$); 115.0036 ($C_4H_3O_4$); 115.03993 ($C_5H_7O_3$); 119.03474; 125.02441 ($C_6H_5O_3$); 127.04015 ($C_6H_7O_3$); 131.03481; 139.00389 ($C_6H_3O_4$); 143.03481 ($C_6H_7O_4$); 145.05037 ($C_6H_9O_4$); 157.01384 ($C_6H_5O_5$); 161.04543 ($C_6H_9O_5$); 163.06107; 205.07155 ($C_8H_{13}O_6$); 423.33044; 439.35764; 509.3996; 599.39862; 833.39819; 879.41077; 907.50928; 925.51624 ($C_{48}H_{77}O_{17}$) | m/z Cloud (8183) CS (22913504) | - |
| | | Cp61 | 6-Deoxy-α-L-mannopyranosyl-(1->3)-[β-D-glucopyranosyl-(1->6)]-1-O-[3,27-dihydroxy-27,28-dioxolup-20(29)-en-28-yl]-β-D-glucopyranose | 10.645 | 955.49066 | 956.49808 | 956.49792 | 0.17 | $C_{48}H_{76}O_{19}$ | 59.8 | 65.15 | 68.99817 ($C_2HO_2$); 71.0138 ($C_3H_3O_2$); 72.99308 ($C_2HO_3$); 73.0294 ($C_3H_5O_2$); 75.0087 ($C_2H_3O_3$); 83.01385 ($C_4H_3O_2$); 85.02946 ($C_4H_5O_2$); 86.00097 ($C_3H_2O_3$); 87.00872 ($C_3H_3O_3$); 89.02438 ($C_3H_5O_3$); 95.01382; 97.02889; 99.00872 ($C_4H_3O_3$); 101.02435 ($C_4H_5O_3$); 111.00822; 112.01656; 113.02438; 119.03484 ($C_4H_7O_4$); 125.02399; 129.01917 ($C_5H_5O_4$); 131.03488 ($C_5H_7O_4$); 139.00366; 143.03526 ($C_6H_7O_4$); 157.01385 ($C_6H_5O_5$); 159.03 ($C_6H_7O_5$); 161.04543 ($C_6H_9O_5$); 179.056 ($C_6H_{11}O_6$); 221.06654 ($C_8H_{13}O_7$); 391.30048; 407.33075; 435.32559; 455.35422; 613.37567; 937.4801 ($C_{48}H_{73}O_{18}$); 955.49054 ($C_{48}H_{75}O_{19}$) | m/z Cloud (8186) CS (22913959) | - |

**Table 1.** *Cont*

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (matched fragments) | Database (m/z Cloud ID; CS ID) | Reference (Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lipids and lipid-like molecules | Prenol lipids (Triterpene saponins) | Cp62 | Dehydrosoyasaponin I | 10.945 | 939.49561 | 940.50317 | 940.50288 | 0.31 | $C_{48}H_{76}O_{18}$ | - | 58.62 | 68.99763; 71.01378 ($C_3H_3O_2$); 71.01736; 72.99299 ($C_2HO_3$); 73.02941 ($C_3H_5O_2$); 75.00866 ($C_2H_3O_3$); 83.01385 ($C_4H_3O_2$); 85.02941 ($C_4H_5O_2$); 86.00053; 87.00872 ($C_3H_3O_3$); 89.02434 ($C_3H_5O_3$); 95.01382; 97.02892; 99.00869 ($C_4H_3O_3$); 101.02431 ($C_4H_5O_3$); 113.02435 ($C_5H_5O_3$); 119.03484; 131.03482 ($C_5H_7O_4$); 143.03464 ($C_6H_7O_4$); 157.01387 ($C_6H_5O_5$); 159.02908; 161.04556 ($C_6H_9O_5$); 179.05524; 221.0666 ($C_8H_{13}O_7$); 421.31259; 437.34155; 507.38394; 921.48059; 939.4953 ($C_{48}H_{75}O_{18}$) | CS (571084) | [53] FDB018884 (Fabaceae) |
| | | Cp63 | Spinasaponin A | 11.018 | 793.43790 | 794.44525 | 794.44517 | 0.10 | $C_{42}H_{66}O_{14}$ | - | 48.65 | 55.01825; 57.03389; 58.00534; 59.01365; 59.01633; 67.01855; 68.99814 ($C_3HO_2$); 71.01382 ($C_3H_3O_2$); 71.01761; 71.01924; 72.99306 ($C_2HO_3$); 73.02941 ($C_3H_5O_2$); 75.00871 ($C_2H_3O_3$); 83.01385 ($C_4H_3O_2$); 85.02947 ($C_4H_5O_2$); 86.00063; 87.00876 ($C_3H_3O_3$); 89.02440 ($C_3H_5O_3$); 95.01384; 99.00874 ($C_4H_3O_3$); 101.02436 ($C_4H_5O_3$); 113.02444; 115.00308; 119.03493 ($C_4H_7O_4$); 129.01895($C_5H_5O_4$); 131.03436; 139.00360 ($C_6H_5O_4$); 157.01416 ($C_6H_5O_5$); 161.04535 ($C_6H_9O_5$); 391.30072; 407.33237; 435.32599; 455.35443; 613.37042; 793.43768 ($C_{42}H_{65}O_{14}$) | CS (390522) | [53] FDB013035 (Food and plants) |
| | Prenol lipids (Triterpenoids) | Cp67 | Ursolic acid | 14.767 | 455.35272 | 456.36035 | 456.35995 | 0.87 | $C_{30}H_{48}O_3$ | 80.5 | 33.33 | 79.9569; 319.22989; 455.35263 ($C_{30}H_{47}O_3$) | m/z Cloud (771) CS (58472) | [89] (Fruits and vegetables) |
| | | Cp58 | Jujuboside B | 10.034 | 1043.54272 | 1044.55054 | 1044.5499 | 0.61 | $C_{52}H_{84}O_{21}$ | - | 50.00 | 72.99304 ($C_2HO_3$); 73.02945 ($C_3H_5O_2$); 75.00871 ($C_2H_3O_3$); 75.01272; 81.03427; 83.01381 ($C_4H_3O_2$); 84.02131; 85.02948 ($C_4H_5O_2$); 86.00098 ($C_3H_2O_3$); 87.00871 ($C_3H_3O_3$); 87.04514; 89.02438 ($C_3H_5O_3$); 95.01382; 97.02940 ($C_5H_5O_2$); 99.00875 ($C_4H_3O_3$); 99.04488 ($C_5H_7O_2$); 101.02434 ($C_4H_5O_3$); 103.04002 ($C_4H_7O_3$); 111.00868 ($C_5H_3O_3$); 112.01649; 113.02441 ($C_5H_5O_3$); 115.00333; 115.03983 ($C_5H_7O_3$); 119.03485; 125.0241 ($C_6H_5O_3$); 129.01906 ($C_5H_5O_4$); 131.03496 ($C_5H_7O_4$); 139.00362; 143.03494 ($C_6H_7O_4$); 145.05052 ($C_6H_9O_4$); 157.01447; 161.04546 ($C_6H_9O_4$); 163.06114; 205.07167 ($C_8H_{13}O_6$); 391.30252; 407.28998; 407.33362; 423.3331; 439.3208; 457.37042; 463.36121; 511.37976; 559.40314; 717.422; 967.52271; 981.54431; 999.52289; 1025.53259 ($C_{52}H_{81}O_{20}$); 1043.54272 ($C_{52}H_{83}O_{21}$); 1044.53027 | CS (24534051) | [90, 91] (*Zizyphus jujube*) |
| | | Cp70 | 18-β-Glycyrrhetinic acid | 15.478 | 469.33173 | 470.33961 | 470.33904 | 1.20 | $C_{30}H_{46}O_4$ | 73.4 | 20.00 | 96.96004; 112.98502; 241.2173; 451.32217; 469.33231 ($C_{30}H_{45}O_4$) | m/z Cloud (1281) CS (9710) | [92] (Licorice root) |

**Table 1.** *Cont*

| Classification | | # | Tentative Identification | RT (min) | Observed mass [M-H]- | Theoretical Molecular weight | Experimental Molecular weight | Δ mass (ppm) | Formula | Match m/z Cloud | FISh Cov. | Spectrum $MS^2$ (matched fragments) | Database (m/z Cloud ID; CS ID) | Reference (Plant/ Food item) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Lipids and lipid-like molecules** | Prenol lipids (Menthane monoterpenoids) | Cp68 | lamesticumin A | 14.773 | 501.35831 | 502.36581 | 502.36551 | 0.60 | $C_{31}H_{50}O_5$ | - | 33.33 | 297.15329; 455.35254 ($C_{30}H_{47}O_3$); 501.31668 | CS (26334699) | [93] (*Lansium domesticum*) |
| **Nucleosides, nucleotides and analogues** | Purine nucleosides | Cp26 | alpha-N-acetyllysine-N(6),N(6)-dimethyladenosine 5'-phosphomorpholidate | 7.951 | 613.25012 | 614.25775 | 614.25739 | 0.58 | $C_{24}H_{39}N_8O_9P$ | - | 7.14 | 58.00585; 59.01366; 59.01639; 69.03403; 71.01379 ($C_3H_5O_2$); 73.02947; 83.04981; 87.04515; 95.04992; 113.06084; 135.08138; 137.09677; 191.14397; 217.15921; 219.13914; 243.13921; 243.17545; 245.15451; 245.19109; 255.175; 257.15463; 257.19125; 261.18561; 269.15436; 273.18628; 275.20148; 285.18509; 291.19501; 301.18179; 303.19638; 321.20648; 337.20349; 343.19171; 347.18622; 361.20053; 365.19684; 389.19666; 407.20776; 449.21552; 509.23941; 527.24872 ($C_{21}H_{36}N_8O_6P$); 569.26031 ($C_{23}H_{38}N_8O_7P$) | CS (26331866) | [72] |
| **Organic acids and derivatives** | Carboxylic acids and derivatives (Tetracarboxylic acids and derivatives) | Cp53 | Succinyldisalicylic acid | 9.664 | 357.06140 | 358.06888 | 358.06867 | 0.58 | $C_{18}H_{14}O_8$ | - | 37.5 | 93.03432 ($C_6H_5O$); 121.02934; 165.01918; 180.05698; 181.06573; 225.05516; 313.07095 ($C_{17}H_{13}O_5$); 357.06122 ($C_{18}H_{13}O_8$) | CS (58553) | [94] (fruit, vegetables, herbs and spices) |

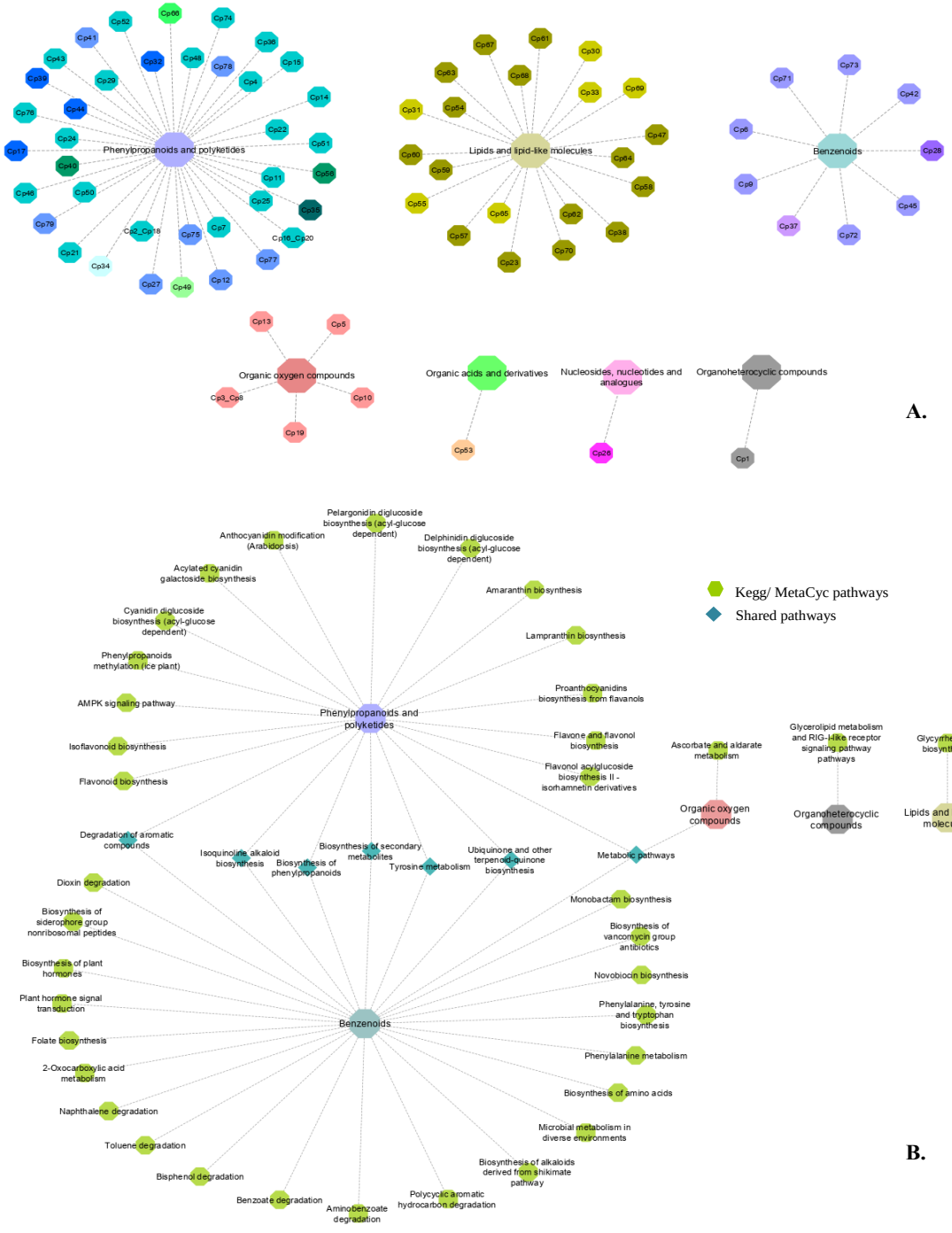



**Figure 1. A.** Classification of metabolites into different superclasses. **B.** Schematic representation of the described pathways in which the different superclasses of metabolites participate.

## 3.2. Effect of contrasting environments and accessions in common bean dry seeds metabolomics diversity

In the present study although no significant differences were found, under contrasting environments, in the overall total phenolic content (TPC) and the total flavonoids content (TFC) determined by spectrophotometric methodologies, the use of hyphenated high-resolution separation techniques with accurate tandem mass spectrometry in conjugation with bioinformatic tools allowed the identification of specific metabolites that could be synergistically involved in common beans heat tolerance, **Table S2**. Under heat stress environmental conditions (Córdoba) the relative percent area of individual compounds classified as a pteridine derivative (Cp1), flavonoids (Cp16, Cp43, Cp51 and Cp52), isoflavonoid (Cp44), coumarin (Cp56), stilbene (Cp35), macrolide (Cp66), organo oxygen compounds (Cp3, Cp13), benzenoids (Cp9, Cp42, Cp45), fatty acyls (Cp65, Cp69), prenol lipids (Cp23, Cp38, Cp47, Cp54, Cp67, Cp68, Cp70) and carboxylic acids (Cp53) was significantly higher than in the milder traditional cropping environment of Cabrela, **Table S2**. The quantified benzenoid compound (Cp72) and cinnamic acid (Cp77) also presented higher concentration in Córdoba than in Cabrela field trial.

Multivariate analysis summarized the common bean dry seeds quantitative metabolomics diversity under contrasting environments (traditional, Cabrela *versus* stressful, Córdoba), **Figure 2**. This multivariate analysis highlighted the existence of two groups of accessions, established along the first component of the bi-dimensional space, which explained 79.8% of the total variance found in the two contrasting environments.

Under the most stressful environmental conditions, Córdoba, the metabolites with higher correlation loading (> 0.5) were classified into pteridines and derivatives (Cp1), organooxygen compounds (Cp13) and benzenoids (Cp42) superclasses. Conversely under the most traditional environment (Cabrela), metabolites included in lipids and lipid-like molecules superclass, such as Cp57, Cp58, Cp59, Cp60, Cp61, Cp62 and Cp63 showed higher relative areas, **Figure 2**, **Table S2**.

In fact, for compounds Cp1 (pteridine derivative), Cp42 (benzenoid) and Cp57 (lipid and lipid-like molecule), the contribution of environmental conditions (40 – 43%) to metabolites' variability was slightly higher than the contributions attributed to genotype or to genotype × environment interaction, **Figure 3** and **Table S3**, which unveiled the importance of these metabolites for common beans' local adaptation.

Although until now no metabolomics study has been performed in common bean accessions to understand the role of specific metabolites in common beans' heat tolerance, the obtained results are aligned with previous studies performed in other plant species. Under heat stress conditions, the development of reproductive organs and the nodulation process in legumes are impaired which decreases respectively the fertilization and the nitrogen fixation [43]. Despite the scarcity of metabolomics studies reporting the specialized effect of individual metabolites in legumes adaptation to challenging environmental stressful conditions, the activation of phenylpropanoid biosynthetic pathway under abiotic stress is well recognized. As a consequence of the phenylalanine ammonia lyase increased activity and polyphenol oxidase decreased activity, phenolic compounds responsible by plant protection against

reactive oxygen species accumulate in plant cells, enabling stress tolerance and adaptation to challenging environments [44]. Triterpene saponins have also a key role on plant growth and nodulation. Nevertheless, further investigation regarding their impact on plant heat-stress tolerance is still required [45]. Regardless of the high relative percent area of lipids and lipid-like molecules superclass, **Table S4**, particularly prenol lipids, **Table S5**, in Cabrela field trial, under heat-stress circumstances (Córdoba), the high percent area of specific lipids and lipid-like metabolites (e.g. Cp23, Cp38, Cp47, Cp54, Cp67, Cp68, Cp70, Cp53) anticipate their important contribute for the establishment and progress of nodulation counteracting the adverse abiotic stress promoted by the temperature rising.

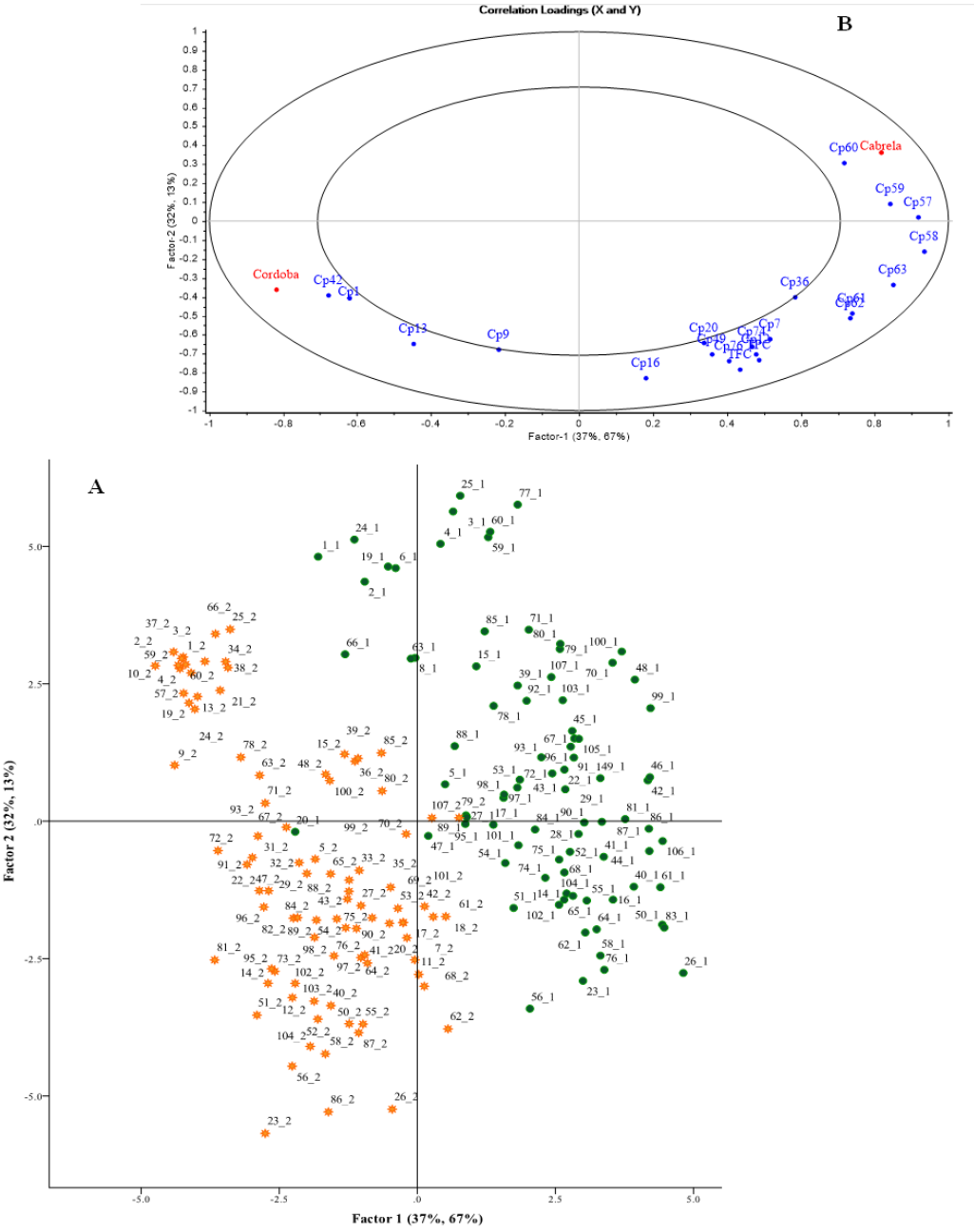



**Figure 2. A.** Score plot of common bean accessions, obtained by partial least square – discriminant analysis (PLS_DA), showing common bean accessions, cropped in Cabrela, ● and Córdoba, ✳. The explained variance (%) of predictors (X variables) and responses (Y variables) attributed to the first and second component, factor # (X%, Y%), are shown in the figure. The different accessions were named by the numbers attributed in **Table S1**, followed after

underscore by the corresponding environment (1-Cabrela and 2-Córdoba). **B.** Correlation loading plot of environment and parameters responsible by common bean varieties' projection, including TPC, total phenolic content; TFC, total flavonoid content; the area of compounds, named in accordance to **Table 1**, quantified by Q-Orbitrap-MS and the absolute concentration of compounds, named in accordance to **Table S3**, quantified by Q-TOF-MS. All the selected parameters, as well as the groups defined by cropping environment were located between the inner and outer (50 and 100%) explained circles. $R^2(X) = 0.6895$; $R^2(Y) = 0.7976$; RMSECV = 0.2310; RMSEC = 0.2247; $Q^2 = 0.7560$ and $R^2$-$Q^2 = 0.042$, difference < 0.3 [26], indicate the quality of the model.

The role of metabolites such as the pteridine derivative (Cp1) has been described, in drought stress conditions, as a co-factor for reactive oxygen species (ROS) scavenging enzymes, e.g. glutathione reductase and NADPH-thiol reductase [46]. Salicylic acid (Cp42) showed, in wheat, the ability of improving photosynthesis under heat stress conditions through enhancement of proline accumulation and inhibition of ethylene production [47]. Metabolomics studies conducted in other plant species such as in the carrots showed the relevance of coumaric and caffeic acid as heat stress protectors [48].

Notwithstanding the significantly high genotype impact and the G × E interaction contribution relatively reduced, < 20%, for the majority of the studied metabolites, **Figure 3** and **Table S3**, in just a few metabolites such as azelaic acid (Cp33), hesperetin (Cp52), succinyldisalicylic acid (Cp53), 6,7-dihydroxy-4-methylcoumarin or isomer (Cp56) and ursolic acid (Cp67), the contribution of G × E interaction to compounds' variability was ≥ 20%.

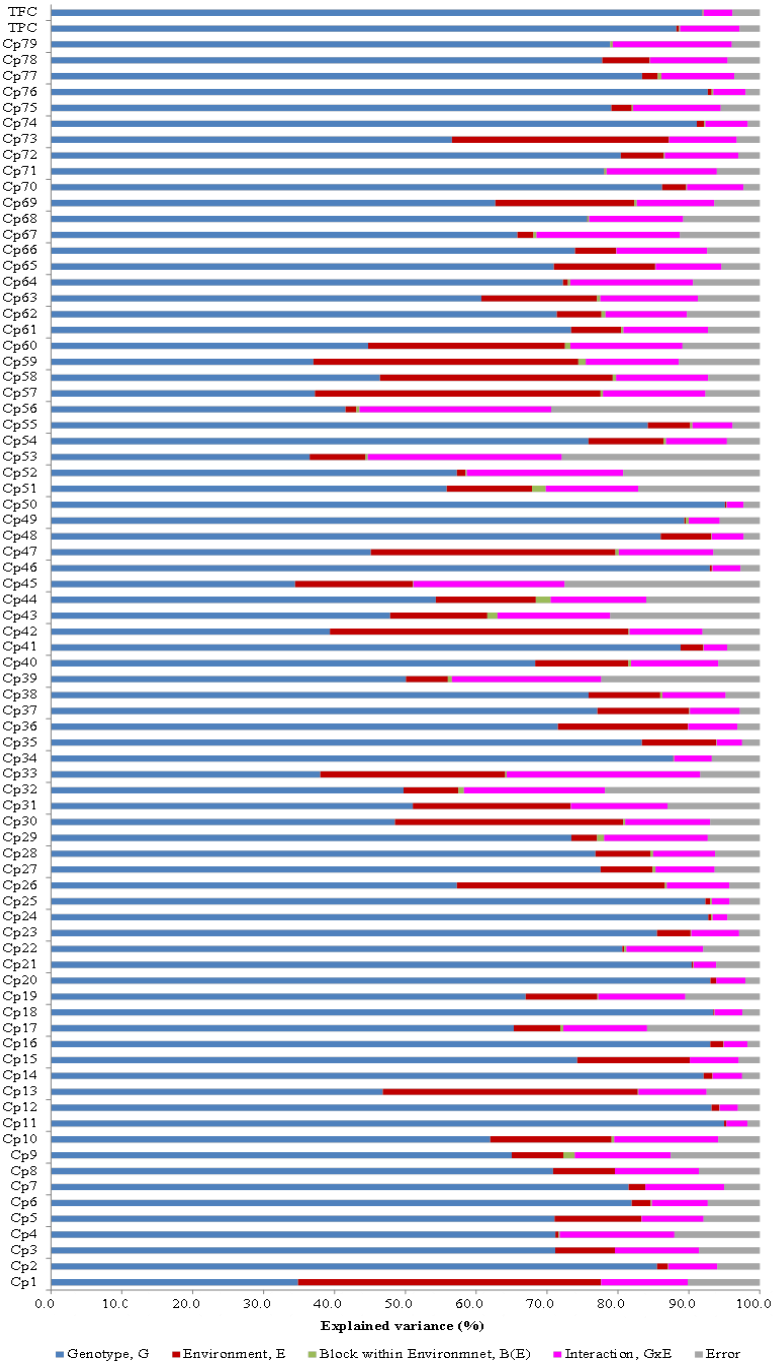



**Figure 3.** Contribution in % of genotype (G), environment (E), block within environment (B(E)), and genotype × environment (G×E) interaction to the variability of the analysed parameters.

These last metabolites could be explored in common bean management and breeding as potential metabolites for specific local common bean adaptation.

When the multivariate analysis was performed by environment, **Figure 4**, several metabolites, as well as, the TPC and TFC parameters were associated to samples' dispersion and contributed to the establishment of three clusters, within each environment. The set of the two first principal components explained 70.3% and 65.8% of parameters' total variability, respectively, in Cabrela (**Figure 4.1.**) and Córdoba (**Figure 4.2.**) environments.

The common bean accessions spatial distribution along the two first principal components was quite similar in Cabrela and Córdoba field trials. On both environments, cluster 3 was separated from the remaining ones along the first principal component (correlation of -0.765 and -0.881 with the first principal component, in Cabrela and Córdoba field trials, respectively). This cluster included accessions morphologically characterized by white seed coat colour with medium or large seed size (92 and 85% of the total accessions within cluster 3 in Cabrela and Córdoba, respectively). When compared to the coloured accessions, the white ones showed globally the lowest content on the different analysed parameters, TPC, TFC, phenylpropanoids and polyketides relative percent area, **Table S6**, as well as the lowest concentrations of most of the quantified phenolic compounds, including benzene compounds (Cp72, Cp73) cinnamic acids (Cp75, Cp78), and flavonoids (Cp74, Cp76, Cp46 and Cp50), $p < 0.05$, **Table S7**.

The observed differences in the TPC and TFC values among the diversity of Portuguese common bean seed coat colours, with white accessions showing the lowest content, has been consistently documented previously in Portuguese [15] varieties. Nevertheless, on both studied environments, white accessions were characterized by the highest relative percent area of organic oxygen compounds (80.5 and 79.6% of the 70 compounds' total area) as well as by the highest content of sinapic acid. As described for Mexican common beans genotypes [49], in the present study, the genotype effect also appeared as the main responsible for TPC, TFC, as well as for the majority of metabolites variability ($Eta^2$ (G) > 50%), **Figure 3**.

The other two clusters, of the three ones identified in each environment, were clearly separated along the second component (clusters 1, correlation of -0.819 and 0.679 in Cabrela and Córdoba, respectively, and cluster 2, correlation of 0.793 and -0.776 in Cabrela and Córdoba, respectively). On both environments these clusters were morphologically characterized by coloured accessions. Similarly to cluster 3 (white accessions), these last two clusters concentrated mostly seeds with large size (83% and 77% of the total accessions included in cluster 1, for Cabrela and Córdoba, respectively, and 50% and 60% of the total accessions included in cluster 2 for Cabrela and Córdoba, respectively). In both environments, cluster 1 gathered the accessions with the highest percent area of the triterpene saponin, Cp54 and cluster 2 the highest percent area of the flavonol (Cp50), supporting the genotype impact in these metabolites' contents. Specific environmental differences were found on the proportion of other individual metabolites among clusters. For

instance, cluster 1, in Cabrela showed the highest percent area of the flavonoid O-glycoside, Cp16, and the hydroxyanthraquinone, Cp37, $p < 0.05$, **Table S8**. Cluster 2, in the same environment, was characterized by accessions with the highest levels of the auronol, Cp34, and the flavonol, Cp46, $p < 0.05$, **Table S8**. In Córdoba, the accessions included in cluster 1 were defined by the highest percent area of the coumaric acid derivative, Cp27, the methoxyphenol, Cp28, and the 7-hydroxycoumarin, Cp40, $p < 0.05$, **Table S8**. In the set of accessions included in Cluster 2, established in Córdoba, the flavonoid-3-O-glycosides, Cp15 and Cp21 showed the highest percent area, $p < 0.05$, **Table S8**. The identification of higher levels of these particular metabolites classified as phenylpropanoids and polyketides, within each cluster, at the different environmental conditions suggested their relevance as biomarkers of common beans adaptability under specific environmental conditions. In fact, phenylpropanoids and polyketides (e.g. phenolic compounds) have been described as fundamental plant metabolites that improve the interaction between plant and rhizobacteria enhancing nutrient uptake and minerals mobilization. As antioxidants these metabolites can also protect plant cells from harmful conditions (e.g. UV radiation, temperature rising) that promote DNA mutations and ROS production [44].

Regarding the gene pool of origin, on both environments, accessions with Mesoamerican origin were characterized by lower percent area of organoheterocyclic compounds (0.2% in Cabrela and 0.9% in Córdoba) and benzenoid compounds (2.0% in Cabrela and 5.0% in Córdoba), than the accessions with Andean and Mixed origins (for

organoheterocyclic compounds, 0.4% in Cabrela and 1.2% in Córdoba, and for benzenoids, 4.3% in Cabrela and 7.0% in Córdoba), $p < 0.05$.

The data collected herein from the metabolomics study and integrated by multivariate analysis showed the richness of accessions such as 15, 39, 70, 80 and 85, in flavonoids and the high level of metabolites, including cinnamic acids and prenol lipids in accessions such as 55, 64, 68, 101 and 104.

These accessions may have interest for future breeding programs focused in the selection of varieties richer in metabolites with potential to induce heat-stress tolerance.

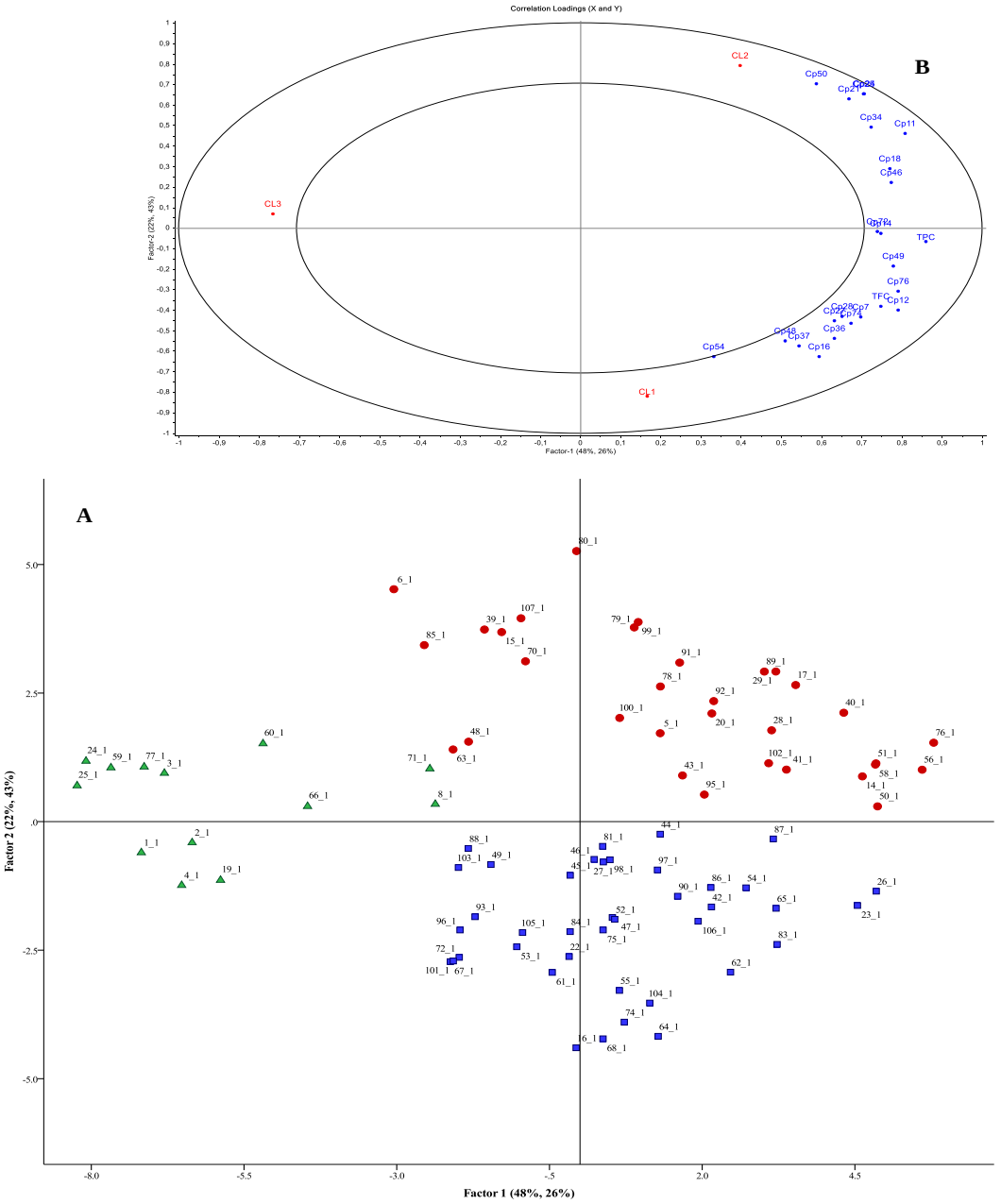



**Figure 4.1. A.** Score plot of common bean accessions, obtained by partial least square – discriminant analysis (PLS_DA), showing common bean accessions (n=86), cropped in Cabrela, grouped into different clusters along the two first factors, cluster 1,■; cluster 2,●; cluster 3,▲. The different common bean accessions were named as reported in **Figure 2**. **B.** Correlation loading plot of clusters and parameters, named in accordance to **Figure 2**. All the parameters, as well as the clusters (CL1, CL2, CL3) were located between the inner and outer (50 and 100%)

explained circles. $R^2(X) = 0.7030$; $R^2(Y) = 0.6917$; RMSECV = 0.2546; RMSEC = 0.2428; $Q^2 = 0.5218$ and $R^2\text{-}Q^2 = 0.1699$, difference < 0.3 [26], indicate the quality of the model.

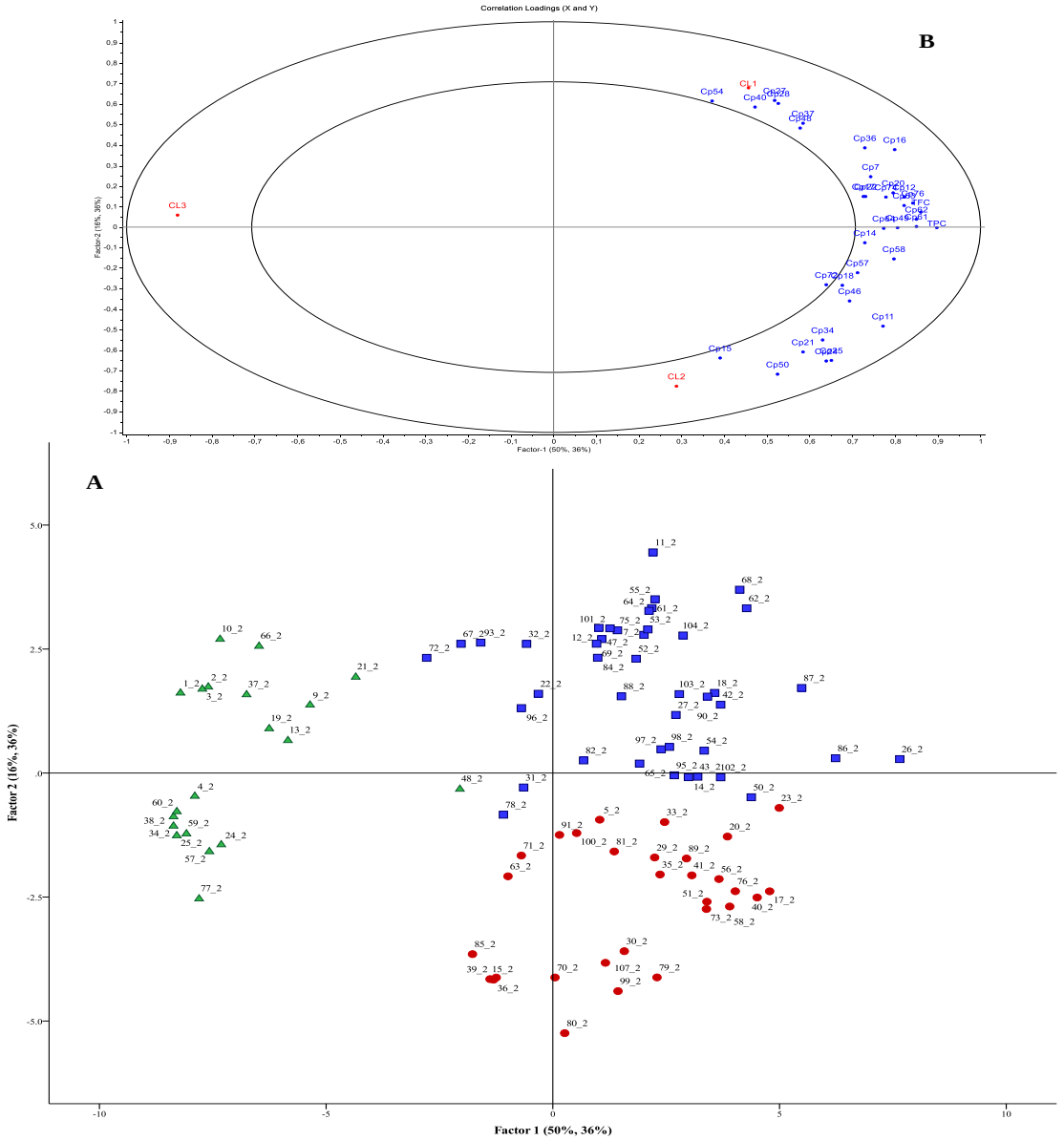



**Figure 4.2. A.** Score plot of common bean accessions, obtained by partial least square – discriminant analysis (PLS_DA), showing common bean accessions (n=93), cropped in Córdoba, grouped into different clusters along the two first factors, cluster 1,■; cluster 2,●; cluster 3,▲. The different common bean accessions were named as reported in **Figure 2**. **B.** Correlation loading plot of clusters and parameters, named in accordance to **Figure 2**. All the parameters, as well as the clusters (CL1, CL2, CL3) were located between the inner and outer (50 and 100%)

explained circles. $R^2(X) = 0.6576$; $R^2(Y) = 0.7118$; RMSECV = 0.2598; RMSEC = 0.2471; $Q^2 = 0.4801$ and $R^2\text{-}Q^2 = 0.2317$, difference < 0.3 [26], indicate the quality of the model.

### 3.3. Integrative approach to metabolite-metabolite interaction

As systematized by the correlation-based network analysis, **Figure 5**, the partial correlations established between the analysed metabolites were, as expected, particularly strong between the metabolites classified into the same superclass. The phenylpropanoids and polyketides superclass, which included the higher number of metabolites analysed, stood out by the high number of significant partial correlations higher than 0.75, $p < 0.05$. As highlighted by the network edges with heavy red colour, **Figure 5**, compounds such as kaempferol (Cp50) established partial correlations higher than 0.75 with astragalin (Cp21), 2'-acetylastragalin (Cp24), luteolin 7-O-(6-O-malonyl-β-glucoside) (Cp25), maesopsin (Cp34), and quercetin (Cp46). The last one was also highly and positively correlated to quercetin-3-β-D-glucoside (Cp18), taxifolin (Cp22) and protocatechuic acid (Cp72). The flavonol compounds, Cp50 and Cp46 share a common molecular backbone C6-C3-C6 consisting of two benzene rings (A and B) connected by a heterocyclic pyrane ring (C) and only few substitutions on the C ring (Cp21, Cp24, Cp18 and Cp22) or on the A (Cp25) rings explain the structure of the highly correlated metabolites. Cp34 and Cp72 classified, respectively, as an aurone flavonoid and as a hydroxybenzoic acid share with the flavonoids Cp50 and Cp46 the same biosynthetic pathway. As shown in **Figure S3**, aurone flavonoids and flavonols are synthesized via the phenylpropanoid pathway from the same precursor, p-coumaroyl-CoA. The dihydroxybenzoic acid, Cp72, can be produced via shikimate/chorismate or via phenylpropanoids [50], sharing

with flavonoids, such as Cp46, a concomitant increase in their synthesis. This highly positive strong interaction was also observed between other metabolites classified as benzenoids (aurantio-obtusin β-D-glucoside, Cp37, homovanillic acid, Cp28 and syringic acid acetate, Cp9) and phenylpropanoids' metabolites (diosmin, Cp48, 5,7-dihydroxy-4-methylcoumarin or isomer, Cp40, sinapoyl D-glucoside, Cp27 and sinapic acid, Cp79), which supported the existence of common precursors in the metabolic routes responsible by the biosynthesis of metabolites classified into the two distinct superclasses.

The negative significant moderate partial correlations (-0.5 to -0.75, $p < 0.05$), highlighted by the network light blue edges, between some metabolites classified into the phenylpropanoids and polyketides superclass (Cp50 *versus* Cp18; Cp50 *versus* kaempferol-3-O-rutinoside, Cp36) showed the complexity on the regulation of metabolites characterized by a similar backbone structure. Possible interconversions based on few substitutions at C, A and/or B rings are responsible by differences in the relative metabolites' proportion, in common bean accessions. For instance, on both environments, cluster 1 grouped samples with higher relative percent area of Cp36 and lower percent area of Cp50. The inverse situation was observed for samples classified into cluster 2. Such difference might be related to the natural variability in the flavanone-3-hydroxylase (F3H) enzymatic activity as well as in the flavonol UDP-glycosyltransferases among the different common bean accessions. As previously reported in safflower (*Carthamus tinctorius* L.) the existence of differential accumulation patterns of flavonoids could be attributed to different levels of F3H expression [51]. F3H participates in flavonoid

biosynthetic pathway acting in the 3-hydroxylation of flavanones into dihydroflavonols. Low expression of F3H could affect downstream the flavonol (e.g kaempferol) content [51]. Moreover the qualitative diversity and the natural variability in the expression levels of flavonol UDP-glycosyltransferases (UGTs) could contribute to explain the accumulation of flavonols' glycosylated forms with concomitant reduction of flavonols upstream the flavonol biosynthetic pathway [52]. Besides the negative correlations between metabolites of the same superclass, there were also negative linear correlations established between metabolites of distinct superclasses, especially between compounds from benzenoids (e.g. Cp37) and lipids superclass (e.g. Cp38), as well as between phenylpropanoids (e.g. Cp66) and lipids superclass (e.g. Cp61), **Figure 5**. A possible displacement of carbon precursors into the metabolic route of benzenoids and phenylpropanoid synthesis with a simultaneous downregulation in lipids and lipid-like molecules synthesis, **Figure S3**, [34] could contribute for the observed differences in the proportion of metabolites belonging to distinct superclasses.

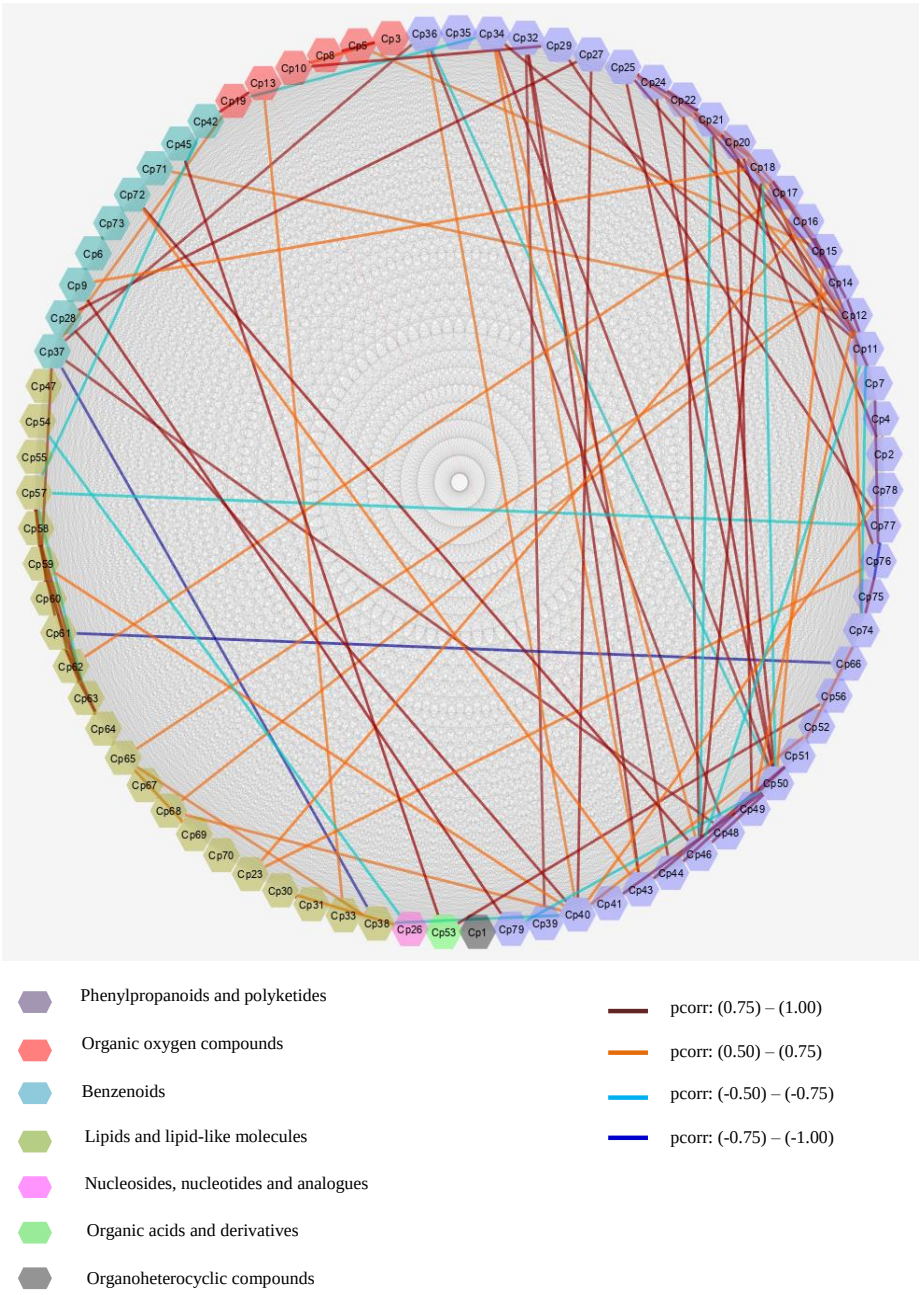



**Figure 5.** Correlation-based network established between metabolites. The analysed metabolites (nodes) were included into distinct superclasses. Only the significant ($p < 0.05$) partial correlations (edges) established between the metabolites were represented. Different colours indicate different pcorr – partial correlations' intensities.

## 4. Conclusions

In the present study conducted with 107 Portuguese common bean accessions, cropped in two contrasting environments, 70 compounds, from an initial dataset of 1122 compounds, classified into seven different superclasses, were annotated. The compounds' annotation, performed by Q-Orbitrap-MS was impaired by the limited diversity of compounds described in available online libraries, as well as by the experimental and reported quality of MS spectra and MS/MS fragmentation spectra. Some of these compounds classified as phenylpropanoids and polyketides as well as lipids and lipid-like molecules were described for the first time in common bean extracts.

The multivariate data analysis showed the contribution of factors such as genotype, environment and genotype × environment interaction to metabolomics variability.

Despite the absence of significant differences in the total phenolic and total flavonoid contents determined in common bean accessions cropped under contrasting environments (traditional, Cabrela, Portugal *versus* heat stress, Córdoba, Spain), there were significant differences in individual metabolites content, namely in benzenoids (e.g. Cp42), lipids and lipid-like molecules (e.g. Cp57, Cp58, Cp59 and Cp60) and in organoheterocyclic compounds (e.g. Cp1).

Considering morphological traits such as seed coat colour, the coloured accessions highlighted, in both environments, as the ones with higher percent area of metabolites, including the phenylpropanoids superclass area. Although white accessions showed globally lower percent

area of metabolites, the white accessions were characterized by a higher proportion of organooxygen compounds. Among coloured common bean accessions, two distinct clusters were defined by PLS-DA analysis. The major differences were found in the percent area of flavonoids, cinnamic acids, phenols and prenol lipids. In relation to the gene pool of origin, accessions with Mesoamerican origin were characterized by a lower percent area of organoheterocyclic compounds and benzenoid compounds than the accessions with an Andean or mixed origin.

For the majority of the studied parameters genotype was the factor with the highest contribution ($Eta^2$ > 50%) suggesting the high potential of the Portuguese common bean germplasm for future breeding programs. Selecting accessions rich in metabolites mainly influenced by genotypic effects will contribute to the development of new varieties with interesting metabolomics profiles regardless of the environmental conditions (breeding for broader use). Conversely, selecting accessions rich in metabolites associated to specific environmental conditions (e.g. salycilic acid, Cp42) may have interest for the production of varieties in challenging heat-stress environments (breeding for local use). The correlation-based network analysis performed in this study summarized the complex interactions established between the metabolites included into the different superclasses, (defined in accordance to the ClassyFire web-based compounds classification) which contributed to elucidate shared metabolic pathways. Moreover, the list of detailed metabolites characterized in common bean accessions, and presented herein, may represent a starting point for future *in vitro* and *in vivo* studies focused on the impact of single

and multiple common beans' metabolites for human health, namely for the prevention of human non-communicable diseases.

## Supplementary material

The following are available online in FigShare repository: https://figshare.com/s/e460f3b814e08eb20668, List of References (Table 1), Figure S1: Venn diagram showing the number of selected compounds related with differences in genotype, cropping environment and common beans gene pool of origin. The number of compounds shared by the different factors is shown in the intersection zones. The number underlined inside squares indicates the number of compounds with acceptable annotations, Figure S2: Molecular structure of identified common bean metabolites, organized into the different compounds' classes, Figure S3: Simplified representation of the metabolic pathways involved in common bean metabolites' synthesis (G-6-P, glucose-6-phosphate; Ribulose 5-P, ribulose 5-phosphate; Erythose-4-P, erithose-4-phosphate; Glyceraldehyde 3-P, glyceraldehyde 3-phosphate; Pentose-P, pentose-phosphate; MEP/DOXP, 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate ; VLCFA, very long chain fatty acids; L-Phe, L-Phenylalanine), adapted [34], Table S1: Morphological aspects of seeds and described gene pool of origin (Mesoamerican; Andean; Mixed) of Portuguese common bean accessions [13], Table S2: Comparison of compounds' relative quantification, %, (average ± standard deviation, SD) determined in common bean accessions, cropped under contrasting environmental conditions, using Orbitrap-MS. a,b significant differences ($p < 0.01$) *Below limit of quantification, Table S3: Detailed information regarding the impact

of genotype (G), environment (E), block within environment (B(E)) and genotype x environment (GxE) interaction in common beans' metabolites variability, Table S4: Relative quantification, % peak area of metabolites' superclasses[1] (average ± SD, standard deviation), SC1–Organoheterocyclic compounds; SC2–Phenylpropanoids and polyketides; SC3–Organic oxygen compounds; SC4–Benzenoids; SC5–Lipids and lipid-like molecules; SC6–Nucleosides, nucleotides and analogues; SC7–Organic acids and derivatives, in Portuguese common bean accessions cropped in contrasting environments (1, Cabrela and 2, Córdoba), determined by Orbitrap-MS. a,b significant differences between average values * Below the limit of quantification, Table S5: Relative quantification, % area of metabolites' classes[1] (average ± SD, standard deviation), C2.1–Flavonoids; C2.2–Isoflavonoids; C2.3–Aurone flavonoids; C2.4–Cinnamic acids and derivatives; C2.5–Coumarins and derivatives; C2.6–Linear 1,3-diarylpropanoids; C2.7–Stilbenes; C2.8–Macrolides and analogues; C4.1–Benzene and substituted derivatives; C4.2–Phenols; C4.3–Anthracenes; C5.1–Fatty acyls; C5.2–Prenol lipids, in Portuguese common bean accessions cropped in contrasting environments (1, Cabrela and 2, Córdoba), determined by Orbitrap-MS. a,b significant differences between average values * Below the limit of quantification, Table S6: Total phenolic content (TPC) in mg GAE/g DW, total flavonoids content (TFC) in mg CE/g DW, relative quantification of metabolites' superclasses in % and quantification of phenolic compounds (Cp71, Cp72, Cp73, Cp74, Cp75, Cp76, Cp77, Cp78, Cp79, Cp46 and Cp50) in µg/g DW, average ± standard deviation (SD), considering the white and coloured common bean accessions cropped in the two contrasting environments (Cabrela and

Córdoba), Table S7: Total phenolic content (TPC) in mg GAE/g DW, total flavonoids content (TFC) in mg CE/g DW and quantification of individual metabolites (Cp71, Cp72, Cp73, Cp74, Cp75, Cp76, Cp77, Cp78, Cp79, Cp46 and Cp50), average ± SD, by UPLC-Q-TOF-MS, in µg/g DW, determined in the Portuguese common bean accessions cropped in contrasting environments (1, Cabrela and 2, Córdoba). a,b significant differences between average values *Below the limit of quantification [25], Table S8: Common bean accessions' clusters, per environment considering the different studied parameters.

## Author Contributions

**C**onceptualization, E.M., M.E.F., M.C.V.P., M.R.B.; funding acquisition: M.E.F., M.C.V.P., M.R.B.; methodology, E.M., A.C.L.G., R.P.F.M.; investigation, E.M., A.C.L.G., I.B., A.B.S., S.T.L.; software, E.M., G.L.E., A.C.L.G., R.P.F., S.T.L.; resources, M.M.V., D.R., M.C.V.P., M.R.B.; project administration, M.C.V.P., M.R.B.; data curation, E. M., G.L.E., M.C.V.P., M.R.B.; formal analysis, E.M.; validation, E.M.; visualization, E.M.; supervision, R.P.F., A.R-M., M.E.F., M.C.V.P., M.R.B.; writing-original draft preparation, E.M.; writing-review & editing, E.M., G.L.E., M.C.V.P., M.R.B.

## Acknowledgments

To the Research Unit of Biotechnology and Genetic Resources germplasm bank, INIAV, Oeiras, Portugal, for providing the common bean samples. To FCT, Portugal, for the financial support in BEGEQA project (PTDC/AGR-TEC/3555/2012), E.M. PhD fellowship (SFRH/BD/89287/2012), as well as

to R&D unit, UIDB/04551/2020 (GREEN-IT – Bioresources for sustainability). The authors acknowledge to the University of Düsseldorf for the collaboration in the experimental work and to COST Action FA1403 (STSM-FA1403-290815-063873) for funding. The authors also acknowledge PORTUGAL 2020 to the Portuguese Mass Spectrometry Network, grant number LISBOA-01-0145-FEDER-402-022125. The project NETDIAMOND (SAICTPAC/0047/2015), financially supported by FEEI (Lisboa 2020 and FCT/POCI-01-0145-FEDER-016385), to the iNOVA4Health (UID/Multi/04462/2013), financially supported by FCT and co-funded by FEDER under the PT2020 Partnership Agreement, as well as to POCI-01-0145-FEDER-029702, funded by FEDER funds through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI) and by national funds (PIDDAC) through FCT/MCTES.

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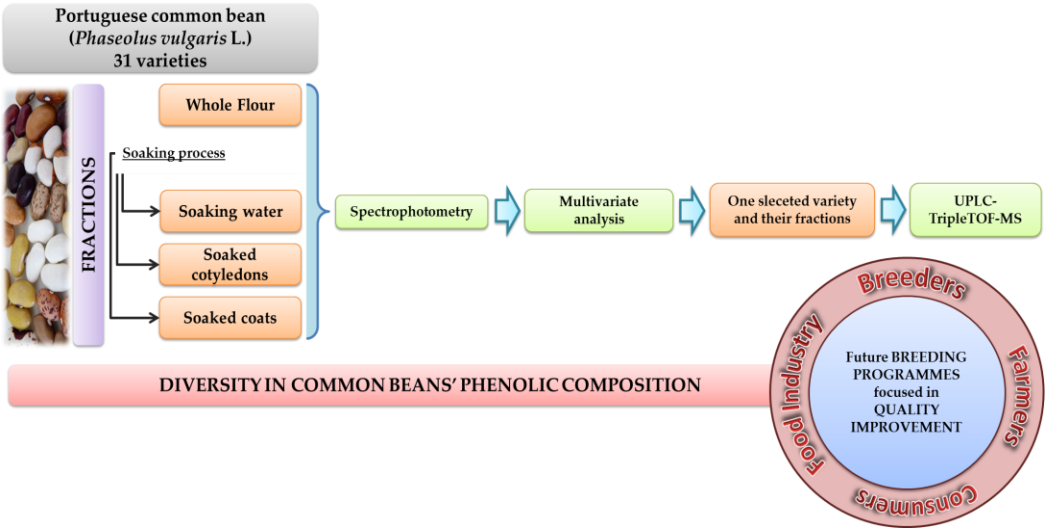
# Chapter IV




This Chapter was submitted and accepted by Foods as,

**Mecha, E.**; Leitão, S.T.; Carbas, B.; Serra, A.T.; Moreira, P.M.; Veloso, M.M.; Gomes, R.; Figueira, M. E.; Brites, C.; Vaz Patto, M.C.; Bronze, M.R. Characterization of soaking process' impact in common beans phenolic composition: contribute from the unexplored Portuguese germplasm. *Foods* **2019**, 8(8), 296; DOI:10.3390/foods8080296.

In this Chapter, Elsa Mecha participated in the experimental work, data analysis, manuscript drafting and final manuscript writing.

## Characterization of soaking process' impact in common beans phenolic composition: contribute from the unexplored Portuguese germplasm

### Abstract

Despite the common beans' nutritional and phytochemical value, in Portugal its consumption decreased more than 50%, in the last decade. The present study aimed to characterize phenolic composition of the Portuguese traditional varieties and corresponding soaked seed fractions (including soaking water). With such purpose, the phenolic composition (total content of soluble phenolics, flavonoids and proanthocyanidins) and *in vitro* antioxidant activity were evaluated in the raw whole flour of 31 Portuguese common bean varieties. The phenolic composition of the soaked fractions was respectively compared to the raw flour. Phenolic compounds' identification and relative quantification were achieved by UPLC-TripleTOF-MS for one representative variety and their fractions. The highest phenolic content was found in coloured varieties and the brown market class highlighted as the richest one. The loss of phenolic compounds to the soaking water was highly dependent on variety. The predominant phenolic compounds' classes were flavan-3-ols (soaking water and coats), flavonols (coats) and phenolic acids (cotyledons). This characterization study showed the diversity on the phenolic composition of Portuguese varieties, and the need to adjust the soaking and peeling processes to the variety (considering the possible loss of potential health promoter compounds, e.g. phenolic compounds).

**Keywords:** *Phaseolus vulgaris*; Portuguese varieties; phenolic compounds; soaking; peeling; spectrophotometry; UPLC-TripleTOF-MS

## 1. Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most widely grown grain legume species (Fabaceae family) cropped across a wide range of different environments, from arid climates to humid tropics [1]. Common beans nourish millions of people, in developing and developed countries. It fulfils 28% of the carbohydrates, 34% of the dietary fiber and 25% of the protein dietary recommended intake (DRIs) values for an average healthy adult (18 – 65 years old) [2-4]. Common beans are also a rich dietary source of minerals (e.g. magnesium, potassium, zinc and copper) and vitamins (e.g. vitamins B1, B6 and folate) [4].

Despite the recommendations, nowadays the consumption of common beans, even in the most traditional markets, known by their Mediterranean diet, is decreasing, mainly as a consequence of dietary habit changes [5, 6]. In Portugal, regardless of the national rich common bean germplasm [7], data from 2007 to 2017, reported a production decrease of 53%. This decrease is dramatic, considering that common bean represent 75% of the Portuguese grain legumes total consumption and the country is producing less than 10% of its intake, relying heavily on imports [8]. Part of the solution may involve the valorization of traditional varieties, in both high and low-income communities.

Grain legume regular consumption has been related to a reduction on the risk of non-communicable diseases (NCD) as cardiovascular diseases, type 2 diabetes *mellitus*, obesity and colon cancer, due to the

dietary fiber content and the presence of phenolic compounds [9-11] (e.g. phenolic acids and flavonoids as flavanols, flavonols, anthocyanins and isoflavones [12]).

Several factors, such as genotype, agronomic practices, climatic conditions or harvest conditions (e.g. maturity state), may influence the phenolic composition of common beans [13]. Also the preparation and cooking processes are critical for the phenolic composition of common beans' food based products. Domestic preparation may include washing, soaking (previously to thermal processing) and peeling procedures [14]. Soaking is one of the most disseminated procedures at domestic and industrial level, since it softens seeds' texture, accelerates the cooking process and increases the drained weight [15, 16]. Additionally, discarding the soaking water improves seeds' nutritional quality by removing, at least partially, some anti-nutritional compounds such as the oligossacharides, stachyose and raffinose, (involved in intestinal gas production) [17], phytic acid and tannins (linked to protein and carbohydrates digestibility impairment) [18], saponins (which may cause bloating symptoms and change cholesterol metabolism, by reducing total and LDL cholesterol without changing HDL cholesterol) and trypsin inhibitors (associated to reduced protein digestibility in monogastric animals) [19]. However, processing without discarding the soaking water may preserve these anti-nutritional compounds, which consumption can be advantageous in the context of NCD (e.g. obesity, hypercholesterolemia, cardiovascular diseases) prevention [20].

Despite the highly genetic and morphological diverse germplasm [7], the phenolic composition of the Portuguese varieties has been

unexplored and so far, no comprehensive characterization focused in the different common bean fractions (soaked coats, soaked cotyledons and soaking water) is available. Although there are several studies regarding the impact of preparation and cooking on the nutritional and protein quality of legumes [21], the effect of soaking process in the phenolic composition is barely known [22] and only a few studies have been dedicated to the distribution of phenolic compounds in the soaked seeds, coats and cotyledons [22].

This study was designed to evaluate the richness of the national common bean genetic resources on phenolic compounds and the effect of the soaking process on the seeds' phenolic composition. The characterization of these bioactive compounds may provide useful information to breeders, in order to ameliorate the commercially available varieties, as well as to elucidate consumers about the importance of consuming this legume in a daily diet. Moreover, a deeper characterization of the phenolic composition in soaked coats, cotyledons and corresponding soaking water, may contribute to change some gastronomic practices, such as the bean seed peeling, commonly used in some countries [14], in order to increment the access to bioactive phenolic compounds. To attain these objectives a collection of 31 Portuguese common bean traditional varieties, representing different market classes, was studied. Spectrophotometric assays were conducted to access phenolic compounds' content and *in vitro* antioxidant activity on common beans whole flour was also evaluated. Mass spectrometry was performed to identify the main phenolic compounds, in a representative common bean variety, before and after the soaking process.

## 2. Materials and Methods

### 2.1. Chemicals

Folin-Ciocalteu's phenol reagent, sodium carbonate (99%), catechin (98%), protocatechuic acid, p-hydroxybenzoic acid, gentisic acid, p-coumaric acid, sinapic acid, ferulic acid, sodium nitrite (97%), aluminium chloride (99.9%), and vanillin (99%) were purchased from Sigma-Aldrich (St. Louis, USA). Procyanidin B2, rutin, epicatechin, kaempferol, quercetin dehydrate, daidzein and genistein were purchased from Extrasynthese (Genay, France). Sulphuric acid (95 – 97%) and gallic acid (98%) were purchased from Fluka (Seelze, Germany). Sodium hydroxide (98%) was purchased from Merck (Darmstadt, Germany). Absolute ethanol (99.9%) and methanol (99.9%) were purchased from Carlo Erba Reagents (Rodano, Italy). Milli-Q® water (18.2 MΩ.cm) was obtained in a Millipore – Direct Q3 UV System equipment (Molsheim, France).

### 2.2. Plant Material

31 different traditional common bean varieties were collected from local farmers in the central region of Portugal [23] and kept in cold storage at the Research Unit of Biotechnology and Genetic Resources germplasm bank, INIAV, Oeiras, Portugal (PRT 005). These varieties were multiplied, in 2010, at ESAC (Coimbra, Portugal) under the same edaphoclimatic conditions, using traditional agronomic procedures for common bean production. Information relative to the collection data (geographical location – latitude, longitude, seed colour and pattern) is presented in **Table 1**.

Seed colour and pattern classes (market classes) were defined visually, based on *P. vulgaris* plant data descriptors [24]. In **Figure 1** one example of each studied common bean market class is shown.

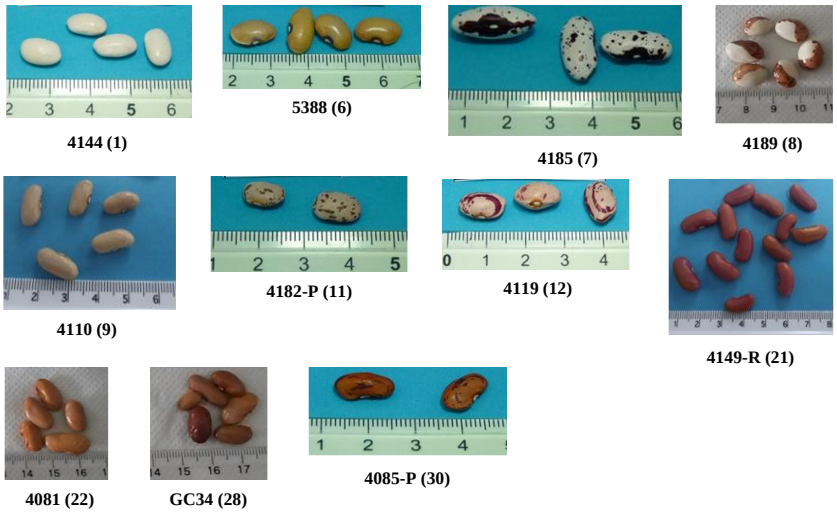



**Figure 1.** Example of (*Phaseolus vulgaris* L.) common beans' varieties morphological aspect (one variety from each colour market class)

**Table 1.** Accession numbers (PRT 005 germplasm bank collection) of Portuguese common bean's varieties, corresponding locations (latitude and longitude), seed colour and pattern

| Sample | PRT 005 accession nº | Latitude | Longitude | Colour | Pattern |
|---|---|---|---|---|---|
| **1** | **4144** | 40° 51' 0'' | 7° 29' 49'' | White | Plain |
| **2** | **5383** | 41° 32' 43'' | 8° 25' 35'' | White | Plain |
| **3** | **4088** | 40° 41' 51'' | 8° 5' 4'' | White | Plain |
| **4** | **1979** | 40° 45' 3'' | 7° 32' 14'' | White | Plain |
| **5** | **5249** | 40° 39' 24'' | 7° 54' 52'' | White | Plain |
| **6** | **5388** | 40° 39' 24'' | 7° 54' 52'' | Yellow | Plain |
| **7** | **4185** | 40° 20' 7'' | 7° 9' 48'' | White background with brown speckles and brown marginal pattern | Speckled with marginal colour |
| **8** | **4189** | 40° 20' 7'' | 7° 49' 48'' | White and brown backgrounds | Spotted bicolour |
| **9** | **4110** | 40° 37' 60'' | 8° 3' 40'' | Pink | Plain |
| **10** | **4179** | 40° 37' 60'' | 7° 23' 34'' | Pink | Plain |
| **11** | **4182-P** | 40° 39' 37'' | 7° 24' 38'' | Pink background with brown stripes | Stripes |
| **12** | **4119** | 40° 39' 24'' | 7° 54' 52'' | Pink background with purple stripes | Stripes |
| **13** | **4097** | 40° 41' 51'' | 8° 5' 4'' | Pink background with purple stripes | Stripes |
| **14** | **4038** | 40° 53' 43'' | 7° 44' 49'' | Pink background with purple stripes | Stripes |
| **15** | **4051** | 40° 52' 50'' | 7° 48' 16'' | Pink background with purple stripes | Stripes |
| **16** | **5389** | 40° 39' 24'' | 7° 54' 52'' | Pink background with purple stripes | Stripes |
| **17** | **4120** | 40° 39' 24'' | 7° 54' 52'' | Red | Plain |
| **18** | **5387** | 40° 32' 19'' | 7° 16' 3'' | Red | Plain |
| **19** | **4070** | 40° 54' 49'' | 7° 58' 32'' | Red | Plain |
| **20** | **5382** | 41° 32' 43'' | 8° 25' 35'' | Red | Plain |
| **21** | **4149-R** | 40° 19´33'' | 7° 41' 16'' | Red | Plain |
| **22** | **4081** | 41° 11' 50'' | 7° 49' 33'' | Light Brown | Plain |
| **23** | **4182-B** | 40° 39' 37'' | 7° 24' 38'' | Brown | Plain |
| **24** | **GC-34** | 40° 32' 19'' | 7° 16' 3'' | Brown | Plain |
| **25** | **GC-35** | 40° 32' 19'' | 7° 16' 3'' | Brown | Plain |
| **26** | **GC-17** | 40° 12' 7'' | 8° 26' 48'' | Brown | Plain |
| **27** | **4194** | 40° 20' 50'' | 7° 51' 26'' | Brown background with dark brown stripes | Stripes |
| **28** | **GC-40** | 40° 32' 19'' | 7° 16' 3'' | Brown background with dark brown stripes | Stripes |
| **29** | **5384** | 39° 14' 12'' | 8° 41' 9'' | Brown background with dark brown stripes | Stripes |
| **30** | **4085** | 40° 41' 51'' | 8° 5' 4'' | Brown background with dark brown stripes | Stripes |
| **31** | **4071** | 40° 54' 49'' | 7° 58' 32'' | Brown background with dark brown stripes | Stripes |

## 2.3. Sample Preparation

### *2.3.1. Whole Seed Flour Extracts*

Dry mature seeds were milled (Falling nº 3100 – Perten, Sweeden) to a particle size of 0.8 mm and stored at -20 °C, until analysis. Extracts were prepared according to Lin et al. [25] with slight modifications. Briefly, one gram of dry whole seed flour was extracted with 20 mL of methanol: water (60:40, v/v) solution, followed by sonication, during 60 minutes. The mixture was centrifuged at 420x g during 15 minutes. The volume was adjusted to 20 mL. Final extract was filtered through a 0.45 µm 13 mm PVDF syringe filter (Filter-Lab®). Before analysis by UPLC-TripleTOF MS, 5 mL of the extracts were concentrated, until dryness, in a SpeedVac (Labconco, Kansas City, USA) and reconstituted in 1 mL of methanol: water (60:40, v/v). Extracts were filtered through a 0.20 µm 13 mm CA syringe filter (LLG-Labware®) and kept at -20 °C, until analysis.

### *2.3.2. Soaking Water*

Soaking process was performed according to AACC [26]. Briefly, 100 dry mature seeds were soaked overnight, for 16 hours, in distilled water, on the proportion of 1 g per 3 mL of water. Soaking waters were collected, filtered through a 0.45 µm 13 mm PVDF syringe filter (Filter-Lab®).

Before analysis by UPLC-TripleTOF MS, 5 mL of the extracts were concentrated, until dryness, in a SpeedVac (Labconco, Kansas City, USA) and reconstituted in 1 mL of Milli-Q® water. Extracts were filtered through a

0.20 µm 13 mm CA syringe filter (LLG-Labware®) and kept at -20 °C, until analysis.

*2.3.3. Coats and Cotyledons Extracts*

After soaking, coats were manually separated from cotyledons and both fractions were dried (Memmert® drying oven) at 30 °C, for an average period of 48 hours. Dried coats (0.12 g) were extracted without previous milling, and dried cotyledons were grounded in a mill (Falling n° 3100 – Perten, Sweeden) with mesh 0.8 mm to obtain cotyledons' flours. The same extraction procedure applied for the common bean whole flour (section 2.3.1) was applied to cotyledons' flour (0.88 g). Dried coats were extracted with the methanol: water (60:40, v/v) solution and the mixture was ground, for 5 minutes, using a T25 Ultra-turrax equipment (IKA®) followed by sonication during 60 minutes. All extracts were prepared as triplicates and filtered through a 0.45 µm 13 mm PVDF syringe filter (Filter-Lab®). Before analysis by UPLC-TripleTOF MS, 5 mL of the extracts were concentrated, until dryness, in a SpeedVac (Labconco, Kansas City, USA) and reconstituted in 4 mL of methanol: water (60:40, v/v). Extracts were filtered through a 0.20 µm 13 mm CA syringe filter (LLG-Labware®) and kept at -20 °C, until analysis.

2.4. Determination of the Phenolic Compounds Content

Total Phenolic Content (TPC) as well as Total Flavonoid Content (TFC) and Total Proanthocyanidin Content (TPAC) were determined, as triplicates, in the whole flour, as well as, in the different fractions (soaking water, whole flour, soaked cotyledons and coats). The content of phenolic

compounds from cotyledons and seed coats were expressed per gram of common bean's dry weight (DW), taking into account the average percent weight of cotyledons (88%) and seed coats (9%) referred to the total seed weight [27]. Seed's dry weight was determined based on the moisture content (%) assessed by Near Infrared (NIR) analyser (MPA; Bruker, Billerica, MA, USA), using the flour calibrations for grain legumes provided by Bruker [28].

*2.4.1. Total Phenolic Content (TPC)*

TPC was determined by the method described by Stamatakis et al. [29], with modifications. Briefly, Folin-Ciocalteu reagent (0.100 mL) was added to 3.5 mL of extracts previously diluted according to the fraction and variety. After 3 min, 0.400 mL of sodium carbonate solution (35%, w/v) was added, and after one hour the absorbance was measured against water, in a Spectrophotometer DU – 70 (Beckman® USA), at 725 nm. Gallic acid was used as the external standard in a concentration range of 1 to 6 mg/L of gallic acid. Results were expressed in milligrams of gallic acid equivalents (mg GAE) per g of seed's dry weight (DW).

*2.4.2. Total Flavonoid Content (TFC)*

TFC was determined by the method described by Çam & Hışıl [30]. Briefly, 1 mL of the extract previously diluted or concentrated, depending on the fraction and variety, was added to 4 mL of Milli-Q® water and 0.300 mL of sodium nitrite (5%, w/v). The mixture was shaken and after waiting 5 minutes, 0.300 mL of aluminum chloride (10%, w/v) was added. After 6 minutes, 2 mL of 1 M sodium hydroxide solution finished the reaction. The

final volume (10 mL) was completed with Milli-Q® water. Absorbance was measured against water, in a Spectrophotometer DU – 70 (Beckman® USA), at 510 nm. *(+)*-Catechin was used as the external standard in a concentration range of 20 to 100 mg/L. Results were expressed in mg of *(+)*-catechin equivalents (mg CE) per g of seed's dry weight (DW).

*2.4.3. Total Proanthocyanidin Content (TPAC)*

TPAC was determined by the vanillin assay following Çam & Hışıl [30] with modifications. Briefly, extracts and soaking water were concentrated, until dryness, in a SpeedVac concentrator (Labconco®). Methanol (5 mL) was added to dried samples, and after shaking in vortex, 1 mL of the supernatant was mixed with 2.5 mL of vanillin 1% in methanol and 2.5 mL of $H_2SO_4$ 25% in methanol. The mixture rested during 15 min at 30 °C and the absorbance measured against methanol in a Genesys 10UV Spectrophotometer (Thermo Spectronic® USA), at 500 nm. *(+)*-Catechin was used as the external standard in a calibration range of 2.5 to 100 mg/L. Results were expressed in mg of *(+)*-catechin equivalents (mg CE) per g of seed's dry weight (DW).

2.5. *In vitro* Antioxidant Activity

The Oxygen Radical Absorbance Capacity (ORAC) assay was applied to evaluate antioxidant capacity of common bean whole flour towards peroxyl radicals. The assay was carried out following a modified method described by Ou et al. [31], in order to measure the ability of antioxidant species, present in the sample, to inhibit Fluorescein (FL) oxidation catalyzed by 2,2'-Azobis(2-amidinopropane) dihydrochloride

(AAPH) – generated peroxyl radicals ($ROO^{\cdot}$). The reaction mixture included 6.3 x $10^{-8}$ M FL, 1.28 x $10^{-2}$ M AAPH (prepared in 75 mM PBS, pH 7.4) and the diluted sample, in a total volume of 1.8 mL. The reaction started by addition of AAPH to the mixture, placed in a 10 mm wide fluorescence cuvette at 37 °C. Fluorescence emitted by the reduced form of FL was measured and recorded every 1 min at the emission wavelength of 515 nm and excitation wavelength of 493 nm (fluorescence spectrophotometer with thermostatic bath, model Cary Eclipse, Varian Ltd., Surrey, UK) for a period of 30 min. PBS was used as blank and 1, 5, 12.5, 25 and 50 M Trolox solutions as control standards. For ORAC analysis only the whole flour extracts were analyzed. All samples, including blank and controls, were analyzed in triplicate. Final ORAC values were calculated using a regression equation established between Trolox concentration and the net area under FL decay curve. Data were expressed in micromoles of Trolox equivalents antioxidant capacity (TEAC) per g of seed's dry weight (DW).

## 2.6. Phenolic Compounds Identification and Relative Quantification

One representative variety with the highest qualitative diversity of phenolic compounds was selected and characterized using a UPLC-TripleTOF 6600 mass spectrometer. Additionally, relative quantification of the identified compounds was also performed.

### *2.6.1. Analysis by UPLC-TripleTOF 6600 Mass Spectrometer*

The UPLC analysis was carried out on UPLC Acquity from Waters. The chromatographic separation was performed on a LiChrospher® 100

RP18 (250 x 4 mm, particle size 5 µm, Merck) thermostated at 35 °C. The injection volume was 20 µL. The flow rate was set at 300 µL/ min. The mobile phase was composed by eluent A (0.5% formic acid + 95.5% MQ® Water) and eluent B (90% acetonitrile + 9.5% MQ® water + 0.5% formic acid). Initially, an isocratic condition, corresponding to 5.6% of eluent B was used for 10 minutes, followed by a gradient elution, 16.7% of eluent B for 30 minutes and 22.2% of eluent B at 45 minutes. The percentage of eluent B remained at 22.2% for 20 minutes followed by an increase to 60.0% at 95 minutes and to 70.0% at 110 minutes. The initial elution conditions were established (5.6% of eluent B) during 20 minutes. The UPLC system was coupled to a TripleTOF 6600 mass spectrometer (SCIEX). The system was tuned using the taurocholic acid solution 2 ng/µL (Ref: 44052241) and calibrated with the ESI negative calibration solution (Ref: 4463277), both solutions from SCIEX.

The detection was performed in a mass range of *m/z* 50.0 – 1000.0. Samples were analyzed in the negative mode with a capillary voltage of +4500 V, using Curtain GasTM at 30 psi, Gas1 (nebulizer gas) at 60 psi and Gas2 (heater gas) at 50 psi. Samples were vaporized at 500 °C. Information Dependent Acquisition mode (IDA) was used to select the 20 most intense ions, with intensity greater than 100 cps. For $MS^2$ experiments it was applied collision energy of -25 V, with a collision energy spread of 15 V. The dynamic background subtraction was chosen. The MS and $MS^2$ data were processed in PeakView 2.1 Software (SCIEX). When possible, the identification of phenolic compounds was performed based on the comparison of the retention time, fragmentation pattern and mass accuracy of available commercial standards. Since for the majority of

compounds there are no commercial standards, the tentative identification was based on the fragmentation pattern, accurate mass measurements (mass error ≤ 5 ppm) and comparison with available literature on phenolic compounds.

The relative quantification was expressed per each fraction analyzed as Compounds' class area = Compounds' class area/ Total quantified area and % Area (each compound in the class) = Compounds' area/ Total quantified compounds' class area, which allowed comparison of the different common bean's fractions, before and after the soaking process, considering the relative abundance of each compounds' class, as well as the abundance of each identified compound in the corresponding phenolic compounds' class.

## 2.7. Data Analysis

All data statistical analyses were conducted in IBM® SPSS® Statistics, version 22. ANOVA test was performed for each parameter analyzed in the extracts, after testing each parameter for the normality (Shapiro-Wilk test). Variables transformation by logarithmic or two-step transformations [32] were performed when the variables were not normally distributed. Multivariate analysis was performed by Principal Component Analysis (PCA) followed by k-means cluster analysis to classify common bean traditional varieties (n=31) based on the PCA solution. The colour and pattern of common bean varieties were superimposed to the distribution of the samples on the bi-dimensional space defined by the two first principal components. The number of clusters was established by comparison of the determination coefficient ($R^2$) obtained for different

clusters' solutions (K=2 to K=6) and the clustering solution confirmed by discriminant analysis. The groups defined by cluster analysis were compared by ANOVA test and significant differences between groups determined by the *post-hoc* Scheffé's test or by the Games-Howell test (depending, respectively, on the acceptance or violation of the homoscedasticity criterion) at a significance level of 5%. Although the multivariate analysis had been developed with some transformed variables, the clusters' characterization was reported taking in account the corresponding results on the non-transformed (original) variables. Correlations between quantitative variables were defined by Pearson's R coefficient [33].

## 3. Results and Discussion

### 3.1. Phenolic Content in Common Beans' Whole Flour, Before Soaking

Phenolic compounds are bioactive molecules that are found in common bean samples. **Table 2** presents a summary of the results obtained for the whole flour, different fractions (coats and cotyledons), before and after the soaking process, as well as for the soaking water. Samples were organized into different colour classes, according to seeds' colour similarity. As only one yellow variety was studied, the results from this sample were excluded from **Table 2**.

As expected, since some of these compounds are known for their contribution to seed colour [39], the coloured market classes were richer in phenolic compounds (including flavonoids and proanthocyanidins). A strong positive correlation was found between TPC in whole flour extracts

and TFC (Pearson's R of 0.890, $p < 0.05$), as well as, between TPC and TPAC (Pearson's R of 0.746, $p < 0.05$), as already described by Nyau et al. [35]. For the white (n=5) and brown varieties (n=10) studied, **Table 2**, the average TPC values were higher than the ones previously described for the brown coloured Zambian varieties [35].

The same was verified for the red varieties (n=5) with TPC, TFC and TPAC average values higher than the ones, previously, reported for a red kidney market class [37]. The differences observed between the measured phenolic content and the values described in the literature [35, 37] can be attributed to different causes, such as common beans' genotypes and geographical origins but, also, to differences in the extraction protocol. Although the extracting solvent used herein and in literature [35, 37] was methanol, there were differences in the proportion of solvent per mass of common beans' flour [35, 37], as well as in the extraction technique applied, herein with ultra-sonication and in Xu et al. [37] with orbital shaker.

**Table 2.** Summary of the results, expressed as the average ± standard deviation (SD), obtained for the spectrophotometric parameters, TPC (Total Phenolic Content), TFC (Total Flavonoid Content), TPAC (Total Proanthocyanidin Content) and ORAC (Oxygen Radical Absorbance Capacity) in the different common bean fractions (WF - whole flour, SW - soaking water, coats and cotyledons). Yellow sample was excluded since only one sample could be classified in such color class

| Parameter | Analyzed fraction | White (n=5) | White and brown (n=2) | Pink (n=8) | Red (n=5) | Brown (n=10) | Described in literature |
|---|---|---|---|---|---|---|---|
| **TPC (mg GAE/g DW)** | **WF (raw)** | 1.38 ± 0.22 **(a)** | 2.73 ± 0.23 **(ab)** | 4.58 ± 0.67 **(bc)** | 4.62 ± 0.70 **(bc)** | 5.12 ± 1.22 **(c)** | 1.59 ± 0.08 (navy [34]); 0.37 ± 0.026 (white [35]); 0.45 ± 0.23 (navy [36]); 3.11 ± 0.14 (dark red kidney [34]); 1.24 ± 0.043 (red [35]); 2.15 ± 0.94 (red kidney[36]); 2.25 ± 0.05 (red kidney [37]); 0.60 ± 0.038 (Brown [35]) |
| | **SW** | 0.12 ± 0.05 **(a)** | 1.20 ± 0.14 **(ab)** | 1.98 ± 0.77 **(b)** | 1.92 ± 0.66 **(b)** | 2.18 ± 0.78 **(c)** | n.d |
| | **Coats** | 0.04 ± 0.00 **(a)** | 2.38 ± 0.82 **(ab)** | 3.42 ± 0.93 **(b)** | 3.77 ± 0.31 **(b)** | 3.71 ± 0.62 **(b)** | 1.20 ± 0.02 (navy); 1.16 ± 0.01 (great northern); 1.88 ± 0.00 (pink); 1.44 ± 0.00 (dark red kidney); 5.53 ± 0.87 (light red kidney); 3.79 ± 0.04 (small red) [38] |
| | **Cotyledons** | 0.87 ± 0.06 **(a)** | 1.01 ± 0.14 **(a)** | 1.02 ± 0.09 **(a)** | 0.96 ± 0.11 **(a)** | 1.06 ± 0.16 **(a)** | 2.00 ± 0.01 (navy); 1.86 ± 0.04 (great northern); 1.97 ± 0.01 (pink ); 2.11 ± 0.06 (dark red kidney); 2.15 ± 0.04 (light red kidney); 2.02 ± 0.08 (small red) [38] |
| **TFC (mg CE/g DW)** | **WF (raw)** | 0.14 ± 0.04 **(a)** | 0.77 ± 0.00 **(b)** | 2.01 ± 0.70 **(c)** | 1.45 ± 0.24 **(c)** | 1.85 ± 0.64 **(c)** | 0.85 ± 0.03 (red kidney [37]) |
| | **SW** | 0.01 ± 0.00 **(a)** | 0.59 ± 0.35 **(ab)** | 1.33 ± 0.49 **(b)** | 1.13 ± 0.22 **(b)** | 1.33 ± 0.55 **(b)** | n.d |
| | **Coats** | 0.01 ± 0.00 **(a)** | 1.67 ± 0.61 **(ab)** | 2.26 ± 0.68 **(b)** | 2.36 ± 0.13 **(b)** | 2.31 ± 0.38 **(b)** | n.d |
| | **Cotyledons** | 0.15 ± 0.01 **(a)** | 0.17 ± 0.01 **(ab)** | 0.21 ± 0.03 **(bc)** | 0.23 ± 0.04 **(bc)** | 0.22 ± 0.04 **(c)** | n.d |
| **TPAC (mg CE/g DW)** | **WF (raw)** | 0.02 ± 0.01 **(a)** | 0.29 ± 0.25 **(ab)** | 0.70 ± 0.49 **(b)** | 0.65 ± 0.37 **(b)** | 0.65 ± 0.39 **(b)** | 0.30 ± 0.03 (red kidney [37]) |
| | **SW** | 0.04 ± 0.01 **(a)** | 0.07 ± 0.03 **(ab)** | 0.62 ± 0.35 **(c)** | 0.46 ± 0.36 **(bc)** | 0.38 ± 0.27 **(bc)** | n.d |
| | **Coats** | 0.01 ± 0.00 **(a)** | 1.24 ± 0.21 **(ab)** | 2.49 ± 1.02 **(bc)** | 2.38 ± 0.70 **(bc)** | 2.60 ± 0.75 **(c)** | n.d |
| **ORAC (µmol TEAC/ g DW)** | **WF (raw)** | 37.35 ± 4.77 **(a)** | 76.00 ± 2.59 **(b)** | 143.22 ± 35.39 **(c)** | 125.46 ± 31.50 **(bc)** | 154.83 ± 40.41 **(c)** | 59.41 ± 3.26 (red kidney [37]) |

Equal letters per parameter (row) indicate absence of significant differences between colour classes ($p > 0.05$); n.d. not described

In the present study, and among the brown varieties, the ones identified as 27, 30 and 31 showed the highest TPC values, contributing for the high variability of such market class (coefficient of variation, 24%). In what concerns the TFC and TPAC values, this market class also showed high variability in TFC and TPAC values (coefficient of variation, 35% and 60%, respectively). From the results obtained, the brown market class was considered a highly valuable class for future breeding programs focused on increasing phenolic compounds content. The role of phenolic compounds in the prevention of chronic diseases has been attributed to their free radicals' scavenging ability [40] responsible by their antioxidant properties. Several different chemical assays such as ORAC (oxygen radical absorbance capacity), HORAC (hydroxyl radical antioxidant capacity), DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing antioxidant power assay) have been used to evaluate compounds' antioxidant activity. The major difference between these assays is the nature of the molecules reduced by the antioxidant compounds. While ORAC measures the ability of antioxidant compounds to reduce the peroxyl radicals, in HORAC the hydroxyl radicals are the reduced compounds. In DPPH the reduced compound is 2,2-diphenyl-1-picrylhydrazyl and in FRAP the complex $Fe^{3+}$ tripyridyltriazine is the reduced one [41].

In the present study, the ORAC method was the selected one, since previous works showed higher correlation with TPC of legumes' extracts [34]. Since the variation between the TPC triplicate values, determined in the whole flour extracts, ranged between 0.7 and 6.6%, for ORAC determination only one extract per variety was randomly analyzed. As

shown in **Table 2**, the pink, red and brown varieties revealed the highest ORAC values, 143.22 ± 35.39, 125.46 ± 31.50 and 154.83 ± 40.41 μmol TEAC/g DW, respectively, and the white varieties the lowest, 37.35 ± 4.77 μmol TEAC/g DW, following the same trend of the phenolic content. These values were higher than the ones obtained for pink and red varieties described by Xu et al. [42], **Table 2**. ORAC values also presented a strong correlation with TPC and TFC values, Pearson's R of 0.909 and 0.918, respectively. Considering that all the common bean varieties were cultivated under the same edaphoclimatic conditions, the variability found in the phenolic compounds must be strongly dependent on the common bean variety.

### 3.2. Phenolic Content in Soaking Water, Soaked Coats and Soaked Cotyledons

In the beans' processing industry, soaking water has been traditionally discarded, remaining one of the most underexplored byproducts. The results obtained in this work show that the percentage of TPC in the soaking water can vary a lot with the common bean variety, regardless of the phenolic content in the raw whole beans. While in a white variety soaking water's TPC represented 5 – 14% of the TPC in the whole flour, for brown varieties the values ranged from 28 to 55% and the highest value, 66%, was determined in the light brown variety (sample 22), **Table S1**. The results obtained, herein, contradict previous results reported for the soaking water of black beans and cream background beans with pink and red stripes [43], described with minor phenolic compound losses, < 2% [13] or with no quantifiable phenolics in soaking water [43]. After the

soaking process, on average, soaked beans showed higher phenolic content (including flavonoids and proanthocyanidins) than the non-soaked whole flour. These results may be attributed to the extraction of soaked coats and soaked cotyledons, performed separately after the soaking process. Such procedure allowed a higher extraction rate of the free phenolic compounds present in coats fraction, since cotyledons removal could eliminate some phenolic-protein interactions [47]. For this reason, despite the phenolic compounds' loss into the soaking water, an overall comparison of common beans TPC in whole flour and in soaked beans (calculated as the sum of the TPC determined, separately, in both soaked fractions, coats and cotyledons) showed that at least 51% of the TPC determined in non-soaked seeds was preserved after soaking, which contradicts the high decrease, -73%, in the TPC of soaked beans reported by Faller & Fialho [48]. The comparison study performed between the phenolic content determined in cotyledons and coats, **Table S2**, revealed that for soaked white beans, cotyledons fraction had higher contribution to TPC (95 – 96%) and TFC (94 – 96%) than coats, which is in accordance to Sutivisedsak et al. [38]. By opposition, in coloured varieties, the coats revealed higher phenolic and flavonoid contents than the cotyledons, which support the consumption of the coloured common bean seeds without peeling as a strategy to preserve common beans' phenolic compounds. The results suggested that the peeling process, traditionally performed in some African countries to prepare porridge and recipes like Akara, Moin Moin and Gbegiri soup, might impair the phenolic content of the final common bean food based products. Therefore, and bearing in mind the impact of the peeling process on the nutritional food quality (by enhancing

protein and carbohydrate digestibility) but also on the elimination of compounds with potential health effect [14], the traditional recipes could be adjusted and reinvented to include or not the peeling process, depending on the populations' nutritional status.

### 3.3. Phenolic Compounds' Characterization

Although the information regarding the total phenolic content has been fundamental to characterize beans' samples, analysis of individual compounds has become mandatory and for that separation methodologies must be used. The individual study of phenolic compounds is challenging as the samples are complex (with a large number of compounds at different concentration ranges) and often there are no commercially available standards. Presently the identification of individual compounds is achieved mostly by mass spectrometry associated to chromatographic techniques. In this work, one representative variety, with high qualitative diversity of phenolic compounds, was selected to identify the individual phenolic compounds present in common bean. The selection was done after the PCA analysis of the measured global parameters, followed by cluster analysis, **Figure 2** and **Table S3**. This procedure allowed classifying common bean samples into three different clusters, **Figure 2** and **Table S4**, which explained 65.9% of the total variance.

The discriminant analysis confirmed the clustering solution, **Table S5**. All the clusters revealed high variability in the phenolic content, **Table S4**. Sample 22, a light brown variety, chosen as a representative variety, was located in cluster 2, in an intermediate position between cluster 1 (characterized by the samples with the lowest phenolic content) and cluster

3 (characterized by the samples with the highest phenolic content in coats' fraction).

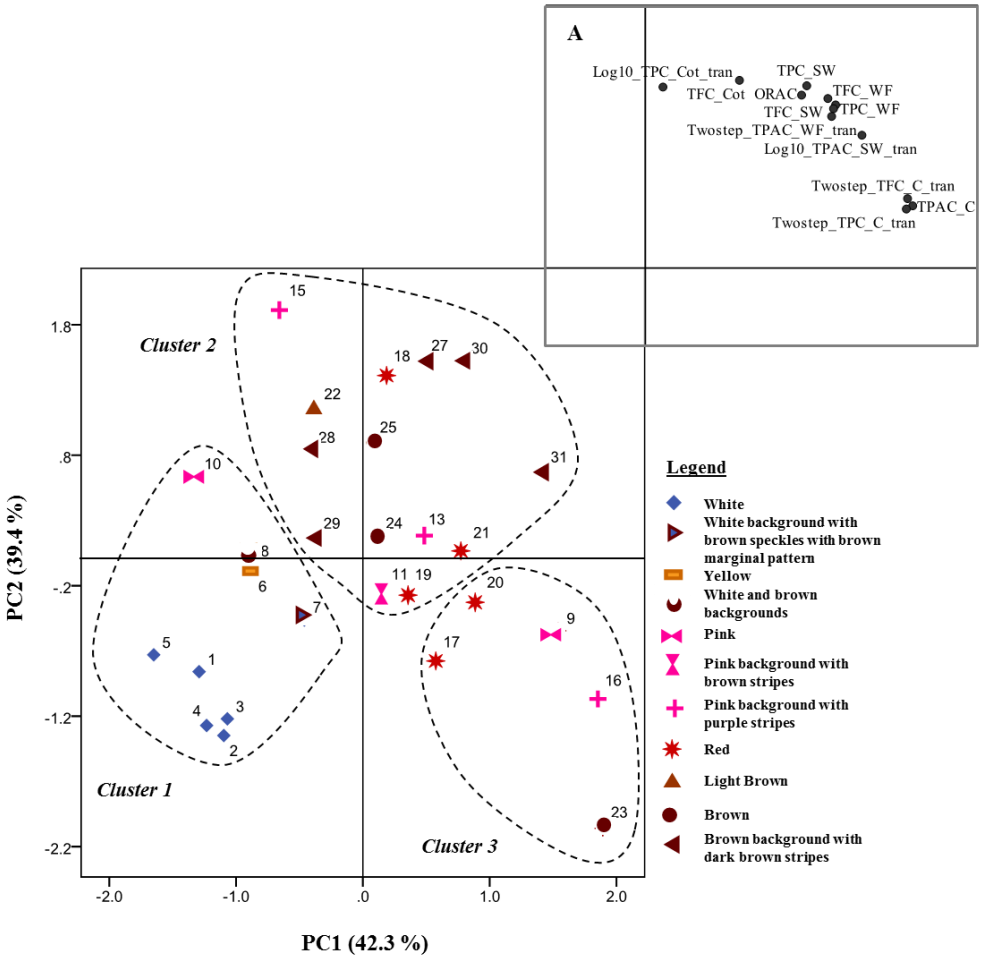



**Figure 2.** Projection of the Portuguese common bean varieties (n=28) in a bi-dimensional space (Principal Component 1 – PC1 and Principal Component 2 – PC2). Colour of samples was depicted and clusters highlighted. **A** – Distribution of the parameters analyzed in the bidimensional space (TPC – Total Phenolic Content; TFC – Total Flavonoid Content; TPAC – Total Proanthocyanidin Content; ORAC – Oxygen Radical Absorbance Capacity; C – Coats; Cot – Cotyledons; SW – Soaking Water; WF – Whole Flour). To achieve normal distribution, the parameters TPC in Cot and TPAC in SW were submitted to the logarithmic ($Log_{10}$) transformation and the parameters TPC in C, TFC in C and TPAC in WF to the two-step transformation, before multivariate analysis. The two-step transformation excluded from analysis samples 12, 14 and 26, since TPC_C, TFC_C and TPAC_WF were out of ranking.

The different fractions of the representative variety were analyzed by UPLC-Triple-TOF-MS, in negative mode, for compounds' identification and relative quantification, as presented in **Tables 3** and **4**. Identification was based on the retention time, mass accuracy, fragmentation pattern and previous description in literature.

The chromatographic profiles of the different fractions, at 280 nm, are compared in **Figures 3A** and **3B**.

Compounds were classified in six different phenolic compounds' classes: hydroxybenzoic acids, hydroxycinnamic acids, flavan-3-ols, flavanones, flavonols, isoflavones, and their structures are shown in supplementary material, **Table S6**. The relative quantification was expressed as the % area of the compounds' class area considering the total area, **Figure 4** and the % area of the identified compound considering the total compounds' class area, **Table 4**.

Hydroxybenzoic Acids

Hydroxybenzoic acids' biological activity on the endothelial dysfunction, blood lipid profile and inflammation indicates their important role in cardiovascular system, as reviewed by Juurlink et al. [63]. These compounds were identified in all common bean fractions analyzed, representing from 0.4% of the total identified compounds, in raw whole flour, to 14.7% of the total identified compounds, in soaked cotyledons.

Among hydroxybenzoic acids, identified in the present study, vanillic acid (2) was mostly abundant in the raw flour and its presence was previously described in dark beans [50]. The soaked coats represented the richest fraction in protocatechuic acid, which was already identified by Xu &

Chang [52] and López et al. [53] in dark, pinto and black beans. Protocatechuic acid-4-O-glucoside (1) and *p*-hydroxybenzoic acid-4-O-glucoside (12) were also identified based on the fragmentation experiments, which showed the product ions *m/z* 153.0229 and *m/z* 137.0304, corresponding to protocatechuic acid and *p*-hydroxybenzoic acid, respectively. The presence of the glycosidic forms of protocatechuic and *p*-hydroxybenzoic acids in legumes was described, for the first time, by Moran et al. [49] in the soybean root nodules. Gentisic acid (27) was identified using an external standard, and is known to be widely distributed in legume species [13, 54]. Because of its water solubility, the glycosidic forms of protocatechuic and *p*-hydroxybenzoic acid, as well as gentisic acid were dominant in soaking water.

Hydroxycinnamic Acids

Hydroxycinnamic acids were mostly present in soaked cotyledons (49.3% of the total identified compounds), as shown in **Figure 4**, and included the *p*-coumaric (41), sinapic (44) and ferulic acid (45), as well as their aldaric derivatives. As described by Aguilera et al. [51] and Dueñas et al. [55], the aldaric derivatives of hydroxycinnamic acids represent the most typical hydroxycinnamic acids in common beans. In the selected light brown variety it was possible to identify, mostly in soaked coats, six isomers of p-coumaroyl aldaric acid, seven isomers of feruloyl aldaric acid and five isomers of sinapoyl aldaric acid. The p-coumaric acid derivative, p-coumaroyl aldaric acid 6, was not found in soaking water. The free hydroxycinnamic acids, p-coumaric and ferulic acids, were mainly detected in the soaking water (3.1% of the total hydroxycinnamic acids), possibly

due to the enzymatic disruption of some glycosidic bonds attached to the phenolic acids in the seeds' cell walls.

Flavan-3-ols

Flavan-3-ols are known as important phenolic compounds in common beans [51], and represented 39.0% and 25.5% of the total compounds identified in soaking water and soaked coats, respectively, **Figure 4**. As shown in **Table 4**, the oligomeric forms of flavan-ols were mostly dimeric and trimeric procyanidins. Although this class of compounds has been assumed as anti-nutritional, nowadays their value has been recognized, not only in the feeding industry, to improve animals' growth and gut health, acting as a promising alternative to antibiotics in poultry [64], but also to human health, as prebiotic compounds in colon and as protective factors against cariogenic bacteria in the mouth (*Streptococcus mutans*) and ulcerogenic *Helicobacter pylori* in the stomach [65].

In the whole flour of the light brown variety analyzed it was possible to identify 5 dimeric procyanidins, at different retention times. Procyanidins dimers B2 and B3 were described previously in pinto beans [51] and the dimer B4 [53] in dark beans. Identification of procyanidin B5 was performed in peanut peel [59] but, as far as we know, this is the first paper that describes the presence of the dimer B5 in common bean. Catechin-3'-O-glucoside (4) was identified and previously described in pinto beans [51]. On the other hand, catechin-7-O-glucoside (17) was tentatively identified herein, for the first time in common beans, and was previously described in cowpea [56].

Mostly present in seeds' coat and soaking water, as previously stated by Aguilera et al. [51], in this compounds' class, the major compounds identified included catechin-3'-O-glucoside, procyanidin B1, and catechin. The presence of such compounds in seeds' coats has been associated to embryos' protection against pathogens, ensuring seeds' germination [66].

In the cotyledons' fraction the most abundant flavan-3-ol was the catechin-3'-O-glucoside. Proanthocyanidins, like procyanidin B1, B3 and B4, were below the detection limit of the method, possibly as a consequence of the crosslinking reactions with proteins, highly accumulated in cotyledons fraction [67] making these compounds not accessible for extraction.

**Table 3.** Phenolic compounds identified in sample's 22 fractions

| | | | | | Soaking water | | | Soaked Coats | | | Soaked Cotyledons | | | Whole Flour | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Compound** | **CF** | **Expected Mass Da** | **Fragments MS$^2$** | **RT Average ± SD (min)** | **Found at Mass Da [M-H]-** | **Error ppm** | **IS** | **Found at Mass Da [M-H]-** | **Error ppm** | **IS** | **Found at Mass Da [M-H]-** | **Error ppm** | **IS** | **Found at Mass Da [M-H]-** | **Error ppm** | **IS** | |
| **Hydroxybenzoic acids** | | | | | | | | | | | | | | | | | |
| **[1]** Protocatechuic acid-4-O-Gl | $C_{13}H_{16}O_9$ | 316.0794 | 108.0241/ 109.0319/ 152.0150/ 153.0229 | 27.8 ± 0.1 | 315.0722 | 0.0 | 0.8835 | 315.0724 | 0.8 | 0.8915 | 315.0716 | -1.8 | 0.8896 | 315.0701 | -6.5 | 0.7409 | [49] |
| **[2]** Vanillic acid | $C_8H_8O_4$ | 168.0423 | 108.0240/ 123.0489/ 124.0157/ 152.0158 | 28.1 ± 0.1 | 167.0351 | 1.0 | 0.9916 | 167.0352 | 1.4 | 0.7308 | 167.0351 | 0.7 | 0.9901 | 167.0351 | 0.8 | 0.9683 | [50, 51] |
| **[5]** Protocatechuic acid* | $C_7H_6O_4$ | 154.0266 | 108.0241/ 109.0325 | 31.4 ± 0.1 | 153.0196 | 1.7 | 0.9835 | 153.0200 | 4.4 | 0.9558 | 153.0195 | 0.9 | 0.9721 | 153.0194 | 0.5 | 0.9770 | [52, 53] |
| **[12]** *p*-Hydroxybenzoic acid-4-O- Gl | $C_{13}H_{16}O_8$ | 300.0845 | 93.0372/ 137.0304 | 36.5 ± 0.1 | 299.0775 | 0.9 | 0.8742 | 299.0770 | -0.8 | 0.8896 | 299.0771 | -0.5 | 0.8573 | 299.0756 | -5.6 | 0.9116 | [49] |
| **[25]** *p*-Hydroxybenzoic acid* | $C_7H_6O_3$ | 138.0317 | 65.0413/ 93.0369 | 41.4 ± 0.1 | 137.0246 | 1.0 | 0.9898 | 137.0245 | 0.7 | 0.9804 | 137.0246 | 1.0 | 0.9797 | 137.0245 | 0.7 | 0.9880 | [51] |
| **[27]** Gentisic acid* | $C_7H_6O_4$ | 154.0266 | 108.0241/ 109.0322 | 43.4 ± 0.1 | 153.0199 | 3.8 | 0.9684 | 153.0195 | 1.2 | 0.9061 | 153.0194 | 0.6 | 0.9798 | 153.0193 | -0.4 | 0.8411 | [13, 54] |
| **Hydroxycinnamic acids** | | | | | | | | | | | | | | | | | |
| **[3]** *p*-Coumaroyl aldaric acid 1 | $C_{15}H_{16}O_{10}$ | 356.0743 | 57.0358/ 59.0152/ 85.0312/ 89.0261/ 119.0370/ 129.0215/ 147.0313/ 191.0233/ 209.0339 | 29.5 ± 0.1 | 355.0673 | 0.6 | 0.9334 | 355.0653 | -4.9 | 0.9543 | 355.0745 | 20.8 | 0.9266 | 355.0714 | 12.3 | 0.9840 | [51] |
| **[6]** *p*-Coumaroyl aldaric acid 2 | $C_{15}H_{16}O_{10}$ | 356.0743 | 57.0363/ 59.0156/ 85.0316/ 129.0216/ 147.0330/ 163.0428/ 191.0235/ 209.0343 | 32.7 ± 0.1 | 355.067 | -0.3 | 0.8553 | 355.0656 | -4.2 | 0.9724 | 355.0657 | -3.9 | 0.8463 | 355.0654 | -4.6 | 0.8660 | [51] |
| **[7]** Feruloyl aldaric acid 1 | $C_{16}H_{18}O_{11}$ | 386.0849 | 57.0362/ 59.0154/ 85.0315/ 129.0216/ 147.0327/ 191.0234/ 209.0339 | 33.4 ± 0.1 | 385.0772 | -1.1 | 0.8642 | 385.0755 | -5.5 | 0.9330 | 385.0761 | -4.0 | 0.8615 | 385.0761 | -3.9 | 0.8802 | [51, 55] |
| **[8]** *p*-Coumaroyl aldaric acid 3 | $C_{15}H_{16}O_{10}$ | 356.0743 | 57.0364/ 59.0159/ 85.0320/ 129.0225/ 147.0336/ 191.0246/ 209.0357 | 34.9 ± 0.1 | 355.0672 | 0.3 | 0.8547 | 355.0659 | -3.4 | 0.9032 | 355.0659 | -3.3 | 0.8706 | 355.0655 | -4.4 | 0.8581 | ND |
| **[9]** Sinapoyl aldaric acid 1 | $C_{17}H_{20}O_{12}$ | 416.0955 | 57.0363/ 59.0155/ 85.0316/ 129.0220/ 147.0329/ 191.0230/ 209.0338 | 35.0 ± 0.1 | 415.0869 | -3.0 | 0.9592 | 415.0878 | -1.0 | 0.9388 | 415.0882 | 0.1 | 0.9354 | 415.0856 | -6.4 | 0.8778 | [55] |
| **[10]** Feruloyl aldaric acid 2 | $C_{16}H_{18}O_{11}$ | 386.0849 | 57.0362/ 85.0312/ 147.0323/ 191.0227/ 209.0339/ 223.0495 | 35.3 ± 0.1 | 385.0761 | -4.0 | 0.9050 | 385.0755 | -5.4 | 0.9710 | 385.0761 | -3.9 | 0.8701 | 385.0756 | -5.3 | 0.8887 | [51, 55] |
| **[13]** Feruloyl aldaric acid 3 | $C_{16}H_{18}O_{11}$ | 386.0849 | 57.0359/ 59.0154/ 85.0311/ 129.0217/ 147.0319/ 191.0228/ 193.0539/ 209.0331 | 36.6 ± 0.1 | 385.0764 | -3.3 | 0.9242 | 385.0753 | -6.0 | 0.9524 | 385.0763 | -3.4 | 0.8808 | 385.0758 | -4.8 | 0.8981 | [51, 55] |

**Table 3.** *Cont.*

| Compound | CF | Expected Mass Da | Fragments MS² | RT Average ± SD (min) | Soaking water | | | Soaked Coats | | | Soaked Cotyledons | | | Whole Flour | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Found at Mass Da [M-H]- | Error ppm | IS | Found at Mass Da [M-H]- | Error ppm | IS | Found at Mass Da [M-H]- | Error ppm | IS | Found at Mass Da [M-H]- | Error ppm | IS | |
| **Hydroxycinnamic acids** | | | | | | | | | | | | | | | | | |
| **[14]** *p*-Coumaroyl aldaric acid 4 | $C_{15}H_{16}O_{10}$ | 356.0743 | 85.0315/ 147.0326/ 191.0233/ 209.0343 | 36.8 ± 0.2 | 355.0665 | -1.6 | 0.8922 | 355.0653 | -4.8 | 0.9581 | 355.0659 | -3.2 | 0.8631 | 355.065 | -5.7 | 0.8873 | ND |
| **[15]** *p*-Coumaroyl aldaric acid 5 | $C_{15}H_{16}O_{10}$ | 356.0743 | 57.0361/ 59.0153/ 85.0315/ 129.0216/ 147.0324/ 191.0234/ 209.0343 | 37.6 ± 0.1 | 355.0664 | -1.9 | 0.8651 | 355.0658 | -3.6 | 0.9319 | 355.0660 | -3.1 | 0.8480 | 355.0655 | -4.5 | 0.8628 | ND |
| **[16]** Feruloyl aldaric acid 4 | $C_{16}H_{18}O_{11}$ | 386.0849 | 57.0361/ 85.0313/ 129.0211/ 147.0322/ 191.0228/ 209.0333 | 38.2 ± 0.1 | 385.0775 | -0.5 | 0.8943 | 385.0762 | -3.7 | 0.9035 | 385.0766 | -2.6 | 0.8748 | 385.076 | -4.3 | 0.8851 | [55] |
| **[19]** Sinapoyl aldaric acid 2 | $C_{17}H_{20}O_{12}$ | 416.0955 | 57.0360/ 59.0154/ 85.0314/ 129.0214/ 147.0327/ 191.0234/ 209.0339/ 223.0661 | 38.7 ± 0.1 | 415.0865 | -4.0 | 0.9481 | 415.0856 | -6.2 | 0.9243 | 415.0855 | -6.4 | 0.9349 | 415.0851 | -7.5 | 0.9162 | [55] |
| **[20]** Sinapoyl aldaric acid 3 | $C_{17}H_{20}O_{12}$ | 416.0955 | 57.0363/ 85.0317/ 129.0220/ 147.0329/ 191.0237/ 209.0343 | 39.4 ± 0.1 | 415.0871 | -2.5 | 0.8585 | 415.0855 | -6.5 | 0.9589 | 415.0859 | -5.5 | 0.8748 | 415.0857 | -6.1 | 0.8622 | ND |
| **[22]** Feruloyl aldaric acid 5 | $C_{16}H_{18}O_{11}$ | 386.0849 | 57.0363/ 85.0315/ 129.0216/ 147.0326/ 191.0232/ 209.0342 | 40.2 ± 0.1 | 385.0774 | -0.7 | 0.8745 | 385.0758 | -4.8 | 0.9144 | 385.0767 | -2.5 | 0.8842 | 385.0762 | -3.6 | 0.8726 | [55] |
| **[23]** Sinapoyl aldaric acid 4 | $C_{17}H_{20}O_{12}$ | 416.0955 | 57.0362/ 59.0154/ 85.0315/ 129.0215/ 147.0324/ 191.0234/ 209.0343 | 40.8 ± 0.1 | 415.0861 | -5.0 | 0.9706 | 415.0853 | -7.1 | 0.9082 | 415.0861 | -4.9 | 0.8771 | 415.0856 | -6.3 | 0.8977 | ND |
| **[26]** *p*-Coumaroyl aldaric acid 6 | $C_{15}H_{16}O_{10}$ | 356.0743 | 57.0361/ 59.0154/ 85.0313/ 129.0212/ 147.0326/ 163.0420/ 191.0232/ 209.0339 | 41.8 ± 0.1 | NF | - | - | 355.0654 | -4.7 | 0.7331 | 355.0658 | -3.6 | 0.8664 | 355.0654 | -4.7 | 0.8717 | ND |
| **[30]** Feruloyl aldaric acid 6 | $C_{16}H_{18}O_{11}$ | 386.0849 | 57.0364/ 59.0157/ 85.0317/ 111.0106/ 129.0218/ 147.0333/ 191.0241/ 209.0348 | 44.5 ± 0.1 | 385.0772 | -1.0 | 0.8900 | 385.0760 | -4.1 | 0.8934 | 385.0766 | -2.8 | 0.9271 | 385.0764 | -3.2 | 0.8801 | ND |
| **[31]** Sinapoyl aldaric acid 5 | $C_{17}H_{20}O_{12}$ | 416.0955 | 57.0361/ 59.0152/ 85.0314/ 129.0219/ 147.0326/ 191.0232/ 209.0339 | 44.8 ± 0.1 | 415.0871 | -2.7 | 0.8610 | 415.0857 | -6.1 | 0.9493 | 415.0863 | -4.7 | 0.8794 | 415.0858 | -5.9 | 0.8865 | ND |
| **[40]** *p*-Coumaric acid* | $C_9H_8O_3$ | 164.0473 | 93.0364/ 117.0369/ 119.0529 | 58.9 ± 0.2 | 163.0407 | 4.2 | 0.9776 | 163.0404 | 1.9 | 0.9503 | NF | - | - | 163.0401 | 0.2 | 0.8870 | [25] |
| **[43]** Sinapic acid* | $C_{11}H_{12}O_5$ | 224.0685 | 93.0361/ 121.0308/ 149.0272/ 163.0423/ 164.05077/ 165.0227/ 193.0175/ 208.0417 | 63.1 ± 0.2 | 223.0613 | 0.6 | 0.9604 | 223.0610 | -1.0 | 0.7187 | 223.0611 | -0.5 | 0.9814 | 223.0605 | -3.1 | 0.9721 | [25] |
| **[44]** Ferulic acid* | $C_{10}H_{10}O_4$ | 194.0579 | 102.9351/ 133.0316/ 134.0400/ 149.0636/ 178.0305 | 63.8 ± 0.2 | 193.0510 | 1.9 | 0.9545 | 193.0506 | -0.3 | 0.8344 | NF | - | - | 193.0507 | 0.3 | 0.9355 | [25, 51] |
| **Flavanols** | | | | | | | | | | | | | | | | | |
| **[4]** (+)-Catechin 3'-O-glucose | $C_{21}H_{24}O_{11}$ | 452.1319 | 137.0279/ 245.0874/ 289.0836/ 299.0835 | 30.8 ± 0.1 | 451.1234 | -2.5 | 0.8674 | 451.1227 | -4.2 | 0.8103 | 451.1220 | -5.8 | 0.8440 | 451.1218 | -6.1 | 0.8412 | [51] |
| **[11]** Procyanidin B1 | $C_{30}H_{26}O_{12}$ | 578.1424 | 125.0272/ 161.0276/ 287.0613/ 289.0771/ 407.0836/ 425.0935/ 451.1095 | 36.3 ± 0.1 | 577.1338 | -2.4 | 0.8707 | 577.1322 | -5.1 | 0.8217 | NF | - | - | 577.1308 | -7.6 | 0.8528 | [51] |

**Table 3.** *Cont.*

| Compound | CF | Expected Mass Da | Fragments $MS^2$ | RT Average ± SD (min) | Soaking water | | | Soaked Coats | | | Soaked Cotyledons | | | Whole Flour | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Found at Mass Da [M-H]- | Error ppm | IS | Found at Mass Da [M-H]- | Error ppm | IS | Found at Mass Da [M-H]- | Error ppm | IS | Found at Mass Da [M-H]- | Error ppm | IS | |
| **Flavanols** | | | | | | | | | | | | | | | | | |
| **[17]** *(+)*-Catechin 7-O-β-D-Gl | $C_{21}H_{24}O_{11}$ | 452.1319 | 245.0860/ 289.0766 | 37.7 ± 0.1 | 451.124 | -1.3 | 0.8408 | 451.1226 | -4.5 | 0.8522 | 451.1216 | -6.6 | 0.9377 | 451.1214 | -7.1 | 0.8410 | [56] |
| **[18]** Procyanidin B2* | $C_{30}H_{26}O_{12}$ | 578.1424 | 125.0270/ 289.0768/ 407.0831/ 425.0936/ 451.1091 | 38.3 ± 0.1 | 577.1334 | -3.0 | 0.8429 | 577.1320 | -5.5 | 0.7773 | 577.1306 | -7.8 | 0.7106 | 577.1306 | -7.8 | 0.8349 | [51] |
| **[21]** Procyanidin C1 | $C_{45}H_{38}O_{18}$ | 866.2058 | 575.1276/ 577.1431/ 695.1510/ 713.1622 | 40.2 ± 0.1 | 865.1957 | -3.3 | 0.7994 | 865.1925 | -7.0 | 0.7427 | 865.1950 | -4.1 | 0.5898 | 865.1907 | -9.0 | 0.8559 | [43, 57] |
| **[24]** *(+)*-Catechin* | $C_{15}H_{14}O_6$ | 290.079 | 109.0321/ 123.0479/ 125.0272/ 137.0274/ 151.0433/ 179.0388/ 203.0753/ 205.0546/ 221.0865/ 245.0866 | 40.6 ± 0.1 | 289.0719 | 0.4 | 0.8594 | 289.0718 | 0.0 | 0.8649 | 289.0711 | -2.1 | 0.9019 | 289.0714 | -1.1 | 0.8714 | [51] |
| **[28]** Procyanidin B3 | $C_{30}H_{26}O_{12}$ | 578.1424 | 125.0267/ 287.0609/ 289.0762/ 407.0829/ 425.0932/ 451.1094 | 43.7 ± 0.1 | 577.1331 | -3.5 | 0.8371 | 577.1312 | -6.9 | 0.8396 | NF | - | - | 577.1290 | -10.7 | 0.9183 | [51] |
| **[32]** Procyanidin B4 | $C_{30}H_{26}O_{12}$ | 578.1424 | 125.0266/ 161.0267/ 287.0598/ 289.0756/ 407.0813/ 425.0923/ 451.1092 | 46.4 ± 0.1 | 577.1327 | -4.2 | 0.8305 | 577.1310 | -7.2 | 0.8814 | NF | - | - | 577.1294 | -9.9 | 0.7277 | [53] |
| **[33]** (-)-Epicatechin* | $C_{15}H_{14}O_6$ | 290.079 | 109.0317/ 123.0476/ 125.0267/ 151.0425/ 203.0749/ 205.0537/ 245.0860 | 46.8 ± 0.1 | 289.0719 | 0.6 | 0.8671 | 289.0716 | -0.4 | 0.8691 | 289.0705 | -4.2 | 0.9701 | 289.0712 | -1.8 | 0.8682 | [58] |
| **[35]** Procyanidin C2 | $C_{45}H_{38}O_{18}$ | 866.2058 | 575.1269 | 48.1 ± 0.2 | 865.1952 | -3.9 | 0.7947 | 865.1920 | -7.6 | 0.7222 | 865.1909 | -8.8 | 0.8242 | 865.1908 | -8.9 | 0.7730 | [57] |
| **[36]** Procyanidin B5 | $C_{30}H_{26}O_{12}$ | 578.1424 | 125.0266/ 287.0603/ 289.0760/ 407.0825/ 425.0924/ 451.1082 | 50.8 ± 0.2 | 577.1334 | -3.0 | 0.8527 | 577.1316 | -6.2 | 0.8447 | 577.1296 | -9.6 | 0.7375 | 577.1308 | -7.6 | 0.8452 | [59] |
| **Flavanones** | | | | | | | | | | | | | | | | | |
| **[29]** Eriodictyol-hexoside 1 | $C_{21}H_{22}O_{11}$ | 450.1162 | 125.0267/ 179.0017/ 243.0698/ 259.0662/ 283.0657/ 287.0615/ 301.0764/ 421.1202 | 43.7 ± 0.1 | 449.1074 | -3.4 | 0.8475 | 449.1054 | -8.0 | 0.8682 | 449.1052 | -8.3 | 0.8673 | 449.1052 | -8.4 | 0.8317 | [51, 55] |
| **[34]** Eriodictyol-hexoside 2 | $C_{21}H_{22}O_{11}$ | 450.1162 | 125.0273/ 259.0672/ 269.0510/ 287.0618 | 47.2 ± 0.1 | 449.108 | -2.0 | 0.8735 | 449.1068 | -4.6 | 0.8607 | 449.1070 | -4.3 | 0.8465 | 449.1061 | -6.2 | 0.8569 | [55] |
| **[37]** Eriodictyol-hexoside 3 | $C_{21}H_{22}O_{11}$ | 450.1162 | 259.0651/ 287.0607 | 53.6 ± 0.1 | 449.1077 | -2.7 | 0.8574 | 449.1060 | -6.6 | 0.9051 | 449.1057 | -7.3 | 0.8843 | 449.1064 | -5.7 | 0.8703 | ND |
| **[38]** Eriodictyol-hexoside 4 | $C_{21}H_{22}O_{11}$ | 450.1162 | 125.0262/ 151.0061/ 152.0143/ 179.0014/ 180.0094/ 269.0497/ 287.0590 | 56.7 ± 0.2 | 449.1078 | -2.6 | 0.8554 | 449.1056 | -7.5 | 0.9268 | 449.1056 | -7.4 | 0.9693 | 449.1062 | -6.1 | 0.8633 | ND |
| **[42]** Naringenin-7-Gl | $C_{21}H_{22}O_{10}$ | 434.1213 | 119.0521/ 151.0057/ 271.0647/ 313.0589 | 60.4 ± 0.2 | 433.1119 | -4.8 | 0.9503 | 433.1107 | -7.6 | 0.8883 | 433.1088 | -12.0 | 0.7686 | 433.1097 | -10.0 | 0.9045 | [53] |
| **[50]** Eriodictyol | $C_{15}H_{12}O_6$ | 288.0634 | 125.0269/ 243.0703/ 259.0659 | 82.7 ± 0.2 | 287.0561 | -0.1 | 0.9315 | 287.0557 | -1.5 | 0.8979 | 287.0547 | -4.8 | 0.9471 | 287.0553 | -2.7 | 0.9044 | [51] |
| **[53]** Naringenin | $C_{15}H_{12}O_5$ | 272.0685 | 107.0160/ 119.0524/ 151.0058/ 177.0228 | 95.4 ± 0.1 | 271.0610 | -0.7 | 0.9873 | 271.0605 | -2.6 | 0.9748 | 271.0605 | -2.7 | 0.9126 | 271.0602 | -3.8 | 0.9438 | [51] |

**Table 3.** *Cont.*

| Compound | CF | Expected Mass Da | Fragments $MS^2$ | RT Average ± SD (min) | Soaking water | | | Soaked Coats | | | Soaked Cotyledons | | | Whole Flour | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Found at Mass Da [M-H]- | Error ppm | IS | Found at Mass Da [M-H]- | Error ppm | IS | Found at Mass Da [M-H]- | Error ppm | IS | Found at Mass Da [M-H]- | Error ppm | IS | |
| **Flavonols** | | | | | | | | | | | | | | | | | |
| **[39]** Luteolin 3',7-di-O-Gl or kaempferol-3',7-dihexoside | $C_{27}H_{30}O_{16}$ | 610.1534 | 284.0373/ 285.0446 | 56.8 ± 0.2 | 609.1442 | -3.1 | 0.8322 | 609.1415 | -7.5 | 0.8720 | 609.1409 | -8.6 | 0.9374 | 609.1417 | -7.3 | 0.8715 | [60] |
| **[41]** Rutin* | $C_{27}H_{30}O_{16}$ | 610.1534 | 284.0370/ 285.0453/ 300.0320/ 301.0386/ 327.0554/607.2499 | 59.9 ± 0.2 | 609.1441 | -3.2 | 0.8302 | 609.1421 | -6.6 | 0.8892 | 609.1418 | -7.1 | 0.8585 | 609.1410 | -8.3 | 0.8494 | [25, 55] |
| **[45]** Kaempferol-3-O-xylosyl-Gl | $C_{26}H_{28}O_{15}$ | 580.1428 | 284.0377/ 285.0453/ 429.0891 | 64.7 ± 0.1 | 579.1335 | -3.5 | 0.8194 | NF | - | - | NF | - | - | 579.1316 | -6.9 | 0.8276 | [25, 61] |
| **[46]** Quercetin-3-O-Gl | $C_{21}H_{20}O_{12}$ | 464.0955 | 300.0318/ 301.0392 | 65.0 ± 0.2 | 463.0867 | -3.2 | 0.8117 | 463.0855 | -5.9 | 0.8163 | NF | - | - | 463.0849 | -7.1 | 0.8265 | [25, 61] |
| **[47]** Quercetin-3-(6-O-acetyl-β-Gl) | $C_{23}H_{22}O_{13}$ | 506.106 | 300.0319/ 301.0399/ 463.0923 | 73.2 ± 0.2 | 505.0970 | -3.5 | 0.8666 | 505.0942 | -9.1 | 0.9576 | NF | - | - | 505.0952 | -7.0 | 0.8700 | [55, 62] |
| **[48]** Kaempferol-3-O-Gl | $C_{21}H_{20}O_{11}$ | 448.1006 | 227.0397/ 255.0363/ 284.0447/ 285.0476 | 78.1 ± 0.1 | 447.0934 | 0.3 | 0.8361 | 447.0918 | -3.4 | 0.8064 | 447.0904 | -6.4 | 0.8319 | 447.0909 | -5.2 | 0.8716 | [25, 55, 61] |
| **[49]** Kaempferol-3-O-acetyl-Gl | $C_{23}H_{22}O_{12}$ | 490.1111 | 255.0339/ 284.0386/ 285.0451 | 82.4 ± 0.1 | 489.1027 | -2.2 | 0.8634 | 489.1004 | -7.1 | 0.8248 | 489.1006 | -6.5 | 0.8577 | 489.1010 | -5.8 | 0.8644 | [61] |
| **[52]** Quercetin* | $C_{15}H_{10}O_7$ | 302.0427 | 107.0143/ 121.0312/ 151.0059/ 179.0017/ 255.2372/ 273.0434 | 91.2 ± 0.1 | 301.0347 | -2.1 | 0.9273 | 301.0353 | -0.2 | 0.8619 | 301.0341 | -4.3 | 0.9211 | 301.0336 | -5.9 | 0.9445 | [25, 51] |
| **[55]** Kaempferol* | $C_{15}H_{10}O_6$ | 286.0477 | 93.0368/ 107.0159/ 151.0062/ 159.0479/ 185.0639/ 187.0428/ 211.0429/ 229.0545/ 239.0385/ 257.0493 | 97.4 ± 0.1 | 285.0407 | 1.0 | 0.8786 | 285.0403 | -0.5 | 0.8557 | 285.0402 | -1.0 | 0.8565 | 285.0400 | -1.6 | 0.8669 | [25, 61] |
| **Isoflavones** | | | | | | | | | | | | | | | | | |
| **[51]** Daidzein* | $C_{15}H_{10}O_4$ | 254.0579 | 209.0643/ 224.0538/ 225.0617 | 88.5 ± 0.1 | 253.0505 | -0.4 | 0.9479 | 253.0503 | -1.2 | 0.8295 | 253.0502 | -1.7 | 0.9510 | 253.0495 | -4.4 | 0.7987 | [61] |
| **[54]** Genistein* | $C_{15}H_{10}O_5$ | 270.0528 | 133.0309 | 96.3 ± 0.1 | 269.0452 | -1.2 | 0.7351 | 269.0447 | -3.1 | 0.9337 | 269.0449 | -2.5 | 0.8803 | NF | - | - | [61] |

CF – Chemical Formula; IS – Isotope Score; RT – Retention Time; SD – Standard Deviation; HBA – Hydroxybenzoic Acid; HCA – Hydroxycinnamic Acid; Gl – Glucoside;

*Compounds identified by comparison with standards; the major fragments are underlined; NF – Not Found; ND – Not Described

**Table 4.** Relative quantification of identified compounds in sample's 22 fractions

| | | Soaking Water | | Soaked Coats | | Soaked Cotyledons | | Whole Flour (Raw) | |
|---|---|---|---|---|---|---|---|---|---|
| **Class** | **Name** | **Area (%, Area/ Total area)** | **%, Area/ Total compounds' class area** | **Area (%, Area/ Total area)** | **%, Area/ Total compounds' class area** | **Area (%, Area/ Total area)** | **%, Area/ Total compounds' class area** | **Area (%, Area/ Total area)** | **%, Area/ Total compounds' class area** |
| **HBA** | [1]Protocatechuic acid-4-O-Gl | 1677120 (2.9) | 43.2 | 85308 (0.6) | 20.5 | 451623 (4.8) | 31.2 | 13153 (0.03) | 8.1 |
| | [2]Vanillic acid | 67863 (0.1) | 1.7 | 4363 (0.03) | 1.0 | 15603 (0.2) | 1.1 | 29105 (0.1) | 17.9 |
| | [5]Protocatechuic acid | 130651 (0.2) | 3.4 | 137055 (1.0) | 33.0 | 9253 (0.1) | 0.6 | 25185 (0.1) | 15.5 |
| | [12]*p*-Hydroxybenzoic acid-4-O- Gl | 1841267 (3.2) | 47.4 | 163157 (1.2) | 39.3 | 949047 (10.1) | 65.6 | 23743 (0.1) | 14.6 |
| | [25]*p*-Hydroxybenzoic acid | 60267 (0.1) | 1.6 | 19416 (0.1) | 4.7 | 13706 (0.1) | 0.9 | 69860 (0.2) | 43.0 |
| | [27]Gentisic acid | 106891 (0.2) | 2.8 | 6345 (0.05) | 1.5 | 7149 (0.1) | 0.5 | 1446 (0.003) | 0.9 |
| | **Total compounds' class area** | 3884059 (6.6) | 100.0 | 415644 (3.0) | 100.0 | 1446381 (14.7) | 100.0 | 162493 (0.4) | 100.0 |
| **HCA** | [3]*p*-Coumaroyl aldaric acid 1 | 26590 (0.05) | 0.6 | 4318 (0.03) | 2.2 | 167283 (1.8) | 3.8 | 114749 (0.3) | 1.5 |
| | [6]*p*-Coumaroyl aldaric acid 2 | 139230 (0.2) | 3.3 | 8798 (0.1) | 4.5 | 42992 (0.5) | 1.0 | 73863 (0.2) | 1.0 |
| | [7]Feruloyl aldaric acid 1 | 97092 (0.2) | 2.3 | 7766 (0.1) | 4.0 | 305601 (3.3) | 6.9 | 689964 (1.7) | 9.2 |
| | [8]*p*-Coumaroyl aldaric acid 3 | 1265546 (2.2) | 30.4 | 22910 (0.2) | 11.7 | 417109 (4.4) | 9.5 | 496423 (1.2) | 6.6 |
| | [9] Sinapoyl aldaric acid 1 | 35954 (0.1) | 0.9 | 1977 (0.01) | 1.0 | 65773 (0.7) | 1.5 | 183550 (0.4) | 2.5 |
| | [10] Feruloyl aldaric acid 2 | 12142 (0.02) | 0.3 | 3078 (0.02) | 1.6 | 137695 (1.5) | 3.1 | 130905 (0.3) | 1.7 |
| | [13] Feruloyl aldaric acid 3 | 66532 (0.1) | 1.6 | 3551 (0.03) | 1.8 | 49572 (0.5) | 1.1 | 139151 (0.3) | 1.9 |
| | [14] *p*-Coumaroyl aldaric acid 4 | 321109 (0.6) | 7.7 | 5263 (0.04) | 2.7 | 9651 (0.1) | 0.2 | 9516 (0.02) | 0.1 |
| | [15] *p*-Coumaroyl aldaric acid 5 | 150575 (0.3) | 3.6 | 17330 (0.1) | 8.9 | 396951 (4.2) | 9.0 | 339760 (0.8) | 4.5 |
| | [16] Feruloyl aldaric acid 4 | 139602 (0.2) | 3.3 | 3934 (0.03) | 2.0 | 213762 (2.3) | 4.9 | 125167 (0.3) | 1.7 |
| | [19] Sinapoyl aldaric acid 2 | 49814 (0.1) | 1.2 | 1705 (0.01) | 0.9 | 55150 (0.6) | 1.3 | 14544 (0.03) | 0.2 |
| | [20] Sinapoyl aldaric acid 3 | 172631 (0.3) | 4.1 | 5470 (0.04) | 2.8 | 242307 (2.6) | 5.5 | 677720 (1.6) | 9.0 |
| | [22] Feruloyl aldaric acid 5 | 470732 (0.8) | 11.3 | 44560 (0.3) | 22.8 | 757939 (8.1) | 17.2 | 1381715 (3.3) | 18.4 |

**Table 4.** *Cont.*

| Class | Name | Soaking Water | | Soaked Coats | | Soaked Cotyledons | | Whole Flour (Raw) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Area (%, Area/ Total area) | %, Area/ Total compounds' class area | Area (%, Area/ Total area) | %, Area/ Total compounds' class area | Area (%, Area/ Total area) | %, Area/ Total compounds' class area | Area (%, Area/ Total area) | %, Area/ Total compounds' class area |
| HCA | [23] Sinapoyl aldaric acid 4 | 171674 (0.3) | 4.1 | 8603 (0.06) | 4.4 | 291186 (3.1) | 6.6 | 747370 (1.8) | 10.0 |
| | [26] *p*-Coumaroyl aldaric acid 6 | NF | NF | 18685 (0.1) | 9.6 | 433166 (4.4) | 9.0 | 213995 (0.5) | 2.9 |
| | [30] Feruloyl aldaric acid 6 | 730915 (1.3) | 17.5 | 28548 (0.2) | 14.6 | 971927 (10.4) | 22.1 | 1644680 (4.0) | 22.0 |
| | [31] Sinapoyl aldaric acid 5 | 185506 (0.3) | 4.5 | 7616 (0.05) | 3.9 | 273562 (2.9) | 6.2 | 465873 (1.1) | 6.2 |
| | [40] *p*-Coumaric acid | 89291 (0.2) | 2.1 | 338 (0.002) | 0.2 | NF | NF | 11431 (0.03) | 0.2 |
| | [43] Sinapic acid | 2151 (0.004) | 0.1 | 418 (0.003) | 0.2 | 8055 (0.1) | 0.2 | 6305 (0.02) | 0.1 |
| | [44] Ferulic acid | 40918 (0.1) | 1.0 | 158 (0.001) | 0.1 | NF | NF | 23022 (0.1) | 0.3 |
| | **Total compounds' class area** | 4168002 (7.1) | 100.0 | 195028 (1.4) | 100.0 | 4839680 (49.3) | 100.0 | 7489702 (18.0) | 100.0 |
| Flavanols | [4] Catechin 3'-O-Gl | 7636898 (13.0) | 33.5 | 1165545 (8.3) | 32.7 | 518148 (5.3) | 86.3 | 3166476 (7.6) | 31.6 |
| | [11] Procyanidin B1 | 4199734 (7.2) | 18.4 | 866524 (6.2) | 24.3 | NF | NF | 1885313 (4.5) | 18.8 |
| | [17] Catechin 7-O-Gl | 1214896 (2.1) | 5.3 | 70095 (0.5) | 2.0 | 10469 (0.1) | 1.7 | 172724 (0.4) | 1.7 |
| | [18] Procyanidin B2 | 926982 (1.6) | 4.1 | 197566 (1.4) | 5.5 | 2303 (0.02) | 0.4 | 468641 (1.1) | 4.7 |
| | [21] Procyanidin C1 | 1216935 (2.1) | 5.3 | 546543 (3.9) | 15.3 | 2018 (0.02) | 0.3 | 265008 (0.6) | 2.6 |
| | [24] Catechin | 5800883 (9.9) | 25.4 | 507760 (3.6) | 14.2 | 60537 (0.6) | 10.1 | 3025744 (7.3) | 30.2 |
| | [28] Procyanidin B3 | 205083 (0.4) | 0.9 | 21266 (0.2) | 0.6 | NF | NF | 38912 (0.1) | 0.4 |
| | [32] Procyanidin B4 | 60776 (0.1) | 0.3 | 8658 (0.1) | 0.2 | NF | NF | 28180 (0.1) | 0.3 |
| | [33] Epicatechin | 559522 (1.0) | 2.5 | 51252 (0.4) | 1.4 | 4432 (0.05) | 0.7 | 353630 (0.8) | 3.5 |
| | [35] Procyanidin C2 | 185749 (0.3) | 0.8 | 25218 (0.2) | 0.7 | 171 (0.002) | 0.0 | 85610 (0.2) | 0.9 |
| | [36] Procyanidin B5 | 820786 (1.4) | 3.6 | 104129 (0.7) | 2.9 | 2311 (0.02) | 0.4 | 522375 (1.3) | 5.2 |
| | **Total compounds' class area** | 22828243 (39.0) | 100.0 | 3564555 (25.5) | 100.0 | 600389 (6.1) | 100.0 | 10012612 (24.0) | 100.0 |

**Table 4.** *Cont.*

| | | Soaking Water | | Soaked Coats | | Soaked Cotyledons | | Whole Flour (Raw) | |
|---|---|---|---|---|---|---|---|---|---|
| **Class** | **Name** | **Area (%, Area/ Total area)** | **%, Area/ Total compounds' class area** | **Area (%, Area/ Total area)** | **%, Area/ Total compounds' class area** | **Area (%, Area/ Total area)** | **%, Area/ Total compounds' class area** | **Area (%, Area/ Total area)** | **%, Area/ Total compounds' class area** |
| **Flavanones** | [29]Eriodictyol-hexoside 1 | 98920 (0.2) | 1.7 | 4350 (0.03) | 1.7 | 4011 (0.04) | 1.3 | 197075 (0.5) | 2.7 |
| | [34] Eriodictyol-hexoside 2 | 5166817 (8.8) | 86.4 | 178852 (1.3) | 70.6 | 272049 (2.8) | 90.9 | 5800730 (13.9) | 80.6 |
| | [37] Eriodictyol-hexoside 3 | 252875 (0.4) | 4.2 | 25333 (0.2) | 10.0 | 12016 (0.1) | 4.0 | 844769 (2.0) | 11.7 |
| | [38] Eriodictyol-hexoside 4 | 177730 (0.3) | 3.0 | 5960 (0.04) | 2.4 | 804 (0.01) | 0.3 | 165914 (0.4) | 2.3 |
| | [42] Naringenin-7-Gl | 10713 (0.02) | 0.2 | 585 (0.004) | 0.2 | 409 (0.004) | 0.1 | 5652 (0.01) | 0.1 |
| | [50] Eriodictyol | 254008 (0.4) | 4.2 | 28839 (0.2) | 11.4 | 1324 (0.01) | 0.4 | 161919 (0.4) | 2.3 |
| | [53] Naringenin | 15799 (0.03) | 0.3 | 9365 (0.1) | 3.7 | 8739 (0.1) | 2.9 | 17103 (0.04) | 0.2 |
| | **Total compounds' class area** | 5976862 (10.2) | 100.0 | 253284 (1.8) | 100.0 | 299352 (3.0) | 100.0 | 7193164 (17.3) | 100.0 |
| **Flavonols** | [39] Luteolin 3,7-di-O-Gl or Kaempferol-3',7-dihexoside | 309103 (0.5) | 1.4 | 20371 (0.1) | 0.2 | 2187 (0.02) | 0.1 | 535830 (1.3) | 3.2 |
| | [41] Rutin | 208461 (0.4) | 1.0 | 20628 (0.1) | 0.2 | 14381 (0.1) | 0.6 | 86221 (0.2) | 0.5 |
| | [45]Kaempferol-3-O-xylosyl-Gl | 2737477 (4.7) | 12.7 | NF | NF | NF | NF | 2217478 (5.3) | 13.2 |
| | [46] Quercetin-3-O-Gl | 1233782 (2.1) | 5.7 | 8850 (0.1) | 0.1 | NF | NF | 1485967 (3.6) | 8.9 |
| | [47]Quercetin-3-(6-O-acetyl-Gl) | 62179 (0.1) | 0.3 | 37 (0.003) | 0.0 | NF | NF | 63797 (0.2) | 0.4 |
| | [48] Kaempferol-3-O-Gl | 1147503 4 (19.6) | 53.2 | 6344589 (45.4) | 66.5 | 438101 (4.5) | 16.8 | 7437720 (17.9) | 44.3 |
| | [49]Kaempferol-3-O-acetyl-Gl | 5393293 (9.2) | 25.0 | 1228219 (8.8) | 12.9 | 1942204 (19.8) | 74.7 | 4250372 (10.2) | 25.3 |
| | [52] Quercetin | 8100 (0.01) | 0.0 | 12113 (0.1) | 0.1 | 10403 (0.1) | 0.4 | 17618 (0.04) | 0.1 |

**Table 4.** *Cont.*

| Class | Name | Soaking Water | | Soaked Coats | | Soaked Cotyledons | | Whole Flour (Raw) | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Area (%, Area/ Total area)** | **%, Area/ Total compounds' class area** | **Area (%, Area/ Total area)** | **%, Area/ Total compounds' class area** | **Area (%, Area/ Total area)** | **%, Area/ Total compounds' class area** | **Area (%, Area/ Total area)** | **%, Area/ Total compounds' class area** |
| **Flavonols** | [55] Kaempferol | 145232 (0.3) | 0.7 | 1903123 (13.6) | 20.0 | 194088 (2.0) | 7.5 | 682007 (1.6) | 4.1 |
| | **Total compounds' class area** | 21572661 (36.9) | 100.0 | 9537931 (68.2) | 100.0 | 2601364 (27.7) | 100.0 | 16777009 (40.3) | 100.0 |
| **Isoflavones** | [51] Daidzein | 2243 (0.004) | 25.2 | 2434 (0.02) | 23.0 | 9867 (0.1) | 34.6 | 538 (0.001) | 100.0 |
| | [54] Genistein | 6664 (0.01) | 74.8 | 8165 (0.1) | 77.0 | 18661 (0.2) | 65.4 | NF | NF |
| | **Total compounds' class area** | 8907 (0.02) | 100.0 | 10599 (0.1) | 100.0 | 28528 (0.3) | 100.0 | 538 (0.001) | 100.0 |
| | **Total area** | 58438734 (100.0) | | 13977041 (100.0) | | 9815694 (100.0) | | 41635518 (100.0) | |

HBA – Hydroxybenzoic Acid; HCA – Hydroxycinnamic Acid; Gl – Glucoside; NF – Not Found; the underlined percentage(s) indicate, per compounds' class the most abundant compound(s) in each fraction

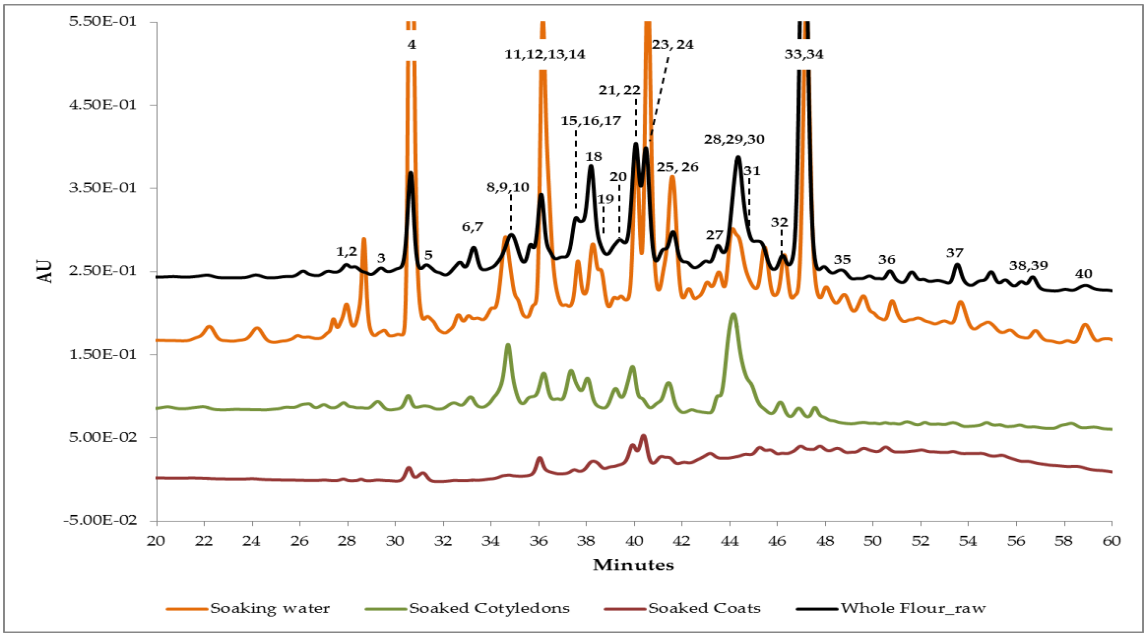



**Figure 3A.** Zoom of the chromatographic profiles of sample's 22 fractions (20 – 60 min) at 280 nm. The numbers highlight the identified compounds in **Table 4**

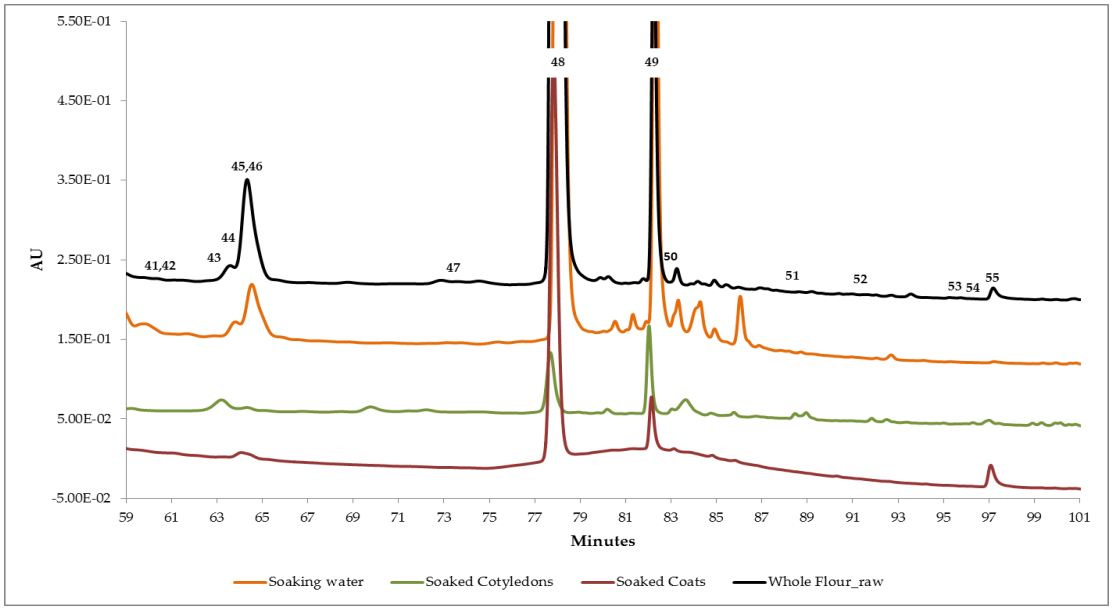



**Figure 3B.** Zoom of the chromatographic profiles of sample's 22 fractions (59 – 101 min) at 280 nm. The numbers highlight the identified compounds in **Table 4**

## Flavanones

The aglycone forms of naringenin (54) and eriodictyol (51) and their glycosidic forms were accurately identified in all the studied fractions. The aglycones were previously described in whole flour of white beans [51]. Compounds 29, 34, 37 and 38 were identified as eriodictyol-hexoside isomers, since they showed a product ion at *m/z* 287.06, which corresponds to the loss of an eriodictyol unit. Aguilera et al. [51] also described in white beans a deprotonated molecule with *m/z* 449 as an eriodictyol derivative. Naringenin-7-glucoside was also previously described [53] in dark beans. In the light brown variety analyzed, this phenolic compounds' class represented 17.3% and 10.2% of the total phenolic compounds, in whole flour and soaking water, respectively. Eriodictyol-hexoside 2 was the most predominant flavanone in all the fractions (more than 70% of the identified flavanones). The aglycones, eriodictyol and naringenin contributed to 15.1% of the flavanones total area in soaked coats fraction, suggesting that these compounds are mostly located in seeds' coat rather than in cotyledons. Recent studies on eriodictyol's cell activity suggest the potential of such compound in immunomodulation, anti-inflammation and antioxidant activity [68], which supports the importance of preserving on food based-products common beans coats naturally rich in these flavanones.

## Flavonols

Flavonols represent one of the most abundant phenolic compounds' classes in common beans [25], especially in soaked coats.

In the present study they represented 68.2%, 40.3% and 36.9% of the total compounds identified in soaked coats, whole flour before soaking and soaking water, respectively, **Figure 4**.

Quercetin (52) and kaempferol (55) were identified, as well as their derivatives. The identification of the product ion at *m/z* 301.04 in the fragmentation of compound (41) was a clear indication for rutin (quercetin-3-O-rutinoside) identification, as described by Lin et al. [25]. Quercetin glycosides (46 and 47) were also identified in the present study based on the characteristic quercetin product ion, *m/z* 301.04, as previously described by Pitura [62], in coloured common bean varieties.

Compound 39 was tentatively identified as luteolin-3',7-di-O-glucoside or as kaempferol-3',7-diglucoside, considering the characteristic fragment ion at *m/z* 285.04. As far as we know, these compounds have not been previously described in common beans, but luteolin-3',7-di-O-glucoside was identified in chickpea [60] and pea [60] and the kaempferol-3',7-diglucoside in broad beans [69].

A recent research highlighted the role of kaempferol as an antioxidant and immunomodulator agent with potential anti-carcinogenic effect in 5-fluorouracil resistant LS174 colon cancer cells [70]. Such beneficial health impact supports the regular consumption of common bean, including the coats' fraction, as a natural rich source of kaempferol aglycone (55) (20.0% of the total flavonols' area). Besides the aglycone form, kaempferol derivatives (46, 49 and 50) were also identified and previously reported in common beans [61]. Kaempferol-3-O-glucoside was the most abundant flavonol in soaked coats, soaking water and whole flour, representing, respectively, 66.5%, 53.2% and 44.3% of the total flavonols'

area. In soaked cotyledons the predominant kaempferol derivative was the kaempferol-3-O-acetyl-glucoside. Similarly to kaempferol, kaempferol-3-O-glucoside has also been described as a biological active agent in inflammatory pathological conditions and as a scavenger of free radical compounds, showing anti-cancer activity in several cancer cell lines [71].

Isoflavones

Isoflavones was the less representative class of phenolic compounds found before and after the soaking process. It represented only 0.3% and 0.1% of the total quantified area, in soaked cotyledons and soaked coats, respectively. Daidzein and genistein were the isoflavones identified after the soaking process, in soaking water, soaked coats and soaked cotyledons and have been described previously in yellow and black common bean varieties [61]. Before the soaking process, only daidzein was identified. As water-soluble compounds isoflavones are present in the soaking water [72]. Nevertheless, this phenolic compounds' class only represented 0.02% of the total compounds diffused into water, indicating its low contribution to the soaking water's phenolic content.

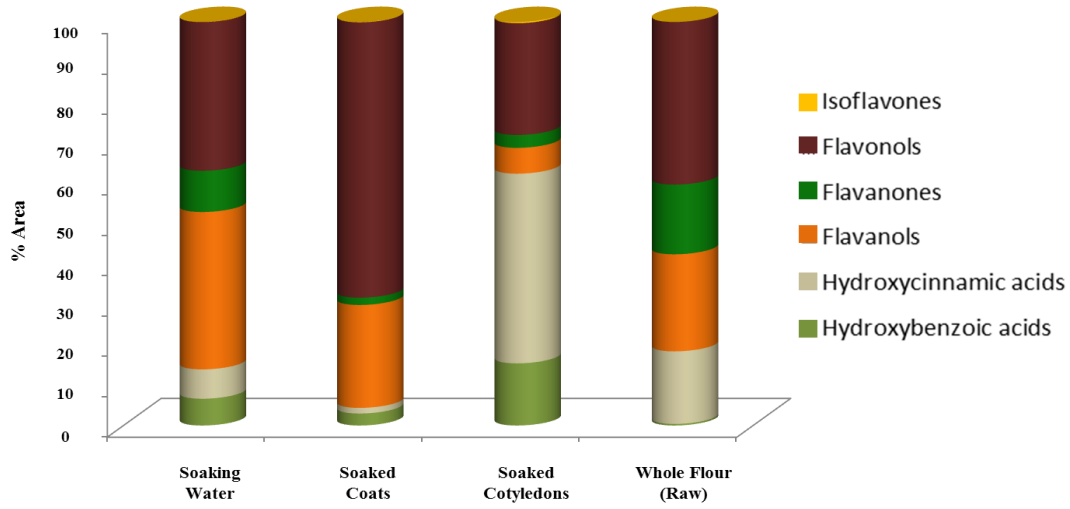



**Figure 4.** % Area of compounds' classes in different sample's 22 fractions

## 4. Conclusions

The results obtained in this study showed that the phenolic compounds' content in the high genetic diverse Portuguese common bean germplasm was quite variable. Such variability allowed studying the impact of the traditional soaking process only with water, overnight, in different common bean varieties. During the soaking period, the percentage of phenolic compounds lost into the water was dependent on common bean's variety. Therefore the soaking process should be adapted to each variety in accordance to the populations' nutritional needs. In over nourished populations could be beneficial to retain the phenolic compounds released into the soaking water, during the cooking process, as a strategy to preserve health promoter compounds. Such information should be passed to consumers and food industry.

This study showed that TPC, TFC, TPAC, and the *in vitro* antioxidant activity (ORAC) were found higher in the coloured varieties than in the white ones. The brown market colour class showed the highest TPC, TFC and TPAC values with high variability. In opposition to the coloured varieties, where soaked coats represented the richest fraction, in white varieties the soaked cotyledons had higher contribution to the total soaked seeds' phenolic content. Despite the phenolic compounds' loss into the soaking water, on average, more than 50% of the phenolic content remained preserved in the soaked seeds, being distributed between the two fractions, coats and cotyledons, in the different common bean varieties.

The use of UPLC-TripleTOF-MS enabled the identification of procyanidin B5 (36), catechin-7-O-glucoside (17) and luteolin-3',7-di-O-glucoside (39) or kaempferol-3',7-diglucoside, for the first time, in common beans. Several p-coumaroyl, feruloyl and synapoyl aldaric acid isomers were also identified, in higher number than the one described in the literature. After the soaking process in cotyledons, the phenolic acids (hydroxybenzoic and hydroxycinnamic acids), represented the predominant class, 64.0% of the total chromatographic area. In soaking water and soaked coats, the sum of flavanols and flavonols' classes represented, respectively, 75.9 and 93.7% of the total areas.

Information about abundance and distribution of phenolic compounds in common bean seeds and fractions represents an affordable approach, especially when commercial standards are not available. Such compounds may be isolated from those fractions, in order to clarify their

individual effect or synergistic impact in *in vitro* and *in vivo* studies of disease models.

In summary, the present study showed for the first time that the Portuguese common bean genetic resources have high diversity in the phenolic composition and associated antioxidant activity, demonstrating high potential for quality improvement through cross/selection-breeding. Additionally, the identification of bioactive phenolic compounds (e.g. procyanidins), in soaking water and soaked coats, with recognized value as prebiotic compounds, in feed and food industry, and health promoting agents, especially in communities with high prevalence of NCD (e.g. obesity or hypercholesterolemia) supports an active discussion on food preparation techniques, such as discarding the soaking water or removing the beans' coats, for the sake of preserving the health properties of beans-based food products. In order to provide complementary information about the relevance of common beans' phenolic compounds in human diet, more work on common beans phenolic compounds' bioaccessibility and bioavailability should be performed.

## Supplementary Materials

The following are available online at http://www.mdpi.com/2304-8158/8/8/296/s1, Table S1: Average values ± Standard Deviation of Total Phenolic Content (TPC), mg GAE/g DW, Total Flavonoid Content (TFC), mg CE/g DW, and Total Proanthocyanidin Content (TPAC), mg CE/g DW, determined in common bean whole flour (WF) and soaking water (SW). ORAC, µmol TEAC/g DW was determined in whole flour (WF). TPC(SW)/ TPC(WF), TFC(SW)/ TFC(WF), TPAC(SW)/ TPAC(WF) are, respectively,

the percentage of TPC, TFC and TPAC released into the soaking water, Table S2: Average values ± Standard Deviation of Total Phenolic Content (TPC), mg GAE/g DW, Total Flavonoid Content (TFC), mg CE/g DW, and Total Proanthocyanidin Content (TPAC), mg CE/g DW, determined in soaked cotyledons (Cot) and coats (C), Table S3: Contribution of the different parameters analyzed in the two first principal components (* loadings ≥ |0.400|), Table S4: Average ± standard deviation values of the parameters analyzed in the different fractions, considering the clusters of common bean varieties defined by multivariate analysis. Different letters indicate values significantly different between clusters (Scheffé's test, $p < 0.05$), Table S5: Discriminant analysis to evaluate the clustering solution, Table S6: Molecular structure of the compounds identified

## Author Contributions

Conceptualization, E.M., M.E.F., M.C.V.P., M.R.B.; funding acquisition, E.M., M.E.F., M.C.V.P., M.R.B.; investigation, E.M., M.E.F., M.C.V.P., M.R.B.; methodology, E.M., S.T.L., B.C., A.T.S.; project administration, M.R.B.; M.C.V.P.; resources, P.M.M., M.M.V., C.B., M.C.V.P., M.R.B.; software, E.M., R.G.; writing – original draft preparation, E.M.; writing—review and editing, E.M., M.C.V.P., M.R.B.

## Funding

FP7-EU project Strategies for Organic and Low-input Integrated Breeding and Management (SOLIBAM); FCT, Portugal for E.M. PhD fellowship (SFRH/BD/89287/2012) and M.C.V.P FCT Investigator Program

Development Grant (IF/01337/2014) and to R&D unit, UID/Multi/04551/2013 (GreenIT).

## Acknowledgments

The authors acknowledge the Research Unit of Biotechnology and Genetic Resources germplasm bank, INIAV, Oeiras, Portugal, for providing common bean samples, and UniMS-Mass Spectrometry Unit, ITQB/IBET, Oeiras, Portugal, for the UPLC-TripleTOF-MS analysis.

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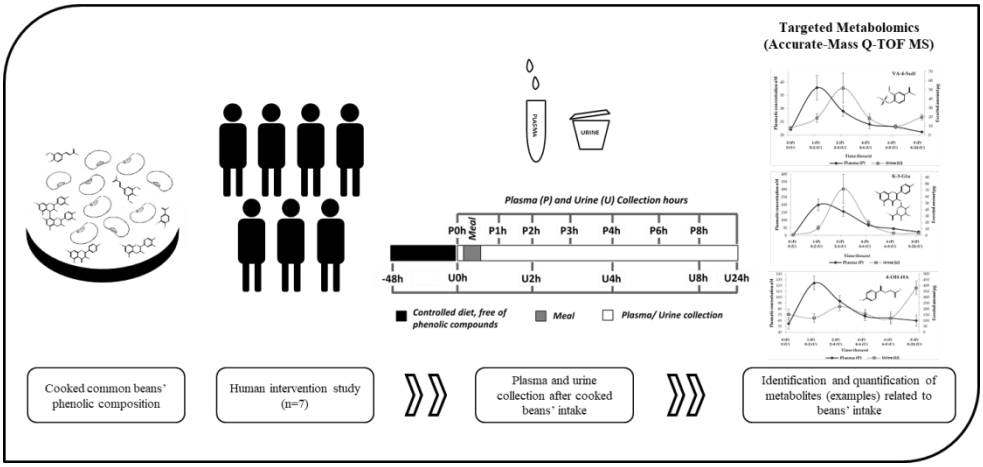
# Chapter V




This Chapter was submitted and accepted by British Journal of Nutrition as,

**Mecha, E.**, Feliciano, R., Rodriguez-Mateos, A., Silva, S., Figueira, M., Vaz Patto, M., & Bronze, M. Human bioavailability of phenolic compounds found in common beans: the use of high-resolution MS to evaluate inter-individual variability. *Br J Nutr* **2020**, 123(3), 273-292; DOI:10.1017/S0007114519002836.

In this Chapter, Elsa Mecha participated in the experimental work, data analysis, manuscript drafting and final manuscript writing.

# Human bioavailability of phenolic compounds found in common beans: the use of high resolution mass spectrometry to evaluate inter individual variability

## Abstract

Although common beans (*Phaseolus vulgaris* L.) are consumed worldwide, studies on the metabolic fate of phenolic compounds from common beans are still very scarce. The present work aimed to study the bioavailability of phenolic compounds in human plasma and urine, after acute consumption of a single meal of cooked common beans. Blood and urine of seven volunteers were collected before (0 h) and at different time points (1, 2, 4, 6 and 8 h for plasma and 0–2, 2–4, 4–6, 6–8 and 8–24 h for urine) after beans' intake. Ultra-high performance liquid chromatography-quadrupole-time of flight-MS (UPLC-Q-TOF-MS) was used for quantification. After beans' intake, 405 ± 3 g containing 188 mg of phenolic compounds (expressed as gallic acid equivalents), there was a significant increase ($p < 0.05$) in the plasma concentration of six metabolites and in the urinary excretion of eleven metabolites. After 1 h post-consumption, metabolites, such as kaempferol-3-O-glucuronide, showed a significant increase in plasma concentration, suggesting kaempferol's glucuronidation in the upper gastrointestinal tract. More than 50% of the total amount of metabolites, such as 4-methylcatechol-O-sulphate and dihydrocaffeic acid-3-O-sulphate, were excreted after 8 h post-consumption, indicating colonic bacterial metabolism of the phenolic compounds. Partial least square-discriminant analysis models clearly showed clusters of metabolites, which contributed to extend the list of compounds, related to cooked common

beans' human intake at different time points and showed the human inter-individual variability in plasma concentration as well as in urinary excreted metabolites, after cooked common beans' intake.

**Key words:** common beans; phenolic compounds; metabolites; plasma; urine; human variability

## 1. Introduction

Approximately 4 billion people in the world depend primarily on a plant-based diet to obtain essential nutrients [1]. Since the 1990s, a growing body of evidence has emerged regarding the health benefits of plant phenolic compounds consumed on a regular basis [2]. Legumes, such as common beans (*Phaseolus vulgaris* L.), have a long shelf life and are a cheap, rich source of macro- (e.g. protein), micronutrients (e.g. folate, Fe, Zn, B vitamin complex) and phytochemical compounds (e.g. phenolic compounds) [3]. The phenolic composition of common beans depends greatly on the genotype, as well as on the environmental conditions and processing techniques applied before consumption [4]. Several epidemiological studies [5], such as the Japanese Collaborative Cohort study [6] and the First National Health and Nutrition Examination Survey Epidemiologic Follow-up Study [7], have reported an inverse association between legume intake and CVD, particularly when consumed more than four times per week. Extracts obtained from white kidney beans have also been proposed as promoters of body weight reduction, and a health claim has been submitted to the European Food Safety Authority panel for consideration. The lack of *in vivo* and human intervention studies,

supporting an explanation for the inhibition of α-amylase activity, led to the rejection of the claimed effect in 2014 [8].

Studies on the bioavailability provide useful information to understand which metabolites from phenolic compounds can be responsible for the bioactivity in the human body. Briefly, after ingestion, phenolic compounds are highly modified, by phase I and II metabolising enzymes, to increase water solubility [9] and only a minor amount of the native compounds appear on systemic circulation. Phase I reactions include oxidation, reduction, hydroxylation, decarboxylation, hydrolysis or a combination of such reactions, catabolised by cytochrome P450 superfamily, as well as by intestinal esterases [10]. Phase II reactions include conjugation reactions with sulphate, glucuronide, methyl groups and amino acids through the action of enzymes such as sulfotransferase, uridine diphosphate-glucosyltransferase, β-glucuronidase, catechol-O-methyltransferase, cholylCoA synthetase and N-acetyltransferase [10]. The unmodified compounds that reach colon, mostly those attached to fiber, are metabolised by the gut microbiota, remaining on bloodstream during a longer period of time, 48 h [11, 12]. Human bioavailability studies, related with phenolic compounds in food, have been performed so far with food items such as olive oil [13, 14], coffee [15, 16], tea [15, 16], chocolate [17], wine [18], berries [19, 20], apple [21], orange [22], almonds [23], wholegrains [11, 24], potato [25], soyabeans [26] among others, but for common beans, which represent one of the most important food items, on a daily basis diet, especially in developing countries, there is a scarcity of studies about phenolic compounds and their metabolites in human intervention studies. To date, only one study has investigated the

bioavailability of phenolic compounds derived from common beans in humans, focusing exclusively in the metabolic fate of the flavonol, kaempferol [27]. The human intervention study described herein aimed to report the metabolism of phenolic compounds after cooked common beans' consumption, in plasma and urine samples, using a targeted metabolomics approach with authentic standards. An assessment of the inter-individual variability in bioavailability is also presented.

## 2. Material and methods

### 2.1. Chemicals

Folin-Ciocalteu's phenol reagent and sodium carbonate (99%) were purchased from Sigma-Aldrich. Methanol (99.9%) was purchased from Carlo Erba Reagents. Acetonitrile for LC-MS Ultra Chromasolv was purchased from Honeywell Riedel-de HaënTM. Milli-Q® water (18.2 MΩ.cm) was obtained in a Millipore – Direct Q3 UV System equipment. L-(+) Ascorbic acid *pro analysis* was purchased from Merck. Pyrogallol-1-O-sulphate, pyrogallol-2-O-sulphate, 1-methylpyrogallol-O-sulphate, 2-methylpyrogallol-O-sulphate, 4-methylcatechol-O-sulphate, 4-methylgallic-3-O-sulphate, catechol-O-sulphate and vanillic acid-4-O-sulphate were kindly provided, and their synthesis has been described elsewhere [12]. Caffeic acid-4-O-β-D-glucuronide, dihydrocaffeic acid-3-O-sulphate, dihydrocaffeic acid-3-O-β-D-glucuronide, caffeic acid-3-O-β-D-glucuronide, dihydroferulic acid-4-O-sulphate, dihydroferulic acid-4-O-β-D-glucuronide, ferulic acid-4-O-sulphate, ferulic acid-4-O-glucuronide, isoferulic acid-3-O-β-D-glucuronide, dihydroisoferulic acid-3-O-sulphate and dihydroisoferulic acid-3-O-β-D-glucuronide were obtained from Toronto Research

Chemicals. Kaempferol-3-O-glucuronide and quercetin-3-O-glucuronide were obtained from Extrasynthese. 3-Hydroxyhippuric acid and 4-hydroxyhippuric acid were purchased from Enamine. Gallic acid, protocatechuic acid, p-hydroxybenzoic acid, sinapic acid, catechin, epicatechin, hippuric acid, o-hydroxybenzoic acid, m-hydroxybenzoic acid, caffeic acid, p-coumaric acid, o-coumaric acid, m-coumaric acid, t-ferulic acid, kaempferol, quercetin and p-hydroxybenzaldehyde were obtained from Sigma-Aldrich Co. Formic acid (98%), orthophosphoric acid (≥85%) and acetic acid (100%) were obtained from Carl Roth, and OASIS HLB Elution plates (2 mg sorbent per well, 30 µm particle size) were from Waters.

2.2. Plant material

Three Portuguese common bean traditional varieties, Patalar, Tarrestre and Moleiro, were compared in terms of phenolic content. These varieties were collected directly from local farmers in the centre-northern region of Portugal: Tarrestre from Arcos de Valdevez, Viana do Castelo; Moleiro from Celorico de Basto, Braga, and Patalar from Sintra, Lisboa, and kept in cold storage at the Germplasm bank located in the Research Unit of Biotechnology and Genetic Resources, INIAV, Oeiras, Portugal (PRT 005). The three varieties were multiplied before analysis at ESAC, using traditional farming techniques. Morphologically, Patalar and Tarrestre dry seeds have a kidney shape with white and brown coat colours, respectively. Moleiro dry seeds are characterised by a cuboid shape and coats with a light brown colour without pattern. Based on the total phenolic content (TPC) determined in the extracts of raw common beans from these

three different varieties, it was possible to select the one with the highest phenolic content.

2.3. Preparation of raw and cooked common beans extracts

In order to prepare extracts of raw beans, part of the raw seeds was grounded in a Falling nº 3100 miller (Perten) to a particle size of 0.8 mm. The other part of the raw seeds was cooked in a pressure cooker, only with water and salt, for 50 min (1 g of dried beans: 4 mL of water: 12 mg of salt). Extracts of raw and cooked beans were prepared according to Lin et al. [28], with slight modifications. Briefly, 1 g of dry whole seed flour and 2.5 g of cooked beans were extracted with 20 mL of methanol: water (60:40, v/v) solution, followed by sonication for 60 min. The mixture was centrifuged at 420× g for 15 min. The final volume was adjusted to 20 mL, using volumetric flasks. Final extracts were filtered through a 0.22 µm 13 mm CA syringe filter (GE WhatmanTM). The extracts prepared in triplicate were kept on glass flasks at –20 °C, until analysis.

2.4. Total phenolic content of raw and cooked common beans extracts

For TPC measurement, the method described by Stamatakis et al. [29] was applied, with some modifications. Briefly, the diluted sample extract (3.5 mL) was mixed to Folin–Ciocalteu's reagent (0.100 mL) and, after 3 min, 0.400 mL of sodium carbonate solution (35%, w/v) was added to the mixture. Absorbance was measured at 725 nm, after 1 h, against water, in a Spectrophotometer DU-70 (Beckman®). Gallic acid was used as the external standard. A blank of water was also prepared in the same conditions. All the measurements were performed in a Spectrophotometer

DU-70 (Beckman®), and the final results were expressed as mg gallic acid equivalents (GAE) per g of seed's dry weight (DW), considering the moisture content of the raw seeds. Moisture content (%) was determined by a Near-IR analyser (MPA; Bruker) with flour calibrations for grain legumes [30].

2.5. Human study design

A human intervention study was designed in order to evaluate the metabolism of phenolic compounds derived from common beans in plasma and urine, after cooked common beans' intake. Seven healthy volunteers (six female and one male), with age between 24 and 40 years old and BMI of 19.9–34.4 kg/m$^2$ were recruited. The sample size was established based on previous bioavailability studies [31, 32] and justified by the nature of the study (accomplishment of a restrictive diet, free of phenolic compounds during 48 h, blood and urine collection). The exclusion criteria for volunteers included the presence of diagnosed disease, use of medication and/or dietary supplements. The procedures were conducted according to the Declaration of Helsinki and the study protocol approved by the Ethics Committee for Clinical Experimentation of the Pharmacy Faculty, University of Lisbon, identification number 03/CEECFFUL/2016. All the volunteers were informed about the aim of the study, and a written informed consent was signed before the study. Volunteers followed a low phenolic diet, 48 h before the intervention day and during the 24 h post-bean consumption, online **Supplementary Tables S1A** and **S1B**. A list of allowed and not allowed food items, online Supplementary **Table S1C**, was supplied to promote volunteers compliance with dietary restrictions [24], particularly on

food items such as coffee, chocolate, fruits, vegetables, wine, beer, juice, olive oil, nuts, tea and whole-grain products, in order to reduce the presence of phenolic compounds in blood and urine and to ensure the provenance of the phenolic compounds from cooked common beans. After an overnight fasting period, venous blood and urine were collected, at baseline level, before ingestion of cooked common beans, and volunteers were asked to report their food intake in the previous period of 48 h in order to check their compliance with the recommended diet. Venous blood was collected in EDTA containing vacutainers, 1, 2, 4, 6 and 8 h after consumption of a single meal of cooked common beans (404.7 ± 2.7 g corresponding to 166.1 ± 1.1 g of raw beans), and urine was collected in different time points (0–2, 2–4, 4–6, 6–8 and 8–24 h). The amount of cooked beans represented a full plate of beans, and volunteers were allowed to add salt according to their taste.

A summary of the study design is shown in **Figure 1**.

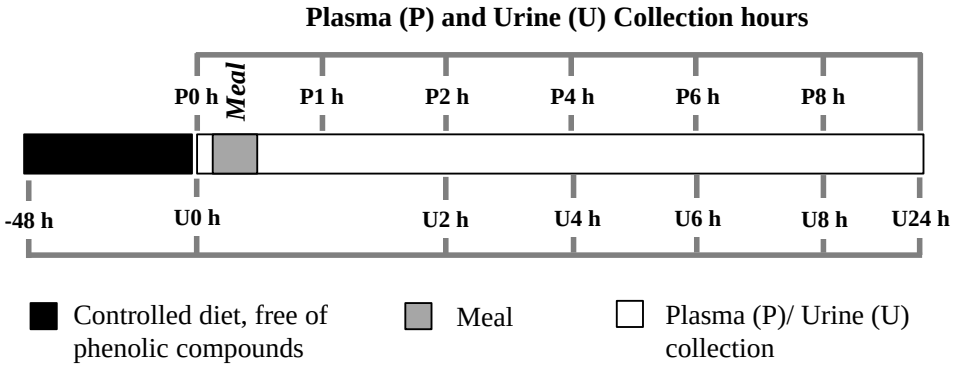



**Fig. 1.** Study design scheme. After a diet free of phenolic compounds for 48 h, plasma (P) and urine (U) were collected at different time points, after a single meal of cooked common beans (404.7 ± 2.7 g).

In order to quantify the amount of excreted phenolic compounds and corresponding metabolites in urine, the total volume of excreted urine

was measured, for each volunteer, at different time points. For one of the seven volunteers, there was no urine collection, in time points 0–2 and 6–8 h. L-(+) Ascorbic acid was added to the urine samples (1 g/ 2 litres of urine) [33]. The venous blood was centrifuged, immediately after collection, at 657× g for 20 min at 4 °C. The supernatant (plasma) was collected, and both plasma and urine samples were stored at –20 °C and analysed according to Feliciano et al. [19].

### 2.6. UPLC-Q-TOF-MS analysis

The common beans' extracts of the selected variety with the highest phenolic content, as well as the human plasma and urine, were analysed by ultra-high performance liquid chromatography-quadrupole-time of flight-MS (UPLC-Q-TOF-MS), using an Agilent 6550 iFunnel Accurate-Mass Q-TOF MS (Agilent), in order to identify and quantify phenolic compounds and their metabolites, through an electrospray interface with Jet Stream technology, after separation on a 1290 Infinity UPLC system (Agilent) in the same conditions as the ones described by Feliciano et al. [19]. A Zorbax Eclipse Plus RRHD column 2.1 × 50 mm, 1.8 mm with a compatible Eclipse Plus guard column 2.1 × 5 mm, 1.8 mm (Agilent) was used for sample analysis. The elution program included 0.1% HCOOH (eluent A) and acetonitrile with 0.1% HCOOH (eluent B), during 10 min, at a flow rate of 0.4 mL/min. Eluent B increased from 1 to 10% during the first 5 min, to 25% at 8 min and to 99% at 9.1 min. This percentage of eluent B stayed constant at 99% during the remaining 0.9 min of analysis. The gradient returned to 1% for 2 min to equilibrate the column. Samples were analysed in the negative mode as described by Feliciano et al. [19]. For data

processing, the MassHunter Workstation Quantitative Analysis software, version B.06.00 (Agilent), was used.

2.7. Identification and quantification of metabolites in common beans' extracts.

Identification of compounds was performed by comparison with the retention time and *m/z* of the authentic standards analysed on the same conditions (gallic acid, protocatechuic acid, p-hydroxybenzoic acid, catechin, epicatechin, p-coumaric acid, t-ferulic acid, sinapic acid, quercetin and kaempferol). Variation of the retention time was calculated as the difference between the average value determined in the standard compound and the average value determined in the sample. The mass measurement error was calculated, to determine the accuracy of the detected *m/z*, following the equation:

$$Error = \frac{Predicted\ m/z - Observed\ m/z}{Predicted\ m/z} \times 1000\,000$$

and the results expressed as ppm (parts per million) (online **Supplementary Table S2**). A retention time variation of 0.2 min and/or a mass error measurement lower than 5 ppm were adopted as quality criteria to identify the metabolites [34]. In order to quantify the phenolic compounds in the extract of the Portuguese common bean variety, calibration curves of the authentic standards were prepared. With exception of the flavonoids, quercetin and kaempferol, prepared in aqueous methanol (50%, v/v), all the standards were prepared in Milli-Q® water. Final results were expressed as μg/g of raw beans in DW. Procyanidins B1 and B2 were quantified using the catechin standard and expressing the final results as

µg of catechin equivalents per g of raw beans in DW. The lowest concentration of the selected range (8–9916 nM), in the different calibration curves, was higher than the limit of quantification defined as a S:N ratio of 10.

2.8. Identification and quantification of metabolites in plasma and urine

Plasma and urine were prepared for analysis as described by Feliciano et al. [19] with slight modifications. Briefly, plasma and urine (1000 µL) were thawed in an ice bath and centrifuged at 15 000× g for 15 min at 4 °C. The supernatant (353 µL) of plasma or urine was diluted with 4% phosphoric acid (353 µL). Each sample was loaded (600 µL) on a 96-well microelution solid phase extraction plate, washed with water (200 µL) and 0.2% acetic acid (200 µL). After eluting with methanol (60 µL), the ninety-six-well collection plate was immediately covered with a polyolefin tape to avoid evaporation and put in the UPLC autosampler, using the UPLC-Q-TOF-MS equipment for the analysis, as described in the UPLC-Q-TOF-MS analysis section. A total of forty compounds (pyrogallol-1-O-sulphate, pyrogallol-2-O-sulphate, 2-methylpyrogallol-O-sulphate, 1-methylpyrogallol-O-sulphate, gallic acid, protocatechuic acid, 4-methylgallic acid-3-O-sulphate, vanillic acid-4-O-sulphate, p-hydroxybenzoic acid, m-hydroxybenzoic acid, o-hydroxybenzoic acid, catechol-O-sulphate, 4-methylcatechol-O-sulphate, 4-hydroxyhippuric acid, 3-hydroxyhippuric acid, hippuric acid, caffeic acid-4-O-β-D-glucuronide, ferulic acid-4-O-glucuronide, dihydrocaffeic acid-3-O-sulphate, dihydrocaffeic acid-3-O-β-D-glucuronide, caffeic acid-3-O-β-D-glucuronide, caffeic acid, dihydroferulic acid-4-O-β-D-glucuronide, dihydroferulic acid-4-O-sulphate, ferulic acid-4-

O-sulphate, isoferulic acid-3-O-β-D-glucuronide, dihydroisoferulic acid-3-O-sulphate, dihydroisoferulic acid-3-O-β-D-glucuronide, p-coumaric acid, ferulic acid, sinapic acid, m-coumaric acid, o-coumaric acid, p-hydroxybenzaldehyde, catechin, epicatechin, quercetin-3-O-glucuronide, kaempferol-3-O-glucuronide, quercetin, kaempferol) were investigated. For quantification purposes, in plasma and urine, authentic standards prepared in Milli-Q® water were used for the calibration curves. The considered concentration range was above the validated method quantification limit, in plasma and urine, determined by Feliciano et al. [35]. The phenolic compounds or their corresponding metabolites with a concentration below the validated method quantification limit, described by Feliciano et al. [35], in at least one volunteer, were not considered for data analysis.

2.9. Data analysis

In plasma samples, the AUC of phenolic compounds and their metabolites was calculated using the PK Solver tool of Microsoft Excel (Microsoft). To determine the amount, in µg, of excreted metabolites, the volume of urine excreted, at each time point, was measured. The urinary recovery (%) was determined as described by Feliciano et al. [36]. Briefly, it was calculated as the ratio between the total amount of excreted metabolites and the TPC consumed in the intervention study. The human inter-individual variability, described as the CV in percentage, was determined using the AUC of each metabolite studied in the plasma and also for the total excreted amount of each metabolite, in urine [37]. Multivariate analysis was applied to describe inter-individual variability and select the most relevant metabolites associated with sample grouping.

Using IBM® SPSS® Statistics, version 22, software, the normality of variables distribution was assessed by the Shapiro–Wilk test (n < 50) at a significance level of 1%. To achieve normality, in some variables, different transformation approaches, such as logarithmic, inverse, squared root or two-step transformation [38], were tested and for principal component analysis (PCA), only the metabolites with communalities higher than 0.5 were considered. PCA in articulation to cluster analysis was used for exploratory analysis of the inter-individual variability. After retaining the PCA scores of the PCA components with higher contribution (>50% of the total variance), different cluster solutions (K = 2, K = 3 and K = 4), obtained by K-means cluster analysis, were applied as Y responses in partial least square regression-discriminant analysis (PLS-DA). PLS-DA amplified group separation allowing the prediction of the clusters' membership. The Unscrambler® X 10.4.1, Camo Analytics Software, was used to select the best model of PLS-DA after full cross-validation. The statistical parameters, correlation coefficient of multiple determination for Y–$R^2$ (Y), correlation coefficient of multiple determination for X–$R^2$ (X), root-mean-square error of calibration and validation – RMSEC and RMSECV, respectively, and cross-validated correlation coefficient – $Q^2$, were assessed to control the quality of the model. The last parameter, $Q^2$, was extrapolated from the equations proposed elsewhere [39], using the following equation:

$$Q^2 = 1 - \frac{RMSECV^2 \times M}{\left[-\frac{RMSEC^2 \times (M-K-1)}{R^2(Y)-1}\right]}$$

Where, RMSECV is the root-mean-square error of cross-validation; RMSEC is the root-mean-square error of calibration; M is the number of samples; K is the number of descriptors; $R^2$ (Y) is the correlation coefficient

for Y. The PLS-DA model was built with all the samples and the most relevant metabolites were selected based on the correlation loadings and weighted regression coefficients. To compare the different clusters, the differences between plasma concentrations, as well as between the excreted amounts of metabolites, were defined by one-way ANOVA and the significantly different clusters identified by *post-hoc* Scheffé or Games–Howell tests (depending on the acceptance or rejection of homoscedasticity between clusters), at a significance level of 5%, using IBM® SPSS® Statistics, version 22, software.

## 3. Results

### 3.1. Selection and characterisation of the Portuguese common bean traditional variety used in the human intervention study

Based on the TPC analysis (**Table 1**), the three Portuguese traditional varieties (Patalar, Tarrestre and Moleiro) were compared.

**Table 1.** Comparison of the moisture content and the total phenolic content (TPC) determined in traditional Portuguese common bean varieties (Averages and standard deviations, SD)

| | | **TPC** (mg GAE/g raw beans dry weight) | |
|---|---|---|---|
| **Traditional variety** | **Moisture content (%)** | **Average** | **SD** |
| *Patalar* | 13.9 | 0.69[a] | 0.04 |
| *Tarrestre* | 14.6 | 2.55[b] | 0.16 |
| *Moleiro* | 13.5 | 3.36[c] | 0.11 |

GAE, gallic acid equivalents.

a,b,c. Different letters indicate significant differences between the average values obtained for each traditional variety ($p < 0.05$).

Moleiro stood out as the variety with the highest TPC value, 3.36 ± 0.11 mg GAE/g DW, $p < 0.05$, therefore, it was the one selected to be used

in the human intervention study. Both extracts obtained from raw and cooked Moleiro beans were analysed by UPLC-Q-TOF-MS, and using commercial standards, it was possible to identify and accurately quantify thirteen compounds (**Table 2** and **Table S2**) belonging to different classes: benzoic acids (gallic acid, protocatechuic acid and p-hydroxybenzoic acid), cinnamic acids (caffeic acid, p-coumaric acid, t-ferulic acid and sinapic acid) and flavonoids such as proanthocyanidins (procyanidins B1 and B2), flavan-3-ols (catechin and epicatechin) and flavonols (quercetin and kaempferol).

The most abundant phenolic compounds, in raw seeds, were catechin, procyanidin B1 and kaempferol representing, respectively, 30, 35 and 13% of the total quantified individual phenolic compounds. After the cooking process, the weight of common bean seeds increased to more than the double, due to the hydration process. In order to compare the phenolic composition of raw and cooked samples, results were expressed per g of raw seeds' DW. As shown in **Table 2**, after the cooking process, the TPC decreased about 61%. Results from quantification of individual phenolic compounds showed that compounds as gallic acid, protocatechuic acid, p-hydroxybenzoic acid, procyanidin B1, catechin, p-coumaric acid, caffeic acid, ferulic acid, sinapic acid, quercetin and kaempferol decreased significantly. In opposition, for epicatechin and procyanidin B2, there was a significant increase in the average contents. For the cooked beans, the most abundant phenolic compounds were catechin and epicatechin corresponding, respectively, to 36 and 22% of the total quantified individual phenolic compounds.

**Table 2.** Phenolic composition of raw and corresponding cooked common beans by ultra-high performance liquid chromatography-quadrupole-time-of-flight-MS (UPLC-Q-TOF-MS): comparison with the published data

| | Experimental data | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Raw | | Lietrature Data | | Cooked | | Literature Data | |
| | Average | SD | Average | SD | Average | SD | Average | SD |
| **Total phenolic content (mg GAE/ g of raw seed DW)** | 3.36[a] | 0.11 | 1.88-3.44 [(40)] | 0.16-0.57 | 1.30[b] | 0.03 | 1.31-2.23[(40)] | 0.16-0.17 |
| **Class** | | | | | | | | |
| **Benzoic acids (µg/g of raw seed DW)** | | | | | | | | |
| **Gallic acid** | 0.19[a] | 0.01 | 83.17[(41)] | 7.4 | 0.07[b] | 0.01 | 38.16[(41)] | 2.6 |
| **Protocatechuic acid** | 5.15[a] | 0.19 | 16.08[(41)] | 3.7 | 3.32[b] | 0.20 | 8.94[(41)] | 0.8 |
| ***p*-hydroxybenzoic acid** | 0.60[a] | 0.00 | 16.59[(41)] | 0.9 | 0.12[b] | 0.02 | 4.66[(41)] | 0.5 |
| **Cinnamic acids (µg/g of raw seed DW)** | | | | | | | | |
| **Caffeic acid** | 0.26[a] | 0.02 | | | 0.21[b] | 0.02 | | |
| ***p*-Coumaric acid** | 0.68[a] | 0.05 | 5.4[(42)] | | 0.31[b] | 0.01 | 3.3[(42)] | |
| ***t*-Ferulic acid** | 4.80[a] | 0.63 | 24.0[(42)] | | 1.13[b] | 0.14 | 18.2[(42)] | |
| **Sinapic acid** | 3.38[a] | 0.17 | 264.0[(42)] | 18.1 | 0.96[b] | 0.07 | 60.70[(42)] | 1.7 |
| **Flavan-3-ols (µg catechin equivalents/g of raw seed DW)** | | | | | | | | |
| **Catechin** | 30.67[a] | 0.47 | | | 16.72[b] | 1.58 | | |
| **Epicatechin** | 2.21[a] | 0.12 | | | 9.92[b] | 0.81 | | |
| **Procyanidin B1** | 36.05[a] | 1.76 | | | 5.58[b] | 1.10 | | |
| **Procyanidin B2** | 3.02[a] | 0.04 | | | 4.98[b] | 0.66 | | |
| **Flavonols (µg/g of raw seed DW)** | | | | | | | | |
| **Quercetin** | 1.34[a] | 0.05 | 10.9[(42)] | | 0.26[b] | 0.03 | 6.5[(42)] | |
| **Kaempferol** | 13.54[a] | 0.55 | 52.3[(42)] | | 2.30[b] | 0.27 | 27.2[(42)] | |

GAE, gallic acid equivalentes; DW, dry weight.

a,b. Average values in a row with unlike superscripts letters are significantly different ($p < 0.05$).

## 3.2. Identification and quantification of metabolites in plasma and urine by UPLC-Q-TOF-MS

Compounds in plasma and in urine were identified, unequivocally in all volunteers, by comparison with the retention time and *m/z* values of the available standards. From the twenty four metabolites identified in plasma (**Table S3**), seventeen were quantified (**Table 3** and **Table S4**), corresponding to five sulphate conjugates, four glucuronide conjugates and three N-containing conjugates. Four compounds were detected as aglycones and one as an aldehyde. Based on the total AUC, **Table 3**, determined for the different quantified compounds in plasma, after 8 h of a single meal of cooked common beans, the most abundant class of phenolic

compounds' metabolites were hippuric acids (71%) followed by catechols (11%), benzoic acids (7%), cinnamic acids (5%), flavonols (3%), benzaldehydes (1%) and pyrogallols (1%). In plasma (**Table 3**), vanillic acid-4-O-sulphate, 4-hydroxyhippuric acid, ferulic acid-4-O-glucuronide, ferulic acid-4-O-sulphate and kaempferol-3-O-glucuronide showed a significant increase 1 h after common beans' consumption and corresponded to 10% of the quantified metabolites. For the other compounds, included in pyrogallols, benzoic acids, benzaldehydes, catechols and hippuric acids classes, the average plasma concentrations were not significantly different at the different collection time points. From the twenty-eight metabolites identified in urine (**Table S5**), twenty-four were quantified (**Table 4** and **Table S6**) in the urine of all volunteers. Nine of them were sulphate conjugates, six were glucuronide conjugates, six were detected in the aglycone form and three in the N-containing form. As shown in **Table 4**, the most abundant class of phenolic compounds' metabolites in urine was hippuric acids (60%) followed by cinnamic acids (25%) and catechols (11%).

*3.2.1. Human inter-individual variability*

There was inter-individual variability in plasma concentration and urinary excretion of phenolic compounds derived from common beans and their metabolites (**Figures 2** and **3**). In plasma, **Table 3**, a variation of 24% was obtained for the total AUC. Considering only the metabolites with a significant plasma increase, the variation of AUC ranged from 13% in vanillic acid-4-O-sulphate to 46% in kaempferol-3-O-glucuronide. In urine, **Table 4**, it was possible to determine a variation of 30% in the volunteers'

excretion, ranging from 19%, in sinapic acid, to 73%, in kaempferol-3-O-glucuronide excretion. Although not specifically related to common beans' intake (without a significant increase in the urinary excretion during the study period), the compounds o-hydroxybenzoic acid, m-hydroxybenzoic acid and dihydroisoferulic acid-3-O-β-D-glucuronide showed the highest inter-individual variability with variations of 106, 101 and 100%, in urine, respectively. PCA was applied to explore the inter-individual variability among the seven volunteers (P1–P7) (**Figure 4**). The plasma samples named P1_1, P6_0 and P7_0 were excluded from the analysis since those samples were out of the rank in the two-step transformation approach. In plasma samples, the three first principal components (PC) explained 71.7% of the total variance with the first two accounting to more than 50% of the variability (57.5%). As suggested by the metabolites (MP) arrangement in the bi-dimensional space defined by the first two principal components (**Figure 4(a)**), the PCA analysis indicated a clear separation between the plasma samples collected in early collection times (1 and 2 h after common beans intake), and the ones collected lately at 4, 6 and 8 h after common beans' intake (**Figure 4(b)**). The selected metabolites, in the PLS-DA analysis, explained 62% of the clusters' variability in the regression model and reinforced the PCA observations (**Figure 5**). As shown in the correlation loadings plot, **Figure 5(a)**, for the first two factors, the variables positioned in the 50–100% explained circle (defined as the space delimited by the outer and inner circumferences), vanillic acid-4-O-sulphate (MP2), located near the cluster 3, and ferulic acid-4-O-sulphate, (MP12), located near the cluster 1, were the main metabolites responsible for samples' classification into two different groups, clusters 1 and 3. The

metabolites ferulic acid-4-O-glucuronide (MP10), 4-hydroxyhippuric acid (MP5) and kaempferol-3-O-glucuronide (MP11) near cluster 3 were also responsible for samples'separation, but with lower discrimination capacity. In the different volunteers, such metabolites were predominant at 1 and 2 h, after common beans' intake (**Figure 5(b)**). The remaining metabolites, hippuric acid (MP7), caffeic acid (MP8) and dihydroferulic acid-4-O-β-D-glucuronide (MP3) contributed mostly to the cluster 2, **Table 5**. m-Coumaric acid (MP9) explained the proximity of samples grouped in clusters 1 and 2. Although not responsible for clusters' separation, 3-hydroxyhippuric acid (MP6) allowed samples' dispersion along factor 2, contributing to the variance within clusters. Regarding the urinary excretion of metabolites, the three first principal components retained 72.9% of the variability and suggested samples' separation into two distinct groups (**Figure 6**). The PLS-DA model with some selected metabolites explained 66% of the samples variability into three different clusters (**Figure 7**). As shown in the correlation plot of the urinary excreted metabolites, **Figure 7(a)**, dihydrocaffeic acid-3-O-sulphate (MU16), 3-hydroxyhippuric acid (MU12), 4-methylcatechol-O-sulphate (MU10) and m-hydroxybenzoic acid (MU7) were responsible for samples' separation into clusters 1 and 2. Cluster 2, highlighted as the one with the highest content on such metabolites, **Table 6**, included the urine samples collected lately, at time point 8–24 h, after common beans' intake (**Figure 7(b)**). By opposition, cluster 1 was the one with the lowest content on such metabolites (**Table 6**). In the 50–100% explained circle, **Figure 7(a)**, the metabolites sinapic acid (MU23) and kaempferol-3-O-glucuronide (MU24) were mostly responsible for sample grouping in cluster 3, which included mainly the

urine samples of different volunteers collected at time points 2–4 and 4–6 h after common beans' intake (**Figure 7(b)**). The metabolites vanillic acid-4-O-sulphate (MU5) and o-hydroxybenzoic acid (MU8) were also related to cluster 3 but with lower discrimination ability. Despite the high concentration of those metabolites in cluster 3, the average value obtained for such cluster was not significantly different from cluster 2 ($p > 0.05$, **Table 6**). For caffeic acid-3-O-β-D-glucuronide (MU17), located in an intermediate position between cluster 2 and cluster 3, the urinary excretion was prolonged on time, from 2–4 to 8–24 h after common beans' intake, allowing clusters 2 and 3 approximation.

## 4. Discussion

As far as we know, this work is the most complete study that has been performed to evaluate the bioavailability of phenolic compounds from cooked common beans, using UPLC-Q-TOF-MS.

Based on the phenolic content of the three Portuguese studied varieties, the Portuguese common bean variety Moleiro was chosen as the variety with the highest TPC (**Table 1**). Despite of the morphological differences in the seed colour, the TPC of Moleiro raw beans, 3.36 ± 0.11 mg GAE/g of raw seed DW (2.91 ± 0.09 mg GAE/g of raw seed fresh weight (FW)), characterised by light brown seeds, was within the range of values described by Heimler et al. [43] for light green, white and yellow varieties (1.17–4.40 mg GAE/g of raw seed FW) and by Silva et al. [40] for pinto varieties, characterised by cream coloured seeds with speckles (**Table 2**).

The cooking process was responsible for changes in the beans' accessible compounds. A reduction in the TPC (–61%) was detected after cooking, and it was comparable to the TPC value described for the pinto cooked beans [40], representing 63–77% of the TPC determined in the raw seeds of pinto beans [41].

Most of the studies on beans are still focused on the TPC and only a few ones [41, 42, 44–49] on the individual phenolic compounds. In our work, the data obtained using UPLC-Q-TOF-MS showed that Moleiro raw beans represented a source of catechin, 30.67 ± 0.47 μg/g of raw seed DW (26.54 ± 0.41 μg/g of raw seed FW), with higher content than the one described by Owino et al. [46] for a pink variety (13.50 ± 0.50 μg/g of raw seed FW), and lower than the value described by de Pascual-Teresa et al. [47] for pinto beans (50.7 μg/g of raw seed FW). It also represented a source of procyanidin B1, 36.05 ± 1.76 μg catechin equivalents/g of raw seed DW (31.20 ± 1.52 μg catechin equivalents/g of raw seed FW), with a lower content than the pinto beans described by Aguilera et al. [45], 41.20 ± 1.85 μg/g (without specification of DW or FW). Furthermore, Moleiro beans were a source of kaempferol, 13.54 ± 0.55 μg/g of raw bean DW (11.72 ± 0.47 μg/g of raw bean FW), with a similar content to the black beans described by Romani et al. [49], 18 ± 0.23 μg/g of raw bean FW, but with a considerably lower content than the average value determined by Diaz-Batalla et al. [42] for Mexican cream-red, black, grey, cream, brown and black-brown varieties (**Table 2**).

**Table 3.** Concentration (nM) of phenolic compounds and their metabolites in **plasma**, before (0 h) and after (1, 2, 4, 6, 8 h) beans' consumption, considering n=7

| Compounds | Plasma Concentration (nM) | | | | | | | | | | | | | | | | | | AUC$_t$ (nM x h) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 h | | | 1 h | | | 2 h | | | 4 h | | | 6 h | | | 8 h | | | | | |
| | Average | SEM | CV % | Average | SEM | CV % | Average | SEM | CV % | Average | SEM | CV % | Average | SEM | CV % | Average | SEM | CV % | Average | SEM | CV % |
| **Pyrogallols** | | | | | | | | | | | | | | | | | | | | | |
| **1-Methylpyrogallol-*O*-sulfate** | 24.1$^{a}$ | 1.3 | 14 | 23.6$^{a}$ | 0.4 | 5 | 23.2$^{a}$ | 0.5 | 6 | 23.0$^{a}$ | 0.2 | 2 | 23.4$^{a}$ | 0.4 | 5 | 23.4$^{a}$ | 0.4 | 4 | 186.6 | 2.8 | 4 |
| **Vanillic acid-4-*O*-sulfate** | 22.1$^{a}$ | 0.5 | 6 | 37.9$^{c}$ | 4.7 | 33 | 29.0$^{b,c}$ | 1.9 | 18 | 24.0$^{a,b}$ | 1.6 | 18 | 23.1$^{a,b}$ | 0.8 | 9 | 21.1$^{a}$ | 0.3 | 3 | 207.8 | 10.4 | 13 |
| ***o*-Hydroxybenzoic acid** | 130.1$^{a}$ | 35.0 | 71 | 205.1$^{a}$ | 54.1 | 70 | 197.8$^{a}$ | 57.0 | 76 | 164.2$^{a}$ | 43.5 | 70 | 145.4$^{a}$ | 47.2 | 86 | 115.5$^{a}$ | 32.1 | 74 | 1301.6 | 366.6 | 75 |
| **Total** | 152.2$^{a}$ | 35.0 | 61 | 242.9$^{a}$ | 58.4 | 64 | 226.8$^{a}$ | 58.7 | 68 | 188.3$^{a}$ | 45 | 63 | 168.5$^{a}$ | 47.2 | 74 | 136.6$^{a}$ | 32.2 | 62 | 1509.4 | 376.3 | 66 |
| **Benzaldehydes** | | | | | | | | | | | | | | | | | | | | | |
| ***p*-Hydroxybenzaldehyde** | 20.9$^{a}$ | 2.3 | 29 | 32.2$^{a}$ | 2.5 | 20 | 33.7$^{a}$ | 3.1 | 24 | 35.1$^{a}$ | 6.2 | 47 | 34.1 $^{a}$ | 5.1 | 40 | 40.4$^{a}$ | 4.3 | 28 | 272.1 | 26.1 | 25 |
| **Catechol-*O*-sulfate** | 48.9$^{a}$ | 11.3 | 61 | 50.6$^{a,b}$ | 7.7 | 41 | 38.9$^{a}$ | 4.5 | 31 | 43.1$^{a}$ | 5.3 | 32 | 77.6$^{a,b}$ | 10.7 | 36 | 95.0$^{b}$ | 16.0 | 45 | 469.9 | 49.1 | 28 |
| **4-Methylcatechol-*O*-sulfate** | 217.5$^{a}$ | 75.9 | 92 | 203.1$^{a}$ | 44.4 | 58 | 160.7$^{a}$ | 35.0 | 58 | 193.9$^{a}$ | 76.1 | 104 | 334.7$^{a}$ | 203.1 | 161 | 278.7$^{a}$ | 106.3 | 101 | 1888.8 | 688.7 | 96 |
| **Total** | 266.4$^{a}$ | 80.8 | 80 | 253.7$^{a}$ | 43.1 | 45 | 199.6$^{a}$ | 36.3 | 48 | 237.0$^{a}$ | 76.8 | 86 | 412.3$^{a}$ | 210.0 | 135 | 373.6$^{a}$ | 109.3 | 77 | 2358.6 | 695.0 | 78 |
| **Hippuric acids** | | | | | | | | | | | | | | | | | | | | | |
| **4-Hydroxyhippuric acid** | 59.8$^{a}$ | 9.5 | 42 | 129.2$^{b}$ | 12.0 | 25 | 98.4$^{a,b}$ | 9.6 | 26 | 72.4$^{a}$ | 7.9 | 29 | 69.2$^{a}$ | 10.2 | 39.0 | 64.8$^{a}$ | 9.6 | 39 | 654.8 | 70.1 | 28 |
| **3-Hydroxyhippuric acid** | 123.5$^{a}$ | 30.3 | 65 | 119.3$^{a}$ | 22.2 | 49 | 103.5$^{a}$ | 19.7 | 50 | 121.7$^{a}$ | 21.6 | 47 | 160.8$^{a}$ | 32.2 | 53 | 154.2$^{a}$ | 27.8 | 48 | 1055.5 | 183.8 | 46 |
| **Hippuric acid** | 1797.9$^{a}$ | 255.1 | 38 | 1507.2$^{a}$ | 189.5 | 33 | 1254.6$^{a}$ | 175.8 | 37 | 1818.2$^{a}$ | 257.9 | 38 | 1859.2$^{a}$ | 260.4 | 37 | 1741.6$^{a}$ | 257.1 | 39 | 13384.3 | 1776.1 | 35 |
| **Total** | 1981.1$^{a}$ | 281.3 | 38 | 1755.7$^{a}$ | 202.1 | 30 | 1456.4$^{a}$ | 187.2 | 34 | 2012.3$^{a}$ | 272.3 | 36 | 2089.3$^{a}$ | 284.1 | 36 | 1960.7$^{a}$ | 273.3 | 37 | 15094.6 | 1896.7 | 33 |
| **Cinnamic acids** | | | | | | | | | | | | | | | | | | | | | |
| **Ferulic acid-4-*O*-glucuronide** | 16.4$^{a}$ | 0.7 | 12 | 59.0$^{c}$ | 5.3 | 24 | 47.5$^{c}$ | 3.7 | 21 | 29.2$^{b}$ | 2.6 | 24 | 24.9$^{b}$ | 1.6 | 18 | 22.6$^{a,b}$ | 1.3 | 15 | 269.1 | 19.9 | 20 |
| **Caffeic acid** | 1.9$^{a}$ | 0.0 | 4 | 1.9$^{a}$ | 0.0 | 4 | 1.9$^{a}$ | 0.0 | 1 | 1.9$^{a}$ | 0.0 | 3 | 2.0$^{a}$ | 0.0 | 4 | 1.9$^{a}$ | 0.0 | 5 | 15.4 | 0.1 | 2 |
| **Dihydroferulic acid-4-*O*-*β*-D-glucuronide** | 44.7$^{a}$ | 4.9 | 29 | 43.2$^{a}$ | 3.9 | 24 | 41.0$^{a}$ | 2.6 | 17 | 45.5$^{a}$ | 3.2 | 19 | 48.1$^{a}$ | 4.4 | 24 | 43.4$^{a}$ | 2.1 | 13 | 357.6 | 25.0 | 19 |
| **Ferulic acid-4-*O*-sulfate** | 11.9$^{a}$ | 0.6 | 14 | 92.6$^{c}$ | 19.8 | 57 | 41.1$^{c}$ | 5.9 | 38 | 20.5$^{b}$ | 2.5 | 32 | 19.9$^{b}$ | 1.6 | 21 | 17.3$^{b}$ | 1.7 | 25 | 258.2 | 33.0 | 34 |
| **Dihydroisoferulic acid-3-*β*-*D*-glucuronide** | 4.0$^{a}$ | 0.2 | 12 | 3.9$^{a}$ | 0.1 | 10 | 3.8$^{a}$ | 0.2 | 12 | 4.1$^{a}$ | 0.2 | 15 | 4.0$^{a}$ | 0.3 | 19 | 4.0$^{a}$ | 0.2 | 16 | 31.8 | 1.2 | 10 |
| ***m*-Coumaric acid** | 22.2$^{a}$ | 0.7 | 8 | 18.8$^{a}$ | 0.7 | 9 | 19.8$^{a}$ | 0.6 | 8 | 20.2$^{a}$ | 1.3 | 18 | 21.7$^{a}$ | 1.1 | 13 | 22.5$^{a}$ | 1.4 | 17 | 165.9 | 6.4 | 10 |
| **Total** | 101.1$^{a}$ | 6.1 | 16 | 219.4$^{c}$ | 18.8 | 23 | 155.0$^{b,c}$ | 7.3 | 13 | 121.4$^{a,b}$ | 5.9 | 13 | 120.5$^{a,b}$ | 6.8 | 15 | 111.8$^{a}$ | 4.2 | 10 | 1098.0 | 50.4 | 12 |
| **Flavonols** | | | | | | | | | | | | | | | | | | | | | |
| **Kaempferol-3-*O*-glucuronide** | 3.9$^{a}$ | 0.0 | 3 | 198.7$^{e}$ | 37.7 | 50 | 156.0$^{d,e}$ | 31.0 | 53 | 67.3$^{c,d}$ | 10.4 | 41 | 43.6$^{b,c}$ | 7.8 | 47 | 22.2$^{b}$ | 4.0 | 48 | 678.6 | 119.0 | 46 |
| **Quercetin** | 2.6$^{a}$ | 0.0 | 2 | 2.7$^{a}$ | 0.0 | 3 | 2.6$^{a}$ | 0.0 | 3 | 2.6$^{a}$ | 0.0 | 3 | 2.7$^{a}$ | 0.0 | 4 | 2.7$^{a}$ | 0.0 | 4 | 21.2 | 0.2 | 3 |
| **Total** | 6.5$^{a}$ | 0.1 | 2 | 201.4$^{e}$ | 37.7 | 50 | 158.6$^{d,e}$ | 31.0 | 52 | 69.9$^{c,d}$ | 10.4 | 39 | 46.3$^{b,c}$ | 7.7 | 44 | 24.9$^{b}$ | 4.0 | 43 | 699.8 | 119.0 | 45 |
| **Sum of phenolic compounds' metabolites in plasma** | 2552.3$^{a}$ | 263.9 | 27 | 2728.8$^{a}$ | 169.2 | 16 | 2253.4$^{a}$ | 133.2 | 16 | 2686.8$^{a}$ | 273.0 | 27 | 2894.5$^{a}$ | 364.6 | 33 | 2671.4$^{a}$ | 304.8 | 30 | 21219.1 | 1902.9 | 24 |

a,b,c,d,e. Average values in a row with unlike superscript letters are significantly different ($p < 0.05$).

**Table 4.** Urinary excretion (amount in µg) of phenolic compounds metabolites determined at different time points*. (Average values with their standard errors; CV %)

| | Excreted amount (µg) | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 h | | | 0-2 h | | | 2-4 h | | | 4-6 h | | | 6-8 h | | | 8-24 h | | | Total excreted µg (24 h) | | |
| **Compounds** | **Average** | **SEM** | **CV%** | **Average** | **SEM** | **CV%** | **Average** | **SEM** | **CV%** | **Average** | **SEM** | **CV%** | **Average** | **SEM** | **CV%** | **Average** | **SEM** | **CV%** | **Average** | **SEM** | **CV%** |
| **Pyrogallols** | | | | | | | | | | | | | | | | | | | | | |
| **Pyrogallol-1-*O*-sulfate** | $0.3^{a,b,c}$ | 0.1 | 51 | $0.8^{b,c,d}$ | 0.1 | 35 | $1.0^{c,d}$ | 0.2 | 64 | $0.3^{a,b}$ | 0.1 | 48 | $0.2^{a}$ | 0.0 | 61 | $1.1^{d}$ | 0.2 | 50 | 3.4 | 0.4 | 33 |
| **Pyrogallol-2-*O*-sulfate** | $3.0^{a,b}$ | 1.0 | 91 | $1.1^{a}$ | 0.3 | 74 | $1.9^{a,b}$ | 0.4 | 49 | $1.5^{a}$ | 0.7 | 121 | $1.5^{a}$ | 0.5 | 87 | $9.2^{b}$ | 2.1 | 59 | 17.8 | 3.2 | 48 |
| **1-Methylpyrogallol-*O*-sulfate** | $6.9^{a,b}$ | 1.3 | 52 | $3.3^{a}$ | 0.9 | 71 | $4.9^{a,b}$ | 1.2 | 62 | $4.0^{a}$ | 1.3 | 84 | $3.6^{a}$ | 1.0 | 69 | $16.5^{b}$ | 4.9 | 78 | 38.3 | 8.1 | 56 |
| **Total** | $10.2^{a,b}$ | 2.2 | 58 | $5.1^{a}$ | 1.1 | 53 | $7.8^{a,b}$ | 1.6 | 52 | $6.6^{a}$ | 1.8 | 74 | $5.3^{a}$ | 1.4 | 66 | $26.8^{b}$ | 6.8 | 67 | 59.5 | 10.4 | 46 |
| **Benzoic acids** | | | | | | | | | | | | | | | | | | | | | |
| **Protocatechuic acid** | $21.3^{b,c}$ | 3.7 | 47 | $17.3^{a,b,c}$ | 4.5 | 64 | $9.3^{a,b}$ | 0.8 | 22 | $5.6^{a}$ | 1.5 | 69 | $5.7^{a}$ | 1.0 | 45 | $41.5^{c}$ | 9.8 | 63 | 97.5 | 15.3 | 41 |
| **Vanillic acid-4-*O*-sulfate** | $9.6^{a}$ | 2.4 | 66 | $18.9^{a,b}$ | 4.7 | 60 | $51.2^{b}$ | 15.0 | 77 | $18.4^{a,b}$ | 4.2 | 60 | $9.6^{a}$ | 1.3 | 32 | $22.4^{a,b}$ | 3.9 | 46 | 126.0 | 20.4 | 43 |
| ***p*-Hydroxybenzoic acid** | $5.1^{a,b}$ | 2.2 | 115 | $3.3^{a,b}$ | 0.9 | 63 | $3.7^{a,b}$ | 0.6 | 43 | $2.6^{a}$ | 0.5 | 51 | $2.3^{a}$ | 0.6 | 62 | $8.5^{b}$ | 1.1 | 35 | 24.6 | 4.5 | 49 |
| ***m*-Hydroxybenzoic acid** | $2.3^{a,b}$ | 1.2 | 142 | $0.6^{a,b}$ | 0.1 | 41 | $0.9^{a,b}$ | 0.1 | 45 | $0.5^{a}$ | 0.1 | 38 | $0.5^{a}$ | 0.1 | 51 | $6.7^{b}$ | 2.5 | 133 | 9.6 | 3.7 | 101 |
| ***o*-Hydroxybenzoic acid** | $0.3^{a}$ | 0.2 | 130 | $1.1^{a}$ | 0.5 | 118 | $3.3^{a}$ | 1.5 | 122 | $0.7^{a}$ | 0.4 | 139 | $0.3^{a}$ | 0.1 | 83 | $1.3^{a}$ | 0.6 | 122 | 6.8 | 2.7 | 106 |
| **Total** | $38.6^{a,b,c}$ | 6.8 | 47 | $41.2^{a,b,c}$ | 8.9 | 53 | $68.3^{b,c}$ | 17.1 | 66 | $27.8^{a,b}$ | 5.4 | 52 | $18.4^{a}$ | 2.2 | 29 | $78.7^{c}$ | 12.9 | 43 | 264.5 | 26.3 | 26 |
| **Catechols** | | | | | | | | | | | | | | | | | | | | | |
| **Catechol-*O*-sulfate** | $23.2^{a}$ | 6.2 | 71 | $8.2^{a}$ | 2.6 | 77 | $17.2^{a}$ | 3.8 | 58 | $14.7^{a}$ | 3.8 | 68 | $14.1^{a}$ | 2.6 | 46 | $93.5^{b}$ | 16.1 | 46 | 167.8 | 24.4 | 39 |
| **4-Methylcatechol-*O*-sulfate** | $126.7^{a}$ | 30.0 | 63 | $71.8^{a}$ | 20.2 | 69 | $85.8^{a}$ | 12.8 | 39 | $99.6^{a}$ | 17.3 | 46 | $131.8^{a}$ | 42.1 | 78 | $586.4^{b}$ | 150.2 | 68 | 1073.0 | 216.1 | 53 |
| **Total** | $149.9^{a}$ | 30.2 | 53 | $80.0^{a}$ | 22.6 | 69 | $103.0^{a}$ | 14.7 | 38 | $114.3^{a}$ | 19.6 | 45 | $145.9^{a}$ | 43.2 | 72 | $679.9^{b}$ | 156.0 | 61 | 1240.8 | 220.0 | 47 |
| **Hippuric acids** | | | | | | | | | | | | | | | | | | | | | |
| **4-Hydroxyhippuric acid** | $142.0^{a}$ | 42.8 | 80 | $122.8^{a}$ | 37.5 | 75 | $207.0^{a,b}$ | 36.5 | 47 | $142.3^{a}$ | 37.3 | 69 | $120.2^{a}$ | 32.8 | 67 | $379.2^{b}$ | 53.1 | 37 | 1078.7 | 185.4 | 45 |
| **3-Hydroxyhippuric acid** | $77.9^{a}$ | 28.0 | 95 | $30.8^{a}$ | 6.9 | 55 | $40.9^{a}$ | 9.3 | 60 | $45.8^{a}$ | 8.4 | 48 | $54.6^{a}$ | 7.9 | 35 | $236.5^{b}$ | 40.1 | 45 | 474.4 | 62.6 | 35 |
| **Hippuric acid** | $597.4^{a,b}$ | 102.7 | 45 | $327.6^{a}$ | 65.2 | 49 | $1079.7^{a,b}$ | 304.0 | 75 | $739.9^{a,b}$ | 189.9 | 68 | $484.3^{a}$ | 175.8 | 89 | $1904.3^{b}$ | 418.4 | 58 | 5017.1 | 836.0 | 44 |
| **Total** | $817.2^{a,b}$ | 139.4 | 45 | $481.2^{a}$ | 103.0 | 52 | $1327.7^{a,b}$ | 337.3 | 67 | $928.0^{a,b}$ | 209.2 | 60 | $659.1^{a}$ | 206.9 | 77 | $2520.0^{b}$ | 463.9 | 49 | 6570.2 | 960.0 | 39 |
| **Cinnamic acids** | | | | | | | | | | | | | | | | | | | | | |
| **Caffeic acid-4-*O*-*β*-D-glucuronide** | $1.9^{a}$ | 0.6 | 81 | $1.2^{a}$ | 0.3 | 62 | $1.6^{a,b}$ | 0.3 | 51 | $1.7^{a,b}$ | 0.3 | 48 | $1.6^{a}$ | 0.2 | 37 | $5.3^{b}$ | 1.1 | 55 | 12.9 | 1.7 | 36 |
| **Ferulic acid-4-*O*-glucuronide** | $13.5^{a,b}$ | 2.3 | 44 | $23.5^{a,b}$ | 3.8 | 40 | $21.0^{a,b}$ | 3.8 | 48 | $11.0^{a,b}$ | 2.1 | 49 | $7.4^{a}$ | 1.1 | 37 | $34.5^{b}$ | 7.9 | 61 | 106.5 | 14.2 | 35 |
| **Dihydrocaffeic acid-3-*O*-sulfate** | $47.9^{a,b}$ | 11.1 | 61 | $28.5^{a}$ | 9.5 | 81 | $23.2^{a}$ | 6.2 | 71 | $41.9^{a}$ | 23.0 | 145 | $68.5^{a,b}$ | 34.2 | 122 | $264.8^{b}$ | 74.9 | 75 | 460.8 | 146.9 | 84 |
| **Caffeic acid-3-*O*-*β*-D-glucuronide** | $0.8^{a}$ | 0.2 | 71 | $1.5^{a,b}$ | 0.3 | 43 | $2.5^{b}$ | 0.2 | 23 | $2.7^{b}$ | 0.4 | 38 | $2.1^{b}$ | 0.4 | 45 | $3.5^{b}$ | 0.7 | 54 | 12.6 | 1.0 | 21 |
| **Dihydroferulic acid-4-*O*-*β*- D-glucuronide** | $85.0^{a}$ | 33.6 | 105 | $37.2^{a}$ | 19.6 | 129 | $26.2^{a}$ | 8.5 | 86 | $38.2^{a}$ | 12.4 | 86 | $29.1^{a}$ | 8.4 | 71 | $63.4^{a}$ | 15.4 | 63 | 269.6 | 71.6 | 70 |
| **Caffeic acid** | $0.1^{a}$ | 0.0 | 41 | $0.1^{a}$ | 0.0 | 46 | $0.2^{a,b}$ | 0.0 | 58 | $0.2^{a,b}$ | 0.0 | 71 | $0.2^{a}$ | 0.1 | 125 | $0.5^{b}$ | 0.0 | 22 | 1.3 | 0.2 | 45 |
| **Dihydroferulic acid-4-*O*-sulfate** | $32.4^{a}$ | 11.1 | 91 | $13.3^{a}$ | 6.6 | 121 | $18.2^{a}$ | 6.4 | 93 | $25.8^{a}$ | 9.1 | 93 | $12.5^{a}$ | 4.0 | 78 | $27.0^{a}$ | 8.0 | | | | |
| **Dihydroferulic acid-4-*O*-sulfate** | $32.4^{a}$ | 11.1 | 91 | $13.3^{a}$ | 6.6 | 121 | $18.2^{a}$ | 6.4 | 93 | $25.8^{a}$ | 9.1 | 93 | $12.5^{a}$ | 4.0 | 78 | $27.0^{a}$ | 8.0 | 78 | 125.4 | 24.5 | 52 |
| **Ferulic acid-4-*O*-sulfate** | $225.2^{a,b}$ | 65.4 | 77 | $313.3^{a,b}$ | 70.7 | 55 | $363.1^{a,b}$ | 82.4 | 60 | $164.0^{a,b}$ | 29.9 | 48 | $79.3^{a}$ | 15.0 | 46 | $477.7^{b}$ | 157.9 | 87 | 1566.5 | 273.9 | 46 |
| **Dihydroisoferulic acid-3-*O*-*β*-D-glucuronide** | $24.0^{a}$ | 11.1 | 123 | $11.4^{a}$ | 6.3 | 137 | $8.7^{a}$ | 3.6 | 111 | $12.7^{a}$ | 4.7 | 98 | $4.0^{a}$ | 2.4 | 145 | $6.3^{a}$ | 2.1 | 86 | 64.9 | 24.6 | 100 |
| **Sinapic acid** | $5.0^{a}$ | 1.0 | 52 | $29.4^{c}$ | 6.7 | 56 | $36.8^{c}$ | 2.5 | 18 | $18.5^{b,c}$ | 2.5 | 35 | $9.5^{a,b}$ | 2.4 | 62 | $20.8^{b,c}$ | 4.9 | 63 | 114.6 | 8.0 | 19 |
| **Total** | $435.9^{a,b}$ | 127.6 | 77 | $459.4^{a,b}$ | 107.3 | 57 | $501.4^{a,b}$ | 92.3 | 49 | $316.7^{a,b}$ | 75.1 | 63 | $214.1^{a}$ | 56.4 | 65 | $903.8^{b}$ | 157.6 | 46 | 2735.1 | 422.2 | 41 |
| **Flavonols** | | | | | | | | | | | | | | | | | | | | | |
| **Kaempferol-3-*O*-glucuronide** | $0.9^{a}$ | 0.1 | 44 | $11.6^{b,c,d}$ | 3.4 | 73 | $63.2^{d}$ | 22.6 | 95 | $18.0^{c,d}$ | 4.0 | 59 | $3.4^{a,b}$ | 1.3 | 95 | $4.7^{b,c}$ | 1.4 | 79 | 99.6 | 27.4 | 73 |
| **Sum of phenolic compounds' metabolites excreted in urine** | $1452.8^{a,b}$ | 251.9 | 46 | $924.5^{a}$ | 244.6 | 70 | $2071.3^{a,b}$ | 424.8 | 54 | $1410.6^{a}$ | 264.6 | 50 | $896.7^{a}$ | 284.8 | 84 | $4213.7^{b}$ | 647.3 | 41 | 10969.6 | 1257.8 | 30 |
| **Urinary recovery (%)** | 0.8 | | | 0.5 | | | 1.1 | | | 0.8 | | | 0.5 | | | 2.2 | | | 5.8 | | |

a,b,c,d. Different letters, per row, indicate significant differences between the average values determined in the different time points for each compound ($p < 0.05$). Equal letters showed non-significant differences

($p > 0.05$). *The average, standard error of mean and the CV (%) were determined for each compound, considering n 7, except at time points 0-2 h and 6-8 h, when it was considered n 6.

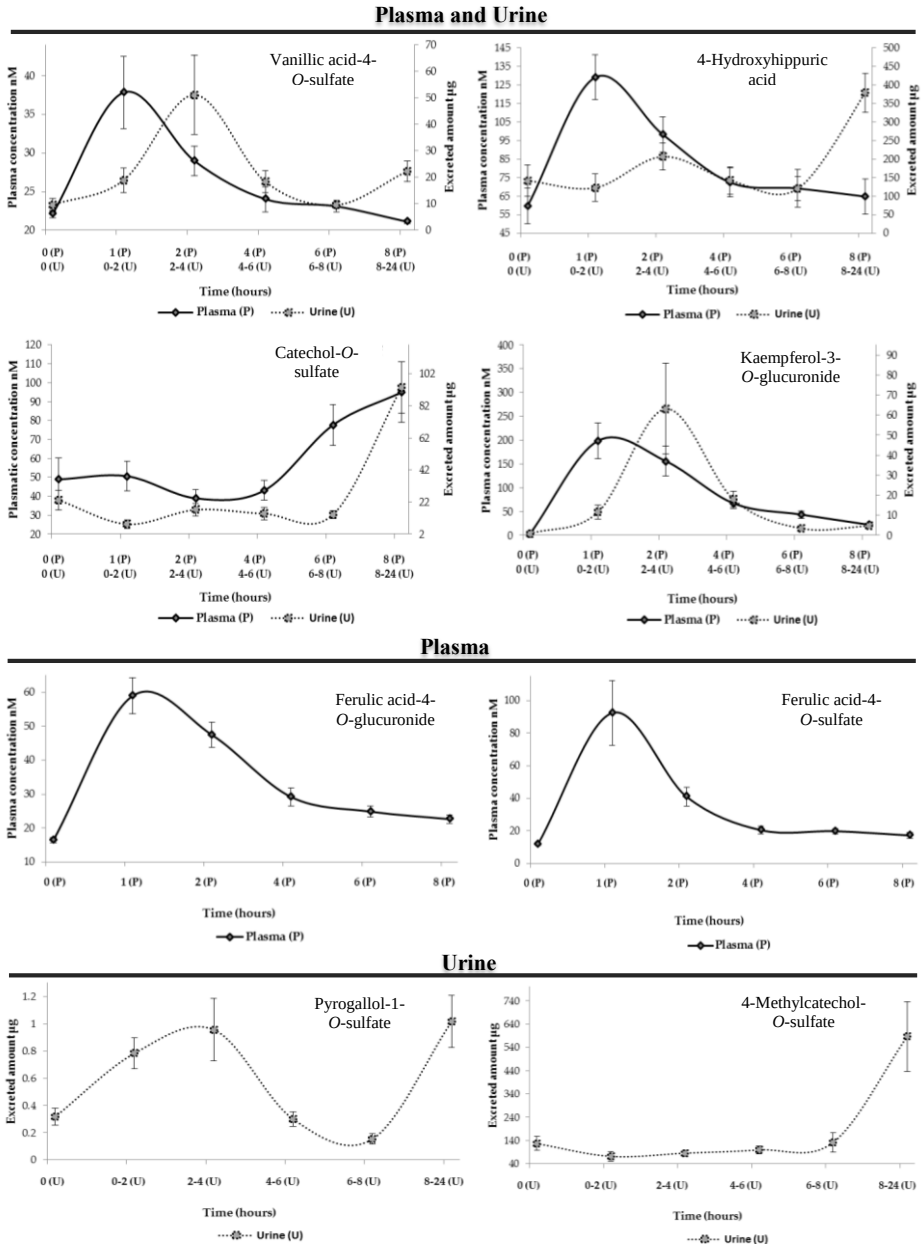



**Figure 2.** Plasma pharmacokinetic and/or urinary excretion profiles of metabolites related to cooked common beans' intake. Data are means (n 7), with standard errors represented by vertical bars.

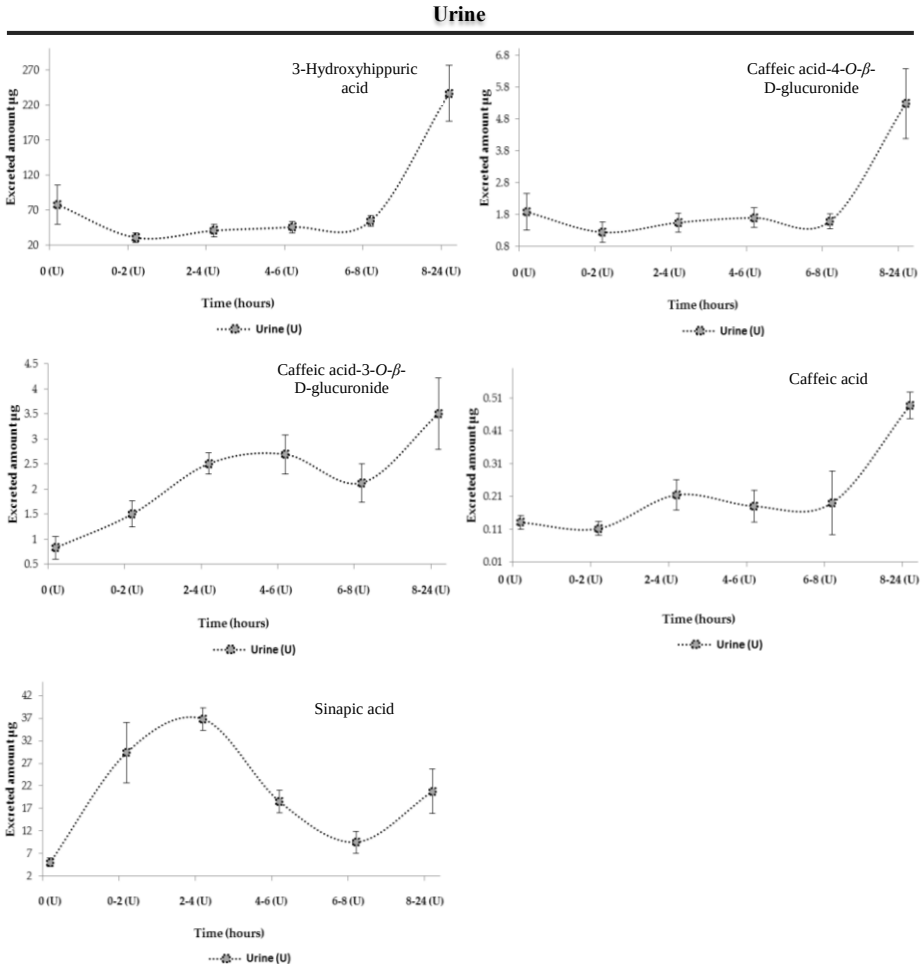



**Figure 2.** (continued)

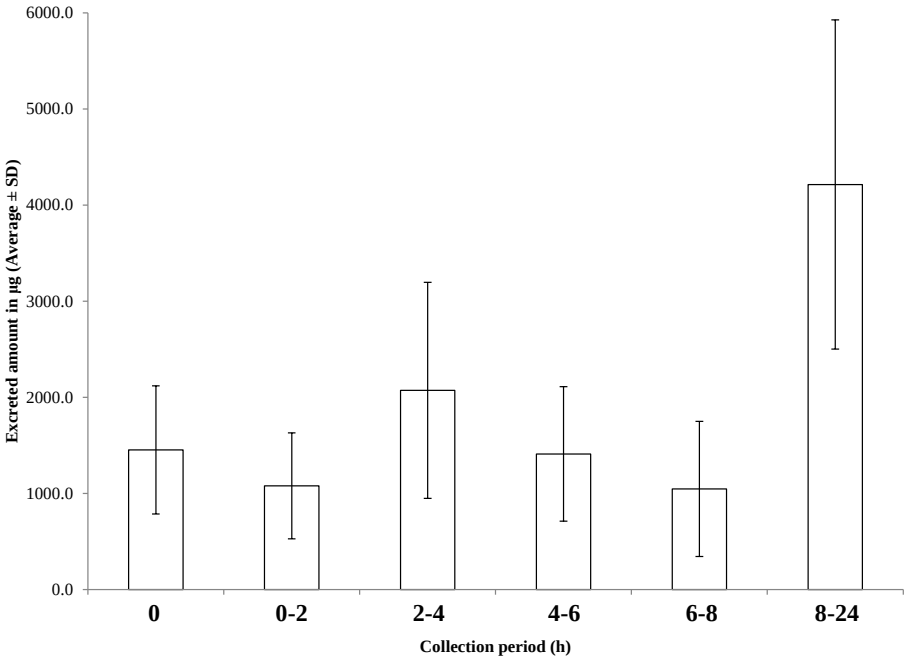



**Figure 3.** Variability of total urinary excretion (averages and standard deviations, in µg) of the phenolic compounds and their metabolites at different time points, before (0h) and after common beans' intake (0-2, 2-4, 4-6, 6-8 and 8-24h), *n 7*

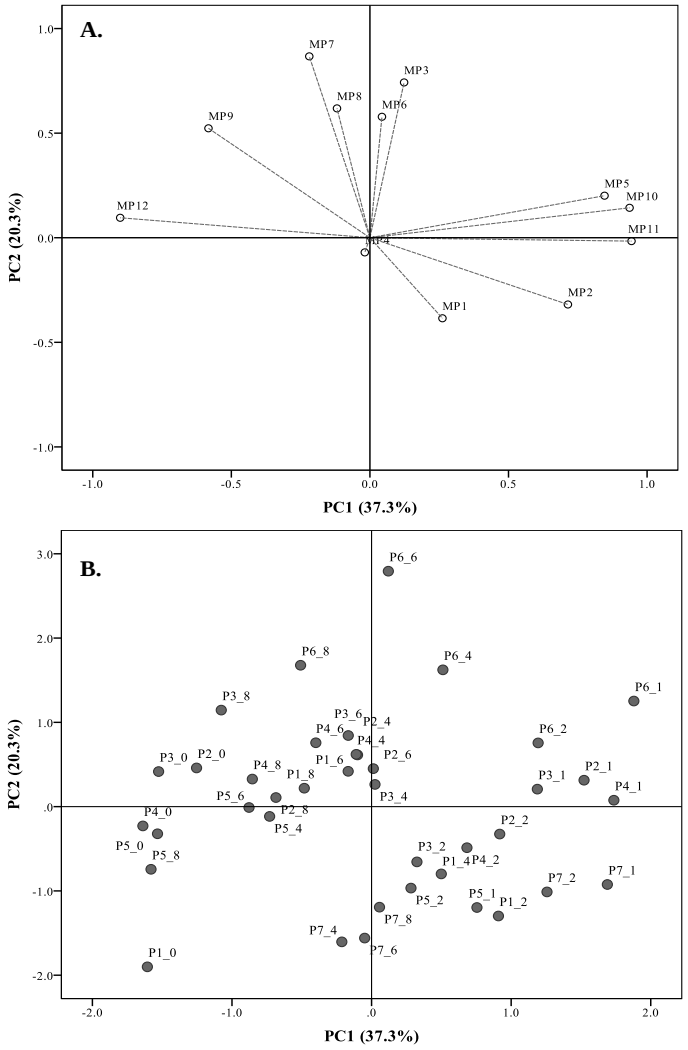
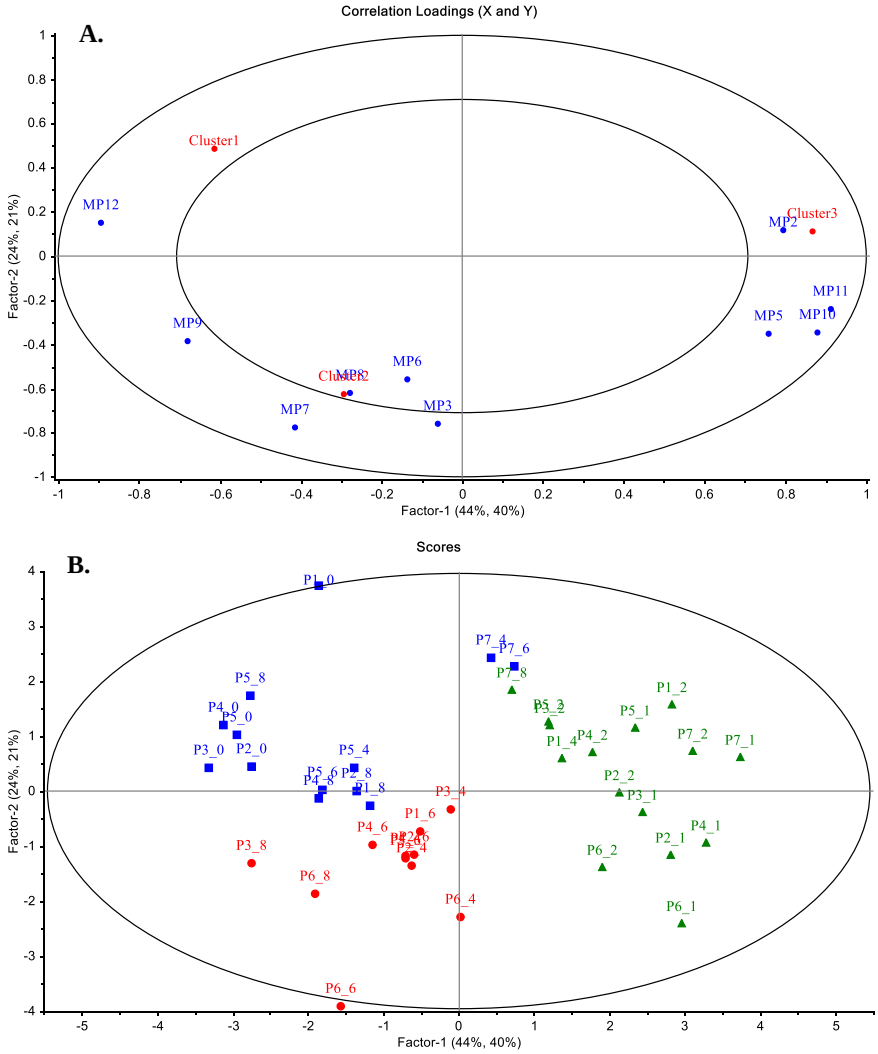



**Figure 4.** Principal component analysis (PCA) of the plasma samples collected before (0 h) and after common beans' intake. **A.** Loading plot of plasma metabolites (PC1 *versus* PC2), MP1 – two step_1-methylpyrogallol-*O*-sulfate; MP2 – two step_vanillic acid-4-*O*-sulfate; MP3 – two-step_dihydroferulic acid-4-*O*-*β*-D-glucuronide; MP4 – *p*-hydroxybenzaldehyde; MP5 – 4-hydroxyhippuric acid; MP6 – 3-hydroxyhippuric acid; MP7 – Hippuric acid; MP8 – caffeic acid; MP9 – *m*-coumaric acid; MP10 – log_ferulic acid-4-*O*-glucuronide; MP11 – log_kaempferol-3-*O*-glucuronide; MP12 – Inverse_ferulic acid-4-*O*-sulfate. **B.** Score plot of the plasma samples distributed in a space defined by the first two principal components (PC1 *v.* PC2). The label attributed to the plasma (P) included a first number, which defined the anonymous identification of each volunteer and after the underscore character the collection time period, meaning for example in the label P1_0 the plasma sample of volunteer 1 collected in the fasting period (0 h).




C.

M=39
K=10
$R^2(X)$= 0.671
$R^2(Y)$= 0.618
RMSEC= 0.337
RMSECV= 0.316
$Q^2$= 0.529
$R^2(Y)$-$Q^2$= 0.09

**Figure 5.** Partial least square – discriminant analysis (PLS-DA) highlighting the plasma samples' clustering. **A.** Correlation loading plot of plasma metabolites, MP2 – two step_vanillic acid-4-*O*-sulfate; MP5 – 4-hydroxyhippuric acid; MP6 – 3-hydroxyhippuric acid; MP8 – caffeic acid; MP9 – *m*-coumaric acid; MP10 – log_ferulic acid-4-*O*-glucuronide; MP11 – log_kaempferol-3-*O*-glucuronide; MP12 – Inverse_ferulic acid-4-*O*-sulfate. **B.** Score plot of plasma samples distributed in the two first factors (cluster 1, ■; cluster 2, ●; cluster 3, ▲). **C.** Quality parameters of the PLS-DA model defined for the plasma samples. The samples' identification was the same of **Figure 4**.

**Table 5.** Plasma concentration of different metabolites (nM) in the described clusters. (Averages and SD-standard deviations)

| | Plasma Concentration (nM) | | | | | |
|---|---|---|---|---|---|---|
| | Cluster 1 | | Cluster 2 | | Cluster 3 | |
| **Metabolites** | **Average** | **SD** | **Average** | **SD** | **Average** | **SD** |
| 1-Methyl pyrogallol-*O*-sulfate (MP1) | 23.30[a] | 0.93 | 22.80[a] | 0.65 | 23.48[a] | 1.18 |
| Vanillic acid-4-*O*-sulfate (MP2) | 21.69[a] | 0.93 | 22.77[a] | 1.92 | 31.10[b] | 7.80 |
| Dihydroferulic acid-4-*O*-*β*-D-glucuronide (MP3) | 39.96[a] | 4.93 | 49.42[b] | 9.49 | 43.38[ab] | 8.52 |
| *p*-hydroxybenzaldehyde (MP4) | 34.47[a] | 15.62 | 34.39[a] | 12.85 | 31.50[a] | 7.93 |
| 4-hydroxyhippuric acid (MP5) | 54.24[a] | 14.97 | 74.23[a] | 27.32 | 109.21[b] | 33.88 |
| 3-hydroxyhippuric acid (MP6) | 113.25[a] | 44.45 | 169.51[a] | 80.66 | 114.75[a] | 52.47 |
| Hippuric acid (MP7) | 1524.96[a] | 538.95 | 2215.68[b] | 370.81 | 1337.44[a] | 508.20 |
| Caffeic acid (MP8) | 1.90[a] | 0.06 | 1.98[b] | 0.08 | 1.88[a] | 0.06 |
| *m*-Coumaric acid (MP9) | 21.85[b] | 3.01 | 22.80[b] | 1.67 | 18.62[a] | 2.20 |
| Ferulic acid-4-*O*-glucuronide (MP10) | 19.93[a] | 4.14 | 28.08[b] | 6.39 | 48.88[c] | 16.15 |
| Kaempferol-3-*O*-glucuronide (MP11) | 24.34[a] | 31.47 | 48.03[b] | 22.93 | 160.68[c] | 97.63 |
| Ferulic acid-4-*O*-sulfate (MP12) | 15.16[a] | 4.54 | 19.34[b] | 4.18 | 54.46[c] | 36.02 |

[a,b,c] Average values in a row with unlike superscript letters are significantly different ($p < 0.05$).

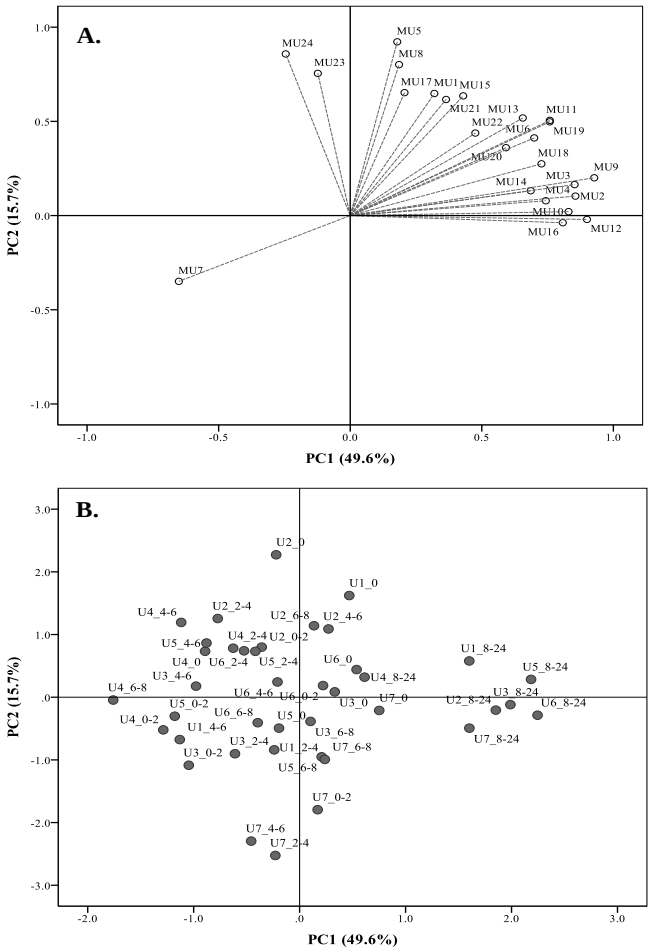
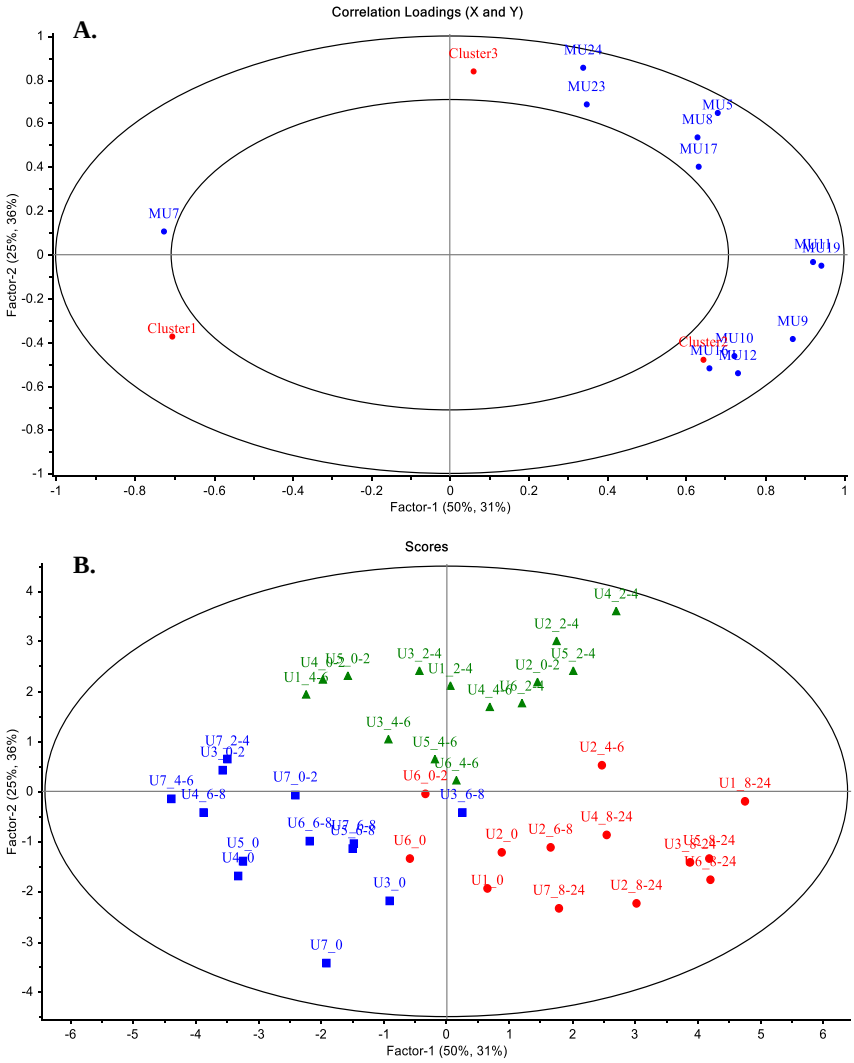



**Figure 6.** Principal component analysis (PCA) of the urine samples collected before (0 h) and after common beans' consumption. **A.** Loading plot of the urinary excreted metabolites (PC1 *versus* PC2), MU1 – log_pyrogallol-1-*O*-sulfate; MU2 – log_pyrogallol-2-*O*-sulfate; MU3 – log_1-methyl pyrogallol-*O*-sulfate; MU4 – log_protocatechuic acid; MU5 – log_vanillic acid-4-*O*-sulfate; MU6 – log_*p*-hydroxybenzoic acid; MU7 – Inverse_*m*-hydroxybenzoic acid; MU8 – log_*o*-hydroxybenzoic acid; MU9 – log_catechol-*O*-sulfate; MU10 – log_4-methylcatechol-*O*-sulfate; MU11 – log_4-hydroxyhippuric acid; MU12 – log_3-hydroxyhippuric acid; MU13 – hippuric acid; MU14 – log_caffeic acid-4-*O*-*β*-D-glucuronide; MU15 – log_ferulic acid-4-*O*-glucuronide; MU16 – log_dihydrocaffeic acid 3-*O*-sulfate; MU17 – log_caffeic acid-3-*O*-*β*-D-glucuronide; MU18 – log_dihydroferulic acid-4-*O*-*β*-D-glucuronide; MU19 – log_caffeic acid; MU20 – squared root_dihydroferulic acid-4-*O*-sulfate; MU21 – log_ferulic acid-4-*O*-sulfate; MU22 – log_dihydroisoferulic acid-3-*O*-*β*-D-glucuronide; MU23 – log_sinapic acid; MU24 – log_kaempferol-3-*O*-glucuronide. **B.** Score plot of the urine samples in the space defined by the two first principal components (PC1 *versus* PC2). For the volunteer 1 the urine samples were not provided at 0-2 h and 6-8 h. The label attributed to the urine (U) samples included a first number, which defined the anonymous identification of each volunteer and after the underscore character the collection time period, meaning for example in the label U1_8-24, the urine sample of volunteer 1 collected in the time period 8 -24 h after common beans' intake.




**C.**

M=40
K=12
$R^2(X)$= 0.751
$R^2(Y)$= 0.665
RMSEC= 0.273
RMSECV= 0.297
$Q^2$= 0.410
$R^2(Y)$-$Q^2$= 0.255

**Figure 7.** Partial least square – discriminant analysis (PLS_DA) highlighting the urine samples' clustering. **A.** Correlation loading plot of some selected urinary metabolites excreted during the study period, MU5 – log_vanillic-4-*O*-sulfate; MU7 – inverse_*m*-hydroxybenzoic acid; MU9 – log_catechol-*O*-sulfate; MU10 – 4-methylcatechol-*O*-sulfate; MU11 – log_4-hydroxyhippuric acid; MU12 – log_3-hydroxyhippuric acid; MU16 – log_dihydrocaffeic acid-3-*O*-sulfate; MU17 – log_caffeic acid-3-*O*-*β*-D-glucuronide; MU19 – log_caffeic acid; MU23 – log_sinapic acid; MU24 – log_kaempferol-3-*O*-glucuronide. **B.** Score plot of urine samples distributed along the two first factors (cluster 1, ■; cluster 2, ●; cluster 3, ▲). For the volunteer 1 the urine samples were not provided at 0-2 h and 6-8 h. **C.** Quality parameters of the PLS-DA model defined for the urine samples. The samples' identification was the same of **Figure 6**.

**Table 6.** Excreted amount of different metabolites (µg) in the described clusters (Averages and SD-standard deviations)

| | Excreted amount (µg) | | | | | |
|---|---|---|---|---|---|---|
| | Cluster 1 | | Cluster 2 | | Cluster 3 | |
| **Metabolites** | **Average** | **SD** | **Average** | **SD** | **Average** | **SD** |
| Pyrogallol-1-*O*-sulfate (MU1) | 0.27[a] | 0.21 | 0.74[b] | 0.49 | 0.75[b] | 0.54 |
| Pyrogallol-2-*O*-sulfate (MU2) | 1.43[a] | 1.25 | 6.74[b] | 5.02 | 1.32[a] | 0.96 |
| 1-Methyl pyrogallol-*O*-sulfate (MU3) | 3.19[a] | 2.31 | 12.82[b] | 10.14 | 4.29[a] | 2.75 |
| Protocatechuic acid (MU4) | 10.24[a] | 7.04 | 32.40[b] | 22.49 | 9.15[a] | 6.98 |
| Vanillic acid-4-*O*-sulfate (MU5) | 7.38[a] | 3.35 | 19.92[b] | 9.37 | 37.65[b] | 30.82 |
| *p*-hydroxybenzoic acid (MU6) | 2.24[a] | 1.21 | 7.17[b] | 4.78 | 3.56[a] | 1.64 |
| *m*-hydroxybenzoic acid (MU7) | 0.60[a] | 0.43 | 3.80[b] | 5.44 | 0.73[a] | 0.32 |
| *o*-hydroxybenzoic acid (MU8) | 0.13[a] | 0.14 | 0.94[b] | 1.19 | 2.38[b] | 2.96 |
| Catechol-*O*-sulfate (MU9) | 9.80[a] | 5.07 | 64.63[b] | 45.04 | 14.77[a] | 8.79 |
| 4-Methylcatechol-*O*-sulfate (MU10) | 103.62[a] | 86.44 | 383.03[b] | 363.89 | 84.60[a] | 39.69 |
| 4-Hydroxyhippuric acid (MU11) | 82.17[a] | 46.27 | 308.02[b] | 147.57 | 177.03[b] | 89.34 |
| 3-Hydroxyhippuric acid (MU12) | 44.36[a] | 25.22 | 168.54[b] | 116.67 | 39.51[a] | 22.98 |
| Hippuric acid (MU13) | 332.10[a] | 291.53 | 1380.20[b] | 993.91 | 918.55[b] | 637.67 |
| Caffeic acid-4-*O*-*β*-D-glucuronide (MU14) | 1.30[a] | 0.51 | 4.07[b] | 2.61 | 1.44[a] | 0.76 |
| Ferulic acid-4-*O*-glucuronide (MU15) | 8.88[a] | 3.29 | 27.30[b] | 17.82 | 19.65[b] | 9.64 |
| Dihydrocaffeic acid-3-*O*-sulfate (MU16) | 29.73[a] | 17.39 | 191.42[b] | 171.53 | 25.07[a] | 19.15 |
| Caffeic acid-3-*O*-*β*-D-glucuronide (MU17) | 1.36[a] | 0.98 | 2.82[b] | 1.76 | 2.44[b] | 0.64 |
| Dihydroferulic acid-4-*O*-*β*-D-glucuronide (MU18) | 16.41[a] | 15.21 | 93.39[b] | 60.24 | 32.85[a] | 26.73 |
| Caffeic acid (MU19) | 0.08[a] | 0.04 | 0.40[b] | 0.19 | 0.19[c] | 0.10 |
| Dihydroferulic acid-4-*O*-sulfate (MU20) | 6.88[a] | 5.41 | 38.34[b] | 24.15 | 20.72[b] | 15.59 |
| Ferulic acid-4-*O*-sulfate (MU21) | 106.10[a] | 65.88 | 393.81[b] | 323.44 | 319.04[b] | 196.18 |
| Dihydroisoferulic acid-3-*O*-*β*-D-glucuronide (MU22) | 1.71[a] | 1.65 | 22.07[b] | 22.32 | 10.35[b] | 9.48 |
| Sinapic acid (MU23) | 12.76[a] | 11.24 | 16.57[ab] | 11.80 | 30.05[b] | 13.65 |
| Kaempferol-3-*O*-glucuronide (MU24) | 2.78[a] | 2.36 | 5.94[a] | 6.77 | 41.70[b] | 46.71 |

Several factors such as the common bean variety, the maturity of seeds at harvest, the climatic conditions, the agronomic practices and the post-harvest storage conditions [46] contribute to explain the differences between the experimental data obtained in the present study and the described data in the literature (**Table 2**). Following the same trend noticed in raw beans, in cooked beans, the compounds, gallic acid, protocatechuic acid and sinapic acid, showed lower amounts than the ones reported by Xu et al. [41] and for the compounds, p-hydroxybenzoic acid, p-coumaric acid,

t-ferulic acid, quercetin and kaempferol, there were also lower amounts than the average values described by Diaz-Batalla et al. [42] (**Table 2**). Despite the differences between the obtained and the described results, possibly explained by differences in the processing conditions, the loss of protocatechuic acid, reported in the present study (–36%), was quite similar to the loss described in Xu et al. [41] study (–44%).

During the cooking process, the instability of the phenolics' chemical structure can contribute to explain the decrease of their content in cooked beans as already reported by Díaz-Batalla et al. [42] for quercetin, kaempferol, p-hydroxybenzoic acid and t-ferulic acid. The high temperature during cooking may cause evaporation of intracellular water, which triggers chemical reactions such as depolymerisation of phenolic compounds attached to polysaccharides and denaturation of proteins linked to phenolic compounds on the cell walls of cotyledons [50]. Those reactions responsible for changes in the cell wall structure may increase the accessibility of some phenolic compounds [51], such as procyanidin B2. As reported for cocoa beans, there are content variations in monomeric and dimeric forms of flavanols at high temperatures (100–140 °C). Such variations can be attributed to epimerisation reactions that may induce losses and increments in flavanol contents [52]. Unlike Kothe et al. [52], who reported for cocoa beans a significant increase of catechin (+240%) after the roasting process, in the present study, in cooked common beans, there was a significant increase of epicatechin (+350%) probably at the expense of procyanidins degradation and catechin epimerisation.

In the present study, the volunteers had straight nutritional recommendations regarding a controlled diet, free of phenolic compounds

during 48 h. After such period, in plasma, the concentration of vanillic acid-4-O-sulphate, 4-hydroxyhippuric acid, ferulic acid-4-O-glucuronide, ferulic acid-4-O-sulphate and kaempferol-3-O-glucuronide increased significantly ($p < 0.05$), 1 h after common beans intake (**Table 3**, **Figure 2**). This pattern was common to all volunteers and allowed to separate the plasma samples in different clusters (**Figure 5(b)**). 4-Hydroxyhippuric acid (derived from conjugation reactions of 4-hydroxybenzoic acid and glycine [53] and/or produced endogenously, from catecholamine's metabolism [54, 55]) has been described in association with different dietary sources (e.g. berries [19], green and black tea [56]). Herein, 4-hydroxyhippuric acid was associated with common beans' intake, considering that a diet, free of phenolic compounds, was performed previously, during 48 h. Additionally, to the 4-hydroxyhippuric acid plasma concentration increase, there was a concomitant increase of kaempferol-3-O-glucuronide (the main quantified flavonol, in plasma, after Moleiro common beans intake), **Figure 2**, which is in accordance with Penczynski et al. [57].

The phase II conjugation reactions with sulphate and glucuronide groups occurred with vanillic acid, ferulic acid and kaempferol in the upper part of the gastrointestinal tract, by sulfotransferases and uridine diphosphate-glucosyltransferase, as suggested by the time at which the maximum plasma concentration of vanillic acid-4-O-sulphate, ferulic acid-4-O-sulphate, ferulic acid-4-O-glucuronide and kaempferol-3-O-glucuronide was reached, 1 h post-consumption. Such results are in accordance with Feliciano et al. [19] and Bresciani et al. [58]. Since no data regarding such metabolites were found in literature after common beans intake, it was necessary to compare the obtained data with results described for different

food matrices by other authors. The maximum plasma concentration of ferulic acid-4-O-sulphate was slightly higher than the value described for whole-grain bread [11] but considerably lower than the one described in berries purée [20] and in cranberries [19]. Catechol-O-sulphate was also associated with common beans intake, but, contrarily to the previous compounds, its plasma concentration increased significantly only 8 h after common beans' intake. Such late increase in catechol-O-sulphate plasma concentration was also reported 7 h after cranberries intake [19]. More similar to cereals than to berries, in beans, the presence of free accessible phenolic compounds available to be metabolised in phase I and II reactions is limited, as a consequence of the strong covalent interactions of phenolic compounds and cell wall glycosides [59].

In urine, a total of twenty-four different metabolites was identified and quantified after common beans' intake, which represented a higher number of compounds than those determined in plasma (**Table 4**).

Contrarily to plasma, in urine, it was possible to detect and quantify compounds such as pyrogallol-1-O-sulphate, pyrogallol-2-O-sulphate, protocatechuic acid, p-hydroxybenzoic acid, m-hydroxybenzoic acid, caffeic acid-4-O-β-D-glucuronide, dihydrocaffeic acid-3-O-sulphate, caffeic acid-3-O-β-D-glucuronide, dihydroferulic acid-4-O-sulphate and sinapic acid. Nevertheless, in urine, the compounds, p-hydroxybenzaldehyde, m-coumaric acid and quercetin, were not quantified (**Table 7**). The absorption of metabolites derived from gut microbiota catabolism [11], such as dihydrocaffeic acid-3-O-sulphate, dihydroferulic acid-4-O-sulphate, only detected after 8 h, could contribute to explain the higher number of compounds in urine. With the exception of vanillic acid-4-O-sulphate,

sinapic acid and kaempferol-3-O-glucuronide (which maximum amounts were excreted earlier than 8 h, at the time point 2–4 h post-consumption, cluster 3) (**Table 4**, **Figures 2** and **7(b)**), for the majority of the metabolites (catechol-O-sulphate, 4-methylcatechol-O-sulphate, 4-hydroxyhippuric acid, 3-hydroxyhippuric acid, caffeic acid-4-O-β-D-glucuronide and caffeic acid), the excreted amount only increased significantly 8 h after common beans intake. For pyrogallol-1-O-sulphate and caffeic acid-3-O-β-Dglucuronide, the urinary excretion peaks were registered at different collection time points, following a multiphasic urinary excretion (**Table 4**). Despite the limited amount of bio accessible phenolic compounds in common beans, the excreted amounts of 4-methylcatechol-O-sulphate, 3-hydroxyhippuric acid, dihydrocaffeic acid-3-O-sulphate, vanillic acid-4-O-sulphate, sinapic acid and kaempferol-3-O-glucuronide were considerably higher after common beans' intake, than after cranberries' juice consumption [36]. Based on the metabolites quantified in urine, 8 h after common beans intake, **Figure 3**, a colonic metabolism, by gut microbiota, is expectable and supported by the phenolic compounds' entrapment in common beans' fiber. For 4-hydroxyhippuric acid, the urinary excretion earlier than 4 h, and at time points higher than 4 h, might be an indication of the metabolite's enterohepatic recirculation or the additional synthesis of the metabolite at the colon. Contrarily to the study conducted by Bonetti et al. [27], which described the urinary excretion of kaempferol, after β-glucuronidase and sulfatase enzymatic hydrolysis, in a percentage of 5.4 ± 5.4% and 6.1 ± 5.5% of the kaempferol consumed in common beans, in the present study, the kaempferol-3-Oglucuronide was the flavonols' metabolite detected and quantified, **Figure 2**, in both plasma and urine,

representing 30% of the consumed kaempferol. In urine, the sinapic acid represented 83% of the consumed sinapic acid. Part of this percentage should derive not only from the native compound present in cooked common beans but also from O-methylation reactions of the cinnamic acids, caffeic acid, p-coumaric and ferulic acids [60] (**Figure 8**).

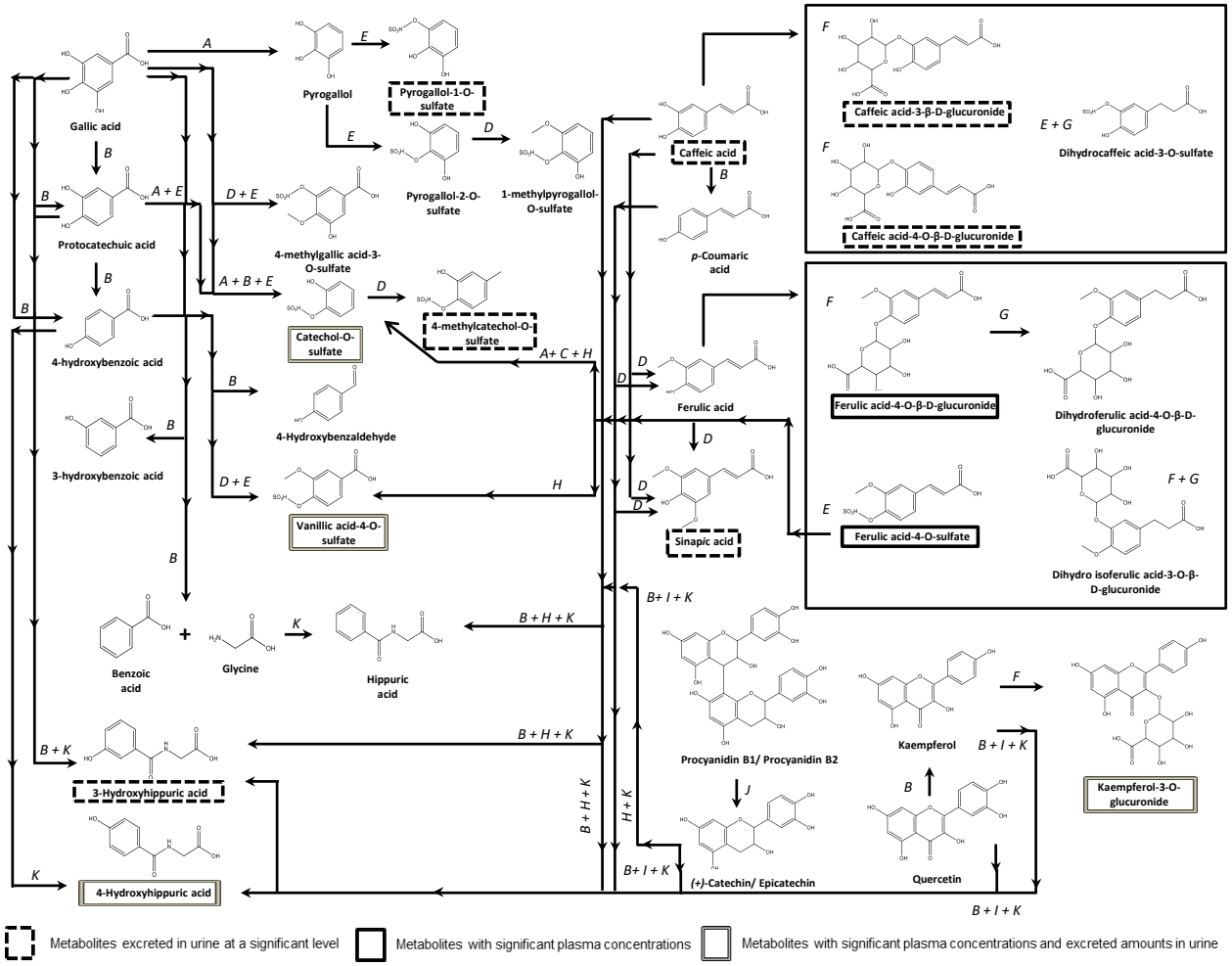



**Figure 8.** Proposed metabolic pathways involved in human metabolism of phenolic compounds from common beans, based on previous literature [15, 19, 59]. A, decarboxylation (phase I); B, dehydroxylation (phase I); C, dealkylation (phase I); D, *O*-methylation (phase II), E, *O*-sulfation (phase II), F, *O*-glucuronidation (phase II); G-reduction (phaseI); H, oxidation of the $C_3$ chain (phase I); I, fission of the C-ring (phase I); J, dimer's cleavage (phase I); K, conjugation with glycine (phase II).

Regarding the human inter-individual variability, the total excreted amount of metabolites (11.0 ± 1.3 mg of excreted compounds) represented only 5.8% of the total phenolic compounds consumed in the common beans portion (187.5 ± 4.0 mg of GAE in 143.8 ± 1.0 g of raw seeds' DW), which was in accordance with the one described for cranberries juice, where 6.2% of the total phenolic compounds consumed is reported, and in line with the low urinary recovery related to other food products rich in (poly)phenols [19]. The low urinary recovery of phenolic compounds could be attributed to the low bioaccessibility of the phenolic compounds derived from cooked common beans. These compounds are mostly entrapped in the dietary fiber (17% of total seed weight) [61] which slows down their absorption and excretion [62], contributing possibly to faecal metabolites, not quantified in the present study. In common beans, the inter-individual variability was evident not only on the plasma concentration but also on the urinary excretion of specific phenolic compounds' metabolites at different time points. The PLS-DA models, defined herein by values of $R^2(Y) > 0.6$ and small differences (< 0.3) [39] between $R^2(Y)$ and $Q^2$, indicated a common metabotype in the different volunteers after common beans' intake. Metabolites such as vanillic acid-4-O-sulphate, 4-hydroxyhippuric acid, ferulic acid-4-O-glucuronide, ferulic acid-4-O-sulphate and kaempferol-3-O-glucuronide were predominant in plasma at early times (1–2 h) after common beans' intake.

In urine, Kaempferol-3-O-glucuronide was also one of the metabolites early excreted (2–4 h) and others, such as 4-methylcatechol-O-sulphate, dihydrocaffeic acid-3-O-sulphate and 3-hydroxyhippuric acid, were only excreted after 8 h of common beans' intake. As reported

previously [37], the variability in specific metabolites, such as kaempferol-3-O-glucuronide, **Table 4**, higher than the variability obtained for the sum of compounds in urine could be an indication about the individual variation on the enzymatic activity and the complex interaction between the gut microbiota (with possible different bacteria compositions) and phenolic compounds from common beans. Several factors could contribute for such variability (e.g. enzyme activity, microbiota composition, gastrointestinal transit time, age, sex and genetics) [63, 64].

In conclusion, to our knowledge, the present work was the first human intervention study developed, through targeted metabolomics, to identify and quantify accurately phenolic compounds and their metabolites, in plasma and/or urine, after cooked common beans' intake. It also explored the effect of the cooking process on phenolic composition of common beans, since it influences the bioaccessibility and consequently the bioavailability of the compounds present in the original raw beans. The metabolites associated with plasma concentration and/or urinary excretion increments, after a diet free of phenolic compounds during 48 h followed by a single meal of cooked beans, were vanillic acid-4-O-sulphate, 4-hydroxyhippuric acid, ferulic acid-4-O-sulphate, ferulic acid-4-O-glucuronide, kaempferol-3-O-glucuronide, pyrogallol-1-O-sulphate, caffeic acid, catechol-O-sulphate, 4-methylcatechol-O-sulphate, 3-hydroxyhippuric acid, caffeic acid-4-O-β-D-glucuronide, caffeic acid-3-O-β-Dglucuronide and sinapic acid. Even if not specific of common beans' intake (it can also derived from other dietary sources), the plasma concentration and/or urinary excretion increase of these compounds, during the study period, made possible to define clusters of metabolites and associate them with

cooked common beans' intake. Most of the metabolites, such as the ones produced from the hydroxycinnamic acids, for example, caffeic acid-4-O-β-D-glucuronide, were excreted during the period of 8–24 h, indicating their persistence in the systemic circulation for a longer period of time. To access the kinetic profile of the metabolites with a return to the baseline level, future studies should be extended to at least 48 h including, if possible, a higher number of volunteers. In order to understand the role of metabolites derived from phenolic compounds of common beans in human health, future *in vitro* and *in vivo* studies regarding the biological activity of the different metabolites (namely those whose concentration increased significantly in plasma and urine) should be performed. Additionally, studies regarding individual differences on microbiota composition and concerning the faecal metabolites obtained after common beans' consumption could also contribute to understand the impact of common beans in human health, especially in gut health.

## Supplementary materials

The following are available online at https://doi.org/10.1017/S0007114519002836, Table S1A: Diet for reduced phenolic compounds intake (To accomplish in the 48 h before the assay), Table S1B: Diet for reduced phenolic compounds intake (To accomplish in the 24 h of the assay day), Table S1C: List of allowed and not allowed food items (To accomplish in the 48 h before the assay and in the 24 h of the assay day), Table S2: Identification of phenolic compounds in Moleiro bean extracts by UPLC-Q-TOF-MS, Table S3: Identification of phenolic compounds and their metabolites in plasma, before (0 h) and after (1, 2, 4,

6, 8 h) cooked common beans' consumption. Results for n=7, P1 – P7, Table S4: Concentration (nM) of phenolic compounds and their metabolites in plasma, before (0 h) and after (1, 2, 4, 6, 8 h) cooked common beans' consumption. Results for n=7, P1-P7, Table S5: Identification of phenolic compounds and their metabolites in urine, before (0 h) and after (0-2, 2-4, 4-6, 6-8, 8-24 h) cooked common beans' consumption. Results for n=7 (U1 – U7), with exception of time points 0-2 h and 6-8 h, only registered for n=6, Table S6: Urinary excretion (amount in μg) of phenolic compounds' metabolites determined at different time points for the different volunteers, U1 – U7.

## Author Contributions

Conceptualization, E.M., M.E.F., M.C.V.P., M.R.B.; funding acquisition, E.M., M.E.F., M.C.V.P., M.R.B.; investigation, E.M.; methodology, E.M., R.P.F., S.D.S., M.R.B.; project administration, M.C.V.P., M.R.B.; resources, M.C.V.P., M.R.B., A.R-M., R.P.F., software, E.M., R.P.F.; writing – original draft preparation, E.M.; writing—review and editing, E.M., A.R-M., S.D.S., M.C.V.P., M.R.B.

## Acknowledgements

The authors acknowledge all the volunteers involved in the study, and also Dr Claudia Nunes dos Santos (iBET) and Dr Rita Ventura (ITQB) for providing some metabolites (pyrogallol-1-O-sulphate, pyrogallol-2-O-sulphate, 1-methylpyrogallol-O-sulphate, 2-methylpyrogallol-O-sulphate, 4-methylcatechol-O-sulphate, 4-methylgallic-3-O-sulphate, catechol-O-sulphate, and vanillic acid-4-O-sulphate) used in the study. This study was

financially supported by FCT Portugal through the BEGEQA project (PTDC/AGR-TEC/3555/2012), a PhD fellowship to E. M. (SFRH/BD/89287/2012) and a FCT Investigator Program Development Grant to M. C. V. P. (IF/01337/2014), R&D unit, UID/Multi/04551/2019 (Green-IT) and COST Action FA1403 (STSM-FA1403-290815-063873).

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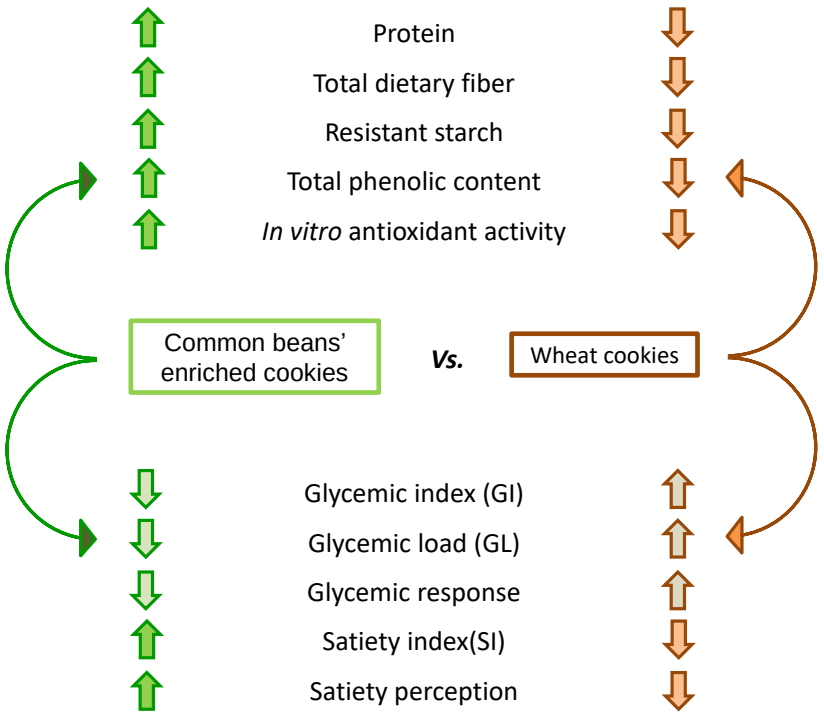
# Chapter VI




The chapter was submitted and accepted by Cereal Chemistry as,

**Mecha, E.**, Correia, V., Bento da Silva, A., Ferreira, A., Sepodes, B., Figueira, M.E., Vaz Patto, M.C., Bronze, M.R. Improvement of wheat cookies' nutritional quality, by partial substitution with common bean and maize flours, sustained human glycemia and enhanced satiety perception. *Cereal Chemistry* **2021**, 00, 1-12; DOI: 10.1002/cche.10460.

In this Chapter, Elsa Mecha participated in the experimental work, data analysis, manuscript draft and final manuscript writing.

# Improvement of wheat cookies' nutritional quality, by partial substitution with common bean and maize flours, sustained human glycaemia and enhanced satiety perception

## Abstract

As a dietetic source of fiber, protein, vitamins, minerals and phenolic compounds, common beans have potential benefits in human health, namely in chronic diseases' prevention (e.g. cardiovascular diseases and colon cancer). Still, legume consumption, especially in European countries is below recommendations. The consumers demand for innovative, attractive legume-based food products suggests a potential future increase in consumption of legumes, especially in modern societies, keen on ready-to-eat foods with known health benefits. With the aim of studying the impact of wheat flour's partial substitution by common bean (56%) and maize (22%) flours in the nutritional composition of formulated cookies and its effect on human glycaemia and consumers' satiety perception, after ingestion, a human intervention study (n=16) was designed. Approved by consumers, common bean enriched cookies were responsible by reducing glycemic response, and by increasing satiety perception. The nutritional composition of common bean enriched cookies contributed to explain these effects. Common bean enriched bakery food products are valuable nutritional options for consumers concerned with satiety and chronic diseases' prevention. This study showed, for the first time, through a human intervention trial the relevance of using legumes (common beans in particular) as alternative ingredients to improve ready-to-eat products' nutritional quality.

**Keywords:** common bean enriched cookies; glycemic response; satiety perception; volunteers; wheat cookies

## 1. Introduction

Nutritionally, legumes are excellent sources of protein, complex carbohydrates, dietary fiber, vitamins, e.g. folate, and minerals, e.g. potassium, magnesium and copper [1]. Despite their recognized nutritional and health benefits, in the Prospective Urban Rural Epidemiology (PURE) study, which involved 135 335 individuals from 18 countries (North America, Europe, South America, the Middle East, South Asia, China, Southeast Asia and Africa), the reported legumes' consumption was lower than 60 g/day, which corresponds to less than one USDA serving size per day, 150 g of cooked beans [2]. Although gastrointestinal discomfort (increased flatulence, stool changes, and bloating) has been identified by consumers as the main reason to avoid eating beans, the low innovation and attractiveness of marketed legume-based food products also contributed to reduce legume consumption in modern societies [3]. Concerning the gastrointestinal discomfort, the individual intestinal response to the presence of fiber, oligosaccharides and resistant starch is quite variable and will decrease overtime as long as legumes remain on diet [4]. While in raw state, legumes, such as common beans, have low digestibility due to protease inhibitors activity, after cooking the serine-protease inhibitors are inactivated by the heat treatment and the activity of digestive enzymes is no longer affected [5]. Nevertheless protease inhibitors and other bioactive secondary metabolites such as phenolic compounds (e.g. flavonoids and tannins) and phytic acid, as well as total

dietary fiber (soluble and insoluble) have also been linked to the prevention of non-communicable diseases such as cardiovascular diseases [6] and colon cancer [7]. Additionally, as a rich source of total fiber and resistant starch, common beans are also known to be responsible for a low glycemic index (GI), 7-42% relative to glucose and 40-59% relative to white bread [8]. In 2019, EAT-Lancet Commission launched the planetary health diet which basically consists on the intake of high quality plant based food products, e.g. fruits, vegetables, legumes, whole grains, nuts, and reduced amounts of animal products, e.g. whole milk, eggs, fish, meat. This diet has been proposed as a flexible diet and aims to improve biomarkers, such as fasting blood glucose concentrations and glycated proteins, HDL-cholesterol concentrations [9, 10], insulin sensitivity and body weight, reducing the risk of non-communicable diseases [11-13]. The dietetic shift, with a decrease by 50% on animal products with a concomitant increase on the consumption of plant based food products, will increase consumers' demand for affordable, diverse, attractive, high quality and sustainable plant derived products [11].

Prejudices regarding culinary use and misleading associations about legumes and socio-economic status must be demystified through education and reinvention of traditional gastronomy, with development of innovative food products adapted to consumers' taste and preferences.

The use of grain legumes in the production of ready-to-eat bakery food products (e.g. pasta, bread, biscuits, breakfast cereals/ snacks), meat derived products (e.g. sausages, nuggets, burgers) and soups will enable all over the world, the increment of plant protein consumption, and the reduction of meat and high carb-derived products in the diet [14]. Common

bean flour has recently been incorporated in enriched biscuits with improved nutritional properties [15], gluten-free cookies [16], and cookies prepared from the flour of germinated beans [17]. Nevertheless, so far no human intervention study was conducted to evaluate the impact of common bean flour as an alternative ingredient, on human glycaemia. In the present study, bean flour was applied as the main alternative ingredient to wheat flour in cookies preparation and the hypothesis that wheat flour's partial substitution, mainly by common bean flour (56%, with a smaller portion, 22%, of maize flour), induces significant modifications in final cookies' nutritional composition, reducing human glycaemia and promoting consumers' satiety perception, after ingestion, was tested.

In comparison with the traditional cookies prepared only with wheat flour, the nutritional composition of both cookies (moisture, protein, total carbohydrates, sugars, total dietary fiber, total fat, saturated, monounsaturated and polyunsaturated fat, salt and energy) as well as the resistant starch, phenolic compounds content and antioxidant activity were evaluated. For the first time the impact of common bean flour together with maize flour, as ingredients in cookies preparation, on the glycemic response, glycemic index (GI), glycemic load (GL), and satiety perception were determined by comparison with the traditional wheat cookies, through a human intervention study with healthy volunteers.

## 2. Materials and methods

### 2.1 Chemicals

Folin-Ciocalteu's reagent, sodium carbonate (99%), gallic acid (≥ 98%), trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid),

fluorescein sodium salt, AAPH (2,2-azobis(2-ethylpropionamidine)dihydrochloride), hydrochloric acid (37%), anhydrous glucose were obtained from Sigma-Aldrich (St. Louis, USA). Milli-Q® water (18.2 MΩ.cm) was obtained in a Millipore Direct Q3 UV system equipment (Molsheim, France). Sodium hydroxide (98%) was purchased from Merck (Darmstadt, Germany). Ethyl acetate, diethyl ether and methanol (99.9%) were purchased from Carlo Erba Reagents (Rodano, Italy). Resistant starch assay kit was purchased from Megazyme International (Bray, Ireland).

## 2.2 Plant Material

Moleiro, a Portuguese plain light brown common bean (*Phaseolus vulgaris* L.) traditional variety collected from a farmer (F. Pinto) in Celorico de Bastos, Portugal, with a reduced genetic variability [18], was selected for the study based on the highest phenolic content reported previously [19]. Moleiro seeds were grounded in a Falling 3100 (Perten, Sweeden) mill to a particle size of 0.8 mm to obtain common bean whole flour. Wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) flours, type 65 and type 70, respectively, were commercially available at supermarket.

## 2.3 Cookies' preparation

Cookies were prepared in accordance to a previous procedure [15] with slight modifications. Whereas for wheat cookies, only wheat flour was used (100% of total added flour), for common bean enriched cookies, after testing different proportions of flours (ingredients) and removing the vanilla extract proposed in the Sparvoli et al., 2016 recipe, the wheat flour was

partially substituted (in 78% of the total added flour) by maize (22%) and common bean (56%) flours, in order to obtain a final proportion of 1: 1: 2.5 in wheat: maize: common bean flours, **Table 1**. This proportion of flours, in combination with the remaining ingredients (eggs, butter and sugar) masked the beany flavor derived from common bean raw flour. All the ingredients were weighed in a kitchen scale (model HR2393, Philips®, Amsterdam, Netherlands) and added in a bowl. Briefly after mixing the ingredients, common bean flour, sugar, salt and yeast, the eggs and the melted butter were added and involved to form dough that was manually kneaded. Finally, the dough was cookie-shaped and baked at 180 °C for 10-15 minutes.

**Table 1.** Ingredients and quantities (g) used for common bean enriched and wheat cookies preparation.

| Ingredients (g) | Common bean enriched cookies (n=6) | Wheat cookies (n=6) |
|---|---|---|
| Wheat flour | 30 | 135 |
| Maize flour | 30 | 0 |
| Bean flour | 75 | 0 |
| Eggs | 55 | 55 |
| White sugar | 75 | 75 |
| Butter | 50 | 50 |
| Salt | 2 | 2 |
| Yeast | 4 | 4 |

## 2.4 Nutritional composition

The nutritional composition (energy, moisture, protein, total carbohydrates, total dietary fiber, total sugars, total fat, saturated, monounsaturated and polyunsaturated fat, ash and sodium) was determined for both types of cookies. Total energy value was estimated using Atwater factor [20]. Moisture was determined by drying samples in an

oven at 105 ºC until constant weight of the samples [21]. Protein was determined by Kjeldahl method [22]. Total carbohydrates were calculated by difference using the equation proposed by USDA [23],

$$\text{Total Carbohydrates (calculated)} = 100 - (\text{Total Protein} + \text{Total Fat} + \text{Moisture} + \text{Ash})$$

and total sugars using high performance liquid chromatography (HPLC) with refractive index (RI) detection [22, 24]. Total dietary fiber was determined using an enzymatic gravimetric method [21]. Total lipids were determined by Soxhlet extraction [21]. The fatty acids composition was determined by gas chromatography (GC) with flame ionization detector (FID) [21]. Ash was determined by samples' incineration [21]. Salt was estimated as equivalent salt content from the sodium content (salt (%) = sodium content (%) x 2.54). Sodium content was determined by atomic absorption spectrophotometry with flame [25]. The analyses were performed in triplicate, considering three cookies from each type.

2.5 Resistant starch

The resistant starch was evaluated in accordance to AACC method 32–40.01 [26] using an assay kit (Megazyme International, Bray, Ireland).

2.6 Phenolic content and antioxidant activity (ORAC) of formulated cookies

The phenolic compounds from common bean enriched and wheat cookies were extracted following a previous procedure [27]. Briefly, 500 mg of cookies were submitted to alkaline hydrolysis for 4 h at room temperature using 10 mL of NaOH (4M). After pH adjustment to 2.0 using HCl (6M), 5 mL were extracted with 5 mL of ethyl acetate and diethyl ether, for four times. After combining the supernatants, 5 mL were evaporated

until dryness, in Speedvac concentrator (Labconco®, Kansas City, MO, USA), and the final residues re-suspended in 1.5 mL of methanol. The extracts were kept at -20 ºC until further analyses.

The total phenolic content (TPC) and the *in vitro* antioxidant activity (ORAC) of the extracts were analyzed using a microplate reader, Bio-Tek Instruments, Winooski, VT, USA, following, for TPC, Folin-Ciocalteu's method and for ORAC the ability of antioxidant compounds in extracts to inhibit fluorescein oxidation catalyzed by peroxyl radicals generated from AAPH (2,2-Azobis(2-methylpropionamidine)dihydrochloride) [28]. The final results were expressed, for TPC, as gallic acid equivalents (GAE) per 100 g of cookies and, for antioxidant activity, as micromoles of trolox equivalents antioxidant capacity (TEAC) per 100 g of cookies.

## 2.7 Glycemic response and glycemic index

A human intervention assay was carried out to evaluate the glycemic response and the glycemic index (GI) of the two types of cookies. The study design was according to the Declaration of Helsinki and the protocol was approved by the Ethics Committee for Clinical Experimentation of the Pharmacy Faculty, University of Lisbon with the approval number 04/CEECFFUL/2019. The study was registered at https://eudract.ema.europa.eu/ with the number 2021-001687-17.

### *2.7.1 Volunteers*

Sixteen volunteers (eleven females and five males) with age between 23 – 32 years old and body mass index (BMI) between 18.5 – 24.9 kg $m^{-2}$ were recruited. All the volunteers matched the inclusion criteria

(non-smokers, no clinical history of disease, not taking drugs responsible by changes in glucose and/or lipid metabolism, nor dietetic supplements). On average, the volunteers consumed legumes once per week. All volunteers were orally and written informed about the experimental procedures, including the cookies main type of flour, and signed an informed consent form.

The volunteers received clear instructions to avoid intense exercise in the day before each intervention, also to avoid alcohol and fiber-rich foods consumption and had to accomplish a fasting period of 12 h from the dinner time to the intervention day.

*2.7.2 Intervention assay*

For data comparison, the human intervention assay was conducted with all volunteers along three intervention assay days always one week apart:

- First Week: two common bean cookies, 85 g (test cookies) and 250 mL of water;
- Second week: two wheat cookies, 85 g (control cookies) and 250 mL of water;
- Third week: 50 g of anhydrous glucose in 250 mL of water.

On the intervention day, after capillary glycaemia measurement at 0 min, corresponding to fasting, using a glucometer (GLUCOCARD ™ SM), a Visual Analog Scale (VAS) questionnaire for the hunger, satiety, fullness and future consumption perception evaluation [29] (using a quantitative scale: 0 to 10 to describe low to high satiety insights) was filled. After 15 minutes, volunteers consumed the cookies in test (two cookies,

approximately 85 g in total or the anhydrous glucose). After cookies ingestion, the capillary glycaemia measurement was performed at different time points (15, 30, 45, 60 and 120 min) and the VAS questionnaire filled. The questionnaires were collected, and it was not possible for the volunteers to have access to them again.

Cookies consumer acceptance was evaluated through another questionnaire, with a qualitative scale: strongly agree, agree or disagree. A neutral answer could also be given.

After expressing the mean glycaemia values as mmol/L, the incremental glycaemia (Δ glycaemia) was determined according to Equation **1**.

$$\Delta\ glycaemia\ \left(\frac{mmol}{L}\right) = Glycaemia\ (t15, t30, t45, t60, t90\ or\ t120) - Fasting\ glycaemia\ (t0),\ \textbf{(1)}.$$

The incremental area under the curve (AUCt) was determined considering only the positive incremental values (above the baseline obtained at the fasting period) on the common bean enriched cookies, wheat cookies and oral glucose assays. The glycemic index (GI), defined as the incremental area under the glycemic response curve (AUCt) elicited by a portion of food containing 50 g of available carbohydrate was expressed as a percentage by comparison with the glycemic response elicited by 50 g of glucose [30, 31], accordingly to Equation **2**.

$$Glycaemic\ index\ (GI) = \frac{Incremental\ mean\ area\ under\ the\ curve\ (AUCt) in\ common\ bean\ cookies\ or\ in\ wheat\ cookies\ assay}{Incremental\ mean\ AUCt\ in\ glucose\ assay} \times 100,\ \textbf{(2)}.$$

The glycemic loading (GL) was also evaluated. It considers the real consumption portion [31] and is calculated accordingly to Equation **3,**

$$Glycaemic\ load\ (GL) = \frac{Glycaemic\ index\ (GI)}{100} \times net\ grams\ of\ carbohydrates\ (Total\ carbohydrates - Total\ fibre),$$ **(3)**.

A satiety index and a satiety quotient (SQ) were used to predict future consumption [32]. The satiety index was computed following the same procedure described for GI, using the satiety ratings instead of glycemic values to determine the AUCt values and compared to the AUCt after glucose intake. The satiety quotient was determined as the difference in the hunger perception before and after cookies consumption divided by the cookies weight.

2.8 Statistical analysis

The area under the curve (AUCt) relative to the glycemic response for 120 minutes in the three assays (with common bean enriched cookies, wheat cookies and oral glucose solution) was calculated using the PK Solver tool of Microsoft Excel (Microsoft) for each volunteer and the mean ± standard error of mean (SEM) values presented. Glycaemia was expressed as mg/dL and converted to mmol/L.

The rating of satisfaction, fullness, hunger and perspective of future consumption obtained at the different time points (0, 15, 30, 45, 60, 90 and 120 min), evaluated with a quantitative scale (varying from 0 to 10), were expressed as mean ± SEM values (n=16) and the mean incremental area under the curve (AUCt) for the satisfaction score was calculated for the cookies and oral glucose assay. Using IBM® SPSS® Statistics, version 22, software, Armonk, NY, USA, the normality of variables distribution was evaluated by Shapiro–Wilk test ($n < 50$) at a significance level of 1%. One-Way ANOVA was applied, at each time point, to compare AUCt mean

values obtained for the three assays in the 16 volunteers. One-way ANOVA was also used to compare the mean values of satiety, fullness, hunger and perspective of future consumption perceptions, at each time point, in the three intervention assay days. After testing for homoscedasticity with Levene´s test, the *post-hoc* Scheffé's test or the non-parametric Games-Howell test (depending, respectively on the existence or absence of homoscedasticity) were performed to establish multiple comparisons between the assays (assay with common beans enriched cookies, wheat cookies or oral glucose solution) at a significance level of 5%.

## 3. Results and discussion

### 3.1 Differences in the nutritional parameters, resistant starch, phenolic content and antioxidant activity of formulated common bean enriched and wheat cookies

The nutritional composition (energy, protein, total carbohydrates, total sugars, total fat, saturated, monounsaturated, polyunsaturated fat, total dietary fiber, and salt) of the test (common bean enriched) and control (wheat) formulated cookies is summarized in **Table 2**. The results were compared with the described values for a commercial wheat cookie (*Bolacha Maria*) [33].

**Table 2.** Nutritional composition, mean values ± standard deviation, in test (common bean enriched) and control (wheat) formulated cookies. [a,b] Significant differences between the two types of cookies, $p < 0.05$.

| | **Common bean enriched cookies (*Per 100 g*)** | **Wheat cookies (*Per 100 g*)** | **Wheat commercial cookies,** *Bolacha Maria* **(*Per 100 g*)** |
|---|---|---|---|
| **Energy (kJ/ kcal)** | 1751 ± 35/ 417 ± 8[a] | 1881 ± 38/ 448 ± 9[b] | 1825/ 436 |
| **Moisture (g)** | 8.30 ± 0.33[b] | 5.20 ± 0.21[a] | |
| **Protein (g)** | 10.1 ± 0.2[b] | 8.0 ± 0.4[a] | 8.4 |
| **Total Carbohydrates (g)** | 57.80 ± 1.16[a] | 66.90 ± 1.00[b] | 72.00 |
| ***Sugars (g)*** | 34.00 ± 2.55[a] | 33.80 ± 2.54[a] | 21.50 |
| **Total Fat (g)** | 14.90 ± 0.75[a] | 16.10 ± 0.81[a] | 12.20 |
| ***Saturated (g)*** | 8.90 ± 0.01[a] | 9.60 ± 0.01[b] | 5.90 |
| ***Monounsaturated (g)*** | 3.64 ± 0.01[a] | 3.98 ± 0.01[b] | 3.40 |
| ***Polyunsaturated (g)*** | 1.12 ± 0.01[a] | 1.12 ± 0.01[a] | 1.30 |
| **Dietary fiber (g)** | 5.70 ± 0.25[b] | 1.60 ± 0.24[a] | 2.10 |
| **Ash (g)** | 3.19 ± 0.11[b] | 2.28 ± 0.08[a] | |
| **Salt (g)** | 1.48 ± 0.10[b] | 0.97 ± 0.06[a] | 1.06 |

As shown in **Table 2**, the nutritional composition of the formulated wheat cookies was similar to the one described for the commercial wheat cookies (*Bolacha Maria*) available at the Portuguese food store markets. The major difference between common beans enriched and wheat cookies was observed in total dietary fiber, which, stood out as the nutritional parameter with highest representativeness, four times more fiber, in the common bean enriched cookies, 5.7% in common bean enriched cookies against 1.6% in wheat cookies. In terms of protein content, the common bean enriched cookies showed higher protein content than the one determined in wheat cookies. Although common bean enriched cookies presented in their formulation 22% of maize flour, the expected nutritional composition of maize flour in terms of fiber and protein contents is similar

to the one described for the wheat flour (for total dietary fiber, 2.6 g/ 100 g in maize flour against 2.9 g/ 100 g in wheat flour and for total protein, 8.3 g/ 100 g in maize flour against 7.8 g/ 100 g in wheat flour) [33], therefore the main difference in the fiber and in the protein contents, between common bean enriched cookies and wheat cookies, could be attributed to the presence of common bean flour (representing 56% of the total added flour), characterized by higher fiber and protein contents than the cereals flours (22.9 g/ 100 g of total dietary fiber and 21.8 g/ 100 g of total protein, in common bean whole grain) [33]. This is also in accordance with a previous study conducted with bakery products developed with faba beans flour, as 50-100% of total added flour [34]. Other source of protein in both cookies derived from eggs. In opposition, the total carbohydrates content was significantly lower in common bean enriched cookies than in wheat cookies. This could be once again, attributed to the presence of common bean flour instead of only wheat flour, as supported by a previous study developed for composite flour cookies prepared from germinated triticale, kidney common bean, and chickpea [35]. The presence of maize flour in common bean enriched cookies was not responsible for the total carbohydrates content decrease since the total carbohydrates content in maize flour is quite similar to that of wheat flour (75.3 g/ 100 g in maize flour and 74.3 g/ 100 g in wheat flour) [33] and higher than the value described for common bean flour's carbohydrates content (42.6 g/ 100 g) [33].

Regarding the carbohydrates nutritional quality, although both cookies (common bean enriched *versus* wheat cookies) had a ratio of total dietary fiber: free sugars less than 0.5, which reflects the high level of free

sugars on cookies formulation, overall the common bean enriched cookies had a better nutritional quality. This was attributed to a lower ratio between total carbohydrates and fiber content (10.1, in common bean enriched cookies against 41.8 in wheat cookies). In fact, the inclusion of common bean flour (56% of the total added flours) instead of only wheat flour improved the final total fiber content to at least 1 g per 10 g of carbohydrates, which according to American Heart Association contributes for the higher nutritional quality of the processed product [36].

Regarding the resistant starch content, legumes (including common beans), compared to other food products, such as cereal-derived products, have been recognized as one of the major natural sources of resistant starch in human diet [37, 38], improving the final resistant starch content of cereal based products, once added to the formulations [39]. The recognized health potential of resistant starch, including in the prevention of colorectal cancer, due to colonic production of small chain fatty acids and promotion of microbiota growth, sparked the interest of the food industry for the use of resistant starch as a food ingredient in the production of food functional products [40]. As shown in **Table 3**, the inclusion of common bean and maize flours in cookies, by comparison to the formulated wheat cookies, improved remarkably the final resistant starch content (twenty six times more resistant starch) of cookies, contributing to enhance the health properties of ready-to-eat food products. The presence of common bean flour also improved the cookies' final phenolic content and antioxidant activity, **Table 3**. Although maize flour, also contributed to total phenolic content of the final formulated cookie, 1.50-2.76 mg/g dry weight [41], the higher proportion of raw common bean

flour compared to the maize flour (2.5:1) and the high level of phenolic compounds in the Moleiro common bean variety, 3.36 mg GAE/g dry weight [19], anticipated an higher contribution of common bean flour to the final phenolic composition of common bean enriched cookies.

**Table 3.** Resistant starch, total phenolic content and *in vitro* antioxidant activity (ORAC) of formulated common bean enriched and wheat cookies. Results expressed as mean ± standard deviation (n=3)

| | **Common bean enriched cookies (*Per 100g*)** | **Wheat cookies (*Per 100g*)** |
|---|---|---|
| **Resistant starch (g)** | 11.24 ± 0.36 | 0.43 ± 0.30 |
| **Phenolic content (mg GAE)** | 83.00 ± 4.00 | 19.00 ± 3.00 |
| **Antioxidant activity (µM TEAC)** | 3471.00 ± 510.00 | 1319.00 ± 145.00 |

GAE- Gallic Acid Equivalent; TEAC – Trolox Equivalent Antioxidant Capacity

Although a decrease in the phenolic content is expected after thermal processing, baking [42], the level of phenolic compounds, derived from common bean flour, contributed for a total phenolic content four times higher in the common bean enriched cookies than in wheat cookies. The same was observed for the *in vitro* antioxidant activity, which was 2.6 times higher for the common bean enriched cookies than for the wheat cookies. The reported high correlation between the total phenolic content and the *in vitro* antioxidant activity determined by ORAC in legumes [43] and in common beans, in particular, makes the association between the total phenolic content and cookies' antioxidant activity predictable.

## 3.2 Human intervention assay

### *3.2.1 Glycemic response, glycemic index and glycemic load*

The glycemic response measured, as capillary glycaemia, in all the volunteers at different time points, 0, 15, 30, 45, 60, 90 and 120 min, in the three human intervention assays (common bean enriched cookies, wheat cookies and oral glucose consumption) is detailed in **Table S1**. The increment on mean capillary glycaemia (*n* 16) values, determined at the different collection points, considering the fasting period as the baseline, is represented in **Figure 1**.

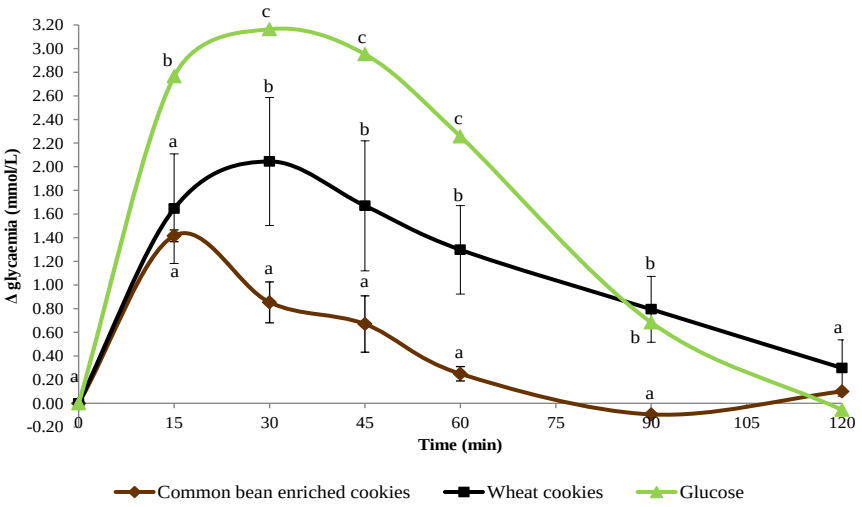



**Figure 1.** Variation of the glycaemia mean value on the common bean enriched cookies and wheat cookies assays considering the oral glucose assay as the reference (Δ glycaemia (mmol/L). Values represent glycaemia increments of mean ± SEM, *n* 16, taking the fasting glycaemia (t0) as the baseline. For clarity in graphical representation the bar errors relative to oral glucose assay were not considered but detailed in Table S1. At each time point, different letters represent significant differences in the glycemic response. Only one letter indicates absence of significant differences between assays.

Assuming the curve of oral glucose as reference, it was clear the lower impact of common bean enriched cookies in capillary glycaemia ($p < 0.05$). The maximum mean glycaemia value in the common bean enriched cookies assay was achieved 15 minutes post-consumption. After this time, the mean glycaemia values started to decrease, reaching the baseline

values (fasting period) 1 hour after consumption and no significant difference was determined between the mean glycaemia values registered at 60, 90, 120 and the values determined at the fasting period, $p > 0.05$. In the wheat cookies assay, the maximum mean glycaemia was reached 30 minutes after cookies ingestion. The major difference between common bean enriched and wheat cookies glycaemia curves was the lower glycaemia impact followed by glycemic stabilization in the common bean enriched cookies assay. In the wheat cookies assay the mean glycemic values remained steadily high during the following 60 minutes ($p < 0.05$), matching the mean glycaemia value obtained in the oral glucose assay at 90 minutes. At 90 and 120 minutes the mean glycaemia values returned to the baseline values at 0 minutes, $p > 0.05$.

The values of the area under the curve (AUCt) during the intervention period (120 minutes), **Table S2**, indicated high inter-variability between volunteers and overall lower AUCt values in the common bean enriched cookies assay (11281.88 ± 270.42) when compared to the wheat cookies assay (13275.94 ± 768.89), $p < 0.05$. This supports the beneficial effects of adding common bean flour to processed bakery products as an inducer of mild/moderate glycemic responses.

Comparing the incremental AUCt obtained in the common bean enriched cookies with the incremental AUCt elicited by oral glucose, a GI of 29% (25% when the measured GI was divided by the factor 1.16 to extrapolate the result for a portion of cookies with 50 g of available carbohydrates) and 71% (53% when the measured GI was divided by the factor 1.34 to extrapolate the result for a portion of cookies with 50 g of available carbohydrates) was determined to common bean enriched and

wheat cookies, respectively. Taking in account the net grams of planned carbohydrates, a GL of 14.24 (13.03 considering the extrapolated GI) and 40.38 (34.61 considering the extrapolated GI) was, respectively, determined in common bean and wheat cookies. Considering the reference values proposed for glycemic index and glycemic load [44], the inclusion of common bean flour in cookies formulation decreased the measured glycemic index from high (GI > 70) to low (GI < 55) and the glycemic load from high (GL > 20) to medium (GL 11 – 19). In a previous study the impact of combining legumes (black beans and chickpea) and cereals as part of a meal in the glycemic response showed the importance of legumes in a meal to reduce post-prandial glucose [45]. The richness of common beans in resistant starch and phenolic compounds, that may form complexes with starch, impairs starch enzymatic digestion and consequently the amount of available glucose in bloodstream. Additionally, the protein increment promoted by the common bean flour contributes to delay gastric emptying and to secrete insulin, which reduce post-prandial glycemic response [31].

### *3.2.2 Satiety perception and consumer acceptance*

Satiety can be defined as the different mechanisms, which puts end to a meal and prevents hunger between meals. Satiation is required to inhibit food intake and involves sensory signals (taste, smell, texture); gastrointestinal signals (gastric distension, release of hormones and peptides, leptin, insulin, cholecystokinin, CCK, glucagon-like peptide, GLP-1, gastric inhibitory peptide, GIP and peptide YY, PYY); rise of glycaemia and cognitive factors (expectations regarding satiating effects, beliefs,

distractions). Inhibiting hunger for a certain period of time can be modulated by the weight, volume, energy and nutrient content of the food product/meal. While the volume affects the early phase of satiety, the nutrient content influences the post-absorptive phase, determining the size of the next meal [32]. The VAS questionnaire applied in previous studies as a reproducible tool to evaluate the impact of resistant starch in satiety, hunger, fullness and evaluation of future consumption [29] was used, herein, to compare consumers' perception of satiety, hunger, fullness and future food consumption on the common bean enriched cookies and wheat cookies assays. For both types of cookies, the consumers' perception of satiety, hunger, fullness and future consumption after oral glucose consumption was applied as the reference. As expected, the satisfaction and fullness ratings (**Figures 2A** and **2B**), **Tables S3**, **S4** and **S5**, obtained after cookies consumption was considerably higher than after an oral glucose solution, $p < 0.05$. The solid state, volume/weight and texture of the cookies contributed to the mouthfeel sensation and satiety effects. As shown in **Table 4**, most of volunteers approved common bean enriched cookies taste, appearance and the mouth sensation. None of the volunteers disagreed with cookies taste or palatability. Although with wheat cookies, the satisfaction and fullness ratings showed a decreasing trend 30 and 15 minutes post-consumption, respectively, with common bean enriched cookies, the satiety (satisfaction and fullness) sensation was stable and perpetuated during a longer period of time, at least during two hours. Moreover, the satiety index determined for common bean enriched cookies (41.3) was 1.6 times higher than the satiety index obtained for wheat cookies (26.5). Complementarily the hunger and the perception of

future consumption ratings (**Figures 2C** and **2D**), **Tables S6** and **S7**, were remarkably lower after common bean enriched cookies intake, $p < 0.05$ and on average sustained for longer time. As shown by the calculated satiety quotient (SQ) values, while for common bean enriched cookies SQ remained at 0.05 /g of cookie from 15 to 60 minutes post-consumption decreasing to 0.03 /g during the remaining 30 minutes of the assay, for wheat cookies SQ always showed a decreasing trend after 30 minutes post-consumption, from 0.05 /g to fasting values at 120 minutes. Compared to the wheat cookies, the higher fiber, resistant starch and protein contents [32] of common bean enriched cookies, and the consumers' expectations and beliefs regarding the idea of a longer satiety period with a food product that includes common bean in its composition, may have contributed to strength the satiety efficiency of common bean enriched cookies. These results reinforce the importance of adding common bean flour as a nutritional strategy to ameliorate the nutritional quality of processed foods, contributing for the reduction of food products (number and portions) consumed between meals.

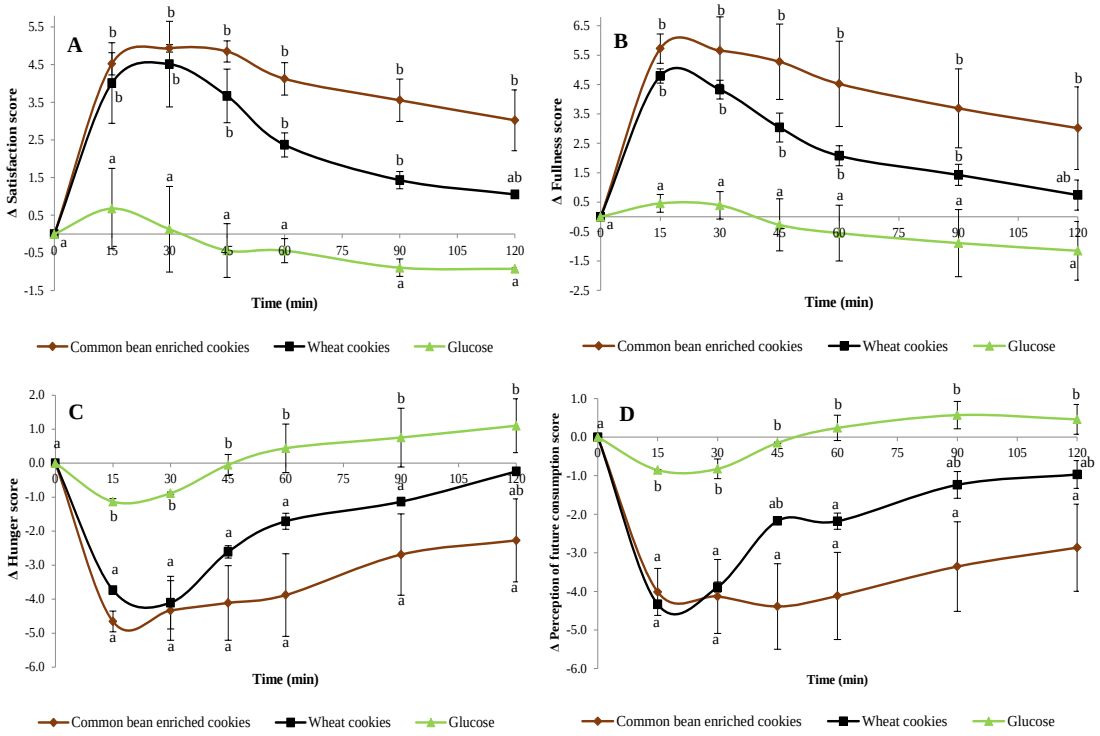



**Figure 2.** Subjective scores (A. satisfaction, B. fullness, C. hungry and D. perspective of future consumption) in 16 healthy volunteers. The results were expressed as the mean (± SEM) differences from the fasting (baseline) values. Different letters indicate significant differences in the scores obtained in the different intervention assays (common bean enriched cookies, wheat cookies and oral glucose), $p < 0.05$. Only one letter indicates absence of significant differences between assays.

**Table 4.** Volunteers' appreciation of common bean enriched and wheat cookies' taste and mouthfeel sensation

| | | *Strongly agreed* | *Agreed* | *Neutral* | *Disagreed* |
|---|---|---|---|---|---|
| **Common bean enriched cookie** | The cookie is tasty | 7 | 8 | 1 | 0 |
| | The cookie feels nice in my mouth | 3 | 13 | 0 | 0 |
| **Wheat cookie** | The cookie is tasty | 2 | 11 | 3 | 0 |
| | The cookie feels nice in my mouth | 1 | 14 | 1 | 0 |

## 4. Conclusions

Despite of the well-recognized nutritional and health benefits of legumes on diet as affordable, sustainable protein alternatives to animal products, legumes' consumption remains well below the recommended legumes' intake, especially in Europe.

The impact of legumes on the prevention of non-communicable diseases and on the improvement of biomarkers such as glycaemia and HDL-Cholesterol should represent a priority goal for a future concerned food industry focused in public health. The inclusion of legumes in processed foods is a strategy to increase legumes' consumption and to ameliorate the nutritional quality of processed food products. In the study conducted herein the nutritional quality, glycemic response and satiety perception after cookies intake was evaluated considering three human intervention assays with healthy volunteers (*n* 16) carried out with cookies formulated with 56% of common bean flour; cookies formulated only with wheat flour and an oral glucose solution, used as reference.

The results showed the richness of common bean enriched cookies in dietary fiber, which accounted for 1 g to 10 g of carbohydrates content and contributed for the carbohydrate's quality of these cookies. With higher protein content, common bean enriched cookies also showed higher resistant starch, and higher phenolic contents as well as higher antioxidant activity than wheat cookies. Well accepted by consumers in terms of taste and mouthfeel sensation, compared to wheat cookies, common bean enriched cookies showed lower glycemic index and lower glycemic load, inducing lower and stable glycemic responses, at least during two hours. The ratings of satiety, fullness, hunger and perspective of future

consumption obtained with common bean enriched cookies reinforced the benefits of including common bean flour as an alternative ingredient to wheat flour in bakery food products and snacks, for the improvement of satiety efficiency allowing a better management of food intake between meals. The use of common bean flour as an alternative ingredient could be adapted in future formulations to populations with specific dietetic needs (e.g. *diabetes mellitus*, and overweight/obese individuals) as a strategy to control food intake, body weight and biomarkers (e.g. glycaemia, glycated hemoglobin), promoting life quality and preventing associated health complications.

## Supplementary materials

The following are available online in FigShare repository: https://figshare.com/s/963acb3beb441edad4d4, Table S1: Capillary glycaemia at different time points on the three intervention assays (common bean enriched cookies, wheat cookies and oral glucose assays) in 16 volunteers. Unless stated otherwise the results were expressed in mg/ dL, Table S2. Area under the curve (AUCt) values obtained on common bean enriched cookies, wheat cookies and oral glucose intervention assays considering the glycemic curves traced during the study period, t0, t15, t30, t45, t60, t90 and t120, Table S3: Subjective satisfaction perception scores, Table S4: Area under the curve (AUCt) values obtained on common bean enriched cookies, wheat cookies and oral glucose intervention assays considering the satisfaction perception ratings during the study period, t0, t15, t30, t45, t60, t90 and t120, Table S5: Subjective fullness perception scores, Table S6: Subjective hunger

perception scores, Table S7: Subjective perspective of future food consumption scores.

## Author Contributions

Conceptualization, E.M., V.C., M.E.F., M.C.V.P. and M.R.B.; funding acquisition, E.M., M.E.F., M.C.V.P. and M.R.B.; project administration, M.E.F., M.C.V.P. and M.R.B.; investigation, E.M., V.C., M.E.F., M.C.V.P. and M.R.B.; methodology, E.M., V.C., A.B.S. and A.F.; resources, M.E.F., M.C.V.P. and M.R.B.; software, E.M. and V.C.; formal analysis, E.M. and V.C.; data curation, E.M., V.C., M.C.V.P. and M.R.B.; validation, E.M., V.C., M.C.V.P. and M.R.B.; visualization, E.M. and V.C.; supervision, B.S., M.E.F., M.C.V.P. and M.R.B.; writing—original draft preparation, E.M. and V.C.; writing—review and editing, E.M., V.C., M.C.V.P. and M.R.B.

## Acknowledgments

To the Research Unit of Biotechnology and Genetic Resources germplasm bank, INIAV, Oeiras, Portugal, for conserving the common bean samples. FCT, Portugal, through BEGEQA project (PTDC/AGR-TEC/3555/2012), E.M. PhD fellowship (SFRH/BD/89287/2012) and R&D unit, UIDB/04551/2020 (GREEN-IT – Bioresources for sustainability) as well as to PORTUGAL 2020, grant number LISBOA-01-0145-FEDER-402-022125. The authors also greatly acknowledge national funding from iNOVA4Health– UIDB/04462/2020, a program financially supported by Fundação para a Ciência eTecnologia / Ministério da Ciência, Tecnologia e Ensino Superior and the funding from INTERFACE Programme, through the Innovation, Technology and Circular Economy Fund (FITEC).

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# Chapter VII: Discussion and future perspectives

## General Discussion

Common bean (*Phaseolus vulgaris* L.) has been recognized as a valuable grain legume to ensure food security and prevent malnutrition worldwide. Sustainable agriculture systems with concomitant legumes cropping should be a priority for countries that aim to achieve the second sustainable developmental goal of FAO, by 2030, mitigating hunger and affording food to the word population [1]. The use of intensive agricultural systems, the dependence on imported grain legumes' supply, the climate changes (rising temperature 2 – 4 °C over the next century), the reduced national market investment in local varieties, the consumers' prejudices regarding grain legumes nutritional value, health impact and culinary use, may jeopardize FAO's goal achievement [2-6]. The lack of investment in intervention studies regarding the impact of common bean in human health, has been threatening the dissemination of meaningful information that ultimately would contribute to increase consumers' acceptance and demand for legume based food-products. Additionally, despite of the recognized genetic diversity, some common bean germplasm like the Portuguese one [7] has been underexplored, compromising their use in the breeding world and putting at risk the survival of legume crops under challenging environmental conditions worldwide.

The studies conducted throughout this thesis aimed to cope with the two major current challenges presented above:

- Improve awareness of food industry and consumers on common beans' importance in a daily based diet;
- Valorisation of underexplored common bean accessions (Portuguese germplasm), through chemical characterization of existent diversity, in order to bring useful information to future breeding programs focused in selecting varieties more adapted to warming environmental cropping conditions.

**More beans for human health**

The consumers' dietary habits have currently changed and, if the busy lifestyle demands for ready-to-eat foods, nowadays consumers do not dismiss the importance of food choices in their health and well-being. Consumers' aware of the relevance of local production for sustainable, food supply systems will demand for national, affordable, sustainable, innovative and healthier ready-to-eat-food products. The COVID-19 pandemic proved that consumers are aware of the relevance of beans in their diet as economical, versatile, shelf-stable and healthy food products. The lack of canned and dry beans in the stores' shelves, especially when the pandemic was an unprecedented event, is a good indication of the consumers' requirements [8]. More than trying to understand the motivational reasons underneath this rampant demand it will be important to understand how to sustain consumers purchase on a routine basis for their versatility, sustainability and healthfulness [8]. Once incorporated in ready-to-eat food products, grain legumes, in general and common beans,

in particular, contribute to increase the consumption of plant-based food products in the diet, which has impact in human health, through the improvement of HDL-Cholesterol level and reduction of glycated proteins [9, 10]. Embracing the challenge of reinventing the traditional gastronomy and developing innovative plant-based food products, in Chapter VI a human intervention study with healthy volunteers was conducted to assess the impact of grain legumes flour in human capillary glycaemia and consumers' acceptance of bakery alternative products. Once used to substitute part of the wheat flour in the traditional wheat cookies, common bean flour can be added to improve protein, fiber (1 g per 10 g of carbohydrates), resistant starch and phenolic composition of traditional cookies, Chapter VI. A fiber:carbohydrates ratio, 1:10, according to the American Heart Association, contributes for the higher nutritional quality of the processed cookies [11]. Increasing protein, resistant starch and phenolic contents contribute to delay gastric emptying, and to enhance insulin secretion, which reduces post-prandial glycemic response [12, 13], allowing mild/moderate glycemic responses and promoting higher satiation perception ratings after common beans enriched cookies consumption [Chapter VI]. Besides exploiting an alternative use of common bean flour in bakery products, Chapter VI contributed to demystify the generalized idea of similarity between legumes' and cereals' nutritional values, showing for the first time through a human intervention study that not only the nutritional profile but also the impact in volunteers' glycaemia and satiation perception are considerably different.

In order to ensure consumers' accessibility to common beans compounds with potential health impact in the prevention of chronic

diseases (e.g. cardiovascular diseases, cancer), food industry and consumers should be aware about the effect of pre-processing methods (soaking and peeling processes) in compounds' (e.g. phenolic compounds) availability. Notwithstanding the negative impact of these compounds in nutrients digestibility [14], and minerals availability, phenolic compounds present in plant food products including in legume-based products, and produced by plants against biotic and abiotic stresses [15], may contribute to the prevention of chronic diseases [16]. Besides phenolic compounds, legumes, in general and common beans, in particular, are also important sources of trypsin inhibitors. These last compounds may interfere with trypsin activity, impairing protein digestibility. Nevertheless, they are inactivated by heating temperatures during the cooking process [17], which improves common beans' protein digestibility [Chapter II]. Well recognized by their impact in human health, when available in diet, phenolic compounds may exert antioxidant, anti-inflammatory [18, 19], anti-hyperglycemic [20], anti-hyperlipidemic [21] and anti-carcinogenic [22] activities. In order to bring light on the diversity of phenolic compounds present in the complex structure of common bean seeds, in Chapter IV the different fractions obtained after the soaking process were characterized using UPLC-Triple-TOF-MS. This allowed elucidating the diversity of phenolic compounds in the different fractions and the importance of avoiding/ adjusting pre-processing methods (e.g. rejection of the soaking water and/or the peeling process) as a strategy to take advantage of the maximum diversity and amount of phenolic compounds in cooked common beans, especially in non-undernourished populations [23]. The percentage of phenolic compounds lost into water was dependent on the common

bean accession, and particularly on the seeds' permeability to water [24]. With higher diversity of phenolic compounds, the seed coats of coloured accessions were particularly rich in flavonols (e.g. Kaempferol). The glycosidic forms of hydroxybenzoic acids and flavanols (including procyanidins) were abundant in the soaking water obtained from the coloured accessions and in the soaked cotyledons fraction the hydroxycinammic acids were the predominant phenolic compounds [Chapter IV]. The chemical structure of flavonoids (O-dihydroxy groups in the B-ring, double bond C2-C3 and the ketone at C4 in C-ring) and the high degree of proanthocyanidins' polymerization, in seed coats and soaking water, anticipate the higher antioxidant activity of these common bean fractions [25, 26]. Along with the soaking procedure, the cooking process, as a consequence of the phenolic compounds' chemical instability during the heating process, also conducts to phenolic content decrease [27]. Nevertheless, changes in the plant cell wall structure may increase the accessibility to some linked phenolic compounds [28]. Even when accessible to consumers, the health benefits of phenolic compounds will always depend on their bioavailability, which is closely related to individual factors such as inter-individual variability in genetics, gender, age, microflora composition, intestinal transit time, enzymatic activity and gastrointestinal pathologies [29]. The scarcity of studies reporting the metabolic fate of phenolic compounds derived from common beans in human bioavailability studies [30] and the diversity of phenolic compounds found in cooked common beans, imposed the urge of conducting in Chapter V, a human intervention study. This was designed to accurately identify and quantify by UPLC-Q-TOF-MS, in plasma and urine,

metabolites derived from phenolic compounds, obtained at different time points, after consumption of a common beans' meal. With few exceptions, in plasma (vanillic acid-4-O-sulphate, ferulic acid-4-O-sulphate, ferulic acid-4-O-glucuronide and kaempferol-3-O-glucuronide) and in urine (vanillic acid-4-O-sulphate, sinapic acid and kaempferol-3-O-glucuronide) [31, 32], the majority of the quantified metabolites only reached the maximum concentration after 8h post-consumption, supporting the impact of gut microbiota in the metabolism of phenolic compounds derived from common beans (e.g. hydroxycinnamic acids). Bound to the cell wall polysaccharides (fiber fraction), in the cooked beans, most of the phenolic compounds will remain unmodified until they reach colon [33, 34]. As the first human intervention study performed, through targeted metabolomics, to assess human bioavailability of phenolic compounds derived from common beans intake, this study identified the metabolites with plasmatic concentration and urinary excretion increments after beans consumption. Since volunteers followed a free phenolic compounds diet in the 48h prior to the study, the metabolites, vanillic acid-4-O-sulphate, 4-hydroxyhippuric acid, ferulic acid-4-O-sulphate, ferulic acid-4-O-glucuronide, kaempferol-3-O-glucuronide, pyrogallol-1-O-sulphate, caffeic acid, catechol-O-sulphate, 4-methylcatechol-O-sulphate, 3-hydroxyhippuric acid, caffeic acid-4-O-β-D-glucuronide, caffeic acid-3-O-β-D-glucuronide and sinapic acid, may have interest as markers of common beans' consumption to exploit in future through *in vitro* and *in vivo* model diseases studies. Although the role of beans in colon health of colorectal cancer survivors has been recognized [35] further studies, focusing especially in the phenolic compounds that reach colon unmodified, should be conducted to understand the complex

interactions between diverse common beans compounds and gut microbiome.

**Providing data for improving beans quality worldwide**

In addition to the diversity of phenolic compounds found in the different common bean fractions separated after the soaking process, the study conducted in Chapter IV with different Portuguese common bean accessions cropped under the same edaphoclimatic conditions, emphasized the existent genetic diversity in the phenolic content (including flavonoids and proanthocyanidins contents) among the set of studied varieties. These findings represent a launching pad to recognize the high value of the underexplored Portuguese common bean germplasm for future human health and breeding studies. Besides the described genetic diversity in phenolic compounds content, studying the behavior of distinct common bean accessions in different environmental conditions through untargeted metabolomics is fundamental for future breeding programs that aim to keep up with consumer and food industry demands, without putting at risk food security, ensuring high quality common beans' production in increasingly challenging environmental conditions.

Heat stress can impair plant growth from germination to reproduction, compromising the productivity of staple crops [36]. It adversely affects the photosynthetic process and the reproductive development. The plants able to cope with increased temperatures will be able to maintain photosynthetic rates, membrane thermostability and pods/ grain production in a changing climate [37]. Breeding for regional environmental adaptation (like for the Mediterranean basis, where the

summer temperatures are 40% larger than the global mean temperature raising) [38], and taking advantage of cross-over genotype x environment interaction effects in breeding programs, still represent barely explored approaches [39]. Understanding the molecular and biochemical mechanisms behind plant heat tolerance is complex and plant breeders need fast-tracking genomic and phenotyping solutions to produce high resilient plants. Over the last decade multi-Omics tools have been developed not only to identify potential biomarkers of food consumption [Chapter V] but also to disclose the genotype x environment interactions and the metabolic networks that tackle abiotic stresses in plants [40]. Most of the genes responsible by heat tolerance are involved in primary (e.g. sugars, amino acids) and secondary (e.g. phenolic compounds, saponins) metabolism of the plant [36]. Sought as an alternative source of protein, underexploited common bean accessions, in Chapters II and III were investigated for their nutritional quality and metabolomics composition, respectively, considering the environmental pressure of warming adverse climate conditions. Regarding the nutritional quality, under the heat stress environment, common bean accessions showed higher protein and lower carbohydrate contents than the accessions cropped under the mild/moderate environment [Chapter II]. As expected above 30-35 °C, the enzymes involved in photosynthesis were repressed and the assimilation/remobilization of nitrogen promoted, ensuring the embryo's survival [6, 41]. Breeding for the improvement of protein quality, more than selecting common bean accessions based on protein content, requires the selection of accessions with higher protein quality in terms of amino acids composition, meaning higher amino acids contents. Studying the diversity

of underexploited accessions through multivariate analysis allowed the identification of the most interesting accessions for future breeding programs focused in improving protein quality [Chapter II]. In an unprecedented study, through untargeted and targeted high-resolution mass spectrometry methodologies allied with bioinformatics tools for data mining, in Chapter III, the metabolites diversity of different common bean accessions submitted to two distinct environmental pressures were analysed. Specific metabolites from pteridines, organooxygen compounds and benzenoids classes were highly correlated to heat stress tolerant common bean accessions. Overall, metabolites from lipids and lipid-like molecules superclass were mostly abundant in the milder environmental conditions [Chapter III]. Data suggested that under abiotic stress carbons are displaced to the shikimate pathway leaving fewer carbons to the route responsible by the lipids and lipid-like molecules synthesis [15]. Some of the phenolic compounds identified in common bean fractions by UPLC-Triple-TOF-MS [Chapter IV] were also identified by UHPLC-Orbitrap-MS (vanillic acid, feruloyl and sinapoyl aladaric acids, procyanidin C1, glycoside derivatives of quercetin and kaempferol, aglycones quercetin and kaempferol and isoflavones, daidzein and genistein) in the diversity of common bean accessions cropped under contrasting environmental conditions [Chapter III]. Although identified by both mass spectrometry methodologies, the contribution of other metabolites, including phenolic compounds, such as salycilic acid, in heat stress tolerance, was quite evident (with correlation higher than 50% with the heat stress environment) and well recognized in the literature. Salycilic acid is an essential secondary metabolite that increases proline accumulation, maintaining

normal cell membrane function and photosynthesis under heat stress environment [42]. Identifying the unique metabolites that are associated to specific environmental conditions and/or influenced by specific genotype x environment interactions is the way forward to assist crop improvement programs. New analytical approaches (e.g. Fourier transformed infrared spectroscopy) to access metabolomics composition (including primary and secondary metabolites) associated with suitable chemometric techniques should be further exploited to generate faster, scalable, and reliable data to assist modern routine breeding strategies. With wide applicability in breeding assistance, the metabolomics data could be complemented with other omics, namely genomics, to bring new insights on the genetic architecture of common beans quality, and sustain the development of selection molecular tools speeding the development of more resilient varieties that cope with new environmental challenges and answer consumers concerns.

In conclusion, in this PhD work, centered in the study of common beans' quality, a multidisciplinary approach was applied, pin-pointing several aspects with interest for breeders/researchers, consumers and the food industry. It discussed, the nutritional and metabolomics of common beans cropped under warming environmental conditions, to answer to future breeding challenges under heat stress environments; the molecular markers of common beans' consumption with potential health effects; the impact of processing methods (soaking and/or coats peeling) in phenolic compounds accessability and the development of common beans enriched ready-to-eat food products. Finnally, throughout this thesis, a global legume-centered prespective was taken in account connecting different

stakeholders that ultimately will contribute for an environmental, sustainable and healthier solution to achieve food security worldwide.

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# Funding acknowledgment

The work developed for this thesis was financially supported by FP7-EU project Strategies for Organic and Low-input Integrated Breeding and Management (SOLIBAM), Fundação para a Ciência e Tecnologia (FCT), through a Ph.D grant, reference SFRH/BD/89287/2012, assigned to Elsa Mecha as well as to BEGEQA project (PTDC/AGR-TEC/3555/2012) and to the research R&D unit, GREEN-IT – Bioresources for sustainability (UIDB/04551/2020). COST Action FA1403 also provided financial support, through a grant reference COST-STSM-ECOST-STSM-FA1403-290815-063873, attributed to Elsa Mecha.



*“In diversity there is beauty and there is strength”*

*Maya Angelou*

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